Novel vaccination strategies against 
*human respiratory syncytial virus*

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Novel vaccination strategies against human respiratory syncytial virus

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<th>Description</th>
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<tbody>
<tr>
<td>ALRI</td>
<td>Acute lower respiratory infection</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BRSV</td>
<td>Bovine respiratory syncytial virus</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional dendritic cell</td>
</tr>
<tr>
<td>cp</td>
<td>Cold passage</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary RNA</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded RNA</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>F</td>
<td>Fusion protein</td>
</tr>
<tr>
<td>FI-RSV</td>
<td>Formalin inactivated RSV vaccine</td>
</tr>
<tr>
<td>FMDV</td>
<td>Foot and mouth disease virus</td>
</tr>
<tr>
<td>G</td>
<td>Attachment protein</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HA&lt;sub&gt;min&lt;/sub&gt;</td>
<td>Codon pair deoptimized hemagglutinin</td>
</tr>
<tr>
<td>HBC</td>
<td>Hepatitis B core protein</td>
</tr>
<tr>
<td>HBC-I</td>
<td>Intermediate hepatitis B core protein (163 aa)</td>
</tr>
<tr>
<td>HBC-L</td>
<td>Long hepatitis B core protein (183 aa)</td>
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<tr>
<td>HBC-S</td>
<td>Short hepatitis B core protein (149 aa)</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonated kidney (cells)</td>
</tr>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>Acronym</td>
<td>Abbreviation and Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HN</td>
<td>Hemagglutinin-neuraminidase protein</td>
</tr>
<tr>
<td>HPAIV</td>
<td>Highly pathogenic avian influenza virus</td>
</tr>
<tr>
<td>HR-A</td>
<td>Heptad repeat A</td>
</tr>
<tr>
<td>HR-B</td>
<td>Heptad repeat B</td>
</tr>
<tr>
<td>iBALT</td>
<td>Inducible bronchus associated lymphoid tissue</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-1-thiogalactopyranisid</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>L</td>
<td>Large polymerase protein</td>
</tr>
<tr>
<td>LAIV</td>
<td>Live attenuated influenza vaccine</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphochoriomeningitis virus</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LRT</td>
<td>Lower respiratory tract</td>
</tr>
<tr>
<td>M</td>
<td>Matrix protein</td>
</tr>
<tr>
<td>M1</td>
<td>Matrix protein 1</td>
</tr>
<tr>
<td>M2</td>
<td>Matrix protein 2</td>
</tr>
<tr>
<td>M2e</td>
<td>Ectodomain of matrix protein 2</td>
</tr>
<tr>
<td>MDA-5</td>
<td>Melanoma Differentiation-Associated protein 5</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darbey Canine Kidney (cells)</td>
</tr>
<tr>
<td>MDV</td>
<td>Master donor virus</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIR</td>
<td>Major immunodominant region</td>
</tr>
<tr>
<td>N</td>
<td>Nucleoprotein (RSV)</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NBe</td>
<td>Ectodomain of the NB protein</td>
</tr>
<tr>
<td>NDV</td>
<td>Newcastle disease virus</td>
</tr>
<tr>
<td>NEP</td>
<td>Nuclear export protein</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain-containing protein</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein (influenza)</td>
</tr>
<tr>
<td>NP&lt;sub&gt;min&lt;/sub&gt;</td>
<td>Codon pair deoptimized nucleoprotein</td>
</tr>
<tr>
<td>NS1</td>
<td>Non-structural protein 1</td>
</tr>
<tr>
<td>NS2</td>
<td>Non-structural protein 2</td>
</tr>
<tr>
<td>P</td>
<td>Phosphoprotein</td>
</tr>
<tr>
<td>PA</td>
<td>Polymerase acidic protein</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular marker</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PB1</td>
<td>Polymerase basic protein 1</td>
</tr>
<tr>
<td>PB2</td>
<td>Polymerase basic protein 2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>PIV</td>
<td>Parainfluenza virus</td>
</tr>
<tr>
<td>Pol</td>
<td>DNA dependent RNA polymerase</td>
</tr>
<tr>
<td>PR8</td>
<td>Influenza A/Puerto Rico/8/34 virus</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PTV</td>
<td>Porcine teschovirus</td>
</tr>
<tr>
<td>rAd</td>
<td>Recombinant adenovirus</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA dependent RNA polymerase</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene-I</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I like receptor</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>SeV</td>
<td>Sendai virus</td>
</tr>
<tr>
<td>SH</td>
<td>Small hydrophobic protein</td>
</tr>
<tr>
<td>SP</td>
<td>Surfactant protein</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded RNA</td>
</tr>
<tr>
<td>T&lt;sub&gt;CM&lt;/sub&gt;</td>
<td>Central memory T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T&lt;sub&gt;EM&lt;/sub&gt;</td>
<td>Effector memory T cell</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>ts</td>
<td>Temperature sensitive</td>
</tr>
<tr>
<td>URT</td>
<td>Upper respiratory tract</td>
</tr>
<tr>
<td>VEE</td>
<td>Venezuelan equine encephalitis virus</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>vRNA</td>
<td>Viral RNA</td>
</tr>
<tr>
<td>vRNP</td>
<td>Viral ribonucleoprotein</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Part 1

Introduction
Chapter 1
Human respiratory syncytial virus

1.1 HRSV history, classification and global burden

HRSV was first isolated in 1956 from a chimpanzee with respiratory symptoms characterized by coughing, sneezing and a nasal discharge containing mucus and pus. It was identified as a virus and named “Chimpanzee Coryza Agent”. Antibodies against this virus were also detected in children and a laboratory worker, suggesting that this virus could also infect humans (1). Later, Chanock et al. isolated the same virus from children with symptoms of a cold. Since the virus was able to form syncytia in human cells in tissue culture the name of the virus was changed to “Respiratory syncytial virus” (2). Soon it became clear that HRSV was a common cause of mild to severe respiratory tract disease.

HRSV is an RNA virus classified in the family of Paramyxoviridae, subfamily Pneumovirinae, genus Pneumovirus (3). Characteristics of the Paramyxoviridae are (I) a non-segmented, single stranded, negative-sense RNA genome; (II) transcription of the genome occurs through a transcriptional stop-start mechanism, which results in multiple mRNA molecules; (III) the replication of the virus occurs in the cytoplasm of the cell; (IV) virions possess a lipid membrane, derived from the host cell by budding of progeny virus; (V) entry in the host cell occurs through a fusion process of the viral and cellular membranes. Other viruses, closely related to HRSV, in the pneumovirus genus are: bovine RSV (BRSV), ovine RSV, caprine RSV and pneumovirus of mice. HRSV can be divided into two major antigenic subgroups, designated A and B, based on in vitro neutralization assays and reactivity with monoclonal antibody panels (4). Within each of the two subgroups, several different genotypes have been identified which can co-circulate at the same time at the same location. Shifts in dominance between the A and B subtypes typically occur every one or two years (5) (Figure 1.1). In contrast to other viruses such as influenza A virus, which change quickly due to strong immune pressure, HRSV evolves slowly and new strains do not immediately dominate during the following epidemic. An example of this is the HRSV B Buenos Aires (BA) strain, which contains a 60 nucleotide duplication in
the G protein: this strain was first described in 1998 and spread throughout the world rather quickly. It wasn’t until 2005 that this strain became the dominant B genotype (6).

HRSV causes seasonal epidemics of respiratory disease during late autumn or winter (Figure 1.1). It is globally the most important cause of acute lower respiratory infections in young children and babies, especially when premature (8). Typically, HRSV infects at very young age: almost every child has been infected during its first year of life, and by the age of two virtually all children have been infected at least once and some even twice (9). A prospective study performed by Glezen et al., of children infected during the first year of life revealed that 47 % and 45 % of these children were re-infected during the second and third year respectively (9). Remarkably, HRSV can infect the neonate, despite the presence of maternally derived HRSV-specific serum IgG antibodies in this population. A recent study showed that in 2005 HRSV was responsible for over 30 million cases of acute lower respiratory tract infections (ALRI) in children younger than five years old (10). Approximately 10% of these children required hospitalization due to ALRI caused by HRSV. Moreover, it is estimated that up to 200,000 children younger than five years old die due to complications caused by HRSV, most of which occur in developing countries (10). Furthermore, severe bronchiolitis due to HRSV infection during infancy has been associated with an increased incidence of recurrent wheezing and asthma in later childhood (11). It is however not clear whether the HRSV infection is the cause of the later asthma, or whether children with a genetic predisposition for asthma are more susceptible to severe HRSV induced bronchiolitis. Re-infections are common in adults (9). The disease symptoms in adults however are less severe than in children, as it mostly manifest as a mild upper respiratory (URT) infection (12). Still, it is seen as the second most important cause of adult respiratory disease, after influenza infections (13). Several groups are more susceptible to severe HRSV bronchiolitis, such as premature born children, children with underlying heart- and/or lung disease, and children with immunodeficiency disorders (14). One study estimated the hospitalization rate during the first year
of life in HRSV infected children with immunodeficiency as high as 38%, compared to 3% for healthy control children (15). Also in immunosuppressed adults, such as leukemia or hematopoietic stem cell transplant patients, HRSV is an important cause of morbidity and mortality, with mortality rates ranging between 80% and 100% in HRSV infected patients (16). Besides an important cause of infant respiratory disease, HRSV is more and more recognized as an important cause of illness in the elderly. In this population, HRSV causes pneumonia, bronchiolitis and exacerbation of chronic obstructive pulmonary disease; conditions that often lead to hospitalization and excess mortality in this age group (17).

Disease symptoms during a primary HRSV infection can vary strongly among individuals. In most young children a primary HRSV infection is restricted to the URT. In 25% to 40% of the cases however the infection spreads to the lower respiratory tract (LRT), resulting in bronchiolitis or pneumonia (9). These children have symptoms such as coughing, sneezing, rhinorrhea, wheezing and fever. In some severe cases of HRSV children experience episodes of apnea (18).

Currently, no vaccine exists that can inhibit HRSV infection. The only preventive measure currently available is a passive immunoprophylaxis therapy. The first developed therapy, named RSV Intravenous Immune Globulin (RSV-IVIG, RespGam™, produced by MedImmune) consisted of human IgG prepared from donor sera that had been screened for high HRSV-neutralizing titers. These antibodies are administered by monthly intravenous infusion, for 4 to 5 months during the HRSV season, to children at risk of developing severe HRSV-induced disease. Given prophylactically, RSV-IVIG reduces the frequency of hospitalization by 55% and the duration of stay in intensive care unit by 97% (19, 20). Since 2004 this therapy is no longer being used and is replaced by Palivizumab (Synagis™, MedImmune) (21, 22). This new treatment consists of an HRSV F protein-specific neutralizing monoclonal antibody that was created by humanizing a mouse monoclonal antibody. Even though Palivizumab is comparable in efficiency of reducing HRSV hospitalization as RSV-IVIG, it is more effective on a weight basis, allowing administration of smaller volumes. In addition, Palivizumab is administered by monthly intramuscular injection rather than an intravenous infusion which takes several hours. Due to high costs associated with the treatment, Palivizumab is only given to children that have a high chance of developing severe disease upon an HRSV infection, such as premature born baby’s or infants with underlying heart or lung disease (23). Table 1.1 gives an overview of current and past prophylactic RSV treatments.
**Table 1.1  Prophylactic RSV treatments**

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Application</th>
<th>Clinical study results, Comments.</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>RespiGam ®</td>
<td>Enriched RSV immunoglobulin, produced from pooled human plasma that contained high RSV neutralizing activity</td>
<td>Approved in 1996 for preterm infants with and without CLD. Contraindicated for use in children with CHD. Intravenous infusion (750 mg/kg).</td>
<td>Reduces incidence of RSV hospitalization by 41%. Total number of RSV hospitalization days reduced by 55%. Significant reduction of disease severity. Drawbacks: large fluid volume necessary for infusion. Risk of transmitting blood-borne infections. Withdrawn from the market in 2004.</td>
<td>19 20</td>
</tr>
<tr>
<td>Palivizumab</td>
<td>Humanized version of the RSV neutralizing antibody MAb 1129. The six CDRs of the mouse MAb were grafted on the framework of a human IgG1 MAb.</td>
<td>Approved in 1998 for premature born infants with CLD or premature birth (≤ 35 weeks gestational age). Approved in 2003 for infants with CHD. Five monthly intramuscular injections (15 mg/kg)</td>
<td>Significant reduction in RSV hospitalization (55% reduction). Significant reduction in total days of hospitalization and in days with increased oxygen administration.</td>
<td>21</td>
</tr>
<tr>
<td>Motavizumab</td>
<td>Derivative of palivizumab with 13 aa changes to try to improve its biological properties.</td>
<td>Not available on the market because clinical trials did not reveal an improvement over palivizumab. Five monthly intramuscular injections (15 mg/kg)</td>
<td>No statistically significant difference in the duration of RSV hospitalization, number of patients requiring supplemental oxygen and intensive care submission between motavizumab and palivizumab.</td>
<td>261 262 263</td>
</tr>
</tbody>
</table>

MAb=monoclonal antibody; CDR= complementary determining region; CLD=chronic lung disease; CHD=chronic heart disease

### 1.2 The HRSV virion and proteins

Human respiratory syncytial viruses are spherical or filamentous particles. Their outer membrane is made up of a lipid bilayer which is derived from the host-cell plasma membrane. The transmembrane glycoproteins F, G and SH protrude through this membrane as spikes. The matrix protein (M) forms a core lining the inside of the viral membrane. A helical nucleocapsid is located within the matrix shell and comprises the viral genome, in association with the nucleoprotein (N), the viral polymerase (L) and the phosphoprotein (P) (Figure 1.2). The HRSV genome consists of a single negative-sense strand of RNA of 15.2kb in length. The genome encodes ten genes in the order 3’ NS1-NS2-N-P-M-SH-F-G-M2-L 5’ (13).
The nucleoprotein (N). The viral nucleoprotein is tightly bound to the viral RNA, to form the viral ribonucleoprotein (RNP). The N protein functions to stabilize the single stranded RNA molecule and most likely it also shields the RNA from host pattern recognition receptors, such as RIG-I and MDA-5, which could initiate the host anti-viral response upon detection of cytoplasmic tri-phosphorylated RNA or dsRNA respectively (13). The RNP is the template for viral transcription.

The large polymerase (L). The L protein performs RNA synthesis, as well as capping and polyadenylation of the mRNA transcripts (25). As the name suggests, this protein is very big: it consists of 2165 amino acids.

The phosphoprotein (P). The P protein is a homotetramer and is an essential co-factor of the L protein. Besides the L protein, it also interacts with the N and the M2-1 protein (26). During RNA synthesis, its function consists of opening the tight structure of the RNP, to allow the L protein to reach the RNA for transcription. It is phosphorylated at several sites, hence its name (27).

The matrix (M) protein. The M protein lines the inner side of the viral membrane. It plays a role in budding of progeny viruses (28). Upon expression of the viral transmembrane proteins, the M proteins tightly assemble at lipid rafts in the membrane, an interaction which is not seen when viral glycoproteins are not present (29).

Figure 1.2 Structure of human respiratory syncytial virus particle. The HRSV virion is made up of a lipid bilayer with 3 glycoproteins inserted into it: the fusion protein, the attachment (G) protein and the small hydrophobic (SH) protein. The matrix protein lines the inner surface of the membrane and surrounds the viral ribonucleoprotein (RNP) complex. The viral RNP consists of the viral RNA surrounded by the nucleoprotein, and is associated with the viral nucleoprotein and large polymerase. The nonstructural proteins NS1 and NS2, and the regulatory proteins M2-1 and M2-2 are not shown. Adapted from (24)
**The M2 proteins.** HRSV encodes two M2 proteins from overlapping reading frames. M2-1 is a homotetramer that binds to N and the viral RNA and functions as a transcription elongation factor: when absent, transcription is terminated prematurely (30, 31). The second M2 protein, M2-2, encoded from a second downstream AUG, is a small, not abundant protein that accumulates during infection. It is responsible for a switch from transcription to replication of the viral genome (32).

**The nonstructural proteins (NS1 and NS2).** Both NS proteins are involved in the inhibition of the host IFN response at multiple levels (reviewed in (13)). Mutant strains lacking one or both NS proteins are viable, indicating that both proteins are dispensable, however such mutants grow much slower in vitro than wild type HRSV and are highly attenuated in vivo (33-35). Besides the direct inhibition at several steps of the IFN pathway, the NS1 protein is also involved in an indirect mechanism of IFN inhibition, through inhibition of RNA synthesis. Overexpression of NS1 protein in a minireplicon system results in inhibition of viral RNA transcription and replication (36), which might serve as a safety mechanism of the virus to prevent massive production of viral RNA which could trigger host pattern recognition receptors (such as RIG-I, MDA-5 or PKR) and would activate the host IFN response. In this way the NS1 is indirectly responsible for inhibition of the host IFN mechanisms.

**The small hydrophobic (SH) protein.** SH is the smallest of the three transmembrane glycoproteins of HRSV: it consists of only 63 amino acids. The monomers aggregate to homopentamers with a pore like structure in the membrane. SH pentamers can function as ion channels, however, the exact function of this in the HRSV replication cycle is still unclear (37, 38). Mutant viruses lacking SH have been developed, indicating that the protein is not essential for virus transcription or replication; however these viruses are attenuated in vivo in mice and chimpanzees (34, 39).

**The attachment glycoprotein (G).** The G protein is a type II transmembrane protein with an N-terminal transmembrane domain, leaving the C-terminal two thirds of the protein oriented externally (13). It is also present in a second, secreted form that arises from transcription starting at a second downstream AUG (40-42), which leaves out the N-terminal transmembrane domain. In vitro it was shown that this protein functions as a decoy, to catch neutralizing antibodies away from the virion (43), however these observation were not confirmed in in vivo studies in mice, and the relevance of this finding for HRSV infection in humans is not known. The G protein is characterized by a high degree of glycosylation, both of the N- and O-type (44). G is the protein with the highest degree of variability among isolates: only 53% of homology is present between subgroups A and B. Also within the same subgroup a reasonable amount of variability can be found: 20% between HRSV A isolates and 9% between isolates of subgroup B (45). One central region in the G protein, comprising residues 164 to 176 however is highly conserved. This region overlaps with a region that contains 4 cysteine residues which form disulfide bonds, resulting in a cysteine noose (46). The most downstream pair of cysteines forms a CX3C motif, which resembles the CX3C motif in the chemokine fractalkine (47). Binding of fractalkine with its receptor CXC3R1, expressed on leukocytes, results in activation of the leukocytes and trafficking to the lung. Interaction of the G protein CX3C motif with this receptor
interferes with CX3CR1 receptor signaling, resulting in less trafficking of leukocytes to the lungs (48). In this way the G protein can interfere with the antiviral T cell response. The G protein functions as the attachment protein for HRSV since G protein specific antibodies can inhibit attachment of virions in vitro, whereas F protein specific antibodies inhibit fusion but not attachment (49). (See below – replication cycle). Together with the F protein, the G protein is the major antigenic target to which antibodies are directed. Remarkably, G protein specific antibodies are less protective and have less neutralizing activity than antibodies targeting the F protein (50).

**The fusion protein (F).** The F protein, the third membrane protein, is present at the viral outer surface as spikes protruding the membrane. F is a type I fusion protein that mediates fusion of the viral membrane with the cell membrane, resulting in the release of the viral nucleocapsid in the cytoplasm. It is also responsible for fusion of an infected cell with a neighboring cell, the so called syncytium formation (51). The F protein is present as homotrimers. Activation of the F protein trimer requires cleavage of the inactive F0 precursor by a host furin-like protease to yield the F1 and F2 chains, which remain linked by disulfide bridges (Figure 1.3). Cleavage occurs at two different sites, hereby releasing a 27 amino acid peptide (p27) of which the function is unknown (52). The F1 polypeptide contains the fusion peptide, followed by a heptad repeat region (HR-A), a globular domain, HR-B, a transmembrane domain and a C-terminus. Together with the G protein, the F protein is the major target of antibodies. Five major antigenic regions have been located in the fusion protein (antigenic site I, II, IV, V and VI) by identifying amino acids that were altered in antibody escape mutants (53, 54). Antigenic site III was mentioned in early studies (55), however in later studies this site was no longer mentioned as an antigenic region in the fusion protein (54). Sites II and IV are the best characterized. Antigenic site II (also called site A) contains amino acids 255 to 275 and is the recognition site for palivizumab (55), a humanized monoclonal antibody used prophylactically against HRSV. Antigenic site IV (also known as site C) includes residues 422 to 438 (53, 55) and is the target site for monoclonal antibodies such as 101F (56) and MAb 19 (55).

![Figure 1.3 Schematic representation of the HRSV fusion protein. Arrows indicate the furin cleavage sites. The peptide p27 is released after cleavage. The F1 and F2 fragments which are produced as a result of furin cleavage are indicated. CT, cytoplasmic tail; SP, signal peptide; TM, transmembrane region. HR-A and HR-B, heptad repeat region A and B.](image-url)
1.3 The HRSV replication cycle

Attachment. For a long time it was thought that attachment occurred through interaction of the G protein with glycosaminoglycans (GAG) (57). The exact cellular receptor though was unknown. Although the G protein has been shown to be important for replication in vivo, it was not essential for replication in vitro: mutants lacking the G protein are viable in cell culture (58-61). Recently however nucleolin was proposed as the cellular receptor for HRSV, through interactions with the fusion protein (62). The results suggest that a more complex process of attachment occurs, in which both the G and the F protein are involved in two independent binding events to both GAGs and nucleolin. The exact mechanism of attachment still needs to be unraveled.

Fusion. Fusion of the viral membrane with the cellular membrane is carried out by the fusion protein in a process similar to other Paramyxoviridae (Figure 1.4). Upon attachment, the metastable fusion protein is triggered to undergo a series of conformational changes, leading to the formation of a stable post-fusion conformation (Figure 1.5) (63, 64). The prefusion form of the F protein is lollipop-shaped with a stalk consisting of a coiled-coil of HR-B and a globular head in which HR-A is packed. During the conformational changes, the fusion peptide (located adjacent to HR-A) is inserted in the cellular membrane. The C-terminal coiled-coil of HR-B then dissociates, wraps around the globular head and joins the HR-A helices, to form the post-fusion six-helix bundle. These rearrangements brings the viral and cellular membrane into proximity, ultimately leading to fusion of both membranes (Figure 1.4) and release of the viral nucleocapsid into the cytoplasm. The remainder of the viral replication cycle takes place in the cytoplasm. Interestingly, the major antigenic sites II and IV, which are present in the pre-fusion form are conserved in the post-fusion conformation (Figure 1.5).

Figure 1.4  Model of F-mediated membrane fusion. (A) The prefusion form of the F protein is lollipop-shaped with a stalk consisting of a coiled-coil of HR-B (blue) and a globular head in which HR-A (green) is packed. (B) During the conformational changes, the fusion peptide (located adjacent to HR-A) is inserted in the cellular membrane and the C-terminal coiled-coil of HR-B dissociates. (C) Structural rearrangements bring the cellular and viral membranes into proximity. (D) After fusion, the F protein adopts the post-fusion conformation consisting of a six-helix bundle made up of the HR-A and HR-B helices. Adapted from (63).
Transcription and translation. Transcription is carried out by the viral L protein, with the help of the co-factors P, N and M2-1 (reviewed in (3)). The viral genome contains a single polymerase entry site at its 3’ end. Between every pair of consecutive genes, conserved gene-end and gene-start sequences are present to guide the viral polymerase during the process of transcription. The gene-end sequence contains several U residues, which leads to polyadenylation of the mRNA transcript. Upon completion of the transcript, the mRNA is released. At this point, half of the polymerases detach from the template strand, which can then only re-initiate transcription at the polymerase entry site, at the 3’ end of the template strand. Only 50% of the polymerases continue along the strand for transcription of the more downstream genes. As a consequence, a gradient in mRNA transcripts exist, with mRNA of the 3’ end genes being more abundantly present than those at the 5’ end (66). An important co-factor in this process of sequential transcription is the M2-1 protein, which functions as a transcription elongation factor. Its absence results in preterm dissociation of the polymerase from the RNA template, leading to incomplete mRNAs and almost complete absence of the more downstream mRNAs (30, 31). Transcription in the presence of M2-1 leads to the production of 10 distinct mRNAs (67). Each of these mRNAs is translated into one protein, except for the M2
mRNA which encodes the M2-1 and M2-2 proteins from overlapping ORFs through a process of re-initiation of translation (68, 69). The HRSV genome contains one exception to the transcription process described above: the transcriptional start sequence of the L gene is 68 nucleotides more upstream than the transcriptional stop sequence of the M2 gene. Studies with mini-replicons have shown that after transcription of the M2 gene the viral polymerase scans the viral RNA in both the upstream and downstream direction to locate the L gene start. This scanning mechanism is thought to be a general process that occurs at each gene junction during sequential transcription (70, 71).

**Replication.** Replication of the viral genome occurs through a full length, positive-sense RNA intermediate. To achieve this, the viral polymerase should “ignore” the gene-end and gene-start signals, resulting in 1 complete RNA strand. This positive-sense RNA strand subsequently serves as a template for the production of a full length, negative sense viral genomic RNA (13). The M2-2 protein is thought to regulate the switch from RNA transcription to RNA replication. Recombinant HRSV lacking the M2-2 protein show a decrease in RNA replication and an increase in viral mRNA synthesis (32). It was suggested that this process is dependent on the intracellular concentration of the M2-2 protein: early in the infection when M2-2 concentration is still low viral mRNA synthesis predominates whereas at later time points during the infection, when the concentration of M2-2 gradually rises, RNA replication is favored, in preparation for viral assembly and budding (32).

**Packing and assembly.** The last step in the viral replication cycle is the packing of the viral genome and proteins near the cell membrane and budding of progeny viruses. Assembly and budding occur at the apical side of polarized cells in filamentous structures (72, 73). The exact mechanism through which assembly of progeny virus occurs is not yet completely understood, however, it is clear that the M and F proteins play an important role in this process. The M protein associates tightly at lipid raft structures in the membrane, only when the viral glycoproteins are present (29). This might be an effect of the F protein cytoplasmic tail, since mutant viruses with a mutation in the F cytoplasmic tail were unable to attract the viral RNP to the site of assembly (74).

### 1.4 The immune response to HRSV infection

Airway epithelial cells form the main target for HRSV infection in the lungs. Once infected, the virus is sensed by these cells and an immune response is started. The immune response to HRSV is complex and the viral proteins are capable of interfering with this response at several levels. The immune response can be roughly divided into an innate immune response, which is a more general defense mechanism independent of the pathogen and an adaptive immune response, which starts off later, however is specific for the pathogen.
The innate immune response

The virus is sensed by the immune system through pattern recognition receptors (PRR), which detect a variety of pathogen associated molecular patterns (PAMP). To date, three classes of PRRs have been identified, including toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptor (NLRs) (75, 76) and HRSV can be recognized by members of all three classes. Binding of HRSV to these PAMPs leads to signaling through NF-κB, mitogen-activated protein kinases (MAPKs) and/or members of the interferon regulatory factor (IRF) family, resulting in the expression of inflammatory cytokines, chemokines and interferons, which activate and/or recruit innate, as well as adaptive, immune cells (77). TLRs are expressed on a wide variety of cells, including eosinophils, neutrophils, dendritic cells (DC), macrophages and epithelial cells. Several TLRs have been shown to be involved in the immune response to HRSV, including TLR2 (78), TLR3 (79), TLR4 (80, 81) and TLR7 (82). While TLR2 and TLR4 are found on the cell membrane, TLR3 and TLR7 are present in intracellular vesicles, such as endosomes.

TLR4 recognizes the HRSV F protein using a CD14 co-receptor. Binding of the F protein by TLR4 leads to NF-κB-mediated secretion of the pro-inflammatory cytokines interleukin (IL)-6 and IL-8 and the regulatory cytokine IL-10 (83). TLR4 engagement by the F protein enhances the expression of TLR4 on epithelial cells (84). Two single nucleotide polymorphism (SNPs) in the TLR4 ectodomain have been associated with an increased risk for severe bronchiolitis and hospitalization in infants (85, 86), suggesting a TLR4 response is necessary for inducing protective immunity to HRSV (87). A second extracellular TLR which reacts to HRSV infections is TLR2. This TLR forms a heterodimer complex with TLR6 (78). Signaling through TLR2/6 in leukocytes results in the production of CCL5, IL-6, and CCL2, neutrophil migration and DC activation.

TLR3 is an intracellular PRR that recognizes double stranded dsRNA. These dsRNA molecules are intermediates of the HRSV replication cycle. The TLR3-mediated response channeled through TRIF (toll-interleukin (IL)-1 receptor domain-containing adaptor) activates both NF-κB and IRF-3, and subsequently drives production of IFN-β, CXCL10, CCL12, and CCL5 (79, 88). By a yet unknown mechanism, the HRSV G protein has been shown to inhibit TLR3/4 mediated cytokine production, through interfering with TRIF signaling (89). Activation through TLR3 leads to a predominantly Th1 response; deletion of TLR3 leads to a Th2 response including the production of IL-5 and IL-13 (90).

TLR7 is present on endosomal membranes and detects single stranded RNA (ssRNA) (91). Plasmacytoid DCs (pDCs) are the major cell type expressing TLR7. Signaling of TLR7 through the MyD88 adaptor protein leads to activation of IRF7 and production of IFN-α (92). TLR7 is responsible for the balance between IL-12 and IL-23 production, which determines the type of immune response induced upon HRSV infection: absence of TLR7 results in enhanced IL-23 production (a Th17 promoting cytokine) and reduced IL-12 production (a Th1 cytokine) (82) resulting in enhancement of HRSV-induced pathology.

RLRs are cytoplasmic viral sensors, present in most cell types including macrophage and conventional DCs (cDCs). MDA-5 and RIG-I, two members of the RLR family can sense HRSV infection, which
activates NF-κB and IRF3 pathways by complexing with the adaptor MAVS (93). RIG-I is activated by 5’ triphosphate structure of viral RNA, both single or double strand (94). Scagnolari et al. (95) reported that the relative gene expression levels of MDA-5 and RIG-I were significantly higher in the HRSV-infected infants than virus-negative infants. HRSV induced RIG-I activation leads to the production of IFN-β, IP-10, and CCL5 expression in the airway epithelial cells (96). The HRSV NS1 and NS2 proteins interfere with RIG-I induced IFN-β production by decreasing the interaction between RIG-I and MAVS (97).

NLRs are a second type of cytoplasmic PRR. NOD2 was well-known, as a sensor for peptidoglycan structures of intracellular bacteria (98) but recent evidence has shown that it can also detect ssRNA. NOD2 activated by HRSV ssRNA translocate to the mitochondria where it interacts with MAVS to induce activation of both IRF3 and NF-κB (99). HRSV infection of NOD2 knock-out mice results in markedly higher body weight loss and severe lung pathology with higher concentrations of pro-inflammatory cytokines, as well as chemokines, than wild-type counterparts. These results demonstrate that, like the RLR receptors, NOD2 can function as a cytoplasmic PRR for HRSV and is important for host defense against HRSV infection.

Activation of PRRs in the epithelial cells or alveolar macrophages induces changes in the cellular expression of genes encoding for a variety of factors, including surfactants, cytokines, chemokines, and cell surface molecules. *In vitro* studies have shown that HRSV infection leads to the secretion of cytokines and chemokines such as RANTES/CCL5, macrophage chemotactant protein 1 (MCP-1), eotaxin-1/CCL11, IL-9, TNF-α, IL-6, IL-1 and CX3CL1 (fractalkine) (100). In HRSV infected patients increased levels of macrophage inflammatory protein (MIP)-1α, RANTES and IL-8 are often found in the upper and lower respiratory tract (101). Chemokines identified in the respiratory tract secretions of children who experience an HRSV infection include CCL3, MIP-1α, CCL2, MCP-1, CCL11 and CCL5. Levels of these chemokines correlate with the severity of HRSV-induced illness, presumably by initiating an augmented inflammatory response to HRSV (102). Chemokine expression by HRSV infected epithelial cells promotes activation of several types of innate immune cells (including neutrophils, eosinophils, NK-cells and monocytes) and their recruitment from the blood to the infected tissue. NK-cells are important effector cells in viral clearance by orchestrating cytotoxic lysis of infected airway cells. Another major function of activated NK-cells is the production of early IFN-γ which primes the subsequent antiviral adaptive Th1-cell and cytotoxic T-lymphocyte (CTL) immune response (13). The chemokines CCL5, CCL11 and MIP-1α have been shown to be involved in lung histopathology, since blocking of these chemokines in mice resulted in reduced lung histopathology (103-105).

Next to chemokine production, sensing of HRSV through the PRRs also induces the production of type I IFNs, of which IFN-α and IFN-β are key members, as well as IFN-λ1, IFN-λ2, and IFN-λ3 (106). These cytokines signal through the IFNAR receptor, which activates the Jak/STAT signaling pathway resulting in the upregulation of interferon-stimulated genes (ISG). Expression of these genes triggers anti-viral functions such as the activation of ribonuclease L which degrades host RNA (107). pDCs are the main producers of IFN-α. In addition, epithelial cells are important producers of type I IFNs (108). Interestingly, the HRSV NS1 and NS2 proteins are involved in impairing IFN secretion by epithelial
cells which inhibits the establishment of an anti-viral state and allows viral replication within infected tissues (106, 109).

Surfactant protein (SP)-A and SP-D may also play an important role in the immune response to HRSV infections. These proteins opsonize the pathogen, thereby promoting its uptake by macrophages and neutrophils (110). In addition, gene polymorphisms in these proteins have been associated with the severity of HRSV-induced disease in infected infants (111, 112).

The adaptive immune response

Activation of DCs leads to the initiation of the adaptive immune response, by presenting pathogen derived peptides to T cells (discussed in detail in chapter 2). The adaptive immune response can be divided in a humoral immune response and a cell-mediated immune response.

While the CTL response is essential for viral clearance (discussed in detail in chapter 2), HRSV-specific antibodies are mainly important in restricting replication and disease upon reinfection with HRSV (113). A linear correlation has been found between the ability of HRSV to infect and the level of HRSV specific serum antibodies in experimentally infected humans (114). HRSV infection induces antibodies to multiple viral proteins; however only F- or G-protein specific antibodies can neutralize the virus and contribute to protection (115, 116). HRSV infection induces both serum IgG and mucosal secretory IgA production. Secretory IgA is mostly important in protecting the URT which cannot easily be accessed by serum IgG antibodies. Although secretory IgA antibodies are short-lived, repeated HRSV infections have been shown to induce a sustained IgA response which can protect against HRSV infection independently of IgG antibodies. Serum IgG antibodies can more efficiently access the LRT and hence are important in providing protection in this compartment (100).

HRSV disease pathology is clinically characterized by airway hyperreactivity (AHR), increased mucus production and inflammation. This is thought to be caused by an altered immune environment due to an imbalance in the CD4⁺ Th1 and Th2 response. Indeed, severe acute HRSV infections are characterized by a Th2 skewed immune response (254). Recently it was shown that IL-17 producing Th17 cells play an important role in the pathogenesis of HRSV-induced disease. Increased IL-17 levels were observed in tracheal aspirate samples from children hospitalized with severe HRSV-induced disease. In addition, neutralization of IL-17 after an HRSV infection in mice led to reduced lung viral load, reduced mucus production and increased CD8+ T cell levels (255). These findings demonstrate that IL-17 plays an important role in the pathogenesis of HRSV infections. This was an interesting result, since IL-17 production has recently been implicated in the development of severe forms of asthma (256). Which again links severe HRSV infections with asthma.

Regulatory T cells (Treg; CD4⁺ Foxp3⁺) play an important role in regulating the adaptive immune response upon an HRSV infection (257). Upon HRSV infection Tregs proliferate and rapidly accumulate in the lung-draining mediastinal lymph nodes and lungs. Interestingly, in vivo
depletion of Tregs in mice prior to HRSV infection results in delayed virus clearance characterized by an early lag in the recruitment of HRSV-specific CD8+ T cells into the lungs (258). Additionally, Treg depletion results in exacerbated disease severity, including increased weight loss, morbidity, and enhanced airway restriction, illustrating the importance of this cell type in regulating the adaptive immune response.

IL-10 is an important regulatory cytokine produced by Foxp3+ Tregs, although also conventional Foxp3− CD4+ T cells have been shown to produce this cytokine (259). Protein levels of IL-10 in the lung increase following acute HRSV infection, with maximum production corresponding to the peak of the virus-specific T cell response. HRSV infection of IL-10-deficient mice results in more severe disease compared with wild type mice and an increase in the magnitude of the HRSV-induced CD8+ and CD4+ T cells (260). In addition, blocking of the IL-10 receptor during an HRSV infection alters the activate T cell subsets, resulting in more IL-17 producing CD4+ T cells and a decrease in the number of Foxp3+ Tregs. These results demonstrate the importance of IL-10 in modulating the adaptive immune response to HRSV infections.

1.5 Animal models for HRSV vaccine studies.

For evaluating new vaccines or antiviral strategies, an in vivo model is indispensable. Even though humans are the only known natural host of HRSV, several animal models are susceptible to HRSV replication, such as mice, cotton rats, lambs, ferrets, guinea pigs and several types of non-human primates. However, no model can completely mimic the disease observed in humans. The disease features, such as viral replication, pulmonary histopathology and immune response, differ between the different models and therefore, the choice of the model should depend on the hypothesis under investigation.

Adult mice.

Mice have the advantage over other animal models that multiple inbred strains are available, allowing studies in a homogenous background. Prince et al. (117) first described HRSV infection in inbred mice in 1979. Several mice strains were tested for their susceptibility to HRSV growth in the nose and lungs. BALB/c mice are the most frequently used inbred strain for HRSV studies, even though DBA/2N mice were most susceptible for HRSV growth both in the lungs and in the nose (117). Mice are semi-permissive for HRSV infection. In BALB/c mice, a high intranasal inoculum, in the range of 10^5 to 10^7 PFU is necessary for evoking LRT disease (118). This high viral load, instilled directly into the airway does not resemble a natural infection in humans, which originates from a small viral inoculum (119). In humans, the virus is first amplified by a couple of rounds of replication in the nose,
before progressing to the lower airways. In contrast, due to the high inoculum, in mice the infection proceeds immediately to the LRT. This difference in the progression of the viral infection may have consequences for the induction of immune responses, which should be taken into account when using the mouse as animal model. The HRSV strain used for infecting mice should also be taken into account. HRSV-A2 and Long strain are the most commonly used laboratory strains, however the clinical isolate Line-19 has been shown to induce substantial mucus production associated with airway hyperreactivity upon infection of mice (120). This Line-19 strain infection in mice is therefore a useful model for studying HRSV-induced pulmonary pathophysiology.

**Neonatal mice.**

Recently, the neonatal mouse has been introduced as a new model to study HRSV infections. Neonatal mice (aged ≤ 7 days) infected with HRSV develop long-term asthma-like symptoms such as increased airway hypersensitivity, extensive mucus production, Th2 cytokine and cellular responses and airway remodeling (121). A secondary HRSV infection in these mice causes severe lung immunopathology, which is not observed when the primary infection occurs in mice older than 7 days of age. Data from these mouse models closely resemble the data from human epidemiological studies (i.e., that the age of initial infection is the major determinant in the persistence of lung dysfunction into early adulthood) (122).

**Cotton rats.**

The cotton rat (*Sigmodon hispidus*) is an interesting model for HRSV because it is highly susceptible to HRSV infection and permissive to HRSV replication. The cotton rat is at least 100-fold more susceptible to HRSV than the mouse (123). HRSV can be detected in both the upper and lower respiratory tract of infected cotton rats at 2 days post infection. The viral lung titer peaks around the 5th day of infection and usually becomes undetectable by the 8th day of infection. HRSV infection in cotton rats induces symptoms similar to those observed in humans, such as proliferative rhinitis, and bronchiolitis (124, 125). Cotton rats have been extensively used to study the development of enhanced disease upon formalin-inactivated RSV (FI-RSV) vaccination (see below). Vaccinated cotton rats develop severe pulmonary inflammation as observed in FI-RSV vaccinated human infants (124). A major disadvantage of the cotton rat model is the limited number of immunological reagents available for these animals.

**Perinatal lambs.**

Perinatal lambs, i.e. both term and preterm born lambs, are an interesting model for infant HRSV disease, mainly because the lamb pulmonary structure closely resembles that of humans. Both term and preterm lambs are susceptible to HRSV infection and develop both URT and LRT disease, with symptoms such as coughing, fever, malaise, bronchiolitis, neutrophil infiltration and syncytium formation (126, 127). The disease is less severe in term born than in preterm born lambs. HRSV readily replicates in the airway epithelium of lambs with peak viral lung titers around day 6 after
infection. On this day, a 30-fold increase in viral RNA levels has been observed, compared to day 3 after infection (126, 127). The larger size of lamb lungs compared to mice lungs makes them a more suitable model for studying lung-function following HRSV infection. In addition, since lamb lung development is similar to infant lung development, this model is interesting to study the importance of the age upon primary HRSV infection (128). As with cotton rats, an important disadvantage of lambs as animal model is the limited availability of immunological reagents. In addition, handling and housing of larger animals is more complex than small animal models (129).

**Non-human primate models for HRSV.**

Because of their genetic and anatomical relatedness to humans, the non-human primates are an interesting model for studying HRSV infections and vaccines. HRSV was first isolated from a chimpanzee, before it was recognized as a human pathogen. Chimpanzees permit HRSV replication with modest increases (approximately 1 Log_{10} increase) of viral titers observed in the nasal or tracheal secretions (130). Upon intranasal HRSV infection chimpanzees develop URT disease symptoms similar to that observed in humans, with symptoms such as rhinorrhea, sneezing and coughing (131). No LRT disease has been reported in chimpanzees. HRSV replication has been demonstrated in several other non-human primates, such as African green monkeys, Bonnet monkeys, owl monkey, rhesus monkey, cebus monkey and squirrel monkey (131-134). However, despite their genetic relatedness to humans, all these animals are only semi-permissive to HRSV (119) and do not develop clinical symptoms. The African green monkey has been most extensively studied. Upon an intranasal and intratracheal infection titers of $10^5$ and $10^3$ PFU per ml can be recovered from the lung and nose respectively (135, 136).

**Human challenge model.**

Experimental infection of healthy human volunteers can be used to study the pathogenesis of HRSV and to evaluate therapeutics or vaccine candidates. This model replicates features of a natural HRSV infection in humans, such as, small inoculum dose, inoculation in the nose (instead of directly in the lungs as in the mouse model), an incubation period and URT symptomatic disease. A limitation of this model is that the study subjects are not HRSV-naïve, since HRSV is ubiquitously present and every adult has been infected at least once, but likely multiple times. Therefore, the URT disease observed might be a consequence of the present neutralizing antibodies, and hence this model will not completely mimic the course of an HRSV infection in HRSV-naïve infants (114, 137). The human challenge model is an important model which can be used for testing new antiviral therapeutics or vaccines.

**Models using non-human pneumoviruses.**

BRSV, a relative of HRSV belonging to the same genus, is a natural pathogen of calves. The disease observed in cattle is almost identical to that observed in humans, varying from mild URT infections to
severe LRT infections with bronchiolitis and pneumonia. This model is of particular interest because it can mimic the FI-RSV induced enhanced disease that was observed in the human clinical trials. Compared to unvaccinated controls, calves vaccinated with FI-RSV vaccine develop significantly greater disease upon a BRSV infection with a Th2 polarized immune response characterized by increased levels of IL-4 and IL-5, decreased levels of IFN-γ and a lack of BRSV specific CD8\(^+\) T cells (138-140). Similarly, Pneumovirus of mice (PVM) is a natural rodent pathogen related to HRSV. The virus induces severe inflammation which, depending on the size of the inoculum may advance to bronchiolitis, pneumonia or even death. The advantage of using this model is that the virus is studied in its natural host, allowing robust replication: an infection with less than 100 PFU results in viral titers peaking at day 7 post infection at \(1 \times 10^8\) PFU/g lung (141).

Studying these viruses in their natural host more closely reflects the pathogenesis of a natural infection and has advanced our understanding of pneumovirus pathogenesis in general. These models are useful to evaluate vaccine concepts which may be applied to HRSV.

1.6 A history of vaccine failures

**Formalin inactivated HRSV vaccine**

When HRSV was discovered, in 1956, vaccination against poliomyelitis was introduced all over the world. One of the polio vaccines had been developed by Dr. Jonas Salk, and consisted of formaldehyde inactivated polio virus (142), a strategy that had previously also been applied to influenza virus in the 1940's when the first influenza vaccines were used clinically. Given the success of this vaccine in inhibiting poliomyelitis the same strategy was applied to other viral infections, such as HRSV. This resulted in the development of the first HRSV vaccine in 1965 (143). The vaccine consisted of formalin-inactivated, in vitro grown HRSV that was concentrated with alum adjuvant for intramuscular administration (FI-RSV). From 1965 to 1967 several clinical trials were performed with the FI-RSV vaccine, in children of various age (144-147). All trials however had the same negative outcome that the vaccine was not strongly immunogenic and poorly protective against a natural HRSV infection. FI-RSV recipients experienced enhanced disease during a subsequent natural HRSV infection and developed symptoms such as bronchiolitis and pneumonia; whereas control vaccinated children mostly developed mild symptoms like rhinitis. In one of the studies up to 80% of the vaccinated children required hospitalization versus 5% in the control group (144). Moreover, two of the vaccinated children in this study died during a subsequent HRSV infection. Histopathological analysis of the lungs of the deceased children demonstrated a strong infiltration of cells in the small bronchioles and the presence of numerous eosinophils (144). Analysis of the induced immune responses revealed that compared to control vaccinated children, FI-RSV vaccinated children
developed a completely different immune response upon a subsequent HRSV infection, including poor induction of neutralizing antibodies (148) and an exaggerated CD4+ T cell response of Th2 subset (149). Later, these results were confirmed by animal models mimicking the effects of Fl-RSV vaccination (reviewed in (150)). Mouse studies confirmed that Th2 type cytokines played an important role and that the induction of enhanced disease could be abrogated by depletion of CD4+ T cells or the Th2 cytokines IL-4 and IL-10 (151, 152). Additionally, the poor stimulation of CD8+ T cells and as a consequence the lack of an appropriate CD8+ T cell memory also contributed to the Th2 biased immune response (153). The lack of neutralizing antibodies is likely a consequence of denaturation of the antigen by the formaldehyde as well as a deficiency in affinity maturation. (154, 155).

As a consequence of the enhanced disease observed upon Fl-RSV vaccination and due to the fact that immunity induced upon a natural HRSV infection is not long lasting, the development of an HRSV vaccine has become a slow and difficult process. Requirements for an HRSV vaccine that is both safe and effective have been proposed. It is suggested that, (i) the induction of a neutralizing antibody response is required for efficacy of the vaccine and (ii) induction of the right type of T cell response (i.e. induction of CD8+ T cells and avoiding Th2 responses) is required for safety of the vaccine (119).

**Live attenuated HRSV vaccines**

After these fatal results with inactivated vaccines, HRSV vaccine development has focused on live attenuated vaccines, since these vaccines induce an immune response that more closely resembles the immune response induced upon a natural HRSV infection (156, 157). Intranasal administration of a live attenuated vaccine has the advantage of being capable of stimulating both a local and a systemic immune response. Additionally, intranasal administration can partly overcome the immunosuppressive effects of maternal antibodies (130). The first generation of live attenuated HRSV vaccines were produced already in the 1960s by serial passages at a suboptimal temperature (cold-passage; cp) (158, 159). Fifty two passages at low temperature (as low as 26°C) resulted in the cp-RSV strain, which, due to the lack of a good animal model for HRSV at that time, was immediately tested in humans (158). The strain was highly attenuated in adults however, for children it was not sufficiently attenuated and symptoms of respiratory tract illness occurred in seronegative children (160), and therefore was not an acceptable HRSV vaccine. A different strategy for generating live attenuated viruses consisted of passaging the virus in the presence of mutagens, followed by identification of mutants with a temperature sensitive (ts) phenotype (160, 161). The first attenuated virus generated with this method was clearly attenuated in adults, however loss of its temperature sensitivity was observed in seronegative children (160, 161). Therefore, a second ts strain (ts-2) was produced which was more stable than ts-1. This ts-2 strain was highly attenuated in primates, however it was poorly infectious and only weakly immunogenic in adults and children (162). A problem which makes the search for an acceptable live attenuated vaccines difficult, is that only a
small window of attenuation exist, often resulting in either under attenuated (cp-RSV and ts-1) or over attenuated (ts-2). Loss of stability is another problem for which solutions need to be found. The cp-RSV mutant was subsequently subjected to chemical mutagenesis to introduce ts mutations. This resulted in several mutants, named cpts530 and cpts248, which were attenuated in mice and chimpanzees and which were genetically more stable than previously generated live attenuated strains (163). These mutants were subjected to a second round of chemical mutagenesis, to introduce additional temperature sensitive mutations. Several candidates were generated (cpts530/1009, cpts 248/955, cpts 248/404) which were much more restricted in replication in the nasopharynx of mice and chimpanzees and which retained their temperature sensitive phenotype in both animal models (164, 165). The cpts248/955 and 530/1009 vaccines were highly attenuated in adults and seropositive children. In seronegative children the cpts248/955 vaccine was not sufficiently attenuated (166). One candidate, cpts248/404 was shown to be protective in chimpanzees (165). This vaccine candidate, after the failed Fi-RSV vaccine, that was the first that was considered safe enough to be tested in seronegative children less than 2 months of age. The cpts248/404 vaccine was immunogenic and was protective against a second dose of the vaccine strain. However a mild nasal congestion was observed in these young children, rendering it unacceptable for use as a vaccine in the most important target group of HRSV (167).

Thanks to the development of reverse genetics for HRSV, the so called ‘second generation’ of live attenuated viruses could be generated more easily by introducing specific mutations into wild type HRSV or by deletion of (fragments of) genes. By introducing the 5 attenuating mutations that were present in the cp-RSV mutant into wild type HRSV, the recombinant rA2cp was generated, which is used as a basis for other strains (168). Attenuating mutations of strains generated by chemical mutagenesis were determined and could be combined to form new combinations of mutations. This resulted in the generation of a new strain designated rA2cp248/404/1030ΔSH, of which it was suggested that the combined effect of several attenuating elements might result in a strain that is more attenuated in humans than the parent cpts248/404 strain (169). The rA2cp248/404/1030ΔSH strain contains a total of 5 different attenuating elements: (I) cp which is based on 5 missense mutations in the N and L proteins and the F glycoprotein that together confer the phenotype of cp-RSV and that are considered to be a single attenuating genetic element (168); (II) ts248, a missense mutation in the L protein (163); (III) ts404, a nucleotide substitution in the gene-start transcription signal of the M2 gene, derived from the mutant cpts248/404 (165, 170); (IV) ts1030, another missense mutation in the L protein, derived from the mutant cpts530/1030 (169) and (V) ΔSH, complete deletion of the SH gene (34). The rA2cp248/404/1030ΔSH strain was evaluated for its safety, immunogenicity and genetic stability in adults and children (171). The vaccine was found to be highly attenuated and immunogenic in adults, seropositive children and seronegative children older than 6 months. This vaccine was the second live attenuated strain found safe enough to be tested in seronegative infants of 1-2 months old. The vaccine was well tolerated and a single dose provided substantial restriction of a second dose, indicative of a certain amount of protection. However only 44% of the infants had a detectable antibody response after two doses (171), whereas high antibody titers were observed in seronegative children older than 6 months. This vaccine was
recently tested extensively (under the name MEDI-559) in a phase I/IIa clinical trial (trial NCT00767416 (172)) for its immunogenicity and for its ability to provide protection against a natural HRSV infection in 5 to 24 months old children (173). The trial was finished in August 2012 but the results on immunogenicity, protection and stability of the virus have not been communicated. The virus that was recovered from children in the first small scale trial did not show any reversions to wild type HRSV, which is highly unlikely given the large number of mutations that are present in the vaccine strain. However, changes in temperature sensitive phenotype were observed in some isolates, but these mutants were not associated with disease and remained attenuated in vivo (171). Similar changes were observed in vitro, when the virus was passaged at 35°C (174). Currently studies are ongoing to improve the stability of this vaccine (175, 176), and again, questions are being raised whether this vaccine will be stable enough for use in young children.

Another strategy for generating attenuated vaccines involve the deletion of other non-essential genes such as NS1, NS2 or M2-2, either on its own or in combination with additional point mutations in other genes. Three vaccines have been created with a deletion of the NS2 gene, rA2cpΔNS2, rA2cpts248/404ΔNS2 and rA2cpts530/1009ΔNS2 (177), the last two based on observations of attenuation of the strains generated through chemical mutagenesis. Since NS2 is involved in the inhibition of the host IFN response, deletion of NS2 would be expected to increase IFN signaling and response during infection and thereby result in enhanced immunogenicity. The rA2cpΔNS2 virus was attenuated in adults and seropositive children, however, since it replicated efficiently in this last population it was not tested in seronegative children (177), as it was known from previous studies that HRSV strains that replicate efficiently in HRSV-seropositive children can retain reactogenicity for HRSV-seronegative children or infants (166). The other two ΔNS2 strains were evaluated in seronegative children. Both rA2cpts248/404ΔNS2 and rA2cpts530/1009ΔNS2 were clearly over attenuated in seronegative children, and evaluation in the target population of infants was not performed (178).

Strains with deletions of NS1 or M2-2, either separately or the combination of both have been tested in monkeys. In contrast to all previously described strains generated through reverse genetics, these strains (rA2ΔNS1, rA2ΔM2-2 (179) and rA2ΔM2-2NS2 (180)) do not contain the cp mutations which originated from the first cp-RSV strain (158). All three strains were attenuated in monkeys and it will be interesting to characterize these vaccines in humans.

**Purified subunit HRSV vaccines**

Since live attenuated vaccines induce an immune response resembling the immune response upon a natural infection, they are the best candidate for use in infants. There is however also need for a vaccine for older children, adults and elderly who are at risk of severe HRSV infection due to an underlying disease or old age. Since several live attenuated vaccine strains have been reported to be too much restricted in replication in these populations other strategies are being investigated. The most studied strategy, together with the live attenuated vaccine was the production of purified
subunit vaccines, consisting of purified HRSV proteins. Several approaches have been developed to produce subunit vaccines. A first approach consisted of F protein purified from infected cells. Three generations of these proteins were produced, designated PFP-1, PFP-2, PFP-3. The three vaccines differ in purification strategy, HRSV strain used for preparation and the adjuvant with which it is formulated (Table 1.2).

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>HRSV strain used for preparation</th>
<th>Purification strategy used</th>
<th>Adjuvant used in clinical trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFP-1</td>
<td>wt HRSV</td>
<td>Immunoaffinity column</td>
<td>Aluminum hydroxide</td>
</tr>
<tr>
<td>PFP-2</td>
<td>wt HRSV</td>
<td>Ion exchange chromatography</td>
<td>Aluminum hydroxide</td>
</tr>
<tr>
<td>PFP-3</td>
<td>cpts HRSV</td>
<td>Ion exchange chromatography</td>
<td>Aluminum phosphate</td>
</tr>
</tbody>
</table>

The purified F protein vaccines have been evaluated extensively in clinical trials in healthy adults, in institutionalized or ambulatory elderly, in pregnant women and in children older than 1 year, either healthy or who had a chronic lung disease (cystic fibrosis or chronic lung disease of prematurity) (181-189). PFP-1 and PFP-2 were well tolerated and immunogenic in healthy adults and children over 12 months of age, as well as in the elderly; however some mild upper respiratory symptoms were observed (181, 184, 186, 189). A phase I clinical trial performed in pregnant women, showed that vaccination with purified F protein vaccines can induce protective antibodies in the infant, by vaccinating the mother during pregnancy. Women were vaccinated with PFP-2 between the 30th and 34th week of pregnancy (185). The vaccine was well tolerated and immunogenic and during the subsequent HRSV season no increase in frequency or morbidity due to respiratory disease was observed in children from immunized women. Mean anti-HRSV antibody titers were increased 4 fold in children born from immunized mothers compared to children from unimmunized mothers. An increase in HRSV neutralizing antibody titer was reported in only 10% of the women and their infants at birth, and at 2 months and 6 months of age (185). PFP-3 was evaluated in children with cystic fibrosis. The vaccine was safe, well tolerated and immunogenic in this group. A fourfold rise in HRSV antibody titer was observed in 67% of the vaccinated children, however, no significant reduction in the number of lower respiratory infections between the vaccine or placebo recipients could be demonstrated (187). Since no significant protective effect could be attributed to the PFP-3 vaccine, the development of these HRSV PFP vaccines is no longer continued.

A comparable approach, using co-purified F, G and M protein was evaluated in elderly > 65 years of age. Several doses were tested, with or without alum as adjuvant. Surprisingly only the highest dose (100 µg of vaccine) given without alum adjuvant, induced a fourfold rise in antibody titer in 58% of the vaccines (190). Further development of this vaccine has also been suspended (13).

Another subunit vaccine, called BBG2Na, is a fusion protein expressed in bacteria that consists of aa 130-230 of the G protein, which contains the central conserved region, fused to the albumin binding
domain of streptococcal protein G. This vaccine induced protection against HRSV-A and –B in both mice and cotton rats (191, 192) without enhanced lung pathology (193). Evaluation of the BBG2Na vaccine in a phase I study in healthy adult volunteers proved the vaccine was safe, well tolerated however only mildly immunogenic (194). When BBG2Na absorbed with alum was tested in young macaques, there was evidence of an enhanced Th2 response, with IL-13 producing T cells and a certain degree of pulmonary eosinophilia (132). Due to adverse events, both in humans and macaques, de vaccine was not further developed.

New technological developments might help the search for a good subunit vaccine. Recently, a post fusion form of the HRSV F protein was produced, which forms stable trimers. This post-fusion form of the F protein still contains (at least some of) the known antigenic regions, as it is recognized by several known monoclonal antibodies (195). Moreover, in rodents it induces neutralizing antibodies that are protective against an HRSV infection (65). More research needs to be done, but until now these post fusion F trimers seem a promising new vaccine candidate.

Recently, the crystal structure of the binding site of motavizumab/palivizumab (196) and MAb 101F (197) on the F protein have been solved. This new knowledge could be the basis for a new emerging vaccine strategy. By building key epitope residues on protein scaffolds, minimalistic subunit vaccines can be produced that only contain the neutralizing epitope. The usefulness of this vaccine strategy has been explored in an HIV vaccine (198).

In order to elicit an optimal immune response, purified subunit vaccines require co-administration of an adjuvant. The adjuvant should be selected carefully, in order to avoid induction of an undesired Th2 type immune response. Several studies have been performed for testing different adjuvants in animal models.

Oien and colleagues compared the use of cholera toxin B subunit (CTB) for intranasal vaccination with alum as a parenteral vaccine adjuvant, in combination with a chimeric F/G glycoprotein vaccine in mice. While both vaccination protocols induced serum antibodies against RSV and protected the lower respiratory tract from RSV infection, only intranasal FG/CTB afforded protection of the upper respiratory tract (248). A purified F protein vaccine in combination with an adjuvant formulation containing caprylic/capric glycerides (CCG) and polyoxyethylene-20-sorbitan monolaurate (PS) was tested as intranasal vaccine in BALB/c mice. This vaccine formulation unduced IgG1, IgG2b and IgA antibodies which was protective against an RSV challenge (249). The BBG2Na vaccine was tested in an adjuvant formulation with dimethyldioctadecylammonium bromide (DDA). This vaccine/adjuvant combination was shown to induce a mixed Th1/Th2 immune response that was protective in both mice and cotton rats (250).

Another interesting promising category of adjuvants consists of synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides (CpG-ODN). These compounds have been reported to induce a Th1 type immune response (251). Compared with immunization with natural F protein adsorbed to aluminum hydroxide (F/AIHO) adjuvant alone, co-administration of F/AIHO with CpG-ODN resulted in an increase in serum IFNγ and anti-F IgG2a titers, while pulmonary IL-5 levels significantly decreased, indicating that the CpG-ODN adjuvant directs the immune response in a Th1
direction (252). In addition, administration of CpG during FI-RSV immunization has been shown to reduce disease upon a subsequent RSV infection by inducing a more Th1 type immune response (253). In addition, Delgado et al. showed that inactivated RSV in combination with a TLR agonist treatment (Poly I:C, LPS and polyU) could protect mice from disease exacerbation by RSV (155). These interesting observation show that the selection of the adjuvant is critical for the outcome of the effects of FI-RSV induced disease exacerbation.

**Live viral vectors as HRSV vaccine**

Several other strategies for the development of an HRSV vaccine have been studied. Most of them have only been evaluated in preclinical studies in mice, cotton rats and/or chimpanzees. Different live viral vectors, including vaccinia virus (199), parainfluenza virus (PIV) (200), sendai virus (201), adenovirus (202-204) and Newcastle disease virus (205), have been tested in animal models. Most of them are immunogenic in different animal models and have the possibility of advancing through clinical trials. Two of these have been tested in clinical trials: PIV and sendai virus. The PIV vaccine, is a chimeric human and bovine parainfluenza virus of type 3, that consists of a bovine PIV backbone with the HN and F proteins replaced by those of the human parainfluenza virus which was additionally engineered to express the HRSV F protein (136, 200, 206). In African green monkeys the vaccine was immunogenic and protective against an HRSV challenge. This vaccine was recently evaluated (under the name MEDI-534) in a clinical phase I study in seronegative children 6 to 24 months of age (NCT00493285; (207)). The vaccine was safe for this population and 50% of the vaccinated children had a serum response after 3 administrations of the highest dose (10⁶ TCID₅₀) (208). Furthermore, the bPIV3 backbone of this vaccine has already been tested in young infants and was found to be safe, immunogenic and stable in this population (209). These results are promising and if the stability results of the MEDI-534 are positive this vaccine might advance to clinical testing in young infants. Another virus, sendai virus (SeV), which is a murine virus that has not been reported to infect humans, has been used as a live vector against human parainfluenza virus type 1, 2 and 3 (210). A recombinant sendai virus expressing the HRSV F protein (SevRSV) has been studied as vaccine against both hPIV-1 and HRSV. In cotton rats, this vaccine elicits HRSV-specific neutralizing antibody and T cell responses (211) and is protective against a variety of HRSV A and B subtypes. In African green monkey studies, immune responses against SeV or SevRSV were generated without adverse events (212, 213). SevRSV conferred complete protection against lower respiratory tract infection after HRSV challenge. No clinically relevant adverse events were demonstrated after either vaccination or challenge in the large animal model. Until now, the SevRSV vaccine has not been tested in clinical trials, however the SeV backbone was well tolerated in adults and children (214). Given these promising results, the SevRSV vaccine might be a good candidate vaccine against HRSV, however until now it is unclear whether this vaccine is stable. Compared to live attenuated HRSV vaccines this kind of vectors, such as bovine PIV, SeV and also Newcastle disease virus (NDV) have the advantage that their native tropism is not human. Therefore these vectors are attenuated in vivo.
Also, since the frequency of pre-existing immunity to these viruses is low, the impact of maternally derived antibodies to the vaccine will be minimal (119). On the other hand, bovine RSV has also been used as a live vector for a recombinant bovine/human RSV vaccine consisting of BRSV expressing HRSV F and/or G protein. However, due to host range restrictions, this virus was not able to replicate well in chimpanzees (215) and hence did not progress to clinical testing in humans.

**Virus-like particle strategies for HRSV vaccines**

Virus-like particles (VLP) are considered a relatively safe vaccination strategy, as there is no concern for viral spread or reversion, which is the case for replicating viruses. One VLP-strategy uses influenza virosomes as carrier for foreign proteins. The virosomes resemble empty influenza shells that are made up of phospholipids and the influenza hemagglutinin and neuraminidase proteins (216). The PEV4 viroosome (Pevion biotech), that carries the HRSV fusion protein, induces a neutralizing antibody response in mice that is protective against an HRSV challenge (217). Interestingly, also empty virosomes were associated with non-specific protection (218). This virosome technology has already been tested in clinical trials for other pathogens, giving the PEV4 virosome vaccine a good chance of advancing to clinical trials. A similar VLP strategy used virus-like particles consisting of influenza matrix protein core and HRSV F or G protein on the surface (219). Vaccination of mice using these VLPs induced a strong IgG2a antibody response that significantly decreased lung viral load upon an HRSV infection. Another interesting VLP technology uses nanoparticles consisting of HRSV F protein (Novavax). The F-protein is engineered to form rosettes (220). These nanoparticles have been tested in a phase I clinical trial in healthy adults, where it was found to be safe and immunogenic. On their website, novavax reports the nanoparticles to induce functional immunity, however, no details have been reported (220, 221). A phase I study in the elderly and a phase II study in healthy women are ongoing (222, 223). Both VLP-based strategies show promising results in animal models and healthy volunteers; however whether a VLP-based HRSV vaccine would be free from disease enhancement in HRSV-naïve infants is unknown.

**Replication defective gene based vectors**

Another vaccination technology of particular interest uses viral derived replication defective gene-based vectors, for the delivery of a gene encoding the vaccine antigen of interest. In particular alphavirus vectors and recombinant adenovirus vectors have been studied as vaccination strategies against HRSV. The advantage of these gene-delivery vectors is that the gene expression of the vaccine antigen occurs in the host cell in a process that closely resembles natural infection resulting in authentic proteins able to elicit both an antibody and a T cell immune response, including CD8+ T cells. Additionally, these vectors are considered to be safe, as they are not capable of replicating (119). Recombinant adenovirus vectors (rAd) are considered an interesting vaccine backbone
candidate, in part for its robust production techniques. A rAd vaccine expressing the codon-optimized soluble F1 part of the HRSV F protein induced a mucosal IgA response in mice, which was protective against HRSV challenge (224). rAd have not been used in clinical trials as an HRSV vaccine, however rAd expressing HIV envelope proteins have been extensively studied in humans and were found to be immunogenic and well tolerated (224, 225). A challenge with the use of rAd of serotype 5 is the high prevalence of immunity against this viral vector in the human population, which dampens immune responses to the vaccine (226). However, children between 6 months and 2 years of age are still rAd5-seronegative (227). Therefore, rAd5 vector expressing HRSV proteins might be a good vaccine candidate in this age group. For adults, or children under 6 months of age, which still possess maternally derived antibodies, other serotypes of rAd can be used, which have a lower seroprevelance (228).

In an alternative strategy, alphavirus vectors, such as Venezuelan equine encephalitis virus (VEE) or Semliki forest virus (SFV) have been used as backbone for replication-defective HRSV vaccines. Their self-amplifying RNA replicon, which significantly increases antigen expression levels, makes these vectors an attractive system. VEE and SFV vectors expressing HRSV F or G proteins have been tested in mice and cotton rats and were found to be immunogenic and protective against HRSV challenge, and, for the VEE vector, this occurred in the absence of Th2-like immunity (229, 230). This technology platform might be a suitable candidate for clinical evaluation in the future.

**DNA vaccination**

DNA vaccination has been of great interest as a vaccine platform for various pathogens, due to its ability to induce both humoral and cellular immune responses (231). Despite promising results in small animal models, first generation DNA vaccines appeared to be only mildly immunogenic in humans (232). However, improvements in the vaccine formulation, the route of administration and the DNA construct itself have resulted in a new generation of DNA vaccines, which might be a more promising strategy (233). Several DNA vaccines, expressing different HRSV proteins have been tested in small and large animal models (234-239). Gene-gun immunization is the most popular method for administration, since it requires smaller quantities of DNA than conventional immunization methods. Gene gun immunization of mice with DNA encoding F or G protein was shown to be immunogenic (240) and protective against HRSV (241, 242). However, this method was associated with an undesirable Th2-biased immune response and enhancement of disease upon subsequent HRSV challenge (243, 244). DNA vaccination in calves using the BRSV F or N protein primed a strong cell mediated immune response. This response protected the calves from developing pneumonic lesions upon a BRSV infection and significantly reduced viral replication. An additional vaccination with a killed-virus vaccine induced high titers of neutralizing antibodies in these calves, which was fully protective following BRSV challenge (245, 246).
### Table 1.3 Overview of RSV vaccination strategies

<table>
<thead>
<tr>
<th>Experimental approach</th>
<th>Preclinical study comments</th>
<th>Clinical study comments</th>
<th>Ref</th>
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<tbody>
<tr>
<td><strong>Non-replicating, inactivated virus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1-RSV</td>
<td>Not immunogenic and poorly protective. Associated with enhanced disease upon subsequent RSV infection, resulting in two deaths.</td>
<td></td>
<td>143-147</td>
</tr>
<tr>
<td><strong>Live-attenuated and genetically engineered RSV derivatives</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cp-RSV</td>
<td>Highly attenuated in adults. Not sufficiently attenuated for seronegative children.</td>
<td></td>
<td>158,160</td>
</tr>
<tr>
<td>ts-1</td>
<td>Attenuated in adults. Loss of temperature sensitivity in seronegative children.</td>
<td></td>
<td>160,161</td>
</tr>
<tr>
<td>ts-2</td>
<td>Highly attenuated in primates</td>
<td>Weakly immunogenic in adults and children.</td>
<td>162</td>
</tr>
<tr>
<td>cpts248/955</td>
<td>Replication restricted to nasopharynx in mice and chimpanzees.</td>
<td></td>
<td>164-166</td>
</tr>
<tr>
<td>cpts248/404</td>
<td>Protective in chimpanzees.</td>
<td>Mild nasal congestion observed in seronegative infants &lt; 2 months. Highly attenuated in adults.</td>
<td>165,167</td>
</tr>
<tr>
<td>rA2cp248/404/1030ΔSH</td>
<td>Highly attenuated. High antibody titers in seronegative children older than 6 months. Phase II clinical trial performed in 2012. Results not yet known.</td>
<td></td>
<td>171</td>
</tr>
<tr>
<td>rA2cpΔNS2</td>
<td>Attenuated in adults and seropositive children, however, replicated efficiently in children.</td>
<td></td>
<td>177</td>
</tr>
<tr>
<td>rA2cpts248/404ΔNS2 and rA2cpts530/1009ΔNS2</td>
<td>Over attenuated in seronegative children.</td>
<td></td>
<td>178</td>
</tr>
<tr>
<td>rA2ΔNS1, rA2ΔM2-2 and rA2ΔM2-2NS2</td>
<td>Attenuated in monkeys.</td>
<td>No clinical studies performed until now.</td>
<td>179</td>
</tr>
<tr>
<td>RSV ΔG</td>
<td>Highly attenuated in cotton rats. Induces long-lasting protective immunity in cotton rats.</td>
<td></td>
<td>247</td>
</tr>
<tr>
<td><strong>Live viral vector-based</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPIV/HPIV3 expressing RSV F protein</td>
<td>Immunogenic and protective in African green monkeys.</td>
<td>Phase I trial in seronegative children (6-24 months old): serum response in 50% of children after 3 doses</td>
<td>206-208</td>
</tr>
<tr>
<td>SeV-RSV F protein</td>
<td>Protective in cotton rats and African green monkeys. Protective against HRSV A and B subtypes.</td>
<td></td>
<td>211-213</td>
</tr>
<tr>
<td>BRSV expressing HRSV F and/or G</td>
<td>Limited immune response in chimpanzees.</td>
<td></td>
<td>215</td>
</tr>
<tr>
<td>Experimental approach</td>
<td>Preclinical study comments</td>
<td>Clinical study comments</td>
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<tr>
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<tr>
<td><strong>Subunit vaccines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFP-1</td>
<td>Tolerated and immunogenic in healthy adults and children &gt; 1 year. Mild upper respiratory symptoms.</td>
<td></td>
<td>186</td>
</tr>
<tr>
<td>PFP-2</td>
<td>Phase II trial in pregnant women: only mild effect on antibody levels of babies at birth.</td>
<td></td>
<td>182-185</td>
</tr>
<tr>
<td>PFP-3</td>
<td>No significant protective effect observed in vaccinated children with cystic fibrosis.</td>
<td></td>
<td>187</td>
</tr>
<tr>
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1.7 Concluding remarks

The development of an HRSV vaccine has been a high priority for many years. The past decades, several different technologies have been studied, and new technologies are still being developed. Currently, our knowledge about immune responses to HRSV, and why an HRSV infection does not cause long lasting protection, is still not complete. In order to develop a safe and effective HRSV vaccine we will need to understand more clearly the immune evasion and immunopathology of HRSV. Given the diversity in immune status of different age groups, distinct HRSV vaccines will need to be developed for each category. Several strategies have aimed at newborns as their primary vaccine target group. However, these often encountered problems due to safety or immunogenicity in this young age group. Therefore, it has been suggested, as a short term success, it might be interesting to aim at developing a vaccine for the children > 6 months of age, of which a considerable number is still HRSV naïve (9). Alternatively, the consequences of herd immunization, or maternal immunization need to be further investigated to clarify whether this might form a (partial) solution to the HRSV burden. Also, in contrast to other difficult vaccine targets, such as HIV, herpes simplex virus or hepatitis C virus, there is a known immune correlate of protection for HRSV, which is the neutralizing antibodies to the F protein. Bearing this in mind, it should be possible to come up with a safe and effective vaccine that can reduce HRSV disease burden.

1.8 References


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PevionBiotech, consulted Dec 2012. Virosomes are the only VLP assembled in vitro, not by host cell http://www.pevion.com/index.php?page=723


Chapter 2  
Are CD8$^+$ T cells the solution for the development of an HRSV vaccine?

2.1 Introduction

Human respiratory syncytial virus (HRSV) was first discovered in the 1950s (1) but after decades of intensive research, a licensed vaccine against this pathogen is still not available. Since antibodies directed against the fusion (F) protein are known to correlate with protection based on epidemiological studies (2), most vaccination strategies developed until now focused on inducing a humoral immune response against F. The induction of a T cell immune response has received a lot less attention in the quest for the development of a safe HRSV vaccine. T cell immunity directed against HRSV has not unequivocally been associated with protection against HRSV; in fact it has also been associated with worsening of disease. Studies in mouse have associated CD4$^+$ Th2 responses with increased HRSV pulmonary pathology. Depletion of the Th2-associated cytokine IL-4 prior to acute HRSV infection led to decreased lung inflammation and mucus production (3-5). Moreover, a Th2 directed immune response is associated with delayed viral clearance in mice (5, 6). In human studies sufficient evidence supports a role for CD8$^+$ T cells in clearance of HRSV from the lungs (7). Therefore a CD8$^+$ T cell response is favored over a CD4$^+$ Th2 immune response in providing protection against an HRSV infection.

In this chapter we try to answer the question whether a cytotoxic T lymphocyte (CTL) response induced by vaccination is a suitable strategy to provide protection against an HRSV infection. First, we describe how a CTL response is induced and how these cells can act to protect the host from invading pathogens. Additionally, we discuss the factors that have to be kept in mind in the development of a CTL based vaccine. Finally, we describe the role of CTLs in controlling HRSV infection and the possibilities for CTL based vaccines against HRSV.
2.2 Activation, proliferation and memory formation of CD8$^+$ T cells

Antigen processing and presentation

Before going into detail on the possibilities for vaccine induced CD8$^+$ T cells in protecting against pathogens, we will first focus on the basics of T cell stimulation: how are CD8$^+$ T cells activated? Two cell types are involved in the activation of T cells; these are (I) an antigen presenting cell (APC) which presents a foreign peptide on a major histocompatibility complex (MHC) molecule and (II) a naïve T cell expressing the T cell receptor (TCR) and a co-receptor molecule (CD4 or CD8) (8). MHC molecules can be divided into two structurally and functionally different classes: class I (MHC I) and class II (MHC II) molecules. The function of both molecules is to present short, pathogen-derived molecules to T cells, which initiates the adaptive immune response (9).

Cellular proteins are constantly degraded by the proteasome into short peptides, a process that seems to closely follow mRNA translation into protein (10, 11). The same happens to pathogen-derived proteins in an infected cell, and many pathogens have actually evolved mechanisms to try to circumvent this host process. With the help of TAPs (transporters associated with antigen processing) these peptides are then transported to the lumen of the endoplasmic reticulum where they are loaded onto the peptide binding groove of the MHC I molecule. Proteins from extracellular pathogens undergo a different fate: these proteins are internalized into the cell in endosome vesicles where they are degraded and loaded on MHC II molecules (9). Noteworthy, through the process of cross-presentation, peptides derived from extracellular pathogens can also be presented on MHC I molecules (12). Recently it has been shown that presentation of virus-derived antigen on MHC-I molecules likely also occurs via cross-presentation through internalisation of apoptotic infected cells by APCs (18). After peptide loading, both classes of MHC molecules/peptide complexes are transported to the cell surface, where they present the foreign peptide to T cells (Figure 2.1).

Both MHC classes are recognized by T cells expressing a specific co-receptor: while MHC II molecules are recognized by CD4$^+$ T cells, MHC I molecules are exclusively recognized by CD8$^+$ T cells. Peptides ranging in length between 8 and 11 residues long can bind the MHC molecule in a groove. This groove has small pockets which, upon binding of a peptide, are occupied by the side chains of this peptide. An MHC I molecule typically has 6 pockets, of which 2 or 3 are important for anchoring the peptide to the MHC molecule (9). Since only specific side chains can fill the pockets, each MHC molecule is specific for a certain type of peptide. In humans, the genes coding for the MHC molecules are called human leukocyte antigen or HLA genes. Many polymorphisms of these genes exist, hence, each individual has her/his own specific MHC molecules to which different peptides can bind (13).
Figure 2.1  **Antigen processing.** Viral infection leads to the expression of viral proteins in the cytoplasm which are degraded into peptides by the proteasome. Selected peptides are then loaded into the endoplasmic reticulum where they are loaded onto newly synthesized MHC class I molecules. The MHC I-peptide complexes are transported through the golgi apparatus to the cellular membrane. Extracellular proteins are taken up by the cell through endocytosis and sequestered into endosomes. MHC class II molecules are synthesised in the endoplasmic reticulum and transported through the Golgi apparatus into lysosomes which fuse with the endosomes. Enzymes brought into this compartment by the lysosomes degrade the engulfed proteins into peptides. Peptides are loaded onto MHC II molecules and transported to the extracellular membrane.

Figure 2.2  **Activation of CD8+ T cells by dendritic cells results in the formation of memory cells.** Schematic representation of the phases during T cell proliferation: (A) activation, (E) expansion, (C) contraction and memory formation. The number of CD8+ T cells is shown in green. The viral load is shown in red.
Activation of naïve CD8+ T cells

Dendritic cells (DC) are the main type of APCs that are responsible for the initiation of an adaptive immune response. In the lung, DCs reside in the interstitium and constantly sample the lung for invading pathogens. DCs are a heterogeneous population of cells represented by two main subsets, the conventional CD11c+ MHC-II+ DC (cDC) and the CD11clow/B220+ plasmacytoid DC (pDC) (14). In addition, cDCs can be further divided into CD11b+CD103+ cDCs (CD11b+cDCs) and CD11b+CD103+ cDCs (CD103+ cDCs). Upon antigen uptake, DCs are activated and migrate to the lung draining lymph nodes (LN) where they can encounter antigen-specific T cells. For influenza virus it was shown that CD103- cDCs are the main cell type that migrates to the LNs and presents antigen on MHC-I molecules for priming and activation of CD8+ T cells, while CD11b- DCs mainly remain in the lung (15, 16). Interestingly, CD103+ DCs preferentially present peptides on an MHC-I molecule through cross-presentation of antigen derived from apoptotic infected cells (17). This is in line with the finding that CD103- DCs are not productively infected by influenza (18). In contrast upon an HRSV infection it was shown that both CD11b+ and CD103+ DCs migrate to the draining lymph nodes and in addition, both subsets are capable of presenting antigen to CD8+ as well as to CD4+ T cells (19). Whether the HRSV-derived antigen presented by CD103+ DCs on MHC-I molecules is derived from direct- or cross-presentation has not yet been clarified. It has been shown that HRSV can directly infect murine and human DCs in vitro. However, only low frequencies of DCs are infected in vitro (i.e. 4%) even at a multiplicity of infection of >20 (20). The in vivo relevance of this infection of DCs by HRSV remains to be established.

Migration of DCs to the lymph nodes serves to concentrate antigen at a site where virus specific T cells can encounter their cognate antigens. When naïve T cells recognize MHC/peptide complexes on APCs with high affinity they are stimulated to initiate an immune response; however, antigen alone (generally referred to as ‘signal 1’) is not sufficient to efficiently activate CD8+ T cells. Indeed, APCs also provide a second and third signal required for CD8+ T cell activation. Signal two, or the co-stimulatory signal consists of the binding of B7 (also known as CD80/86), CD70 or OX-40L on the APC to CD28, CD27 or OX-40, respectively on the T cell and provides the signal necessary for clonal expansion of the T cell (21, 22). Absence of the co-stimulatory signal leads to anergy, for instance, when cognate antigen is presented by an epithelial cell. A third signal shapes the overall magnitude of the primary response and the formation of memory. This signal consists of cytokines, such as IL-12 and type I interferons (IFN-α and IFN-β), which impact the CD8+ T cell activation and expansion; however, requirements for these cytokines are highly pathogen-dependent (23). To avoid tolerance, naïve T cells reside in lymphoid tissues, where they only encounter ‘professional’ APCs expressing the necessary costimulatory signals (24). Encounter by a naïve CD8+ T cell of an APC providing al 3 stimulatory signals leads to the activation of the CD8+ T cell, resulting in proliferative expansion and differentiation into effector cells (22, 25). Activated T cells migrate from the LN to the site of infection where they can exert their effector functions. The chemokine receptor CXCR3 has been shown to be involved in the process of migration to the lungs. CXCR3+/ mice show a decreased infiltration of CD8+ T cells in the lungs upon viral infection (26). In addition, inhibition of CXCL10, the
ligand for CXCR3 results in enhanced disease severity, impaired viral clearance and decreased numbers of HRSV specific CD8\textsuperscript{+} T cells in the lungs upon HRSV infection (27).

Activated CD8\textsuperscript{+} T cells become cytotoxic T cells (CTL) capable of eliminating pathogen-infected target cells that express the same MHC/peptide complex on their cell surface (9). The effector functions of CTLs include (I) lysis of infected cells through a perforin-dependent release of granzyme-containing granules or (II) the induction of apoptosis of infected epithelial cells through Fas-Fas Ligand interactions (28). These effector functions can be exerted in the absence of the co-stimulatory signals. Activated CD8\textsuperscript{+} T cells produce pro-inflammatory cytokines such as IFN-γ, IL-2 and TNF. In addition, IL-10, a regulatory cytokine with anti-inflammatory properties is produced by activated CD8\textsuperscript{+} T cells (29). Production of IL-10 occurs at the time of initial influx of effector T cells in the lungs with maximum production corresponding to the peak of the virus-specific T cell response (30). This cytokine has been shown to be involved in limiting the inflammatory component of the host immune response to the pathogen and thereby preventing damage to the host and maintaining critical lung function (30, 31). HRSV infection of IL-10\textsuperscript{−/−} mice resulted in more severe disease with enhanced weight loss, delayed recovery and greater cell infiltration of the respiratory tract without affecting viral load (32). In addition, clinical studies have documented that certain polymorphisms in the gene encoding the regulatory cytokine IL-10 are associated with the development of severe bronchiolitis in HRSV infected infants (33, 34). Even though effector T cells are the primary source of IL-10 after HRSV infection, it should be noted that also other cell types, such as B cells and multiple cell types of the innate immune system can produce IL-10 (35). Production of this regulatory cytokine by CD8\textsuperscript{+} T cells requires exposure of the T cells in the infected lungs to IL-2 derived from antiviral CD4\textsuperscript{+} effector T cells (29) (see below).

Characteristics of memory CD8\textsuperscript{+} T cells

After removal of the pathogen, the effector phase is followed by a contraction phase and the formation of memory cells (Figure 2.2) (22, 25). Memory CD8\textsuperscript{+} T cells, generated after vaccination or after a first encounter with a pathogen have a number of advantages over naïve CD8\textsuperscript{+} T cells with respect to clearing a second infection with this pathogen. Whereas as few as 100 to 1000 naïve CD8\textsuperscript{+} T cells specific for a given epitope are present in naïve mice, the antigen specific memory CD8\textsuperscript{+} T cell population after vaccination or infection is at least 100 to 500 times larger (36, 37). Next to these changes in absolute number, these epitope-specific memory CD8\textsuperscript{+} T cells also possess certain characteristics which distinguish them from naïve CD8\textsuperscript{+} T cells. First, they can rapidly produce cytokines such as IFNγ, TNF or IL-2. Also, memory CD8\textsuperscript{+} T cells can kill infected target cells immediately, without the need for prior co-stimulation. In addition, these memory CD8\textsuperscript{+} T cells exhibit more robust killing capacities due to increased granzyme B expression (22). Finally, memory CD8\textsuperscript{+} T cells can reside in peripheral tissues, such as the lung, liver and skin, much closer to the site of pathogen invasion compared to the draining lymph node (38). Kimpen et al. showed that a secondary HRSV infection in mice induces an accelerated lymphocyte response.
(both CD4+ and CD8+) compared to a primary infection, which is also greater in absolute number of T cells (39, 40).

Memory CD8+ T cells established after infection or vaccination can be divided in two categories, based on the expression of the surface molecules L-selectin (CD62L) and chemokine receptor CCR7. Central memory T (T_{CM}) cells which are CD62L+CCR7+, localize preferentially to lymphoid tissues and can circulate throughout the blood, whereas effector memory T (T_{EM}) cells are CD62LCCR7 and preferentially localize to the peripheral tissues (38). A recall response upon a secondary encounter with the pathogen occurs in three distinct phases, with different memory CD8+ T cells involved in each phase (38). Antigen-specific tissue resident T_{EM} cells will be the first to respond to the invading pathogen, such as a virus and start eliminating virus-infected cells. These T_{EM} cells will rapidly proliferate to generate a secondary effector response. The second line of response consist of the recruitment of antigen-specific and –nonspecific memory T from the blood to the infected tissue. The minor fraction of these cells that are antigen-specific can also proliferate and initiate a secondary effector response upon antigen encounter. Finally, the last phase consist of large numbers lymphoid resident T_{CM} cells that have been activated by circulating APCs which migrate through the bloodstream to the peripheral tissue. Together, these three phases contribute to the rapid control and elimination of the invading pathogen. It has been shown that after a primary HRSV infection in mice memory T cells reside in both the lungs and the lung draining lymph nodes.

The cytokine IL-2 plays an important role in regulating CD8+ T cells both through optimizing effector generation and memory differentiation. It affects CD8+ T cells during different phases, including expansion phase, contraction phase and memory generation. Following viral infection, the primary expansion of the CD8+ T cell population is about threefold lower in IL-2−/− mice than in IL-2-competent controls, and this results in less efficient viral clearance by virus-specific CTLs (41). In addition, IL-2 treatment of HRSV infected mice results in enhancement of the frequency of IFN-γ-producing HRSV-specific CD8+ effector T cells and increase in numbers of memory T cells. This resulted in reduced weight loss and illness after HRSV infection in mice (42). IL-2 also plays a role in determining whether an activated CD8+ T cell differentiates into either a short-lived effector T cell or a long-lived memory T cell. Following initial activation, CD8+ T cells upregulate the expression of CD25 (i.e. the IL-2 receptor α-chain). Some cells only express CD25 during a short period of time, after which these cells upregulate CD62L. After the contraction phase, these cells adopt a long-lived T_{CM} phenotype. In contrast, cells expressing CD25 for a longer period of time, and hence received strong IL-2 signals, differentiate into short-lived effector CD8+ T cells, which do not remain after the contraction phase (41, 43). Both CD4+ and CD8+ T cells produce IL-2 during viral infection. It has been shown that CD8+ T cells respond to IL-2 from both autocrine origin (i.e. produced by CD8+ T cells) and paracrine origin (i.e. produced by CD4+ T cells) (44).
2.3 T cell-based vaccines

The aim of CD8$^+$ T cell based vaccines is to present pathogen derived epitopes to the immune system in the absence of disease, resulting in the activation of CTLs and the production of a memory response which, upon a second encounter with the same antigen results in rapid removal of the pathogen. This rapid elimination of the pathogen is a result of the higher number of antigen specific T cells and the faster and more robust response of these memory T cells. Because of this mode of action, T-cell based immunity can not prevent the infection by the pathogen but rather requires a degree of pathogen-derived gene expression before the T cells can recognize the cognate antigen in the context of MHC-molecules on the surface of the affected cell. Unlike vaccines that aim at inducing a strong antibody response, induction of an effective memory CD8$^+$ T cell response by vaccination is more complicated. Several factors should be considered when developing a CD8$^+$ T cell based vaccines.

First, one should bear in mind that the antigen(s) delivered by the vaccine should be presented by an MHC I molecule on an APC, either through direct presentation or cross presentation, in order to stimulate a strong immune response. For this reason, live vaccine vectors should be preferred over recombinant protein vaccine. These vectors can be divided into two categories: (I) replication competent vectors, such as live attenuated vaccines or replication competent recombinant vectors and (II) replication defective vectors, either recombinant vectors or DNA. Direct presentation would require the APC to be infected directly by this vaccine vector. Recently however it was shown that CD103$^+$ DC are not easily infected by influenza virus (which likely will also be the case for other viruses) and that these cells present foreign peptides through cross presentation rather than through direct presentation (18).

Most vaccines currently in use require repeated administration in order to generate a robust, long lasting immune response. Thus, human vaccination will likely require repeated administration with T cell vaccines. Classical vaccines boost the immune response by administering the same vaccine, i.e. homologous boost. This strategy would not be suitable for generating memory T cell responses, especially when using viral vectors, since the robust cellular and humoral immune response to the vector would lead to a rapid neutralization and elimination of the vector upon a second encounter. This would not lead to the desired boosting of the memory response to the antigen encoded by the vector. Therefore, a heterologous prime-boost strategy would be more suited for generating strong memory CD8$^+$ T cell responses using viral vectors. Of note, the effect of boosting on the T cell memory is pathogen dependent an may either be favourable or not (45). For instance, for the chronic Lymphocytic Choriomeningitis Virus (LCMV) infection it has been shown that repeated antigen exposure does not result in increased number of memory CD8$^+$ T cells, rather it drives the CD8$^+$ T cells into a terminally differentiated state which is unresponsive to re-stimulation with antigen (45, 46). The same study showed however that this was not observed for a number of acute infections, such as with *Listeria monocytogenes*, *Vaccinia virus* or an acute LCMV infection (45).
The diversity in human HLA proteins poses another difficulty to the development of CD8+ T cell based vaccines. For the efficient induction of a T cell response in a broad human population it will be necessary to include multiple CTL epitopes matching different haplotypes. This is in strong contrast to the situation in most pre-clinical studies, which are often performed in inbred mice strain. Here, the haplotype of the population is known and is identical for each mouse. Including a single epitope of the pathogen in the vaccine would already be sufficient for efficient CTL induction in inbred mice. Therefore, it is usually essential to include complete and as much pathogen-derived T cell antigens as possible in the vaccine to be developed. Whereas lots of HRSV CTL epitopes are already known for different mice strains (47), human CTL epitopes are still being discovered. Using computer algorithms HRSV specific human CTL epitopes specific for different haplotypes are being mapped to the different HRSV proteins, which can be of use for designing a human CTL based HRSV vaccine (48, 49). Until now, human CTL epitopes for HRSV have been mapped to the F, N, M, M2-1 and SH proteins (50-56). Two studies performed in South-Africa revealed that for the epitopes in the N and F proteins which are commonly recognized by the HLA subtypes in the South-African population, no variation in these epitopes was found in HRSV isolates over multiple years (52, 57). This suggests that immune selection of HRSV strains resulting in induction of CTL escape mutations is not common. This however does not exclude that immune pressure as a consequence of a potential HRSV CTL vaccine may result in CTL escape variants. Another point that should be taken in consideration is the immunodominance of the CTL epitopes. Indeed, it has been shown that strong immunodominant epitopes elicit high frequencies of responding cells and limit the response to subdominant epitopes. Immunodominance in HRSV has been extensively studied in a hybrid mouse model. For dominance studies in HRSV, the CB6F1/J hybrid mouse model is often used. This mouse strain is the F1 generation of a BALB/c x C57BL/6 mating and therefore these mice express both H-2b (derived from C57BL/6 background) and H-2d (derived from BALB/c background) class I MHC molecules. Despite being dominant in the parent H-2b C57BL/6 strain (58), the D8M187-195 epitope is subdominant to the KdM282-90 epitope in the H-2d/b hybrid mouse (59). Additionally, it was shown that mutating the subdominant D8M187-195 epitope enhanced the response to the dominant KdM282-90 epitope, whereas mutation of the dominant epitope resulted in a subdominant epitope becoming the dominant one (60, 61). Vaccination can change the immunodominance pattern: vaccination with a subdominant epitope of a pathogen results in enhanced numbers of primed T cells specific to this epitope which upon subsequent encounter with the pathogen, can dominate the response if they outnumber the dominant epitope. An advantage of CD8 T cell based vaccines is that the antigen epitope can be derived from any internal or external protein of the pathogen, if it can be presented by MHC I molecules. In contrast, vaccines that induce antibody responses can exclusively use epitopes derived from external pathogen proteins, or pathogen proteins expressed on the surface of the infected cell.
2.4 Control of HRSV by CD8\(^+\) T cells: do mice fool us?

CD8\(^+\) T cells with a TCR directed against viral epitopes that are presented in an MHC class I context on the surface on infected cells are key for clearing viral infections (62). Clearance of HRSV from the infected host is no exception to this paradigm. HRSV-challenge experiments in laboratory mice have been instrumental to support this statement. First evidence for the role of CD8\(^+\) T cells in the clearance of HRSV came from adoptive transfer studies. Here, transfer of HRSV primed T cells (derived from the spleens of HRSV infected mice) to athymic nu/nu BALB/c mice or immunodeficient gamma irradiated mice, results in clearance of HRSV from the lungs by day 15 after HRSV infection, whereas without T cell transfer, the virus remains present beyond day 15 after infection (63). Clearance can be achieved by transfer of either CD4\(^+\) or CD8\(^+\) T cells separately, or by the combination of both. However CD4\(^+\) T cell are less efficient in clearing HRSV than CD8\(^+\) T cells.

The role of CD8\(^+\) T cells upon an HRSV infection is however still somewhat controversial since it is not completely clear whether they are in part also responsible for the lung damage observed upon an HRSV infection. The answer to this question is not clear cut, as the effects of the CD8\(^+\) T cells differ in mice and in humans. CD8\(^+\) T cells from an HRSV-specific CD8\(^+\) T cell line transferred to gamma irradiated mice were responsible for rapid clearance of the virus from the lungs of persistently infected mice, however, this was associated with augmented and sometimes lethal respiratory disease (64). The amount of lung damage correlated with the number of cells transferred: lower numbers of CTLs transferred produced less severe disease. Also in normal, immunocompetent mice, transfer of HRSV specific CTLs was associated with clearance of HRSV from the lungs and was again accompanied by acute and sometimes lethal respiratory disease (64). Depletion studies, performed by Graham and colleagues confirmed that T lymphocytes are responsible for both viral clearance and lung pathology and that CD8\(^+\) T cells appear to be the important mediator of illness (65). Depletion of the CD8\(^+\) lymphocyte population during primary infection resulted in modest illness and early recovery. Also in rechallenge studies, mice in which CD8\(^+\) T cells were present (but CD4\(^+\) T cells and HRSV specific antibodies were absent) experienced severe illness. These results suggest that in mice CD8\(^+\) T cells are important for viral clearance, however also are responsible for the lung pathology.

These findings in mice are in strong contrast with results observed in humans where there is no clear evidence that CD8\(^+\) T cells play a role in lung pathology (66). The most important target group of HRSV are very young infants (younger than 6 months of age). Little is known about immune responses elicited in these young children, although it is generally accepted that the infant immune system is still developing and reacts differently and mostly less effective to a variety of infections than does the adult immune system (67). Infant immune responses are mainly biased in the Th2 direction, which, for HRSV infections, is considered as unwanted. It has however been shown that a CD8\(^+\) T cell response can be found in infants experiencing severe disease due to HRSV. Activated, HRSV-specific CD8\(^+\) T cells with an effector phenotype could be found in the blood and BAL of these infants (66). No correlation could be found between the severity of disease and the magnitude of the CD8\(^+\) T cell response in these infants, which suggests that the disease is not caused by a strong CD8\(^+\) T
cell response, as seen in mice (66). Furthermore, in timing, the peak of the CD8\(^+\) T cell response does not coincide with the peak of disease severity. In a study performed by Lukens et al. (7) the kinetics of the HRSV viral load in the lungs and the CD8\(^+\) T cell numbers in the blood were followed daily, during hospitalization of infants. They observed that peak viral load occurred early after hospitalization or even before hospitalization, with a peak of disease severity 2-3 days after admission. In contrast, the levels of CD8\(^+\) T cells in the blood peaked around day 11-15 after onset of the primary symptoms, which correlates with the recovery from disease and not with the peak of disease symptoms (7). Moreover, reports have demonstrated that fatal cases of HRSV infections are characterized by high viral titers and near absence of pulmonary infiltration of T cells or the cytokines they produce (68). Taken together, all these studies demonstrate that CD8\(^+\) T cells play a role in viral clearance and that they are not the cause of the observed lung pathology, which is in contrast to the observations in mice.

An intriguing feature of HRSV infection is the susceptibility of previously infected individuals to reinfection with antigenically closely related viruses or the identical virus strain (69). In a human challenge model, almost 50% of adults with high levels of circulating neutralizing antibodies could be re-infected with HRSV of the same serotype within two months of natural infection (70). These observations suggest that the duration of protective immunity to challenge HRSV infection is short-lived and that the formation of a long-lived memory T cell response (CD4\(^+\) and/or CD8\(^+\)) after HRSV infection is lacking. Little is known about the formation of a proper memory CD8\(^+\) T cells in humans. One study reported that memory CD8\(^+\) T cells are present in healthy elderly people, however in smaller numbers compared to influenza specific CD8\(^+\) T cells (71). This is an indication that a memory response does exist, however is not effective at preventing reinfections (at least in the elderly). There was a tendency towards lower numbers of HRSV specific CD8\(^+\) T cells with increasing age, which might explain the poor protection against HRSV reinfection and the increased risk to develop an HRSV-related severe illness in this population. With respect to the formation of a memory response, Braciale and colleagues studied the response to the M2\(_{82-90}\) epitope, the major target of CD8\(^+\) T cells (72) in BALB/c mice upon HRSV infection (73). By comparing tetramer staining of M2\(_{82-90}\) specific T cells with functional assays they found that a large number of M2\(_{82-90}\) specific T cells expanded and infiltrated into the lungs upon HRSV infection, only half of these possessed effector activity. These cells were impaired in cytokine synthesis, ex vivo cytolytic activity as well as development of memory cells in the lung. It was suggested that the impaired effector phenotype was due to defective TCR signaling. The same discrepancy between tetramer staining and intracellular IFN-\(\gamma\) production was found for the subdominant F\(_{85-93}\) epitope. These results suggest that in BALB/c mice, an HRSV infection can suppresses T cell effector function and the establishment of a long lived lung memory response through its effect on TCR dependent signaling. Later, these results were questioned by an different group, who found that the impairment of the CD8\(^+\) T cell response is not typical for HRSV but rather specific for the lungs (74): the same effect was observed in the lungs after influenza infection. It is not known whether such impairment of CD8\(^+\) T cells in the lungs is also present upon HRSV infection in humans and whether or not this could contribute to the absence of long-lasting protection to HRSV.
2.5 The role for CD8\(^+\) T cells in protecting against vaccine induced enhancement of disease

The first HRSV vaccine trials, using formalin inactivated HRSV (FI-RSV) vaccine had a fatal outcome, with vaccinated children experiencing more severe disease than unvaccinated controls, upon HRSV infection. In experimental mouse models, this vaccine-associated enhancement of disease can be mimicked and such a model has been used to try to recapitulate and understand the unfortunate outcome of the clinical use of FI-RSV. Analysis of the induced immune response upon vaccination with FI-RSV vaccine revealed that the vaccine efficiently primed B cells and CD4\(^+\) T helper 2 cells, but only weakly primed a CD8\(^+\) memory response (75, 76). This is an expected consequence of the use of an inactivated vaccine, since it is generally assumed that antigen from injected inactivated vaccines can only be processed in the MHC I presentation pathway via cross-priming, which is usually far less efficient than direct-priming, when the antigen is present and processed for MHC I presentation from within the cell, for example when the antigen is delivered by a life viral vector. In mice, the same enhancement of disease after an HRSV challenge was observed when the mice had been vaccinated with vaccinia virus expressing HRSV G protein (vacv-G) (77). Similar to the FI-RSV vaccine trial, the immunity induced by vacv-G vaccination consisted of only a memory CD4\(^+\) T cell response, with no detectable memory CD8\(^+\) response. This was in contrast to vaccination with vaccinia expressing other HRSV proteins (F, N or M2): these mice developed both CD4\(^+\) and CD8\(^+\) memory T cell responses, and did not show any enhancement of disease upon HRSV infection. Interestingly, immunization with vacv-G in which the CD8\(^+\) T cell epitope of the M2.1 protein was inserted resulted in the formation of both a memory CD4\(^+\) and a memory CD8\(^+\) immune response (78). These mice were devoid of enhancement of disease upon HRSV infection. These results were further confirmed by studies in CD8\(^+\) T cell deficient mice (beta 2 microglobulin knock-out mice): priming with Vacv-F in these mice results in enhancement of disease, presumably due to the absence of CD8\(^+\) T cells; an observation that was not found in wild type mice primed with vacv-F upon HRSV infection. In another study, vacv-F immunized mice develop enhanced disease, characterized by pulmonary eosinophilia, when CD8\(^+\) T cells were depleted by anti-CD8 antibodies (79). Together, these studies show that a CD8\(^+\) memory response plays an important role in preventing enhanced disease that is associated with certain vaccines upon HRSV infection. In addition, this ameliorating effect by HRSV-specific CD8\(^+\) T cells is dose-dependent: the ability of CD8\(^+\) T cells to control enhanced disease depends on the number of CD8\(^+\) T cells present in the lungs before or early after HRSV infection: reducing the number of HRSV-M2 specific CD8\(^+\) T cells on day 3 after HRSV challenge to 2 x 10\(^4\) cells per lung resulted in enhancement of disease, whereas 8 x 10\(^4\) HRSV-M2 specific CD8\(^+\) T cells present in the lung on day 3 after HRSV challenge was sufficient to inhibit disease enhancement (80).

Given the observation that enhancement of disease due to FI-RSV vaccination is characterized by the absence of CD8\(^+\) T cells, and that this enhancement of disease can be abolished when sufficient CD8\(^+\) T cells are present before the HRSV infection it is clear that a vaccination strategy that induces a CD8\(^+\)
memory T cell response is desired for maximally avoiding the possibility of enhancement of disease upon a subsequent HRSV infection.

### 2.6 CD8\(^+\) T cells as a correlate of protection in HRSV vaccines

Even though most vaccination strategies aim at inducing a protective antibody response, several studies have been performed to investigate the possibility of CD8\(^+\) T cells to provide protection against HRSV. In a study by Voges and colleagues, recombinant sendai virus (a *Respirovirus*) expressing HRSV F protein (SeV F) or soluble HRSV F protein (SeV sF) were used as vaccine (81). These recombinant sendai viruses induced both an antibody response and a CTL response to the HRSV F protein and protected against an HRSV challenge 3 weeks after vaccination. The CD8\(^+\) T cell component induced by SeV F and Sev sF vaccination provided protection against an HRSV challenge in B cell deficient MB1 mice, 3 weeks after vaccination. These data show that CTLs are sufficient to provide protective immunity against HRSV, shortly after vaccination. The formation of memory T cells and the protection at later time points were not examined. Instead of using a complete HRSV protein, several studies used only a CTL epitope of the HRSV M2-2 protein. A DNA plasmid containing a minigene encoding the HRSV M2\(_{82-90}\) CD8\(^+\) T cell epitope was used for DNA vaccination in BALB/c mice. Intradermal injection with this plasmid induces an M2\(_{82-90}\) specific CD8\(^+\) T cell response that provided significant reduction in viral load upon HRSV challenge 3 weeks later (82). Intranasal administration of this DNA in nanoparticles composed of chitosan could also induce a protective M2\(_{82-90}\) specific CD8\(^+\) T cell response (83); however, HRSV challenge was performed only 14 days after the last vaccination and the memory response induced by this DNA vaccine was not assessed.

For the induction of a long-lasting memory response, a peptide encoding this same M2\(_{82-90}\) CD8\(^+\) T cell epitope was formulated with different adjuvants. Upon administration of the M2\(_{82-90}\) peptide with an enterotoxin-based adjuvant LTK63, the CD8\(^+\) T cells remained detectable in the spleen more than 100 days after vaccination (84). Unfortunately, resistance to HRSV afforded by this vaccine is accompanied with enhanced weight loss upon an HRSV infection. Alternatively, a fusion protein consisting of the M2\(_{82-90}\) epitope fused to the carrier protein DsbA (disulphide bond isomerase) of E. coli and the fusion peptide of measles virus F1 protein can induce CD8\(^+\) T cells which remained detectable in the spleen until at least 123 days after vaccination (85). These CD8\(^+\) T cells possessed ex vivo cytolytic activity, but the ability to protect against an HRSV challenge 123 days after vaccination was not examined. In contrast to the previous study mentioned, this last vaccine did not prime for enhanced weight loss upon an HRSV infection. It is unclear why one vaccine causes weight loss while the other does not. Simmons et al. (84) believe that the excessive TNF production during viral infection, which is partly driven by IFN-\(\gamma\) producing CTLs can have detrimental consequences, such as the observed weight loss. This could be influenced by the adjuvant used in the first study, which
indicates that the selection of an appropriate adjuvant may be crucial when inducing a CTL response by peptide vaccination.

A recent study tried to answer the question whether memory CD8\(^+\) T cells induced by vaccination can still provide sufficient protection long after the vaccination. A vaccine termed TriVax, which consists of a peptide representing the immunodominant M2\(_{82-90}\) CD8\(^+\) T cell epitope, the Toll-like receptor agonist poly(I·C), and a costimulatory anti-CD40 antibody, was used as a T cell vaccine (86). The vaccine efficiently induced HRSV specific effector and memory CD8\(^+\) T cells in BALB/c mice. It was reported that vaccinated mice were protected against HRSV infection, as they efficiently cleared HRSV four days after challenge; however, it should be noted that challenge HRSV infection was performed only six days after vaccination, which is at the moment when CTL numbers are increasing, due to vaccination. Protection against HRSV challenge by memory T cells was only partial: an HRSV challenge 42 days after TriVax vaccination could reduce the viral lung titer 4 days after challenge by only 1 Log\(_{10}\).

To explore the possibilities of inducing a CD8\(^+\) T cell response against HRSV using a live viral vector, we created a recombinant influenza virus carrying the HRSV F\(_{85-93}\) CTL epitope in the NA stalk (PR8/NA-F\(_{85-93}\) virus). Infection of mice with this virus showed that an HRSV specific CTL response was efficiently induced and upon an HRSV challenge 4 weeks after the vaccination these CTLs were capable of reducing the HRSV replication in the lungs of the mice (87). When postponing the HRSV challenge until 50 days after the vaccination we also observed a reduction of the viral lung titer by 1 Log\(_{10}\).

These results demonstrate that CD8\(^+\) T cells induced by vaccination can contribute to protection against an HRSV infection. However, more studies will need to be performed to sort out how a long-lasting memory response could be achieved and whether CD8\(^+\) T cells alone can provide sufficient protection

### 2.7 Conclusions

After the failure of the formalin inactivated HRSV vaccine, it became clear that the development of a safe and effective HRSV vaccine would likely require a long path to success, which today still has still not reached an endpoint. Sufficient evidence is present that supports the role for CD8\(^+\) T cells in viral clearance in humans. In addition, the studies summarized here suggest that induction of a CD8\(^+\) memory T cell response is necessary to avoid the induction of a harmful Th2 CD4\(^+\) memory response that is responsible for enhancement of disease during an HRSV infection. Furthermore, the formation of a long lasting T cell memory response after an HRSV infection is lacking. Therefore, we believe that induction of a CD8\(^+\) T cell memory response will be essential for effective protection against HRSV. Several strategies for the induction of CD8\(^+\) T cells against HRSV by vaccination have been explored.
These studies have learned us that not the induction of a T cell response per se is a challenge, but rather the formation of long-lasting protective CD8+ memory response. Therefore, in order to develop a safe and effective HRSV vaccine, a better understanding of the formation of memory response to HRSV CTL epitopes will be necessary. Ideally, an HRSV vaccine should be administered to the infant as early as possible. Since the potential use of a non-replicating HRSV vaccine is still being held back by the early studies that showed that a formalin-inactivated vaccine caused an abnormal and pathologic immune response, a replicating vaccine is currently the best option. Live attenuated vaccines eg. against rotavirus, measles virus, mumps virus and rubella virus are already routinely being used in early childhood vaccination programs and offer opportunities for the acceptance of a potential live attenuated HRSV vaccine to be included in the early childhood vaccination program.

2.8 References


Chapter 3
Recombinant influenza viruses as live viral vector vaccines

3.1 Influenza virus

The classification, nomenclature and global burden of influenza viruses

Influenza viruses belong to the family of the *Orthomyxoviridae*, members of which are characterized by a segmented, negative-sense, single stranded RNA genome. They are divided into three genera, influenza A, B and C, based on antigenic differences in the nucleoprotein (NP) and the matrix protein (M). Influenza A viruses are further categorized based on the subtypes of membrane glycoproteins hemagglutinin (HA) and neuraminidase (NA). Until now 17 HA subtypes (H1-H17) and 9 NA subtypes (N1-N9) have been reported (1). The last HA subtype (HA17) was only recently discovered in an influenza A virus isolated from yellow shouldered bats in Guatemala (2). While all of these subtypes can be found in their natural reservoir, aquatic wild birds, only 3 HAs (H1, H2 and H3) and 2 NAs (N1 and N2) are capable of circulating in humans. Sporadically, there have been cases reported of human infection with an avian H5N1 virus but until now these viruses have not been reported to transmit from one person to another. A common nomenclature is used for naming different influenza virus isolates. This name consists of the subtype, the species of origin (if not human), the place of origin, the isolate number, the year it was isolated, and for influenza A viruses, the HA and NA subtype. For example A/Puerto Rico/8/34 (H1N1) is isolate number 8 of a human influenza virus, isolated in Puerto Rico in 1934 and it has a subtype 1 HA and subtype 1 NA glycoprotein (3).

Influenza is an important cause of respiratory infections. Yearly epidemics, caused by influenza A and B viruses result in 3-5 million clinical infections and 250.000-500.000 fatal cases (4). In humans, influenza virus infects the respiratory tract leading to disease which is characterized by symptoms such as fever, aching muscles, headache, malaise, cough, sore throat and rhinitis (5). Most people
recover within one or two weeks, without the need for medical treatment, however in risk groups, such as very young children, the elderly, immunocompromised people or people with other infections, influenza infection can lead to severe pneumonia and even death.

**Antigenic variation of influenza viruses**

Due to the constant pressure by neutralizing antibodies, and thanks to the error-prone viral replication machinery, point mutations occur in the influenza HA and NA, resulting in virus which can evade existing antibodies. This change, called antigenic drift, is the reason why influenza vaccine composition has to be adapted yearly. A second method through which change arises in influenza viruses occurs when a person is simultaneously infected by two different viruses. Due to the segmented nature of the influenza viral genome, exchange of gene segments can occur between these two viruses. This rearrangement of genes is called antigenic shift, and can result in a new pandemic when an antigenically distinct virus arises that can transfer between humans and to which little or no protective immunity is present in the human population (6, 7). These variations are interesting when using influenza as a viral vector for vaccination, because antigenically unrelated strains can be used for priming and boosting vaccinations.

**The influenza virion structure**

Influenza virions are spherical or filamentous particles with the spherical particles in the order of 100 nm of diameter and the filamentous often in excess of 300 nm in length. The lipid bilayer of the viral particle is acquired from the apical cell membrane during the process of budding and is covered with three membrane proteins (Figure 3.1). These proteins are the large glycoproteins HA and NA, present in a four to one ratio, and the smaller transmembrane channel protein M2, which is present at approximately 1 M2 protein per 10 to 100 HAs. HA is present as homotrimers and is important during the first steps of the influenza infection cycle. First, it is responsible for the binding to the host cell through the binding of sialic acids on carbohydrate side chains of cellular glycoproteins. Second, after the virion is taken up into the cell in an endosome, HA mediates fusion of the viral and endosomal membranes, to release the viral genome in the cell cytoplasm (8, 9). NA is present as homotetramers (10, 11) and possesses sialidase activity, which, upon the budding of a new virus particle, is used to cleave terminal sialidase activity which is essential for the spread of newly formed virions. NA removes sialic acid residues form both viral and host glycoproteins, to prevent the newly formed virus particles from aggregating to each other or to the cell membrane (12, 13). Additionally, NA is shown to be responsible for degradation of mucins in the respiratory tract, allowing binding of the virus to the respiratory epithelium (14). The M2 protein forms homotetramers that act as an ion channel (15, 16). It has a small ectodomain, consisting of only 23 amino acids, which are highly conserved among different influenza A viruses from different hosts.
The inner side of the lipid bilayer is lined by a shell formed by the matrix 1 protein (M1). The M1 protein is one of the most abundant proteins present in the viral particle. It is responsible for the shape and structure of the virion (17). Within the matrix core there are the nuclear export protein (NEP; formerly known as nonstructural protein 2) (18) and the viral RNA genome (vRNA) that is surrounded by numerous nucleoproteins (NP) and is associated with the heterotrimeric RNA dependent RNA polymerase (RdRp) consisting of two basic and one acidic subunit (PB1, PB2 and PA) (17). Together, the vRNA, NP, PB1, PB2 and PA form the viral ribonucleoprotein complex (RNP). The nonstructural protein 1 is only expressed in the infected cells and is not included in the virion. It counteracts the interferon-induced host immune response (19). Several other non-structural accessory proteins have been described for influenza A viruses, such as PB1-F2, N40 and PA-X. PB1-F2 is present in some, but not all influenza isolates. It is located in the mitochondria and multiple functions for this protein have been described: (1) The protein can induce apoptosis in a cell type-dependent manner, (2) PB1-F2 is able to promote inflammation, and (3) finally it up-regulates viral polymerase activity by its interaction with the PB1 subunit (20, 21). PB1 N40 represents an N-terminally truncated version of the PB1 protein which lacks transcriptase function but is still able to interact with the other subunits of the RdRp complex. The function of PB1 N40 is still unclear (22) although this protein appears to be nonessential for viral replication. Via a mechanism of ribosomal frameshifting, a second protein, termed PA-X, is encoded from the PA gene segment. This protein comprises the endonuclease domain of the viral PA protein with a different C-terminal domain and functions to repress cellular gene expression. In a mouse model, PA-X acts as a virulence-modulating factor to decrease pathogenicity (23). Intriguingly, PA-X and PB1-F2 exert opposite effects on pathogenesis. Presumably, the two proteins work together to optimally modulate host immunity (24). Recently, two other N-terminally truncated forms of PA, which were named PA-N155 and PA-N182, have been described. These N-truncated PAs did not show polymerase activity when expressed together with PB1 and PB2; however, mutant viruses lacking the N-truncated PAs
replicated more slowly in cell culture and had lower pathogenicity in mice than did wild-type virus (25).

The influenza genome structure and its transcription and replication

The influenza A viral genome consists of eight negative sense RNA strands. The segments are numbered in decreasing order of size. These segments encode minimum 10 proteins but some strains encode 11 or 12 proteins. The segments 1, 4, 5 and 6 each encode a single protein: PB2, HA, NP and NA. Segment 2 encodes the PB1 protein in all influenza viruses. In some influenza A viruses this protein also encodes two accessory proteins: PB1-F2 (20, 21) and PB1 N40 (22) from overlapping reading frames. Segment three that encodes PA from the full length mRNA also encodes up to 3 accessory proteins via ribosomal frameshifting (PA-X) (23) or through alternative start codons (PA-N155 and PA-N182) (25). Segment seven encodes two proteins: M1 is encoded from the full length mRNA, whereas the M2 protein is encoded by a spliced mRNA. The 8th segment also encodes two proteins in the same way as the M segment: NS1 is produced from full length mRNA whereas NS2 is produced from a spliced mRNA.

The 5’ and 3’ ends of each vRNA segment form a helical hairpin, which is associated with one RdRp complex; the remainder of the vRNA is covered with NP molecules. The negative sense coding sequence of the viral genome is flanked at both the 3’ and 5’ end by a noncoding region. The ultimate 13 nucleotides on the 5’ end and 12 nucleotides on the 3’ end are highly conserved among viral strains and among the different vRNPs (26-28). These sequences are partly complementary and base-pair to form a short double stranded RNA stretch (29) which serves as the promoter for the viral RdRp (30, 31). Even though they are not translated, these non-coding sequences are indispensable for the virus; besides sequences involved in transcription of the genome, these regions also contain packaging signals, for the incorporation of the vRNPs in the newly formed virion (32). The viral vRNA serves as template for both the transcription and replication of the viral genome, processes which are performed by the viral RdRp complex, built up of PB1, PB2 and PA. mRNA transcripts contain a 5’ CAP structure and a 3’ poly-A tail. This CAP is ‘stolen’ from a cellular pre-mRNA, by the viral RdRp and serves as primer which is subsequently elongated to positive sense mRNA (33). The production of the poly-A tail occurs through stuttering of the RdRp at a poly U stretch at the 5’ end of the vRNA (34).

The process of viral replication also uses the negative sense vRNA as template. For replication the vRNA is copied by the viral RdRp complex into a positive sense complimentary RNA strand (cRNA). This cRNA is a complimentary copy of the complete vRNA, including non-coding regions at both ends of the vRNA. In contrast to mRNA, this cRNA is not capped nor does it contain a poly-A tail. The cRNA in turn serves as template for the production of a new full length vRNA, again produced by the viral RdRp complex (1). These vRNA molecules are then covered by NP molecules and attach to one viral RdRp complex, which is essential for its incorporation in progeny virions. Understanding of the transcription and replication process was crucial for the development of reverse genetics systems for the generation of recombinant viruses form plasmid DNA.
3.2 Reverse genetics techniques for the generation of recombinant influenza viruses

The RNP transfection method

For using influenza virus as a vaccine vector, reverse genetics techniques have been developed, allowing the production of recombinant influenza viruses which contain foreign proteins or epitopes. Several generations of techniques have been developed. In 1989 it was discovered that the vRNP, consisting of vRNA complexed with NP proteins and a viral polymerase complex is the minimal essential unit necessary for the production of viral proteins and vRNA (35). This discovery soon led to the first reverse genetics technique: the RNP transfection method (32). A foreign gene, flanked by the 5’ and 3’ non-coding regions of the influenza A NS gene, under control of a T7 promoter was incubated with the purified NP, PB1, PB2 and PA proteins and the T7 RNA polymerase. This led to the in vitro transcription of the gene into viral like RNA and the assembly of vRNPs. These RNPs, containing the foreign gene sequence were transfected into cells co-infected with influenza helper virus, to provide the remaining vRNPs. The recombinant RNA is amplified, expressed, and packaged into progeny virus particles, which can be passaged several times. The drawback of this system is that a mixture of helper virus and recombinant virus is obtained and selection is needed for the production of pure, recombinant virus. Without selection, the recombinant virus is usually lost after several passages (32). A more advanced system, first described by Neumann and colleagues, no longer made use of in vitro transcription of vRNA. Instead, the vRNPs were reconstituted in cells by a DNA-dependent RNA polymerase I (Pol I) driven system (36). Pol I is abundantly present in cells and transcribes ribosomal RNA, which, like vRNA does not contain a cap or a poly A tail. The gene of interest, present in a DNA plasmid between the Pol I promoter and terminator, was transfected to cells and subsequently transcribed to vRNA by the Pol I. Packaging of the vRNA into vRNPs still required the polymerase and NP proteins. Provision of these 4 essential proteins occurred through the infection with a helper virus or by transfection of plasmids expressing these proteins, under control of a DNA-dependent RNA polymerase II (Pol II) driven promoter. For generation of recombinant influenza virus a helper virus was still required. Nevertheless, selection was still required for the isolation of pure recombinant virus. At that time, selection systems for only six of the eight RNA systems had been described. For two of the influenza genes, PB1 and PA, it was not possible to generate a genetic mutant at that time (reviewed in (37)). As selection techniques were elaborative, a new, selection-free method would be an advantage.
A plasmid only system

A breakthrough in the generation of recombinant influenza viruses came in 1999, with the development of the first, “plasmid only” rescue system for influenza. This first generation of plasmid only reverse genetics consisted of 17 plasmids: 8 plasmids containing the viral genome and 9 expression plasmids for the production of the viral proteins (38, 39). The expression plasmids used the pol II promoter for mRNA and protein expression, resulting in 9 viral proteins. The M1 and M2 proteins were synthesized form different plasmids. NS1 protein was not produced, since it is a non-structural protein that is not necessary to generate new progeny virus. To generate influenza vRNA, the positive sense sequence of the influenza gene segments was cloned behind a Pol I promoter. Two different methods used either the Pol I terminator (38) or the hepatitis delta virus ribozyme (39) for generating the correct 3’ end. The latter contains autocatalytic activity, which splices the RNA transcript at a defined site. After transcription by Pol I, these vRNA fragments contain the exact 3’ and 5’ viral ends that are recognized by the influenza polymerases and NP proteins which assemble to vRNPs. The aggregation of all viral proteins and the vRNPs near the membrane results in budding of recombinant progeny virus. Even though this system required the introduction of 17 plasmids into the same cell, high viral titers, up to 10^{7} PFU/ml culture supernatant could be recovered (37). With this new system, for the first time, virus could be generated starting from “scratch”, i.e. only DNA. In contrast to previous systems, now virus with changes in any of the 8 segments could be produced, including PB1 and PA, at least if the introduced mutations or changes did not affect important steps in the viral replication cycle. This system was easy to use, since only molecular cloning, DNA purification and transfection techniques were needed. Additionally, the need for selection could be circumvented, since no helper virus was used and hence only pure recombinant virus is generated. In fact, the minimal number of plasmids necessary for the generation of influenza virus was 12: only PB1, PB2, PA and NP were necessary as expression plasmids (38). Upon transfection of these 12 plasmids, a first round of transcription would be performed by the cellular Pol II resulting in the expression of PB1, PB2, PA and NP. The presence of these 4 proteins ensures further transcription and expression of the other viral proteins. However, expression of more than these 4 proteins from expression plasmids increased the viral titer that could be recovered.

The eight-plasmid system

In 2000 Hoffmann and colleagues described a new influenza A virus reverse genetics system, combining the Pol I and Poll II transcription units in one, ambisense Pol I/Poll II cassette (40). This system consisted in 8 plasmids carrying each one of the influenza gene segments which provide both the vRNA and the viral proteins from the same plasmid. In these plasmids negative sense vRNA was produced under control of a human Pol I promoter and murine Pol I terminator. This transcription unit was in turn flanked by a truncated immediate early Poll II promoter of human cytomegalovirus and a polyadenylation signal from bovine growth hormone (bgh) in the opposing direction of the Pol I
unit, such that positive sense mRNA was produced by Pol II (Figure 3.2). As Pol I terminator, only a truncated form of 33 bp of the murine Pol I terminator was used, as it was observed that nucleotide sequences in the full length 174 bp terminator could interfere with Pol II driven protein expression. (40). Interestingly, two different types of mRNA are produced with this system. A first mRNA species is transcribed by the cellular Poll II polymerase and contains sequences of the pol I promoter and terminator in its non-coding regions. A second type of mRNA is produced by the viral polymerases, from the negative sense vRNA, which in turn was produced by the Pol I polymerase. This second mRNA species does not contain additional 3’ and 5’ non-coding regions.

Figure 3.2 Schematic representation of the pol I/pol II transcription system for the synthesis of vRNA, mRNA and viral proteins. The cDNA of each of the eight viral gene segments, together with its 5’ and 3’ non coding regions (NCR) is inserted between the human pol I promoter (Pol I) and murine pol I terminator (T). This pol I transcription unit is flanked, in the opposite orientation, by the human cytomegalovirus pol II promoter (Pol II) and the bovine growth hormone gene polyadenylation signal (pA). Upon transfection of eight plasmids to a human cell line, transcription from the pol I promoter results in negative vRNA transcripts, which contain the viral NCRs. Pol II transcription yields positive sense mRNA that has a 5’ CAP and a 3’ poly-A tail. This mRNA is translated into at least 10 viral proteins.

Variations of the plasmid only system

Later, multiple systems have been reported which reduced the number of plasmids that need to be co-transfected. For example, in 2005, Neumann. et al. reported a 3 or 4 plasmid system consisting of 1 plasmid containing all 8 Pol I transcription cassettes combined with a plasmid containing the PB1, PB2 and PA Pol II expression cassette and a third plasmid containing the NP Pol II expression cassette (41). Alternatively, the Pol I cassettes could be separated on two plasmids, one carrying the HA and NA gene sequence and the other carrying the other 6 gene sequences. This system would be ideal for rapid generation of new vaccine strains. It has the advantage over the eight plasmid system that
fewer plasmids need to be transfected, which is required for the transfection of cell types such as Vero cells that have been approved for the production of vaccines but are difficult to transfect (42). An ultimate reduction in the number of plasmids resulted in a one-plasmid system, in which the pol I/pol II ambisense cassettes for PB1, PB2, PA and NP were combined with the Pol I cassettes of HA, NA, M and NS into one large plasmid (43). A major drawback of these 3-, 4- or 1-plasmid systems is that these systems use large plasmids, up to 23.6 kb in size, which are susceptible to intraplasmid recombination. This is most likely a consequence of the large number of repetitive structures present in these plasmids. Growth of large plasmids at room temperature (41), or the use of low copy number plasmids (43) have been reported to overcome this problem, however this makes it more difficult to produce these plasmids. For this reason the eight plasmid system, which uses smaller plasmids is currently still widely being used for the rapid production of recombinant influenza viruses.

Due to the species specificity of the Pol I promoter there is a restriction on the cell line that can be used for recombinant virus production with the plasmid-only based systems. The first systems that possessed a human Pol I promoter used HEK293T cells, as these cells are easily transfectable (38). However, these cells do not support multiple rounds of influenza replication, because they detach upon addition of high concentrations of trypsin to the cell culture, which is required for the cleavage of the HA protein, to generate infectious virus particles (44). In contrary, MDCK cells are commonly used to grow influenza virus, however, these cells have a very low transfection efficiency, which is undesirable when multiple plasmids need to be transfected into a single cell. Additionally, MDCK cells do not support transcription from a human Pol I promoter, making these cells not suitable for generation of recombinant influenza virus from plasmid only rescue system. Therefore, combination of these cell types in coculture was used to increase the yield of influenza viral titer (40). In this coculture, transfection of plasmids resulted in recombinant virus produced by a single round of virus growth in HEK cells, which after addition of trypsin was capable of replicating to high titers in MDCK cells. Plasmid-based transfection systems were also adapted to multiple cell types, by changing the Pol I promoter used. Cell types used, other than human cell lines, include monkey cells (Vero) (39, 41), canine cells (MDCK) (45) and avian cells (CEF, QT6) (43, 46). One study reported the generation of recombinant influenza virus using the human Pol I promoter in MDCK cells (47). This is the only exception to the species specificity of the Pol I promoter that was reported. To overcome the species specificity, a universally applicable plasmid-based reverse genetics system was designed using the bacteriophage T7 promoter for the expression of vRNAs instead of the Pol I promoter (48). This system is dependent on the expression of the T7 polymerase in the cells, which can be achieved by transfection of a plasmid expressing the T7 polymerase. Even though this system is still dependent on transfection of multiple plasmids, it allows the production of recombinant influenza virus in cell types from multiple species.

Next to the systems mentioned here, also reverse genetics 8 plasmid and 7 plasmid systems have been constructed for the generation of influenza B virus and influenza C virus respectively (49, 50).
A plasmid-free reverse genetics system

Recently, Chen et al. reported the generation of a plasmid-free reversed genetics system (51). By PCR, using primers that contain the sequence of the pol I promoter and terminator, they generated 8 PCR amplicons consisting each of 1 viral gene flanked by the pol I promoter and terminator. Similarly, 4 pol II PCR amplicons were generated containing the 3 polymerase genes and NP flanked by the pol II promoter and bgh poly A site. Transfection of these 12 PCR products into co-cultured HEK293T/MDCK cells resulted in detectable, albeit low levels or viable influenza A virus. This plasmid free system may be interesting when cloning difficulties are encountered. A drawback of this system is that internal primers in the influenza genes are needed to generate PCR amplicons. This requires sequence information, which may not always be available for new viral strains.

3.3 Applications of the reverse genetics system

A new strategy for the production of influenza vaccines

The plasmid based reverse genetics systems have become a useful tool for generating recombinant influenza viruses, which can be used for several purposes. By site-directed mutagenesis in a gene of interest, the function of a specific gene or a gene domain can be elucidated. Also for the generation of new vaccine strains this system has proven to be useful. Two different types of influenza vaccine are currently licensed and used for the prevention of seasonal influenza infection. Inactive influenza vaccines that are delivered by an intramuscular injection have been popularly used world-wide for the prevention of annual influenza epidemics. Alternatively, live vaccines are given by an intranasal route and have the advantage to stimulate a broader immunological response than the inactive vaccines providing cross-protective immune responses (52, 53). The production of both vaccines requires a reassortant vaccine strain that matches the current infection. The classical method for generating such a reassortant strain is based on the co-infection of a circulating strain with a high growth laboratory strain such as A/Puerto Rico/8/34 or with a live attenuated master strain and the selection of the reassortant of a particular 6:2 ratio, that possesses the NA and HA gene from the circulating strain and the other six genes from the master strain (54). The method is very laborious and time-consuming and may not meet the tight schedule for the production of a vaccine in a short period of time (55). In contrasts, the reverse genetic approach allows direct generation of the 6:2 recombinant vaccine strains without the need for selection, which can be achieved in a few weeks (56, 57). Until now seasonal influenza vaccines continue to be produced via the classical way. However, during the 2009 so called swine flu or Mexican flu pandemic, caused by the A/California/07/2009 H1N1v virus, reverse genetics has been successfully used, alongside the
classical reassortant based method, in an attempt to rapidly produce a vaccine before the pandemic reached its peak (57). In the end, both vaccines were manufactured and used for prevention of the swine flu pandemic. Due to biosafety concerns, classical reassorting techniques cannot be used for the generation of vaccine strains against highly pathogenic avian influenza viruses (HPAIV), as these vaccine viruses would still contain the polybasic cleavage site in HA, which is characteristic for HPAIV, and is responsible for their pathogenicity (57, 58). Removing a stretch of basic amino acids from the cleavage site of HA results in attenuation of the HPAIV (59) which is suited for use as a vaccine strain (58, 60). Several of this avian influenza vaccine strains have been used as seed strain for the production of avian flu vaccines (57) which have been tested in human clinical trials (61, 62).

Influenza as a recombinant viral vector vaccine for other pathogens

In addition, plasmid based reverse genetics systems have become interesting for the use of influenza as a recombinant viral vector expressing foreign proteins or peptides to induce immunity against a variety of diseases. Influenza is particularly interesting as viral vaccine vector, for several reasons. First, since no DNA intermediates are produced during viral replication, there is no fear for integration of viral DNA in the host genome, which could result in adverse mutations or cancer. Second, influenza is known to induce a strong humoral and cellular immune response, which is a requirement for a good vaccine. Another advantage of influenza vectored vaccines is that they may be used frequently. As a consequence of its continuously evolving NA and HA genes, changing in these proteins in the vaccine vector would result in a new vaccine, to which no immunity is present. A practical advantage of the use of influenza as viral vector, is the availability of large scale production, in embryonated chicken eggs. Additionally, since live attenuated influenza vaccines are already being used in the human population, it is not unlikely that other influenza vector based vaccines would be accepted for human use. And finally, the reverse genetics plasmid based systems have made influenza an easy to manipulate vector, as changes only need to be introduced in DNA plasmids. With the advent of reverse genetics techniques, several recombinant influenza viruses expressing foreign peptides or proteins have been created (for a review, see (63, 64). These have been used for providing protection in experimental animal models against a wide range of infectious diseases such as HIV (65, 66), tuberculosis (67, 68), or malaria (69-71) and even as an anti-tumor therapy (72, 73). Several different strategies have been used, with differences in the protein used to insert the foreign sequence and differences in the length of the insertion, and in the way the foreign sequence is expressed, leading to differences in attenuation of the resulting recombinant virus. Indeed, it has been shown that most of the insertions result in a virus that is viable, however, attenuated in vitro and/or in vivo, compared to the wild type virus. The foreign proteins or peptides are expressed either as fusions to one of the influenza proteins, or as a separate open reading frame in a bicistronic gene, or even as a completely independent genome segment. The proteins most frequently used for insertion of foreign sequences are HA, NA and NS and will be discussed in more
Determining the regions that allow insertion of foreign sequences, without affecting the protein function, has been critical for the construction of recombinant viruses.

**Hemagglutinin**

The influenza HA forms mushroom-shaped trimers on the surface of the virion. These consist of a globular head and a stem. HA is known for its induction of neutralizing antibodies and the antigenic structure of different HA molecules has been extensively studied. Five antigenic regions, named A to E, have been mapped to the HA protein (74, 75) (Figure 3.3). Antigenic site A is centered around a protruding loop containing residues 133 and 137 and 140–146; site B is centered on a loop of residues 155–160 and an α-helix at residues 186–197; site C comprises the surface around the bonded cysteine residues 52 and 277; site D is located near the interface between monomer subunits; and site E is near the bottom of the globular domain between sites A and C. Since HA is the major antigenic protein of the influenza virus, this protein is of particular interest for the insertion of foreign epitopes for the induction of antibody responses.

![Figure 3.3 Model of influenza HA antigenic structure.](image)

Foreign B cell epitopes have been inserted with success into sites B and E, resulting in antibody mediated protection against pathogens such as HIV (65, 66), *Pseudomonas aeruginosa* (77) and *Plasmodium falciparum* (71). The HA protein was not only successful for the induction of B cell responses; also introduction of foreign cytotoxic T lymphocyte (CTL) epitopes into antigenic site B or E induced an effective immune response against the foreign epitope (70, 71, 78, 79). A recombinant influenza virus carrying a CTL epitope of the rodent malaria parasite *Plasmodium yoelii* in its antigenic site E was shown to induce a CD8+ T cell response that was protective against *P. yoelii* (70). This was the first report showing that a recombinant influenza virus can induce a protective T cell response.
against a heterologous pathogen. Besides small B- and T-cell epitopes, also large polypeptides have been expressed as fusion proteins with HA. For example, Li and colleagues succeeded in fusing a fragment of 140 amino acids, representing the receptor binding domain of *Bacillus anthracis* PA protein to the HA protein, resulting in a functional HA and a recombinant virus which remained stable after multiple passages. The foreign protein was fused behind the fusion peptide of HA at the N-terminus of the HA1 domain; a region known to allow large insertions. Immunization of mice with plasmids encoding the recombinant HA and the recombinant virus induced protective antibody responses against both influenza and *Bacillus anthracis* (80).

**Neuraminidase**

The NA is the second major glycoprotein that forms spikes on the viral membrane. The gene encoding NA is 1,413 nucleotides long and codes for a protein of around 470 amino acids (11); however, differences in length occur between different subtypes and even within viruses belonging to the same subtype. NA is present as mushroom-shaped, homotetramers on the virion (11). It is built up of 4 different parts, which are, (I) a short highly conserved cytoplasmic tail, (II) a hydrophobic transmembrane domain, (III) a stalk region and (IV) a globular head which possesses the enzymatic activity (10). The stalk, the cytoplasmic domain and transmembrane domain are not essential for the neuraminidase to be active: globular head domains separated from the rest of the protein is still enzymatically active (81). The sequence of the stalk region is highly variable between viruses of different subtypes. Additionally, differences in stalk length due to deletions or insertions are frequently found in nature (82, 83). For example, deletions of about 20 amino acids in the stalk of NA are characteristic for the adaptation of influenza viruses from aquatic birds to domestic poultry, such as chickens (84, 85). These observations led to the idea that the stalk of NA might tolerate the insertion of additional, foreign sequences, which was confirmed by Castrucci and coworkers (86). Later, Luo and colleagues described that insertions of up to 41 amino acids in the stalk of NA did not abolish the formation of infectious progeny virus (87). Soon, this resulted in the development of the first influenza viral vector expressing a foreign epitope in NA. This vector, constructed by Castrucci and colleagues contained a CTL epitope of the lymphocytic choriomeningitis virus (LCMV) and protected mice against an otherwise lethal LCMV infection (88). Additionally they showed that insertions of up to 58 amino acids into the stalk are tolerated by NA, opening windows for insertions of large foreign epitopes; however, until now only short T cell epitopes have been reported as insertions into the NA stalk for generating influenza viral vectors. T cell epitopes inserted in the NA stalk have been reported to protect against a variety of infectious diseases, such as malaria (69), LCMV (88), Chlamydia (89), Herpes (90) and others. Additionally, recombinant influenza viruses expressing a foreign epitope in the NA stalk have been reported as an attractive candidate for anti-tumor vaccines. A recombinant influenza virus expressing a CTL epitope of the proto-oncogene HER-2 was shown to specifically induce CTLs against this tumor associated gene (72). Another study, which used a recombinant influenza viral vector expressing a CTL epitope of β-galactosidase in the NA stalk, showed that CTLs induced by this vector mediated the regression of established pulmonary metastasis in mice (91) and tumor clearance (92). Using a recombinant influenza virus containing the
OVA-derived K\(^2\)-SIINFEKL epitope in the neuraminidase stalk, Dolan and colleagues demonstrated that this epitope is rapidly presented at the cell surface by MHC-I molecules: the first peptide-MHC complexes were detected already 3h after infection (93).

HA is the major antigenic protein of influenza viruses, and substantially less antibodies are directed to the NA. This might explain why insertions in the stalk of NA has been almost exclusively limited to T cell epitopes (reviewed in (63)) and little insertions of foreign B cell epitopes for the induction of an antibody response have been reported. One study using the NA stalk for the insertion of the Flag epitope showed that this region is not suited for the induction of B cell responses. Mice immunized with the Flag expressing virus did not mount a detectable antibody response (94). Different approaches have been used for the insertion of larger foreign protein fragments into the NA gene segment. A first approach expresses a foreign protein from a bicistronic gene segment using a duplication of the NA 3’ noncoding region (95, 96). In contrast to the strategies described earlier, this approach does not produce a fusion protein, but rather results in the production of 2 separate proteins: a wild type influenza NA protein and a reduced but significant expression of a foreign protein. These recombinant influenza viruses harboring a bicistronic NA gene fragment however show a reduced growth rate \textit{in vitro}, which correlated with reduced expression of NA protein in infected cells (95). These recombinant viruses have been shown to effectively induce both B- and T-cell mediated immune responses to the foreign protein in mice (96). A similar strategy uses an internal ribosomal entry site (IRES) for the expression of a second protein (97). A last approach uses the foot and mouth disease virus (FMDV) 2A protease recognition site, between the foreign sequence and the NA sequence (98, 99). Upon translation, this sequence is self-cleaved, resulting in 2 separate proteins. This strategy has also been used for the generation of a reporter influenza virus, carrying the enhanced green fluorescent protein (eGFP) sequence in the NA gene segment (100).

\textbf{Non-structural protein 1}

A last gene that is commonly used for the insertion of foreign sequences is the influenza NS gene segment. This gene codes for the NS1 protein from collinear mRNA and the NEP protein from a spliced mRNA (101, 102). NS1 has a length of 230-237 aa, depending on the strain. The NS1 protein can be divided into three domains: an N-terminal RNA binding domain (aa 1 – 73) (103), a larger effector domain (aa 74 – 207) and a short C-terminal tail (23 to 30 aa in length). Full-length NS1 proteins are present in the cell as homodimers (104). NS1 is not a structural component of the virion but is present in very high levels in the infected cells. The non-structural NS1 protein is a non-essential accessory protein that has multiple functions during the viral infection cycle. The major function of the NS1 protein consists of antagonizing the anti-viral state in the host by inhibiting the production of IFN-\(\alpha\) and IFN-\(\beta\) and the antiviral effects of IFN-induced proteins. Additionally, NS1 also plays an import role in other aspects of the viral replication cycle, such as RNA replication, viral protein synthesis and general host-cell physiology (reviewed in (105)). The second protein, encoded from a spliced mRNA of segment 8 is the 121 aa long NEP (previously referred to as NS2). This protein, as the name says, is involved in the export of newly synthetized vRNP complexes from the nucleus (106). NEP consists of an N-terminal domain (aa 1-53) which binds to Exportin 1 (also known
as Chromosome Region Maintenance protein 1 (CRM1)) and a C-terminal domain (aa 54-121) that binds to influenza M1 protein, which in turn is bound to the vRNPs (107). Recent studies have suggested that NEP may have multiple functions during the influenza virus replication cycle. In addition to nuclear export of vRNPs, it has been demonstrated that NEP contributes to the viral budding process through interaction with a cellular ATPase. Furthermore, studies have demonstrated that NEP is involved in regulating the accumulation of viral RNA species, which might lead to a switch from viral transcription during early viral replication to the production of genomic vRNPs later in the replication cycle (reviewed in (108)). The NS gene is of particular interest for the insertion of foreign epitopes or proteins, because of several reasons. First, due to the small size of both NS1 and NEP, large sequences can be inserted in the gene segment (109). Second, since NS1 is non-essential, deletion of this gene (by replacing it with foreign sequences) does not affect the viability of the virus in IFN deficient systems (110). The same is true for truncations of NS1. In fact, viruses with NS1 deletions or truncated NS1 proteins are attenuated and replicate less efficiently in vivo (111); which might be an advantage, if one aims at constructing a live attenuated influenza viral vector. Mice infected with influenza viruses lacking (a part) of the NS1 protein, experience reduced or no illness as a results of a more robust host IFN response. As a consequence of this strong host immune response, T and B cell responses are efficiently induced (112, 113) which are effective in providing protection against a subsequent influenza challenge (111). Influenza viruses possessing a C-terminally truncated NS1 have been studied extensively for their potential as live attenuated influenza vaccine, for instance against highly pathogenic avian influenza viruses (114). Another advantage of the NS gene for inserting foreign sequences is that a functional NEP, which is essential for virus replication, can still be produced even when NS1 is changed, by introducing a FMDV 2A autoproteolytic cleavage site in between both coding sequences. Alternatively, NEP production is not affected when foreign sequences are inserted between nucleotides 57 and 528 of the NS gene, which corresponds to the NS1 coding region which is not present in NEP, due to splicing of the mRNA. For the expression of foreign epitopes or proteins from the NS gene similar strategies have been used as for the expression of foreign proteins from the NA gene (see above). These include fusions to the NS1 protein (115), insertion a caspase recognition site (109) or the FMDV 2A autoproteolytic cleavage site (116) between NS1 and the foreign protein, or alternatively, the insertion of an overlapping stop-start codon (UAAUG) between both coding sequences (117). A recombinant influenza virus which expresses a truncated NS1 protein (125 amino acids) fused to the FMDV 17-amino-acid self-cleaving 2A site and the 137 C-terminal amino acids of the HIV-1 Nef protein, was able to induce HIV-specific immune responses. Even though the virus was highly attenuated in vivo (it did not replicate in the mouse respiratory tract) it induced significant Nef-specific B cell and CD8+ T cell responses (116). The same approach, using a virus with a truncated NS1 protein of 125 aa expressing the Mycobacterium tuberculosis ESAT-6 protein (95 aa) was shown to induce a CD4+ Th1 immune response which was protective against M. tuberculosis in mice and guinea pigs (67, 68). The immune response could be enhanced by administering a booster vaccination with an influenza virus of a different subtype than the priming vaccination. Insertion of GFP in the NS gene results in the generation of a reporter virus which can be used to follow the course of in influenza infection in vivo and in vitro (109, 118).
Another interesting application uses recombinant influenza viruses for the expression of biologically active molecules, such as cytokines (117, 119). Kittel et al. generated a recombinant influenza virus with a truncated NS1 (aa 1-125) that encodes interleukin 2 (IL-2) in a bicistronic reading frame (117). Upon infection of mice with this virus, IL-2 is expressed and acts as an adjuvant enhancing the immunogenicity of the virus. This approach resulted in an enhanced CD8+ T cell immune response to the viral antigens. Similarly, the IL-2 coding sequence was added to the NS1 gene of the cold adapted influenza strain which is currently being used for the generation of live attenuated influenza vaccines (LAIV) (54, 120). The expression of IL-2 from this enhanced the immunogenicity of this LAIV in mice, resulting in enhanced mucosal IgA production and an enhanced CD8+ T cell response. This might form an interesting strategy for the improvement of the immunogenicity of LAIVs. The expression of immunomodulatory cytokines from a virus lacking the NS1 protein (delNS1) has also been shown to be an interesting anti-cancer tool. One of the functions of NS1 is to inhibit PKR. As a consequence, delNS1 viruses can no longer replicate in normal, PKR expressing cells, but are still replication competent in PKR-deficient cells. Activated ras, which can be found in malignant tumor cells, induces an inhibitor of PKR (121). Hence, cells expressing ras are permissive for delNS1 viruses, resulting in lysis of these tumor cells. This delNS1 virus was shown to repress tumor growth in a SCID mouse model (122). The oncolytic effect could be further enhanced by the expression of biologically active IL-15 from the NS gene (73). This immunomodulatory cytokine is known to activate tumor specific CTLs and NK cells (123), and the delivery of this cytokine specifically in tumor cells through a delNS1 virus might provide an interesting anti-tumor strategy.

**Influenza with additional gene segments**

A different strategy for creating recombinant influenza viruses is by adding an extra gene segment carrying a foreign gene to the virus. In fact, the first report of a reverse genetics technique using the RNP transfection method, generated such a virus expressing the chloramphenicol acetyltransferase (CAT) gene instead of the NS genes (32). Crucial for the generation of these recombinant viruses is the characterization of packaging signals required for incorporation of the influenza gene segments in the virion (124). Even though the process of packaging was not completely understood at that time, Luytjes et al. succeeded in incorporating a foreign gene in the influenza virion, by flanking the non-viral CAT sequence with the viral non-coding regions of the NS gene segment (32). Shortly after this first recombinant virus, Enami et al. reported the creation of the first influenza virus carrying 9 instead of the normal 8 gene segments (125). This 9th segment can be of use for generating bivalent vaccines against 2 pathogens, or against 2 different influenza strains, for instance, by incorporating an additional HA gene of a second influenza strain as was demonstrated by Gao et al. (126). Stability of the resulting recombinant viruses remains an important factor. For example, for the 8-segment influenza virus carrying the CAT coding sequence it was reported that CAT activity dropped after as little as 3 passages (32). In contrast, the 9-segment virus produced by Enami et al. stably replicated. This is most likely explained by the fact that the 9-segment virus has a selective advantage over the same virus with only 8 segments, because it carried an extra NS1 gene, which complemented for the loss of function of NS1 in the parental, 8-segment virus (125).
3.4 Conclusion

Influenza viruses are capable of inducing strong and long-lasting humoral as well as cellular immune responses. Therefore, manipulated influenza viruses expressing selected antigens from a foreign pathogen have the potential to elicit humoral and cellular immune responses to this pathogen. This was made possible by the development of reverse genetics techniques. The generation of recombinant influenza virus relies on the expression of the polymerase and NP proteins, in the presence of the viral RNA genome. The plasmid only rescue systems form an attractive platform for manipulating influenza virus genes and generating recombinant viruses. These systems have been used for the generation of recombinant influenza viruses expressing a variety of B and T cell epitopes or polyproteins derived from foreign pathogens. Protection has been shown in several cases. For the efficient induction of a T cell response, a live viral vector, such as a recombinant influenza virus, is an ideal vehicle, since it delivers the T cell epitope inside the cell, a perquisite for its processing and presentation on the cell surface. It should be noted, that a prime/boost vaccination strategy might be necessary in most cases for induction of a strong long lasting immune response. In this respect, priming with recombinant influenza virus expressing malaria antigens, followed by a boost with recombinant vaccinia virus expressing the same antigen, provided the first evidence of such a strategy in animal models (70, 127). Alternatively, as a result of the constantly occurring antigenic drift, influenza strains with different antigenic characteristics, which do not cross-protect could be used for prime/boost vaccination strategies. Recombinant influenza viruses with truncated or deleted NS1 proteins form an even more attractive strategy for the use of influenza as recombinant viral vector for foreign epitopes. Indeed, as a result of their decreased anti-IFN effect, these viruses induce a potent immune response in the host, with minimal morbidity, due to in vivo attenuation.

The time between the emergence of a new (possible pandemic) influenza strain and the availability of a vaccine is crucial in the fight against a possible new influenza pandemic or epidemic. With the development of reverse genetics techniques for influenza viruses it has now become faster and easier to develop such vaccine strains for the production of vaccines. The limitations of the 8 plasmid system, which are, the transfection of multiple plasmids into one cell and the use of a cell line not approved by the FDA for vaccine production (HEK293T cells) has already been tackled. By reducing the number of plasmids for transfection to 3 or even 1 it is possible to have high yield of recombinant influenza virus in the FDA approved Vero cell line (42). The reverse genetics system has already been used for the production of a vaccine strain against the 2009 Mexican flu, and will likely be used more frequently in the future for the production of influenza vaccine viruses.

Highly pathogenic H5N1 viruses are lethal to human and embryonated chicken eggs and therefore, vaccines against these viruses cannot be produced by traditional methods. The reverse genetics system made it possible to replace the cleavage site of the HPAIV HA protein with that of less pathogenic avian strains. These viruses are now safe to be handled by human and are no longer lethal, when produced in embryonated chicken eggs (128).
3.5 References


Part 2

Aims and objectives
Aims and objectives

Infection with respiratory syncytial virus (RSV) is a major cause of severe pneumonia and bronchiolitis in new-born babies. Preventive measures available for this pathogen consist of monthly injections with monoclonal antibodies directed against RSV. However, due to its high cost, this treatment is only routinely being applied to children having an increased risk of developing severe RSV-induced disease. In addition, the protection provided by this treatment is partial and only temporary (1). Therefore, there is an urgent need for effective preventive measures against RSV. A vaccination that protects infants from becoming infected with RSV would be an ideal solution.

Strategies for vaccine development explored in the past mainly involved the use of live attenuated RSV or subunit vaccines. However, these vaccines were not suitable as a vaccine for use in infants because they were either not sufficiently attenuated or did not provide a significant level of protection (2). Therefore, the main aim of this thesis is to explore novel strategies for developing a safe and effective RSV vaccine.

The level of RSV neutralizing antibodies has been shown to correlate with protection against RSV infection in experimentally infected adults (3). In addition, high levels of maternally derived antibodies correlate with protection in infants (4) and passive administration of antibodies reduces the risk of acquiring severe RSV-induced disease (1). Therefore, the first approach explored in this research aims at inducing a significant level of RSV neutralizing antibodies (Chapter 4). To achieve this, selected peptides of the RSV fusion protein were genetically fused to virus-like particles consisting of Hepatitis B virus core protein monomers, which results in the presentation of the peptide in a highly repetitive manner to the immune system.

An increasing number of data supports the rationale that, for an efficient vaccine against RSV, the induction of a T cell immune response will be mandatory. In RSV infected infants, the increase in RSV specific CTLs coincides with convalescence, indicating a role for CD8$^+$ T cells in clearing the virus from the lungs (5). Moreover, reports have demonstrated that fatal cases of RSV infections are characterized by high viral titers and near absence of pulmonary infiltration of T cells or the cytokines they produce (6). In addition, the induction of a CD4$^+$ Th2 immune response by vaccination has been shown to lead to exacerbation of disease upon a subsequent RSV infection (7). The induction of a CD8$^+$ T cell response has been shown to redirect this harmful response to a more balanced immune
response in mice (8). Therefore, the second vaccine approach described in this research explored the possibility of inducing an RSV specific CTL immune response by using a recombinant influenza virus that expresses an RSV CTL epitope. The protective efficacy of this vaccine against an RSV infection was evaluated in vivo in BALB/c mice (Chapter 5).

Since the recombinant influenza virus is a live, replicating virus used as vaccine vector a last aim of this research was to develop a safe variant of this vaccine that is harmless to the mice, yet still potently induces immunity against an RSV infection. We explored the use of passive anti-M2e antibody treatment prior to infection with the recombinant influenza virus (Chapter 5). In addition, two strategies for developing an attenuated variant of the influenza virus were assessed (Chapter 6). Moreover, one of these attenuated viruses was engineered to express GFP protein in the infected cells. The possibility to use this virus as an in vitro and in vivo reporter virus was examined (Chapter 6).

References

Part 3

Results
Chapter 4
Design of a virus-like particle based HRSV vaccine

Author contributions: Xavier Saelens, Bert Schepens and Sarah De Baets designed and discussed the experiments. Sarah De Baets performed all the experiments.
4.1 Introduction

Despite decades of research, there is currently no licensed safe and effective HRSV vaccine available. Throughout the years, several different approaches have been developed, however, none were successful, due to different reasons. First attempts, with formalin-inactivated whole HRSV as a vaccine, were only poorly protective and even resulted in exacerbated disease upon a subsequent HRSV infection (1). Later attempts with subunit vaccines consisting of purified F protein (PFP-1, PFP-2 and PFP-3) or the conserved central domain of the G-protein fused to the albumin-binding domain of streptococcal G-protein (BBG2Na) were discontinued for several reasons. The PFP-3 vaccine was tested in a clinical phase III trial in children 1 to 12 years of age with cystic fibrosis, but even though the vaccine was well tolerated and induced an increase in neutralizing HRSV antibodies, this response was not associated with significant protection (2). A clinical phase III trial with the BBG2Na vaccine had to be stopped due to unexpected adverse events (3). A different strategy consisted of the production of live, attenuated virus vaccines by cold-adaptation and chemical mutagenesis of the virus. The initial live attenuated viruses were further modified through genetic engineering, by deletion of non-essential genes or adding additional attenuating mutations. One such virus, rA2cpts248/404/2030/ΔSH (MEDI-559) is currently being tested in a phase I/IIa trial in 5 to 24 months old children (4). Despite the fact that MEDI-559 has been shown to be immunogenic in a small scale trial in infants and has an acceptable safety profile (5), stabilization of the temperature sensitive phenotype seems to be difficult, due to the emergence of additional mutations (4). A difficult challenge regarding the development of a live attenuated vaccine remains to find a balance between under- and over-attenuation, resulting either in an unfavorable safety profile or in insufficient protection. Recent progress in the development of an HRSV vaccine include the use of chimeric viruses and replication-competent and –incompetent vectors (Table 1.3; reviewed in (6)). Due to problems with stability, safety and potency in the above mentioned attempts, the search for an HRSV vaccine is still ongoing.

Antibodies induced upon an HRSV infection are mostly short lived and are poorly protective against reinfections. Despite the defective natural immunity induced by an HRSV infection, neutralizing antibodies play an important role in protecting against an HRSV infection. In animal models, HRSV F- and G-protein can induce long lived protection through neutralizing antibodies (7, 8). In human, high titers of HRSV neutralizing antibodies tend to correlate with protection of adults against an experimental HRSV challenge (9), although the level of preexisting antibodies is not fully predictive of susceptibility to HRSV infection. Also in children the amount of HRSV neutralizing antibodies inversely correlates with susceptibility to severe HRSV infection (10, 11). The observation that neutralizing
antibodies can provide a substantial protection against HRSV infections was a rational for the
development of a prophylactic treatment consisting of an IgG preparation containing high levels of
HRSV neutralizing antibodies derived from human donors (Respigam) (12), which can substantially
reduce HRSV incidence in high risk children. Nowadays this treatment is replaced by a humanized
monoclonal antibody treatment Palivizumab (also known as Synagis; Medimmune, Gaithersburg,
Maryland, USA)(13). Efficacy studies with Palivizumab have shown that a high level of circulating
antibodies is sufficient to prevent lower respiratory illness in infants (13) (Table 1.1).
Together with the attachment G-protein, the fusion F-protein is a major glycoprotein expressed as a
homotrimer at the surface of the HRSV particle. The F-protein is responsible for the fusion of the viral
membrane with the host cell membrane. Each F-monomer is expressed as an inactive precursor,
which needs to be cleaved at two sites by a host furine protease to liberate the so called fusion
peptide (14) (see chapter 1, Figure 1.3). After insertion of the fusion peptide in the host membrane,
the metastable pre-fusion form is activated and a series of conformational rearrangements leads to
fusion of the viral membrane with the host membrane (15, 16). After fusion, the F-protein acquires a
highly stable post-fusion conformation, which is characterized by a 6-helix bundle (see chapter 1,
Figure 1.4). Neutralizing antibodies have been mapped to three major antigenic regions in the fusion
protein: antigenic site I, II and IV, by identifying amino acids that were altered in in vitro selected
antibody escape mutants (17). Sites II and IV are the best characterized. Antigenic site II (also called
site A) contains amino acids 255 to 275 and is the recognition site for palivizumab (18), the
humanized monoclonal antibody used prophylactically against HRSV. More recently the structure of
motavizumab, a more potent derivative of palivizumab, in complex with a synthetic peptide
revealed that the peptide that corresponds to residues 254 to 277 (NSELLSLINDMPITNDQKKLMSNN)
forms the recognition site for this monoclonal antibody. However, modeling suggested that besides
this peptide, the antibody also binds to other residues which are present on an adjacent monomer in
the F-protein trimer, indicating that this antibody recognizes a quaternary epitope (19). Antigenic site
IV (also known as site C) includes residues 422 to 438 (17, 18) and is the target site for monoclonal
antibodies such as 101F (20) and MAb 19 (18). For these two antigenic sites (site II and IV) it has
recently been shown that they are present in both the pre- and the post-fusion conformation of the
F-protein (21) and that the well-characterized monoclonal antibodies that bind to these regions, such
as palivizumab/motavizumab and 101F can bind to both conformations. Whether the pre-fusion or
the post-fusion state is the better vaccine candidate is still being discussed (21-23).
There is an increasing interest in the use of VLPs as vaccines for different pathogens. Currently,
licensed VLP-based vaccines for human use are available against Hepatitis B virus and human
papilloma virus (24, 25). VLPs consist of structural proteins without the incorporation of a viral
genome. VLPs mimic infectious viruses but are not capable of performing multiple rounds of
infection as infectious viruses, yet they retain the superb antigenicity of viruses, thereby potentially
yielding safer vaccines (24). As potential immunogens that mimic the optimally spaced array of
repetitive elements characteristic of virus surfaces, VLPs can elicit strong humoral response by
efficiently cross-linking the immunoglobulin receptors of B cells. Interaction of B-cells with VLPs,
therefore, efficiently induces antibodies against the highly repetitive and orderly displayed viral
capsid proteins. Thus, we believe that a VLP expressing fragments of the HRSV fusion protein might be an attractive strategy for designing an effective HRSV vaccine.

We explored a vaccination strategy against HRSV that is based on the induction of humoral immunity to small peptide-epitopes instead of complete HRSV G- or F-proteins. Since small peptides on itself are only poorly immunogenic, the peptides were presented at the surface of a virus-like particle (VLP) consisting of Hepatitis B virus core protein (HBc) monomers. HBc VLPs have been extensively evaluated by our research group, for the development of a universal influenza vaccine based on linking the ectodomain of the influenza A virus M2 protein (M2e) to the HBc monomer (26-29). By linking short peptides of the HRSV fusion protein to HBc, we aim at inducing a protective, antibody response directed against specific epitopes of the HRSV fusion protein.

4.2 Results

Selection of the epitopes

The fusion protein (F-protein) of HRSV is highly conserved between isolates of the HRSV A and B subgroups. Up to 91% of the amino acid sequence is identical between HRSV A and B isolates, rendering this protein a good candidate for the selection of epitopes that can induce cross-protective immunity against HRSV A and B subtypes. The selection of epitopes was mainly based on previously described neutralizing epitopes, categorized into three antigenic sites (17). Additionally, since the VLPs will be produced in *E. coli* the peptides were selected based on the absence of glycosylation sites. Known glycosylation sites are: Asn27, Asn70 and Asn500.

A first peptide that was selected is the sequence from amino acid (aa) 260 to 298 of the HRSV fusion protein. This highly conserved region contains antigenic site II (18) and forms the recognition site for two known neutralizing antibodies (47F and L4) (30, 31). Furthermore, this region also contains the residues that are substituted in mutants that are resistant to neutralization by Palivizumab antibodies (32, 33). Structural data confirm that this region lies at the outer surface of the protein and is easily accessible to antibodies in the post-fusion state. Based on homology models with the parainfluenza virus type 3 F-protein pre-fusion state it is suggested that this region is also accessible to antibodies when it is in the pre-fusion state (21).

The HBc carrier protein

To render the peptides immunogenic they were coupled to virus-like particles consisting of monomers of the hepatitis B virus (HBV) core (HBc) protein. This is a well-characterized carrier protein that has been successfully used for a variety of antigens (34-36); one such example is the
fusion of the influenza A virus M2 ectodomain (M2e) to the HBc monomer, which has been extensively studied by our research group (27-29, 37). HBc is a 183 amino acid long structural protein (Figure 4.1 A), that assembles into dimers which form the viral capsid of the HBV particle. The N-terminal part of HBc (amino acid residues 1-140) represents the assembly domain, as this region is necessary to form dimers that can self-assemble into capsids. The carboxy-terminal part (residues 150-183) of HBc is a protamine-like domain that is arginine-rich and binds the viral genome in the HBV particle (38). Upon expression of HBc in *E. coli*, this domain can bind to host RNA, which can function as an adjuvant to stimulate the immune response into a, for an HRSV vaccine desired, Th1 direction (39). However, since particles containing the C-terminal part are more complex to characterize and to purify, we also explored two shorter, C-terminally truncated particles consisting of either 149 aa (HBc-S) or 163 aa (HBc-I). An extra cysteine was engineered at the C-terminus of these shorter particles, to ensure stability of the particle, which in the full length protein is accomplished by the C-terminal cysteine at position 183. A similar approach to display M2e from an HBc-I based VLP has been reported recently by our group (68).

![Figure 4.1](image)

**Figure 4.1 Structure of hepatitis B core.** (A) Primary structure of hepatitis B core (HBc) protein. (B) Structure of an HBc monomer, residues 1-142. (Adapted from pdb file 1QGT). (C) Structure of an HBc dimer (pdb 1QGT). (D) Cryo-electron microscopy picture of an HBc particle. The major immunodominant region (MIR) is indicated in red (panel A and B); The cysteines at position 61 that form a disulfide bridge are indicated in blue (panel A and C)
The 3-dimensional structure of a HBc monomer is made up of alpha-helices that are connected by loops (Figure 4.1 B) (40). HBc dimers are characterized by a central bundle of 4 helixes that are connected by a disulfide bridge between the cysteine residues at position 61 in each monomer (Figure 4.1 C). The loops that connect the anti-parallel helices within an HBc monomer are oriented distal from the inner capsid core and thus are located at the top of the spikes that are formed by the bundle of 4 helixes in each HBc dimer. The tips of these spikes are known to be very immunogenic and hence form the major immunodominant region (MIR) of the VLP (Figure 4.1 B). Insertion of heterologous epitopes in these loops or replacement of parts of the loop by heterologous epitopes usually does not interfere with particle formation and has been exploited to create highly immunogenic recombinant VLPs (41, 42). Besides the MIR, we also explored addition of peptides at the N-terminal part of the HBc monomer. Because of the repetitive pattern in which the epitopes are presented, optimal stimulation of B-cells can be obtained both in T-cell dependent and independent manner (43).

Expression system

For heterologous expression of HBc in *E. coli*, the HBc coding sequence was cloned into the pLT32h vector, behind the Pl promotor of bacteriophage λ. Expression was performed in *E. coli* B. strain (BL21 CodonPlus) bacteria that stably contain the repression plasmid pICA2. At low temperature, protein expression is blocked by the temperature sensitive λcI857 repressor, present on the pICA2 plasmid. Protein expression can be achieved by raising the cultivation temperature to 42°C, which inactivates the λcI857 repressor. Additionally, expression may also be achieved at low temperature, by the addition of isopropyl-β-D-1-thiogalactopyranisid (IPTG). This molecule inhibits the expression of a lacI repressor from the Lac promotor. This leads to the activation of the expression of an anti-repressor of cI857, from the pN25/O2 promotor hereby no longer inhibiting expression of the HBc protein from the Pλ promotor (44).

Analysis of protein expression

A first series of three expression vectors was produced using the aforementioned F260-298 peptide. This epitope was cloned into the loop of the HBcS, HBcI and the full length HBc protein (HBcL), replacing amino acids 78-82, hereby aiming to produce particles with the F260-298 sequence protruding from the tips of the spikes of the VLPs (Table 4.1, n° 1-3).

For expression and purification, these three plasmids were each transformed to pICA2 BL21 *E. coli*. Protein expression was induced either at 28°C by the addition of IPTG or by raising the culture temperature to 42°C. After fractioning the bacteria by sonication, the soluble fraction was separated from the insoluble pellet fraction by centrifugation. Analysis of the induced HBc fusion proteins containing the F260-298 epitope, either by western blot or by SDS-PAGE revealed that all the protein
was present in the insoluble pellet fraction (Figure 4.2). No difference was observed between the two induction methods. Since re-solubilizing the protein is a difficult process with low success rate, we searched for a new strategy that would yield more soluble protein. No expression was observed for the HBcl-F\textsubscript{260-298}-Loop construct, but this was due to a technical issue. We could later confirm expression of this construct.

Table 4.1  Overview of the HBc constructs

<table>
<thead>
<tr>
<th>Number</th>
<th>Name of the construct</th>
<th>Epitope used</th>
<th>Position of the epitope in HBc</th>
<th>HBc variant used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HBcS-F\textsubscript{260-298}-Loop</td>
<td>F 260 - 298</td>
<td>Loop</td>
<td>Short</td>
</tr>
<tr>
<td>2</td>
<td>HBcl-F\textsubscript{260-298}-Loop</td>
<td>F 260 - 298</td>
<td>Loop</td>
<td>Intermediate</td>
</tr>
<tr>
<td>3</td>
<td>HBclL-F\textsubscript{260-298}-Loop</td>
<td>F 260 - 298</td>
<td>Loop</td>
<td>Long</td>
</tr>
<tr>
<td>4</td>
<td>N-F\textsubscript{260-298}-M2e-HBcs</td>
<td>F 260 - 298</td>
<td>N-terminus</td>
<td>Short</td>
</tr>
<tr>
<td>5</td>
<td>N-F\textsubscript{260-298}-M2e-HBcl</td>
<td>F 260 - 298</td>
<td>N-terminus</td>
<td>Intermediate</td>
</tr>
<tr>
<td>6</td>
<td>N-F\textsubscript{260-298}-M2e-HBcl</td>
<td>F 260 - 298</td>
<td>N-terminus</td>
<td>Long</td>
</tr>
<tr>
<td>7</td>
<td>N-F\textsubscript{234-303}-M2e-HBcl</td>
<td>F 234 - 303</td>
<td>N-terminus</td>
<td>Intermediate</td>
</tr>
<tr>
<td>8</td>
<td>N-F\textsubscript{234-485}-M2e-HBcl</td>
<td>F 234 - 485</td>
<td>N-terminus</td>
<td>Intermediate</td>
</tr>
<tr>
<td>9</td>
<td>N-F\textsubscript{420-441}-M2e-HBcl</td>
<td>F 420 - 441</td>
<td>N-terminus</td>
<td>Intermediate</td>
</tr>
<tr>
<td>10</td>
<td>HBcl-F\textsubscript{420-441}-Loop</td>
<td>F 420 - 441</td>
<td>Loop</td>
<td>Intermediate</td>
</tr>
</tbody>
</table>

**Figure 4.2**  SDS-PAGE and western-blot analysis show that chimeric HBc particles expressing F\textsubscript{260-298} in the loop precipitate upon sonication. Plasmids carrying HBcS-F\textsubscript{260-298}-Loop (1), HBcl-F\textsubscript{260-298}-Loop (2) or HBclL-F\textsubscript{260-298}-Loop (3) were transformed to BL21 *E. coli* and protein expression was induced using IPTG or enhancement of the growth temperature to 42°C. Not induced bacteria transformed with HBcS-F\textsubscript{260-298}-Loop were included as negative control. Protein expression was analyzed by SDS-PAGE (A) or western blot (B). (S = soluble fraction; P = pellet, insoluble fraction)
Next, the same $F_{260-298}$ epitope was fused behind the first methionine of all 3 versions of the HBc protein. The ectodomain of the influenza A virus M2 protein (M2e) was added between the $F_{260-298}$ epitope and the HBc sequence, in an attempt to enhance the solubility of the expression fusion protein (Table 4.1, n° 4-6). Fusion of this epitope to HBc had proven in the past to result in a soluble fusion product upon expression in *E. coli* (28). Analysis of the expressed fusion products however revealed that again all the protein was present in the insoluble pellet fraction (Figure 4.3). The addition of M2e to the fusion product did not result in more soluble protein.

![Figure 4.3](image)

**Figure 4.3** SDS-PAGE analysis shows that chimeric HBc particles expressing $F_{260-298}$ at the N-terminus of M2e-HBc precipitate upon sonication. Plasmids carrying N-$F_{260-298}$-M2e-HBcS (4), N-$F_{260-298}$-M2e-HBcl (5) and N-$F_{260-298}$-M2e-HBcL (6) were transformed to BL21 *E. coli* and protein expression was induced using IPTG or enhancement of the growth temperature to 42°C. Not induced bacteria transformed with HBcS-$F_{260-298}$-Loop were included as negative control. Protein expression was analyzed by SDS-PAGE. (S = soluble fraction; P = pellet, insoluble fraction)

### Comparison of different sonication buffers

Until now, sonication of all bacteria was performed in a sonication buffer (50mM TRIS, 25mM NaCl, 1mM EDTA – pH 8.0; Table 4.2, n° 1) that has been used in the past for sonication of M2e-HBc VLPs. Since optimizing of sonication conditions might result in more soluble protein we tested sonication buffers with different salt concentration or at a slightly higher pH (pH 8.5) (Table 4.2, n° 1-4).

The expression of the fusion proteins HBcl-$F_{260-298}$-Loop and N-$F_{260-298}$-M2e-HBcl was induced by addition of IPTG. A control construct, containing three copies of the M2e from the A/Chicken/Vietnam/35/2004 (H5N1) strain at the N-terminus of HBc was used as positive control. Analysis of the expressed fusion proteins, either by western blot or SDS-PAGE revealed the HBcl-$F_{260-298}$-Loop and N-$F_{260-298}$-M2e-HBcl VLPs are present in the pellet fraction under all tested conditions (Figure 4.4). In contrast, for the control construct, a large fraction of the expressed protein was present in the soluble fraction.
<table>
<thead>
<tr>
<th>N°</th>
<th>Buffer name</th>
<th>pH</th>
<th>Buffer composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TRIS Buffer 1</td>
<td>8.0</td>
<td>50 mM TRIS 1 mM EDTA 50 mM NaCl</td>
</tr>
<tr>
<td>2</td>
<td>TRIS Buffer 2</td>
<td>8.0</td>
<td>50 mM TRIS 1 mM EDTA 25 mM NaCl</td>
</tr>
<tr>
<td>3</td>
<td>TRIS Buffer 3</td>
<td>8.0</td>
<td>50 mM TRIS 1 mM EDTA 5 mM NaCl</td>
</tr>
<tr>
<td>4</td>
<td>TRIS Buffer 4</td>
<td>8.5</td>
<td>50 mM TRIS 1 mM EDTA 25 mM NaCl</td>
</tr>
<tr>
<td>5</td>
<td>Piperazine buffer</td>
<td>10.0</td>
<td>20 mM Piperazine 1 mM EDTA 50 mM NaCl</td>
</tr>
<tr>
<td>6</td>
<td>MES buffer</td>
<td>6.0</td>
<td>50 mM MES 1 mM EDTA 10 mM NaCl</td>
</tr>
<tr>
<td>7</td>
<td>Diethanolamine buffer</td>
<td>8.5</td>
<td>50 mM Diethanolamine 1 mM EDTA 25 mM NaCl</td>
</tr>
</tbody>
</table>

Figure 4.4 SDS-PAGE analysis shows that chimeric HBc particles expressing F<sub>260-298</sub> precipitate upon sonication using different sonication buffers. Plasmids carrying N-F<sub>260-298</sub>-M2e-HBcI (A) and HBcI-F<sub>260-298</sub>-Loop (B) and N-(VN_M2e)<sub>2</sub>-HBcI (C) were transformed to BL21 E. coli and protein expression was induced using IPTG. A control construct, containing three copies of the M2e from the A/Chicken/Vietnam/35/2004 (H5N1) strain at the N-terminus of HBc was used as positive control (C). The buffer used for sonication is mentioned above the figure: (1) 50mM TRIS, 5mM NaCl, pH 8.0; (2) 50mM TRIS, 25mM NaCl, pH 8.0; (3) 50mM TRIS, 50mM NaCl, pH 8.0; (4) 50mM TRIS, 25mM NaCl, pH 8.5; Protein expression was analyzed by SDS-PAGE. (S = soluble fraction; P = pellet, insoluble fraction)

Searching for new epitopes

After analyzing in silico models of the HRSV fusion protein we noted that the chosen epitope only consist of a fragment of a structural domain of the complete protein. This may have prompted misfolding of the protein, which might be an explanation for the insolubility of the VLPs. Therefore, we continued by selecting three new epitopes; this time taking into account the different structural domains of the F-protein.

A first epitope that was selected contains the sequence of amino acid residues 234 to 303. This epitope is slightly longer than the previously used epitope, however, in contrast to the first epitope, this epitope forms one structural domain within the F-protein, which might facilitate proper folding of the protein. As a second option, we chose to attach almost the complete F-protein sequence to HBc (F<sub>234-485</sub>). This sequence contains most of the known antigenic regions of the F-protein, which might result in high immunogenicity of the VLP. The coding sequence of both peptides, followed by
an M2e sequence, was inserted at the N-terminus after the first methionine of HBcI (Table 4.1, n° 7 & 8). As third option we chose again for a short peptide of the HRSV F-protein: the region from aa 420 to 441 \( F_{420-441} \) which contains antigenic site IV (45), the target site of several monoclonal antibodies such as MAb19 (18) and 101F (20). This peptide is highly conserved among isolates of HRSV and does not contain any glycosylation sites. This \( F_{420-441} \) sequence, followed by the M2e sequence was inserted at the N-terminus of the HBcI protein (Table 4.1, n° 9). Analysis of the produced proteins, upon expression in BL21 \( E. coli \) again revealed that the first two constructs \( (N-F_{234-303}-M2e-HBcI \) and \( N-F_{234-485}-M2e-HBcI \) did not result in any soluble fusion protein (Figure 4.5 & Figure 4.6). Analysis of the expressed proteins of the third new construct \( (N-F_{420-441}-M2e-HBcI) \) however revealed that a small fraction of the protein was in the soluble fraction upon induction with IPTG (Figure 4.7).

**Figure 4.5**  SDS-PAGE analysis shows that chimeric HBc particles expressing \( F_{234-303} \) precipitate upon sonication using different sonication buffers. Plasmid carrying \( N-F_{234-303}-M2e-HBcI \) was transformed to BL21 \( E. coli \) and protein expression was induced using IPTG. The buffer used for sonication is mentioned above the figure: (1) 50mM TRIS, 5mM NaCl, pH 8.0; (2) 50mM TRIS, 25mM NaCl, pH 8.0; (3) 50mM TRIS, 50mM NaCl, pH 8.0; Protein expression was analyzed by SDS-PAGE. (S = soluble fraction; P = pellet, insoluble fraction)

**Figure 4.6**  SDS-PAGE and western-blot analysis show that chimeric HBc particles expressing \( F_{234-485} \) at the N-terminus precipitate upon sonication using different buffers. Plasmid carrying \( N-F_{234-485}-M2e-HBcI \) was transformed to BL21 \( E. coli \) and protein expression was induced using IPTG (A & C) or enhancement of the growth temperature to 42°C (B & D). The buffer used for sonication is mentioned above the figure: (1) 50mM TRIS, 5mM NaCl, pH 8.0; (2) 50mM TRIS, 25mM NaCl, pH 8.0; (3) 50mM TRIS, 50mM NaCl, pH 8.0; (4) PBS. Protein expression was analyzed by western blot and SDS-PAGE. (S = soluble fraction; P = pellet, insoluble fraction)
Figure 4.7 Western-blot analysis shows that a small fraction of chimeric HBC particles expressing F\textsubscript{420-441} at the N-terminus is present in the soluble fraction. (A) Plasmids carrying N-F\textsubscript{420-441}-M2e-HBCl, N-F\textsubscript{260-298}-M2e-HBCl (as negative control) and N-(VN\_M2e\textsubscript{2})-HBCl (+) were transformed to BL21 E. coli and protein expression was induced using IPTG. Sonication was performed in buffer 1 (50mM TRIS, 5mM NaCl, pH 8.0). (B) Plasmids carrying N-F\textsubscript{420-441}-M2e-HBCl and N-(VN\_M2e\textsubscript{2})-HBCl (+) were transformed to BL21 E. coli and protein expression was induced using IPTG. The buffer used for sonication is mentioned above the figure: (1) 50mM TRIS, 5mM NaCl, pH 8.0; (2) 50mM TRIS, 25mM NaCl, pH 8.0; (3) 50mM TRIS, 50mM NaCl, pH 8.0; (4) PBS. Protein expression was analyzed by western blot. (S = soluble fraction; P = pellet, insoluble fraction)

Given the relative success we had with the F\textsubscript{420-441} epitope we also cloned this epitope into the immunodominant loop of HBCl (Table 4.1, n° 10). Unfortunately, for this construct we did not observe any protein in the soluble fraction after sonication (Figure 4.8). Since the best result was observed with N-F\textsubscript{420-441}-M2e-HBCl when a sonication buffer of pH 8.5 was used, we hypothesized that a change of pH in the sonication buffer might result in more soluble protein. To test this hypothesis we sonicated bacteria, that expressed either the F\textsubscript{420-441} epitope at the N-terminus in sonication buffers consisting of different buffering agents (Table 4.2, n° 4-7). However, the change in pH did not result in condition in which the majority of the protein is present in the soluble fraction (Figure 4.9).

Figure 4.8 Western-blot analysis of chimeric HBC particles expressing F\textsubscript{420-441} in the loop. Plasmids carrying HBCl-F\textsubscript{420-441}\_Loop, and N-(VN\_M2e\textsubscript{2})-HBCl (+) were transformed to BL21 E. coli and protein expression was induced by enhancing the growth temperature to 42°C or by the addition of IPTG, or both (indicated in the figure). Sonication was performed in buffer 1 (50mM TRIS, 5mM NaCl, 1 mM EDTA, pH 8.0). Protein expression was analyzed by western blot.
Figure 4.9 Western-blot analysis of chimeric HBc particles expressing F_{420-441} at the N-terminus. Plasmid carrying N-F_{420-441}-M2e-HBcI was transformed to BL21 E. coli and protein expression was induced by enhancing the growth temperature to 42°C. Sonication was performed in different sonication buffers, indicated in the figure: (1) 50mM TRIS, 5mM NaCl, 1 mM EDTA, pH 8.0; (4) 50mM TRIS, 25mM NaCl, 1 mM EDTA, pH 8.5. (5) 20mM Piperazine, 50mM NaCl, 1 mM EDTA, pH 10.0; (6) 50mM MES, 10mM NaCl, 1 mM EDTA, pH 6.0; (7) 50mM Diethanolamine, 25mM NaCl, 1 mM EDTA, pH 8.5; Protein expression was analyzed by western blot.

Since no condition could be found in which the fusion of an epitope of the HRSV F-protein to HBc VLPs resulted in soluble particles, a possible solution might be to search for different carrier proteins, which might result in a more soluble fusion product.

4.3 Discussion

Since humanized monoclonal antibodies directed to the HRSV F protein are a known correlate of protection and are capable of reducing HRSV-associated hospitalization (13), we thought that inducing an antibody response through vaccination might be a feasible strategy for preventing, or at least reducing disease incidence caused by HRSV. Earlier studies using purified F protein to induce an antibody response by vaccination were discontinued, because the proteins were not immunogenic enough to induce a strong antibody response in humans, which correlated with protection (2). Therefore, any novel strategy should be capable of inducing a strong immune response. We hypothesized that coupling of an HRSV fusion protein epitope to VLPs made up of the HBc monomers would be a good candidate vaccine. VLP vaccines generally consist of one or more structural viral proteins, without the presence of a viral genome. Hence these particles cannot replicate, and form a potentially safer vaccination strategy.

HBc monomers aggregate to form VLPs which have been shown to be very immunogenic, because of the highly repetitive pattern in which the foreign antigen is presented to the immune system, which allows both T-cell dependent and T cell independent stimulation of the immune system. These VLPs have been used with success in our lab, for the production of a universal influenza vaccine (27, 29, 37, 46), by coupling the influenza M2e to HBc. Applying the same strategy to HRSV epitopes seemed straightforward with little difficulties. However, the opposite turned out to be true. We experienced
difficulties in producing VLPs that did not precipitate. Upon separation of the soluble fraction from the precipitated fraction, after sonication, most of the VLPs containing HRSV derived peptides were present in the insoluble fraction whereas only a minor fraction of control M2e-VLPs precipitated. Having a soluble VLP is necessary for further purification processes, especially if one wants to produce this vaccine on a large scale. From the performed experiments it cannot be concluded whether VLPs are directly produced in insoluble inclusion bodies or whether the VLPs precipitate during sonication. We tried if changing the buffer solution in which the sonication was performed would change the outcome. Buffers with different pH were tested, however, this did not result in any VLP product that was highly soluble. When looking into the structure of the F protein, we realized that the first chosen peptide might experience difficulties to fold properly, since the peptide was only a part of a structural domain. Therefore we tried coupling several different fragments of the HRSV F protein to HBc. A variety of fragments, ranging from peptides of only 20 aa in length to almost the complete F protein were coupled to HBc; however, again this did not resolve the solubility problem. M2-VLPs are highly soluble, since most of these proteins were always present in the soluble fraction, after sonication. This brought us to the idea that fusion of insoluble HRSV peptides to the soluble M2e peptide, which in turn is fused to HBc might enhance the solubility of the VLPs. But again this did not result in any highly soluble VLP product. Also, changing of the position of the peptide in the HBc monomer to the N-terminus instead of insertion in the MIR did not change the solubility of the VLP product.

It remains unclear why VLPs with HRSV proteins precipitate so easily and the difference with M2e-VLPs is remarkable. The hydrophobicity of the selected peptides might play an important role in determining the solubility of the HBc VLP. When examining the hydrophobicity, using the Kyte & Doolittle prediction method (47) (Protscale, http://web.expasy.org/cgi-bin/protscale/protscale.pl) we noticed that, with the exception of some N-terminal amino acids, the M2e sequence is hydrophilic. Most of the HRSV F protein derived peptides however show some degree of hydrophobicity. The largest fragments used (F_{234-485} and F_{234-303}) show alternating hydrophobic and hydrophilic regions. Also the F_{260-298} peptide contains several hydrophobic regions. These hydrophobic regions might be involved in the precipitation of the VLPs expressing these peptides. The smallest peptide (F_{420-441}) however is mostly hydrophilic, with only a small hydrophobic sequence. This is comparable to the situation of M2e, but still this F_{420-441} peptide does not result in soluble VLPs. Therefore, other mechanisms might be involved in determining the solubility of the produced VLPs.

When inserting peptides or larger antigen fragments in HBc it is essential that these foreign sequences fold properly. A native folding of the inserted sequence is of utmost importance for the induction of a humoral immune response through vaccination. Especially when inserting foreign fragments in the HBc MIR, the structure of this loop might impose difficulties: the N- and C-terminal ends of the MIR are in close proximity and the same would be needed for the foreign sequence in order for it to fold properly. Insertions at the N-terminus require less stringent structural specifications, since the foreign sequence is only attached to HBc with its C-terminus. Nevertheless misfolding of the foreign sequence is still possible. From the experiments performed it is not possible to conclude whether the foreign sequences were folded properly and it is not excluded that
improper folding is the reason for the insolubility of the VLPs. Insolubility of the VLPs has been reported for other epitopes (48, 49). Additionally, the length of the epitope does not seem to be of importance, as epitopes of less than 10 residues long have generated insoluble VLPs. It has been reported that it is possible to purify the VLPs from the inclusion bodies using urea for denaturation followed by a refolding step. Purifying the HBC VLPs carrying HRSV F protein peptides from the inclusion bodies might be a solution for generating soluble HRSV peptide-VLPs (50). Alternatively, if the insolubility arises already during translation, than codon harmonization might improve folding (51). It is known that folding of a polypeptide occurs co-translationally and the speed of translation may influence the folding: increasing translation rate may result in fewer proteins that are correctly folded (52). In addition, the translation-elongation rate varies during the process of translation: because of differences in the number of genes present for each tRNA, certain codons are ‘slow’ while other are ‘fast’ (53, 54). Moreover, the available tRNA pools are different between species causing the same protein to be synthesized at a different rate in bacteria compared to eukaryotes (55). Therefore, the process of sequence harmonization changes the mRNA codons (without affecting the aa sequence) based on the available expression-host tRNA pools, to mimic the translation rate of the protein in the original organism (51). This was shown to result in more efficient folding of a luciferase, when expressed in bacteria.

As an alternative to the genetic fusion of heterologous protein sequences to HBC, chemical fusion might be used. Using a modified HBC particle that has been engineered with an acceptor lysine residue in the MIR, synthetically produced peptides can be linked to the particle (56). This strategy can overcome folding difficulties observed in the genetic fusion strategy. Controlling the number of peptides that become chemically linked to a HBC particle however is difficult and therefore this strategy is less suited for possible clinical applications.

Besides hepatitis B virus, other viruses have been used for producing VLPs expressing heterologous epitopes such as tobacco mosaic virus (57), alfalfa mosaic virus (58, 59), cowpea mosaic virus (60, 61) and potato virus X (62). VLP vaccines have been described for HRSV. In one study VLPs were constructed using Newcastle disease virus NP, M and hemagglutinin-neuraminidase (HN) proteins, with the ectodomain of HN replaced by the HRSV G protein ectodomain (63). These particles induced a robust HRSV specific antibody response which was protective in mice upon an HRSV infection. Two different groups studied the potential of an alfalfa mosaic virus expressing HRSV G protein derived epitopes (64, 65). These vaccines induced high levels of serum G protein specific antibodies in mice and in non-human primates, that were protective against an HRSV challenge. A novel virus-like particle based vaccine platform uses a modified alphavirus (Alphavax, North Carolina, USA). In contrast to previously described VLP vaccines, these Alphavax particles do not express heterologous antigen on the particle surface but rather carry a modified viral RNA genome that carries the coding sequence of the heterologous antigen (66). Therefore these particles are commonly referred to as VRPs (virus replicon particles). Vaccination with these VRPs results in abundant expression of the heterologous antigen in the infected cell, which induces both an antibody and a T cell immune response. Due to the absence of structural genes – these genes have been replaced by the coding sequence of the heterologous antigen – these VRPs can not replicate in vivo and are considered safe.
Such VRPs have been developed for different pathogens and phase I clinical trials have been conducted with success with vaccines against influenza, cytomegalo virus, HIV as well as cancer. Alphavax particles carrying the HRSV F protein have been shown to induce antibody and T cell immunity which is protective against an HRSV infection in mice and cotton rats (67). These examples suggest that VLP-based vaccine has potential as an HRSV vaccine strategy. Future research on the use of VLPs for HRSV vaccines, whether based on HBc or other viral proteins, might result in the development of a potent and safe HRSV vaccine.
4.4 Materials and methods

Construction of expression vectors.

For expression of recombinant fusion proteins, the pl-promotor driven pLT32H (44) expression plasmid containing the HBc sequence was used. The selected HRSV epitopes (see Table 4.1) were amplified from HRSV-derived cDNA using PCR techniques and subsequently inserted either in the immunodominant loop or at the N-terminus of HBc, using classical restriction digest and ligation. For cloning into the loop, the selected epitope was inserted in frame between the BstXI and NheI restriction site, replacing amino acids 78-82 of the HBc sequence. For N-terminal fusions, the corresponding HRSV epitopes were inserted between the NdeI and ClaI restrictions sites, replacing aa 1-4 of the original HBc sequence. For M2e-HBc fusions to an HRSV epitope, the corresponding HRSV epitopes was fused in frame with the M2e-HBc sequence after the initial methionine, using fusion PCR. The resulting fusion product was subsequently cloned into the pLT32H vector, using the NdeI and HindIII restriction sites.

Expression of chimeric HBc proteins.

For expression of the chimeric HBc proteins, the pLT32H-HBc plasmids carrying an HRSV epitope were transformed to BL21 Codon Plus bacteria (Stratagene) containing the transcription regulatory plasmid pICA2. A 3ml preculture grown over night at 28 °C in Luria broth medium was diluted 40x in 15ml fresh medium. The cultures were re-incubated at 28°C until an OD at 600nm was reached between 0.6 and 0.8. At this point, the expression of the chimeric proteins was induced either by the addition of 1 mM isopropyl 1-thio-β-d-galactopyranoside (IPTG), or by raising the culture temperature to 42°C. After 4h of incubation the bacteria were pelleted by centrifugation (6000 × g, 10 min, 4 °C). The bacterial pellet was resuspended in the desired sonication buffer, in 1/15 of the initial culture volume and sonicated. Bacterial debris was pelleted by centrifugation (20,000 × g, 1 h, 4 °C). The supernatant and pellet fraction were loaded separately on a 12% SDS-PAGE gel. The proteins on the gel were either stained directly with coomassie brilliant blue solution or transferred to nitrocellulose membrane and detected using an anti-hepatitis B-core IgG polyclonal antibody (HBP-023-9 Gentaur).
4.5 References


Chapter 5
Recombinant influenza virus carrying the human respiratory syncytial virus $F_{85-93}$ CTL epitope reduces HRSV replication in mice

Author contributions: Xavier Saelens, Bert Schepens and Sarah De Baets designed the experiments. The results were discussed by Xavier Saelens, Bert Schepens, Michael Schotsaert, Kenny Roose, Walter Fiers and Sarah De Baets. Sarah De Baets performed all the experiments. Koen Sedeyn assisted with isolating mice organs. Flow cytometry samples were measured and analyzed by Pieter Bogaert. The manuscript was written by Sarah De Baets and edited by Bert Schepens and Xavier Saelens.
Recombinant influenza virus carrying the RSV F85-93 CTL epitope reduces respiratory syncytial virus replication in mice

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5.1 Abstract

Human respiratory syncytial virus (HRSV) is the leading cause of lower respiratory tract infections in infants worldwide. Despite decades of research there is still no registered vaccine available for this major pathogen. We investigated the protective efficacy of a recombinant influenza virus PR8/NA-F85-93 that carries the HRSV CD8\(^+\) T cell epitope F85-93 in its neuraminidase stalk. F85-93-specific CTLs were induced in mice after a single intranasal immunization with PR8/NA-F85-93 virus, and these CTLs provided a significant reduction in the lung viral load upon a subsequent challenge with HRSV. To avoid influenza-induced morbidity, we treated mice with M2e-specific monoclonal antibodies before PR8/NA-F85-93 virus infection. Treatment with anti-M2e antibodies reduced the infiltration of immune cells in the lungs upon PR8/NA-F85-93 infection, whereas formation of inducible bronchus associated lymphoid tissue was not affected. Moreover, this treatment prevented body weight loss, yet still permitted the induction of HRSV F-specific T cell responses and significantly reduced HRSV replication upon challenge. These results demonstrate that it is possible to take advantage of the infection-permissive protection of M2e-specific antibodies against influenza A virus to induce heterologous CD8\(^+\) T cell mediated immunity by an influenza A virus vector expressing the HRSV F85-93 epitope.
5.2 Introduction

Human respiratory syncytial virus (HRSV) is the most important cause of acute lower respiratory infections in babies, especially when premature, and young children (1). Almost every child has been infected before the age of two years and will very likely be re-infected several times more with HRSV during its further life (2). It is estimated that each year over 30 million infections with HRSV result in acute lower respiratory infections (ALRI) in children younger than five years (3). Approximately 10% of children in this age group suffering from ALRI due to HRSV require hospitalization. Moreover, it is estimated that up to 200,000 children younger than five years die due to complications caused by HRSV, most of which occur in developing countries (3). Furthermore, severe HRSV infection during infancy has been associated with an increased incidence of recurrent wheezing in later childhood (4). Also in the elderly, HRSV causes pneumonia, bronchiolitis and exacerbation of chronic obstructive pulmonary disease, conditions that often lead to hospitalization and excess mortality in this age group (5).

Despite the disease burden caused by HRSV, no licensed HRSV vaccine is currently available. The development of a safe vaccine is difficult, since natural HRSV infections occur at very young age and do not provide long lasting protective immunity. The inability of natural infections to evoke protective immunity in the absence of significant antigenic drift, might in part be attributed to the ability of HRSV to evade the host immune response at different levels (reviewed in (6)). The main mechanism for evasion of the host innate immune response by HRSV is the inhibition of type I interferon (IFN) production and IFN-associated genes. The HRSV genome encodes two non-structural (NS) proteins, NS1 and NS2, that collaborate to suppress both the synthesis and the function of type I IFN, through the transcription factors IFN regulatory factor 3 (IRF-3) and signal transducer and activator of transcription 2 (Stat-2) (7, 8). This suppression of the type I IFN response contributes to the inhibition of CD8+ and CD4+ T cell responses (9, 10).

A clinical trial with a formalin-inactivated HRSV virion-based vaccine (FI-RSV) in the 1960’s did not evoke protective immunity but led to enhanced disease upon infection (11). A possible explanation for this adverse response could be that the FI-RSV vaccine strongly skews the immune response in an undesired allergic-like Th2 direction, which leads to enhanced infiltration of eosinophils and neutrophils into the lungs upon HRSV infection, causing severe lung damage. Such a strong Th2 response blunts the CD8+ T cell response, thereby compromising viral clearance from the lungs (12). Since that fatal trial, it is generally believed that HRSV vaccines that induce a strong Th2 biased immune response should be avoided.

Past attempts to produce an HRSV vaccine focused mainly on inducing neutralizing antibody responses. However, it has been suggested that an antibody response might not be sufficient for protection (as reinfections occur throughout life) and that a vaccine that elicits both an antibody and a T cell response might be more effective (13). Multiple reports have consistently demonstrated that fatal or severe lower respiratory tract HRSV infections are characterized by high viral titers and near absence of pulmonary infiltration of T cells or the cytokines they produce (14). Moreover, a possible
role for T cells in the clearance of HRSV is supported by the observation that viral clearance from the lungs occurs once a potent T cell response is induced (15, 16). Mouse studies have indicated that both CD8+ and CD4+ T cells are essential for the clearance of HRSV (17, 18). In addition, CD8+ T cells have been shown to mediate protection in animals immunized with several candidate HRSV vaccines, such as BCG-RSV (19).

Therefore, we hypothesized that priming for an HRSV CD8+ T cell response might be an attractive strategy for an HRSV vaccine. These primed CD8+ T cells could promote rapid clearance of the virus from the lungs and potentially prolong HRSV-specific T cell memory. It has been shown that priming for an HRSV KdM282-90-specific CD8+ T cell memory response abrogates the induction of an undesirable Th2 response in a model of Fl-RSV primed mice (12, 20, 21). However, Ruckwardt et al. reported that an augmented response to the dominant KdM282-90 epitope exacerbated illness upon HRSV infection, whereas mutating the dominant KdM282-90 epitope enhanced the response to the subdominant D8M187-195 epitope with significantly less illness upon HRSV infection (22). Moreover, also CD8+ T cells specific for the subdominant KdF85-93 epitope can reduce lung eosinophilia if the total number of F85-93-epitope specific CD8+ T cells is increased early after HRSV infection (23). These results suggest that a vaccination strategy that induces an immune response to a subdominant HRSV cytotoxic T lymphocyte (CTL) epitope, such as the KdF85-93 epitope, could contribute to viral clearance without exacerbating illness.

Here, we used a recombinant influenza virus as a live viral vector for mucosal delivery of the HRSV KdF85-93 CTL epitope. Influenza virus is an interesting vaccine vector candidate because it induces both humoral and cellular immune responses (24) and because it can be modified by reverse genetics (25). We produced recombinant A/Puerto Rico/8/34 influenza virus carrying the HRSV F85-93 CTL epitope in the stalk of the neuraminidase and tested its protective efficacy against HRSV in BALB/c mice. We show that F85-93-specific CTLs are induced in the mice upon a single intranasal immunization with PR8/NA-F85-93 virus and that these CTLs were associated with a significant reduction in the lung viral load upon HRSV challenge. We further optimized the vaccination strategy by passive administration of IgG2a monoclonal antibodies directed against the extracellular domain of influenza matrix protein 2 (M2e) to suppress morbidity associated with PR8/NA-F85-93 virus infection.

5.3 Results

Generation of recombinant influenza viral vector for delivery of an HRSV F CTL epitope.

To evaluate whether a recombinant influenza virus harboring an HRSV CTL epitope can provide protection against HRSV, we used a reversed genetics system for PR8 influenza (25) to generate the
following: (i) a virus containing the H-2d-restricted F85-93 CTL epitope (KYKNAVTEL) of the HRSV fusion protein (PR8/NA-F85-93 virus); (ii) a virus with an H-2d-restricted CTL epitope (IYSTVASSL) of the influenza virus hemagglutinin HA518-526 (PR8/NA-HA518-526 virus). These F- and HA-derived CTL epitopes were cloned in the neuraminidase (NA) coding sequence as replacements of part of the NA-stalk by replacing amino acids (aa) 65 to 71 with a 15-aa sequence containing the CTL epitope extended with the 3 naturally flanking amino acids both C- and N-terminally (Figure 5.1 A). Both viruses could be rescued and were further subcloned by two rounds of limiting dilution. The presence of the epitope in the viral genome was confirmed by RT-PCR followed by sequence analysis (Figure 5.1 B).

Figure 5.1 Characterization of recombinant PR8/NA-F85-93 and PR8/NA-HA518-526 influenza viruses. (A) Schematic representation of wild type and mutant neuraminidases. CT, cytoplasmic tail; TM, transmembrane domain. (B) RT-PCR of a NA fragment containing the insertion site of the epitope: a band shift from 170 to 207 bp is seen when the epitope is inserted. (C) Neuraminidase activity assay on live, purified virus. Fluorescence of the cleaved MUNANA substrate was measured every 2 min during 3 h. (D) Plaque phenotypes of the PR8/NA-F85-93 and PR8/NA-HA518-526 influenza viruses do not differ from the wild type virus plaques in an MDCK plaque assay. (E) In vitro growth kinetics. MDCK cells were infected with a multiplicity of infection of 0.001 of wild type PR8, PR8/NA-F85-93 or PR8/NA-HA518-526 virus. A sample was taken at 0, 4, 8, 12, 24 and 48 h post infection. The viral titer in the samples was determined by TCID₅₀ assay.
To test whether insertion of the epitopes affects the activity of the NAs \textit{in vitro}, we performed a neuraminidase activity assay on whole, purified virus (Figure 5.1 C). The number of virions was normalized by using equal amounts of hemagglutination units of the three viruses that were compared. Both PR8/NA-F\textsubscript{85-93} virus and PR8/NA-HA\textsubscript{518-526} hydrolyzed the MUNANA substrate at a slower rate than NA from wild type PR8 virus, suggesting a lower specific activity of both mutant NAs. The activity of NA-F\textsubscript{85-93} was 40\% of the activity of the wild type NA, whereas the activity of NA-HA\textsubscript{518-526} was even lower (15\% of the activity of wild type NA). In a plaque assay the plaques of wild type PR8, PR8/NA-F\textsubscript{85-93} and PR8/NA-HA\textsubscript{518-526} virus were similar in size (Figure 5.1 D).

To determine the \textit{in vitro} viral growth kinetics, MDCK cells were infected with a multiplicity of infection of 0.001 of wild type PR8 virus, PR8/NA-F\textsubscript{85-93} virus, or PR8/NA-HA\textsubscript{518-526} virus. At various times after infection the viral titer in the supernatant was quantified by TCID\textsubscript{50} assay. The growth kinetics of PR8/NA-F\textsubscript{85-93} virus and PR8/NA-HA\textsubscript{518-526} virus resembled that of wild type PR8 virus (Figure 5.1 E).

**Figure 5.2** PR8/NA-F\textsubscript{85-93} virus infection induces F\textsubscript{85-93} specific CTLs in mice. BALB/c mice (6 per group) were infected with 5 \times 10\textsuperscript{3} PFU of PR8/NA-F\textsubscript{85-93} or PR8/NA-HA\textsubscript{518-526} Virus. Spleens were isolated 10 days post infection and stimulated with HRSV F\textsubscript{85-93} peptide (panels A, B and C) or influenza NP\textsubscript{155-163} peptide (panel D). After restimulation with HRSV F\textsubscript{85-93} peptide IFN\gamma production in splenic F\textsubscript{85-93}-specific CD\textsuperscript{8\textsuperscript{+}} T cells was determined with (A) ELISPOT assay and (B) flow cytometry. (C) Representative dot plots showing IFN\gamma positivity in splenic CD\textsuperscript{8\textsuperscript{+}} T cells, after restimulation with HRSV F\textsubscript{85-93} peptide. (D) Splenocytes were restimulated with influenza NP\textsubscript{155-163} peptide. The percent of NP\textsubscript{155-163} specific CD\textsuperscript{8\textsuperscript{+}} T cells was determined by flow cytometry.
PR8/NA-F\textsubscript{85-93} virus infection induces HRSV F\textsubscript{85-93}-specific cytotoxic T lymphocytes.

We next investigated whether F\textsubscript{85-93}-epitope specific CTLs can be retrieved from mice that had been exposed to PR8/NA-F\textsubscript{85-93} virus. BALB/c mice were immunized with 5 x 10\textsuperscript{3} PFU of PR8/NA-F\textsubscript{85-93} virus or, as a control, PR8/NA-HA\textsubscript{518-526} virus, and spleens were isolated 10 days later. F\textsubscript{85-93}-epitope specific IFN\gamma ELISPOT analysis and intracellular cytokine staining illustrated that F\textsubscript{85-93}-epitope specific CTLs were induced in mice that had been exposed to PR8/NA-F\textsubscript{85-93} virus but not in mice that had been exposed to PR8/NA-HA\textsubscript{518-526} virus (Figure 5.2 A-C). No significant difference in response to the influenza specific NP\textsubscript{155-163} epitope was detected (Figure 5.2 D).

To compare the pathogenicity of PR8/NA-F\textsubscript{85-93} and PR8/NA-HA\textsubscript{518-526}, we infected BALB/c mice with 10\textsuperscript{3}, or 10\textsuperscript{4} PFU of wild type PR8 virus, or 10\textsuperscript{3}, 10\textsuperscript{4} PFU of PR8/NA-F\textsubscript{85-93} or PR8/NA-HA\textsubscript{518-526} virus. Interestingly, both PR8/NA-F\textsubscript{85-93} and PR8/NA-HA\textsubscript{518-526} virus caused less morbidity than wild type PR8 virus (Figure 5.3 A-C). A tenfold higher inoculum dose of PR8/NA-F\textsubscript{85-93} and PR8/NA-HA\textsubscript{518-526} was needed to cause morbidity resembling that caused by wild type PR8 virus (approximately 30% bodyweight loss). PR8/NA-HA\textsubscript{518-526} was slightly more pathogenic than PR8/NA-F\textsubscript{85-93} (Table 5.1). Splenocytes were isolated 14 days after infection with these different doses and cellular responses were analyzed using an F\textsubscript{85-93}-peptide specific IFN\gamma ELISPOT assay. We observed F\textsubscript{85-93}-epitope specific CTLs in mice that had been exposed to PR8/NA-F\textsubscript{85-93} virus. The number of spots, reflecting the number or F-epitope specific T cells, correlated with the viral dose used for inoculation (Figure 5.3 D). Interestingly, the number of IFN\gamma spots after infection of mice with as little as 100 PFU of PR8/NA-F\textsubscript{85-93} was similar to that after infection with 1 x 10\textsuperscript{6} PFU of HRSV. This is most likely due to the fact that PR8/NA-F\textsubscript{85-93} virus is able to replicate in mice, whereas the mouse is far less permissive for HRSV.

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<th>Table 5.1</th>
<th>Survival of BALB/c mice infected with different doses of PR8/NA-F\textsubscript{85-93} or PR8/NA-HA\textsubscript{518-526} or with wild type PR8 virus</th>
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Immunization with PR8/NA-F_{85-93} virus reduces HRSV replication in challenged mice.

Next, we evaluated the ability of the PR8/NA-F_{85-93} virus to protect mice against an HRSV challenge. Eight-week-old BALB/c mice received a single intranasal immunization with PR8/NA-F_{85-93} virus, PR8/NA-HA_{518-526} virus, HRSV, or PBS. Ten days after the immunization, the spleens of the mice were isolated and splenocytes were stimulated for 12 h with the F_{85-93} peptide (KYKNAVTEL) for IFNγ ELISPOT analysis. F_{85-93}-epitope specific CD8^+ T cells were activated in mice exposed to PR8/NA-F_{85-93} and to a lesser extent in HRSV-exposed mice, but no F_{85-93}-epitope specific splenocytes were observed in the control groups (PR8/NA-HA_{518-526} and PBS) (Figure 5.4 A). Four weeks after immunization, the mice were challenged with 1 x 10^6 PFU of HRSV-A2. Lung homogenates were prepared four days after infection and the HRSV titer was determined by plaque assay. The mice that had received the PR8/NA-F_{85-93} virus as a T cell vaccine had a significantly lower HRSV lung titer than the control groups (PR8/NA-HA_{518-526} and PBS). Median lung titer was 19- and 31-times lower than in mice immunized with PR8/NA-HA_{518-526} or PBS, respectively, indicating that the induced CTLs reduced...
HRSV replication in the challenged mice (Figure 5.4 B). Remarkably, we repeatedly observed that mice that were vaccinated with PR8/NA-HA_{518-526} generally had a lower pulmonary HRSV titer than PBS treated mice. This could indicate that influenza induces a pathogen independent immune response that can reduce HRSV replication.

![Figure 5.4](image)

**Figure 5.4** Immunization with PR8/NA-F_{85-93} virus reduces HRSV replication in challenged mice. BALB/c mice were infected with the indicated viruses or with PBS and then challenged intranasally with 1 x 10^6 PFU of HRSV-A2. (A) Spleens were isolated 10 days after infection, restimulated ex vivo with the F_{85-93} peptide, and the number of F_{85-93}-epitope specific splenocytes was counted by IFNγ ELISPOT assay. (B) Lung HRSV titers were determined by plaque assay 4 days after HRSV challenge. Statistical significance was determined by using a two-sided Mann-Whitney U test; ** p < 0.01.

Anti-M2e antibodies reduce infiltration of immune cells in the lungs after PR8/NA-F_{85-93} challenge without impairing iBALT formation.

We investigated whether we could reduce the morbidity induced by the PR8 vector but retain F_{85-93}-specific CD8^+ T cell induction and protection against HRSV infection. We previously described a universal influenza A vaccine based on M2e (27), and recently reported that this vaccine prevents morbidity, but in accordance with the infection permissive nature of M2e-based immune protection (28), it allows the induction of cross-reactive T cells upon challenge with influenza virus (29). Here, we used passive transfer of monoclonal antibodies directed against M2e one day before infection with the PR8-based CTL-delivery vectors to diminish the morbidity caused by the influenza viral vector. To better understand the effect of intranasal instillation of anti-M2e antibodies on an influenza infection, we first characterized the immune response in the lungs of the mice by examining bronchoalveolar lavage (BAL). Mice were treated with 1 µg IgG2a anti-M2e antibodies or control antibodies directed against the ectodomain of the NB protein of influenza B (anti-NBe) and 24 h later they were challenged with 5 x 10^3 PFU of PR8/NA-F_{85-93} virus. Mice treated with polyclonal anti-PR8 mouse serum and mock challenged mice (anti-NBe treated, PBS challenged) were included as controls. BAL fluid was collected five days after influenza infection. Mice treated with anti-NBe showed a strong infiltration of immune cells in the lungs (Figure 5.5 A). This infiltration was
significantly less in anti-M2e treated mice. Infiltration of all cell types was strongly reduced in anti-M2e treated mice compared to anti-NBe treated mice, except for resident alveolar macrophages (Figure 5.5 B-C). These effects were reflected in the viral lung titer five days after influenza infection: viral lung titer in anti-M2e treated mice was significantly lower than in anti-NBe treated mice. However, anti-M2e immunity is not neutralizing in contrast to the polyclonal post-PR8 challenge serum (Figure 5.5 D). Infection with influenza virus is known to induce the formation of inducible bronchus associated lymphoid tissue (iBALT). These tertiary lymphoid structures can contribute to protection by promoting T- and B-cell mediated immune responses (30). Therefore, we also investigated if iBALT is still formed after PR8/NA-F<sub>85-93</sub> challenge in mice pretreated with anti-M2e antibodies. Germinal center formation, a prime hallmark of iBALT, was analyzed on day 26 after infection with PR8/NA-F<sub>85-93</sub>. By flow cytometry we determined the number of IgM/IgD<sup>+</sup> B cells expressing GL7 and Fas. Interestingly, iBALT formation in anti-M2e treated mice was comparable to that in anti-NBe treated mice (Figure 5.5 E). These results show that by combining the PR8/NA-F<sub>85-93</sub> virus with anti-M2e pre-treatment, we can eliminate the disadvantages of the vaccine virus (i.e. lung inflammation and substantial viral replication) without preventing the formation of iBALT.

Figure 5.5 Anti-M2e antibodies reduce infiltration of immune cells in the lungs after PR8/NA-F<sub>85-93</sub> challenge without impairing iBALT formation. Mice were immunized with anti-M2e antibodies or control anti-NBe antibodies 1 day before infection with PR8/NA-F<sub>85-93</sub>. Mice treated with polyclonal anti-PR8 mouse serum and mock challenged mice (anti-NBe treated, PBS challenged) were included as controls. (A) The total number of cells in the BAL fluid was determined on day 5 after influenza infection. Bars represent average + SD. (B-C) BAL cellular composition determined by flow cytometric enumeration of eosinophils (CD3ε<sup>-</sup> CD11c<sup>-</sup> MHC-II<sup>-</sup> CD11b<sup>med</sup> SSC<sup>hi</sup> CCR3<sup>+</sup>), neutrophils (CD3ε<sup>-</sup> CD11c<sup>-</sup> MHC-II<sup>-</sup> CD11b<sup>hi</sup> SSC<sup>med</sup> CCR3<sup>-</sup>), resident alveolar macrophages (AM) (CD3ε<sup>-</sup> CD11c<sup>+</sup> autofluo<sup>hi</sup> CD11b<sup>hi</sup>), recruited AM (CD3ε<sup>-</sup> CD11c<sup>+</sup> autofluo<sup>med</sup> CD11b<sup>-</sup>), dendritic cells (DC) (CD3ε<sup>-</sup> CD11c<sup>+</sup> autofluo<sup>lo</sup> MHC-II<sup>hi</sup>), CD4<sup>+</sup> T cells (CD3ε<sup>+</sup> CD4<sup>+</sup>) and CD8<sup>+</sup> T cells (CD3ε<sup>+</sup> CD8<sup>+</sup>). Bars represent the number of cells + SD. (D) Influenza lung viral titer was determined by plaque assay 5 days after PR8/NA-F<sub>85-93</sub> challenge. (E) Formation of iBALT was analyzed by counting the number of Fas<sup>+</sup>GL7<sup>+</sup> IgM<sup>-</sup> IgD<sup>-</sup> B cells in the lungs 26 days after challenge. Bars represent average + SD; Bdl. = below detection limit. Statistical significance was determined by using a two-sided Mann-Whitney U test; ** p < 0.01.
Treatment with anti-M2e antibodies prevents PR8/NA-F85-93-induced morbidity but still allows the induction of F85-93-specific CD8⁺ T cell responses that reduce HRSV lung viral load.

We next investigated whether anti-M2e treatment followed by a PR8/NA-F85-93 infection still allows the induction of F85-93-specific CD8⁺ T cell responses that are capable of reducing HRSV lung viral load. Mice received a single intranasal dose of 1 µg of an anti-M2e antibody or irrelevant control anti-NBe antibody followed 24 h later by an intranasal influenza virus infection (PR8/NA-F85-93 or PR8/NA-HA518-526). Mock immunized mice received anti-NBe antibodies followed by administration of PBS. As shown in Figure 5.6 A, anti-NBe treated mice displayed up to 25% weight loss after infection with PR8/NA-F85-93 or PR8/NA-HA518-526.

Figure 5.6  Treatment with anti-M2e antibodies prevents PR8/NA-F85-93-induced morbidity but still allows the induction of F85-93-specific CD8⁺ T cell responses and reduced HRSV lung viral load. Mice were immunized with anti-M2e antibodies or control anti-NBe antibodies 1 day before infection with PR8/NA-F85-93 or PR8/NA-HA518-526, and 7 weeks later they were challenged with 1 x 10⁶ PFU of HRSV-A2. (A) Bodyweight after PR8/NA-F85-93 or PR8/NA-HA518-526 infection. Graph shows average relative body weight +/- SD. (B) One day before HRSV challenge, splenocytes were isolated and restimulated ex vivo with F85-93 peptide for 12 h. Percentage IFNγ positive CD8⁺ T cells was determined by flow cytometry. (C) Lung HRSV titers were determined by plaque assay 5 days after HRSV challenge. Statistical significance was determined by using a two-sided Mann-Whitney U test; * p < 0.05; ** p < 0.01.
In contrast, mice receiving anti-M2e lost very little weight after infection, and both anti-M2e treated groups differed significantly from the corresponding groups that received anti-NBe treatment (two-way ANOVA p<0.0001). Fifty days after the primary infection with the PR8 vectors, the CTL memory response in the spleen was analyzed by flow cytometry. F85-93-epitope specific CTLs were induced in the PR8/NA-F85-93 infected groups after anti-M2e treatment or anti-NBe treatment (Figure 5.6 B). Although anti-M2e antibody treatment almost completely prevented body weight loss upon PR8/NA-F85-93 infection, the induction of F85-93-epitope specific CTL responses was only partially affected. Fifty one days after the immunization, mice were challenged with HRSV and the viral lung titer was determined five days later. In anti-NBe treated mice, viral lung titer was reduced in the PR8/NA-F85-93 infected group relative to the PR8/NA-HA518-526-infected group. A significant reduction in viral lung titer was also observed in anti-M2e treated PR8/NA-F85-93-infected mice, compared to anti-M2e treated mice that had been vaccinated with PR8/NA-HA518-526. As in previous experiments, in all groups the lung viral titer was lower than in the PBS immunized group. These results demonstrate that a reduction in HRSV viral load can be achieved by taking advantage of the infection-permissive protection of M2e antibody pretreatment against influenza A virus to induce CD8⁺ T cell mediated immunity by an influenza A virus vector expressing the HRSV F85-93 epitope.

### 5.4 Discussion

We evaluated the vaccine potential of a recombinant influenza virus encoding the H-2d F85-93 CTL epitope of the HRSV F protein. We demonstrate that a single intranasal immunization with this virus induces a potent F85-93-epitope specific CD8⁺ T cell response in mice. HRSV clearance was enhanced in mice immunized with PR8/NA-F85-93 virus but not in control, PR8/NA-HA518-526 and PBS immunized mice. Importantly, the enhanced clearance in PR8/NA-F85-93 immunized mice could not be explained by differences in replication efficacy of the recombinant viruses: PR8/NA-F85-93 and control PR8/NA-HA518-526 virus replicate with similar efficacy in mice and induce similar bodyweight loss.

It has been reported that an enhanced illness is observed upon overcompensation of the CTL response by the immunodominant Kd M282-90 epitope (22). This is mainly due to a difference between quantity and quality of the M282-90 specific CTLs: less than 50% of the M282-90 specific CTLs produce effector cytokines (IFNγ, IL-2 and TNF-α) (23). In a side by side comparison, F85-93-specific CTLs have been shown to contain a higher frequency of cells capable of co-producing these effector cytokines, suggesting that F85-93 Specific CD8⁺ T cells exhibit greater cytokine production capacity compared to their M282-90 counterparts. Based on these arguments we reasoned that inducing an F85-93 specific CTL response would result in at least comparable, if not greater levels of functional CTLs compared to M282-90 specific CTLs, whereas the F85-93-specific CTL responses would not be harmful to the mice.
Remarkably, we repeatedly observed that mice vaccinated with PR8/NA-HA\textsubscript{518-526} generally had lower pulmonary HRSV titers than PBS treated mice, indicating that influenza induces a pathogen-independent immune response that can partly protect against an HRSV infection. It is not clear whether this effect is a result of a nonspecific innate or adaptive immune response. Similar effects have been reported for several pathogens. For example, BCG, the live attenuated vaccine against tuberculosis, induces nonspecific protection against other infections (31). In this example nonspecific innate immune responses through epigenetic reprogramming of monocytes lie at the basis of the pathogen-independent protection (31). This innate imprinting or innate education has been defined as “the long term modification of a microenvironment, which will consequently lead to a nonspecific, but more protective, immune phenotype to a subsequent pathogen” (32). On the other hand it has been reported that in a model of G-protein primed mice an influenza infection can reduce the severity of G-protein vaccination induced enhancement of disease (illness, lung eosinophilia, and weight loss) upon an HRSV infection (33). This effect is most likely mediated by the adaptive immunity, as an activation of influenza specific T cells, possibly through the mechanism of bystander activation, is observed upon a secondary HRSV infection. Additionally, the formation of iBALT might contribute to nonspecific protection (34) (discussed more in detail below).

To prevent influenza induced morbidity, we further optimized our vaccination strategy by passive administration of IgG2a monoclonal antibodies directed against M2e to control morbidity associated with PR8/NA-F\textsubscript{85-93} virus infection. In contrast to vaccination with conventional HA-based influenza vaccines, M2e based vaccination with M2e-VLPs (virus-like particles) is not sterilizing (28, 29). Immunity induced by M2e-VLPs allows limited virus replication, and hence viral antigen processing and presentation to the host immune system, which leads to the induction of a functional influenza-specific T-cell response (29). In agreement with previous results, we observed that passively transferred anti-M2e monoclonal antibodies protected the mice against weight loss following PR8/NA-F\textsubscript{85-93} infection and allowed the induction of an F\textsubscript{85-93}-epitope specific CTL response, which correlated with reduced HRSV lung viral load upon a subsequent HRSV infection. Additionally, anti-M2e antibody treatment was associated with reduced infiltration of immune cells into the lungs after PR8/NA-F\textsubscript{85-93} infection. However, the formation of iBALT, a hallmark of influenza infection, was not affected. These tertiary lymphoid structures might contribute to protection, as they can initiate a localized immune response consisting of B and T cells (30, 34). A recent study demonstrated that iBALT induced by protein cage nanoparticles protects against multiple respiratory viruses, at least until 35 days after immunization with the nanoparticles (34). This is the first report of nonspecific protection evoked by iBALT. In our study we could confirm the presence of iBALT 26 days after PR8/NA-F\textsubscript{85-93} virus infection; however we did not investigate whether this persists until the moment of HRSV infection, which is 51 days after the influenza A virus infection. If iBALT is still present upon HRSV infection, this might contribute to the nonspecific protection observed upon PR8/NA-HA\textsubscript{518-526} infection.

The bodyweight loss caused by the influenza vaccine vector might be prevented or reduced by administering the virus as a live attenuated influenza vaccine (LAIV). Since 2003, LAIV (Flumist\textsuperscript{®}) has been approved in the USA as an influenza vaccine for healthy persons aged 2 to 49 years (35). In
Europe, the European Medicines Agency has approved Fluenz® only for healthy individuals aged 2 to 18 years (36). Compared to an inactivated influenza vaccine, LAIV has the advantage of inducing an immune response that more closely resembles the immune response induced by natural infection (37). Besides a local mucosal immune response, the LAIV also induces a systemic cellular immune response (38). An alternative strategy for producing live attenuated influenza virus was recently described by Mueller and colleagues (39). Using a computer algorithm, the A/PR/8/34 PB1, NP and HA genes were codon pair-deoptimized, while the wild type protein sequence was not affected. This leads to an in vivo attenuation of the virus in mice due to a less than optimal arrangement of codon pairs. Such deoptimized live attenuated viruses are highly unlikely to revert back to original virulence because that would involve hundreds of nucleotide mutations (39).

To adapt the vaccination strategy for humans, one or several human CTL epitopes have to be introduced into the influenza virus. It has been shown that up to 58 aa can be incorporated into the stalk of the neuraminidase (40). In this way, several human epitopes can be included in the NA to generate a vaccine that covers most human HLA types. Alternatively, large fragments can be introduced in the NS1/NS2 gene fragment. Manicassamy et al. reported the production of an influenza virus containing GFP in the NS segment. This virus has the additional advantage of being attenuated in vivo (41).

In conclusion, we describe a novel vaccine approach against HRSV that is based on the induction of a CTL immune response. Our results demonstrate that it is possible to take advantage of the infection-permissive protection of M2e-specific antibodies against influenza A virus to induce CD8+ T cell mediated immunity by an influenza A virus vector expressing the HRSV F85-93 epitope.

## 5.5 Acknowledgements

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5.6 Materials and methods

Cell lines and viruses. Madin-Darby canine kidney (MDCK) cells, African green monkey kidney (Vero) cells, HEp-2 cells and HEK293T cells were cultured in DMEM supplemented with 10% FCS, non-essential amino acids, L-glutamine, sodium-pyruvate and penicillin/streptomycin at 37°C in 5% CO₂. HRSV-A2 (ATCC VR-1540) was propagated on HEp-2 cells and quantified by plaque-titration on Vero cells. Influenza viruses were grown on MDCK cells in serum-free cell culture medium in the presence of 2 µg/ml TPCK-treated trypsin (Sigma). Virus was pelleted from culture supernatant by overnight centrifugation at 25,000 x g.

Construction of PR8/NA-F₈₅-₉₃ and PR8/NA-HA₅₁₈-₅₂₆. Recombinant viruses were rescued using the influenza A/Puerto Rico/8/34 based reverse genetics system (25). Using fusion PCR, the Kᵈ-restricted CTL epitope of the HRSV fusion protein (F₈₅-₉₃) and the KᵈHA₅₁₈-₅₂₆ CTL epitope of influenza A hemagglutinin were cloned into pHW196-NA plasmid by replacing the region encoding aa 65 to 71 of the NA coding sequence with a sequence encoding 15 aa containing the CTL epitope extended with the 3 naturally flanking amino acids both C- and N-terminally. To generate recombinant virus, 1 µg of each of the seven pHW-plasmids (pHW191-PB2, pHW192-PB1, pHW193-PA, pHW194-HA, pHW195-NP, pHW197-M, pHW198-NS) was transfected together with one of the NA plasmids (pHW196-NA, pHW196-NA-F₈₅-₉₃ or pHW196-NA-HA₅₁₈-₅₂₆) in a HEK293T/MDCK cell co-culture using calcium-phosphate co-precipitation in Optimem. After 36 h, TPCK-treated trypsin (Sigma) was added to a final concentration of 2 µg/ml. After 72 hours, the medium was collected. The presence of the virus in the medium was confirmed by hemagglutination of chicken red blood cells. After clonal selection by two rounds of limiting dilution, the virus was amplified on MDCK cells and the viral titer was determined by plaque assay. The presence of the epitope in the viral genome was confirmed by RT-PCR followed by sequence analysis.

Influenza plaque assay. MDCK cells were seeded in complete DMEM in six-well plates at 5 x 10⁵ cells per well one day before infection. The next day, cells were washed once with serum-free medium and incubated with a ten-fold dilution series of the virus in 500 µl medium. After 1 h of incubation at 37°C, medium was removed and replaced by an overlay of 0.8% Avicel RC-591 (FMC Biopolymer) in serum-free medium with 2 µg/ml TPCK-treated trypsin (Sigma). After 72 hours of incubation at 37°C, the overlay was removed, and the cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Plaques were stained with an anti-M2e IgG1 mouse monoclonal (final concentration 0.4 µg/ml) followed by a secondary anti-mouse IgG HRP-linked antibody. TrueBlue peroxidase substrate (KPL) was used for visualization.

HRSV plaque assays were carried out on Vero cells, seeded 1 day before infection at 20,000 cells per well in a 96-well plate. Cells were infected with a 3-fold dilution series of lung homogenate or BAL fluid, in Optimem. After 3 hours incubation at 37°C, the inoculum was removed and replaced with an
overlay of 0.8% Avicel RC-591 (FMC Biopolymer) diluted in cell culture medium containing 2% FCS. After 4 days of incubation at 37°C, the overlay was removed, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Plaques were stained using goat anti-HRSV serum (AB1128, Chemicon International) followed by a secondary anti-goat IgG HRP linked antibody and visualized by the addition of TrueBlue peroxidase substrate (KPL).

**Neuraminidase activity assay.** Forty microliters of virus diluted in PBS was added to 9 µL assay buffer (1 M NaAc, 10 mM CaCl₂, 5% butanol) and 1 µl 5 mM 2′-(4-Methylumbelliferyl)-α-D-N-acetyleneuraminic acid substrate (MUNANA, Sigma) in a 96-well plate. Fluorescence was measured every 2 min in a cytofluometer (excitation at 360 nm, emission at 460 nm) for 3 h. The background (fluorescence measured in a sample containing 40 µl PBS combined with substrate and assay buffer) was subtracted from each measurement. Using a standard curve of free 4-methylumbelliferone, the amount of released 4-methylumbelliferone in the sample for each timepoint was calculated. The number of NA units in the sample was calculated by dividing the amount of free 4-methylumbelliferone by the duration (in minutes). For relative activities, the highest amount of NA units during the 3 h (correlating to the highest turnover rate of the enzyme) was divided by the highest amount of NA units in the wild type NA sample. To confirm that equal quantities of virus were tested, the viral titer was checked by agglutination with chicken red blood cells and by plaque assay.

**Immunizations and HRSV challenge of mice.** Eight-week-old female BALB/c mice were housed in specified pathogen free conditions and used in all experiments. Under mild isoflurane anesthesia the mice were immunized by intranasal administration with 5 x 10⁴ PFU PR8/NA-F₈₅-₉₃, 5 x 10³ PFU PR8/NA-HA₅₁₈-₅₂₆ virus or 1 x 10³ PFU PR8 wild type virus diluted in 50 µl PBS. Passive immunization experiments were performed by giving the mice while under slight isoflurane anesthesia a single intranasal (i.n.) dose of 50 µl of PBS containing 1 µg of monoclonal antibody. An IgG2a monoclonal antibody directed against M2e or an IgG1 monoclonal antibody directed against the ectodomain of the influenza B virus NB protein (NBe) were used for passive immunization before influenza A virus administration. Both antibodies were purified from hybridoma supernatants.

Challenge with 1 x 10⁶ PFU HRSV-A2 was performed under slight isoflurane anesthesia by i.n. administration of 50 µL virus suspension diluted in PBS. Mice were killed on day four or five post challenge (as indicated). Lungs were removed and homogenized in 1 ml of HBSS containing 20% sucrose using a Heidolph RZR 2020 homogenizer. Homogenates were cleared by centrifugation (1,000 x g for 15 min at 4°C) and supernatant was used for quantitation by plaque assay.

**IFNγ enzyme-linked immunospot assay (ELISPOT).** At various time points after immunization (mentioned in the figure legends) spleens were removed aseptically. Splenocytes were isolated and red blood cells were lysed in NH₄Cl red blood cell lysis buffer. IFNγ ELISPOT assay was performed according to the manufacturer’s instructions (U-Cytech Biosciences). Briefly, maxisorp 96-well plates were coated overnight with anti-IFNγ monoclonal antibody at 4°C. The next day, plates were blocked
and 3 x 10^5 cells were seeded per well in 100 µL culture medium (RPMI + 10% FCS + L-glutamin + penicillin/streptomycin) supplemented with H-2^d-restricted HRSV-F protein-derived (KYKNAVTEL) restimulation peptide at a final concentration of 5 µg/ml. After 12 h of restimulation at 37°C, the cells were removed, the plates were washed, and IFNγ trapped on the plates was visualized using biotinylated polyclonal anti-IFNγ antiserum. The spots were counted using an inverted light microscope.

**Intracellular cytokine staining.** For intracellular cytokine staining (ICS), 2 x 10^6 splenocytes were seeded in 96-well suspension plates in 200 µL culture medium (RPMI + 10% FCS + L-glutamine + penicillin/streptomycin) supplemented with restimulation peptide at a final concentration of 5 µg/ml. After 12 h of restimulation at 37°C with HRSV-F protein-derived KYKNAVTEL (F85-93) or NP derived TYQRTRALV (NP155-163) peptides, GolgiPlug (BD) was added at a final concentration of 1 µg/ml and the cells were incubated for another 4 h at 37°C. After restimulation, cells were incubated with anti-mouse CD16/CD32 antibody (BD) to avoid nonspecific staining of immune cells. Staining was performed with anti-CD8a-FITC, anti-CD3ε-PE (both from BD) and LIVE/DEAD® fixable Aqua Dead Cell stain (Molecular probes) for 30 min. Cells were then fixed with 2% paraformaldehyde, permeabilized with Perm/Wash buffer (BD), and stained with anti-IFNγ Alexa Fluor® 647 (BD) for 30 min. IFNγ^+^CD8^+^T cells were quantified on an LSRII flow cytometer (BD, San Jose, CA) and analyzed with FACSDiva software (BD).

**Analysis of pulmonary cell infiltration.** Five days after influenza virus infection, mice were killed with ketamine/xylazine and lungs were washed through the trachea with 3 ml of HBSS + 5 mM EDTA. The first 0.5 ml was collected separately, centrifuged for 5 min at 400 x g, and supernatant was used for viral quantification. The pelleted cells from the first BAL fluid collection were added to the rest of the BAL fluid. Cells were incubated with anti-mouse CD16/CD32 antibody (BD) to avoid nonspecific staining of immune cells and stained with anti-CD3ε-FITC, anti-CD4-PerCP, anti-CD11c-APC, anti-CD11b-APC-Cy7 (all BD), anti-CD8a-PE-Cy7, anti-MHC-II-eFLUOR 450 (both eBiosciences), and CCR-3-PE (R&D Systems). Bronchoalveolar lavage (BAL) immune cell composition was determined on an LSR-II flow cytometer (BD, San Jose, CA) by analyzing cellular autofluorescence and surface expression of CD3ε, CD4, CD8a, CD11b, CCR3, MHC-II, and CD11c, similar to the protocol described in Bogaert et al, (26) using FACSDiva software (BD).

**Analysis of induced bronchus-associated lymphoid tissue (iBALT) formation.** Twenty six days after influenza infection, mice were terminally anesthetized and the lungs were removed. Lungs were grinded with the plunger of a syringe and passed through a 70-µM filter to produce single-cell suspensions. Red blood cells were lysed in NH_4Cl red blood cell lysis buffer. They were incubated with anti-mouse CD16/CD32 antibody (BD) to avoid nonspecific staining of immune cells and stained with anti-IgM-PerCp-Cy5.5, anti-IgD-PE, anti-B220-Alexa Fluor® 700, anti-CD3ε-APC, anti-Fas-PE-Cy7, anti-GL7-FITC (all BD) and LIVE/DEAD® fixable Aqua Dead Cell stain (Molecular probes) for 30 min. IgD
IgM^+ Fas^+ GL7^+ B-cells were quantified on an LSRII flow cytometer (BD, San Jose, CA) and analyzed with FACSDiva software (BD).

### 5.7 References


5.8 Additional data

Construction of a recombinant influenza virus expressing the immunodominant M2<sub>82-90</sub> CTL epitope

In BALB/c mice, the CTL epitope in the M2.1 protein (M2<sub>82-90</sub>) is immunodominant over the other epitopes (1). Immune responses to the M2<sub>82-90</sub> epitope (SYIGSINNII) have been studied more extensively than those against the F<sub>85-93</sub> epitope. However, a strong immune response to the immunodominant M2<sub>82-90</sub> epitope has been shown to be involved in enhancement of disease whereas an immune response to a subdominant epitope can make a significant contribution to viral clearance without exacerbating illness (2). Therefore, we were interested in comparing the immune responses to both epitopes, when inserted in the NA of a recombinant influenza virus.

For the construction of recombinant PR8 virus containing the HRSV M2<sub>82-90</sub> CTL epitope (PR8/NA-M2<sub>82-90</sub>) we used the same strategy as previously described for PR8/NA-F<sub>85-93</sub> and control PR8/NA-HA<sub>518-526</sub> virus (3): the region encoding aa 65 to 71 of the NA coding sequence was replaced with a sequence encoding 15 aa containing the CTL epitope extended with the 3 naturally flanking amino acids both C- and N-terminally. However, even after numerous attempts, we did not succeed in generating recombinant influenza virus containing the M2<sub>82-90</sub> epitope in the NA stalk (Figure 5.7).

![Figure 5.7 PR8/NA-M2<sub>82-90</sub> virus could not be rescued.](image)

**Figure 5.7 PR8/NA-M2<sub>82-90</sub> virus could not be rescued.** Wild type PR8 virus, or recombinant PR8/NA-HA<sub>518-526</sub> and PR8/NA-M2<sub>82-90</sub> were generated by transfecting 1 µg of each of the seven pHW-plasmids (pHW191-PB2, pHW192-PB1, pHW193-PA, pHW194-HA, pHW195-NP, pHW197-M, pHW198-NS) together with one of the NA plasmids (pHW196-NA, pHW196-NA-HA<sub>518-526</sub> or pHW196-NA-M2<sub>82-90</sub>) in a HEK293T/MDCK cell co-culture. A setup without NA (7 plasmids) was used as negative control. After 36 h, TPCK-treated trypsin (Sigma) was added to a final concentration of 2 µg/ml. The viral titer in the culture supernatant was determined with a TCID<sub>50</sub> assay at 24 (blue), 48 (red) and 72 (green) hours post infection.
Sequence analysis did not reveal any abnormalities in the coding sequence of NA-M2$_{82-90}$, although we did note that an extra N-linked glycosylation site was created due to the introduction of the epitope. To analyse protein expression and enzymatic activity, the NA-M2$_{82-90}$ was cloned into a potent eukaryotic expression vector (pCAXL) containing a chicken β-actin/rabbit β-globin hybrid promoter and the human cytomegalovirus immediate early promoter enhancer. Note that this step was required because the pHW-plasmids, used for generating recombinant virus express only low levels of proteins in the cells, also for WT NA, which cannot be detected easily by western blot (data not shown). The pCAXL vectors containing the coding sequence of wild type NA, NA-M2$_{82-90}$, NA-F$_{85-93}$ or NA-HA$_{518-526}$ were transfected to HEK293T cell cultures and the expression of the different NAs was analyzed by western blot (Figure 5.8). NA-M2$_{82-90}$ was expressed at similar levels as the other NAs. Next, to examine the enzymatic activity of NA-M2$_{82-90}$ we performed an NA-activity assay on the same samples. This assay revealed that NA-M2$_{82-90}$ did not possess any NA enzymatic activity (Figure 5.9), which explains why no recombinant PR8/NA-M2$_{82-90}$ virus could be recovered.

**Figure 5.8** NA-M2$_{82-90}$ is expressed and migrates slower in SDS-PAGE. HEK293T cells were transfected with a pCAXL expression vector containing wild type NA, NA-F$_{85-93}$, NA-HA$_{518-526}$ or NA-M2$_{82-90}$. A setup with an empty pCAXL expression vector was used as negative control. 48 hours after transfection the cells were harvested by a brief treatment with EDTA. After centrifugation for 10 min. at 400 x g the pellet was resuspended in 200µl PBS. A 40 µl sample of the cells was loaded on an SDS-PAGE and transferred to a nitrocellulose membrane via western blot. NA was detected using a monoclonal antibody directed against NA.

**Figure 5.9** NA-M2$_{82-90}$ is not enzymatically active. HEK293T cells were transfected with a pCAXL expression vector containing wild type NA (blue diamonds), NA-F$_{85-93}$ (red squares), NA-HA$_{518-526}$ (green triangles) or NA-M2$_{82-90}$ (purple circles). A setup with an empty pCAXL expression vector (black dashed line) was used as negative control. The enzymatic activity was measured using an NA-activity assay.
This lack of NA activity could be a consequence of the introduction of an extra N-glycosylation site in the NA stalk, which is present at the border between the M2_{82-90} epitope and its naturally flanking 3' amino acid (5'VLE SYIGSINNITKO 3'). Indeed, analysis of the expressed NAs on western blot showed that NA-M2_{82-90} migrates slightly slower compared to the other 3 NA-variants (Figure 5.8). The 3 amino acids flanking the M2_{82-90} epitope at the 5' and 3' end in the M2 protein were introduced in NA together with the epitope, to retain as much as possible the processing of the peptide as it was in the M2 protein. Removing these 3 amino acids at the 3' end removes the glycosylation site but this might negatively affect the proper processing of the peptide and the presentation on MHC-I molecules, as has been reported for other epitopes (4-6). Also mutating these residues could impact the dominance pattern (4), making it difficult to draw conclusions when comparing this M2_{82-90} epitope with the F_{85-93} epitope. Therefore it was decided to continue only with the PR8/NA-F_{85-93} virus.

The absence of NA activity due to the introduction of an extra glycosylation site is remarkable. The NA stalk contains several glycosylations, which do not interfere with its enzymatic activity. In addition, NAs of different subtypes differ in the length of the stalk and the number of glycosylations, yet still as these different subtypes are found in nature; this difference does not affect the activity. Moreover, secreted stalkless NA, which only contains the globular head, is still enzymatically active (7). Therefore, a possible explanation for the absence of NA activity might be that the additional glycosylation interferes with the formation of an NA tetramer, which is the enzymatically active unit.

**Boosting the F_{85-93} CTL response by DNA vaccination.**

It was noted (in the first part of this chapter), that the number of F_{85-93} specific CTLs 50 days after PR8/NA-F_{85-93} infection was significantly lower in anti-M2e IgG treated mice compared to anti-NBe IgG treated mice (Figure 5.6, panel B). In addition, upon an HRSV infection the CTLs of anti-M2e treated PR8/NA-F_{85-93} infected mice were not associated with reduced viral lung replication, in contrast to the controlled replication observed in anti-NBe treated PR8/NA-F_{85-93} infected mice (Figure 5.6, panel C). Therefore, we were interested in analyzing the magnitude of the recall response during an HRSV infection. Mice were treated with anti-M2e antibodies or control anti-NBe antibodies one day before infection with PR8/NA-F_{85-93} or PR8/NA-HA_{518-526}, and seven weeks later they were challenged with 1 x 10^6 PFU of HRSV-A2. Five days after HRSV challenge the spleens of the mice were isolated and the splenocytes were *in vitro* restimulated with F_{85-93} peptide. The number of F_{85-93} specific activated CD8^+ T cells was determined by intracellular cytokine staining. We observed significantly lower numbers of activated F_{85-93} specific CTLs in anti-M2e treated PR8/NA-F_{85-93} infected mice compared to anti-NBe treated PR8/NA-F_{85-93} infected mice (Figure 5.7). This supports our previous observations that less CTLs are present in the anti-M2e treated PR8/NA-F_{85-93} infected group and that these mice are less well protected against an HRSV challenge. We hypothesized that, due to the less severe immune response, as a consequence of the anti-M2e treatment, memory CTLs are less well formed in the anti-M2e treated PR8/NA-F_{85-93} infected group. Boosting the immune response might solve this problem.
Antibodies: αα αα M2e αα αα M2e αα αα NBe αα αα NBe αα αα NBe

Challenge: PR8/F 85-93 PR8/HA 518 PR8/F 85-93 PR8/HA 518 PBS

Figure 5.10 Treatment with anti-M2e antibodies reduces the number of activated F 85-93-specific CD8+ T cells in PR8/NA-F 85-93-vaccinated mice upon HRSV infection. Mice were immunized with anti-M2e antibodies or control anti-NBe antibodies 1 day before infection with PR8/NA-F 85-93 or PR8/NA-HA 518-526, and 7 weeks later were challenged with 1 x 10^6 PFU of HRSV-A2. Five days after HRSV challenge, splenocytes were isolated and restimulated ex vivo with F 85-93 peptide for 12 h. Percentage IFNγ positive CD8+ T cells was determined by flow cytometry. Statistical significance was determined by using a two-sided Mann-Whitney U test; ** p < 0.01.

To test this hypothesis we performed a prime-boost experiment. A heterologous boost will be necessary since boosting the immune response with the same PR8/NA-F 85-93 virus would lead to rapid neutralization and elimination of the virus due to the antibodies directed against the influenza HA and NA evoked by the first infection. We therefore chose to boost the immune response by DNA vaccination, using the pCAXL vectors containing the recombinant neuraminidases. The mice were primed as previously described. The anti-M2e treated PR8/NA-F 85-93 infected group and the anti-NBe treated PR8/NA-F 85-93 infected group were divided into two: one half received a boost by DNA vaccination with the pCAXL-NA-F 85-93 plasmid and the other half of each group received a boost with pCAXL-NA-HA 518-526 plasmid. The mice of the anti-M2e treated PR8/NA- HA 518-526 infected group and the anti-NBe treated PR8/NA-HA 518-526 infected group all received a boost with the pCAXL-NA-HA 518-526 plasmid. Five weeks after the boost, the mice were challenged with 1 x 10^6 PFU of HRSV-A2. The numbers of F 85-93 specific CTLs was determined one day before and 5 days after HRSV challenge. On the latter date also the lung HR SV titer was determined. We observed a clear effect of the DNA boost (however, not significantly different) in the mice that were treated with anti-NBe antibodies (Figure 5.11 A). Before as well as after challenge the anti-NBe treated PR8/NA-F 85-93 infected mice that received a pCAXL-NA-F 85-93 plasmid boost had higher CTL numbers than the anti-NBe treated PR8/NA-F 85-93 that received an irrelevant boost with pCAXL-NA-HA 518-526 plasmid. The effect of this boost on clearance of HRSV from the lungs is less clear (Figure 5.11 B). In most mice, the viral lung titer was below detection level on day 5 after challenge, except for one mouse in the anti-NBe treated PR8/NA-F 85-93 infected pCAXL-NA-F 85-93 boosted group. For the mice that received an anti-M2e treatment the outcome is different. In these mice, it seems that the DNA boost did not have an effect, both on the level of CTL numbers (Figure 5.11 A) and the viral titer in the lung (Figure 5.11 B).
It should be noted that for the intracellular cytokine assay before challenge, the background level (i.e. the number of F\textsubscript{85-93} specific CTLs in the PR8/NA-HA\textsubscript{518-526} infected, pCAXL-NA-HA\textsubscript{518-526} boosted groups) was high, making it difficult to draw conclusion from this assay. Since these are results from only one experiment, it would be necessary to repeat the experiment to conclude whether or not the DNA boost has a positive effect on the number of F\textsubscript{85-93} specific CTLs and consequently on the reduction in lung viral titer after HRSV challenge.

![Graph A](image1)

**Antibodies:** αα αα M2e  αα αα M2e  αα αα M2e  αα αα NBe  αα αα NBe  αα αα NBe  αα αα NBe

**Challenge:** PR8/F\textsubscript{85}  PR8/F\textsubscript{85}  PR8/HA\textsubscript{518}  PR8/F\textsubscript{85}  PR8/F\textsubscript{85}  PR8/HA\textsubscript{518}  PBS

**Boost:** NA/F\textsubscript{85}  NA/HA\textsubscript{518}  NA/HA\textsubscript{518}  NA/F\textsubscript{85}  NA/HA\textsubscript{518}  NA/HA\textsubscript{518}  PBS

![Graph B](image2)

**Antibodies:** αα αα M2e  αα αα M2e  αα αα M2e  αα αα NBe  αα αα NBe  αα αα NBe  αα αα NBe

**Challenge:** PR8/F\textsubscript{85}  PR8/F\textsubscript{85}  PR8/HA\textsubscript{518}  PR8/F\textsubscript{85}  PR8/F\textsubscript{85}  PR8/HA\textsubscript{518}  PBS

**Boost:** NA/F\textsubscript{85}  NA/HA\textsubscript{518}  NA/HA\textsubscript{518}  NA/F\textsubscript{85}  NA/HA\textsubscript{518}  NA/HA\textsubscript{518}  PBS

Figure 5.11 Boosting with the pCAXL/NA-F\textsubscript{85-93} vector does not enhance CTL numbers and protection against HRSV in anti-M2e treated PR8/NA-F\textsubscript{85-93} immunized mice. Mice were immunized with anti-M2e antibodies or control anti-NBe antibodies 1 day before infection with PR8/NA-F\textsubscript{85-93} or PR8/NA-HA\textsubscript{518-526}, and and received a boost with a pCAXL vector containing either NA/F\textsubscript{85-93} or NA/HA\textsubscript{518-526} 2 weeks after the infection. 5 weeks after the boost, mice were challenged with 1 x 10\textsuperscript{6} PFU of HRSV-A2. (A) One day before HRSV challenge, splenocytes were isolated and restimulated ex vivo with F\textsubscript{85-93} peptide for 12 h. Percentage IFN\gamma positive CD8\textsuperscript{+} T cells was determined by flow cytometry. (B) Lung HRSV titers were determined by plaque assay 5 days after HRSV challenge. Statistical significance was determined by using a two-sided Mann-Whitney U test; ** p < 0.01.
5.9 Additional materials and methods

Production of recombinant influenza viruses was performed as described in the main text.

Construction of pCAXL plasmids. Recombinant NAs were expressed using pCAXL plasmid, which is a derivative of pCAGGS. For ease of cloning, the restriction sites Nhel, SacI, NotI and MluI were inserted in the multi cloning site of pCAGGS at restriction site XhoI. This modified vector was named pCAXL. The neuraminidase sequences were amplified from the pHW196, pHW196-NA-F\textsubscript{85-93}, pHW196-NA-M2\textsubscript{82-90} and pHW196-NA-HA\textsubscript{518-526} plasmids and inserted in the pCAXL vector between the MluI and XhoI sites.

Expression analysis of recombinant NAs
For expression test, HEK293T cells were seeded in 6 well plates at 2 x 105 cells per well in complete DMEM medium (DMEM supplemented with 10% FCS, non-essential amino acids, L-glutamine, sodium-pyruvate and penicillin/streptomycin). 24h later the cells were transfected with 125µg of the pCAXL plasmids using calcium phosphate precipitation. After 48h cells were washed once with PBS and detached by a brief treatment with 200µL EDTA, which was removed by centrifugation (5 min at 400 x g). Cell pellets were resuspended in 200 µL PBS. Expression of the recombinant proteins was analyzed using SDS-PAGE and western blot. NA was visualized using a mouse anti-NA antibody (isolated and produced in our lab).

Neuraminidase activity assay on transfected cells. Cells were transfected and detached as described above under ‘Expression analysis of recombinant NAs’. A 40µL sample of the PBS containing the cells was added to 10µL of 1M NaAc, 10mM CaCl2, 5% butanol) and 5mM 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid substrate (Sigma) in a 96-well plate. Fluorescence was measured every 2 minutes in a cytofluometer (excitation at 360 nm, emission at 460nm) for 3h.

Mice, vaccination and challenge.
Vaccination and challenge were performed as described for the main text. For DNA vaccination LPS free plasmids were purified using EndoFree Plasmid Giga Kit (Qiagen). DNA was diluted in LPS free PBS to a concentration of 1 mg/ml. Mice were anesthetized by intraperitoneal injection of a ketamine and xylazine mixture (100 µg/g and 10 µg/g body weight, respectively) diluted in 150 µl PBS. Mice were immunized by intramuscular injection in the quadriceps of the hind legs (50 µl/leg).

Intracellular cytokine staining was performed as described in the main text.
5.10 Additional references


Chapter 6

Strategies to attenuate PR8/NA-\(F_{85-93}\) virus

Author contributions: Xavier Saelens, Bert Schepens and Sarah De Baets designed and discussed the experiments. Sarah De Baets performed all the experiments. Anouk Smet constructed the plasmid pHW-NS1(1-73)-GFP-NS2.
6.1 Introduction

We have reported the construction of a recombinant influenza A virus carrying the H-2d restricted CTL epitope of the HRSV fusion protein (F\textsubscript{85-93}), which induces an HRSV F\textsubscript{85-93}-specific CD8\textsuperscript{T} T cell response, that can reduce HRSV replication in BALB/c mice (1). To reduce morbidity associated with the live viral vector, we took advantage of the infection-permissive nature of the immune protection afforded by anti-M2e antibodies. This means that our aim was to control morbidity induced by the PR8/NA-F\textsubscript{85-93} virus by co-administration of an M2e-specific IgG2a monoclonal antibody, without significantly compromising the F\textsubscript{85-93}-specific CD8\textsuperscript{T} T cell response. However, combining the anti-M2e antibodies with the PR8/NA-F\textsubscript{85-93} virus complicates the vaccination strategy to a certain extent. In addition, it is highly unlikely that a live virus without a stable attenuated genotype would be accepted by FDA or another regulatory agency to be used as a prophylactic vaccine. Therefore, we explored strategies to make an attenuated variant of the PR8/NA-F\textsubscript{85-93} virus.

One way to make an attenuated virus is by reassorting with the cold adapted, temperature sensitive A/Ann Arbor/6/60 strain. This strategy is currently applied for the production of live attenuated influenza vaccine (LAIV). Since 2003, LAIV (Flumist\textsuperscript{®}) has been approved in the USA as influenza vaccine for healthy persons aged 2 to 49 years (2). In Europe, the European Medicines Agency has approved Fluenz\textsuperscript{®} only for healthy individuals aged 24 months to 18 years (3). Classical or reverse genetics based reassorting of a circulating human influenza strain with the A/Ann Arbor/6/60 master donor virus (MDV), results in a virus that contains the two surface glycoproteins neuraminidase and hemagglutinin of the circulating strain and the six internal gene segments of the MDV, thereby retaining its attenuated phenotype (4). Compared to an inactivated influenza vaccine, LAIV has the advantage of inducing an immune response that more closely resembles the immune response detected after natural infection (5). The LAIV is administered via a nasal spray, which induces an mucosal immune response, directly at the site of entrance of the respiratory pathogens. Besides this local mucosal immune response it also induces a systemic immune response, which includes the induction of a cellular immune response (6).

Recently a computer aided, rational design strategy was developed for the attenuation of viruses (7). Besides a bias for codon usage, each species also has a certain bias for codon-pair usage, meaning that certain codon-pairs are used more or less frequently than statistically expected. The central idea of codon-pair deoptimization is to recode and synthesize a viral gene (or complete genome) in a way that preserves the wild type amino acid sequence while rearranging synonymous codons to create a suboptimal arrangement of codon pairs. This results in a slower rate of protein synthesis of such a protein. It has already been shown for polio virus and influenza virus that such codon-pair biased
viruses are attenuated in vivo, but are still capable of eliciting a protective immune response against a challenge with the wild type virus (7, 8).

Modifying the gene carrying the coding information of the influenza non-structural protein 1 (NS1) can also result in an attenuated virus (9). The main function of the NS1 protein is the inhibition of the host type I interferon (IFN) mediated immune response. Modification of this gene, such as mutations, truncations or deletions results in a robust local immune response, which limits the viral replication and the associated disease. Interestingly, despite the significant reduction in viral replication, live attenuated viruses with truncated NS1 proteins still prime long-lived T- and B- cell responses (10). NS1 modified, attenuated viruses form a promising new vaccination strategy, which has been proven to be protective against influenza in mice, pigs, horses, chickens and macaques (9, 11).

Reporter influenza viruses have been created by inserting the GFP coding sequence in the NA or NS gene segment (12-14). Such viruses express GFP in infected cells in vitro, as well as in vivo in animal models. These viruses are a useful tool for example for following the course of an influenza infection in animal models or for screening of influenza anti-viral drugs. However, it should be noted that these viruses are attenuated in vivo in the mouse model.

Here we report the construction of two types of live attenuated PR8 viruses: one with codon-pair deoptimized NP and HA gene segments and a second virus with a truncated NS1 open reading frame that was furthermore expressing GFP. Our aim was to assess the induction of F85-93 specific T cell responses using these live viruses in naïve laboratory mice. The addition of GFP also allowed to detect the differences in the course of an influenza infection between mice infected with virus carrying full length NS1 and mice infected with attenuated viruses carrying a truncated NS1 sequence.

6.2 A codon-pair deoptimized variant of PR8/NA-F85-93 virus is slightly attenuated in vivo.

Construction and in vitro characteristics of PR8/NA-F85-93 viruses carrying codon-pair deoptimized HA and/or NP genes.

Mueller et al. previously reported the construction of an influenza virus carrying the codon-pair deoptimized gene sequences of HA, NP and/or PB1. They kindly provided us the pDZ-NP\textsuperscript{mn} and pDZ-HA\textsuperscript{mn} containing the coding sequences for codon-pair deoptimized NP and HA derived from influenza A/Puerto Rico/8/34 (PR8) strain. Using the reversed genetics 8-plasmid system for the generation of reassortant influenza PR8 virus, we constructed viruses containing one or both codon-pair
deoptimized genes, together with NA-F_{85-93}, NA-HA_{518-526} or wild type NA, combined with the five other wild type genes (PB2, PA, NP, M and NS). This resulted in 9 different viable viruses (Table 6.1).

Table 6.1  Overview of the constructed codon-pair deoptimized viruses

<table>
<thead>
<tr>
<th>Virus name</th>
<th>NA</th>
<th>NP</th>
<th>HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR8/NA-F_{85-93}/NP^{min}/HA^{min}</td>
<td>NA-F_{85-93}</td>
<td>NP^{min}</td>
<td>HA^{min}</td>
</tr>
<tr>
<td>PR8/NA-F_{85-93}/NP^{min}</td>
<td>NA-F_{85-93}</td>
<td>NP^{min}</td>
<td>HA wt.</td>
</tr>
<tr>
<td>PR8/NA-F_{85-93}/HA^{min}</td>
<td>NA-F_{85-93}</td>
<td>NP wt</td>
<td>HA^{min}</td>
</tr>
<tr>
<td>PR8/NA-HA_{518-526}/NP^{min}/HA^{min}</td>
<td>NA-HA_{518-526}</td>
<td>NP^{min}</td>
<td>HA^{min}</td>
</tr>
<tr>
<td>PR8/NA-HA_{518-526}/NP^{min}</td>
<td>NA-HA_{518-526}</td>
<td>NP^{min}</td>
<td>HA wt.</td>
</tr>
<tr>
<td>PR8/NA-HA_{518-526}/HA^{min}</td>
<td>NA-HA_{518-526}</td>
<td>NP wt</td>
<td>HA^{min}</td>
</tr>
<tr>
<td>PR8/NA wt/NP^{min}/HA^{min}</td>
<td>NA wt</td>
<td>NP^{min}</td>
<td>HA^{min}</td>
</tr>
<tr>
<td>PR8/NA wt/NP^{min}</td>
<td>NA wt</td>
<td>NP^{min}</td>
<td>HA wt.</td>
</tr>
<tr>
<td>PR8/NA wt/HA^{min}</td>
<td>NA wt</td>
<td>NP wt</td>
<td>HA^{min}</td>
</tr>
</tbody>
</table>

The virus was collected after 3 days and transferred to culture flasks, for expansion on MDCK cells. We observed that the viruses containing one or two codon-pair deoptimized genes induced less cytopathic effect on the cells than similarly generated wild type PR8 virus. Remarkably, the MDCK cells did not detach from the culture flask, even though virus was present at high titers in the culture supernatant. This could indicate that the viruses are attenuated in vitro. After growth for 7 days on MDCK cells, the viral titer present in the supernatant of the cells was determined by plaque assay.

The viruses containing both codon-pair deoptimized genes in combination with wild type NA or, NA-HA_{518-526} seemed to grow slower than viruses containing only one codon-pair deoptimized gene. This however was not observed for PR8/NA-F_{85-93}/NP^{min}/HA^{min}, which grew even to a higher titer than PR8/NA-F_{85-93}/NP^{min} and PR8/NA-F_{85-93}/HA^{min} (Figure 6.1). However, kinetics studies starting off with low MOI will be required to determine what the effect is of the codon-pair deoptimized genes on in vitro replication efficiency of the recombinant viruses.

Plaque phenotypes revealed some effects of the deoptimization of NP and HA. For all 3 types of NA, viruses with NP^{min} and HA^{min} have much smaller plaque phenotypes than the corresponding viruses with wild type NP and HA proteins (Figure 6.2). Similar to the viral titers, this effect was most pronounced in the viruses expressing NA-HA_{518-526} or wild type NA. The virus with wild type NA and NP but with HA^{min} (PR8/NA wt/HA^{min}) also had a smaller plaque size than wild type PR8 virus, which was not the case for virus with NP^{min} and HA wild type (PR8/NA wt/NP^{min}) indicating that the smaller plaque size is most likely a result of the presence of HA^{min}.  

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Figure 6.1  Plaque assay of codon pair deoptimized virus production. Recombinant A/Puerto Rico/8/34 viruses were generated using the reverse genetics 8 plasmid system. All viruses contain wild type PB2, PA, NP, M and NS genes, combined with wild type or codon pair deoptimized (min) NP and/or HA in combination with NA-85-93, NA-HA518-526 or wild type NA. Supernatant containing the virus was collected 3 days after transfection and 1/25 was transferred to MDCK cells. Virus was collected 7 days later and a plaque assay was performed to determine the viral titer.

Figure 6.2 Recombinant viruses with codon pair deoptimized NP or HA or both have smaller plaque phenotypes. Recombinant A/Puerto Rico/8/34 viruses were generated using the reverse genetics 8 plasmid system. All viruses contain wild type PB2, PA, NP, M and NS genes, combined with wild type or codon pair deoptimized (min) NP and/or HA in combination with NA-85-93, NA-HA518-526 or wild type NA as indicated in the borders of the figure. Virus was transferred to MDCK cells and after 1h of incubation an overlay of 0.8% avicel was added to the cells. After 2 days of incubation the plaque assay was developed. (N.D. : not determined)
In vivo attenuation study.

Next, we investigated whether these viruses are attenuated in vivo, compared to PR8/NA-F_{85-93}, which was used in previous in vivo studies (Chapter 5). Our hypothesis was that the presence of the codon-pair deoptimized genes would result in a virus that was more attenuated in vivo than PR8/NA-F_{85-93} and that we might be able to completely abolish body weight loss caused by the influenza vector. Mice received an intranasal infection with doses ranging from $5 \times 10^3$ PFU to $5 \times 10^5$ PFU of PR8/NA-F_{85-93}/NP^{\text{min}}$/HA^{\text{min}}$, PR8/NA-F_{85-93}/NP^{\text{min}}$, or PR8/NA-F_{85-93}/HA^{\text{min}}$. Bodyweight was measured daily and compared to mice that received an intranasal infection with $5 \times 10^3$ PFU or PR8/NA-F_{85-93}.

![Graph A](image1)

**Figure 6.3** Recombinant PR8 viruses expressing codon pair deoptimized NP and/or HA genes are not attenuated in vivo. Mice (n = 3 per group) received an intranasal infection with doses ranging from $5 \times 10^3$ PFU to $5 \times 10^5$ PFU of PR8 virus with NA-F_{85-93} and NP^{\text{min}}$ or HA^{\text{min}}$ or both codon-pair deoptimized genes, as indicated in the figure legends. Bodyweight was measured daily and compared to mice that received an intranasal infection with $5 \times 10^3$ PFU of PR8/NA-F_{85-93}. (A) Relative body weight of mice infected with $5 \times 10^5$, $5 \times 10^4$ or $5 \times 10^3$ PFU of PR8/NA-F_{85-93}/NP^{\text{min}}$/HA^{\text{min}}$. (B) Relative bodyweight of mice infected with $1.63 \times 10^4$, $5 \times 10^3$ PFU PR8/NA-F_{85-93}/NP^{\text{min}}$ or $5 \times 10^3$ PFU PR8/NA-F_{85-93}/HA^{\text{min}}$. (Group average +/- SD.)
We did not observe any \textit{in vivo} attenuation of the PR8/NA-F_{85-93}/NP^{min} or PR8/NA-F_{85-93}/HA^{min} virus (Figure 6.3 A). On the contrary, PR8/NA-F_{85-93}/NP^{min} infection even appeared to induce more morbidity than PR8/NA-F_{85-93} infection in mice, as both administered doses (1.63 \times 10^4 \text{ and } 5 \times 10^3 \text{ PFU}) were lethal to the mice. The virus containing two codon-pair deoptimized genes (PR8/NA-F_{85-93}/NP^{min}/HA^{min}) was slightly attenuated compared to PR8/NA-F_{85-93} (Figure 6.3 B). Here, a dose of 5 \times 10^4 \text{ PFU} of PR8/NA-F_{85-93}/NP^{min}/HA^{min} results in a comparable mean bodyweight loss as when a 10-fold lower dose (5 \times 10^3 \text{ PFU}) is given of PR8/NA-F_{85-93}. With a dose of 5 \times 10^3 \text{ PFU} of PR8/NA-F_{85-93}/NP^{min}/HA^{min} the mean bodyweight of the mice only dropped until 90\% of the initial bodyweight.

\section*{6.3 A GFP-reporter influenza virus that is attenuated \textit{in vivo}}

One of the major functions of the influenza NS1 protein is the inhibition of the host type I IFN response. The NS1 protein consist of an N-terminal RNA binding domain (aa 1-73) followed by an effector domain (aa 74-207) and a short C-terminal region (+/- 20 aa) (15). It has been shown that viruses expressing NS1 proteins that are progressively truncated from the C-terminus replicate less efficiently and hence are attenuated \textit{in vivo} (10). Remarkably, viruses expressing only the N-terminal RNA-binding domain are most attenuated, even though they replicate to higher titers in the lungs, compared to viruses with less truncated forms of NS1 (10). Despite the reduced replication of these viruses \textit{in vivo}, a long-lived protective immune response is still induced in the mice. Again, the shortest versions of NS1 (aa 1-73) were capable of inducing the strongest T cell response, which correlates with their better replication efficiency \textit{in vivo} (10). We therefore were interested in creating a virus containing such a truncated NS1(1-73). This cannot be achieved by just truncating the NS gene segment, since influenza virus also encodes NEP from the same gene segment: NS1 is produced from full length NS mRNA and NEP from a spliced mRNA. It has been previously shown that NS1 and NEP can be expressed as a single polyproteins, separated by a foot and mouth disease (FMDV) 2A autoproteolytic cleavage site (16). Cleavage at this 2A site occurs during translation, resulting in two separate proteins. We applied this strategy for the expression of NS1(1-73) and NEP. In addition, we were interested in creating a reporter influenza virus with this adapted NS gene segment. The GFP coding sequence was introduced between the NS1(1-73) and NEP coding sequences. An FMDV 2A autoproteolytic cleavage site was inserted between NS1(1-73) and GFP, while the latter was separated from NS2 by a porcine teschovirus-1 (PTV-1) 2A cleavage site (Figure 6.4). The coding sequence of an HA tag was fused to NS1, for ease of protein detection. Finally, a dimerization domain of Drosophila melanogaster kinesin motor protein (Dmd) was also added to NS1. This adapted NS gene segment was named NS1(1-73)-GFP-NS2.
Figure 6.4  
Schematic representation of the promoters and coding sequences of the pHW-NS1(1-73)-GFP-NS2 plasmid for the generation of reporter GFP influenza virus. A truncated NS1 gene (aa 1-73) is fused to an HA tag (HA) and a *Drosophila melanogaster* kinesin motor protein dimerization domain (Dmd). The GFP coding sequence was inserted between the coding sequence of truncated NS1 (1-73) and full length NS2. A foot and mouth disease (FMDV) 2A cleavage site is inserted between NS1(1-73) and GFP and a porcine teschovirus-1 (PTV-1) 2A cleavage site is inserted between GFP and NS2. The first methionine codon of the 3 proteins is indicated (ATG). Coding sequences are flanked by the NS non coding regions (NCR). These sequences are inserted in the pol I/pol II expression cassette in the pHW2000 expression vector of the reverse genetics 8 plasmid system.

Figure 6.5  
Characterization of the NS and NA segments of the recovered viruses by RT-PCR. RNA was extracted from virus particles after 1 passage on MDCK cells and cDNA was prepared in the presence (with RT) or absence (No RT) of reverse transcriptase. The cDNA was amplified by PCR using primers specific for the NS segment (A) or primers lining the insertion site in NA (B). Plasmids were used as positive control for the PCR (Lanes 1, 2, 8 and 9). 1 = plasmid pHW198-NS; 2 = plasmid pHW-NS1(1-73)-GFP-NS2; 3 = PCR containing no cDNA, only primers; 4 = PR8/NA-F_{85-93}/ NS1(1-73)-GFP-NS2; 5 = PR8/NAwt/ NS1(1-73)-GFP-NS2; 6 = PR8/NA-F_{85-93}/ NSwt; 7 = PR8wt; 8 = plasmid pHW196-NA; 9 = plasmid pHW NA-F_{85-93}.
Generation of a recombinant reporter influenza virus expressing GFP, that has a truncated NS1 gene.

Using the before mentioned reversed genetics 8-plasmid system, we constructed reassortant A/PR8 influenza virus containing the NS1(1-73)-GFP-NS2 gene segment with either wild type neuraminidase (PR8/NAwt./NS1(1-73)-GFP-NS2 virus) or NA-F85-93 (PR8/NA-F85-93/NS1(1-73)-GFP-NS2 virus). Several attempts to produce virus with the NS1(1-73)-GFP-NS2 gene segment in combination with NA/HA518-526 failed. Both reassortant viruses were further subcloned by two rounds of limiting dilution. The presence of the NS1(1-73)-GFP-NS2 gene segment and of NA-F85-93 or wild type NA in the viral genome was confirmed by RT-PCR (Figure 6.5) and by sequence analysis of the RT-PCR product.

*In vitro* characterization.

To assess whether the deletion of a fragment of the NS1 gene, or the insertion of GFP affected the viral replication, we first performed a plaque assay to spot differences in plaque morphology. We noticed that the plaques of both PR8/NAwt./NS1(1-73)-GFP-NS2 virus and PR8/NA-F85-93/NS1(1-73)-GFP-NS2 virus had a thinner halo than plaques of wild type PR8 virus (Figure 6.6), indicating that the changes made in the NS gene segment may have an effect on the *in vitro* viral fitness. Next, we compared the growth kinetics on MDCK cells of PR8/NAwt./NS1(1-73)-GFP-NS2 virus and PR8/NA-F85-93/NS1(1-73)-GFP-NS2 virus with wild type PR8 virus. Cells were infected with a MOI of 0.001 and at indicated time points after infection the viral titer in the supernatant was determined by TCID\textsubscript{50} analysis. In this multi-cycle replication assay (due to the addition of trypsin to the culture), both recombinant viruses showed similar growth kinetics to wild type PR8 virus (Figure 6.7).

To test whether GFP was expressed in the infected cells we performed fluorescent microscopy. MDCK cells were infected with either one of the GFP expressing viruses or wild type PR8 virus and at 10h post infection the cells were stained for NP expression. We observed GFP signal in all NP expressing cells, indicating that GFP is expressed in all infected cells, both for PR8/ NA-F85-93/NS1(1-73)-GFP-NS2, and PR8/NAwt./NS1(1-73)-GFP-NS2 (Figure 6.8). The FMDV 2A autoprotyolytic cleavage site inserted between the NS1 and GFP sequence and the PTV-1 2A cleavage site inserted between GFP and NS2 should ensure that 3 individual proteins are produced. We performed western blot analysis on PR8/NA-F85-93/NS1(1-73)-GFP-NS2 and PR8/NAwt./NS1(1-73)-GFP-NS2-infected cells to assess whether the cleavage between these 3 proteins is complete. Detection with an anti-GFP antibody revealed multiple bands on the blot: two major bands of approximately 50 kDa and 27 kDa and a smaller band around 25 kDa (Figure 6.9). The higher band most likely corresponds to the complete, uncleaved polyprotein, whereas the exact identity of the two lower bands is less clear. This indicates that, in contrast to what we expected, the cleavage at the 2A cleavage site is only partial, or doesn’t occur at all. Detection of the same blot with an anti-NS1 monoclonal only revealed a band in the wild type PR8 infected cells, since the epitope of this monoclonal lies in the C terminal region of the protein, which is removed in the viruses carrying the NS1(1-73)-GFP-NS2 gene segment.
An HA-tag was inserted behind the NS1 coding sequence to facilitate detection of this protein. However, even after multiple attempts, no protein could be detected with anti-HA antibodies (data not shown).

For other GFP-carrying influenza reporter viruses it has been reported that the GFP-positivity is often lost during multiple rounds of replication (13). To assess the stability of the PR8/NA-F<sub>85-93</sub>/NS1(1-73)-GFP-NS2 and PR8/NAwt./NS1(1-73)-GFP-NS2 the virus was passaged multiple times over MDCK cells. After each round of replication, during which virus replicated for approximately 3 days on the cells, a sample was taken from the medium. Some of these intermediate samples were subsequently used to infect cells, which, after 10 h of infection were fixed and analysed for the expression of GFP and NP by fluorescent microscopy. We did not observe an increasing number of NP positive, GFP negative cells, in later passages, compared to the starting virus, indicating that both PR8/NA-F<sub>85-93</sub>/NS1(1-73)-GFP-NS2 and PR8/NAwt./NS1(1-73)-GFP-NS2 can stably transfer the GFP gene sequence to progeny virus during in vitro replication (Figure 6.10).

Figure 6.6 Plaque assay of recombinant viruses. Plaques of recombinant PR8 viruses PR8/NAwt./NS1(1-73)-GFP-NS2 and PR8/NA-F<sub>85-93</sub>/NS1(1-73)-GFP-NS2 have a smaller halo than plaques of wild type PR8 virus in an influenza plaque assay.

Figure 6.7 PR8/NAwt./NS1(1-73)-GFP-NS2 virus and PR8/NA-F<sub>85-93</sub>/NS1(1-73)-GFP-NS2 virus show similar in vitro growth kinetics as PR8 wild type virus. MDCK cells were infected in duplicate with a multiplicity of infection of 0.001 of wild type PR8 virus, PR8/NAwt./NS1(1-73)-GFP-NS2 virus and PR8/NA-F<sub>85-93</sub>/NS1(1-73)-GFP-NS2 in a total volume of 10 ml. A 200µl sample was taken at 0, 4, 8, 12, 24 and 48 h post infection. The viral titer in the samples was determined by TCID<sub>50</sub> assay.
Figure 6.8  GFP expression is observed in PR8/NA-F_{85-93}/NS1(1-73)-GFP-NS2 and PR8/NAwt./NS1(1-73)-GFP-NS2 infected cells. MDCK cells were infected with PR8/NA-F_{85-93}/NS1(1-73)-GFP-NS2 (A), PR8/NAwt./NS1(1-73)-GFP-NS2 (B) or wild type PR8 (C) virus and at 10h post infection the cells were stained with anti-NP (red) and DAPI (blue). The GFP signal is shown in green. An overlay of the three colours is shown in the right panel.

Figure 6.9  Expression analysis of NS1 and GFP in PR8/NA-F_{85-93}/NS1(1-73)-GFP-NS2 and PR8/NAwt./NS1(1-73)-GFP-NS2 infected cells. MDCK cells were infected with wild PR8/NA-F_{85-93}/NS1(1-73)-GFP-NS2, PR8/NAwt./NS1(1-73)-GFP-NS2 or wild type PR8. After 11h of infection, cells were lysed and expression of GFP (A) and NS1 (B) proteins was analysed on western blot. Not infected cells were used as negative control. 1 = PR8/NA-F_{85-93}/NS1(1-73)-GFP-NS2; 2 = PR8/NAwt./NS1(1-73)-GFP-NS2; 3 = wild type PR8; 4 = not infected MDCK cells.
Figure 6.10 PR8/NA-F_{85-93}/NS1(1-73)-GFP-NS2 and PR8/NAwt./NS1(1-73)-GFP-NS2 can stably transfer the GFP gene sequence to progeny virus. PR8/NA-F_{85-93}/NS1(1-73)-GFP-NS2 (A) and PR8/NAwt./NS1(1-73)-GFP-NS2 (B) were serially passaged over MDCK cells. Supernatant containing virus from passage 3, 7 and 9 was used to infect MDCK cells. After 10h of infection the cells were fixed, stained and analysed by fluorescent microscopy for the expression of with NP (red) and GFP (green). An overlay of both signals combined with nuclear DAPI signal (blue) is shown in the right panel.
Analysis of *in vivo* attenuation.

We hypothesised that the replacement of a part of the NS1 gene by GFP would cause an *in vivo* attenuation of the influenza virus. To test this, BALB/c mice (n = 6 per group) received $1 \times 10^3$, $1 \times 10^4$ or $1 \times 10^5$ PFU of PR8/NA-F<sub>85-93</sub>/NS1(1-73)-GFP-NS2 or PR8/NAwt./NS1(1-73)-GFP-NS2. Body weight was measured daily during 14 days after challenge and weight loss was compared to mice that received $5 \times 10^3$ PFU of PR8/NA-F<sub>85-93</sub> or $1 \times 10^3$ of wild type PR8 virus. A 10-fold higher inoculum dose of PR8/NAwt./NS1(1-73)-GFP-NS2 was needed to reach comparable morbidity as wild type PR8 virus (approximately 70% of initial bodyweight) (Figure 6.11 A). PR8/NA-F<sub>85-93</sub>/NS1(1-73)-GFP-NS2 virus however appeared to be at least 100-fold attenuated: here a dose of $1 \times 10^5$ PFU reduced the mean body weight up to 80% of the initial body weight, whereas a dose of $5 \times 10^3$ PFU of PR8/NA-F<sub>85-93</sub> already induces body weight loss up to 70% of the initial weight (Figure 6.11 B). These results confirm our hypothesis that both PR8/NA-F<sub>85-93</sub>/NS1(1-73)-GFP-NS2 and PR8/NAwt./NS1(1-73)-GFP-NS2 are attenuated *in vivo*.

Analysis of the number of induced F<sub>85-93</sub>-epitope specific CTLs should clarify if the attenuation of the PR8/NA-F<sub>85-93</sub>/NS1(1-73)-GFP-NS2 virus also has an effect on the induction of an adequate CTL response.

![Figure 6.11](image)

**Figure 6.11** PR8/NA-F<sub>85-93</sub>/NS1(1-73)-GFP-NS2 and PR8/NAwt./NS1(1-73)-GFP-NS2 are attenuated *in vivo* in BALB/c mice. Mice (n = 6 per group) received $1 \times 10^3$, $1 \times 10^4$ or $1 \times 10^5$ PFU of PR8/NA-F<sub>85-93</sub>/NS1(1-73)-GFP-NS2 (A) or PR8/NAwt./NS1(1-73)-GFP-NS2 (B). Body weight was monitored daily during 14 days after challenge and weight loss was compared to mice that received $5 \times 10^3$ PFU of PR8/NA-F<sub>85-93</sub> (A) or $1 \times 10^3$ of wild type PR8 virus (B).
In vivo cell tropism of GFP reporter PR8 virus.

Next we were interested in analysing the possibility to use these GFP-carrying viruses as in vivo reporter virus in mice. BALB/c mice (n = 2 per group) were infected with $10^4$ PFU of wild type PR8 virus or $10^6$ PFU of PR8/NA-F85-93/NS1(1-73)-GFP-NS2 or PR8/NAwt/NS1(1-73)-GFP-NS2. Two days after infection the lungs of the mice were isolated and we identified the specific cell types infected by the virus by counting the number of GFP positive cells using multicolour flow cytometry. GFP positive cells could be detected in the lungs of mice infected with PR8/NA-F85-93/NS1(1-73)-GFP-NS2 and PR8/NAwt/NS1(1-73)-GFP-NS2, while these cells were not present in mice infected with PR8 wt virus. For mice infected with either of the GFP viruses we found that on average 25% of the epithelial cells (CD45-) were GFP positive (Figure 6.12). For all types of immune cells (CD45+) analysed, including B cells, CD8+ T cells, CD4+ T cells, CD11c+ DCs and granulocytes we found that only minor fractions (mostly below 1%) were GFP positive; however, there was still a clear difference compared to the mice infected with PR8 wt virus. For all the immune cell types tested we noted that a smaller number of cells was GFP positive in PR8/NA-F85-93/NS1(1-73)-GFP-NS2 infected mice, compared to PR8/NAwt/NS1(1-73)-GFP-NS2 infected mice. This is likely because of the more attenuated phenotype of PR8/NA-F85-93/NS1(1-73)-GFP-NS2 in mice, compared to PR8/NAwt/NS1(1-73)-GFP-NS2.

Figure 6.12 PR8/NA-F85-93/NS1(1-73)-GFP-NS2 or PR8/NAwt/NS1(1-73)-GFP-NS2 infection results in GFP expression in vivo in BALB/c mice. Mice (n = 2 per group) were infected with $10^5$ PFU of PR8/NAwt/NS1(1-73)-GFP-NS2 (black) or $10^6$ PFU of PR8/NA-F85-93/NS1(1-73)-GFP-NS2 (grey) or $10^7$ PFU of wild type PR8 virus (white). Two days after infection the lungs of the mice were isolated and the number of GFP positive cells was determined using multicolour flow cytometry. GFP expression was analysed in (A) immune cells (CD45+), including B cells, CD8+ T cells, CD4+ T cells, CD11c+ DCs and granulocytes and (B) epithelial cells (CD45-).
6.4 Discussion

A live attenuated HRSV vaccine has the best chance of becoming accepted as a vaccine against HRSV in young children (17). Since the recombinant influenza virus we previously constructed (PR8/NA-F85-93 virus; see chapter 5) is not attenuated in vivo, we searched for methods to generate an attenuated variant of this vaccine virus, that would be capable of inducing a strong CTL response against HRSV, in the absence of vector-induced disease. Here we explored the use of two different strategies to genetically attenuate the PR8/NA-F85-93 virus.

The first strategy for generating attenuated viruses uses the technique of codon-pair deoptimization (7, 8). We created recombinant virus containing codon-pair deoptimized NP and/or HA gene sequences. In vitro these codon-pair deoptimized viruses behaved clearly different from wild type PR8 or PR8/NA-F85-93 virus, since we observed less cytopathic effect on MDCK cells and a distinct plaque phenotype. In addition, it was difficult to grow these viruses to high titers on MDCK cells. This was in contrast to the in vivo behaviour of the viruses: we only observed mild in vivo attenuation.

Viruses containing only one codon-pair deoptimized gene (PR8/NA-F85-93/NPmin and PR8/NA-F85-93/HAmin) were not at all attenuated. The virus containing two codon-pair deoptimized genes (PR8/NA-F85-93/NPmin/HAmin) was 10 times more attenuated than the parent PR8/NA-F85-93 virus. This is in strong contrast to the results reported by Mueller et al., who showed that addition of one of the codon-pair deoptimized genes already had a 10- or 30- fold increase of the median lethal dose (LD50) for PR8-NPmin and PR8-HAmin respectively (8). It should be noted that the codon-pair deoptimized viruses in our experiment were not pelleted by a centrifugation step but were administered to the mice as diluted cell supernatant. It is not ruled out that soluble factors, produced by the infected cell could have an impact on the immune response of the host. From the in vivo attenuation study we concluded that addition of two codon-pair deoptimized genes (NPmin and HAmin) confers a 10-fold attenuation, however the virus did not meet our expectations of an in vivo attenuated virus that no longer induces body weight loss at high titers. Therefore, one would likely need to include the codon-pair deoptimized PB1 gene sequence (PB1min), since this gene reportedly confers a 500-fold attenuation on its own, according to Mueller et al., and even a 13 000-fold attenuation when combined with NPmin and HAmin (8).

A second method used for attenuating the PR8/NA-F85-93 virus consisted of truncating the NS1 protein of the virus. Truncations of NS1, which is responsible for inhibition of the host immune response, has been shown to attenuate the virus replication in vivo (10). Here we report the construction of two viruses containing a truncated NS1, consisting of the N terminal amino 73 aa of NS1. In addition, we introduced GFP and designed an expression strategy for GPF as well as for NEP that is independent of mRNA splicing (Figure 6.4). We indeed observed that a 10- and 20-fold higher dose PR8/NAwt./NS1(1-73)-GFP-NS2 and PR8/NA-F85-93/NS1(1-73)-GFP-NS2 respectively had to be given to the mice to achieve similar body weight loss as PR8/NA-F85-93, confirming our hypothesis that both PR8/NA-F85-93/NS1(1-73)-GFP-NS2 and PR8/NAwt./NS1(1-73)-GFP-NS2 would be attenuated in vivo. In contrast, we did not observe any in vitro attenuation: wild type PR8 virus and mutant NS1(1-
73)-GFP-NS2 viruses showed similar growth characteristics on MDCK cells. This corresponds to previous observations by Wang et al. who found that carboxy-terminal truncations of NS1 did not affect virus replication in vitro but did result in attenuation in mice (18). In addition, the MDCK cells used stably express the V protein of parainfluenza virus type 5. This protein binds to the IFN-inducible RNA helicase, mda-5, hereby inhibiting IFN signalling (19). This should allow more efficient growth of viruses which do not possess an IFN-antagonising protein, such as the described influenza A viruses with truncated NS1 proteins.

Besides a truncated NS1 to attenuate the virus, PR8/NAwt./NS1(1-73)-GFP-NS2 and PR8/NA-F85-93/NS1(1-73)-GFP-NS2 also contain an additional sequence in the NS gene segment, coding for GFP. It has been shown that insertion of GFP in the influenza genome results in a virus expressing GFP in infected cells. Such viruses can be employed as a reporter virus to follow the course of an infection (13). Here we show that also viruses with a truncated NS1 (PR8/NAwt./NS1(1-73)-GFP-NS2 and PR8/NA-F85-93/NS1(1-73)-GFP-NS2) can still be utilized as a reporter virus both in vitro and in vivo. We show that in vivo, non-immune cells (CD45-) are the major cell type that is infected by influenza, whereas only small fractions (on average less than 1%) of the different types of immune cells (CD45+) are infected. This is not surprising, since epithelial cells are the primary target cell for influenza viruses (20). Our results are somewhat different from those reported earlier: Manicassamy et al. observed for some immune cell types up to 10% of GFP positive, i.e. influenza infected cells when infecting mice with their NS1-GFP reporter virus (13). This difference might be a result of the truncated NS1 which is present in our PR8/NAwt./NS1(1-73)-GFP-NS2 and PR8/NA-F85-93/NS1(1-73)-GFP-NS2 viruses, whereas a full length NS1 was present in the NS1-GFP reporter virus used by Manicassamy et al., most likely resulting in a more infectious virus. It should be noted that in our study only 1 timepoint after infection was measured. This does not provide any information about the progress of the infection, or for example if infected DCs have migrated from the lungs to the lymph nodes. A kinetics study, both in the lungs and the draining lymph nodes will give a more thorough understanding of the progress of the influenza infection. In addition, a side by side comparison of our virus with truncated NS1 with a GFP containing virus with wild type NS1 will allow a better understanding of the effects of the truncated NS1 on the immune system.

When working with recombinant viruses, stability of the virus is an important issue that needs to be taken into account. Manicassamy et al. reported that in a multicycle in vitro assay after 12 h already 5-10% of the virus was no longer GFP-positive (13). In addition, they observed that 5-30% of the virus present in the lungs of mice was GFP-negative, indicating that these viruses are likely to contain mutation or deletions in the GFP sequence. Similarly Kittel et al. reported the appearance of NS-GFP deletion mutants in high frequency after passage on MDCK cells, with complete loss of GFP positivity after several passages (12). These GFP-negative viruses can arise due to selective pressure for viruses containing mutations in the GFP sequence resulting in more fit viruses. We did not observe such selective pressure for GFP-negative viruses in vitro: after 10 consecutive passages over MDCK cells PR8/NAwt./NS1(1-73)-GFP-NS2 and PR8/NA-F85-93/NS1(1-73)-GFP-NS2 were still GFP positive. This difference might be an effect of the dimerization domain which was added to the PR8/NAwt./NS1(1-73)-GFP-NS2 and PR8/NA-F85-93/NS1(1-73)-GFP-NS2 virus, and which has been reported to (partially)
restore the NS1 function (18). We did not yet assess the in vivo stability PR8/NAwt./NS1(1-73)-GFP-NS2 and PR8/NA-F85-93/NS1(1-73)-GFP-NS2 in mice. We did not determine the in vitro or in vivo stability of the viruses containing codon-pair deoptimized genes. It is not excluded that mutations occur in these viruses, however, it is unlikely that these viruses can revert back to wild type virus, since this would theoretically involve hundreds of mutations.

A live attenuated influenza virus carrying an HRSV CTL epitope, as described here may be used as a vaccine against influenza as well as HRSV. First, however, it needs to be explored whether these attenuated viruses still induce a potent CTL response in mice, which is comparable to the parent, non-attenuated virus. Live attenuated vaccines are already being used for influenza vaccination. In addition, a live vaccine against HRSV has the highest possibility of being approved for use in young infants, compared to inactivated vaccines (17). The latter is discouraged for use in young infants after the dramatic results of the inactivated FI-RSV vaccine trial (21). Besides the FI-RSV vaccine, the only type of vaccine that has been tested in 1-2 months old infants are live inactivated HRSV vaccines (22, 23).

6.5 Materials and methods

Cell lines and viruses. Madin-Darby canine kidney (MDCK) cells and HEK293T cells were cultured in DMEM supplemented with 10% FCS, non-essential amino acids, L-glutamine, sodium-pyruvate and penicillin/streptomycin at 37°C in 5% CO₂. Influenza viruses were grown on MDCK cells in serum-free cell culture medium in the presence of 2 µg/ml TPCK-treated trypsin (Sigma).

Construction of the plasmid pHW-NS1(1-73)/GFP/NS2. The plasmid pHW-NS1(1-73)/GFP/NS2 was constructed using the following strategy: the coding sequence of the HAtag/Dmd/FMDV-2A was ordered from Genscript and cloned into the pcDNA3 vector using the restriction sites NotI and XbaI. The coding sequence of the first 73 aa of NS1 was picked up by PCR from the pHW198-NS plasmid and cloned in front of the HAtag using BamHI and EspEI. This NS(1-73)-HAtag/Dmd/FMDV-2A was cloned into the pHW2000 plasmid using the sites BamHI and MunI. The Quantum SuperGlo GFP coding sequence (derived from Qbiogene vector pQBI25-fc1) was cloned behind the FMDV-2A cleavage site using BglII and EcoRI. The PTV-1 2A cleavage site was fused to the NS2 coding sequence (by fusion PCR) and cloned behind the GFP coding sequence using the EcoRI and BstEII restriction sites.

Production of recombinant viruses. Recombinant viruses were rescued using the influenza A/Puerto Rico/8/34 based reverse genetics system (24). To generate recombinant virus, 1 µg of each of the
seven pHW-plasmids (pHW191-PB2, pHW192-PB1, pHW193-PA, pHW194-HA, pHW195-NP, pHW197-M, pHW198-NS) was transfected together with one of the NA plasmids (pHW196-NA, pHW196-NA- 
F85-93 or pHW196-NA-HA318-526) in a HEK293T/MDCK cell co-culture using calcium-phosphate co-
precipitation in Optimem. For generation of codon-pair biased viruses, the plasmids pHW193-PA and 
pHW194-HA were replaced by pDZ-NPmin and pDZ-HAmin (a kind gift from Dr. E. Wimmer) (8). The 
plasmid pHW198-NS was replaced by the plasmid pHW-NS1(1-73)-GFP-NS2 for production of 
attenuated GFP reporter viruses. 
After 36 h, TPCK-treated trypsin (Sigma) was added to a final concentration of 2 µg/ml. After 72 
hours, the medium was collected. The presence of the virus in the medium was confirmed by 
hemagglutination of chicken red blood cells. The virus was amplified on MDCK cells and the viral 
titer was determined by plaque assay. The presence of wild type or mutant NA and NS segments in 
the viral genome were confirmed by RT-PCR and sequence analysis. The sequence of the primers is 
available on request.

*In vitro growth kinetics.* MDCK cells (seeded at 4 x 10^6 cells per 9 cm dish) were infected in duplicate 
with a multiplicity of infection of 0.001 of wild type PR8 virus, PR8/NAwt/NS1(1-73)-GFP-NS2 virus 
and PR8/NA-F85-93/NS1(1-73)-GFP-NS2 in a total volume of 10 ml. After one hour of incubation the 
inoculum was removed and replaced by 10 ml of serum-free medium with 2 µg/ml TPCK-treated 
trypsin (Sigma). A 200µl sample was taken at 0, 4, 8, 12, 24 and 48 h after removal of the inoculum. 
The viral titer in the samples was determined by TCID₅₀ assay.

*Influenza plaque assay.* MDCK cells were seeded in complete DMEM in six-well plates at 5 x 10^5 cells 
per well one day before infection. The next day, cells were washed once with serum-free medium 
and incubated with a ten-fold dilution series of the virus in 500 µl medium. After 1 h of incubation at 
37°C, medium was removed and replaced by an overlay of 0.8% Avicel RC-591 (FMC Biopolymer) in 
serum-free medium with 2 µg/ml TPCK-treated trypsin (Sigma). After 72 hours of incubation at 37°C, 
the overlay was removed, and the cells were fixed with 4% paraformaldehyde and permeabilized 
with 0.2% Triton X-100. Plaques were stained with an anti-M2e IgG1 mouse monoclonal (final 
concentration 0.4 µg/ml) followed by a secondary anti-mouse IgG HRP-linked antibody. TrueBlue 
peroxidase substrate (KPL) was used for visualization.

*Immunization mice.* Eight-week-old female BALB/c mice were housed in specified pathogen free 
conditions and used in all experiments. Under mild isoflurane anesthesia the mice were immunized 
by intranasal administration with the indicated doses of recombinant or wild type PR8 virus diluted in 
50 µl PBS.

*Immunofluorescence.* MDCK cells (seeded on glass coverslips at 2 x 10^4 cells per well in a 24 well 
plate) were infected with a MOI 5 of PR8/NA-F85-93/NS1(1-73)-GFP-NS2, PR8/NAwt/NS1(1-73)-GFP-
NS2 or wild type PR8 virus. After 10h the cells were fixed with 4% paraformaldehyde, permeabilized 
with 0,2% Triton X-100 and stained with goat anti-influenza RNP, diluted 1/2000 (NR-4282; NIH
Biodefense and Emerging Infections Research Resources Repository, NAIAD). As secondary antibody Alexa Fluor® 568 rabbit anti-goat IgG was used. The coverslips were mounted with DAPI-containing vectashield (Enzo Life Sciences, VC-H-1200).

**Analysis of GFP expression using western blot.** MDCK cells (seeded at $2 \times 10^5$ cells per well in a 6 well plate) were infected with a MOI 5 of PR8/NA-F$_{R5.93}$/NS1(1-73)-GFP-NS2, PR8/NAwt/NS1(1-73)-GFP-NS2 or wild type PR8 virus. Not infected MDCK cells were included as negative control. 10h later, the cells were lysed on ice for 30 min. in 200 µl lysis buffer (20mM Tris pH8, 200mM NaCl, 0,5% NP40, 1 mM EDTA pH 8 with protease inhibitors (Complete; Roche Diagnostics N.V. Belgium)). Laemlli was added and the sample was boiled for 10 min. The proteins were separated using SDS-PAGE and GFP was visualised by western blot, using a monoclonal mouse anti-GFP antibody (A11121; Molecular probes), followed by a sheep anti-mouse HRP-linked secondary antibody and detection using ECL western blotting substrate (Pierce; Cat. No. 32106).

**Flow cytometry.** Two days after influenza infection, mice were terminally anesthetized and the lungs were removed. Lungs were grinded with the plunger of a syringe and passed through a 70-µM filter to produce single-cell suspensions. Red blood cells were lysed in NH$_4$Cl red blood cell lysis buffer. Cells were incubated with anti-mouse CD16/CD32 antibody (BD) to avoid nonspecific staining of immune cells and stained with anti-CD4-PerCP, anti-B220-Alexa Fluor® 700, anti-CD11c-APC, anti-CD11b-APC-Cy7 (all four BD), anti-CD8-PE-Cy7, anti-CD45-PE, anti-CD3-eFluor 450 (all three eBioscience) and LIVE/DEAD® fixable Aqua Dead Cell stain (Molecular probes) for 30 min. The number of GFP positive lung cells was determined on an LSR-II flow cytometer (BD, San Jose, CA) by analysing surface expression of CD45, CD3, CD4, CD8, B220, CD11b, and CD11c, similar to the protocol described in Bogaert et al, (25) using FACSDiva software (BD).

### 6.6 Acknowledgements

We thank Anouk Smet for constructing the plasmid pHW-NS1(1-73)/GFP/NS2. We are grateful to Dr. Eckhard Wimmer for providing us the expression plasmids with the codon-pair deoptimized HA and NP genes.
6.7 References


Part 4

Summary, general discussion

and future perspectives
Summary, general discussion
and future perspectives

Prevention strategies for severe disease caused by human respiratory syncytial virus (HRSV) are urgently needed. The currently used preventive measure consists of monthly intramuscular injections of a monoclonal antibody (Palivizumab) directed against the F protein of HRSV. This intervention is associated with a significantly reduced number of hospitalisation days due to HRSV infections: per 100 patients the total number of hospitalisation days is 36.4 for treated vs. 62.6 for non-treated patients (1, 2). However, the Palivizumab administration cannot be used for every individual (2). Due to the high costs of this treatment, it is only recommended for use in children that are at high risk for severe HRSV disease, such as premature babies and children with congenital heart and lung disease (3). Yet, in this high risk group, Palivizumab is not capable of preventing severe HRSV-associated disease in each treated patient (2). Therefore, the development of a safe and effective vaccine, that can prevent severe HRSV-associated disease would mean a major breakthrough.

Since monoclonal antibodies directed against the fusion protein are a known correlate of protection, a logic strategy for preventing HRSV associated disease would be to induce such antibodies by an active vaccination protocol, a canonical strategy that has been used successfully in the past to control most (childhood) infectious diseases such as polio and hepatitis A and B virus infection. However, history has shown that for HRSV the situation is not just ‘as simple as it seems’. Vaccination of children using a formalin-inactivated HRSV (FI-RSV) vaccine, a strategy that had been applied with success for the development of other childhood vaccines, resulted in severe disease upon an HRSV infection in 80% of the vaccines and two children died (4). This severe disease was characterized by poor functionality of the induced antibodies and a CD4+ Th2 biased immune response (5, 6). Since these fatal clinical trials it became clear that an HRSV vaccine that induces IL-4 producing CD4+ T cells should be avoided in HRSV-naïve infants.
A virus-like particle based vaccine candidate for HRSV.

A first vaccination strategy described in this thesis builds upon the knowledge that antibodies directed to the F protein are a known correlate of protection. We aimed at inducing a strong antibody response, by linking HRSV F protein derived peptides to the virus-like particle (VLP) carrier made up of hepatitis B virus core (HBc) proteins. We hypothesized that the highly repetitive structure in which the HRSV peptides can be presented at the surface of the HBc particle, would be an approach with a high chance of success for the induction of a strong antibody response. Furthermore, this approach might work in the presence or absence of T helper cells. In addition, since VLPs cannot replicate – they consist of structural proteins without a functional genome – this type of vaccine carrier is considered safer than replicating viral carries, which still (partly) retain the ability to cause disease. Vaccine safety is a prime factor when very young children are the target group. Therefore we explored the use of HBc-based VLPs as a candidate for a novel vaccination strategy against HRSV. This technique has been successfully used in the past by our lab for the development of a universal influenza A virus vaccine, based on the ectodomain of the matrix 2 protein (7-10). Two types of peptides were coupled to the VLP by genetic fusion. First we chose small peptides derived from regions in the F protein with known binding sites of neutralizing antibodies. As a second antigen source, we linked larger protein fragments of the F protein, which likely would more closely mimic the natural conformation of full length F protein, to the HBc carrier. All chosen antigens were free of possible N-linked glycosylation sites. These HRSV-derived peptides or domains were genetically fused to the major immunodominant region (MIR) of HBc as well as at the N-terminus of the HBc monomer. Unfortunately, none of the attempted strategies used to fuse the HRSV derived antigens to the HBc carrier resulted in a VLP that was soluble. Changing of the salt concentration or the pH of the buffer used for sonicating the bacteria in which the VLP is produced, did not result in a more soluble VLP product. This imposes severe problems for the upscaling of the production process and the subsequent purification of the VLP vaccine. A possible answer to the question why these HRSV peptides lead to insoluble VLPs might be found in the hydrophobicity of the used peptides. We noticed that the M2e peptide, which has been coupled with success to HBc VLPs is highly hydrophilic, whereas most of the HRSV peptides that we used are predicted to be extremely hydrophobic. These hydrophobic regions which are present at the surface of the VLP might result in a particle with low solubility in aqueous solutions. We attempted to enhance the solubility of the VLP product by fusing the M2e coding sequence behind the HRSV peptides at the N-terminus of HBc. But again this did not result in any highly soluble VLP product. An alternative virus-like particle based vaccine platform uses a modified alphavirus (Alphavax, North Carolina, USA). These so called virus replicon particles (VRPs) carry a modified viral RNA genome that carries the coding sequence of the heterologous antigen, resulting in abundant antigen expression in the infected cell upon vaccination with such a VRP vaccine (11). Both strategies, VLPs and VRPs have advantages compared to the other. A major advantage of the VRPs is that a non-human virus is used as basis for the VRP, such as Equine encephalitis virus, which ensures that no pre-existing vector immunity is present that can blunt the immune response in humans. An advantage of the VLPs is its
highly repetitive superficial exposition of the foreign antigen, which allows cross-linking of the B cell receptor and activation of the B cell completely independent of T cell help (12, 13) as well as presentation in a T cell dependent way, which allows affinity maturation and isotype switching. Since we did not succeed in producing particles that were satisfactorily soluble to proceed to in vivo testing in mice, the question whether this vaccination strategy could provide protection against HRSV remains unanswered. It is likely that, for preventing HRSV infection, the induction of a CD8+ T cell response by a vaccine will be required. Nevertheless, a vaccination strategy as described here, which only induces a robust antibody response, without a CD8+ T cell compartment, may also be beneficial. Due to adverse events that occurred in the past, non-replicating vaccines for HRSV are unlikely to be accepted for use in young infants however, these vaccines can be used for vaccinating adults or older children. A strategy which has been proposed that might benefit from such an antibody-inducing vaccine is the “herd immunization” (14) strategy. This strategy is based on the observation that infants are often infected by a close relative, such as an older sibling, a parent or somebody else with whom he lives together. These older people, when infected with HRSV, often experience only mild symptoms and hence are not aware that they could be the source of infection for the infant. It has been shown, for viruses other than HRSV, that large scale vaccination of the people in a community decreases the chances of the non-vaccinated, in this case the infant, to become infected (14). Another strategy that would benefit from an antibody-inducing vaccine is the maternal vaccination strategy. It has been shown that HRSV-specific maternal antibodies, that have been transferred to the infant during pregnancy, correlate with a reduced number of HRSV cases during the first weeks after infant birth (15). Using a purified F protein vaccine (PFP-2) it was shown that vaccination of the mother during pregnancy enhances the antibody levels in the infant (16). Such a vaccination strategy might be useful in providing a significant amount of protection during the first weeks of life, when the child is still too young for vaccination.

A live attenuated influenza virus as HRSV vaccine candidate.

An increasing amount of data supports the idea that, for an efficient vaccine against HRSV, the induction of a T cell immune response will be mandatory. First, in early vaccine trials using FI-RSV vaccine children suffering severe HRSV induced disease had developed a weak CTL response (17). Second, in a model of vaccine enhanced disease in mice it has been shown that CTLs can inhibit the development of an undesired CD4+ Th2 immune response (17-19). Third, whereas in mice CTLs have been shown to be associated with enhancement of disease (20), in humans they have been shown to peak during convalescence (21), and hence are not the cause of observed lung damage. And last, in humans and mice CTLs have been attributed a role in clearance of HRSV from the lungs of infected patients or animals (20, 21).

To explore the possibilities of inducing a CD8+ T cell response against HRSV using a live viral vector, we created a recombinant influenza virus carrying the HRSV F_{85-93} CTL epitope in the NA stalk (PR8/NA-F_{85-93} virus). Infection of mice with this virus showed that an HRSV specific CTL response was
efficiently induced and this in a dose dependent manner. We showed that upon a subsequent HRSV challenge these CTLs were capable of reducing the HRSV replication in the lungs of the mice, indicating that the CTLs induced by the vaccine can provide considerable protection against HRSV. Since the vaccine is a live virus that is capable of evoking disease, it is desirable to suppress the infectivity of this virus to eliminate vector-induced disease. To attain this, we pretreated the mice with a monoclonal antibody directed against the influenza M2e. In contrast to neutralizing antibodies, these anti-M2e antibodies are infection permissive, allowing at least one round of replication, which is required for efficient presentation of the CTL epitope and induction of a CTL response. Indeed we observed that even though anti-M2e antibody pretreatment completely abrogates weight loss caused by the vaccine vector, an HRSV specific CTL response is still efficiently induced. In addition, following an HRSV challenge these CTLs are capable of reducing HRSV replication in the lungs of the mice.

Interestingly, we repeatedly observed that mice infected with a control influenza virus, that does not carry an HRSV CTL epitope have a reduced lung viral load compared to PBS treated mice, indicating that influenza can partly protect against an HRSV infection. This heterologous immunity, or the immunity that can develop to one pathogen after an encounter with a non-related pathogen, is a common feature that has been demonstrated for a variety of pathogens, including parasites, protozoa, bacteria and viruses (22). It has been observed between closely related species but also between unrelated pathogens and can lead to either diminishing or, as is the situation for an influenza infection followed by HRSV, enhancement of protective immunity. This heterologous immunity can be mediated either by effects of the innate immunity or by effects of the adaptive immune response. One possibility could be the existence of cross-reactive T cells between influenza and HRSV. Such cross-reactive CD8\(^+\) T cells have been reported between several pathogens, both in mice and in humans (summarized by Welsh et al. (22)). For example, in hepatitis C virus (HCV) naïve individuals T cells were found that reacted to stimulation with the HCV HLA-A2 restricted epitope NS3\(_{1073-1081}\). It appeared that these T cells were memory T cells specific for the influenza A virus NA\(_{231-239}\) epitope, which were able to cross react with the HCV NS3\(_{1073-1081}\) epitope (23). Until now, no cross-reactive epitope between HRSV and influenza has been reported. Alternatively, the heterologous immunity might be a result of bystander activation of CD8\(^+\) T cells. This has been observed in the model of HRSV-mediated disease enhancement in G-protein primed mice: here, a prior influenza infection can diminish the enhancement of disease, which is normally observed upon HRSV infection of G-protein primed mice (24). Also, epigenetic reprogramming of monocytes upon BCG vaccination has shown to result in a pathogen non-specific protection, which in this case is mediated by the innate immune system (25). It is not clear which arm of the immune response is involved in the non-specific protection against HRSV.

Noteworthy, only partial protection against HRSV by the recombinant influenza virus could be demonstrated, that is, at day 4 or 5 after HRSV challenge, the virus could still be recovered from the lungs. Possibly, the number of CD8\(^+\) T cells induced by vaccination is not high enough or the memory formation is not effective. However, it might be a property that is characteristic to the mouse model of HRSV. Indeed, to achieve sufficient infection in the mice, a high dose of HRSV (typically > 10\(^6\) PFU)
is needed. This is in contrast to a natural infection in humans, which likely originates from a rather small inoculum (26). It is possible that, in humans the CD8+ T cells induced by vaccination might be sufficient to clear the infection, before the virus reaches high titers. Alternatively, a boost vaccination has been shown to increase memory formation, which might result in a better protection (27). We performed such a boosting experiment, using DNA vaccination for the boost, however we did not observe an enhancement of the number of CD8+ T cells, when used in combination with anti-M2e pretreatment; whereas without the anti-M2e treatment, the effect of the boost was more clear. This is an interesting observation and suggests that the treatment with anti-M2e antibodies drives the CD8+ T cell response in the direction of terminally differentiated effector T cells, which cannot be boosted by a second antigen exposure.

Combining the recombinant influenza virus with M2e pretreatment in humans would be an unpractical solution. This would require people to possess anti-M2e antibodies, either through active vaccination, or through passive administration of monoclonal antibodies as used in the mice study described above. Particularly in young infants an active influenza vaccination preceding the HRSV vaccination would extend the period that the child is highly susceptible to HRSV, which is not recommended. Passive administration of monoclonal antibodies would be a faster option, however such a vaccination strategy remains complex and has not been used for other pathogens. Currently it seems rather unlikely that anti-M2e treatment followed by vaccination with the recombinant influenza virus would be an acceptable vaccination strategy for HRSV in infants. Therefore a more practical strategy for reducing vector induced morbidity would be to attenuate the vector. In this thesis we explored two different strategies for producing an attenuated influenza virus. The first strategy consisted of a virus with codon-pair deoptimized HA and NP genes (28). While preserving the wild type amino acid sequence, codon pair deoptimization results in a virus with sub-optimal codon-pairs, causing slower protein synthesis of the deoptimized genes and as a consequence, reduced viral replication. Such an attenuated recombinant influenza virus with codon-pair deoptimized HA and NP and with the HRSV F85-93 CTL epitope in the NA stalk was only mildly attenuated in mice. However, addition of a third codon pair deoptimized gene, such as PB1 has been reported to dramatically increase the attenuation of the virus in vivo (29).

A second strategy used for attenuating the influenza viral vector is by truncating the NS1 protein. Viruses with C-terminally truncated NS1 proteins have been shown to be attenuated in vivo in multiple animal models (30, 31). In addition, we added a GFP coding sequence to the NS1 gene sequence, not so much to attenuate the virus, but rather to follow the course of the infection with this recombinant vector both in vitro and in vivo. We generated such a recombinant influenza virus with the HRSV F85-93 CTL epitope in the NA stalk and a truncated NS1 followed by the GFP coding sequence. We succeeded in rescuing this virus and demonstrated that it was viable, stable and attenuated approximately 10-fold compared to parental virus in vivo in mice. A live attenuated influenza virus carrying an HRSV CTL epitope, as we described may be used as a vaccine against influenza as well as HRSV. First, however, it needs to be explored whether these attenuated viruses still induce a potent CTL response in mice, which is comparable to the parent, not-attenuated virus.
As with any new technology, before the PR8/NA-F<sub>85-93</sub> virus can be used as a live vaccine for the human population, some issues need to be resolved. Stringent safety requirements must be met for viruses that, in their natural state, have the potential to infect humans. Since the PR8/NA-F<sub>85-93</sub> virus does not contain any attenuating element, it is rather unlikely that this virus will be accepted in this form for human use, even in M2e immune humans. Attenuating elements will be required, certainly for use in young infants. Even though LAIV are already being used for influenza vaccination, they have not been accepted for children younger than 2 years old (32). This implies that before a recombinant influenza virus, carrying any foreign protein, whether derived from HRSV or from a different pathogen, will be accepted as childhood vaccine, outstanding safety will need to be proven. However, “if” an attenuated recombinant influenza virus expressing HRSV CTL epitopes could be generated that meets the safety criteria and induces sufficient protective immunity, such a vaccine has a high chance of being accepted for use in young infants (26): after the fatal FI-RSV trials, only live vaccines have been approved for testing in young infants of 1-2 months old (33, 34).

An additional drawback of influenza virus as vaccine vector is its limited coding capacity, implying little space for foreign antigens. To use influenza as vector for inducing HRSV CTL immunity in humans, several human epitopes need to be included to generate a vaccine that covers most human HLA types. The number of residues that can be inserted in the NA stalk is limited (35, 36) and other strategies are required to accommodate large foreign sequences. Replacing a complete gene segment by a series of human CTL epitopes could be promising strategy (37, 38). This strategy only requires the inclusion of the appropriate segment specific packaging signals and providing the deleted gene in trans. Additionally, this strategy could (partly) overcome safety concerns (as mentioned above) since the deletion of an essential gene renders the virus replication defective in humans.

The first recombinant influenza virus expressing foreign antigens has been described more than 20 years ago, however, since then, no recombinant influenza virus expressing foreign epitopes has advanced to clinical testing. This is an indication that the use of influenza as live viral vector is a tough challenge. Alternatively, other viruses could be used, that are more likely of becoming accepted as recombinant vectors. Recombinant viruses such as Adenovirus, Vaccinia virus and Modified vaccinia virus Ankara are currently already being tested in clinical trials as vaccine against various pathogens including HIV, influenza virus, <i>Plasmodium falciparum</i> and <i>Mycobacterium tuberculosis</i> amongst others (39). The main advantage of influenza virus over these other viral vectors is the antigenic variability of influenza viruses, which can be of particular importance when a vaccination requires one or multiple boosts. If the same virus is used for boosting as for the priming immunization, the vaccine can be neutralized rapidly by antibodies directed to the viral surface proteins that were generated during the priming vaccination. When using influenza as viral vector, this can be simply overcome by boosting with a vaccine strain with a different neuraminidase and hemagglutinin subtype than the vaccine strain that was used for priming.
The future HRSV vaccine: will it ever be found?

More than 60 years of vaccine research has not lead to a suitable vaccine candidate against HRSV. Why is it so difficult to find a vaccine that protects against HRSV? There are several factors that complicate the development of a vaccine. First, the lack of a good animal model, that faithfully reproduces the disease in humans is a major drawback. HRSV animal studies can give us a good clue of what ‘might’ happen in humans, however they rarely provide the complete picture, and often, different results are observed in animals than in humans. A good example is the fact that CD8⁺ T cells can contribute to disease in the mouse model (20), whereas this has not been seen in humans (40). Since the development of a single animal model that completely mimics the human situation is a utopia, testing the vaccine candidates in multiple animal models is the only possibility. A second factor complicating HRSV vaccine development is linked to the main target group of the vaccine, i.e. infants of 1-2 months old. The immune system in these young children is less well developed as in adults, and is mainly biased in the Th2 direction, which should be avoided for HRSV vaccines. It should be noted however, that this Th2 bias is more pronounced in mice, and is less explicit in human new-borns. Rather the CTL response in new-borns is diminished in magnitude compared to adults and, when formulated correctly a Th1 response can be evoked (41). In addition, at very young age, most infants still possess maternally derived anti-HRSV antibodies, that may shield off vaccines, and hence prevent efficient vaccination (42). However, this does not mean that vaccination at this young age is excluded: in Belgium, as in many other countries, vaccination is started at the age of 6 weeks (43). In accordance with the WHO guidelines for vaccination (44), at the age of 6 to 8 weeks old children receive vaccines against Rotavirus, Polio, Diphtheria, Tetanus, *Haemophilus influenzae*, Pertussis and Hepatitis B (43). In addition, children born from infected mothers may receive a Hepatitis B or BCG vaccine as early as 24h after birth (45, 46). These data indicate that it is possible to vaccinate children during the first two months of life. A third and maybe the biggest factor complicating HRSV vaccine development is the complexity of the virus itself and the immune response it induces. HRSV has developed many strategies to evade the innate immunity of the host. For example, the non-structural proteins, NS1 and NS2 collaborate to inhibit expression of IFN-associated genes through IRF3 and Stat2 (47, 48). Also, HRSV inhibits TLR signalling through MyD88 and MAVS and it interferes with RIG-I signaling (49, 50). Many other examples exist, and a thorough understanding of these processes and their consequences for immunity is needed to be able to design a safe HRSV vaccine. In addition, HRSV does not evoke long term protective immunity. A better understanding of memory formation after HRSV infection is needed to design vaccines that efficiently induce memory formation. And last, we need to understand the processes related to the enhancement of disease, associated with the FI-RSV vaccine. Only when the origin of this exacerbation is completely understood, we can rationally design vaccines without this complication. When we look back at the past vaccine attempts against HRSV, we can roughly categorize them into three groups. A schematic representation is given in figure 7.1. On one side of the spectrum we find the non-replicating vaccines, with the FI-RSV at the extreme end. This vaccine caused exacerbation of disease and hence is not suitable as an HRSV vaccine. In the same group, but a bit more to the right,
we find the subunit vaccines, which in some cases were also found to induce enhanced disease, however, not as bad as the FI-RSV vaccine. On the other end of the spectrum we find the second group of vaccines, i.e. the live attenuated HRSV vaccines. The first generations of these vaccines suffered from safety issues: when not sufficiently attenuated, these vaccine viruses could still cause disease. These two groups form the extremes of the spectrum, which are considered not suited as an HRSV vaccine candidate. In between these two categories, we find a big group with a variety of different types of vaccine strategies (such as recombinant viral vectors, virus-like particles, non-replicating viral vectors etc.) of which it is less clear where exactly on the scale they should be placed. The narrow green zone in the middle, between the inactivated and the live vaccines represents the “ideal” HRSV vaccine, that has not been discovered yet. This ideal vaccine should induce an antibody response and a T cell response, and provide long term memory, without the risk of enhancement of disease. Although, due to the different immune status of various age groups, one vaccine that will be suitable for every individual will be rather unlikely. It is likely that specific vaccines for each of these age groups will need to be developed. Future will tell if it is possible to develop a safe and effective HRSV vaccine.

Figure 7.1 Schematic representation of the vaccine candidates for HRSV.
Samenvatting, algemene discussie en toekomstperspectieven

Er is dringend nood aan preventieve maatregelen tegen het humaan respiratoir syncytieel virus (HRSV). De enige beschikbare preventieve maatregel op dit moment bestaat uit een maandelijkse intramusculaire injectie van een monoclonaal antilichaam (Palivizumab) gericht tegen het HRSV F eiwit. Deze maatregel zorgt voor een significante reductie in het aantal hospitalisatie dagen als gevolg van een HRSV infectie: het totaal aantal hospitalisatie dagen voor 100 patiënten wordt gereduceerd van 62,6 dagen naar 36,4 dagen als gevolg van de palivizumab behandeling (1, 2). De behandeling met palivizumab kan echter niet gebruikt worden voor alle kinderen (2). Door de hoge kostprijs wordt de behandeling enkel geadviseerd voor kinderen die een hoog risico lopen op complicaties als gevolg van een HRSV infectie, zoals prematuur geboren baby’s, of kinderen met aangeboren hart en long afwijkingen (3). Maar zelfs in deze hoge risico groep kan een Palivizumab behandeling niet elk kind beschermen tegen ernstige ziekte als gevolg van een HRSV infectie (2). Daarom zou de ontwikkeling van een veilig en doeltreffend vaccin, dat zware ziekte als gevolg van een HRSV infectie kan verhinderen, een grote doorbraak betekenen.

Aangezien het algemeen geweten is dat antilichamen gericht tegen het HRSV F eiwit correleren met bescherming, zou een logische strategie om HRSV infecties te verhinderen eruit kunnen bestaan om zulke antilichamen op te wekken door middel van een actief vaccinatie protocol; een strategie die in het verleden met succes is toegepast voor het onderdrukken van de meeste (kinder-)ziektes zoals bvb. deze veroorzaakt door het polio virus of het hepatitis A en B virus. De geschiedenis heeft ons echter geleerd dat de situatie voor HRSV “niet zo simpel is als het lijkt”. Vaccinatie van kinderen met een formaline-geïnactiveerd HRSV (FI-RSV) vaccin - een strategie die reeds met succes was toegepast voor het ontwikkelen van andere vaccins – leidde tot ernstige ziekte ten gevolge van een HRSV infectie in 80% van de gevaccineerde kinderen met zelfs 2 doden tot gevolg (4). Kenmerkend voor deze zware ziekte was de zwakke neutraliserende activiteit van de opgewerkte antilichamen en de aanwezigheid van Th2 CD4+ immuun cellen (5, 6). Sinds deze fatale klinische studies was het duidelijk dat een HRSV vaccin dat IL-4 producerende CD4+ T cellen opwekt absoluut moet vermeden worden bij HRSV-naïeve baby’s.
**Een virus-achtig partikel vaccin-kandidaat tegen HRSV.**

Een eerste vaccinatie strategie maakt gebruik van het feit dat antilichamen gericht tegen het HRSV F eiwit bescherming kunnen bieden tegen een HRSV infectie. Het doel was om een antilichamen op te wekken door korte peptiden van het HRSV F eiwit te koppelen aan een dragermolecule, nl. een virusachtig partikel (VLP) opgebouwd uit monomeren van het hepatitis B virus kern eiwit (HBC). Hierbij was onze hypothese dat de sterk repetitieve structuur waarin de HRSV peptiden gepresenteerd worden aan het oppervlak van het VLP een goede strategie zou vormen die een grote kans maakte voor het opwekken van een sterk antilichaam-gemedieerd immuun antwoord. Deze strategie zou bovendien kunnen werken zowel in aan- als afwezigheid van helper T cellen. Aangezien VLP-gebaseerde vaccins niet meer in staat zijn, het vaccin bestaat nl. enkel uit structurele eiwitten zonder een functioneel genoom – het vaccin bestaat nl. enkel uit virale dragers die nog de mogelijkheid bezitten om te repliceren en bijgevolg nog (gedeeltelijk) in staat zijn om ziekte te veroorzaken. Vaccin veiligheid wordt beschouwd als een belangrijke factor wanneer jonge kinderen de doelgroep vormen. Daarom onderzochten we de mogelijkheden van een HBC-gebaseerd VLP vaccin als kandidaat voor een nieuwe HRSV vaccinatie strategie. Deze techniek werd in verleden reeds met succes toegepast in onze onderzoeksgroep voor het ontwikkelen van een vaccin tegen influenza A virus, gebaseerd op het ectodomein van matrix 2 eiwit (7-10). Voor het HRSV vaccin werden twee soorten peptiden gekoppeld aan het VLP door middel van genetische fusie. Ten eerste werd gekozen voor korte peptiden afkomstig van regio’s in het F eiwit die epitopen bevatten van reeds gekende neutraliserende antilichamen. Als tweede bron van antigen gebruikten we grote fragmenten van het F eiwit welke mogelijk de natuurlijke conformatie van het complete F eiwit beter benaderen. De gekozen antigenen bezitten geen N-gekoppelde glycosylatieplaatsen. Deze peptiden of domeinen afgeleid van HRSV werden genetisch gefusioneerd zowel aan de immunodominante lus (MIR) van HBc als aan het N-terminale uiteinde van de HBc monomeer. Helaas resulteerde het koppelen van ieder van deze peptiden aan HBc in een onoplosbaar VLP product. Wijzigingen van de zoutconcentratie of de pH van de sonicatiebuffer waarin de bacteriën gesoniceerd werden bood geen oplossing en resulteerde niet in een meer oplosbaar product. Dit zorgt voor moeilijkheden bij het opschalen van het productieproces en de zuivering van het vaccin. Een mogelijke verklaring voor deze onoplosbaarheid kan liggen in de hydrofobiciteit van de gebruikte peptiden. Het M2e peptide, dat reeds met succes aan HBc VLPs werd gekoppeld is sterk hydrofiel, terwijl de meeste HRSV peptiden die gebruikt werden eerder een hydrofoob karakter vertonen. These hydrofobe gebieden die zich aan het oppervlak van het VLP bevinden kunnen ervoor zorgen dat het partikel moeilijk oplosbaar is in waterige vloeistoffen. In een poging om de oplosbaarheid te verhogen plaatsten we de coderende sequentie van M2e na deze van het HRSV peptide aan de N-terminus van HBc. Opnieuw resulteerde dit niet een product dat goed oplosbaar was. Een alternatieve strategie maakt gebruik van een gemodificeerd alfavirus als drager. Deze virus replicon partikels (VRPs) bezitten een gemodificeerd RNA genoom dat de coderende sequentie van een heteroloog antigen draagt, wat bij vaccinatie met dit partikel resulteert in overvloedige expressie van dit antigen in de geïnfecteerde cel (11). Beide strategieën, VLPs en VRPs hebben hun voordelen.
ten opzichte van de andere. A groot voordeel van de VRP strategie is dat wordt gebruik gemaakt van een niet-humaan virus, zoals een Equine encephalitis virus, hetgeen ervoor zorgt er geen immuniteit bij de mensen aanwezig is die het effect van het vaccin kan onderdrukken. VLPs echter bezitten de mogelijkheid om een immuun antwoord op te wekken in afwezigheid van helper T cellen. Een voordeel van VLPs is het sterk repititieve patroon waarin vreemde antigenen worden gepresenteerd aan het immuun systeem wat zorgt voor rechtstreeksse crosslinking van B cel receptoren en de activatie van de B cel zonder de hulp van T cellen (12, 13) alsook voor T cel afhankelijke immuunstimulatie wat zorgt voor affiniteitsmaturatie en isotype switch.

Aangezien we er niet in geslaagd zijn om partikels te produceren die geschikt waren om te testen in vivo in muizen, kunnen we geen antwoord geven op de vraag of deze vaccinatie strategie bescherming kan bieden tegen een HRSV infectie. Het is echter niet ondenkbaar dat het opwekken van een CD8⁺ T cel immuun antwoord noodzakelijk is om een HRSV infectie tegen te gaan. Toch biedt ook een vaccin zoals dit hier voorgesteld, dat enkel een antilichaam-gemedieerd immuun antwoord opwekt zonder een CD8⁺ T cel component, mogelijkheden. Ook al is het onwaarschijnlijk dat niet-levende vaccins zullen geaccepteerd worden voor gebruik in jonge kinderen, toch kunnen deze vaccins gebruikt worden voor het vaccineren van volwassenen en oudere kinderen. Een strategie waarvoor dit soort antilicham-inducerende vaccins een voordeel kan bieden is de zogenaamde “kudde vaccinatie” strategie (14). Deze strategie is gebaseerd op de vaststelling dat babies vaak geïnfecteerd worden door de naaste familie, zoals een oudere broer of zus, een ouder of iemand anders met wie hij samen woont. Doordat deze oudere personen vaak slechts milde symptomen vertonen bij een HRSV infectie zijn ze er zich niet van bewust dat ze een bron van infectie kunnen zijn voor de baby. Voor andere virale infecties werd reeds aangetoond dat het vaccineren van een grote populatie de kansen op infectie van niet-gevaccineerden, in dit geval de baby, drastisch doet afnemen (14). Een andere strategie waarvoor een antilichaam-opwekkend vaccin zinvol kan zijn is de maternale vaccinatie strategie. Er werd reeds aangetoond dat de overdracht van HRSV-specifieke antilichamen van de moeder naar de baby tijdens de zwangerschap correleert met een verlaagde kans op HRSV infectie van de baby tijdens de eerste weken na de geboorte (15). Bovendien werd in een klinische studie bevestigd dat vaccinatie van zwangere vrouwen met een gezuiverd F eiwit vaccin kandidaat (PFP-2) zorgde voor een verhoogd niveau aan HRSV-specifieke antilichamen bij hun baby (16). Deze vaccinatie strategie kan interessant zijn om de baby gedurende de eerste weken van zijn leven, wanneer het kind nog te jong is voor vaccinatie, een zekere mate van bescherming te bieden tegen een HRSV infectie.

**Een levend verzwakt influenza virus als HRSV vaccin kandidaat.**

Een als maar toenemende hoeveelheid data steunen het idee dat het opwekken van een T cel immuun antwoord essentieel zal zijn voor het ontwikkelen van een doeltreffend HRSV vaccin. Ten eerste heeft men vastgesteld dat de FI-RSV gevaccineerde kinderen die zwaar ziek werden ten
gevolge van een HRSV infectie nauwelijks een CTL immuun antwoord hadden opgewekt (17). Ten tweede werd aangetoond dat CTLs in een muismodel van vaccinatie-geinduceerde ziekte-verergering de ontwikkeling van zware ziekte konden verhinderen (17-19). Ten derde werd aangetoond dat de piek in het CTL immuun antwoord bij de mens voorkomt tijdens het herstel van de ziekte en niet tijdens de piek van de ziekte, zoals bij muizen (20, 21). Als laatste heeft men zowel bij de muis als bij de mens kunnen aantonen dat CTLs noodzakelijk zijn om het virus volledig te elimineren uit de longen van geïnfecteerde dieren of patiënten (20, 21).

Om na te gaan of het mogelijk is een CD8\(^+\) T cel immuun antwoord op te wekken door middel van een levende virale vector hebben we een recombinant influenza virus gemaakt dat het HRSV F\(_{85-93}\) cytotoxische T lymfociet (CTL) epitoop draagt in de stam van het neuraminidase eiwit. Wanneer muizen met dit virus geïnfecteerd werden werd op een CTL immuun antwoord opgewekt dat dosis-afhankelijk was. Bij een daaropvolgende infectie met HRSV waren deze CTLs in staat om de vermenigvuldiging van het virus in de longen af te remmen, wat aantoont dat de opgewekte CTLs een zekere mate van bescherming kunnen bieden tegen een HRSV infectie. Aangezien het vaccin bestaat uit een levend virus dat nog in staat is om ziekte te veroorzaken is het wenselijk om de infectiviteit van dit virus te onderdrukken en zodoende de vector-geinduceerde ziekte te voorkomen. Dit werd bereikt door de muizen voorafgaand aan de vaccinatie te behandelen met een monoklonaal antilichaam dat gericht is tegen het influenza M2e. In tegenstelling tot neutraliserende antilichamen zijn deze anti-M2e antilichamen infectie-permissief, hetgeen noodzakelijk is voor de correcte presentatie van het CTL epitoop op MHC-I moleculen en het opwekken van een CTL immuun antwoord. Terwijl het gewichtsverlies ten gevolge van de influenza infectie volledig kon worden onderdrukt, werd nog steeds een HRSV specifiek CTL immuun antwoord opgewekt. Bovendien zijn deze CTLs nog steeds in staat om de vermenigvuldiging van HRSV in de longen van de muis te onderdrukken bij een HRSV infectie.

We stelden herhaaldelijk vast dat muizen die gevaccineerd werden met een controle influenza virus dat geen HRSV CTL epitoop bevat, eveneens in mindere mate in staat waren om de vermenigvuldiging van HRSV in de longen te onderdrukken, in vergelijking met muizen die PBS toegediend kregen. Dit zou erop kunnen wijzen dat een influenza infectie gedeeltelijk kan beschermen tegen een HRSV infectie. Dit soort heterologe immuniteit, nl. de bescherming tegen één pathoheen die opgewekt wordt door een infectie met een ander niet verwant pathoheen werd reeds herhaaldelijk waargenomen tussen verscheidene pathoheen zoals parasieten, protozoa, bacteriën en virussen (22). Dit fenomeen werd reeds waargenomen zowel tussen verwante als niet verwante pathoheen en kan leiden tot hetzij een verminderen hetzij een verbetering van de bescherming, zoals hier wordt waargenomen voor een influenza infectie gevolgd door een HRSV infectie. Deze heterologe immuniteit kan een gevolg zijn van processen van hetzij het aangeboren immuun antwoord, hetzij het adaptief immuun antwoord. Het bestaan van kruis-reagerende T cellen tussen influenza en HRSV zou een mogelijke verklaring kunnen zijn voor de geobserveerde bescherming. Het bestaan van dit soort kruis-reagerende CD8\(^+\) T cellen werd reeds aangetoond tussen verschillende
pathogenen, zowel van muis als van mens oorsprong (samengevat in Welsh et al. (22)). Bijvoorbeeld, in hepatitis C virus naïeve personen werden T cellen waargenomen die reageerden bij stimulatie met het HCV HLA-A2 gerestricteerde NS3_{1073-1081} epitoot (CVNGVCWTV). Verder onderzoek toonde aan dat het hier influenza A virus NA_{231-239} (CVNGSCFTV) specifieke geheugen T cellen betrof, die in staat waren te kruis-reageren met het HCV NS3_{1073-1081} epitoot. (23). Tot op heden werden nog geen kruis-reagerende T cellen waargenomen tussen influenza en HRSV. Een alternatieve verklaring voor de heterologe bescherming die werd waargenomen zou omstaander CD8^+ T cel activering kunnen zijn. Dit werd eerder reeds waargenomen in een model van HRSV-gemedieerde verergering van ziekte in muizen geprimed met het HRSV G eiwit: hier zorgde een voorafgaande influenza infectie ervoor dat de G-eiwit afhankelijke ziekteverergering, die normaal wordt waargenomen bij een influenza infectie van G-eiwit geprimeerde muizen, werd onderdrukt (24). Bescherming tegen niet-verwante pathogenen werd eveneens waargenomen na een BCG vaccinatie. Dit is een gevolg van het aangeboren immuunsysteem en wordt veroorzaakt door epigenetische herprogrammering van monocyten (25). Het is niet duidelijk welke arm van het immuunsysteem verantwoordelijk is voor de niet-specifieke bescherming tegen HRSV, ten gevolge van een influenza infectie.

We konden met het recombinant influenza virus slecht gedeeltelijke bescherming tegen HRSV aantonen: op dag 4 of 5 na infectie kon nog steeds HRSV uit de longen van de muizen geïsoleerd worden. Mogelijk is dit te verklaren doordat het aantal CD8^+ T cellen dat gegenereerd werd door de vaccinatie niet voldoende hoog was, of doordat geheugen T cellen niet efficiënt gegenereerd werden. Anderzijds is het ook mogelijk dat dit een inherente eigenschap is van het HRSV muis-model: om voldoende infectie te bereiken worden muizen met een hoge dosis HRSV geïnfecteerd (vaak > 10^6 PFU), hetgeen in sterk contrast is met een menselijke HRSV infectie, die vaak ontstaat van een klein inoculum (26). Het is mogelijk dat bij de mens de CD8^+ T cellen gegenereerd door vaccinatie met het PR8/NA-F_{85-93} virus in staat zijn om het virus te elimineren alvorens het hoge titers bereikt. Tevens werd reeds aangetoond het genereren van geheugen T cellen kan bevorderd worden door een booster vaccinatie, hetgeen mogelijks tot betere bescherming kan leiden (27). Wij voerden zo’n booster vaccinatie uit door middel van DNA vaccinatie maar we stelden vast dat dit niet resulteerde in een verhoging van het aantal CD8^+ T cellen bij muizen die een anti-M2e behandeling kregen. Enkel bij muizen die niet met enkele antilichamen behandeld werden was een effect van de boost vaccinatie merkbaar. Dit is een interessante vaststelling die suggereert dat de behandeling met M2e immuun serum de CD8^+ T cellen duwt in de richting van terminale differentiatie, welke niet meer kan geboost worden door een tweede blootstelling aan antigeen.

De combinatie van een recombinant influenza virus met een voorafgaande M2e behandeling is een eerder onpraktische oplossing voor een HRSV vaccin bij de mens. Hiervoor zou de te vaccineren persoon anti-M2e immunititeit moeten bezitten, verkregen door hetzij actieve influenza vaccinatie of door middel van passieve toediening van anti-M2e antilichamen zoals in de muizenstudie werd gebruikt. In het bijzonder bij jonge kinderen is een actieve influenza vaccinatie voorafgaand aan de HRSV vaccinatie niet aangewezen, aangezien dit de periode dat het kind extreem vatbaar is voor HRSV infecties zou verlengen. Het passief toedienen van monoclonale antilichamen zou een snellere
mogelijkheid zijn. Deze strategie werd tot op heden echter nog niet toegepast voor een menselijk vaccin waardoor het eerder onwaarschijnlijk lijkt dat de combinatie van een anti-M2e voorbehandeling gevolgd door een vaccinatie met het recombinant influenza virus zal aanvaard worden als HRSV vaccin voor jonge kinderen. Daarom zou afzwakking van de vector een alternatieve oplossing kunnen bieden voor het onderdrukken van de vector-gemedieerde ziekte. In deze thesis werden twee strategieën voor het verzwakken van influenza virus onderzocht. In een eerst strategie werd een influenza virus gegeenereerd met een codon-paar gedeoptimaliseerd HA en NP gen (28). Bij codon-paar deoptimalisatie wordt de wild type aminozuur sequentie van de eiwitten behouden, echter door de niet-optimale codon paren verloopt synthese van deze eiwitten op een trager tempo met verminderde virus verminderdering tot gevolg. Een recombinant influenza virus met het HRSV F_{85-93} CTL epitoop in de NA stam en een codon-paar gedeoptimaliseerd HA en NP gen was echter slechts matig verzwakt in muizen. Doch er werd eerder reeds aangetoond dat toevoegen van een derde codon-paar gedeoptimaliseerd gen, bvb. PB1, de in vivo afzwakking van het recombinant virus drastisch verhoogt (29).

Een tweede strategie die resulteert in een afgezwakte influenza virale vector is het inkorten van het NS1 eiwit. Men heeft reeds aangetoond dat influenza virussen met NS1 eiwitten ingekort langs de cytoplasmatische zijde verzwakt zijn in meerdere diermodellen (30, 31). Bijkomend hebben we de coderende sequentie van GFP toegevoegd aan het NS1 gen, dit niet zozeer als additioneel afzwakkend element, maar hoofdzakelijk om het verloop van een infectie met dit recombinant vector zowel in vivo als in vitro te kunnen volgen. Zo'n verzwakt recombinant influenza virus werd gecreëerd met een verkort NS1 gevolgd door de GFP coderende sequentie, in combinatie met een neuraminidase eiwit met het HRSV F_{85-93} CTL epitoop in de stam. We slaagden erin dit virus te genereren en konden aantonen dat dit virus leefbaar is, stabel is in vitro en ongeveer tienvoudig verzwakt in muizen. Een levend verzwakt influenza virus dat een HRSV CTL epitop draagt, zoals hier werd beschreven, kan gebruikt worden als een vaccin tegen zowel HRSV als influenza. Eerst zal echter moeten nagegaan worden of deze verzwakte virusen nog steeds een CTL immuun antwoord opwekken tegen HRSV dat van vergelijkbare grootte orde is als bij het niet-verzwakt virus.

Zoals bij elke nieuwe technologie, zullen eerst een aantal zaken opgehelderd moeten worden alvorens het PR8/NA- F_{85-93} virus zal kunnen gebruikt worden als levend vaccin in de menselijke populatie. Virale vectoren die in hun normale toestand de mogelijkheid bezitten om mensen te infecteren moeten voldoen aan sterke veiligheidsvereisten. Aangezien het PR8/NA- F_{85-93} Virus geen verzwakkingsinsectenteen bezit is het eerder onwaarschijnlijk dat dit virus onder deze vorm zal geaccepteerd worden voor gebruik in mensen, zelfs in M2e immune mensen. Elementen die het virus afzwakken zijn noodzakelijk, zeker wanneer het vaccin bestemd is voor gebruik bij jonge kinderen. Levend verzwakte influenza virussen worden reeds gebruikt als vaccin tegen influenza, maar niet bij kinderen onder de leeftijd van 2 jaar (32). Dit toont aan dat, voordat een recombinant influenza virus, als drager van een vreemd antigeen, hetzij van HRSV afgeleid, hetzij van een ander pathogeen, kan gebruikt worden als vaccin bij kinderen, er zal moeten aangetoond worden dat het vaccin extreem veilig is. Echter, “als” een verzwakt recombinant influenza virus, dat HRSV CTL epitopen
presenteert, kan gevonden worden dat voldoende veilig is en een sterk immuun antwoord opwekt, heeft zo’n vaccin een grote kans om geaccepteerd te worden als vaccin voor jonge kinderen (26): immers, de enige vaccines die na de fatale klinische proeven met het FI-RSV vaccin werden gebruikt bij kinderen jonger dan 2 maand waren levende verzwakte virussen (33, 34).

Een bijkomend nadeel van het gebruik van influenza virus als vaccin drager is zijn beperkte coderende capaciteit, wat inhoudt dat er weinig ruimte is voor het aanbrengen van vreemde antigenen. Om influenza virus te gebruiken als vector voor het opwekken van een HRSV CTL immuun antwoord bij de mens moeten meerdere menselijke CTL epitopen aangebracht worden in het virus die kunnen herkend worden door de meerderheid van de HLA subtypes. Het aantal residuen dat kan worden aangebracht in de NA stam is echter beperkt (35, 36). Andere strategieën zullen nodig zijn om grote inserties toe te laten. Het vervangen van een volledig influenza gen door een reeks menselijke CTL epitopen is een mogelijkheid (37, 38). Hiervoor hoeft men enkel de nodige inpak signalen van het influenza gen-segment aan te brengen en het verwijderde gen te voorzien in trans. Bovendien omzeilt men zo (gedeeltelijk) de veiligheidsproblemen, omdat de deletie van een essentieel gen ervoor zorgt dat het influenza virus zich niet meer kan vermeerderen in mensen.

Het eerste recombinante influenza virus dat vreemde antigenen draagt werd reeds meer dan 20 jaar geleden beschreven, maar tot op heden is nog geen recombinant influenza virus getest voor gebruik bij mensen. Dit toont aan dat het gebruik van influenza virus als levende virale drager een moeilijke uitdaging is. Als alternatief zouden andere virussen kunnen gebruikt worden die een grotere kans hebben om aanvaard te worden voor menselijk gebruik. Recombinante virussen als Adenovirus, Vaccinia virus en Modified vaccinia virus Ankara worden momenteel reeds getest in klinische proeven als vaccin tegen o.a. HIV, influenza virus, Plasmodium falciparum en Mycobacterium Tuberculosis (39). Het grote voordeel van influenza virussen tegenover deze andere virussen voor het gebruik als virale vector is de antigenische variabiliteit die influenza virussen bezitten. Dit is vooral van belang wanneer één of meerdere boost vaccinaties nodig zijn. Indien hetzelfde virus wordt gebruikt voor de boost vaccinatie als tijdens de eerste vaccinatie kan het vaccin snel geneutraliseerd worden door antilichamen gericht tegen de oppervlakte eiwitten van het vaccin virus, die werden opgewekt tijdens de eerste vaccinatie. Wanneer mijn influenza gebruikt als virale vector kan dit probleem vermeden worden door te boosten met een vaccin stam die een verschillend neuraminidase en hemagglutinine eiwit bezit als de stam die werd gebruikt voor de eerste vaccinatie.

**Het toekomstige HRSV vaccin: zal men het ooit vinden?**

Meer dan 60 jaar aan onderzoek heeft nog niet geleid tot de ontwikkeling van een geschikt kandidaat HRSV vaccin. Waarom is het zo moeilijk om een vaccin te ontwikkelen dat beschermt tegen een HRSV infectie? Er zijn verschillende factoren die de ontwikkeling van een HRSV vaccin bemoeilijken. Een eerste groot nadeel is het ontbreken van een goed diermodel dat de ziekteverschijnselen van een HRSV infectie in mensen waarheidsgetrouw kan nabootsen. HRSV dierenproeven kunnen ons aanwijzingen geven van wat er mogelijks kan gebeuren bij mensen, maar ze geven ons zelden het
volledige plaatje, en vaak worden er verschillende resultaten waargenomen in dieren ten opzichte van mensen. Een goed voorbeeld hiervan is dat CD8+ T cellen bij muizen bijdragen tot de pathologie (20), terwijl dit nog niet werd waargenomen bij mensen (40). Aangezien het ontwikkelen van één enkel diermodel dat alle ziekteverschijnselen bij een humane HRSV infectie volledig kan nabootsen een utopie is, blijft het testen van vaccins in meerdere diermodellen de enige overgebleven mogelijkheid. Een tweede factor die de ontwikkeling van een HRSV vaccin bemoeielt houdt verband met de belangrijkste doelgroep voor dit vaccin, nl. pasgeboren baby’s van 1 à 2 maanden oud. Het immuun systeem in deze jonge kinderen is nog onderontwikkeld vergeleken met volwassenen en het immuun antwoord dat wordt opgewekt is hoofdzakelijk gestuurd in de Th2 richting, hetgeen moet vermeden worden voor HRSV vaccins. Hierbij moet wel opgemerkt worden dat deze dominantie van het Th2 immuun antwoord meer uitgesproken is bij jonge muizen dan bij baby’s. Bij deze laatste wordt vooral vastgesteld dat het CTL immuun antwoord minder groot is vergeleken met volwassenen, maar onder de juiste omstandigheden is het mogelijk om een Th1 immuun antwoord op te wekken (41). Tevens bezitten jonge kinderen vaak nog materiale anti-HRSV antilichamen, die mogelijks vaccins kunnen afschermen van het immuun systeem en zo efficiënte vaccinatie verhinderen (42). Dit betekent echter niet dat het onmogelijk is kinderen te vaccineren op jonge leeftijd: in België wordt, net als in vele andere landen, gestart met vaccinatie vanaf de leeftijd van 6 weken (43). De richtlijnen van het WHO schrijven voor dat kinderen op de leeftijd van 6 à 8 weken mogen gevaccineerd worden tegen rotavirus, polio, difterie, tetanus, Haemophilus influenzae, kinkhoest en hepatitis B (44). Bovendien kunnen kinderen van de moeder geïnfecteerd zijn met hepatitis B of Mycobacterium tuberculosis een vaccin krijgen tegen deze ziekte zodra ze 24 uur oud zijn (45, 46). Deze gegevens tonen aan dat het mogelijk is om kinderen reeds in de eerste twee maanden van hun leven te vaccineren. Een derde en misschien wel belangrijkste factor die de ontwikkeling van een HRSV vaccin bemoeielt is het HRSV virus zelf en het immuun antwoord dat het opwekt. HRSV heeft meerdere strategieën ontwikkeld om te ontkomen aan het aangeboren immuun systeem van de gastheer. Zo werken de niet-structurele eiwitten, NS1 en NS2, samen om expressie van interferon-geassocieerde genen te verhinderen door interacties met IRF-3 en Stat2 (47, 48). Bovendien verhindert HRSV signalering via TLRs door interacties met MyD88 en MAVS en verstoort het signalering via RIG-I (49, 50). Er bestaan vele andere voorbeelden en de doorgronden van deze processen en de gevolgen die ze hebben op het immuun antwoord is noodzakelijk voor de ontwikkeling van een veilig HRSV vaccin. Bovendien wekt HRSV geen langdurig immuun antwoord op. Daarom is een beter begrip van de vorming immunologisch geheugen na een HRSV infectie noodzakelijk voor de ontwikkeling van vaccins die efficiënt langdurige immuniteit opwekken. Tenslotte is het noodzakelijk om de processen die verantwoordelijk zijn voor de verergering van de ziekte na een FI-RSV vaccinatie te doorgronden. Alleen wanneer de oorzaak van deze ziekteverergering duidelijk is zal het rationeel ontwerpen van een vaccin zonder deze complicatie mogelijk zijn.

Wanneer we terugkijken naar alle reeds geprobeerde vaccin strategieën tegen HRSV kunnen we deze ruwweg indelen in drie categorieën. Een schematische voorstelling is weergegeven in figuur 7.2. Langs één zijde van het spectrum vinden we de geïnactiveerde vaccins, met het FI-RSV vaccin aan het
extreem linkse uiteinde. Omwille van de ziekteverergering die door dit vaccin werd veroorzaakt is het niet geschikt als HRSV vaccin. In dezelfde groep, maar iets meer naar rechts, vinden we de geïnactiveerde subunit vaccins, waarvan bij sommigen eveneens ziekteverergering werd vastgesteld, evenwel niet zo extreem als bij het FI-RSV vaccin. Aan de andere zijde van het spectrum vinden we de tweede groep vaccins, nl. de levend verzwakte HRSV vaccins. De eerste generatie van deze vaccins hadden te kampen met veiligheidsproblemen: indien niet voldoende verzwakt was het vaccin nog in staat om ziekte te veroorzaken. Deze 2 groepen vormen de uiteinden van het spectrum en zijn niet geschikt als HRSV vaccin. Tussen deze twee groepen vinden we de derde groep die bestaat uit allerlei vaccinatie strategieën (zoals recombinante virale vectoren, virus-achtige partikels, virale vectoren die niet meer in staat zijn te vermeerderen etc.) waarvan het minder duidelijk is waar exact ze op de schaal moeten geplaatst worden. De smalle groene zone, centraal gelegen tussen de geïnactiveerde vaccins en de levende verzwakte virussen stelt het “ideale” HRSV vaccin voor, dat tot op heden nog niet werd gevonden. Dit ideaal vaccin moet naast een antilichaam immuun antwoord een T cel immuun antwoord opwekken en langdurige bescherming garanderen, zonder risico op ziekteverergering. Hierbij moet wel opgemerkt worden dat, omwille van de verschillen in immunologische staat van de verschillende leeftijdsgroepen het eerder onwaarschijnlijk is dat één vaccin bescherming zal kunnen bieden aan iedereen. Waarschijnlijk zal voor elke leeftijdsgroep een verschillend vaccin moeten ontwikkeld worden. De toekomst zal uitwijzen of het mogelijk is om een veilig en doeltreffend HRSV vaccin te vinden.

Figuur 7.2 Schematische weergaven van kandidaat HRSV vaccins.
References


Part 5

Addenda
Curriculum vitae

Contact information

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2005 - 2007  **Master Biotechnology**, Universiteit Gent, Belgium; Great distinction
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2003 - 2005  **Bachelor Biology**, Universiteit Gent, Belgium; Great distinction
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2009 & 2010  Practical course Molecular biology I (Bachelor Biotechnology)

Publications


Conference proceedings and oral presentations

Oral presentations

**16th DMBR workshop, Inflammation and immunity Research Program**

**Doctoraatssymposium faculteit wetenschappen, Ugent**

**Inflammation and vaccination seminar, Ugent faculty of veterinary medicine**

Posters

**8th International respiratory syncytial virus symposium**
Santa Fe, New Mexico, USA. September 27-30, 2012.

Other

**7th International respiratory syncytial virus symposium**

**Vaccine symposium on infection and immunity, UMC University**

**2nd International workshop on humanized mice**
Nanobodies® Specific for Respiratory Syncytial Virus Fusion Protein Protect Against Infection by Inhibition of Fusion

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(See the editorial commentary by Graham, on pages 1648–50.)

Despite the medical importance of respiratory syncytial virus (RSV) infections, there is no vaccine or therapeutic agent available. Prophylactic administration of palivizumab, a humanized monoclonal RSV fusion (F) protein–specific antibody, can protect high-risk children. Previously, we have demonstrated that RSV can be neutralized by picomolar concentrations of a camelid immunoglobulin single-variable domain that binds the RSV protein F (F-VHHb nanobodies). Here, we investigated the mechanism by which these nanobodies neutralize RSV and tested their antiviral activity in vivo. We demonstrate that bivalent RSV F–specific nanobodies neutralize RSV infection by inhibiting fusion without affecting viral attachment. The ability of RSV F–specific nanobodies to protect against RSV infection was investigated in vivo. Intranasal administration of bivalent RSV F–specific nanobodies protected BALB/c mice from RSV infection, and associated pulmonary inflammation. Moreover, therapeutic treatment with these nanobodies after RSV infection could reduce viral replication and reduced pulmonary inflammation. Thus, nanobodies are promising therapeutic molecules for treatment of RSV.

Infection by respiratory syncytial virus (RSV) is the leading cause of acute lower respiratory tract disease in children worldwide. RSV infects approximately 90% of all children at least once before the age of 2 [1]. As RSV infections themselves do not evoke long-living immune protection, they recur throughout life, causing also considerable morbidity and mortality in the elderly [2]. It has been estimated that RSV infects about 64 million people annually and results in 160 000 deaths. Although most RSV infections cause only moderate symptoms, about 0.5% of children with a primary RSV infection require hospitalization because of bronchiolitis or pneumonia. RSV infections in early life are associated with long-term respiratory distress [3].

Despite the major importance of RSV infections, no vaccine or effective antiviral therapy is available. However, a particular prophylactic treatment can reduce RSV-associated hospitalization of high-risk infants by 55% [4]. This prophylactic therapy is based on monthly intramuscular administration of large amounts (15 mg/kg) of a humanized monoclonal antibody, palivizumab (Synagis), which is directed against an epitope in the antigenic region II of the RSV fusion protein. Because this treatment is very expensive, it cannot be used generally [5].

Camelids and sharks express not only conventional antibodies but also functional antibodies composed only of heavy chains (HcAbs) [6]. In these antibodies, antigen binding is confined to single-variable domain (VHH). Recombinant VHH molecules, called nanobodies, can
Hepatitis B core-based virus-like particles to present heterologous epitopes

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Since the first effort to recombinantly express the hepatitis B core protein (Hbc) in bacteria, the remarkable virion-like structure has fuelled interest in unraveling the structural and antigenic properties of this protein. Initial studies proved Hbc virus-like particles to possess strong immunogenic properties, which can be conveyed to linked antigens. More than 35 years later, numerous studies have been performed using Hbc as a carrier protein for antigens derived from over a dozen different pathogens and diseases. In this review, the authors highlight the intriguing features of Hbc as carrier and antigen, illustrated by some examples and experimental results that underscore the value of Hbc as an antigen-presenting platform. Two of these Hbc fusions, targeting influenza A and malaria, have even progressed into clinical testing. In the future, the Hbc-based virus-like particles platform will probably continue to be used for the display of poorly immunogenic antigens, mainly because virus-like particle formation by Hbc capsomers is compatible with nearly any available recombinant gene expression system.

HBV is a remarkable virus in many respects. First, the virus is responsible for a truly global disease burden: more than two billion people have been or are currently infected with HBV. Approximately 400 million of these people are unable to clear the virus and become chronically infected. Eventually, such a chronic infection will lead to liver cirrhosis or hepatocellular carcinoma (HCC), which, in the absence of treatment, is usually fatal. It is estimated that each year 600,000 people die as a result of liver failure caused by HBV infection [1]. Second, whereas many disease-causing viruses have been identified based on Koch's postulates expanded by those of Rivers [2] — that is, by showing a causal connection between disease and pathogen — HBV was discovered indirectly in a quest for new antigens present in human serum. One such antigen, named the 'Australia' antigen, was discovered by Blumberg et al., and it was initially thought that this antigen was associated with leukemia [3]. However, soon after its discovery, the 'Australia' antigen turned out to correlate with so-called serum hepatitis, for example, caused by transfusion of, in hindsight, HBV-contaminated blood [4]. Independent of Blumberg's work, Prince reported the discovery of an antigen that was associated with serum hepatitis; later, this antigen turned out to be identical to the 'Australia' antigen [5]. In addition, the genomic replication strategy of HBV and related viruses classified as Hepadnaviridae is remarkable. The majority of infectious HBV virions (42–47 nm in diameter) contain a circular DNA genome with an approximate size of 3200 nucleotides. One strand of the genomic DNA is complete, whereas the complementary strand is incomplete (Figure 1A). As a result, the HBV virion-associated genome is partially double-stranded, leaving approximately a third of the DNA single stranded. Despite their DNA genome, members of the Hepadnaviridae replicate through a reverse transcription step. In the infected hepatocyte, a progenomic RNA (pgRNA) is synthesized by the host cell RNA polymerase. This progenomic RNA is then used as a template for the viral reverse transcriptase to generate progeny genomic DNA — that is, partially double-stranded DNA. The 90 kDa hepatitis polymerase initiates reverse transcription by a self-priming mechanism involving the hydroxyl group of tyrosine residue 65 [4,7]. As
Dankwoord

Eindelijk, het is af! Ik kan het afronden van dit doctoraat niet beter vergelijken als met een zware beklimming, te voet of met de fiets, zoals ik er al wel enkele gedaan heb: het is lastig, onderweg stel je jezelf de vraag ‘waarom’ je dit precies doet, je twijfelt, maar er zijn anderen die je overhalen toch door te zetten en de opluchting eens je de top bereikt is onbeschrijfelijk. Ik ben een aantal mensen dan ook terecht een bedankje verschuldigd voor de hulp en steun om deze ‘top’ te bereiken.

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wie ik altijd terecht kon voor info over statistiek, facs, T-cellen en zo veel meer. Hij heette me de kneepjes van het T-cel werk geleerd. Ik herinner me nog toen we onze eerste elispots dededen. We hebben toen allebei tot vrij laat bezig gezeten in TC, al was het bij jouw nog een pak later (of zal ik zeggen vroeger) dan bij mij. Lang leve de gigantische muisexperimenten. Samen met Bert ook een beetje het ‘vuil brein’ van het labo. Ettelijke keren heb ik, toen ik weer eens een smerig lachse vanuit jullie richting hoorde, me afgevraagd waarover dat precies ging. Best dat ik het niet altijd verstaan heb, denk ik ;('. Aan de ‘verre’ overkant van het labo hebben we dan nog Kenny en Anouk, een onafscheidelijk duo. Ik moest bekennen, heel in het begin dacht ik dat jullie een koppel waren: elkaar constant plagen, precies een verliefd stel :-p . Kenny, (of is het Kelly?) door jouw is het allemaal begonnen. Door de thesis bij jouw had ik de smaak te pakken en wou ik absoluut verder doen. Bedankt ook voor je hulp en advies de afgelopen jaren. Anouk, nog zo iemand als Tine, die continu bereid is te helpen. Wat zou het labo zijn zonder jouw... en de koffie/koekskes? Bij mij was het niet zo zeer de koffie, maar eerder de witte chocotofs die verslavend waren ☺. We delen ook de liefde voor onze favoriete assay: de NA-assay ;-) . Lei, I think you’re the next in line. Good luck with your project. When is that first manuscript coming up? Iebe, KiJoon en Liesbeth, bedankt voor de fijne momenten samen en veel succes met jullie projecten.

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Aan iedereen: Bedankt!

Sarah