Chapter 1

General Introduction

1.1 Classification and characteristics

Human respiratory syncytial virus (hRSV) is a member of the family *Paramyxoviridae,* subfamily *Pneumovirinae*. This subfamily is divided in two genera: the genus *Pneumovirus* includes hRSV, bovine RSV (bRSV) and pneumonia virus of mice (PVM), the genus *Metapneumovirus* includes avian pneumovirus (APV) and tentatively also the human metapneumovirus (hMPV). hRSV is a single stranded negative-sense RNA virus, containing a non-segmented genome of 15.222 nucleotides encoding nine structural and two non-structural proteins [1]. The hRSV virion consists of a nucleocapsid contained within a lipid envelope and appears as irregular spherical particles ranging in diameter from 150 to 300 nm (figure 1). hRSV strains can be divided into two different subgroups, hRSV-A and -B, on basis of their reaction patterns with monoclonal antibody panels and nucleotide sequence differences between several of their genes [2-4].

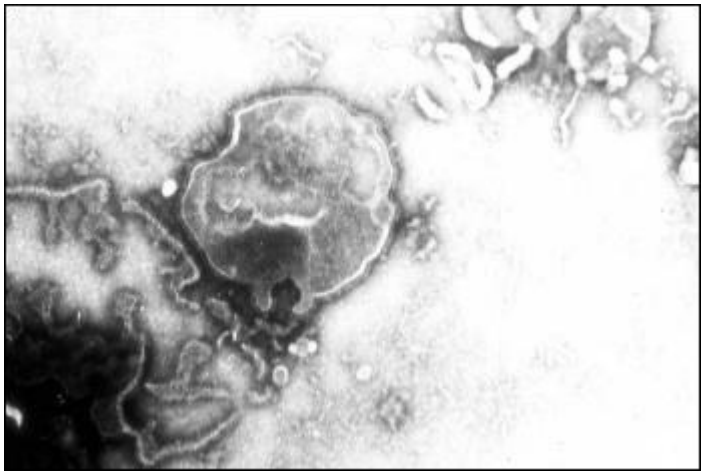

Figure 1: Negative contrast electron microscopic image of an hRSV virion.

The virus envelope contains three virus encoded transmembrane glycoproteins, the fusion (F) protein, the attachment (G) protein and the small hydrophobic (SH) protein. The F protein mediates fusion of the virus membrane with the host cell membrane, resulting in cell entry of the nucleocapsid. In addition, fusion of membranes of infected cells with those of neighboring cells can result in the formation of multinucleated syncytia [5]. The G protein is heavily glycosylated [6] and the ectodomain has, due to its high contents of serine, threonine and proline residues, structural similarities with mucins. It is involved in host cell attachment [7,8], and is thought to facilitate passage of the virus through the mucus-barrier [9]. The third envelope protein is the SH protein, of which the function remains unknown. Syncytium formation using plasmid-expressed F, G and SH was most efficient when all three proteins were present, suggesting a role for SH in enhancing the function of F and/or G [10]. However, both G and SH are

dispensable for hRSV replication in cell culture [8,11]. The matrix (M) protein, a nonglycosylated protein located on the inner side of the envelope, is thought to have two general functions: transcriptional inactivation of the nucleocapsid before packaging and association of the nucleocapsid with the newly developed envelope [1]. The major nucleocapsid (N) protein, the phosphoprotein P and the large polymerase subunit L are the viral proteins necessary and sufficient for RNA replication [12], with the anti-termination factor M2-1 being essential for viral viability [13]. The function of the nonstructural proteins NS1 and NS2 is largely unknown, but virions lacking one of these two proteins are attenuated *in vitro* and *in vivo* [14,15]. Finally, the M2-2 protein is a regulatory protein, which down-regulates transcription and up-regulates RNA replication [16].

1.2 Epidemiology and clinical manifestations

hRSV has a worldwide distribution and is one of the main causes of respiratory tract infections. It causes yearly epidemics in the winter season of moderate climate zones (figure 2) and in the rainy season of tropical climate zones [1]. Viruses of subgroup A or B can co-circulate during one epidemic, and epidemics may be dominated by either subtype. At three years of age all infants have been infected at least once, and re-infections continue to occur throughout life [17].

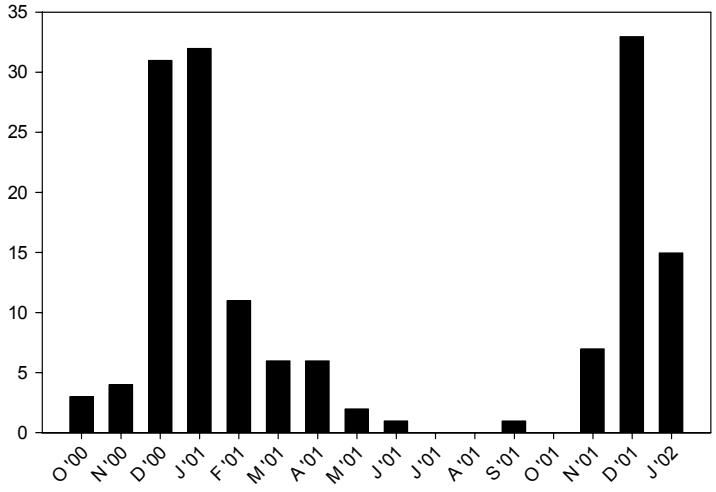

Figure 2: Monthly hRSV isolations as detected at the Department of Virology of the Erasmus MC Rotterdam from October 2000 to January 2002, illustrating the yearly winter outbreaks of RSV (taken from B.G. van den Hoogen *et al.* 2003 J Infect Dis **180**).

The incubation time from hRSV infection to onset of clinical signs is 4-5 days [1]. Relatively mild common cold-like upper respiratory disease can be seen in patients of all ages, but hRSV is also among the most important causes of severe lower respiratory tract disease, especially in preterm infants

[18], infants with underlying cardiac or respiratory disease [19,20], immunocompromised individuals and the elderly [17].

Clinical features seen during relatively mild hRSV infection are similar in all age groups and are generally limited to the upper respiratory tract [21]. Fever, irritating nonproductive cough and/or transient wheezing may be present, but the infection rarely leads to serious complications [22]. In severe hRSV disease the infection also involves the lower respiratory tract, patients may need mechanical ventilation, and in some cases outcome can be fatal. When compared with adults, the elderly have a higher risk (30-40%) for involvement of the lower respiratory tract upon hRSV infection, with rales and wheezing as the most common symptoms, but fever, cough and nasal discharge are also seen [23,24]. After hRSV infection in immunocompromised individuals upper respiratory tract infection precedes lower respiratory tract disease and acute lung injury [25]. Histopathology from autopsied cases has shown diffuse alveolar damage, severe squamous metaplasia, multinucleated giant cells, and intra-cytoplasmic inclusion bodies [25-27].

1.3 Immunity to hRSV

Host resistance to virus infections is mediated by both the innate and the adaptive immune response. Recent data suggest that early inflammatory and immune events play an important role in the outcome of acute hRSV infections. The importance of the adaptive immune response is illustrated by the failure of hRSV clearance in immunocompromised individuals [28,29]. Although complete protection from infection may not exist, hRSV-specific immunity can protect from severe lower respiratory tract disease.

1.3.1 Innate immunity

Upon infection of the local respiratory epithelium of the upper airways, the virus spreads along the respiratory tract. As a consequence, a number of molecules are produced by the epithelial cells, including potent immunomodulatory and inflammatory mediators such as cytokines (IL-1, TNF-α, IL-6 and IL-11), chemokines (IL-8, GRO, MCP-1, MIP-1α, RANTES), type I interferons (IFN-β/α) and growth factors (GM-CSF, G-CSF) [30,31]. In addition, type II alveolar epithelial cells are able to produce opsonins such as complement [32] and surfactant proteins [33] responsible for serum-independent phagocytosis of pathogens by neutrophils, monocytes and macrophages. Therefore, respiratory epithelial cells appear to be ideally located and armed to function as initiators of host defense mechanisms by regulating the prototypic cellular elements of the innate immune response, and may dictate the nature of the specific adaptive immune response to the virus [34].

Cytokines and chemokines produced upon infection can induce migration of eosinophils and neutrophils from the bloodstream into the infected tissue [35,36]. Neutrophils are the predominant airway leukocytes in hRSV bronchiolitis. In a study of 14 intubated infants they constituted 93%

and 76% of the inflammatory cells recovered from the upper and lower respiratory airways, respectively [37]. The cytotoxic effect of neutrophils is maximized by their retention at the site of infection. Neutrophils bind to ICAM-1, which is expressed on hRSV-infected respiratory epithelial cells [38], by a process that is both dose- and time-dependent [36,39]. As a consequence, prolonged presence of virus or viral antigens results in recruitment of more neutrophils to the airways.

Eosinophils can kill hRSV-infected cells by cytotoxic products, which are released by degranulation [40]. This degranulation can be activated by hRSV-infected cells in the appropriate environment of inflammatory mediators, but these infected cells can also activate uninfected epithelial cells, thus triggering further eosinophil degranulation [41]. Eosinophil-cytoxicity against bystander activated cells may in some cases contribute to pathogenesis of hRSV infection.

1.3.2 Adaptive immunity

Different compartments of the adaptive immune system are important in the immune response to hRSV. Virus-specific serum and secretory antibodies can neutralize the virus, and in very young infants maternally-derived specific antibodies are of importance [42]. The cellular immune response to hRSV includes hRSV-specific HLA class I-restricted $CD8^+$ cytotoxic T lymphocytes (CTL) and HLA class II-restricted $CD4^+$ T helper (Th) lymphocytes. HLA class I molecules are present on almost all nucleated cells, and bind small (8-11 amino acids) peptides that are derived from proteins in the cytosol after proteolitic degradation [43-45]. As a consequence, virus-specific CTL usually recognize only infected cells. HLA class II molecules are present on specialized antigen presenting cells (APC) like dendritic cells (DC), macrophages and B cells, and bind longer (9-30 amino acids) peptides that are derived from proteins that are taken up from the extracellular environment by endocytosis [46]. As a consequence, virus-specific Th cells can recognize both infected APC and APC pulsed with inactivated antigen.

Both HLA class I and class II molecules are highly polymorphic, and a large number of alleles are present in the human population [47]. For both classes three genes exist: HLA-A, -B and -C for class I and HLA-DP, -DQ and -DR for class II. Depending on homo- or heterozygosity, each individual therefore carries three to six alleles of both classes. T cells recognize peptides that bind to one of these alleles, and are educated to discriminate between self and non-self HLA/peptide complexes, demonstrated by the crucial importance of HLA molecules in transplantation medicine [47].

1.3.2.1 Humoral immune response to hRSV

hRSV-specific virus neutralizing (VN) antibodies are present in the sera of all full-term newborns, due to the transplacental transfer of maternal antibodies [48]. Their levels decline during the first few months of life, and subsequently increase again as a consequence of natural infections with

hRSV [49,50]. In very young infants hRSV-specific antibody levels produced after infection usually remain low [51,52]. This limited response may be due to either the immaturity of the infant's immune system or the suppressive effect of maternally-derived antibodies [53]. At older ages, specific antibody titers rise after each hRSV infection, and although high levels of specific antibodies can be present in humans, these are usually not high enough to protect against infection [34]. Antibodies are developed to most of the structural proteins, but only antibodies specific for the F or G glycoprotein neutralize hRSV [1] and when transferred passively to animals only these antibodies confer protection [54]. It has been shown both in animal models and in infants that high levels of hRSV-specific VN antibodies protect against the development of severe lower respiratory tract infection [55,56], and reduce viral loads in infected premature infants [57,58].

1.3.2.2 Cellular immune response to hRSV

The importance of the cellular immune response to hRSV is illustrated by the absence of severe respiratory disease during early life in infants with agammaglobulinemia [59], in contrast to prolonged shedding of virus [28] and in some cases giant cell pneumonia [60,61] in individuals with a compromised cellular immune system. In addition, mice were shown to clear hRSV after adaptive transfer of specific $CD4^+$ or $CD8^+$ T cells [62].

Upon hRSV infection, both specific $CD4^+$ and $CD8^+$ T cell responses are generated, which can be directed to almost all proteins encoded by the hRSV genome. However, no MHC class I-restricted CTL responses specific for the hRSV G protein have been described in either mice or humans [63,64], but G-specific $CD4^+$ T cell responses are readily detectable in infected mice [65,66]. For humans, only a few hRSV-specific HLA class I-restricted CTL epitopes (table I), and no human HLA class II-restricted Th cell epitopes have been described.

Table I: HLA class I-restricted hRSV-specific T cell epitopes

Protein	Amino acid sequence	HLA restriction	Reference
F	R_{109}ELPRFMNYT_{118}	A*0101	[67]
N	N_{267}PKASLLSL_{275}	B7	[68]
N	V_{255}MLRWGVLA_{263}	B*08	[69]

Recently, van Bleek *et al.* [70] described the human CD4 response to the hRSV F protein using IFN-γ ELISPOT. By testing a set of overlapping peptides, the authors were able to demonstrate *ex vivo* F-specific CD4 memory T cell responses in peripheral blood mononuclear cells (PBMC) collected from adults, which were mostly restricted by HLA-DR and in a few cases by HLA-DQ. In this study epitope mapping was not performed. Previously, Levely *et al.* [71] demonstrated similar hRSV F-specific CD4 responses by proliferative assays.

In mice more T cell epitopes have been described: MHC class I-restricted epitopes have been identified for the hRSV F protein [72,73] and the M2 protein [74], while a class II-restricted epitope was described for the attachment G protein [66].

1.3.2.3 Mucosal immunity

The immune response to pathogens that enter the body at mucosal sites includes aspects of both the innate and the adaptive immune system [75]. Of the adaptive mucosal response to respiratory viruses, secretory IgA may be one of the most important mediators of protection to reinfection [76,77]. These molecules are actively transported to the mucosa and respiratory lumen, and may neutralize the virus before it is able to establish infection. In some studies in animals and humans a correlation was observed between hRSV-specific nasal IgA levels and protection [78]. However, due to the transient nature of specific IgA responses the protective effect is only short lasting.

1.4 hRSV pathogenesis and immunopathogenesis

Infections with hRSV are among the most important causes for severe lower respiratory tract disease in young infants, immunocompromised individuals and the elderly [17], and the virus can also cause influenza-like disease in adults [79,80]. However, the question remains why the majority of infections cause limited or no clinical signs, while a small subset of the infections is associated with severe disease. A first possible explanation is that some individuals could have a physiological predisposition, e.g. in the form of a low maternally-derived VN antibody titer or a pre-existent lung function abnormality [81]. Secondly, the infectious dose may be of importance [82], and also concurrent infection with other agents could result in enhancement of disease [83]. Finally, it has been hypothesized that the hRSV-specific immune response itself may contribute to the severity of the disease, or in other words that certain manifestations of hRSV-mediated disease are of immunopathological nature. The latter hypothesis is often associated with the hypothesis that hRSV-mediated lower respiratory tract disease during early life could induce, or be a marker for, an atopic asthmatic phenotype [84].

The immunopathology hypothesis to explain the pathogenesis of natural severe hRSV-mediated bronchiolitis should be clearly distinguished from the pathogenesis of hRSV vaccine-mediated enhanced disease. This was first observed in the 1960s, when infants vaccinated with a formalin-inactivated alum-precipitated whole virus preparation (FI-hRSV) were found to be predisposed for severe disease upon subsequent natural hRSV infection [85]. It is now generally accepted that the FI-hRSV-mediated enhanced disease had an immunopathological basis [86](see below).

1.4.1 Specific immune responses in infants with hRSV bronchiolitis

Information about the specific immune response during hRSV-mediated severe lower respiratory tract infection in humans is limited [87-89]. Infection with hRSV at a young age (between 6 weeks and 9 months of age) normally induces a cellular immune response associated with a combined Th1/Th2 (or Th0) cytokine profile. In some studies severe hRSV lower respiratory tract infections were found to be associated with a skewed Th2 response [87,89-94]. However, others have failed to confirm this observation [88,95-97].

1.4.2 A role for hRSV in the development of asthma or allergy?

Whether hRSV infection at a young age is involved in the development of atopic disease, like asthma and allergy, remains a subject of debate. Sigurs *et al.* found an association of severe hRSV infection at young age, subsequent episodes of wheezing and the development or exacerbation of asthma at the age of seven years [98], especially in infants with a family history of atopy. Also, Stein *et al.* showed an association between severe hRSV infection in infancy and wheezing in later childhood, but this association was absent by the age of thirteen years [99]. The potential role of mild hRSV infections in infancy in the development of atopic disease at later age is even more unclear. Forster *et al.* have shown that mild hRSV infections at a young age can promote aeroallergen sensitization during the first year of life, but they were unable to show atopic manifestations during the first two years of life [100].

It has been hypothesized that the putative relationship between hRSV and atopic disease has an immunological basis [101-106]. Studies in a mouse model of airway hyperresponsiveness have shown that hRSV challenge in ovalbumin (OVA)-sensitized mice enhances and prolongs airway inflammation and airway hyperresponsiveness, and that recurrent hRSV infections in sensitized mice shift the immune response toward Th2 immune responses, airway inflammation and airway hyperresponsiveness [107-109]. Studies in rodent models have shown that the production of IL-13, which is induced during hRSV infection in the airway, is a key factor in these processes [110-113]. IL-13 is also a key regulator in the pathogenesis of asthma [110]. In humans, IL-4 was described to play a role in episodes of wheezing [114], but whether IL-13, a cytokine also binding to the IL-4α receptor [115] and expressed by Th2 cells in patients with asthma, is also involved in this process remains to be determined [116,117].

1.4.3 hRSV vaccine-mediated enhanced disease in infants

Because of the high morbidity and mortality associated with hRSV infections, substantial efforts have been invested in the development of a vaccine [118,119]. However, a number of factors hamper the development of hRSV vaccines. Firstly, natural hRSV infections usually do not induce protective immunity [34,120], so the vaccine should induce more protective immune responses. Secondly, since a large part of the morbidity is seen in

very young infants, the vaccine should be able to mount an immune response at an early age and in the presence of maternally-derived antibodies [121]. Finally, vaccination trials with FI-hRSV in the 1960s resulted in enhanced disease upon subsequent natural hRSV infection in the next winter season [85,122]. Although the vaccine induced an antibody response to hRSV, this could not protect the infants. Instead, the infants showed a pulmonary hypersensitivity response, resulting in hospitalization of 80% of the youngest infants vaccinated and eventually the death of two of these infants [85]. Histopathological evaluation of sections of the lungs showed inflammation around the small airways, with infiltration of neutrophils, eosinophils and mononuclear cells [64,123]. The observations with FI-hRSV in infants and subsequent studies in laboratory animals have clearly illustrated that antigenic priming can result in either protective or disease-enhancing immune responses, depending on properties of the host, the immunizing agent and the route of administration [124].

1.4.4 hRSV vaccine-mediated enhanced disease in laboratory animals

Studies using murine models of hRSV infection have demonstrated that FI-hRSV is a strong inducer of Th2 cells, which seemed to be the most important mediators of the pulmonary pathology [86]. The non-replicating FI-hRSV vaccine does not induce HLA class I-restricted hRSV-specific CTL, which are not only crucial in clearance of infection but also produce IFN-γ which counteracts the development of a skewed Th2 response. The presence of alum in the vaccine further pushed the immune response to a skewed Th2 phenotype. This hypothesis was further supported by studies with recombinant vaccinia virus (rVV) expressing the F (rVV-F) or the G (rVV-G) gene of hRSV. If BALB/c mice were primed by dermal scarification with rVV-F and subsequently challenged with hRSV, normal immune responses with a balanced Th1/Th2 phenotype and no lung pathology were observed. However, when mice were primed with rVV-G they showed a skewed Th2 response associated with production of IL-4, IL-5 and IL-13, and pulmonary eosinophilia [125]. As mentioned before, the hRSV G protein could not be shown to induce CTL responses in BALB/c mice, thus mimicking the response induced by the FI-hRSV vaccine. Moreover, if mice were primed with an rVV-G in which a CTL epitope from the hRSV M2 protein was cloned, the induction of skewed Th2 responses and pulmonary eosinophilia was completely abolished [126]. The importance of $CD8^+$ CTLs was also illustrated by the observation that induction of a vigorous influenza virus-specific $CD8^+$ T cell response prior to sensitization with FI-hRSV prevented pulmonary eosinophilia after challenge with hRSV and attenuated the recruitment of inflammatory cells [127]. In a recent study in cynomolgus monkeys (*Macaca fascicularis*), FI-hRSV-mediated hypersensitivity to hRSV challenge proved to be associated with the induction of IL-13 producing Th2 cells after vaccination [128]. However, besides the specific cellular immune response, it has also been proposed that immune complexes

could play a role in the pathogenesis of hRSV vaccine-mediated enhanced disease [129].

1.5 hRSV vaccine development

Since the failure of the FI-hRSV vaccine in the 1960s, several studies have focused on the development of a live attenuated hRSV vaccine. However, it has generally been difficult to find a proper balance between attenuation and immunogenicity [130]. Whereas the initial strategy focused on the development of cold-passaged temperature-sensitive (cpts) mutant viruses, new technology has allowed the generation of mutant or chimeric viruses by using reverse genetics [130]. As an alternative for attenuated hRSV vaccines, live vectors mediating the expression of hRSV genes [131-136] as well as new generation non-replicating candidate hRSV vaccines have been considered [137,138]. A selection of the current approaches in hRSV vaccine formulation is shown in table II. Two of these vaccination approaches which were evaluated in macaques in the framework of the present thesis will be discussed in more detail below.

Table II: A selection of current approaches in hRSV vaccine formulation

Vaccine	Formulation	Reference
Subunit vaccines	purified F protein	[139,140]
	chimeric F / G protein	[141,142]
	bacterial-expressed central conserved domain of hRSV G fused to albumin-binding domain of streptococcal protein G (BBG2Na)	[137,143]
DNA encoding hRSV genes	F protein	[144,145]
	G protein	[146]
	mucosal delivery of DNA	[147]
Attenuated viruses	temperature-sensitive mutants	[148,149]
	gene deletion mutants	[150]
Genetically engineered viruses	chimeric hRSV-A / hRSV-B	[151]
	chimeric bovine / human parainfluenza type 3 expressing the hRSV F and G genes	[152]
	chimeric bRSV / hRSV	[153,154]
Recombinant live virus vectors expressing hRSV genes	rVV	[155]
	rMVA	[156]
	recombinant adenovirus	[132]
	recombinant vesicular stomatitis virus	[133]
	recombinant alphavirus	[134]

1.5.1 Subunit vaccine BBG2Na

BBG2Na is a subunit hRSV vaccine candidate based on a recombinant prokaryote-expressed protein [143]. It consists of the central conserved domain of the hRSV G protein (G2Na, amino acids 130-230) fused to the albumin-binding region of the streptococcal protein G (BB), and is formulated in alum. In different animal models it has been shown that vaccination with BBG2Na induced protective immunity to both subgroups of hRSV and no signs of FI-hRSV-like enhanced immunopathology was seen [143,157-160]. BBG2Na was also capable of inducing protective immunity in 1-week-old mice in the presence of high levels of hRSV A-specific maternal antibodies [161]. Evaluation of BBG2Na in a phase I/II study in healthy young adults showed that it was safe, well tolerated and immunogenic [137]. In a subsequent multi-center phase III study the vaccine was tested in the elderly, but results of this trial have not yet been made public. No standards are available for the pre-clinical information required before a candidate hRSV vaccine can proceed to clinical trials in seronegative infants, but the evaluation of efficacy and especially safety in this target group will be crucial also for all non-replicating hRSV vaccine candidates.

1.5.2 MVA as a vector for hRSV gene delivery

Modified vaccinia virus Ankara (MVA) is a replication-deficient poxvirus, which was used during the late stage of the smallpox eradication campaign. When used as a recombinant vector, rMVA induced similar levels of the inserted genes as compared to the fully replication-competent VV strains [162,163], and induced equal or better humoral and cellular immune responses in animals [164-166]. Since MVA is replication-deficient in most mammalian cells, it ensures safe usage of this vector, which was demonstrated in a safety study using immunocompromised macaques [167]. In addition, the vaccination dose can be relatively high as compared to replication-competent VV, which could contribute to overcoming the maternal antibody barrier. Replication-deficient vaccine vectors expressing hRSV genes represent attractive candidates for hRSV vaccine development. In the first place they can safely be used in preterm infants, immunocompromised patients or the elderly, who are all important target groups for a candidate hRSV vaccine. In addition, gene delivery using a viral vector would result in the *de novo* production of viral proteins by the vaccinee, resulting in presentation of hRSV-derived epitopes to both HLA class I- and class II-restricted T cells antigen and thus the induction of a balanced immune response.

1.6 Animal models in RSV research

Over the past decades a number of animal models have been used to reproduce hRSV vaccine-mediated enhanced disease, to study the pathogenesis and immunopathogenesis of this disease and to evaluate new candidate hRSV vaccines. Although few animal species have a similar susceptibility to hRSV infection as humans, studies performed in these

models can provide clues on how to improve current vaccines and vaccination strategies. In addition to hRSV models in different animal species, another interesting model is experimental infection with bRSV in calves, which allows pathogenesis studies in a natural host. However, hRSV disease is a multifaceted disease of which the clinical manifestations largely depend on age, genetic makeup and immunological status, both in humans and in animals. There is not a single human subpopulation in which all forms of hRSV disease are manifest, and equally there is no animal model, which can manifest all forms of hRSV pathogenesis or disease. Since different animal models have different strengths and weaknesses, the choice for a particular model will depend on the study objective in question.

1.6.1 Mice

The most extensively studied models for hRSV infection and pathogenesis are the mouse models, of which the BALB/c strain is the most often used [125]. The strength of this model is that it allows the study of hRSV in SPF inbred animals, for which a multitude of immunological backgrounds and reagents are available. Adoptive transfer studies can be performed, and the existence of many genetically modified or gene knockout strains allow evaluating the role of specific immunological molecules in the immune response to hRSV. Many studies of hRSV vaccine-mediated immunopathology were also performed in this species. However, a disadvantage is that the BALB/c mouse has a tendency towards Th2 responses, and several of the observations made in these animals could not be reproduced in other mouse strains [168]. Although it remains difficult to extrapolate results from mice to humans, these models can still help us to understand specific mechanisms involved in hRSV pathogenesis and immunopathogenesis.

1.6.2 Cotton rats

hRSV infection in cotton rats (*Sigmodon hispidus*) was first described by Dreizin *et al.* [169], and since cotton rats appeared to be about 100-fold more permissive than mice they seemed to be a good model to study hRSV infection and vaccine-mediated immunopathology [170,171]. Vaccination of cotton rats with FI-hRSV and subsequent hRSV infection indeed resulted in alveolitis and interstitial pneumonitis [172], and vaccination with new generation candidate vaccines resulted in protection from infection [141,143,173]. A weakness of the cotton rat model is the relative lack of reagents for characterization and quantification of the immune response, although these are currently being developed [174]. In addition, no congenic, transgenic or knockout cotton rats are available.

1.6.3 bRSV infection in calves

Human and bovine RSV are closely related viruses, and infections with these viruses in their respective hosts are associated with a similar pathogenesis. Infections with bRSV follow a seasonal pattern, and are the

major cause of respiratory disease in calves during the first year of life [175]. In addition, enhanced disease has been observed in animals vaccinated with inactivated bRSV vaccines after subsequent natural bRSV infection [176]. Experimental infections have been described in conventional and SPF calves, and the pathology of FI-bRSV-mediated enhanced disease could be reproduced [177,178]. However, characterization of specific immune responses in this species is difficult due to a relative lack of reagents.

1.6.3 Non-human primates

Monkeys are of special interest for the study of hRSV infection and immunopathogenesis, because of their close phylogenetic relationship to humans. However, only few species have a susceptibility to hRSV comparable to humans. The best model may be the chimpanzee (*Pan troglodytes*) [179], but for ethical, financial and other practical reasons it is difficult to use this species in an experimental animal model. Another species which was shown to have relatively good susceptibility to hRSV is the African green monkey (*Cercopithecus aethiops*), in which FI-hRSV-mediated immunopathology could be reproduced [180]. However, few immunological reagents are available for this species. Other monkey species used for experimental hRSV infection include owl monkeys (*Aotus trivirgarus*) [181,182], squirrel monkeys (*Samiri sciureus*) [131], rhesus monkeys (*Macaca mulatta*) [183], bonnet monkeys (*Macaca radiata*) [184,185] and cynomolgus monkeys (*Macaca fascicularis*) [128]. The latter three macaque species are of special interest, since many macaque-specific immunological reagents are currently available. A disadvantage of all non-human primate models is that ethical and financial constraints often limit the number of animals per group, which can result in statistically insignificant data. On the other hand, a range of clinical samples can be collected longitudinally, which may provide both qualitative and quantitative virological and immunological parameters of infection.

1.7 Outline of this thesis

Although T cells are considered to play a role in both protective and disease-enhancing immune responses, hRSV-specific T cell-mediated immunity is still poorly understood. In the present thesis hRSV-specific cellular immune responses were investigated in subjects with mild upper respiratory tract infections either or not caused by hRSV, and in patients with a severe hRSV bronchiolitis. In addition, the hRSV-specific T cell response was studied at a protein-specific and at a clonal level. Finally, the safety and efficacy of two new candidate hRSV vaccines were tested in a non-human primate model.

To study the role of the cellular immune response in the pathogenesis of hRSV-induced disease of different clinical severities, cytokine production by specific T cells obtained from different compartments was studied. In chapter 2, systemic (PBMC) and local (nasal) T cells were stimulated with autologous B-lymphoblastic cell lines (BLCL) either or not infected with

hRSV, and Th1 and Th2 cytokine producing cells were quantified in ELISPOT assays.

To study the hRSV-specific cellular immune response at the clonal level and to identify new T cell epitopes, T cell clones were generated. In Chapter 3 the identification of four new hRSV-specific T cell epitopes is described, two of which were restricted over HLA class I and the other two over HLA class II. In addition, hRSV-specific memory T cell responses to the hRSV F and G proteins were studied in rMVA-stimulated human PBMC bulk cultures.

In chapter 4, the evaluation of the safety and efficacy of two candidate new hRSV vaccines BBG2Na and rMVA-F/G (rMVA mediating the expression of the hRSV F and G genes) in a vaccination / challenge model in cynomolgus monkeys is described.

Finally, chapter 5 provides a summarizing discussion of the thesis.