

Chapter 5

Summarizing Discussion



To date, only few studies have been performed to characterize the human respiratory syncytial virus (hRSV)-specific cellular immune response in humans. The experiments described in the present thesis were carried out to evaluate the role of specific T cells in protection from and immunopathogenesis of hRSV infection. In chapter 2, the hRSV-specific T cell response was studied in infants with or without laboratory-confirmed hRSV infections of different clinical severity. In chapter 3, the hRSV-specific T cell response was investigated in infants and healthy young adults. HLA class I- and class II-restricted T cells were studied at a clonal level and in bulk cultures. In chapter 4, the safety and efficacy of two candidate hRSV vaccines were evaluated in a vaccination/challenge model in macaques.

An immunological link between hRSV infection and atopic disease?

Several studies have suggested a relationship between hRSV infections in early life and the development of atopic disease such as asthma and allergy [98,99]. However, whether this relationship is causal, or results from a common physiological predisposition of certain infants continues to be subject of debate [194,195]. On the one hand, it has been hypothesized that a skewed virus-specific T cell response associated with the production of Th2-like cytokines such as IL-4, IL-5 and IL-13 could influence maturation of the immune system [246]. On the other hand, infants with a family history of atopy (FHA) have been shown to have an increased risk to develop atopic sensitization [98], and these infants may also be more prone to severe hRSV infections.

A prospective cohort study entitled "Viral infections in the immunopathogenesis of allergy in early childhood" (in Dutch abbreviated as Vigall) was performed by the Erasmus MC departments of Pediatrics, Otorhinolaryngology and Virology. The aim of Vigall was to evaluate the causal relationship between viral respiratory infections and the development of the immune system and the clinical expression of allergic disease in infants. To this end, clinical samples were collected from infants with or without FHA at the age of 6, 12, 18 and 24 months, and during the acute and convalescent phase of each upper respiratory tract infections (URTI) during their first two years of life.

Different approaches were used to assess epidemiological, virological and immunological characteristics of the infants in the Vigall cohort. Results of these studies were also described in the following PhD theses [247,248].

In the present thesis, the clinical samples obtained in the Vigall project were used to characterize the RSV-specific cellular immune response, both local and systemic, upon laboratory-confirmed hRSV infection. Unfortunately, in 129 infants only twenty hRSV-induced URTI and two lower respiratory tract infections (LRTI) were identified, almost exclusively in infants with FHA. As a result, it became virtually impossible to study the hRSV-specific immune response in relation to genetic predisposition. Therefore, we decided to investigate, with the available samples, the hRSV-

specific T cell response in infants with hRSV- or non-hRSV-induced URTI, in comparison with infants hospitalized with hRSV-induced LRTI.

hRSV-specific T cells: a role in severity of hRSV infection?

A number of studies have evaluated 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 important [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. In other words, certain manifestations of hRSV-mediated disease are of immunopathological nature. The latter hypothesis is often associated with the hypothesis that hRSV-mediated LRTI during early life could induce, or be a marker for, an atopic asthmatic phenotype [84]. However, results from the collective studies addressing the phenotype of T cells during hRSV-mediated LRTI are conflicting [87-97].

In the study described in **chapter 2**, significantly higher frequencies of specific T cells were detected in the infants hospitalized with hRSV bronchiolitis than in infants with mild hRSV-mediated URTI or non-hRSV-mediated URTI. However, the infants in the bronchiolitis group were significantly younger than those in the two URTI groups, prohibiting a direct comparison of these responses. Detection of Th1 and Th2 cytokine producing cells in PBMC and nasal T cells showed that there were few qualitative differences between these three groups: all infants showed a mixed Th1/Th2 response upon infection, without significant differences between the groups.

However, IL-13 producing hRSV-specific T cells were mainly detected in samples collected from infants with severe hRSV LRTI, during the convalescent phase of infection. This cytokine could be the link between more severe hRSV infections at young age and the subsequent exacerbation of allergic asthma at later age, since IL-13 appeared to play a central role in the pathogenesis of asthma and atopic disease [110-113,197]. Recurrent hRSV infections in infants producing IL-13 upon infection could lead to boosting the IL-13 response after each infection, eventually leading to an increase of IL-13 producing T cells and the development of asthma. However, large-scale prospective studies will be required to test this hypothesis.

hRSV-specific T cells: analysis of clones and bulk cultures

In the studies described in **chapter 3**, two approaches were used to study the hRSV-specific T cell response. The first approach was to identify hRSV-specific HLA class I- and class II-restricted T cell epitopes by limiting

dilution analyses. To this end, T cell lines were enriched for specific T cells by stimulation with autologous hRSV-infected B-lymphoblastic cell lines (BLCL-hRSV). IFN- γ ELISPOT assays were used as a read-out for specific responses.

In **chapter 3.1**, CD8⁺ HLA class I-restricted T cell clones (TCC) from two infants were generated four weeks after a severe laboratory-confirmed hRSV infection. Two different hRSV-specific CD8-TCC were generated from each patient, with specificities for the hRSV F and 1B or the F and 1C protein, respectively. The F-specific CD8-TCC were selected for further characterization using a set of overlapping peptides. Both CD8-TCC were found to recognize minimal epitopes of nine amino acids: CD8-TCC-1 recognized F₁₁₈₋₁₂₆ (RARRELPRF) and was restricted over HLA-B*57, while CD8-TCC-2 recognized F₅₅₁₋₅₅₉ (IAVGLLLYC) and was restricted over HLA-C*12.

In **chapter 3.2**, two CD4⁺ HLA class II-restricted TCC were generated from nasal brush cells or PBMC collected from two infants during the acute or convalescent phase of infection, respectively. CD4-TCC-1 was specific for the matrix (M) protein, recognized a minimal epitope of nine amino acids and was restricted over HLA-DPB1*1601. The minimal epitope of this clone was either M₂₄₇₋₂₅₅ (TRFAIKPME) or M₂₄₈₋₂₅₆ (RFAIKPMED). CD4-TCC-2 was specific for the G protein, recognized a minimal epitope of nine amino acids (G₁₆₃₋₁₇₁, FHFVEVFNFV), and was restricted over HLA-DPB1*0401 and 0402.

Interestingly, the G-specific CD4-TCC recognized an epitope located in the central conserved domain of the hRSV G protein (conserved in both hRSV group A and B viruses), and its restriction element HLA-DP4 has a high global allelic frequency [236]. Since in mouse models of hRSV disease it has been suggested that G-specific MHC class II-restricted T cells may play a role in disease enhancement [64,191], we set out to test the hypothesis that the ability to present this epitope could be a factor in determining whether some infants develop more severe hRSV disease than others. However, functional studies using BLCL from infants with mild or severe hRSV-induced LRTI as APC to the G-specific CD4-TCC suggested that severity of disease is not linked to the allelic frequency of HLA-DP4.

Whether the frequency of G₁₆₃₋₁₇₁-specific T cells influences the outcome or hRSV disease remains to be determined. Studies addressing this assumption should be performed *ex vivo* to exclude phenotype or specificity changes due to culturing or stimulating hRSV-specific T cells. Since it is difficult to perform large scale *ex vivo* T cell studies on patient materials from infants with mild or severe hRSV disease, due to sample size, a way out for this problem would be the use of tetramers in combination with the G-specific HLA-DP4-restricted epitope. However, while the use of tetramer-peptide complexes for HLA class I-restricted T cells is widely accepted and used [249,250], the use of HLA class II tetramer-peptide complexes is still limited and mainly based on the HLA-DRB1* and -DQB1* alleles [251-254]. It would be of interest to use tetramers based on the HLA-DPB1*0401

or 0402 alleles to study the frequency of peptide-specific T cells in infants with either mild or severe LRTI.

From an evolutionary point of view, the presence of a conserved T cell epitope restricted by the highly frequent HLA-DP4 is difficult to understand. For influenza viruses and other viruses, it has been described that point mutations in an epitope can result in escape from HLA-binding or T cell recognition, resulting in a selective growth advantage for the virus [255]. The fact that the HLA-DP4-restricted epitope is located in the central conserved region of the G protein could mean that mutations in this epitope are restricted by conformational constraints and would alter the function of the G protein or even lead to loss of function. Even so, the resulting immunological pressure from the response to this epitope in the majority of the human population has been insufficient to reduce or prevent circulation of hRSV. In contrast, if the G₁₆₃₋₁₇₁-specific T cell response would play a role in the pathogenesis of hRSV and would be related to the induction of severe disease, it could be hypothesized that the circulation of hRSV in the human population would have caused a negative selection pressure on the allelic frequency of HLA-DP4. The result of this putative selection pressure is not reflected by the high allelic frequency of HLA-DP4.

In **chapter 3.3** T cell responses directed to the hRSV transmembrane glycoproteins F and G were studied in PBMC bulk cultures, after enrichment by stimulation with autologous cells infected with recombinant modified vaccinia viruses (rMVA) mediating the expression of F (rMVA-F) or G (rMVA-G). Using this approach two different questions could be posed as addressed below: how diverse is the epitope usage of the hRSV F-specific T cell response, and does the hRSV G protein contain HLA class I-restricted CTL epitopes?

HLA class II-restricted F-specific T cell responses are highly diverse

Previous studies [67] and those described in **chapter 3.1** of this thesis identified three HLA class I-restricted CTL epitopes in the hRSV F protein. F-specific HLA class II-restricted memory T cell responses have been studied in bulk cultures using overlapping peptides spanning the F protein [70,71]. These studies identified a number of antigenic regions containing T cell epitopes. The approach used in **chapter 3.3** of the present thesis using enrichment of specific T cells by stimulation with autologous rMVA-F-infected cells resulted in identification of both CD4⁺ and CD8⁺ T cell responses, although the majority of specific T cell lines contained F-specific CD4⁺ T cells. Many of the antigenic regions identified overlapped with those described previously, although a number were newly identified as shown in figure 1. This figure also clearly illustrates that especially the Th epitope-containing regions cover almost the complete F sequence.

Although precursor frequencies were not determined, in donor 3 eleven F-specific T cell lines were obtained from eleven original rMVA-F-stimulated wells containing 50,000 PBMC each. These T cell lines displayed

responses to ten different antigenic regions, suggesting that the frequency of these precursor cells in PBMC may be in the order of magnitude of $1:10^4$ - $1:10^5$. If the precursor frequencies would be substantially higher, it could be expected that T cells of one specificity (or at least a restricted number of specificities) would have been found in each culture. This assumption is supported by the fact that stimulations with the positive peptides in PBMC immediately followed by IFN- γ ELISPOT did not result in detectable specific responses using 150.000 cells per well (results not shown).

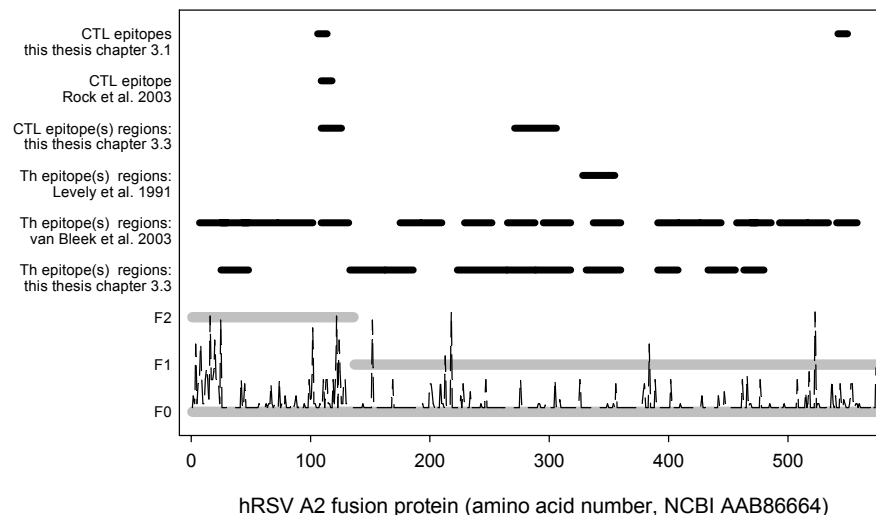


Figure 1: graphical representation of T cell epitopes or epitope(s) containing regions in the hRSV F protein. Gray lines represent the hRSV A2 F0 precursor-protein and its subunits F1 and F2. The dotted line represents a variability plot, showing for each amino acid position how many of 30 hRSV F protein sequences from GenBank were not identical to the A2 sequence. The black lines in the upper part of the figure represent the epitopes or antigenic regions identified by Rock *et al.* [67], Levely *et al.* [71], van Bleek *et al.* [70] or in the present thesis, chapter 3.1 [226] or chapter 3.3.

Does the hRSV G protein contain HLA class I-restricted CTL epitopes?

Stimulation of PBMC with autologous rMVA-F infected cells not only resulted in the identification of several antigenic regions containing Th epitopes as described above, but also three antigenic regions containing HLA class I-restricted CTL epitope(s). In contrast, similar stimulations with rMVA-G infected cells resulted in the identification of G-specific CD4⁺ T cells only.

HLA genotyping of the individuals used in **chapter 3.3** showed that donor 2 had the HLA-DPB1*0401/0402 genotype and rMVA-G-stimulated bulk cultures generated from this donor all recognized a 15-mer peptide comprising the G-specific epitope described in **chapter 3.2** (G₁₆₂₋₁₇₅). Although donors 1 and 3 were both HLA-DPB1*0201 positive, the G-specific T cell lines of these two donors did not recognize this peptide. In

almost all cases G-specific responses were directed to the central conserved region of the G protein (G₁₃₀₋₂₃₀). However, this does not exclude that the variable regions of the G protein do not induce Th cell responses. Due to the high variability in the G protein, the G sequences of the viruses that have infected the adult donors during their lifetime were probably relatively distant from that of the A2 virus used to generate the rMVA-G virus. Therefore, if Th responses to the variable regions of the G protein existed in these donors, they were simply not stimulated by the rMVA-G virus.

One of the initial goals of the study described in **chapter 3.3** was to identify possible G-specific CD8⁺ CTL responses. It has been described that G-specific CTL do not exist in BALB/c mice, nor have they ever been detected in humans [63,64]. rMVA-G infection of APC could lead to presentation of HLA class I-restricted epitopes, and could therefore be used to enrich PBMC bulk cultures for G-specific CTL. However, although in some cultures low G-specific responses were detected, these all proved to be false positive, since the results could not be reproduced and restimulation with rMVA-G-infected APC did not result in outgrowth of specific cells. Since these results were only generated in three individuals, we can not conclude that G-specific CTL are not present in humans. Optimization of the restimulation protocol using rMVA-F infected cells will have to be performed, especially addressing the use of antigen presenting cells (APC) such as monocytes or dendritic cells. Using rMVA-G infected APC in an optimized protocol and a higher number of donors will allow more conclusive statements about the presence of G-specific CTL.

The efficacy and safety of two new candidate hRSV vaccines

Since the vaccination failures in the 1960s, in which a formalin-inactivated alum-formulated hRSV had been used [85], a number of studies have been or are performed on the development of new and safe RSV vaccines [118,119]. Results from animal models of FI-hRSV-mediated enhanced disease suggested that a predominant Th2 response in the absence of a counterbalancing CTL response was one of the factors predisposing for enhanced disease upon hRSV infection [86,126,128]. Therefore, it can be hypothesized that live vaccines or recombinant viral vectors, capable of inducing both class I- and class II-restricted responses, would be better vaccine candidates than inactivated vaccines [131-134,136], but also new generation inactivated vaccines are currently tested [137]. In **chapter 4** the evaluation of the safety and efficacy of two candidate new hRSV vaccines in infant macaques is described: in **chapter 4.1** a subunit vaccine based on the G protein of hRSV [242] and in **chapter 4.2** rMVA mediating the expression of the hRSV F and G genes (rMVA-F/G) [257]. A large number of hRSV vaccination and challenge studies have been carried out by other groups in mice and cotton rats. Since correlates of FI-hRSV-mediated immunopathology have been or are being established in these species, they allow comparison of the immunopathological safety of candidate new

vaccines with FI-hRSV. However, it remains difficult to extrapolate results obtained from SPF inbred rodents to humans. A hRSV vaccination and challenge model in infant cynomolgus macaques was previously developed at Erasmus MC, in which components of FI-hRSV-mediated immunopathology could be reproduced and correlates of enhanced disease could be detected [128]. This model will be useful for preclinical studies with new candidate hRSV vaccines before proceeding to clinical trials in seronegative infants and was used for the evaluation of safety and efficacy of the two new candidate RSV vaccines mentioned above.

Vaccination of infant macaques induced hRSV-specific IgG in 3 of 4 BBG2Na and 4 of 4 rMVA-F/G animals. However, specific IgG responses in all BBG2Na and 2 of 4 rMVA-F/G animals were considered gray zone according to the kit's manufacturer. Levels of vaccine-induced virus neutralizing (VN) antibodies were low in 2 of 4 rMVA-F/G-vaccinated animals and undetectable in the other two rMVA-F/G-vaccinated and all BBG2Na-vaccinated animals. These data are in contrast with results published earlier. Vaccination with BBG2Na induced hRSV-specific IgG and/or neutralizing antibodies in mice, cotton rats and African green monkeys [143,241]. Wyatt *et al.* [156] were able to show high antibody levels in mice vaccinated with a double recombinant rMVA-FG. In addition, Stittelaar *et al.* [229] showed high levels of VN antibodies after vaccination of cynomolgus monkeys with rMVA expressing the measles virus fusion protein (F) and haemagglutinin (H) genes.

Proliferative as well as cytokine responses were also difficult to detect after vaccination with BBG2Na or rMVA-F/G. Vaccination with rMVA-F/G resulted in detectable proliferative responses in only 2 out of 4 animals after vaccination, but cytokine responses were not detected during both the vaccination and challenge period. In BBG2Na-vaccinated animals proliferative responses were also detected in 2 out of 4 animals after vaccination, but these proliferative responses were also accompanied by the production of low levels of IL-13. These results, low antibody levels and low/no proliferative responses, could be explained by the vaccination strategy or vaccination dose especially for rMVA-F/G. Nilsson *et al.* [244] showed that vaccinating three times with rMVA-SIV resulted in high levels of antibodies and proliferative responses in 3 of 4 animals and that vaccinating with rMVA-SIV twice followed by a boost with protein resulted in high levels of antibodies and proliferative responses in all animals. Perhaps a combination of the two hRSV vaccines evaluated in **chapter 4** in a prime-boost vaccination regime could provide a promising vaccination strategy resulting in induction of HLA class I- and class II-restricted T cell responses in combination with high VN antibody levels. In such a scheme, the rMVA-F/G vaccination should be given as prime, while BBG2Na and/or a purified F protein vaccine [138-140] could be considered as boost. However, although such a strategy might result in proof of principle, application in young infants would give substantial practical constraints.

Upon challenge with hRSV no differences in pulmonary hRSV load could be detected between vaccinated and control animals. This could partly be explained by the high challenge dose given to the animals, although with the same dose partial protection was previously detected in FI-hRSV-primed infant macaques [128]. Also, the challenge was given intra-tracheally to ensure delivery of the virus into the lungs, which perhaps renders the model artificial when compared to the natural route of hRSV infection.

Specific antibody responses detected in vaccinated animals after challenge were generally higher and appeared earlier than those in the control animals, indicating that vaccination with both rMVA-F/G and BBG2Na had primed for a secondary immune response. Low levels of eosinophils were detected in broncho-alveolar lavages of 2 of 4 BBG2Na-vaccinated animals and in none of the Rmva-F/G-primed animals.

Conclusions

The results presented in this thesis show that hRSV infection in humans results in a multifaceted immune response, which cannot be described as purely Th1- or Th2-like. However, the observed higher level of IL-13 producing hRSV-specific T cells in infants hospitalized with severe hRSV bronchiolitis could provide a clue for an immunopathological mechanism of natural hRSV-mediated severe disease. Another hRSV-specific immunological factor potentially involved in the pathogenesis of severe hRSV disease could be the frequency and/or phenotype like those of HLA-DP4-restricted T cell responses directed to the conserved region of the RSV G protein. The BBG2Na- and rMVA-F/G-based vaccination strategies evaluated in infant macaques resulted in low VN and cellular immune responses and no detectable protection. A combination of both approaches in a prime-boost regime could possibly increase vaccine immunogenicity, but in this case the immunopathological safety would again have to be evaluated in different animal models.

