Human RSV-specific T cells

Cover design	Peter Kaldeway en Laura Landberg
Layout	Fay Koen Tjoa en Peter Kaldeway
Print	Grafisch Bedrijf Ponsen & Looijen
ISBN-10:	90-393-4413-2
ISBN-13:	978-90-393-4413-2

Cover design: De vele handen symboliseren de intensieve samenwerking waardoor dit proefwerk tot stand is gekomen. Bovendien is een handafdruk persoonlijk en uniek, net zoals het menselijke afweersysteem, waardoor verschillen in ziekte-ernst tijdens RSV-infecties mogelijk verklaard kunnen worden.

Human RSV-specific T cells

Humane RSV-specifieke T-cellen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus,
prof.dr. W.H. Gispen,

ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op vrijdag 15 december 2006
des ochtends te 10.30 uur

door:

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geboren op 20 januari 1974 te Zevenaar

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The printing of this thesis was financially supported by:
Eijkman Graduate School, Dr. Ir. van de Laar Stichting, J.E. Jurriaanse Stichting, Wilhelmina kinderziekenhuis, Abbott, ALTANA Pharma bv, B. Braun Medical B.V., Becton Dickingson, Boehringen Ingelheim, Cara C'air BV, CenE bankiers, Friso kindervoedingen Frieslandfoods, GlaxoSmithKline BV, IVAX Farma B.V., Merck Dharp & Dohme BV, Nestlé Nederland BV. Nederlands vaccin Instituut, Novo Nordisk Farma B.V., Pfizer bv, Sanquin Reagents.

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Introduction

An introduction to life

Introduction: The role of T cells during viral infections

T cell immunity plays a central role in controlling viral infections. $CD4^+$ T cells, or helper T cells, provide B cell help, and create an inflammatory milieu by producing a broad repertoire of cytokines and chemokines that attract inflammatory cells and control the immune response. Effector $CD8^+$ T cells mediate cytolytic activity against infected cells and thus inhibit viral replication and spreading. The cytolitic activity of $CD8^+$ T cells is accomplished via two major pathways. The first pathway involves the release of toxins contained in cytoplasmic granules like perforin and granzymes that induce apoptosis of the target cell. The second pathway involves the ligation of death receptors Fas/FasL on the target cell which results in classical caspase-dependent apoptosis. Besides the necessity of lymphocytes in controlling infection, the inflammatory response and CTL mediated tissue damage can also induce immune pathology. This interplay between virus and immune response is discussed in more detail in **chapter 2**.

T cells express a T cell receptor (TcR) recognizing specific peptides presented by the Major Histocompatibility Complex (MHC) molecules on antigen presenting cells (APCs). Structures on both the MHC molecule as well as the presented peptide determine the contact area specifically recognized by the TcR. Viral proteins that are synthesized in the infected cell are degraded into peptides that are loaded onto MHC class I complexes in the endoplasmatic reticulum. These MHC class I molecules are in man encoded by human leukocyte antigens (HLA) and from both parents a set of 3 different HLA molecules (an A, B and C allele) is inherited. These 6 HLA molecules differ in their peptide binding groove, thus being able to bind a diverse repertoire of peptides. Therefore, the peptides of a virus that are presented on MHC class I molecules vary depending on the HLA-type of the infected donor. In **chapter 3** we identified 5 new RSV-specific epitopes for common HLA alleles, which allowed us to study RSV-specific T cell immunity in specific patient populations.

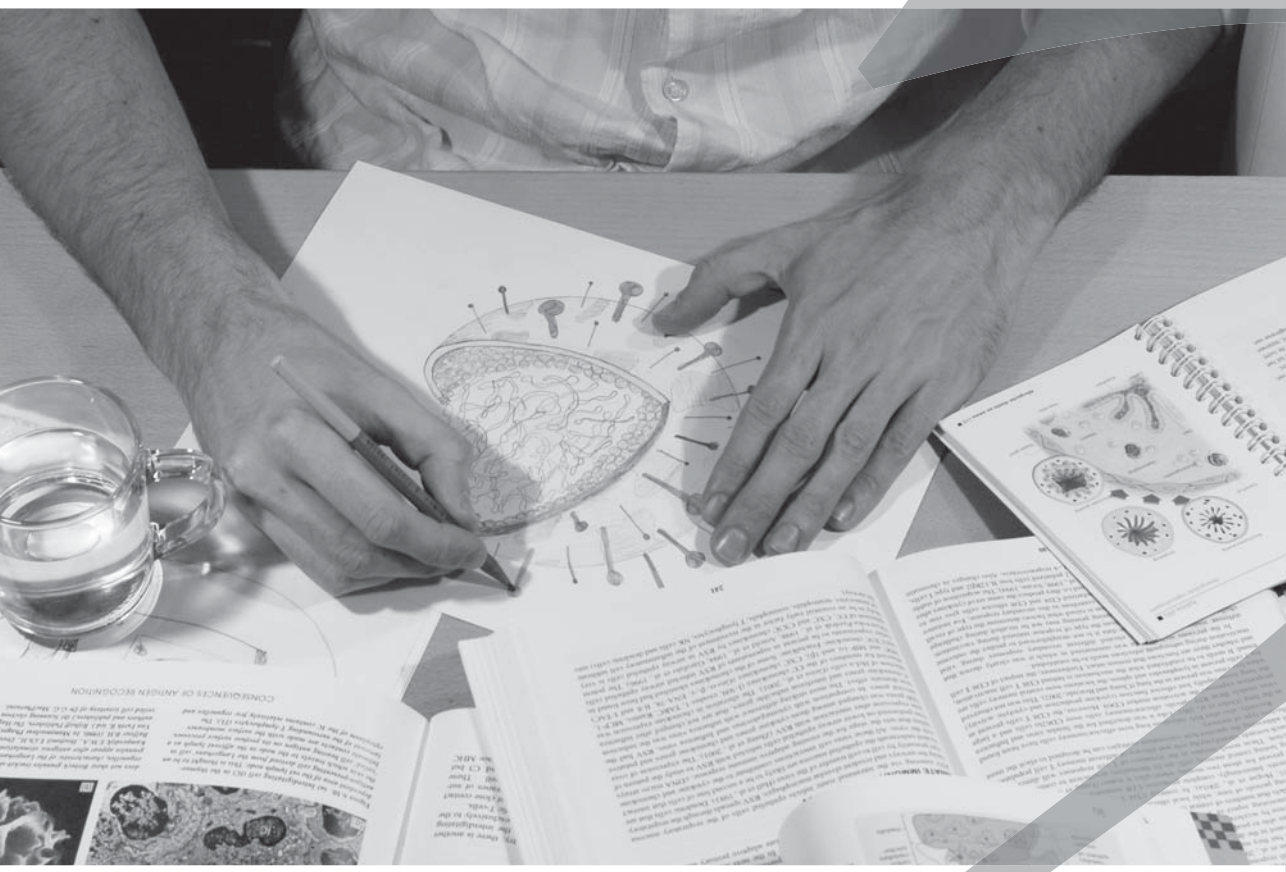
In **chapter 4** two overlapping HLA-DP4 $CD4^+$ T cell epitopes were identified. They were tested for their ability to induce cytokine responses in PBMC of healthy adults and infants with severe primary RSV infection. This study showed that overlapping peptides induced distinct patterns of cytokine production.

To gain more insight in the balance between virally induced and immune mediated pathology during RSV infection, primary $CD8^+$ T cell responses were studied in infants on mechanical ventilation due to RSV infection (**chapter 5**). Total $CD8^+$ T cells and RSV-specific $CD8^+$ T cells were characterized during the course of disease in bronchoalveolar lavage and PBMC and compared to T cell populations in healthy age matched controls.

In **chapter 6** we studied virus-specific responses and the contribution of bystander activation during respiratory infections. Upon respiratory viral infection $CD8^+$ T cells specific for heterologous infections disappear from the blood and migrate to the lungs. In this study we followed $CD8^+$ T cell responses throughout different respiratory virus infections during one winter season in a group of twenty children with a tracheostoma. Tracheal aspirate was collected at several time points during infection. With this study we revealed insight in the dynamics of total $CD8^+$ T cell responses and virus-specific $CD8^+$ T cell responses during secondary respiratory infections.

While RSV causes only mild symptoms of upper respiratory tract infection in healthy adults, it can cause severe morbidity and mortality in the elderly, immune compromised individuals and patients with chronic obstructive pulmonary disease (COPD). In **chapter 7** we compared the frequencies of virus-specific $CD8^+$ memory T cells in the blood of healthy adults and susceptible individuals i.e. the elderly and patients with COPD. In addition we studied the functional capacities of those cells.

Chapter 8 is a general discussion on the role of $CD8^+$ T cells during RSV infection, and the contributions our work supplied extending the insights described in the literature obtained with mouse models.



Pathogenesis of respiratory syncytial virus bronchiolitis; immunology and genetics

J. Heidema, J.L.L. Kimpen and G.M. van Bleek.

The microbe-host interface in Respiratory Tract Infections. Edited by J.L.L. Kimpen and O. Ramilo. 2005 Horizon Bioscience p233-252

A literature study

Summary

Bronchiolitis induced by infection with respiratory syncytial virus is a common cause for hospitalization in infants under 6 months of age. Moreover, epidemiological studies show a relationship between early lower respiratory disease as a result of RSV infection and development of asthma-like symptoms during childhood. An effective and safe licensed vaccine for RSV is not available. A formalin-inactivated whole RSV vaccine (FI-RSV) used in a trial performed in the 1960s primed vaccinees for substantial immune pathology following infection with the natural virus. This event has set back the development of novel vaccines. The mechanism behind FI-RSV induced enhanced disease as well as the mechanism of severe disease during primary infections is still poorly understood. The present review summarizes our current understanding of possible elements of the adaptive and innate immune responses that may determine the balance of efficacious immunity versus virus induced or immune mediated pathology.

RSV: the viral structure

Respiratory SyncytiaI virus (RSV) is an enveloped negative stranded RNA pneumovirus belonging to the family *paramyxoviridae*. The nonsegmented genome consists of ten genes that encode 11 proteins. The gene order is NS1 (nonstructuraI 1), NS2, N (nucleocapsid), P (phosphoprotein), M (matrix), SH (small hydrophobic), G (attachment protein), F (Fusion), M2-1/M2-2 and L (polymerase). The virus is composed of a lipid bilayer derived from the infected ceIl that contains the three transmembrane proteins F, G and SH. The bi-layer encapsulates the nucleocapsid core of N, P, L and the viral RNA. The matrix protein M connects the nucleocapsid to the viral membrane. M2-1 and M2-2 are small proteins that regulate RNA transcription. NS1 and NS2 are nonstructural proteins that are abundantly expressed in infected cells. These proteins enhance Interferon resistance of the virus (1;2). As Interferons enhance both innate and adaptive immune responses and can also inhibit cell growth and promote apoptosis (3), resistance to IFN's will be of great benefit for the virus, augmenting replication and survival. The major surface glycoproteins G and F are the major proteins involved in viral infection. The G protein binds to a host cell receptor, followed by fusion of the virus and cell membranes mediated by the F protein, which results in the penetration of the nucleocapsid complex into the cytoplasm. The F and G proteins are also the major targets for neutralizing antibodies to the virus.

Epidemiology

RSV causes yearly epidemics of respiratory disease during the winter season in moderate climates and during the wet season in tropical regions (4). Nearly 70% of infants are infected in their first year of life and about half of these children are re-infected in the next season. Throughout life everyone is re-infected multiple times (5-7). It has been shown in experimental settings, when human volunteers were re-infected multiple times with the same virus strain (at short time intervals of 4, 8, 14, 20 and 26 months), that each challenge resulted in infection in at least one fourth of the subjects. Re-infection occurred despite the presence of high levels of neutralizing antibodies (6) This clearly demonstrates that immune memory established during natural infection is incomplete.

RSV infections widely vary in disease severity, causing a broad range of symptoms varying from a mild cold to severe lower respiratory tract (LRT) disease. About 3% of infants that are infected with RSV require hospitalization (8), mostly due to respiratory failure or feeding problems. Of hospitalized infants with RSV infection 7-21% require mechanical ventilation (9). Peak incidence of severe LRT disease is around two-three months of age (10) or when corrected for prematurity before 45 weeks of post-conceptional age (11). Premature birth, congenital heart dis-

ease and pre-existing lung conditions are risk factors for severe disease (12;13). However, most hospitalized children do not belong to one of these categories (6).

The virus spreads through aerosols and contact with contaminated surfaces. Upon infection the virus spreads from the upper respiratory tract deeper into the airways, where it infects and replicates in epithelial cells in the small airways as well as the alveolar epithelium. Once these sites are infected the disease manifests as a bronchiolitis that is accompanied by wheezing. During the frequent episodes of re-infection the disease is usually mild and confined to the upper respiratory tract, both in children and adults. However, it may still be "one of the worst colds of the season" and in a small proportion of healthy adults it can cause severe pneumonia (14-16). In elderly people RSV disease is associated with increased morbidity and mortality (17-19). However, in this patient group other factors like chronic lung disease, heart failure and bacterial super-infections contribute to severe pathology.

Infants that experienced lower respiratory tract disease during primary RSV infections are at increased risk of developing asthma-like symptoms during early childhood (20;21). These childhood episodes of wheezing usually disappear between age 8-13 (22). It is currently not well understood what determines this association between RSV infections and later asthma-like symptoms. It may be possible that genetic and/or environmental factors that determine the type of immune response during primary RSV infections may also contribute to the development of asthma. The observation that the risk for asthma development was higher in children of families with a history of allergic disease strengthens this genetic link hypothesis (21). Furthermore, the fact that similar to the increased frequency of allergic disease in western society also the incidence of severe RSV infections has increased, strengthens the suggestion of a common cause (8). An alternative explanation for the high incidence of wheezing illness during childhood after severe RSV bronchiolitus during infancy is a possible causal relationship i.e: severe RSV infections might affect the immature immune- and respiratory systems and induce susceptibility to developing later respiratory disease.

Mechanisms of RSV induced disease

Effective immune responses against respiratory viruses usually require the contribution of the adaptive humoral and cellular responses in addition to innate responses against the virus. Ideally the virus is eradicated before it causes substantial cytopathology with a minimum of collateral damage caused by the host inflammatory response. However when this balance is disturbed disease may result. In patient groups that are immune compromised, such as patients with

severe combined immune-deficiency (SCID) and recipients of allogenic bone marrow transplants or lung transplants, direct cytopathology induced by the virus causes high morbidity and mortality (23-26). They show prolonged shedding of the virus and the characteristic syncytial formation that is induced by the fusion protein of RSV (27).

On the other side of the balance one finds immune pathology. Immune mediated pathology was observed in a patient group that was vaccinated with a formalin-inactivated whole virus vaccine in the1960s. Exposure of vaccinated children to natural RSV infection resulted in enhanced morbidity and mortality in this group compared with children immunized with control virus preparations. Especially in the youngest children (0-6 months) the effect was dramatic: 80% of the children needed hospitalization (28;29) and some infants died. In contrast to the immune-compromised patients the vaccinated children had less pronounced virus induced cytopathology. Rather, the mechanism of the enhanced disease appeared to be the immune response induced during viral infection, which was characterized by a high Iymphoproliferative response to viral antigens (30). In lung sections of the few patients that died a cellular infiltrate was found around the small airways that consisted of mononuclear cells, and abundant neutrophils and eosinophils (29). From the observations in these two patient groups two conclusions can be made: 1. T cells are necessary to clear RSV from the infected lung tissue. 2. In certain circumstances the T ceIl response may cause significant immune pathology.

There is still little knowledge about the mechanism behind severe pathology during primary RSV infections in infants. Developmental immaturity of the cellular immune response may prevent effective viral clearance. Alternatively, the immature immune system may respond to the virus but in an undesirable fashion. It is known that the immature immune system is strongly biased to the induction of Th2 type $CD4^+$ T cell responses (T-helper cells producing cytokines like Interleukin-4 (IL-4), IL-5, IL-6, IL-13) that are also associated with atopy and asthma (31;32). During fetal life Th1 immune responses are suppressed to prohibit rejection of the placenta (33). After birth Th1 function is progressively upregulated by stimuli derived from the extra-uterine environment like stimuli from the intestinal microflora (34). However, the kinetics of this cytokine switch differs between individuals being slowest in children with high risk for atopy. It is possible that such a bias may contribute to more severe disease, because Th2 type cells seem to play an important role in enhanced disease models in rodents. Moreover, it has been found that eosinophils degranulate in the respiratory tract during RSV infections (35;36), which may be a further indication that Th2 type immune responses are involved.

The contribution of high viral load to severity of disease or a relation with different virus strains is still a matter of debate (37-40). Some studies emphasize the role of the immune system with no contribution of high viral load (41), while others

find higher RSV titers in nasopharyngeal samples of mechanically ventilated children compared to children that did not require ventilation (42).

Studying immune responses in small infants is difficult. While the ideal object for research would be the lung tissue at the time of primary infection, ethical reasons prohibit this. Information obtained from studies using peripheral blood or bronchoalveolar lavage samples may not be representative for the processes that occur in the tissue. In a few autopsies peri-bronchiolar and peri-vascular infiltrates were found that were dominated by Iymphocytes, whether these were virus-specific has not been elucidated (41;43). In bronchoalveolar lavage (BAL) samples of RSV patients neutrophils appear to be the dominant cell type present, while T cells are only found at levels up to 4% (44). No information is yet available about specificity and function of these T cells. Due to the difficulties of studying RSV-specific immune responses in infants a lot of research has focused on animal models.

The mechanism of enhanced disease: animal studies

The dramatic outcome of the FI-RSV vaccine trial in the 1960s has set back the development of a RSV vaccine tremendously. Understanding the mechanism of vaccine enhanced illness in RSV has since then become an essential step in the design of new vaccines. Thus, considerable effort has been directed towards unraveling the mechanism of enhanced disease in different animaI models. FI-RSV vaccination caused enhanced disease upon infection with natural virus in cotton rats (45), mice (46-48), and monkeys (49). Studies performed in BALB/c mice and cotton rats have addressed the contribution of humoral and cellular immune responses to enhanced disease. In mice pre-vaccinated with FI-RSV that were subsequently challenged with live virus a clear immunization effect was observed in the lung. Virus titers were reduced in the lung in FI-RSV immune mice upon challenge with live RSV as compared to mice primed with a FI-treated control virus preparation (parainfluenza type 3, PIV3). RSV-specific antibodies were produced, but these were not the cause of disease enhancement as was shown by the absence of enhanced disease in T cell depleted mice (50). This result is in agreement with studies performed in cotton rats in which was shown that passively transferred immune sera from FI-RSV vaccinated animals failed to induce enhanced disease upon RSV challenge (46). However, the crucial role of $CD4^+$ T cells in disease enhancement in these rodent models was clearly demonstrated by the observation that in vivo $CD4^+$ T cell depletion of FI-vaccinated mice just before live virus challenge completely prevented immune pathology. Depletion of $CD8^+$ T cells had no effect.

Vaccination of mice with FI-RSV followed by live virus challenge caused a pulmonary inflammatory response, which involved the recruitment, on day 4 and 8 after RSV challenge, of high numbers of granulocytes, eosinophils and $CD4^+$ T cells into the lung. In comparison with mice experiencing a secondary RSV infection a decrease in the numbers of $CD8^+$ T cells was observed (47;50;51). Furthermore, the mice showed increased levels of mRNA expression in the lung for IL-5 (correlating with eosinophil influx), IL-10 and IL-13 (all Th2 type cytokines) and decreased mRNA levels for IL-12 (a Th1 related cytokine) (47). From these observations the conclusion was drawn that $CD4^+$ Th2 type cells played a crucial role in FI-RSV vaccine mediated enhanced disease.

The role of individual viral proteins in enhanced disease

The contribution of viral components to the enhanced disease was studied with recombinant vaccinia viruses expressing individual RSV proteins (52-55). Priming with the different vaccinia recombinants by skin scarification resulted in very different outcomes during live virus challenge. In BALB/c mice the vaccinia-G recombinant primed for an inflammatory response in the lung characterized by a high influx of eosinophils, that appeared very similar to the inflammation caused after FI-RSV priming and pointed to the involvement of a Th2 type response. (53;55). Other recombinants (Vac-F, Vac-N, Vac-P, Vac-M22) caused mixed Th1/Th2 or polarized Th1 responses. The Th2 response in Vac-G primed BALB/c mice is directed against a single epitope within the G-protein encompassing amino acid residues 183-195 (G183-195) (56-59). The T cells responding against this peptide are dominated by cells expressing a single T ceIl receptor $V\beta$ chain: $V\beta14$ (60). In mice strains of different MHC type ($H\text{-}2^k$, $H\text{-}2^d$ or $H\text{-}2^b$) (MHC: Major Histocompatibility Complex, the genes that encode the molecules that present antigens to T cells) or background (=non-MHC genes) the outcome of priming with vaccinia recombinants is different (61). In $H\text{-}2^k$ mice of different background none of the vaccinia recombinants primed Th2 responses, while in $H\text{-}2^b$ mice the outcome depended on the background genes (among $H\text{-}2^b$ strains: 129 and BALB. b mice are susceptible to eosinophila, while C57BL/6 are resistant) (56;61). AII mice carrying $H\text{-}2^d$ on several different backgrounds were susceptible to enhanced disease. Thus both MHC type and non-MHC genes contributed to the susceptibility of the mouse strains for enhanced disease.

The differences in the sensitive versus resistant mice strains appeared to coincide with a higher CD8/$CD4^+$ T ceIl ratio of virus-specific T cells in the lungs after live RSV challenge in the resistant strains. Interestingly, in C57BL/6 mice that were resistant to eosinophilia induction, the in vivo depletion of $CD8^+$ T cells rendered them susceptible to eosinophilic inflammation (61;62). RSV-G does not prime $CD8^+$ T ceIl responses in different mice strains (55;63). Incorporation of a strong $CD8^+$ T ceIl epitope derived from the M22 protein of RSV in the Vac-G vaccine induced a strong $CD8^+$ T ceIl response in BALB/c mice that were thereafter protected against enhanced disease (63). Further studies implicated INF-γ as the effector cytokine produced by $CD8^+$ T cells that influenced the Th2/Th1 balance of

G specific $CD4^+$ cells (62). Of Note: priming mice with a strong CD8 epitope alone or the transfer of RSV-specific CTL to naive mice, results in accelerated clearance of RSV. However, also a strong biased CTL response can cause severe lung pathology, dramatic weight loss and a strong cellular infiltrate of predominantly neutrophils (64).

The vaccination effect of the Vac-G recombinant was in different studies measured by the level of eosinophilia induction which correlates with the production of IL-5, a Th2 type cytokine (65-67). However, when $CD4^+$ T cell responses were scrutinized more closely it became clear that eosinophilia induction indeed correlated with an enhanced ratio of Th2/Th1 cytokines produced in the lung, but that Th1 effector cells remained also present (59;68). In the BALB/c model both Th1 and Th2 cells were found within the $V\beta14$ $CD4^+$ T cell subset recognizing a single antigenic epitope (60). Thus the Th2 skewing effect of vaccination with G does not seem to be dependent on a single epitope, nor a specific subset of $CD4^+$ T cells, but rather the setting (cytokine environment, $CD8^+$ T cell presence) during the primary or secondary T cell response.

The experiments performed in mice that were described in the previous section appear to show some contradictory observations. It remains an unanswered question why IFN-γ produced by $CD8^+$ T cells would be able to ameliorate enhanced disease while INF-γ, produced by $CD4^+$ T cells in significant quantities in the lung, is not preventing eosinophilia. Clearly, there are factors contributing to the ultimate disease process that are missed by a too narrow focus of the studies performed, and/or in vitro measurements of cytokine production may not reflect the true local compartimentalized responses.

Vaccine composition and route of administration affect the recall immune response after G protein vaccination

It is further important to mention that the route of vaccination as well as the formulation of the G antigen are also important factors that determine the outcome of the immune response after challenge with RSV. The G protein when used in priming experiments in the context of vaccinia by means of scarification of the skin elicits a strong primary $CD4^+$ Th1 response (69). However, upon recall after challenge with live virus the resulting secondary response contains a strong Th2 component that is responsible for enhanced disease (67). On the other hand when vaccinia-G is applied by a different route, intra-peritoneal injection in stead of scarification of the skin, the secondary response in BALB/c mice is not accompanied by eosinophilia.

The G protein of RSV is a type II highly glycosylated membrane protein. An interesting feature of the protein is the existence of a second initiation codon in the transmembrane region that allows the secretion of a soluble form of the G protein from infected cells (70). When this secreted form of G was expressed in a vaccinia virus recombinant it turned out to be a more potent inducer of IL-5 and eo-

sinophilia than a second construct expressing the gene for the membrane bound form of the G protein missing the second initiation codon. (65;67). When a native purified G protein was used in vaccination studies provided in different adjuvants, it caused a Th2 (alum) or Th1 (QS21) $CD4^+$ primary T cell response. However, these vaccination procedures both primed the mice for a Th2 recall response after an intra nasal challenge with RSV (66). Also G protein prepared in PBS sensitized different inbred mice strains for type II T cell responses (71). In contrast, a recombinant fusion protein (BBG2NA), containing the amino acid residues 130-230 comprising the central conserved region of RSV-G, fused to an albumin binding domain of streptococcal protein G formulated in alum elicits a primary Th2 response. However, the secondary response after intra nasal RSV challenge did not cause eosinophilia though it still enhanced production of Th2 cytokines (72). Even in neonatal BALB/c mice that show more strongly polarized Th2 responses to protein vaccinations, the BBG2NA vaccine did not sensitize for eosinophilia (73).

It is not directly clear why the differences that are described in the previous sections occur. There may be a difference in antigen presentation of different forms of the G protein: soluble versus membrane bound, or the presence or absence of glycosylation in native versus recombinant forms of G. Furthermore, the route of administration may impact on the dose of antigen available for antigen presentation, and the antigen presenting ceIl population involved in the initiation of the T ceIl response.

In summary: the outcome of the secondary immune response after priming with RSV-G depends on the route of administration and the context in which the T ceIl response is primed. Importantly, there is no absolute correlation between the type of immune responses during priming (Th1 versus Th2) with the type of immune response after challenge with live RSV. This fact has important implications for vaccine design, because it focuses the attention upon the absolute necessity to understand the link between primary and localized secondary immune responses, in order to understand the consequences of a vaccination approach.

Primary and secondary T cell responses during respiratory infections

Several parameters may influence the nature of a secondary immune response. I. The composition of the memory response that is induced after vaccination, i.e.: the involvement of B cell, $CD4^+$ and $CD8^+$ T cell responses. II. The extent to which memory T cells are polarized. III. The presence of local memory cells in the tissue. Both primary and secondary responses are regulated by IV. The innate immune effects of the invading virus in the local tissue, i.e. the effect this might have

on immune cell recruitment through the local production of chemokines (soluble proteins that provide chemotactic signals for immune cells) or local T cell polarization by cytokines. V. immune evasion strategies of the virus.

After intra-nasal infection with a respiratory virus the virus spreads to the lung. Here dendritic cells (DC) that are located underneath the epithelial mucosa internalize the antigen and transport it to the draining lymphnodes (74). Recently, interesting new data has been gathered about the dynamics of T ceIl responses in vivo using T ceIl receptor transgenic mice and MHC tetramers (tools that allow the tracking of T cells with a particular specificity). From these studies it became clear that primary T cell responses are initiated in the draining lymphnodes. T cells stimulated via the T cell receptor by antigen-MHC complexes start to proliferate when appropriate co-stimulatory signals are also provided. After several rounds of proliferation the antigen specific T cells acquire effector function and the ability to leave the lymphnodes and migrate to inflamed tissues (75-77). Cells that are found in the lungs during and after a viral infection display a highly activated phenotype. They exhibit several effector functions, including the ability to secrete cytokines and the lysis of infected epithelial cells by perforin/granzyme or Fas/FasL mediated mechanisms (76;78;79).

After clearance of infectious virus the inflammation resolves. The number of virus-specific T cells dramatically declines, leaving behind a much smaller population of memory cells (76). These memory cells are responsible for an accelerated response during a next encounter with the virus (80). The persisting memory cells are not a uniform population. They differ in phenotype, location and function (81). A central memory T cell population is characterized by expression of CD62L and CCR7, surface expressed molecules that play a role in locating these cells to the secondary lymphoid tissues (76;82). Recently, it has become clear that also in peripheral tissues like the lung a set of memory T cells remains present, for a period of time after infectious virus has been cleared (79;83-87). These cells the so-called effector memory cells are characterized by an highly activated phenotype (78;83). These cells are able to contribute to the recall response during re-infection, although they may not be able to divide locally (83). Therefore, they may not be able to contribute to a sustained response, but they may contribute by reducing the viral load until the effector cells that are activated in the draining lymphnodes reach the infected lung. Furthermore, because T cells are able to produce chemokines they may contribute to the efficacy of the secondary response by accelerating the recruitment of lymphnode derived effectors.

Another interesting aspect of T cell memory is the observation made in influenzavirus infection models that numbers of central memory $CD8^+$ T cells in the lymphnodes remain stable for long periods of time, while local effector memory responses wane (83;88). In contrast $CD4^+$ T cell responses wane in both compartments. Interestingly, complete protective $CD8^+$ mediated immunity to influenzavirus is present for about two months where-after it wanes (89). Thus, the long

lasting central memory cells are by themselves insufficient to confer complete protection. These novel insights in the dynamics of T cell responses will direct future research towards identifying the role and efficacy of different memory T cell populations and addressing the question how vaccination strategies can be designed to elicit the most effective type of memory.

Recently, also after RSV infections long term effector/memory cells have been found in the lung (90) Like it was described for the sendaivirus and influenzavirus models these RSV-specific effector/memory cells were CD62lo cells and a large proportion expressed the activation marker CD69. However, the $CD8^+$ T cells present in the lung tissue rapidly lost effector function (cytokine production and cytolytic activity) when live virus was present in the lung (91). This was not observed for T cells present in lymphnodes and spleen, the locations were central memory cells are found. It needs to be established what the mechanism is behind $CD8^+$ T cell inactivation, and whether there are functions that remain intact. Furthermore, the impact of $CD8^+$ T cell inactivation during subsequent infections needs to be established.

In the previous sections data was summarized in which it was clearly shown that different routes of vaccination elicited different secondary responses during viral challenge. Also from the available data it is not straightforward to predict the outcome of a secondary immune response from the type of response initiated during vaccination. It appears that a type 2 response during priming may not be recalled during challenge and vice versa. Therefore, the question is raised what factors determine the type of recall response and which are the cells that contribute to this secondary response. For instance it has been described that cytokine polarized $CD4^+$ and CD8 effector cells give rise to memory cells in vivo that remain polarized i.e. they produce the same set of cytokines after restimulation with antigen (92;93). The acquisition of stable polarization has a molecular basis. Type 2 polarized cells lose IL12Rβ2 and type I cells, while retaining the IL-4 receptor loose IL-4 responsiveness. Also changes in chromatine structure, stable demethylation of cytokine gene promotors and type specific transcription factor expression, all contribute to the maintenance of cytokine gene expression patterns (94-96).

While certain cells may be committed to a certain cytokine expression pattern, also highly divided and highly activated cells exist that are not committed to a particular T cell lineage (97) and some may become polarized in the environment of the secondary challenge. Interestingly vaccination with soluble proteins in adjuvant resulted in a high number of these in vivo primed, uncommitted memory cells (98).

The observation that after different RSV vaccination approaches the cytokine profiles during priming did not correspond with the cytokine profile found in the lung during challenge infections with RSV may be explained in two ways. First, a population of unpolarized memory cells is reactived and polarization occurs

locally in the lung. Innate immune effects of the virus may create an inflammatory environment that influences T cell polarization during this phase of the response. A second explanation is selective homing of specific T cell subsets to a local inflammatory site. Th1 and Th2 cells as well as Tc1 and Tc2 cells express different combinations of chemokine receptors that direct their migration into the inflamed tissue (97). Thus the local inflammation induced by a pathogen may by means of creating a particular cytokine and chemokine environment affect the secondary immune response. In the next section novel insights into the innate mechanisms by which RSV may regulate adaptive primary and secondary immune responses are described.

Innate immunity against RSV

RSV entering via the intranasal route infects epithelial cells of the respiratory mucosa lining the airways and resident alveolar macrophages. RSV spreads through the respiratory tract primarily by cell to cell transfer of the virus (6). Dendritic cells that are located underneath the epithelial mucosa are likely to be a second line of cells that interact with the virus. All these virus cell interactions contribute to the cytokine and chemokine milieu that sets the stage for the ensuing adaptive immune response. cDNA micro arrays were performed on lower airway epithelial cells infected with RSV to study the pattern of gene expression after interaction with RSV (99). The expression of over 1200 genes was changed among them cytokine genes and chemokine genes and putative antiviral genes. In comparison with cytomegalovirus and influenzavirus, RSV had the most dramatic effect. The most striking difference with influenzavirus was the induction of chemokine genes and interferon inducible genes, that are not changed after influenzavirus infection of HeLa cells (100). The alteration in chemokine expression was observed for members of the CC chemokines (I-309, exodus-1, TARC, Rantes, MCP-1, MDC and MIP-1α and-1β), CXC chemokines (Gro-α,-β,-γ, ENA-78, Il-8 and I-TAC) and CX3C chemokine: Fractalkine. Some of the chemokines identified had been found in earlier experiments to be present in supernatants of RSV-infected airway epithelial cells (101-103). The potent induction of CC, CXC and CX3C chemokines by RSV in airway epithelial cells is very likely to be an essential early factor in the recruitment of the inflammatory cell infiltrate (of monocytes, neutrophils, eosinophils, T lymphocytes, NK cells and dendritic cells) into the airways.

RSV components involved in innate immune responses

In the previous experiment virus infection of epithelial cells was studied for its effect on global gene expression. However no detailed study was made into the exact requirements of virus interaction with the cells, i.e. whether surface binding suffices to induce a subset of genes and/or whether infection with live virus was required. In a recent study performed in mice the transcription factor nuclear

factor (NF)-κB appeared to play a crucial role in controlling the expression of RSV-induced immuno-modulatory genes (104). Two mechanisms contribute to the RSV-induced NF-κB responses in the lung. The first response is an early response found in alveolar macrophages, which is independent of virus replication but needs the presence of a functional Toll-like receptor-4 (TLR4). (Toll like receptors are a family of receptors recognizing specific molecular patterns on pathogens. Triggering of these receptors initiates natural and adaptive immune responses). The second response requires viral replication in epithelial/and or inflammatory cells. In previous in vitro studies the involvement of the viral surface protein F has been shown in the induction of IL-6 release by human and murine monocytes which was mediated via the TLR4 (105). This viral interaction with TLR4 further facilitated the trafficking of NK cells and $CD14^+$ monocytes to the airways as well as the local expression of the cytokine Interleukin-12. These innate effects of RSV appeared to be essential for an efficient clearance of the virus from the lungs (106). Early recruitment of NK cells to the lung and the production of Interferon-γ by these cells was shown to inhibit lung eosinophilia in mice (107).

Interestingly the other viral surface proteins: G and/or SH appeared to counteract the recruitment of NK cells and neutrophils to the lung and alter tumor necrosis factor and Interferon-γ expression in mice models. This was shown in the primary as well as during secondary immune responses against a cold adapted virus strain that was missing both the G and SH proteins (108). The altered cellular recruitment was possibly the result of an altered chemokine expression pattern (109). How the G or SH protein affect the local chemokine environment is not clear. For the G protein a CX3C (fractalkine) chemokine mimicry has been described and fractalkine receptor binding occurred in vitro (110). In Balb/c mice the CX3C motif within the G protein plays a crucial role in enhanced disease induction observed after FI-RSV vaccination (111). However, whether in vivo interaction with Fractalkine receptors is crucial for this effect rather than the involvement of this region in an immunodominant epitope recognized by $CD4^+$ T cells, needs further investigation.

The link between migratory capacity of T cell effector subsets and the ensuing immune response

The pattern of chemokines expressed directs the infiltration of inflammatory cells into the tissue. Different cell types express different combinations of chemokine receptors that direct their recruitment. Thus, the character of the immune response in the inflamed tissue depends on the array of cells that is recruited. Besides the innate effects of the virus, recruited activated immune cells themselves also contribute to the chemokine profile locally. For instance $CD8^+$ T cells induce the secretion of monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) in alveolar epithelial cells via membrane bound TNF-α. Thus, it can be envisioned that pre-existing memory influences the recall response. Differences in chemokine expression patterns have indeed been observed after intranasal challenge of FI-RSV or live RSV primed mice (112).

FI-RSV primed mice showed much stronger expression of eotaxin and monocyte chemotactic protein 3 (MCP-3) that are potent eosinophil and lymphocyte chemoattractants, while RSV primed mice showed a higher expression of lymphocyte or monocyte chemoattractants (MIP-1β, MCP-1 and MIP-2).

Concluding remarks and future directions

The new insights on the role of the innate immune response and the complex networks of immune cells that contribute to the host immune response against a simple virus like RSV are fascinating. However, it still remains an enormous challenge to translate the newly obtained knowledge i. to a better understanding of the crucial components contributing to severe primary infections in men and ii. to the development of save and effective vaccines. Clearly, careful evaluation of some of the information obtained with rodent models is required. Recent studies with a primate model suggested for instance that the role for humoral responses during FI-RSV mediated enhanced disease was more prominent than observed in cotton rats and mice (113;114). The mouse is also not a good model for primary RSV disease, because the virus replicates poorly in mouse cells. Therefore, models of natural virus/host interactions: pneumovirus of mouse-PVM (115-117) and bovine RSV in cattle (118;119) Antonis, et al. J. Virol. In press) may contribute to the definition of parameters important for disease development during primary infections with pneumoviruses.

Recently several research groups invested in the characterization of antigenic epitopes that are recognized by human $CD4^+$ and $CD8^+$ T cells (120-123). In combination with novel techniques to assay T cell responses (elispot, MHC tetramers, intracellular cytokine staining) this may lead to a better insight in the contribution and the nature of $CD4^+$ and $CD8^+$ T cell responses during primary host responses against RSV, as well as in the dynamics of memory T cell responses in youths, adults and elderly people.

The link of RSV bronchiolitis with later development of asthma has clearly been demonstrated in epidemiological studies. The explanation that both RSV bronchiolitis and asthma are caused by a similar immune mechanism and/or share a similar genetic susceptibility seems plausible. Some experimental evidence now strengthens this hypothesis. Studies have been performed on the contribution of polymorphisms in immune response genes. Focus of these studies was on a Th2 or Th1 bias of immune responses. A relationship has been found with IL-6 and IFN-γ genotypes that impacted on illness severity in adults experimentally exposed to RSV (124). In children with severe RSV bronchiolitis variants of IL-4 and IL-4 receptor-α and IL-10 alleles were linked to severity of disease (125). Obviously newly identified genes that are involved in asthma development, like for instance

the Tim gene family, and components of innate immune response that are affected upon RSV infection of lung epithelium or antigen presenting cells, will be the next targets for genetic analysis.

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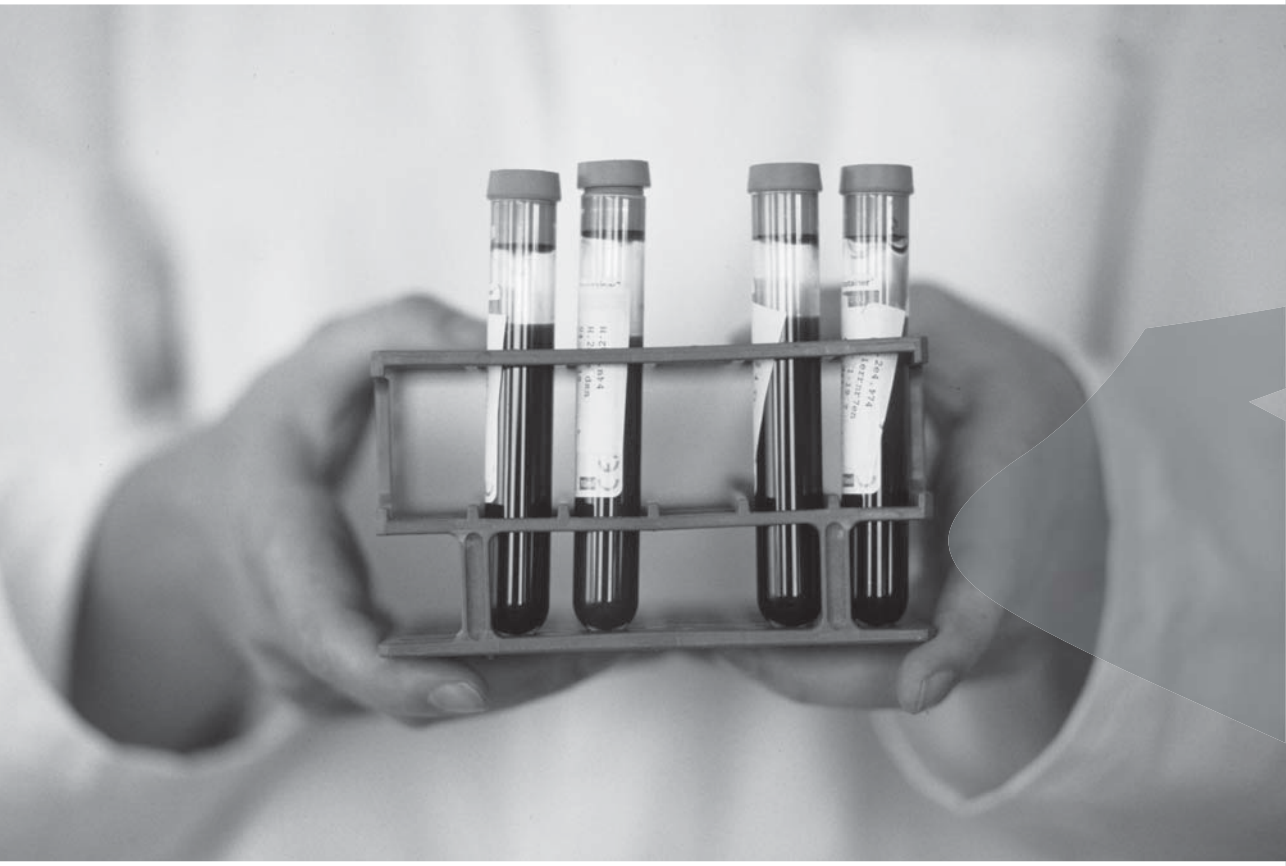

HLA-DP4 presents an immunodominant peptide from the RSV G protein to $CD4^+$ T cells

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Virology. 2004 Sep 1;326(2):220-30

PBMC of healthy donors

Abstract

$CD4^+$ T cells play a crucial role during virus infections, by producing antiviral cytokines and by regulating humoral and cellular immune responses. Unfortunately however, exaggerated $CD4^+$ T cell responses can cause significant immune mediated disease as was observed during RSV infections in children previously vaccinated with a formalin-inactivated virus in the 1960's. It has been observed that vaccination with the G protein of RSV tends to prime mice for a similar Th2 mediated enhanced disease. Whether the G protein may play a role in enhanced disease in man is unclear. In the present study we identified an immunodominant epitope in the conserved region of the G protein encompassing amino acid residues 162-175. This epitope is presented in the context of HLA-DPB1*0401 and DPB1*0402 the most prevalent HLA class II alleles. Importantly, in some patients a mixed Th1/Th2 response against this epitope was found in bronchoalveolar lavage samples during primary RSV infections.

Introduction

Respiratory Syncytial Virus is a major cause of childhood lower respiratory tract infections. Seventy percent of all children are infected in their first year of life, and nearly all children by age three (1). Although the majority of patients suffer a relatively mild upper respiratory tract infection, 3% of infected children are hospitalized and require oxygen support (2). Risk factors for a severe RSV infection are pre-maturity, congenital heart disease and bronchopulmonary dysplasia, although genetic predisposition also seems to play a role (3). Unfortunately however, the precise mechanism(s) of severe lower respiratory tract disease remain unknown.

T cells play an important role in clearing the virus from the infected lung. However, T cells may also be involved in immune pathology that can be associated with respiratory disease. An involvement of $CD4^+$ T cells with a Th2 phenotype has been postulated as a possible cause of severe pathology. In children with severe RSV infections, it has been observed that $CD4^+$ T cell responses in peripheral blood tend to produce higher levels of Th2 cytokines than in control populations (4;5). However, these results could not be reproduced by others (6;7). Moreover, the presence of eosinophil cationic protein in broncheolar alveolar lavage fluid and nasal secretions of children with severe RSV infections also suggests the involvement of local Th2 responses, i.e., in the infected lung (8). By producing cytokines such as IL-5 and IL-13, Th2 cells could be responsible for the recruitment of eosinophils into the lung. However, no direct evidence exists that Th2 cells and Th2 cytokines are indeed present in the lungs of patients with severe RSV infections.

In order to evaluate the role of $CD4^+$ T cells in the immunopathological processes during severe RSV infection, it is essential to characterize the antigenic specificities of $CD4^+$ T cell responses, and to determine the nature and magnitude of such responses in the infected lung. For the fusion (F) protein of RSV, we have recently identified a panel of immunodominant epitopes that are recognized by memory $CD4^+$ T cells obtained from healthy adult blood donors (9). These peptides were localized across the entire length of the F protein. For the second major surface protein of RSV, the glycoprotein (G), we focussed our epitope mapping studies on the conserved antigenic loop of this highly polymorphic protein. Within this region, several antigenic peptides have been described that induce $CD4^+$ T cell responses in mice with different genetic backgrounds (10-12). Moreover, it has been found that these antigenic peptides derived from the G protein all have the tendency to induce, to some extent, Th2 type T cell responses. Similarly, in one human study with short term T cell lines established after stimulation of PBMC with purified RSV F and G proteins the G specific lines produced Th2 type cytokines, whereas F stimulated PBMC cultures produced Th1 type cytokines (13).

Here we present the characterization of a dominant epitope that is presented in the context of HLA-DPB1*0401 and *0402. These HLA class II molecules are present worldwide in an allelic frequency of 20-60% and cover about 75% of the

caucasian population (14;15). Although the DP4 binding motive F_1-F_6 is present twice within the peptide sequence (15;16), our data suggest the predominant use of only one frame. Interestingly, two size variants of the peptide induce different cytokine profiles. These observations indicate that the exact length of the naturally produced peptide and the cognate T cell repertoire, may, in part, determine the outcome of the $CD4^+$ T cell response after natural infection in terms of cytokine profiles.

Participants, materials, and methods

Peripheral blood mononuclear cell preparation

Buffy coats were obtained from healthy adult blood donors. Since (i) all children have been exposed to RSV by the age of three and (ii) adults are re-exposed to RSV every two to three years, we assumed that all adult donors have been primed with RSV and harbor memory responses. PBMC were isolated by density centrifugation with lymphoprep (Nycomed Pharma). PBMC where either used fresh or thawed from cryopreserved samples (liquid nitrogen, in RPMI, 30% fetal bovine serum (HyClone) supplemented with 10% DMSO).

Synthetic Peptides

Peptides were synthesized by standard solid-phase Fmoc chemistry. The sequences of the peptides used are given in Table I. Peptides were either made with a free carboxy terminus or with an amino-terminal amide, as indicated in Table I.

Table I: Amino acid sequences of G peptides

G162-175	DFHFEVFNFVPCSI
G165-179	FEVFNFVPCSICSNN
G168-183	FNFVPCSICSNNPTCW
G171-187	VPCSICSNNPTCWAICK[1]
G174-189	SICSNNPTCWAICKRI
G177-194	SNNPTCWAICKRIPNKKP
G179-194	NPTCWAICKRIPNKKP[1]
G183-197	WAICKRIPNKKPGKK
G185-199	ICKRIPNKKPGKKTT

Variant sequences

G163A	DAHFEVFNFVPCSI[1]
G165A	DFHAEVFNFVPSCI[1]
G168A	DFHFEVANFVPCSI[1]
G170A	DFHFEVFNAVPCSI[1]
G162-175a	DFHFEVFNFVPCSI[1]

[1]Peptide amides

Patient studies

The patients included were admitted to the pediatric intensive care unit of the Wilhelmina Children's Hospital in the winter season of 2002-2003. The infants were under 3 months of age and required mechanical ventilation because of respiratory failure due to RSV lower respiratory tract infection. PBMC of the children were prepared using the same procedure that was applied for adult blood samples. Nonbronchoscopic bronchoalveolar lavage (NB-BAL) was performed twice as described before (17) using 1ml/kg saline at room temperature. Collected samples were directly placed on ice. Specimens were filtered through 70μM cell strainers (Falcon, BD) to remove mucus. No DTT was used to dissolve the mucus in order to avoid possible denaturation of cell surface proteins involved in T cell receptor- target cell interaction. Cells were cryopreserved in 90% fetal bovine serum with 10% DMSO in liquid nitrogen. Before the assays PBMC's and BAL were quickly thawed and washed twice. Cells were plated in duplicate at $5x10^6$ cells/well in 96-wells round bottom plates in AIM-V medium (Gibco) supplemented with 2% human pooled serum (HPS) and 40 units/ml recombinant human IL-2. 1μM of the appropriate peptide was added to the cells in a total volume of 225μl. Cells were allowed to proliferate for 10 days with medium replacement when needed. On day 10 cells were split into two new wells and restimulated with the same peptide in a total volume of 100μl medium. After 48 hours supernatants were sampled and cytokines were measured using the Luminex multiplex cytokine assay. Healthy donors and parents of patients gave their informed consent. The study was approved by the Medical Ethical Committee of the University Medical Center of Utrecht.

HLA-DPB1 sequencing based typing (SBT)

SBT was performed according to the previously published approach (18;19). Full exon 2, containing all allele representative polymorphism was sequenced and ambiguous allele combinations resolved by group specific amplification and subsequent sequencing (20).

Intracellular staining of peptide-specific T cells for flow cytometry

PBMC were plated in a 96 well round bottom plate (Costar) ($0.5x10^6$ cells/well) in AIM V medium supplemented with P/S, 2% HPS, 40 units/ml IL-2 and costimulatory antibodies (anti-CD28 and anti-CD49d). Cells were either stimulated with 1μM of peptide or not stimulated (negative control). After one hour of incubation at 37ºC 10μg/ml of Brefeldin A (BD PharMingen) was added to accumulate cytokines in the cell. After 5 hours, 2mM of EDTA was added in order to arrest activation and to remove adherent cells from the well. After incubating 20 minutes at room temperature, cells were washed twice in FACS buffer (PBS containing 0.02% azide, 2% FCS and 2 mM EDTA). Then phycoerythrin (PE)-labeled anti-CD3 mAb (BD-PharMingen) and Cy5-labeled anti-CD8 mAb (BD-PharMingen) for surface staining were added. After 30 minutes of incubation on ice, cells were washed twice in ice cold FACS buffer. Cells were permeabilized and fixated using FACS permeabilizing/fixation solution (perm/Fix) (BD-PharMingen). Cells were stained intra-cellularly after an additional wash in Perm-wash (BD-PharMingen) with fluo-

rescein isothiocyanate (FITC)-labeled IFN-γ mAb (BD-PharMingen clone 340449) for 30 minutes on ice. Cells were washed three times in perm-wash and fixated in 1% paraformaldehyde/PBS for 20 minutes. Cells were resuspended in FACS buffer and kept at 4ºC until analysis. Cell staining was analysed on a FACS-Calibur using CellQuest software (BD Bioscience, Mountain View, CA). To evaluate whether the G epitopes were presented during infection, PBMC were infected with RSV at a m.o.i. of 5 and cultured in 24 well plates ($1x10^6$/well) in AIM-V medium with 2% (HPS), (P/S) and 40 units/ml of recombinant human IL-2. After 10 days of proliferation, cells were either stimulated with the peptide or not restimulated (negative control). On day 8 PBMC of the same donor were depleted for $CD3^+$ T cells using negative selection MACS separation columns (Miltenyi Biotec, Germany). The remaining cells were infected with RSV at a m.o.i. of 5 two days prior to use, thus allowing the APC to present RSV epitopes. On day 10 these APC were added to the PBMC that had been stimulated with RSV on day 0. Stimulation with peptide or virus infected APC was allowed for 5 hours in the presence of Brefeldin-A. Intra- and extracellular staining was performed as described.

Elispot assay

Elispot assays for INF-γ production were performed as described before, using antibodies 1-D1K and 7-B6-1-biotin (Mabtech) for coating and detection, respectively. Monoclonal antibodies used for the blocking of antigen presentation by HLA-DR (B8.11.2), HLA-DQ (SPVL3) and anti HLA-DP (B7/21) (21), were used as diluted culture supernatants. After coating of the filtration plates with 1-D1K, the plates were washed thoroughly and blocked with RPMI 1640 containing 10% fetal bovine serum. $2.5x10^5$ PBMC were then added to the plates together with the indicated amount of peptides. When antibody blocking was performed the cells and blocking antibodies were pre-incubated for 30 minutes at 37°C, after which the peptides were added to the cultures. Cells were cultured with antigen for 24 hours at 37°C in a humidified incubator, before the addition of detection antibodies. In most experiments peptides were titrated in the presence of a constant concentration of blocking antibody. The result of antibody blocking presented in the figures is the lowest peptide concentration that still induces (close to) maximal levels of interferon-γ production. Data are represented as the number of spots per 10^6 PBMC, minus the background of unstimulated samples.

Multiplex cytokine assay

Multiplex cytokine analysis was applied using a procedure that has been described in detail (22). This procedure is a particle based elisa type assay based on the bioplex system and employing the luminex multi- analite profiling technology. In our laboratory this technique is used to identify 17 different cytokines within a single sample. The antibody pairs used for the experiments in the present study are all the same as described earlier (22). For our application we reduced the number of cytokines to 7 (Interleukin (IL)-2, IL-4, IL-5, IL10, IL-13, TNF-α, IFN-γ. Antigen stimulation was performed with PBMC, $5x10^5$ or 10^6 per well, in 96 well round bottom plates (costar) in triplicate in RPMI 1640 supplemented with penicil-

lin/streptomycin and 10% fetal bovine serum. Peptides were added to the wells at concentrations given in the figure legends. Culture supernatants were routinely sampled at 24, 48 and 72 hours. In the figures cytokine concentrations present in culture supernatants harvested after 72 hours are depicted. Cytokines that were not produced above background levels are omitted from the figures.

Results

Identification of RSV-specific T cell epitopes

To identify RSV cross-reactive CD4+ T cell epitopes, a set of overlapping peptides covering the conserved loop area (amino acid residues 162-199) of the RSV G protein was synthesized (Table I). PBMC of healthy adult blood donors that differed in HLA class II haplotypes were tested in an interferon-γ elispot assay for their response against this set of peptides. Eight out of ten donors from this panel recognized a peptide spanning amino acid residues 162-175 (G162-175, Table II). An overlapping peptide, G165-179, was recognized by the same donors. A second antigenic region was found in the stretch of amino acid residues 177-197 where two sequential peptides, G177-194 and G183-197, shared amino acid residues 183-194. The first epitope G162-175 was recognized at levels of 17-200 spots per 10^6 PBMC. The epitope in the second region was recognized at levels between 16 and 56 spots per 10^6 PBMC. These frequencies are in the same order of magnitude as the CD4+ T cell responses that we have found against epitopes from the fusion protein of RSV (9).

Table II: IFN-γ elispot assay performed with PBMC of healthy adult blooddonors.

DONOR	VB-7	CH-1	VB-2	VB-5	VP-1	MP-4	VB-4	VB-6	VB-11	CE-8
HLA-DRB1	1101	15	0701	0401	1302	1301	1101	0401	15	03
	15	16	1101	0403	15		1301	B4101	16	
HLA-DPB1	0401	0402	0401	0401	0501	0401	0401	0402	0401	0901
		02012	0402		03011	02012	03011	1401		01011
G162-175	129[1]	27	47	200	2	26	52	17	23	1
G165-179	96	14	14	130	4	nd	nd	2	20	0
G168-183	5	1	17	4	6	nd	nd	6	5	nd
G174-189	10	5	1	-2	2	nd	nd	2	2	nd
G177-194	56	19	19	20	4	16	20	-2	-6	-2
G183-197	53	-1	9	12	2	nd	nd	-2	-4	nd
G185-199	20	4	-3	-4	2	nd	nd	-7	2	nd

[1]Number of spots per 10^6 PBMC minus background values of unstimulated samples (nd:notdone).

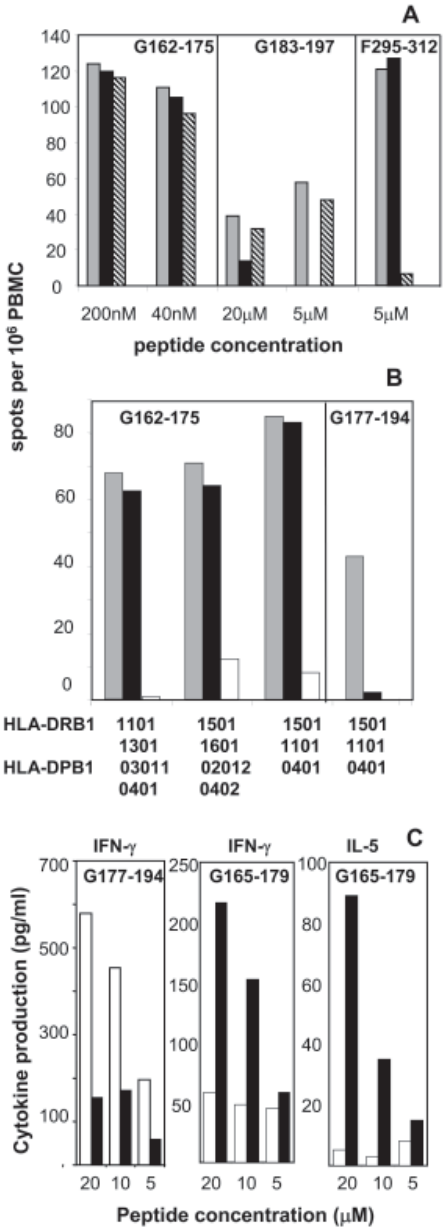

Figure 1: *HLA molecules involved in the presentation of G epitopes. Inhibition of IFN-γ production by MHC class II specific antibodies B8.11.2 (anti HLA-DR), SPVL3 (anti HLA-DQ) and B7/21 (anti HLA-DP).* ***a:*** *IFN-γ elispot assay with PBMC of donor VB7 (HLA-DRB1*1101,*1501, DPB1*0401) against peptides G162-175 and G183-197. PBMC of donor VP1 (HLA-DRB1*15,*1302, DQB1*06, DPB1*03011,*0501) were tested in the same assay against a control peptide F295-312 that was identified by us before and is presented by HLA-DQ.* ***b:*** *IFN-γ elispot assay performed with different donors (HLA class II shown in the Figure) against peptide G162-175 at 500nM and peptide G177-194 at 5μM.* ***c:*** *Cytokine production by PBMC of donor VB-7 stimulated with peptides G177-194, and peptide G165-179. Grey bars no blocking antibody added, black bars B8.11.2, hatched bars SPVL3 and white bars B7/21.*

RSV-G epitopes are presented by HLA-DP and HLA-DR

To confirm that the T cell responses were in fact MHC class II restricted and to determine the restriction elements, we performed T cell stimulation assays in the presence of blocking antibodies with specificity for HLA-DR (B8.22.1), HLA DQ (SPVL2) and HLA-DP (B7/21). As shown in Figure 1, our data revealed that the epitope(s) in peptides G162-175 and G165-179 was presented by HLA-DP and that the epitope(s) in peptides G177-194/G183-197 was presented by HLA-DR. Since our donor panel had not initially been typed for HLA-DP, all donors were subjected to DNA typing. The donors responding to peptides G162-175 and G165-179 all shared HLA-DPB1*0401 or B1*0402, whereas the non-responders did not express the DP4 alleles (Table II). Three of the responding donors were homozygous for HLA-DPB1*0401, providing conclusive evidence that this allele binds and functionally presents the peptide. The second antigenic region contains peptides that could be presented by several HLA-DR molecules that were not further identified at this point. Given the distribution of positive ELISPOT signals, it seems likely that this area contains either multiple or promiscuous epitopes (Table II).

When PBMC of healthy adult HLA-DP4 positive donors were stimulated for 5 hours with peptide G162-175 the T cell response as measured by intracellular staining for IFN-γ was low (Figure 2b). However, when PBMC were cultured with RSV for ten days, T cells were expanded that produced IFN-γ upon re-stimulation on day ten with RSV-infected antigen presenting cells or peptide G162-175 (Figure 2d, e). This result proves that peptide G162-175 or a closely related peptide is indeed naturally processed and presented on RSV-infected APC.

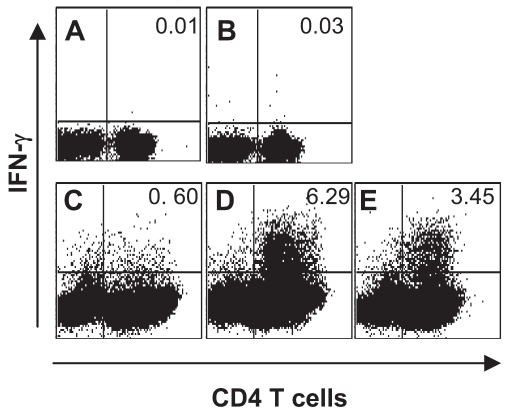

Figure 2: *Natural processing and presentation of a G162-175 related sequence in RSV-infected cells. Intracellular staining for IFN-γ was performed on* ***a:*** *unstimulated PBMC,* ***b:*** *PBMC stimulated for 5 hours with G162-175, and C, D, E in PBMC cultures grown for 10 days in the presence of RSV and recombinant IL-2, after a short 5 hour re-stimulation with* ***d:*** *virus infected APC or* ***e:*** *G162-175.* ***c:*** *Cell cultures that were not restimulated on day 10. The numbers in the upper right quadrants are the percentage of IFN-γ producing CD4⁺ T cells in a live cell and lymphocyte/lymphoblast gate.*

Cytokine production induced by G162-175 and G165-179

Because we used the interferon-γ elispot assay to measure peptide-specific responses, it cannot be excluded that T cells producing other cytokines (e.g., Th2-type) upon T cell receptor triggering were missed. Therefore, we tested the T cell response against the G peptides in selected donors by measuring a set of cytokines in culture supernatants, using a multiplex cytokine assay (luminex, (22)). Although these experiments did not result in the identification of additional dominant epitopes (data not shown), we found that stimulation with the G162-175 and G165-179 peptides elicited strikingly different cytokine profiles. For donor VB-7 (Table II) we observed that stimulation with G165-179 yielded IL-5 and IL-13 and only minor quantities of IFN-γ (Figure 3b). In contrast G162-175 induced production of predominantly IFN-γ. A different pattern was found in a second donor (Figure 3a, donor VB-5): here, stimulation with G165-179 led to reduced levels of IFN-γ compared to G162-175, and no upregulation of Th2-type cytokines. In both cases, however, the Th1/Th2 cytokine ratio differed between G162-175 and G165-179 (Figure 3). These cytokine patterns were highly reproducible over a broad peptide dose range, suggesting that rather than the antigen dose the structural

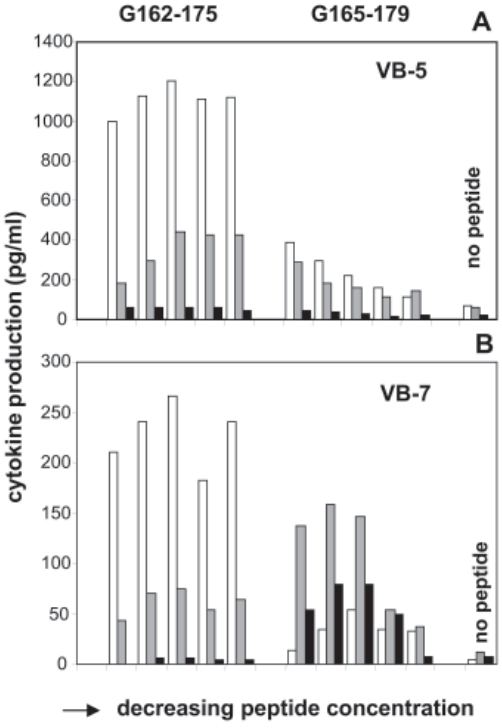

Figure 3: *Cytokine production of PBMC of donor VB-5 (HLA-DRB1*0401,*0403,DPB1*0401) and VB-7 (HLA-DRB1*1501*1101, DPB1*0401) upon stimulation with G162-175 and G165-179. The peptide concentrations tested were 20,10,5,2.5, 1 and 0 μM. White bars IFN-γ, grey bars IL-13 and black bars IL-5.*

features of the HLA/peptide/T cell receptor complex or the phenotypes of the different memory pools determined the pattern of the response.

Peptide G162-175: one or two binding registers?

For HLA-DP, a binding motif has been described that consists of two phenylalanine amino acid residues spaced six positions apart (15;16). This Phe-1/Phe-6 (F1-F6) motif is present twice within the antigenic peptide G162-175 and once in G165-179 (Figure 4a). A favored secondary anchor (valine at position nine) is present only in the amino-proximal motif. Because peptide G165-179 is able to stimulate T cell responses in most donors that are HLA-DPB1*0401 positive, it is evident that the carboxy proximal motif F165/F170 is used for binding to the groove of this HLA molecule. However, the different cytokine patterns found in the responses of donors VB5 and VB7 against the overlapping DP4-restricted peptides might be an indication that the G162-175 peptide can also bind to HLA-DP4 in an alternative fashion. In other words, the G162-175 peptide could comprise two overlapping HLA-DP restricted epitopes. It seems likely that different sets of T cells would respond to the peptides, depending on which register was used for HLA-binding. However, it is also conceivable that G162-175 harbors only a single epitope, but that the extra N-terminal amino acid residues present in peptide 162-175 or the additional C-terminal residues in G165-179 affect the binding with either the HLA molecule or the T cell receptor.

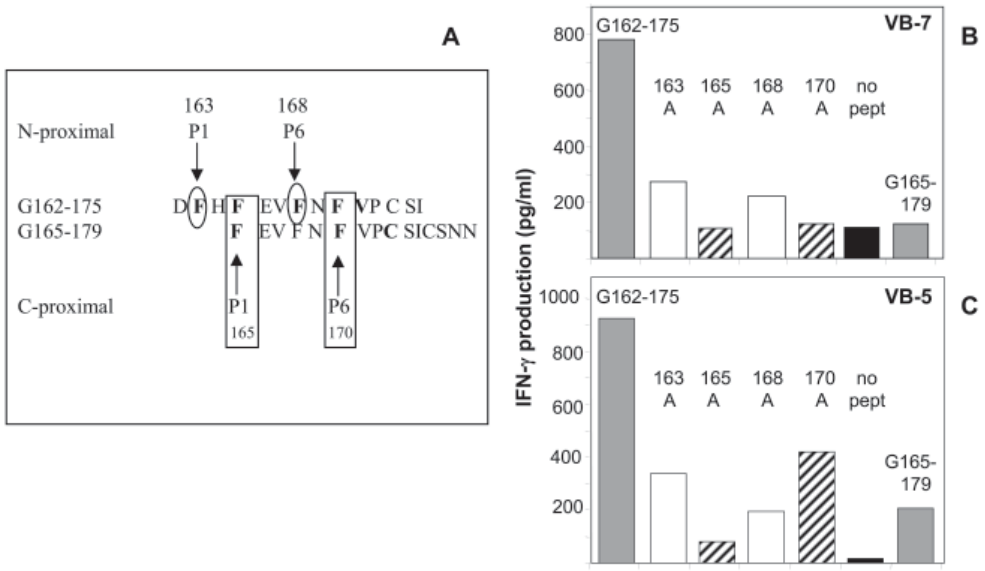

Figure 4a: *Cartoon of the two possible binding registers within peptide G162-175. Only the second C-proximal binding register is present in peptide G165-179.* ***b*** *and* ***c:*** *IFN-γ production upon stimulation by G162-175 (grey bars), mutant peptides with Ala replacements in the N-proximal binding motif (white bars) and Ala substitutions in the second, C-proximal binding register (hatched bars), and G165-179 (grey). Black bars: no peptide added. Peptides were tested in a concentration of 5μM.*

In order to distinguish between these possibilities we synthesized variant peptides with single alanine substitutions at the positions of the four potential phenylalanine anchor residues (positions 163, 165, 168 and 170). We used these variant peptides to stimulate $CD4^+$ T cells and measured cytokine production by luminex. When fresh PBMC from donor VB-7 were stimulated for 72 hours with the peptides containing substitutions at positions 165 or 170, no cytokine production could be measured (Figure 4b). The peptide variants with Ala replacements in the first (amino-proximal) binding register (positions 163 and 168) did induce IFN-γ production, albeit at lower amounts than the wild type peptide. None of the peptide variants induced IL-5 production (data not shown). Slightly different results were obtained for donor VB-5: whereas Ala-substitution at position 165 abrogated IFN-γ production, the effect was less obvious for the Ala-substitution at position 170, contrasting with the data for donor VB-7 (Figure 4c). Mutating F163 and F168 led to reduced levels of IFN-γ production, similar to donor VB-7. From this experiment, it was concluded that peptides mutated in the amino-terminal binding register (positions 163 and 168) still bound to HLA-DP. The F-residue at position 165 was clearly essential for binding in both donors, implicating this amino acid as a crucial anchor residue, consistent with the fact that this anchor residue is present in the only motif that is represented in peptide G165-179. However, the residual response in donor VB-5 against the 170A mutant peptide, showed that measurement of T cell responses against the Ala substituted peptides was not a sensitive enough procedure to conclusively determine the involvement of the position of a mutated amino acid residue in a putative binding motif. Therefore, the diminished response against the peptides that were mutated in the NH_2 proximal motif can not be interpreted as an effect on MHC interaction. Thus, at this point we were still unable to exclude the possibility of the second binding register in peptide G165-179.

To further clarify this issue, we obtained short-term $CD4^+$ T cell lines after stimulation of PBMC with peptide G165-179. We found that these G165-179 stimulated T cells produced cytokines IFN-γ, IL-13 and IL-5 upon re-stimulation with peptide G162-175, showing that within peptide G162-175 the second, C proximal binding register is indeed used for HLA binding (Figure 5a-d). The reverse experiment, using peptide G162-175 for primary stimulations and G165-179 for restimulation, revealed that the secondary IFN-γ response against G165-179 strongly resembles the recall response with peptide G162-179 (Figure 5e). No Il-13, IL-5 or IL-10 response was detected in this case. This result suggested that the response against G162-175 is dominated by T cells that recognize this peptide in the second, i.e. C-terminal binding register (See further discussion).

Patients with acute severe primary RSV infections respond to peptide G162-175

To evaluate the contribution of $CD4^+$ T cells specific for the HLA-DP restricted G epitopes in severe primary RSV infections we tested these peptides in peripheral blood mononuclear cells and in bronchoalveolar lavage (BAL)-samples of patients.

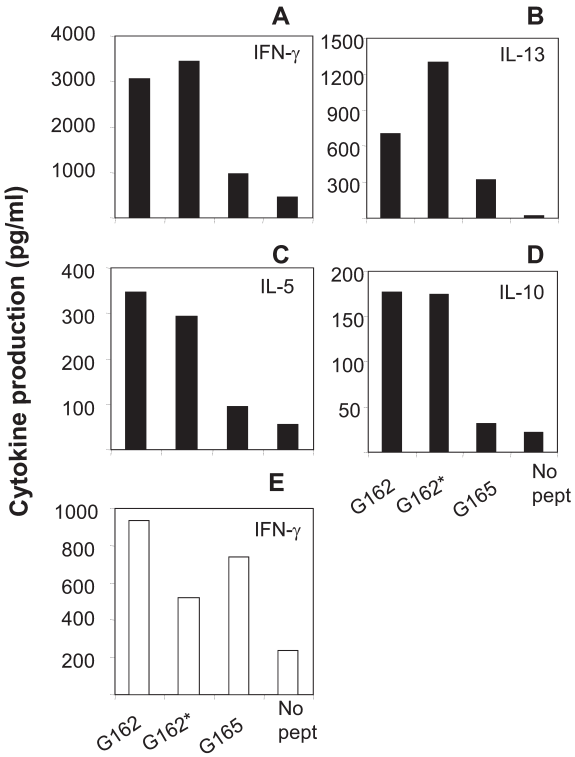

Figure 5: *Cytokine production by in vitro cultured PBMC of donor VB-7. PBMC stimulated once with G165-179 (**a-d**) and with G162-175 (**e**) were from day 5 onwards expanded in IL-2. On day 11 after primary stimulation the cells received a second stimulus with either G162-175 (G162), the amide form of G162-175 (G162*) or with G165-179 (G165), or were left unstimulated. Cytokines were measured by luminex 72 hrs after restimulation. The cultures that received as primary stimulation peptide G162-175 did not produce significant amounts of IL-13, IL-5, IL-10.*

The patients were infants, younger than 3 months of age, requiring mechanical ventilation due to RSV positive bronchiolitis. The HLA-DP haplotype was not known for these patients. Unfortunately, short term stimulation of PBL or BAL samples directly ex vivo did not induce cytokine levels above the detection limit of the luminex assay. However, after 10 days of culturing the cells in the presence of the G peptides, epitope-specific responses were clearly present in cultures of BAL cells from two out of five patients tested (Figure 6) and not in cultures from PBMC (data not shown). In one patient we detected IFN-γ, TNF-α, IL-13 and some IL-10 upon a second in vitro restimulation on day 10 with peptide G162-175. IL-4 and IL-5 were not detected. The cultures of the second patient showed IL-5, IFN-γ, Il-10 and IL-13 production but no TNF-α production upon re-stimulation with peptide G162-175. The culture of the second patient also responded to re-stimulation with peptide G165-179. These results demonstrate that responses against the G epitope are elicited during primary infections. Future studies will address the contribution and type of G specific CD4+ T cell responses in larger patient populations.

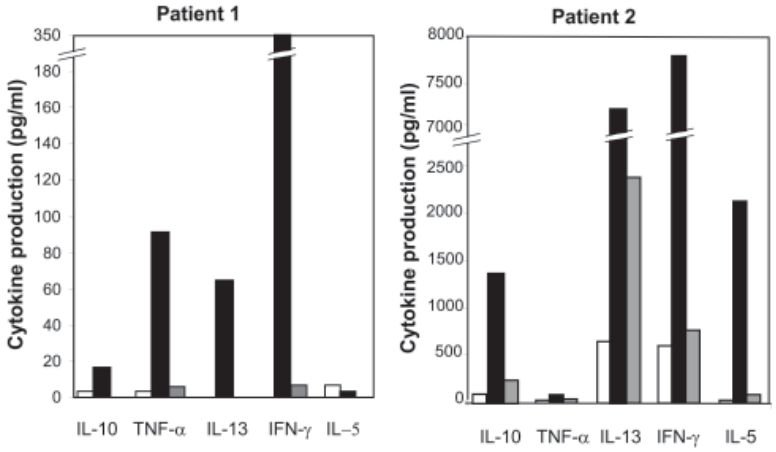

Figure 6: *Cytokine production of non-bronchoscopic BAL samples of two patients with RSV. Cells were stimulated in vitro for 10 days with peptides G162-175 or G165-179. After a restimulation for 24 hours with the same peptides used during primary stimulation, cytokines were assayed in the culture supernatants by luminex. White bars: no peptide, black bars: peptide G162-175, grey bars :G165-179.*

Discussion

In the present study, we have characterized antigenic epitopes in the conserved region of the RSV G protein, which are recognized by human $CD4^+$ T cells. Two regions were found, each containing two overlapping peptides that were able to induce a response in T cells of healthy blood donors without prior in vitro culture of the cells. The fact that one of the epitopes is presented by HLA-DP4 is important, since it allows a further analysis of G-specific $CD4^+$ T cell responses in a large group of patients because HLA-DP4 is widely expressed within the world population. Moreover, we were able to measure responses against G162-175 in almost every HLA-DP4 positive adult donor, which indicates that the response to this peptide is a general event and that the epitope should be qualified as immunodominant. Two donors that responded to peptide G162-175 (CH-1 and MP-4, Table II) expressed HLA-DPB1*02012 in addition to a DP4 allelic product. DPB1*02012 differs in only one amino acid residue from DPB1*0402. At this point we can not exclude the possibility that also HLA-DPB1*02012 can functionally present the immunodominant G epitope.

One of the most striking observations of our study is that two different donors homozygous for HLA-DPB1*0401 responded with different cytokine patterns against peptide G165-179. This could suggest that the responding T cell repertoire present in a certain individual could, in part, determine the type of immune response elicited against RSV. Because we measured direct ex vivo T cell responses, it seems likely that we observed memory T cell responses caused by previous exposure to RSV. Therefore, the differences in the cytokine profiles for the two donors could be caused by differences in the composition of the memory pools, i.e.

reactivation of Th1 type cells in one donor and Th2 type cells in the other donor. However, it is also possible that not pre-existing (memory) T cell polarization but the structural features of the interaction of the MHC/G165-179 complex with the memory T cell receptors determined the outcome of the Th2 type response. This second interpretation that in vitro (re-) stimulation with length variants of epitopes results in qualitative different T cell responses would imply that the memory populations are still subject to final polarization. Careful examination of T cell receptor usage in the responding T cell populations would possibly clarify this issue. Interestingly, during the review process of our manuscript a report was published by the Waal et al describing T cell clones that recognized the same region of the RSV G protein that we found (23). Using overlapping peptides de Waal et al found that two T cell clones recognized slightly different minimal epitopes encompassing amino acid residues 164-170 and 166-171 respectively. The first minimal epitope is only completely contained in peptide G162-175, and the second in both G162-175 and G165-179. These two clones show that T cells that recognize this area of the G protein may do so requiring different contact residues in the peptide. It is unclear however whether this different fine specificity could be responsible for the different cytokine profiles that we observed when PBMC of different donors were stimulated with peptides G162-175 or G165-179.

The choice of differentiation into either Th1 or Th2 cells is a crucial step that determines the direction of the subsequent adaptive immune response. Many factors can influence this choice, such as (i) the local cytokine environment during T cell priming, (ii) the duration of the contact between the T cell receptor and its ligand and (iii) the quality of co-stimulatory signals (24). The duration of contact is influenced by the density of peptide-MHC ligand on the antigen presenting cell, the stability of the interaction between peptide and MHC and the duration of the half-life of T cell receptor-MHC complexes. Upon interaction of T cells with APC reciprocal activation of both cell types occurs. Increased binding affinity between the T cell receptor and the peptide MHC complex increases the expression of CD40 ligand on the T cell, which induces improved conditioning of the antigen presenting cell as can be measured by increased production of IL-12 and altered expression of co-stimulatory molecules B7-1 and B7-2 (25). MHC class II bound peptides are presented on the cell surface as large nested sets that vary in length at the NH_2 and COOH termini. Thus, outside of the HLA class II binding core variable numbers of flanking amino acid residues can be present. These flanking regions can impact dramatically on MHC-peptide stability (26). Moreover, a substantial number of T cells are functionally dependent on recognition of peptide flanking regions (27-29). Thus the exact length of a peptide that is naturally presented is a crucial factor that can influence the nature of the immune response. Therefore, elution of the naturally processed epitope(s) from HLA-DP molecules, after exposure of antigen presenting cells to RSV or soluble G protein is required to prove the relevance of the observation that different cytokine responses are induced by synthetic epitopes.

Compared to HLA-DR, HLA-DP is less frequently reported as antigen presenting molecule. However, the list of antigenic peptides that are functionally presented is growing steadily and covers different types of antigenic sources varying from viruses (30-32), fungi (33) and tumors (34;35), to mycobactera (36) and house dust mite (37) antigens. Also, there have been some reports of disease associations with HLA-DP molecules (38;39). HLA-DR, DQ and DP are expressed to different levels, with DR being most abundantly expressed on most APC (40;41). Both HLA-DR and HLA-DP are also constitutively expressed on airway epithelial cells (42). The expression of the different HLA class II molecules is differently regulated (43-49). However, to our knowledge there are no clear indications that antigen presentation by different HLA isotypes induces a distinct type of immune response.

In murine models of RSV infection, it has clearly been shown that, in comparison to the other viral proteins, the G protein has a stronger tendency to sensitize mice for Th2 type $CD4^+$ responses, which are associated with enhanced disease after viral challenge (50-52). However, the potential role of pathogenic G-specific $CD4^+$ T cell responses should not be over-interpreted. First, in mice it is clear that both the route of administration of the protein vaccine and the form in which it is applied, affects the outcome of the ensuing immune response (53;54). Second, in the setting of primary or secondary RSV infection, the (virus-induced) G-specific $CD4^+$ T cell response did not appear to cause significant immune pathology. Finally, enhanced disease in mice after FI-RSV vaccination can also be primed with recombinant formalin-inactivated (FI)-virus that lacks the G and SH proteins (55). Therefore, the G protein does not seem to play a unique role as the primary cause of enhanced disease in mice. We found Th2 cytokines in only one of the two patients that responded to the G epitopes. Further studies with more patients are currently being conducted in our laboratory to determine the relative contribution of Th1 and Th2 responses against G and F derived epitopes during primary RSV infections.

For future vaccine development, the role of T cells during severe primary RSV infections in infants needs to be addressed, as well as the role of specific viral components therein. The results of previously published studies addressing the role of Th2 type cytokines during RSV disease are conflicting. There are reports that suggest a Th2 skewed T cell responsiveness during RSV infections (4;56;57) while others report predominant Th1 type responses in RSV patients (6;7). Part of the reason for these discrepancies may be the source of tissue sampled, i.e., peripheral blood versus nasal secretions or bronchoalveolar lavage, which are used for the assays. Moreover, different methods of analysis - T cell stimulation assays or direct cytokine measurements- may have contributed to the different results. From mouse studies it was learned that local T cell responses in the lung may differ from the type of T cell response observed in the spleen (52). Likewise, it can be expected that the analysis of antiviral T cell responses in peripheral blood of patients may not properly reflect the events in the lung. Knowing the antigenic

epitopes presented by HLA may contribute to clarifying the role played by T cells in the lung and this knowledge may guide the development of vaccines.

Acknowledgements

This project was supported by the Breedtestrategie, a research initiative from Utrecht University, in conjunction with The Netherlands Vaccine Institute and ID-Lelystad. The authors wish to thank Rein Zaat from the Bloodbank Utrecht for providing us with buffy coats (research project B95.004x) and Rogier van Gent and Ruurd van der Zee for their contributions to the work.

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Human CD8$^+$ T cell responses against five newly identified respiratory syncytial virus-derived epitopes

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J. Gen. Virol. 2004. 85: 2365-2374

Serious labwork in progress

Summary

CD8$^+$ T lymphocytes play a major role in the clearance of respiratory syncytial virus (RSV) infections. To be able to study the primary CTL response in RSV-infected children, epitopes presented by a set of commonly used HLA alleles (HLA-A1, -A3, -B44 and -B51) were searched for. Five epitopes were characterized derived from the matrix (M), non-structural (NS1) and second matrix (M2) proteins of RSV. All epitopes were shown to be processed and presented by RSV-infected antigen-presenting cells. HLA-A1 tetramers for one of these epitopes derived from the M protein were constructed and used to quantify and phenotype the memory CD8$^+$ T cell pool in a panel of healthy adult donors. In about 60% of the donors, CD8$^+$ T cells specific for the M protein could be identified. These cells belonged to the memory T cell subset characterized by expression of CD27 and CD28, and down-regulation of CCR7 and CD45RA. The frequency of tetramer$^+$ cells varied between 0.4 and 3 per 10^4 CD8$^+$ T cells in PBMC of healthy asymptomatic adult donors.

Introduction

Respiratory syncytial virus (RSV) is a pneumovirus in the family *Paramyxoviridae.* Infections with RSV occur in yearly epidemics (1) causing mild colds in immunocompetent adults and in most children. However, about 3% of infants under the age of 1 year develop severe bronchiolitis or pneumonia requiring hospitalization (2). The immunological basis for the different susceptibility to RSV infection, especially in infants, remains unclear. About 50% of children who had RSV bronchiolitis during infancy have subsequent episodes of wheezing in the first decade of life (3-5).

$CD8^+$ T cells play an important role in the host defence during most viral infections. Indeed it was shown that in RSV infections $CD8^+$ T cells are necessary to clear the virus. While healthy children clear RSV in 7-21 days, children with disorders of T cell mediated immunity shed the virus for months (6). Also in murine models, depletion of $CD8^+$ T cells either by using monoclonal antibodies (mAb) or by removing the thymus causes persistent shedding of virus, while transfer of small numbers of $CD8^+$ T cells results in viral clearance within days (7). However, $CD8^+$ T cells can also augment disease in a dose dependent manner (8-11). Thus, a delicate balance exists between effective viral eradication and immune pathology.

Several studies have demonstrated a RSV-specific $CD8^+$ T cell response in infants after primary RSV infection (12;13). In these studies the specific CTL responses were mainly detected in children with mild clinical disease. It was also found that the $CD8^+$ T cell memory response after primary RSV infection might be short lived, as the memory $CD8^+$ T cell frequency in the pre-season of the second year was low. Other groups showed a higher frequency of RSV-specific $CD8^+$ T cells in infants with severe RSV infections compared to those experiencing mild disease (14). As there was a significant difference in age and timing of sampling between the studies, this may account for the differences found.

To study the role of $CD8^+$ T cells in pathogenesis and protection of RSV disease, the identification of CTL epitopes might be of great value. This will allow for the monitoring of the acute and memory CTL cell response in RSV-infected patients and during vaccine trials. So far a few RSV epitopes recognized by human $CD8^+$ T cells have been identified (15-18). However, it would be useful to characterise a broader repertoire of epitopes for highly prevalent HLA alleles. In the present paper we identified 5 new RSV-specific CTL epitopes. Potential HLA class I binding peptides as predicted by the computer selection program SYFPEITHI were tested by stimulating peripheral blood mononuclear cells (PBMC) of healthy adult blood donors and measurement of IFN-γ production by $CD8^+$ T cells using intracellular staining assays. Most donors of different age groups responded to one or more of the RSV epitopes depending on the HLA alleles expressed by each individual. We further showed that all epitopes are also presented on the cell surface of RSV-in-

fected cells. The memory T cells found in these adult donors had down-regulated CD45RA, expressed CD28 and CD27 and were $CCR7^{low}$. The frequency of memory T cells varied between 0.4 and 3 per 10^4 $CD8^+$ T cells.

Participants, materials, and methods

Peripheral blood mononuclear cells (PBMC)

Buffycoats were obtained from HLA-typed healthy adult blood donors who gave informed consent. As all children have been infected with RSV by the age of three and will be re-infected every two to three years thereafter, all adult donors have been primed with RSV. PBMC's were isolated by Ficoll-Paque gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden) and used immediately or stored in liquid N_2.

Virus and peptides

The human RSV-A2 strain (RSV-A2) stock was grown on Hep-2 cells, PEG purified and titrated by plaque assay. Candidate peptides to be tested were selected based upon their HLA binding profile using the computer selection program SYFPEITHI (19). Possible RSV derived CTL epitopes for the HLA-A1, -A3, -B8, -B44 and -B51 alleles were selected. Peptides with a score >24 were synthesised by standard solid-phase Fmoc chemistry. The purity of the peptides varied between 50-90%, as determined by analytical reverse-phase high performance liquid chromatography.

Tetramers

Labeled HLA-B7 tetramers containing the published peptide NPKASLLSL ($NP_{306-314}$) derived from the nucleoprotein (NP) of RSV were purchased from Proimmune (Oxford, UK)(16). The HLA-A1 tetramer containing peptide YLEKESIYY ($M_{229-237}$) was constructed by the CLB (Amsterdam, The Netherlands).

Intracellular staining of peptide-specific T cells for flow cytometry

To identify dominant epitopes presented by MHC class I molecules, we screened candidate peptides for their ability to induce IFN-γ production in $CD8^+$ T cells. PBMC's were cultured in 24 well plates ($1x10^6$/well) in AIM-V medium (Gibco) supplemented with 2% human pooled serum (HPS), penicillin and streptomycin (P/S) and 40 units/ml of recombinant human Interleukin 2 (IL-2). Peptides were added to a final concentration of 1µM. After 10 days of culture, cells were plated in a 96 well round bottom plate (Costar) ($0.5x10^6$ cells/well) in AIM-V medium supplemented with P/S, 2% HPS, 40 units/ml IL-2 and costimulatory antibodies (anti-CD28 and anti-CD49d). Cells were either restimulated with 1µM of the same peptide, 1µg/ml staphylococcal enterotoxin B (SEB) or not restimulated (negative control). After one hour of incubation at 37ºC 10µg/ml of Brefeldin A (BFA) (BD

PharMingen) was added to accumulate cytokines in the cell. After 5 hours, 2mM of EDTA was added in order to arrest activation and to remove adherent cells from the well. After incubating 20 minutes at room temperature, cells were washed twice in FACS buffer (PBS containing 0.02% azide, 2% FCS and 2 mM EDTA). Then phycoerythrin (PE)-labeled anti-CD3 mAb (BD-PharMingen) and Cy5-labeled anti-CD8 mAb (BD-PharMingen) for surface staining were added. After 30 minutes of incubation on ice, cells were washed twice in ice cold FACS buffer. Cells were permeabilized and fixated using FACS permeabilizing/fixation solution (perm/Fix) (BD-PharMingen). Cells were stained intra-cellularly after an additional wash in Perm-wash (BD-PharMingen) with fluorescein isothiocyanate (FITC)-labeled IFN-γ mAb (BD-PharMingen clone 340449) for 30 minutes on ice. Cells were washed three times in perm-wash and fixated in 1% paraformaldehyde/PBS for 20 minutes. Cells were resuspended in FACS buffer and kept at 4ºC until analysis. Cell staining was analysed on a FACS-Calibur using CellQuest software (BD Bioscience, Mountain View, CA). A response was considered to be positive when the number of $CD8^+$ T cells producing IFN-γ upon antigen stimulation was at least three times higher than the number of cells producing IFN-γ without restimulation with antigen.

We confirmed that the epitopes that were identified were indeed presented in the context of the HLA alleles used in the epitope prediction programme SYFPEITHI. To this purpose we stimulated cells of two donors that were respectively HLA-A3, A11, B51, B35 (donor 8) and HLA-A2, -B44, -C5 (donor 9) with the epitopes $M2_{151-159}$ (predicted to be HLA-A3 restricted), $M2_{64-72}$ (B44), $NS1_{41-49}$ (B51) and $M_{195-203}$ (B51). Ten days after peptide stimulation the responder T cell cultures were restimulated with APC that only shared the HLA allele used for epitope prediction, or with APC lacking this HLA allele but instead sharing some other class I molecules with the responder cells. APC used were PBMC that were depleted of CD3 cells using MACS separation (Miltenyi Biotec, Germany). The APC were loaded with the peptide epitopes for one hour in medium without serum, whereafter unbound peptides were removed by extensive washing. Unloaded antigen presenting cells served as negative controls. IFN-γ production by responder T cells was measured after 5 hrs by intracellular staining.

Presentation of the dominant epitopes during RSV infection

PBMC were infected with RSV at a m.o.i. of 5 and cultured in 24 well plates ($1x10^6$/well) in AIM-V medium with 2% (HPS), (P/S) and 40 units/ml of recombinant human IL-2. After 10 days of proliferation, cells were either stimulated with the appropriate peptide or not restimulated (negative control). Cells stimulated twice at day 0 and 10 with the same peptide served as a positive control. The HLA-A1 tetramer was used to identify $M_{229-237}$ specific T cells 10 days after infection of PBMC's with RSV. Extra-cellular staining of $M_{229-237}$ $tetramer^+$ cells with anti-CD3 and anti-CD8 was performed as described in the previous section.

To identify the response against whole RSV virus in previously stimulated PBMC we infected PBMC with RSV at a m.o.i. of 5. Cells were cultured as described above. On day 8 PBMC of the same donor were depleted for $CD8^+$ T cells using negative selection MACS separation columns (Miltenyi Biotec, Germany). The remaining cells were infected with RSV at a m.o.i. of 5 two days prior to use, thus allowing the APC to present RSV epitopes. On day 10 these APC were added to the PBMC that were stimulated with RSV on day 0. Stimulation was allowed for 5 hours in

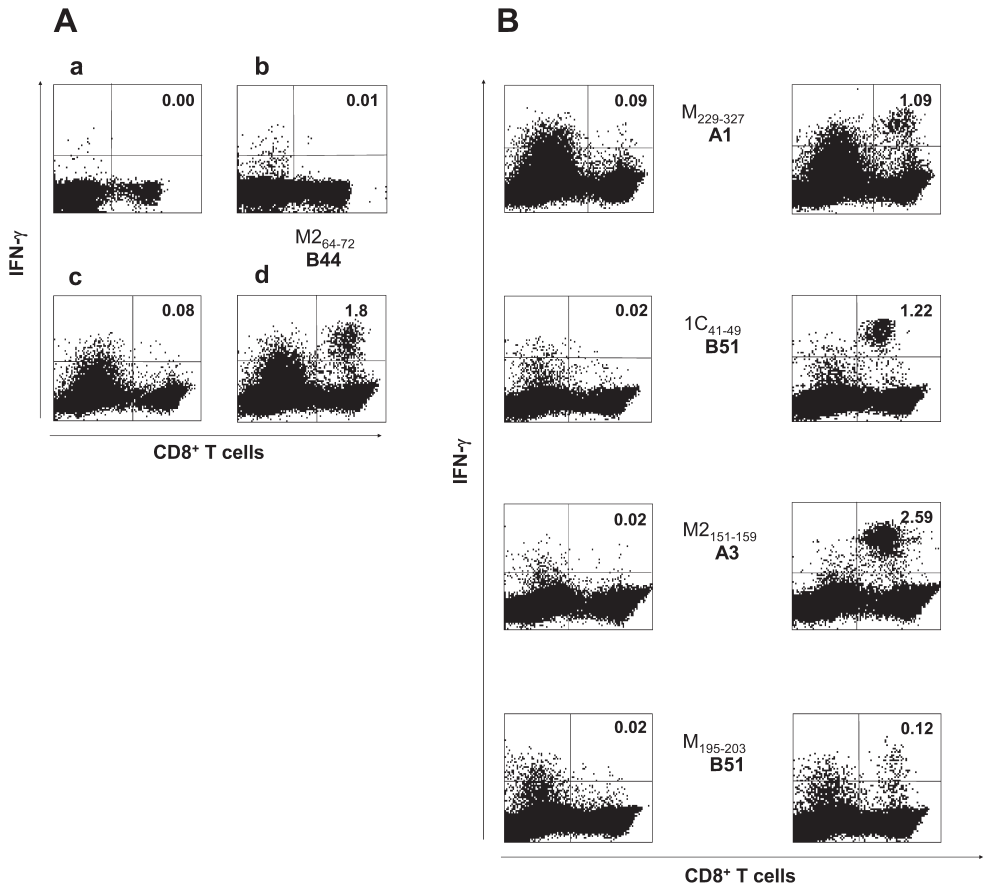

Figure 1a: *Frequency of IFN-γ producing $CD8^+$ T cells after stimulation of PBMC with the $M2_{64-72}$ -peptide for different time periods. Intracellular IFN-γ production was measured directly after 5 hours (a: no peptide added and* ***b:*** *1μM peptide $M2_{64-72}$) or, in cultures grown for 10 days with peptide $M2_{64-72}$ and IL-2, after a short restimulation (5hrs) with the same peptide (**d**). The negative control for the 10 day time point was stimulated on the first day with the $M2_{64-72}$-peptide, but not restimulated on day 10 (**c**).*
Figure 1b: *Frequency of IFN-γ producing $CD8^+$ T cells after stimulation with different peptides. PBMC were stimulated with the selected peptides on day 0 and further cultured in IL-2 for 10 days. Cells were either restimulated on day 10 (right column) or not (left column). In both Figure **1a** and **1b**, $CD3^+$ cells were gated in a lymphocyte/lymphoblast gate. The percentage of IFN-γ producing $CD8^+$ T cells in this gated population is given in the upper right quadrant of the figures.*

the presence of Brefeldin-A. Intra- and extra-cellular staining was performed as described before.

Phenotyping of tetramer$^+$ T cells
PBMC's of HLA-A1 positive donors were washed in FACS buffer and blocked in blocking buffer (FACS buffer containing 10% HPS) for 20 minutes on ice. Cells were stained with 5µl of allophycocyanin (APC)-labelled HLA-A1 tetramer containing the $M_{229-237}$ peptide. After 20 minutes of incubation at room temperature, cells were stained with different extracellular mAbs (anti-CD8-PerCP, -CD45RA-PE, -CD27-FITC, -CD28-FITC, -CCR7-PE (BD-PharMingen)). An additional 20 minutes later, cells were washed twice in FACS buffer and immediately used for FACS analysis.

Results

Selection of peptides containing HLA binding motifs using the SYFPEITHI prediction program
We used the SYFPEITHI prediction program ((19), *http://www.uni-tuebingen.de/uni/kxi*) to select possible RSV derived CTL epitopes for 5 different HLA alleles: HLA-A1, -A3, -B8, -B44 and -B51. This program predicts the probability for peptides to be presented by certain HLA class I molecules, based on the presence of HLA binding motifs. HLA alleles were selected based on a high prevalence in the Caucasian population. The 8 highest scoring peptides for each HLA allele were tested for their capability to stimulate IFN-γ production by CD8$^+$ T cells in PBMC samples from healthy adult blood donors. We found that the frequency of RSV-specific CD8$^+$ T cells was too low in most donors to measure a significant direct response upon short (5 hours) peptide stimulation when using intracellular staining for IFN-γ (Figure 1a-b). Therefore, we cultured the PBMC's for different time periods with the peptides and determined that the best response was found after allowing the peptide-specific T cells to proliferate for 10 days in the presence of recombinant interleukin-2 (IL-2) (Figure 1a-d, 1b and Table I). As the IFN-γ production of CD8$^+$ T cells will have ceased 10 days after initial peptide stimulation, cells were restimulated at this time with the same peptide for 5 hours before measuring IFN-γ production. PBMC's that were stimulated on day 0 but not restimulated on day 10 served as the negative control (Figure 1a-c). Except for HLA-B8 at least one epitope was identified per HLA class I allele using this procedure (Figure 1a, b and Table I). For the HLA-B51 allele a second less dominant epitope was also identified.

All the functional epitopes were screened in at least three donors with different HLA types. The magnitude of responses between different donors varied, as well as the dominance of the response against the different epitopes. However, for all

Table I: Peptide specific CD8$^+$ T cell responses in PBMC cultures

Peptide	Sequence	HLA[1]	Score[2]	HLA type donor	Response[3]
$M_{229-237}$	YLEKESIYY	A1	32	D1:A1,A3,B7,B37	0.02-0.21
				D2:A1,A2,B44,B57	0.07-1.14
				D3:A1,A29,B44,B57	0.13-1.16
				D4:A1,A32,B7,B35	0.03-0.59
				D5: A1,A11,B8,B51	0.08-20.19
$M2_{151-159}$	RLPADVLKK	A3	29	D1:A1,A3,B7,B37	No response
				D6:A1,A3,B7,B51	0.04-0.12
				D7:A3,A31,B7	0.07-2.0
				D8:A3,A11,B35,B51	0.09-2.38
$NP_{306-314}$[4]	NPKASLLSL	B7	23	D1:A1,A3,B7,B37	0.24-0.46
				D4:A1,A32,B7,B35	0.03-0.61
$M2_{64-72}$	AELDRTEEY	B44	27	D9:A2,B44	0.20-1.65
				D2:A1,A2,B44,B57	0.06-1.65
				D3:A1,A29,B44,B57	0.20-1.45
				D10:A2,B44	0.24-2.20
				D6:A1,A3,B7,B51	No response
$NS1_{41-49}$	LAKAVIHTI	B51	28	D8:A3,A11,B35,B51	0.04-1.20
				D5:A1,A11,B8,B51	0.05-8.30
				D11:A2,A25,B51,B18	0.03-0.26
				D6:A1,A3,B7,B51	0.04-0.14
$M_{195-203}$	IPYSGLLLV	B51	26	D8:A3,A11,B51,B35	0.02-0.12
				D5:A1,A11,B8,B51	0.07-1.30
				D11:A2,A25,B51,B18	0.04-0.17
				D6:A1,A3,B7,B51	No response

[1]HLA allele used for peptide prediction.
[2]prediction score in SYFPEITHI for binding to HLA allele (maximum score 32).
[3]IFN-γ response of PBMC stimulated with peptide on day 0 and subsequently cultured for ten days with IL-2. The percentage of IFN-γ positive CD8$^+$ T cells at day 10 was compared for samples that received no second stimulation at day 10 (first number) and samples that were restimulated at this time point for 5 h with the peptide (second number).
[4]peptide described by Goulder et al. (16).

peptides it was found that all donors responding to a peptide expressed the HLA molecules used for epitope predictions, while other HLA alleles were not shared in all responding donors (Table I). These experiments strongly suggested that the dominant epitope was specifically presented by the predicted MHC allele.

HLA restriction of RSV derived epitopes

We next set out to affirm that the HLA molecules used for epitope predictions were indeed the restriction elements for the 5 newly identified epitopes**.** To address this issue, we measured T cell responses when the peptides were presented by APC that only shared the HLA allele used for epitope prediction with the T cells. APC that had other HLA molecules in common with the responder T cells, but lacked

the expression of the HLA molecules used for epitope predictions, were used as a control. We only found a positive response when the HLA molecule that was used for epitope prediction was present on the APC (Figure 2). This procedure unequivocally proved that peptide $M2_{151-159}$ was presented by HLA-A3, peptide $M2_{64-72}$ was presented by HLA-B44, and that peptide $NS1_{41-49}$ and $M_{195-203}$ were presented by HLA-B51. For HLA-A1 we prepared a tetramer containing $M_{229-237}$. This tetramer recognized an expanded population of $CD8^+$ T cells in PBMC cultures that had been cultured with the $M_{229-237}$ peptide and IL-2 for ten days (Figure 3-a). This $tetramer^+$ population was not present in PBMC that were not stimulated with the peptide or in peptide stimulated PBMC cultures of a donor that lacked the HLA-A1 molecule. Thus this result confirmed that HLA-A1 was the molecule that presented $M_{229-237}$.

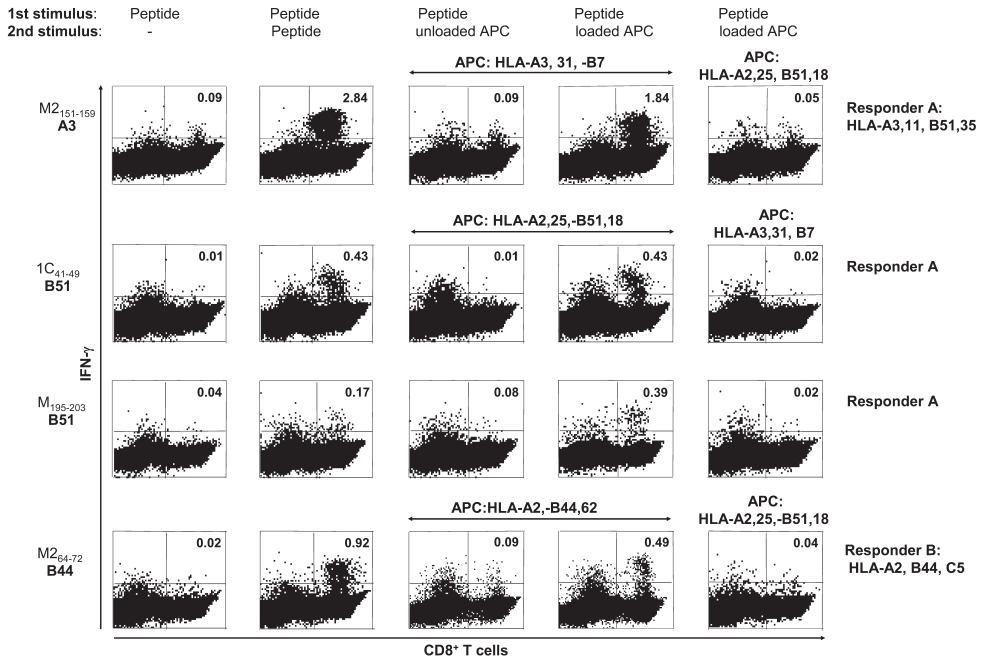

Figure 2: *The epitopes are recognized by T cells in the context of the HLA alleles used for epitope prediction. Responder PBMC were cultured with peptide $M2_{151-159}$ (HLA-A3), $NS1_{41-49}$ (HLA-B51), $M_{195-203}$ (HLA-B51) or $M2_{64-72}$ (HLA-B44) for 10 days in the presence of IL-2. PBMC partly HLA matched with responder cells were depleted for $CD3^+$ T cells and loaded with the 4 different peptides as indicated in the figures. Antigen presenting cells that were positive for the HLA molecule that was used for peptide predictions were also used unloaded as negative control. Responder cells were stimulated with the peptide-pulsed antigen presenting cells for 5 hrs after which intracellular IFN-γ staining was performed. PBMC cultures that were not restimulated served as a negative control (left column) while PBMC cultures to which the peptides were directly added served as a positive control (2^{nd} column from the left). $CD3^+$ cells were gated in a lymphocyte/lymphoblast gate. The percentage of IFN-γ producing $CD8^+$ T cells in this gated population is given in the upper right quadrant of the figures.*

All epitopes identified were presented on cells infected with RSV

When synthetic peptides are used to activate T cells, results should be treated with caution because a positive response does not indisputably prove that the particular peptide of the virus was originally responsible for the induction of the memory T cells that are found in the donor. Similar results may have been obtained if the peptide happened to elicit a cross reactive T cell population that originated from

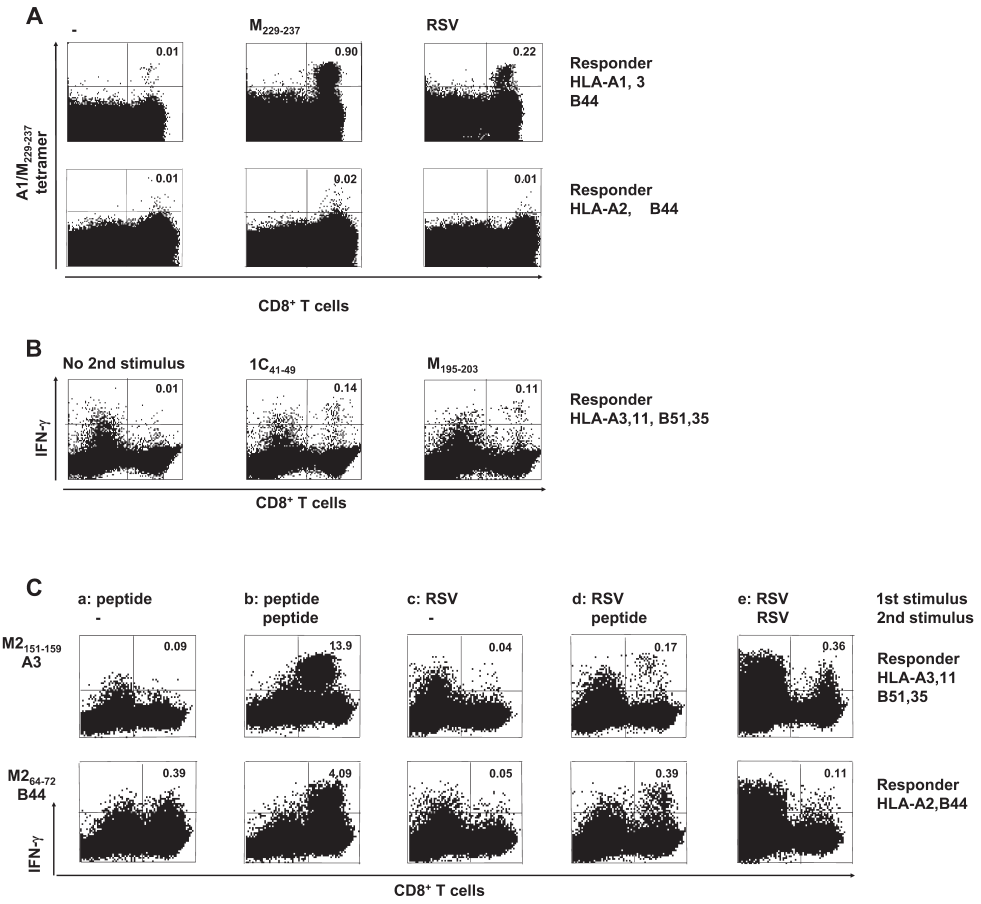

Figure 3: *The dominant epitopes are presented on cells infected with RSV. (**a**) PBMC of an HLA-A1 positive donor were stimulated with RSV m.o.i. 5, peptide $M_{229-237}$ (HLA-A1) or left unstimulated and cultured in the presence of IL-2. After culturing for 10 days peptide-specific cells were identified by extracellular staining with the $M_{229-237}$ containing tetramer. An HLA-A1 negative donor served as a negative control. (**b**) PBMC of donor 8 (HLA-A3, 11, B51, 35) were infected with RSV m.o.i. 5 at day 0 and cultured with IL-2 for 10 days. On day 10 the cells were either not restimulated or restimulated with the peptide $NS1_{41-49}$ (HLA-B51) or $M_{195-203}$ (HLA-B51). Cells were intracellular stained for IFN-γ production. (**c**) PBMC of 2 different donors were stimulated with peptide $M2_{151-159}$ or peptide $M2_{64-72}$ or RSV m.o.i. 5 at day 0 and expanded in the presence of IL-2. On day 10, cells were either not restimulated (1 and 3), restimulated with peptide (2 and 4) or restimulated with autologous APC infected with RSV (5). Cells were intracellular stained for IFN-γ production. In all parts of the Figure $CD3^+$ cells were gated in a lymphocyte/lymphoblast gate. The percentage of IFN-γ producing $CD8^+$ T cells is given in the upper right quadrant of the figures.*

a response against a different (viral) antigen. Therefore, we checked whether the dominant epitopes were processed and presented by antigen presenting cells after infection with RSV. In PBMC cultures grown for ten days with live RSV $CD8^+$ T cells recognized by the HLA-A1/ $M_{229-237}$ tetramer were expanded (Figure 3-a). Thus, the HLA-A1 restricted peptide was indeed processed and presented by antigen presenting cells exposed to the virus. The specificity of the response measured by tetramer staining is confirmed by the lack of staining in PBMC of a HLA-A1 negative donor cultured with RSV.

To prove that also the other peptides were presented on virus infected cells PBMC cultured for 10 days with RSV were restimulated with the dominant peptides or left unstimulated at this time point. Intra-cellular IFN-γ production was measured after 5 hours of incubation with peptides. In RSV-infected cultures a clear response could be detected upon restimulation with peptide, although the magnitude of the response was lower than in cultures that where stimulated both at day 0 and day 10 with the peptide (Figure 3-b,c).

The lower epitope specific T cell response in PBMC cultures stimulated with RSV is not due to interference of other dominant epitopes

The observation that the T cell response against a particular epitope is higher in cultures stimulated with the peptide compared to cultures stimulated with whole virus can be explained in several ways. One explanation may be that, within the context of the total repertoire of peptides displayed on APC exposed to whole virus, the T cell response against some epitopes might be masking the response against others. However, when RSV-infected PBMC cultures were restimulated with APC infected with RSV, the T cell response detected was still lower than the response measured upon restimulation with the peptide (Figure 3). Therefore, we concluded that in RSV-infected PBMC cultures T cell activation is less efficient compared to T cell activation induced by stimulation with peptides. Hence, a lower epitope specific T cell response after RSV infection did not appear to reflect subdominance of the epitopes that were identified in this study.

RSV-specific responses can be detected in 50-60% of randomly chosen healthy adults

The donors that were used for the experiments in Figure 1 and Table I had been used before to identify epitopes recognized by RSV-specific $CD4^+$ T cells. They were selected from a slightly larger panel because they had readily detectable RSV-specific $CD4^+$ T cell responses. We found in this pre-selected panel in almost every donor a response against one of the five epitopes that we have identified. To establish whether in a panel of randomly chosen healthy adults responses against the newly identified HLA-A1 epitope were a general event, we performed an experiment in which PBMC of 13 additional donors were stimulated with the A1 peptide and cultured for ten days with recombinant IL-2. At day ten expanded $CD8^+$ T cells specific for the $M_{229-237}$ peptide were visualised by tetramer staining. By this procedure we could detect responses in seven out of thirteen HLA-A1 posi-

tive donors (Table II). Similarly, we found a response against $NP_{306-314}$ in four out of six HLA-B7 positive donors.

The frequency and phenotype of RSV-specific memory CD8$^+$ T cells

The frequency of RSV-specific memory CD8$^+$ T cells in PBMC was low. It was necessary to culture PBMC with antigen in vitro in order to detect RSV-specific CTL responses using intracellular staining assays. To determine the frequency of CD8$^+$ memory T cells we performed direct tetramer staining experiments using PBMC of 5 healthy HLA-A1 positive donors. By this procedure we determined that the frequency for the HLA-A1 epitope $M_{229-237}$ varied between 0.4 and 3 per 10^4 CD8$^+$ T cells. γAll tetramer$^+$ cells were CD45RA dull, CD27 positive, CD28 positive and about 80% were CCR7 negative, thus resembling an extra lymphoid memory T

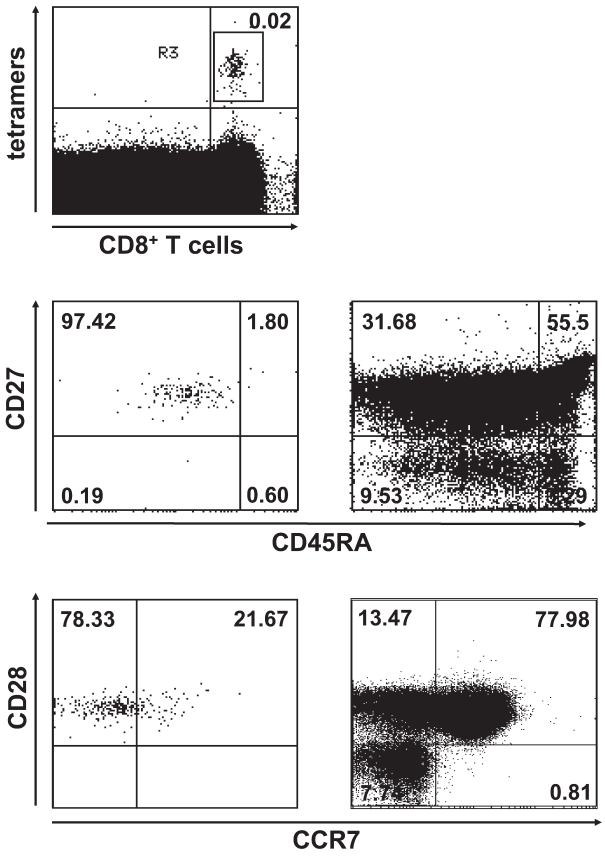

Figure 4: *Frequency and phenotype of RSV-specific CD8$^+$ T cells. PBMC of healthy HLA-A1 positive adults were extra-cellular stained for tetramer HLA-A1/$M_{229-237}$, CD8 and a combination of differentiation markers. The position of the quadrant was determined on a live gate (right figures), while the differentiation of tetramer specific cells was determined on a live tetramer$^+$ gate (left figures). This experiment was repeated in 5 HLA-A1 positive donors.*

Table II: CD8$^+$ T cell responses against peptides $M_{229-237}$ and $NP_{306-314}$ in PBMC of randomly chosen healthy adult donors

Donor	% tetramer^{+ve}/CD8^{+ve}
HLA-A1	
d13	4.01[1]
d14	0.40
d15	0.98
d16	0.09
d17	0.04
d18	2.80
d19	0.06
d20	<0.01
d21	<0.01
d22	<0.01
d23	<0.01
d24	<0.01
d25	<0.01
HLA-B7	
d26	1.11
d27	0.29
d28	0.08
d29	0.16
d30	<0.01
d31	<0.01

[1]Percentage of proliferating tetramer$^+$ cells of total CD8$^+$ T cells after 10 days of stimulation with peptide and IL-2.

cell phenotype. A representative example of the experiments performed is shown in Figure 4.

Discussion

In the present paper we report the identification of 5 novel RSV-derived epitopes that are presented on the surface of RSV-infected cells by HLA class I molecules, A1, A3, B44, B51 (2x). These epitopes are recognized by CD8$^+$ memory T cells present in peripheral blood of healthy adult blood donors. We performed this identification focussing on some of the most prevalent HLA alleles to ensure that the information obtained would be applicable in a large population, in future studies aimed at unraveling the mechanism of the development of RSV-specific primary and memory immune responses. To our knowledge five RSV-derived epitopes that are recognized by human CD8$^+$ T cells have been described before. The first epi-

tope that was identified was HLA-B7 restricted. This epitope was found by testing a series of overlapping peptides derived from the nucleoprotein of RSV in elispot assays using PBMC of healthy adults (16). Using a similar procedure a HLA-B8 restricted epitope in the N protein and a HLA-A1 restricted epitope in the F protein were identified (17;18). Two additional epitopes, derived from the F protein were found to be presented by HLA-B57 and HLA-C12, relatively uncommon HLA alleles (15). These latter epitopes were recognized by T cell clones, that were cultured from PBMC of RSV-infected children. Our newly defined epitopes thus add to this repertoire two epitopes derived from the matrix protein, two derived from the M2 protein and one from the NS1 protein. Interestingly, an early study using vaccinia recombinants claimed that human T cell responses directed against the RSV N, SH, F, M, M2 and NS1 molecules are most prevalent (20). The responses against the NS1 and P proteins were each found in one donor out of the panel of nine donors tested. However, the HLA type of donors was not revealed in this paper. Hence, we do not know whether the HLA molecules for which we determined the epitopes in the present paper were expressed in this donor population, and if so how frequent.

The epitope prediction program SYFPEITHI proved very useful to select candidate epitopes recognized by $CD8^+$ T cells. Only for HLA-B8 we did not find a new peptide among the top eight of predicted peptides. Indeed the HLA-B8 restricted nonamer epitope $N_{255-263}$ that was described in the literature by Venter et al. had a SYFPEITHI score of 19 (maximum score for HLA-B8 is 33), ranking this peptide number 74 (Venter *et al*., 2003). Of the peptides that we identified in the present paper three had the highest score in the SYFPEITHI programme ($M_{229-237}$/HLA-A1; $M2_{64-72}$/HLA-B44; $NS1_{41-49}$/HLA-B51). The other two peptides were among the seven highest ($M2_{151-159}$/HLA-A3) and four highest scoring peptides ($M_{195-203}$/HLA-B51). The SYFPEITHI score for the $NP_{306-314}$ peptide described by Goulder et al. was 23, the maximum score for HLA-B7, which was shared by three other peptides (16). However, in our experiments only the $NP_{306-314}$ peptide induced IFN-γ production in $CD8^+$ memory T cells (data not shown). Rock et al characterized a decamer epitope $F_{109-118}$ in the F protein of RSV (17). A shorter nonamer version of this peptide could also induce a CD8T cell response but was somewhat less effective. The SYFPEITHI scores for these peptides were respectively 4 and 22 ranking the nonamer peptide number 19. By our selection method we would have overlooked these epitopes. In conclusion, the SYFPEITHI programme is a useful tool to predect candidate CD8T cell epitopes. However, not all epitopes will be succesfully predicted.

We found that memory CD8T cells specific for RSV epitopes are present in low numbers in healthy adult donor blood. In order to detect CD8T cell responses by measuring the production of interferon-γ with intracellular staining, an in vitro expansion of CD8T cells was necessary to measure responses above the detection limit of the assay. In Table II we show the results of this type of experiment performed in a small set of randomly chosen HLA-A1 and HLA-B7 donors. In about

sixty percent of the donors we found a response against the $M_{229-237}$ and $NP_{306-314}$ peptides, meaning that responses against these epitopes are probably frequently occurring events in the population. Some of the PBMC used in these experiments were isolated from donor blood sampled outside the RSV season, for some other donors during the RSV season. However, at this point we have not found a direct relation between the level of the responses and the time the blood samples were taken. To answer the question whether there is a difference in the magnitude of the T cell response during and outside the RSV season one needs to study RSV-specific T cell responses in one patient at the different time points, rather than comparing responses of different individuals. Therefore, we are addressing this issue more in depth in a study that is currently underway in our laboratory, whereby the $CD4^+$ and $CD8^+$ T cell responses against RSV are monitored at different time points during and outside the RSV season in a panel of healthy adult donors.

We directly compared the magnitude of the CD8T cell response against the published HLA-B7 epitope with the magnitude of the response of the HLA-A1 restricted epitope from the matrix protein of RSV described in the present paper. When tested in donors that express both HLA-A1 and HLA-B7, peptide-specific CD8responses against both peptides were comparable. Moreover, the number of tetramer specific T cells we found was similar as described by Goulder et al., who first used the $B7/NP_{306-314}$ tetramer (16).

In donors that expressed more than one of the HLA alleles presenting known epitopes, T cell responses against two or more epitopes could often be detected. However, we also found donors that carried the right HLA restriction elements who did not respond against the newly identified epitopes. It may be that in these donors the RSV-specific T cell frequency was too low to detect a response even after expansion of CD8T cells. Whether these donors were low responders for RSV, or whether other dominant epitopes were recognized, is not known. This issue was not further pursued, because of the inefficient expansion of $CD8^+$ T cells in PBMC cultures exposed to intact RSV. We showed that in cultures that had been stimulated with the intact virus $CD8^+$ T cells could be expanded that recognized the newly identified epitopes. However, it appeared that $CD8^+$ T cell expansion by culturing PBMC with virus is less efficient than the T cell expansion obtained with synthetic epitopes. Thus, from these experiments we could not make a reliable estimation of the contribution of the T cells that recognized the single epitopes to the total memory T cell response.

Several factors might explain the sub-optimal expansion of T cells after culturing PBMC with intact virus. First, RSV only efficiently enters monocytes when PBMC are exposed to the virus, while synthetic peptides will be presented on all MHC class I positive cells. Thus the number of APC expressing peptide MHC complexes will be much higher when peptides are added to the PBMC cultures. Second, the number of MHC peptide complexes per cell might be higher on peptide loaded cells than after intracellular processing of viral proteins. Third, RSV is known to

have a suppressive effect on T cell proliferation (21;22). Thus, the presence of live virus might be responsible for an inefficient expansion of CD8T cells in the cultures. This might result in an underestimation of the total numbers of CD8T cells that are specific for the virus. A fourth factor might be the presence of multiple viral epitopes presented on APC after infection with virus. T cells specific for the different epitopes thus should have to compete for the interaction with the APC and/or different epitopes might have to compete for binding to a certain MHC allele. However, these latter phenomena are less likely to have contributed to the lower peptide-specific CD8T cell responses that we observed within RSV stimulated PBMC cultures. We found that the response was also low against virus infected target cells, i.e cells expressing the same epitope repertoire to which the T cells reacted initially (Figure 3-c).

The use of MHC tetramers has provided the technical means to phenotype $CD8^+$ T cells during different stages of the immune response. Thus, phenotypic information has been obtained during primary responses and in the memory stage of the immune response against different chronic virus infections, HCMV, EBV, HCV and HIV (23-27). In the late chronic stage of infection $CD8^+$ T cells specific for these viruses differ significantly with respect to the surface expression of CD27 and CD28 molecules, that were used as markers to type the maturation stage of the memory T cells. In this study we showed that RSV-specific $CD8^+$ T cells are CD45RA dull, CD27 positive, CD28 positive and CCR7 negative. According to the expression of these markers the phenotype of RSV-specific cells resembles that of influenzavirus A, EBV and HCV specific $CD8^+$ memory T cells (23;27;28). All donors analyzed for the frequency of RSV-specific $CD8^+$ T cells directly *ex vivo*, were healthy and showed no signs of respiratory infections. Blood was sampled outside the RSV season. These factors indicate that the RSV-specific $CD8^+$ cells in the peripheral blood are "resting" memory cells. Overall, these observations suggest that healthy adult donors indeed have an antiviral immunity to RSV, characterized by the presence of low frequency IFN-γ producing CD8, $CD45RA^-$, CD28, CD27 and $CCR7^-$ T cells.

In conclusion, we identified 5 new human RSV derived $CD8^+$ T cells epitopes that were HLA-A1, -A3, -B44 or -B51 restricted, a significant addition to formerly published epitopes because they are presented by HLA-alleles that are highly prevalent in the Caucasian population. The knowledge of antigenic epitopes will allow us to address the role of RSV-specific $CD8^+$ T cells in the delicate balance of controlling infection and the possible role these cells may have in immune-mediated pathology during primary RSV infection.

Acknowledgements

We thank Rein Zaat from Bloodbank Utrecht for providing us with buffycoats (research project B95.004x) and Prof. R.A. van Lier and Dr. R.G van der Most for critical reading of the manuscript. We thank Dr. H.G. Otten for HLA-typing of one of the blood donors.

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CD8$^+$ T cell responses in bronchoalveolar lavage fluid and PBMC of infants with severe primary respiratory syncytial virus infections

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Manuscript submitted

Smiling again after recovering from a severe RSV-infection

Summary

A protective role for $CD8^+$ T cells during viral infections is generally accepted, yet little is known about how $CD8^+$ T cell responses develop during primary infections in infants, their effectivity, and how memory is established after viral clearance. We studied $CD8^+$ T cell responses in bronchoalveolar lavage samples and blood of infants with a severe primary RSV infection. RSV-specific $CD8^+$ T cells with a highly activated effector cell phenotype: $CD27^+CD28^+CD45RO^+CCR7^-CD38^+HLA\text{-}DR^+$granzyme-$B^+CD127^-$ could be identified in BAL and blood. A high proportion of RSV-specific $CD8^+$ T cells proliferated and functionally responded upon cognate peptide stimulation. The percentage of $CD8^+$ T cells specific for single RSV epitopes during acute infections was about ten-fold higher compared to the average levels of epitope specific memory $CD8^+$ T cells in disease free adults, and further increased in BAL during convalescence. RSV-specific T cell numbers peaked in blood around day 9-12 after the onset of primary symptoms, which coincided with the total peak of activated effector $CD8^+$ T cells in blood. A substantial fraction of these activated $CD8^+$ T cells was specific for different viral epitopes than those used in the HLA-tetramers. Thus, despite the very young age of the patients a robust virus-specific $CD8^+$ T cell response was elicited against a localized respiratory infection.

Introduction

Respiratory syncytial virus (RSV) is the most common cause of severe lower respiratory tract infections in infants. 50-70% of all children becomes infected during the first year of life, mostly resulting in symptoms of mild upper respiratory tract infection. However, a considerable proportion of infants develops lower respiratory tract disease and 1-3% of the entire birth cohort requires hospitalization because of respiratory distress and feeding problems (1;2). Of these hospitalized children about 10% needs mechanical ventilation (3;4). Morbidity is highest during the first six months of life and in infants with chronic lung disease of prematurity, congenital heart disorders, immune system disorders and in infants that were born preterm.

Since the implementation of non-bronchoscopic bronchoalveolar lavage (NB-BAL) in very young infants, several groups have studied the cellular immune response to primary RSV infection in ventilated patients (5-7). It has consistently been shown that neutrophils are the predominant cells in NB-BAL, accounting for up to 85% of the total cell count, and that the total number of lymphocytes is low (0.1 to 9%). Interestingly, postmortem studies of children who died from RSV infection show a lymphocytic peribroncheolar infiltrate in the lungs (8).

The role of $CD8^+$ T cells, usually appreciated as a crucial component of an antiviral immune response, has not been extensively addressed during human RSV infections. Studies in mouse models and in patients with deficiencies in T cell responses provided evidence for an important role for $CD8^+$ T cells in clearance of the virus (9;10). However, it has been shown in the mouse model that they can also result in immunepathology (11-13). Indications for immune mediated pathology in humans were also found during a vaccine trial in the 1960s, in which infants were vaccinated with formalin-inactivated whole cell virus. Exposure of vaccinated infants to natural RSV infection resulted in enhanced morbidity and mortality (14;15). In previous studies several groups have described the presence of RSV-specific T cells in peripheral blood and/or nasal washes of RSV-infected infants (16-20). However, these studies used in vitro expansion protocols to be able to enumerate and functionally test virus-specific T cells. Moreover, to our knowledge no information besides differential cell counts is available on T cells present in the airways during RSV infections in infants. With the introduction of HLA-class I tetrameric complexes a method of direct detection of virus-specific cells was introduced. The recent characterization by our group (21) and others (22-25) of several dominant RSV T cell epitopes provided the necessary tools to study the role of $CD8^+$ T cells during RSV infection.

In the present study we describe our investigation into the dynamics and function of the primary $CD8^+$ T cell response in the airways and peripheral blood during a localized severe respiratory infection.

Participants, materials, and methods

Study population and sample collection

Thirty-two infants admitted to the pediatric intensive care unit of the Wilhelmina Children's Hospital were included during the winter seasons of 2002-2006. They all required mechanical ventilation because of respiratory failure due to RSV lower respiratory tract infection. RSV status was confirmed by indirect immunofluorescence or PCR on nasopharyngeal aspirates. Excluded were children above 52 weeks of age, and infants with known immune deficiencies.

The control group consisted of 18 infants that underwent surgery for non-respiratory pathology. The control group in which we determined tetramer specificity was born after the RSV season and was included before the next season had started. We also studied the presence of RSV-specific T cells in the tracheostomy aspirate of two children respectively one and three months after they had been admitted for a proven primary RSV infection. Sputum was obtained by direct suction through the tracheostomy opening.

From every infant 1.5 ml of heparinized blood was drawn for HLA-typing. From 4 children blood was drawn at 3-4 different time points after intubation: directly after inclusion, a time point in between, on release from the intensive care and during the convalescence phase on the visit to the outpatient's clinic. From 3 other patients 3ml of blood was drawn on release from the intensive care. Nonbronchoscopic bronchoalveolar lavage (NB-BAL) was performed as described previously (6). In patients NB-BAL was performed prior tot routine bronchial toilet as soon as the clinical situation allowed for it, just before extubation and at a time point in between. In controls NB-BAL was performed within 5 minutes after intubation. Samples were directly placed on ice. Specimens were filtered through 70μm cell strainers (Falcon, BD) to remove mucus. No dithiothreitol was used to dissolve the mucus in order to avoid possible denaturation of cell surface proteins involved in T cell - target cell interaction. Cells were immediately used for FACS staining. Parents of patients and controls gave their informed written consent. The study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht.

Direct HLA-typing of the patients

To be able to perform HLA-tetramer staining of fresh BAL samples, immediate limited HLA-typing was performed. Therefore, 50µl of whole blood was stained with a panel of HLA specific antibodies (IHB-Hu-037 for HLA-A1/A36, and IHB-Hu-035 for HLA-B7 kindly supplied by Dr. A. Mulder, LUMC Leiden, The Netherlands). After 30 minutes of incubation at room temperature cells were labeled with FITC-labelled Fab fragments (IgG1 for IHB-Hu-035 and IgMκ for IHB-Hu-037, DAKO F0315 and F0317). After another 30 minutes of incubation at room temperature cells were washed in FACS-buffer (PBS containing 2% Fetal Calf Serum (FCS), 0.1% sodiumazide and 2mM EDTA). Erythrocytes were lysed using lysis buffer (BD

Bioscience) during 15 min. Cells were washed twice in FACS buffer and analyzed by FACS Calibur flow cytometer and CellQuest software (BD Biosciences). The HLA type of the patients was confirmed in a later stage by PCR-SSP using cultured EBV B cell lines of the patients. PCR-SSP was performed according to the instructions of the manufacturers (Biotest, Dreieich, Germany).

Phenotyping of CD8⁺ T cells and RSV-specific T cells

To maximize cell viability, BAL samples were processed directly after sampling. PBMC's were isolated by Ficoll-Paque gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden) stored in liquid nitrogen and stained at the same time after all samples of one patient were collected. Cells were washed in FACS buffer and blocked in FACS buffer containing 10% pooled human AB serum (HPS) for 30 minutes on ice. Cells were stained with 10µl of the appropriate HLA-A1, or -B7 tetramer. The allophycocyanin (APC)-conjugated HLA-B7 tetramer containing the peptide NPKASLLSL ($NP_{306-314}$) and the HLA-A1 tetramer containing peptide YLEKESIYY ($M_{229-237}$) were purchased from Sanquin (Amsterdam, The Netherlands).

After 20 minutes incubation at room temperature, cells were washed and further stained with different labelled extra-cellular mAbs. FITC-labelled anti-CD3, -CD4, -CD8, -CD14, -CD16, -CD19, -CD25, -CD27, -CD45RA, -CD45RO, -CD69, PE-labeled anti-CD3, -CD4, -CD8, -CD14, -CD27, -CD28, -CD38, -CD45RO, -CD56, -CD69, -CD127, -HLA-DR, -CCR7 and APC-labelled anti-CD3, -CD8, -CD28 were all purchased from BD-PharMingen (San Jose, California) except CD8-FITC (Sanquin, Amsterdam, specifically used in combination with tetramers) and CD127-PE (Immunotech, Marseille, France). For intracellular staining cells were permeabilized and fixed using FACS permeabilizing/fixation solution (perm/Fix) (BD-PharMingen). Cells were stained intra-cellularly with FITC-labelled anti Ki-67, PE-labelled anti-GranzymeB or their isotype control (BD-PharMingen). For surface stained samples 7AAD (BD-PharMingen) was added just before FACS analysis to visualize cell viability. Cell staining was analyzed on a FACS-Calibur using CellQuest software (BD Bioscience, Mountain View, CA).

IFN-γ and CD107a and b expression upon peptide stimulation

PBMC or filtered BAL cells were washed and plated in a 96 well round bottom plate (Costar, $1.0x10^6$ cells/well) in AIM-V medium supplemented with P/S, 2% HPS, 40 units/ml IL-2 and costimulatory antibodies (anti-CD28 and anti-CD49d). Cells were either stimulated with 1µM of the HLA-A1 ($M_{229-237}$, YLEKESIYY) or HLA-B7 ($NP_{306-314}$, NPKASLLSL) RSV peptide or not stimulated (negative control). FITC labelled CD107 a and b was directly added. Every condition was represented in 4 wells to have a total amount of $4x10^6$ stimulated or unstimulated cells. After one hour of incubation at 37ºC 10µg/ml of Monensin (BD PharMingen) was added to accumulate cytokines in the cell. After 4 hours, 2mM of EDTA was added in order to arrest activation and to remove adherent cells from the well. After incubating 20 minutes at room temperature, cells were washed twice in FACS buffer. Then

cells were stained with APC labelled anti-CD8 mAb (BD-PharMingen) and intracellular with PE-labelled IFN-γ specific mAb (BD-PharMingen, clone 340449).

To study the $CD8^+$ T cell responses against antigen presenting cells exposed to whole virus, monocyte derived dendritic cells were used. The culture procedure and infection of dendritic cells was performed as described before (26). As control cells we used immature DC cultured for 48 h. with TNF-α and IL-β to obtain mature DC with a similar pattern of maturation markers as the RSV-infected DC. Infection levels and maturation status were checked by measuring the expression of the viral F protein at the cell surface and the expression level of costimulatory molecules CD80/86 and HLA class II as well as the mature DC marker CD83 (26). $1x10^5$ DC and $5x10^5$ patient PBMC were cocultured for 5 hours. Total RSV-specific responses were compared to the responses against synthetic peptides. These peptides (1μM) were added to cocultures of PBMC and uninfected DC. After one hour brefeldin A was added to the cultures to accumulate IFN-γ inside the responding T cells. Surface and intracellular stainings were further performed as described above.

Statistical analysis

Differences in absolute cell numbers and percentages of activation and differentiation were assessed with the non-parametric Mann Whitney U rank sum test. Tests were two tailed and a p value equal or less than 0.05 was regarded as statistically significant.

Table I: FACS analysis of BAL samples of RSV-infected children

Cell marker	% of cells[1]	Range	Range of absolute BAL cell number (105/ml)
CD16 (granulocytes[2])	76.4	65-97	1.5-10.9
CD14 (monocytes)	25.0	19-36	0.4-4.5
CD19 (B-cells)	0.35	0.1-0.5	0.02-0.5
CD56 (NK-cells)	0.4	0.3-0.4	0.1-0.6
CD3 (T-lymphocytes)	1.9	0.4-4.4	0.3-5.9
$CD4^+$ of CD3	28.1	21-39	0.05-1.2
CD8 of CD3	62.2	50-80	0.2-3.0

[1]The percentage of cells from total live cells, based on FCS/SSC and 7AAD negative-staining (mean of tested patients).
[2]CD16 is mainly expressed on granulocytes but also on minor populations like macrophages and natural killer cells.

Results

Highly activated effector CD8+ T cells accumulate in the airways during acute RSV infections

During the RSV seasons of 2002-2006 NB-BAL samples were collected from 32 patients on mechanical ventilation for RSV bronchiolitis and from 18 healthy controls. The average age of admission was 8.7 (SEM 1.3) weeks for the patients and 13.9 (SEM 2.3) weeks for the controls, this was a non-significant difference. The mean time from onset of clinical symptoms to intubation was 6.3 days (range 2-21) and the mean time of ventilation was 11.1 days (range 1.9-19.8). We studied the BAL leukocyte composition in detail by cytospin (not shown) and FACS analysis (Table I). CD16 was found on 76% of the cells, the majority accounting for the abundant neutrophil population. All monocytes (25%) were HLA-DR posi-

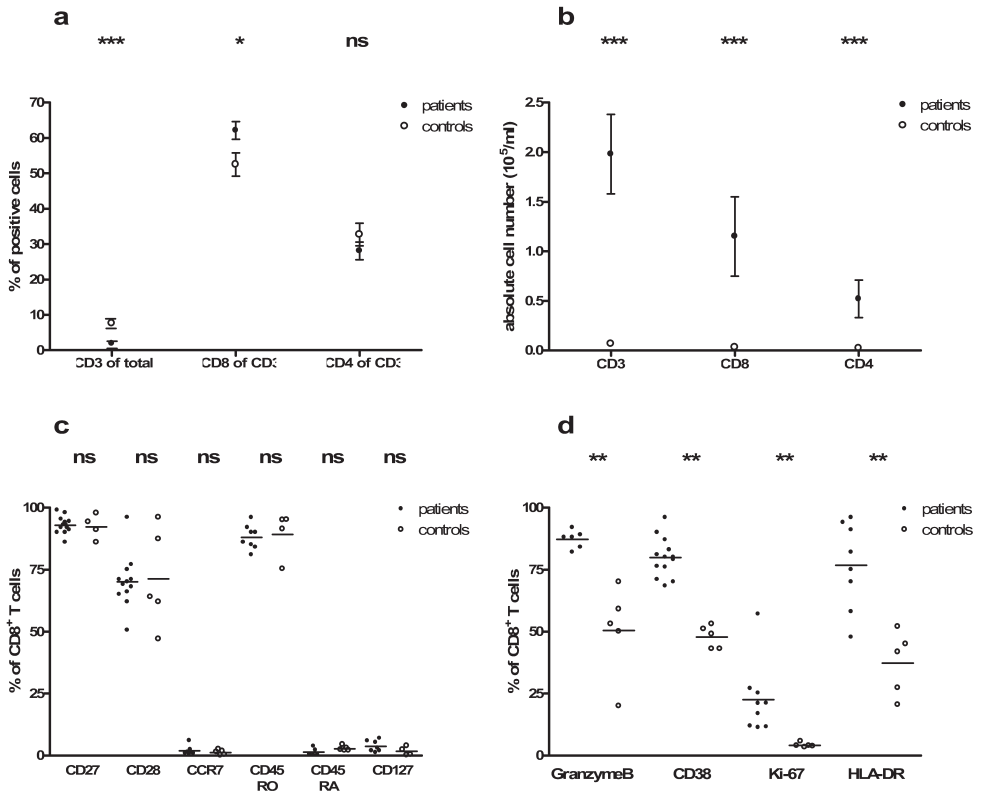

Figure 1: *Lymphocyte populations in BAL samples.* ***a:*** *Percentage of CD3+ lymphocytes of total cells in BAL. CD4+ and CD8+ cells are depicted as the percentage of total live CD3+ BAL cells.* ***b:*** *Absolute cell number of CD3+, CD3+CD8+ and CD3+CD4+ lymphocytes in BAL.* ***c:*** *Differentiation and* ***d:*** *activation markers on CD8+ T cells present in BAL samples. Percentages of marker positive live CD8+ T cells are shown, based on negative 7AAD staining and positive CD8+ staining. ns: not significant, * <0.03, ** <0.006, *** <0.001*

tive. Only small fractions of B cells (0.35%), and natural killer cells (0.4%) were detected. Due to the enormous influx of neutrophils the percentage of lymphocytes in patients was lower than in healthy controls. However, the absolute number of both $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes was much higher in patients because of the higher cellularity of the samples which was $6.6x10^6$ (range 0.6-$19.9x10^6$) in patients versus $0.21x10^6$ (range 0.05-$0.55x10^6$) in controls (Figure 1a,b). Although the increase in $CD8^+$ T cells was larger than the increase in $CD4^+$ T cells, the difference in CD4/CD8 ratio was not significant (patients 0.48 versus controls 0.67, p value 0.13).

To study the $CD8^+$ T cell response during primary RSV infection in the lungs in more detail, we first focused on characteristics of the whole $CD8^+$ T cell population in RSV positive patients, and compared them to healthy controls. In contrast to PBMC of young infants, most $CD8^+$ T cells in the lung had the effector/memory phenotype ($CD45RA^-$, $CD45RO^+$, $CCR7^-$, $CD27^+$ and $CD28^+$) both in the RSV-infected group and in the control group (Figure 1c, d). A large proportion of $CD8^+$ T lymphocytes was highly activated in both patients as well as controls, with upregulation of CD38 and HLA-DR and GranzymeB, but the percentage of HLA-DR and CD38 positive cells i.e. the phenotype of effector cells, was significantly higher in patients (Figure 1d). CD127, the IL-7 receptor that is expressed on naïve and memory T cells and down-regulated on effector cells, was found on 3.7% (range 1.2-6.9%) of the BAL $CD8^+$ T cells. These stainings were performed on several time points after intubation. There was no correlation for any marker with time after intubation, duration of ventilation or severity of disease (need of oxygen/pressure of ventilation, data not shown). These data suggested that $CD8^+$ T cells only migrated to the lower airways, in patients as well as controls, when they had differentiated into effector/effector-memory cells.

During severe primary infection RSV-specific $CD8^+$ T cells as visualized by HLA tetramers have an activated phenotype

To measure RSV-specific T cells in the airways during severe RSV infection we used HLA-A1 and HLA-B7 specific tetramers containing previously characterized RSV epitopes (21;23). Both epitopes are derived from a well conserved region of the RSV genome, and no strain differences have so far been reported. In 22 out of 23 infants that expressed either HLA-A1 or HLA-B7 (confirmed by PCR-SSP) we were able to detect an RSV-specific $CD8^+$ T cell response, although in low percentages (average 0.58% of $CD8^+$ T cells). T cells of the healthy controls that had never been exposed to RSV did not stain positive with the tetramers (Figure 2a and b). Specificity of the staining by the tetramers was further confirmed by the absence of staining in BAL samples of patients that did not express the relevant HLA-type (Figure 2c). There was no correlation found between the percentage of RSV-specific $CD8^+$ T cells and time after intubation, age, the duration of mechanical ventilation or severity of disease. To determine the differentiation and activation status of RSV-specific cells, BAL samples were stained with tetramers and different cell markers (Figure 2d-l). The RSV-specific T cells had the phenotype

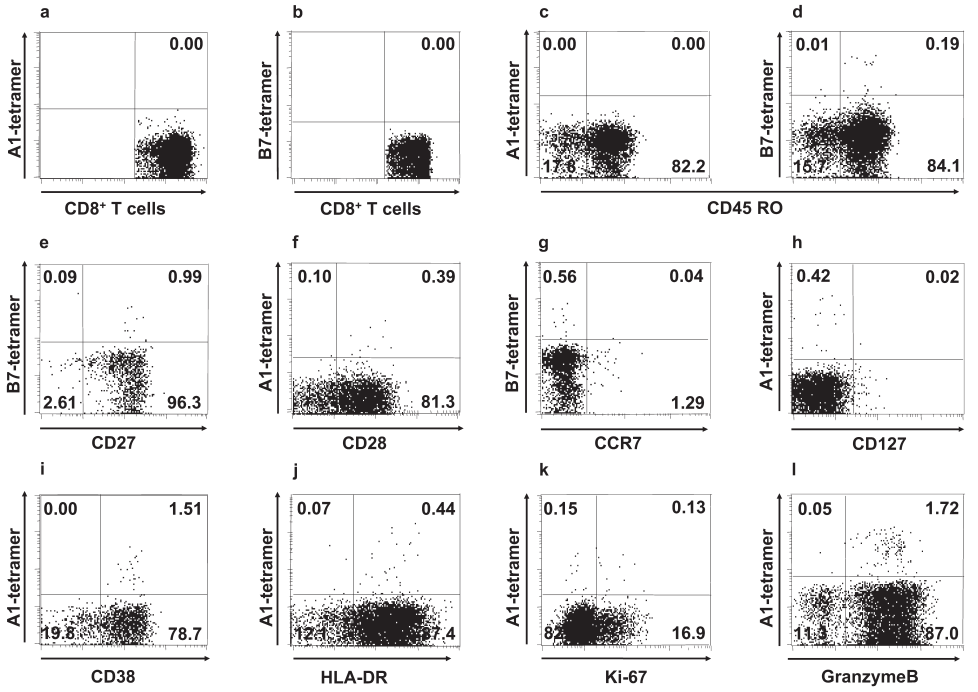

Figure 2: *Differentiation and activation markers on RSV-specific (tetramer+) CD8+ T cells of RSV-infected patients. The pictures show representative data from several patients. Due to the limited amount of material and low percentages of tetramer+ cells only a limited number of surface markers could be stained in a single BAL sample. However, each marker was tested in at least 5 different patients with similar results. Panel **a** and **b** show respectively HLA-A1+ and HLA-B7+ control patients that had never been exposed to RSV. Panels **c** and **d** show the staining in an HLA-A1- -B7+ RSV patient. Panels e-l show the staining in RSV patients that were HLA positive for the tetramer used. In all patients a HLA mismatched tetramer was applied as a negative control, example shown in panel c and d where an HLA-A1-, HLA-B7+ patient is stained with respectively an RSV-specific HLA-A1 and HLA-B7 tetramer. The population shown was gated for live CD8+ T cells.*

of activated effector cells: CD45RO+, CD27+, CD28+, CCR7-, similar to the total CD8+ T cell population. The percentage of activated CD38+/granzyme B+ cells was higher in the tetramer+ fraction compared to the total CD8+ T cell population (Figure 3). Most CD8+ T cells (>93%) were CD127 negative, at all time points during ventilation. To study whether CD8+ T cells were proliferating, tetramer stained cells were stained for Ki-67. Forty-eight percent (range 30-61%) of tetramer specific CD8+ T cells positively stained for Ki-67 indicating that they were actively proliferating cells.

RSV-specific T cells in BAL are functional

The absolute number of virus-specific T cells that we detected with tetramer staining in BAL was very low. It has recently been described in mice that during the early phase of infection CD8+ T cells transiently lose their capability to bind tet-

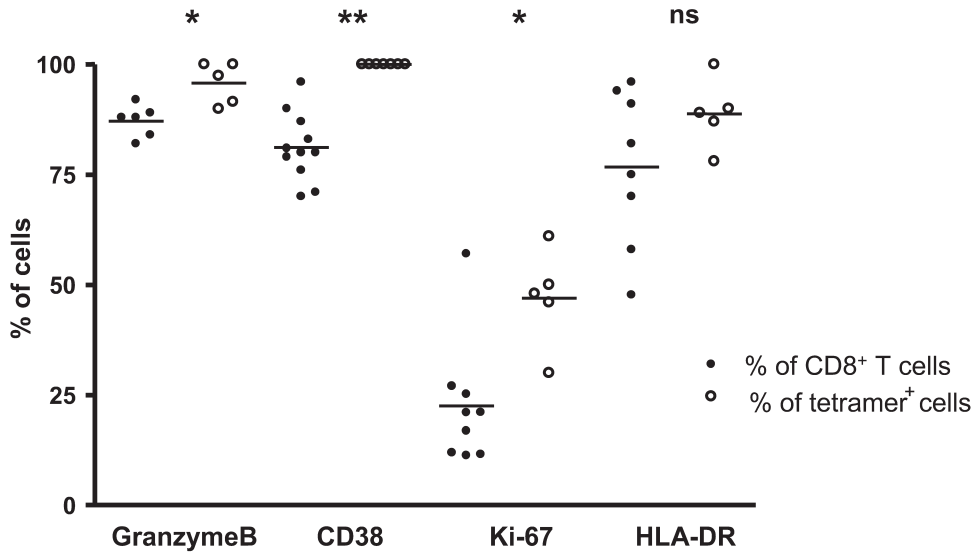

Figure 3: *Activation markers on RSV-specific CD8⁺ T cells in BAL samples of RSV-infected infants. ● The percentage of positive cells of total live CD8⁺ T cells. ○ The percentage of positive cells in the tetramer⁺ CD8⁺ T cell population. Ns not significant, * ≤ 0.05, ** <0.001.*

ramers possibly due to a change in cell surface T cell receptor organization that interferes with tetramer binding (27). This is associated with a transient lower IFN-γ production of these cells. Furthermore, functional inactivation of CD8⁺ T cells has been observed in the lungs of RSV-infected mice (28). In these experiments quantitation of virus-specific T cell responses by functional measurements underrated the attracted virus-specific cells as enumerated by tetramer staining. For these reasons we also quantitated the number of RSV epitope specific T cells based on functional responses. In 4 patients we tested the cell surface expression of CD107a and b granular membrane proteins that are transiently expressed on the cell surface during degranulation and perforin release by cytotoxic T cells (29), and the intracellular expression of IFN-γ after cognate peptide stimulation. These cell numbers were compared with the number of tetramer⁺ cells in the same patients (Figure 4). We found no differences between the number of CD107 positive cells and the number IFN-γ positive cells and both correlated closely with the number of tetramer⁺ cells.

Another explanation for the small numbers of tetramer⁺ cells might be the time and place of sampling. In the mouse it was observed that effector T cell numbers in BAL increased when the peak of the response in tissue had subsided (30). During the last RSV season two children with a tracheotomy came to our hospital that had recovered from a severe primary RSV infection respectively one and three months earlier. In these two cases we had the opportunity to sample tracheal aspirate at a later time point than the BAL samples that we obtained from ventilated patients

that were usually extubated around day 15 after onset of symptoms. Indeed in these two patients we found higher percentages of tetramer⁺ CD8⁺ T cells: 3.7 and 6.7% after respectively one and three months (Figure 5) than in any of the HLA-A1 positive patients tested at earlier time points during infection, where the RSV-specific response never exceeded 1.8%.

Numbers of activated CD8⁺ T cells in PBMC of RSV-infected infants peak at the time of extubation

To study CD8⁺ T cell responses in blood, PBMC's from four patients were isolated on admission, on extubation and during the convalescence phase when infants visited the outpatient's clinic. In 3 infants that were ventilated for more than a week, a 4th time point was included during the ventilation period. All four patients showed a peak in tetramer specific cells by the time of extubation which coincided with the peak of total activated T cells (Figure 6a and b). The RSV-specific cells initially lost CD127 expression which they partly regained during convalescence,

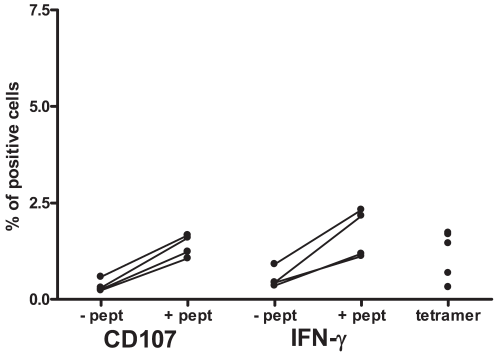

Figure 4: *CD107 and IFN-γ production upon peptide stimulation versus tetramer staining. BAL cells of RSV-infected infants were stained with CD107a and b and either stimulated with peptide or left unstimulated. After 5 hours cells were stained for IFN-γ. Cells were gated on a CD8⁺ gate and a live lymphocyte gate based on the forward/side scatter. Numbers were compared to tetramer⁺ cells in the same patients.*

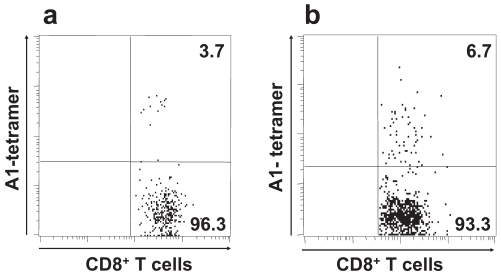

Figure 5: *RSV-specific CD8⁺ T cells during convalescence. One (***a***) and three (***b***) months after a severe primary RSV infection, the number of RSV-specific CD8⁺ T cells is increased compared to RSV-specific CD8⁺ T cell numbers early during primary RSV infection. Cells were obtained by suction through a tracheostoma and gated for live CD8⁺ T cells.*

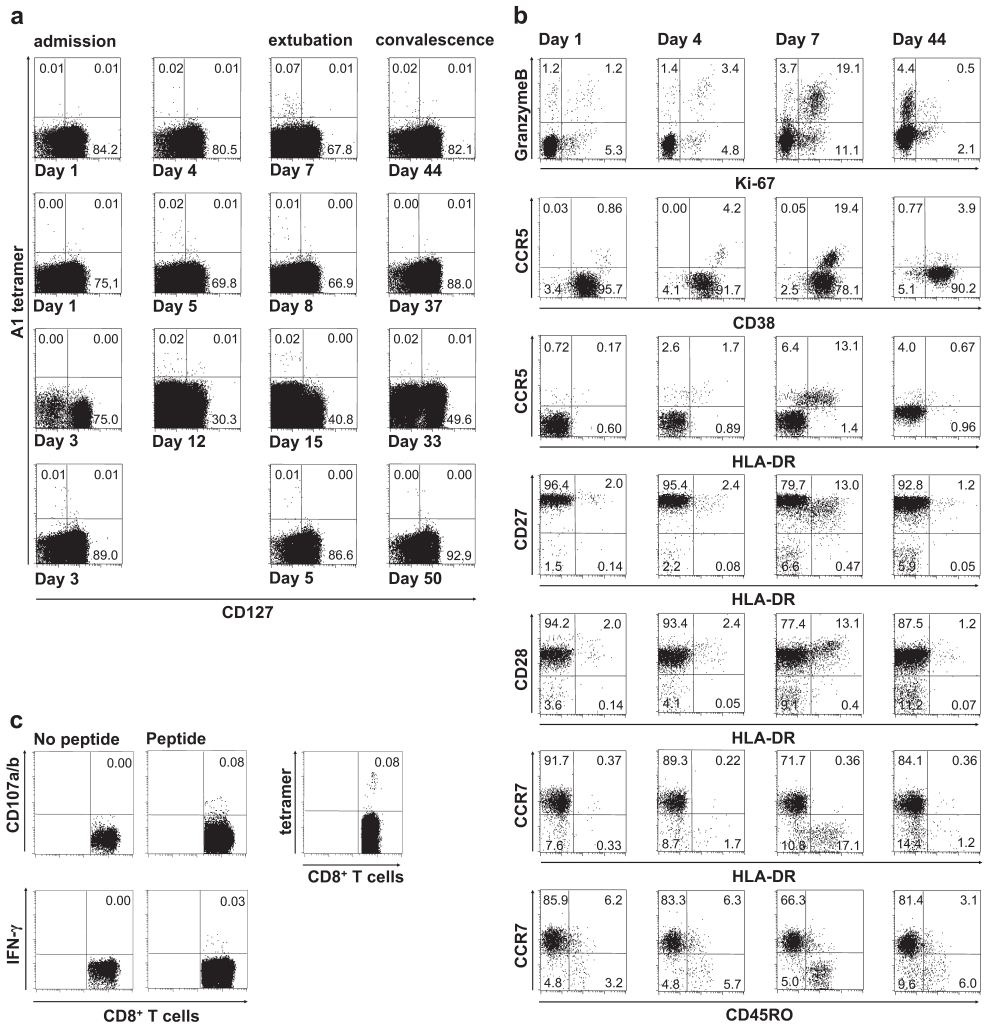

Figure 6: *PBMC of RSV-infected infants show late activation in a significant percentage of CD8⁺ T cells.* ***a****: CD8⁺ T cell kinetics in 4 RSV-infected infants. PBMC were drawn on admission, release and during the convalescence phase, and in case of longer duration of ventilation at a fourth time point in between. Day 1 indicates the first day after intubation (3-5 days after onset of clinical symptoms). Cells were gated on a CD8⁺ gate and a live lymphocyte gate based on the forward side scatter.* ***b:*** *differentiation and activation status of CD8⁺ T cells in a single representative patient during RSV infection. Day 1 indicates first day after intubation (for this particular patient 5 days after onset of clinical symptoms). Similar kinetics of up- and down regulation of activation and differentiation markers was observed in all 4 patients tested. However, the magnitude of the response varied.* ***c:*** *PBMC of RSV-infected infants were stained with CD107a and b and either stimulated with peptide or medium. After 5 hours cells were stained for IFN-γ. Cells were gated on a CD8⁺ gate and a live lymphocyte gate based on the forward side scatter. Numbers were compared to tetramer⁺ cells in the same patients. One representative experiment out of three is shown.*

therefore presumably destined to become memory cells (31). In agreement with these findings is the observed up-regulation of CCR7 on a subpopulation of the HLA-DR positive CD8+ T cells at this time point.

All infants showed exactly the same pattern of activation markers which is represented in Figure 6b. Proliferating CD8+ T cells were present early after intubation as measured by Ki-67 expression and the fraction of Ki-67+ cells increased to reach a peak at the time of extubation. At this time point we also found the highest numbers of activated CD8+ T cells as measured by expression of HLA-DR, GranzymeB, chemokine receptor CCR5 that facilitates trafficking to the lungs, and by downregulation of the IL-7 receptor CD127 and the lymphnode homing chemokine receptor CCR7. As previously shown, all PBMC in the infants were CD38 positive. However, at the same time of gaining CCR5 expression, CD8+ T cells in PBMC upregulated CD38 two to three fold compared to resting T cells. Co-stimulatory molecule/differentiation marker CD27 was downregulated two to three fold and CD28 upregulated two to three fold during the peak of the effector phase of the response, compared to the mean fluorescence intensity of naïve CD8+ T cells. From the kinetic studies we further observed that Ki-67 expression preceded the expression of HLA-DR and GranzymeB in the activated CD8+ T cell population. Importantly, the kinetics of the RSV-specific T cells closely resembled the kinetics of the total activated CD8+ T cell population. Using intra-cellular staining for IFN-γ and CD107 upregulation upon cognate peptide stimulation we found similar percentages of RSV-specific T cells as detected by tetramer staining (example shown in Figure 6c). In summary these observations implicated that in peripheral blood the peak of the total activated CD8+ T cell numbers as well as the RSV-specific T cell numbers coincided with the time of recovery.

Expansion of RSV-specific T cells in peripheral blood

The total number of activated CD8+ T cells in the peripheral blood of the patients as determined by different surface marker combinations: GranzymeB+/Ki-67+ cells, HLA-DR+/CD27^{low} or CD38^{hi}/CCR5+ cells was in general at least 100 fold higher than the number of virus-specific CD8+ T cells that we could detect with single tetramers. This observation might suggest that only an extremely small fraction of the virus-specific response was visualized by the tetramers that incorporated single RSV epitopes. To address this issue we performed experiments that could give an impression of the magnitude of the RSV-specific component in the total activated CD8+ T cell population. We previously showed that monocyte derived dendritic cells could be infected with RSV. Although the infection with the virus strongly inhibits the ability of such DC to support proliferative responses in T cells, the class I antigen presentation route was intact and virus-specific memory T cells responded functionally with the production of IFN-γ to stimulation with infected DC (26) and *unpublished results*). Because we had only small amounts of cryopreserved PBMC of the patients available, we used monocyte derived dendritic cells of adult blood donors that partially matched the HLA type of the patients as antigen presenting cells. Infected and uninfected DC were cocultured

with PBMC samples of the patients that were obtained at the time of discharge of the intensive care. The fraction of IFN-γ producing RSV-specific T cells was compared to the total fraction of activated $CD8^+$ T cells. We found in 3 patients that the T cell responses induced by RSV-infected partly HLA matched dendritic cells were respectively 45% and 27% of the $CD38^{hi}/CCR5^+$ $CD8^+$ T cells in two patients and 17% of $GranzymeB^+/Ki\text{-}67^+$ in the third patient (Figure 7). The responses against synthetic peptides that were earlier identified as dominant epitopes in adult memory responses were around the detection limit of the assay. These data confirm that virus-specific $CD8^+$ T cell responses were substantially higher than the responses visualized with tetramers.

In BAL we did not find any correlation between the magnitudes of T cell responses with disease severity. However, we noticed that in the small group of patients that

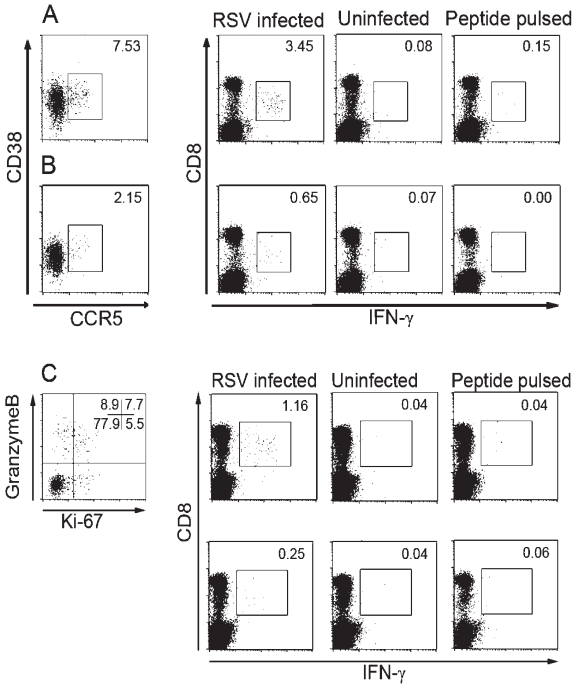

Figure 7: *$CD8^+$ T cell responses against the total repertoire of viral epitopes presented on RSV-infected dendritic cells. 1x105 RSV-infected DC, uninfected DC, or uninfected DC + peptide, were co-cultured with 5x105 patient PBMC for 5 hours. PBMC samples were taken at the time of discharge from the pediatric intensive care unit.* ***a:*** *HLA type of patient (responder T cell population): HLA-A2,A25,B7,B18; HLA type DC (antigen presenting cells,): HLA-A2,A25,B18,B51 (9.0% expressed RSV-F at the cell surface).* ***b:*** *HLA-type of patient: HLA-A2,A24,B7,B51; HLA type DC: HLA-A2,A25,B18,B51. c.: HLA type of patient: HLA-A1,A68,B8,B51 cultured with DC of two different HLA types: upper row: HLA-A2,A68,B62,B51 (infection level 19.3%) and lower row HLA-A1,A10,B8,B45 (infection level 6.8%). To compare these responses to the response against a single dominant peptide, PBMC's of the patients were stimulated with the following peptides:* ***a:*** *$NS1_{33\text{-}41}$ KLIHLTNAL restricted to HLA-A2,* ***b:*** *$NS1_{41\text{-}49}$ LAKAVIHTI restricted to HLA-B51, and for* ***c:*** *HLA-$A1^+$ DC $M_{229\text{-}37}$, YLEKESIYY and on the HLA-$B51^+$ DC peptide $NS1_{41\text{-}49}$ LAKAVIHTI.*

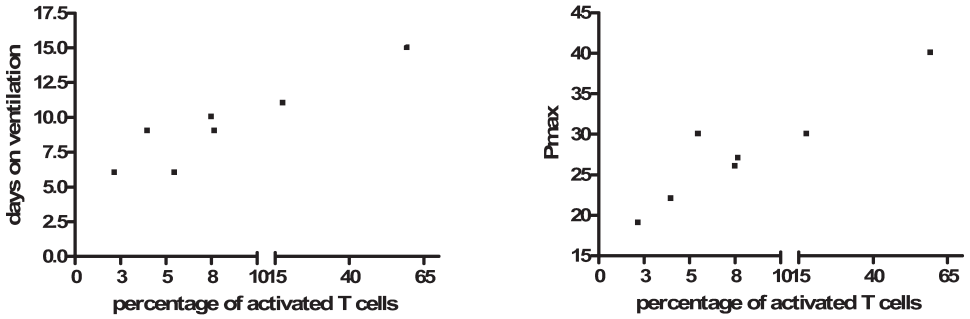

Figure 8: *The percentage of activated T cells in PBMC correlates with severity of disease. The percentage of activated CD 8+ T cells at time of extubation was determined by double stainings: HLA-DR+ CCR7-, CCR5+ CD38high, GranzymeB+ Ki-67+ CD8+ T cell numbers were plotted against days on mechanical ventilation and maximum ventilation peak pressure required.*

were used for PBMC analysis there was a positive correlation between the magnitude of the T cell response and the severity of disease as measured by days of ventilation (correlation coefficient 0.9, $p<0.01$) and maximum ventilation peak pressure required (correlation coefficient 0.9, $p<0.01$) (Figure 8).

Discussion

In the present study we show that substantial numbers of $CD8^+$ T cells infiltrate the airways during severe primary RSV infections. Furthermore, we positively identified RSV-specific $CD8^+$ T cells in this local site as highly activated effector type cells. These cells were functional based on the fact that they showed evidence of degranulation as assessed by cell surface expression of CD107 a and b upon cognate peptide exposure, as well as the ability to produce IFN-γ. As expected in a peripheral site (32;33), the $CD8^+$ T cells had an activated phenotype in both patient and control BAL samples. Most cells were $CD45RO^+$, $CD28^+$, $CD27^+$, $CCR7^-$, $CD127^-$. The high proportion of $CD8^+$ T cells in the BAL of patients expressing GranzymeB and HLA-DR and a somewhat lower expression of CD27 (34) further indicated that preferentially highly differentiated effector type $CD8^+$ T cells migrated to the airways. These findings resemble those of a recent murine study showing that predominantly the effector $CD8^+$ T cell population migrated from alveolar capillaries into the lung interstitium (35). It was shown in this study that this process was dependent on G protein-coupled receptor triggering in T cells. In non-inflamed lungs constitutive expression of RANTES/CCL5 by airway epithelial cells might explain a continuous recruitment of effector cells from the circulation that express CCR5 the receptor for RANTES. RSV infection leads to increased levels of RANTES expression in the lungs which might explain the en-

hanced recruitment of $CCR5^+$ effector T cells to the lungs during an ongoing RSV infection (36;37).

Naïve and memory T cells depend on IL-7 for maintenance. IL-7Ra (CD127) expression is downregulated on effector T cells during acute infection and can therefore be used as a marker of effector cells (38). After clearing of antigen, memory T cells re-express CD127 (39). We found that in both the control group and patients $CD8^+$ T cells in BAL were CD127 negative. Recently, de Bree et al. evaluated the presence of virus-specific T cells in lung tissue of elderly lung carcinoma patients, that had at the time of lobotomy no symptoms of upper respiratory tract infection (34). They found a higher frequency of T cells directed against cleared respiratory viruses in lung tissue compared to blood. It was shown that virus-specific T cells had an activated phenotype, $CD45RO^+$ $CD27l^{ow}$, but in contrast to the situation of acute RSV infection shown by us, the expression of CD127 was high which reflected the memory status of these cells. The enrichment of RSV-specific-specific $CD8^+$ T cells in lung tissue that was not observed for CMV- and EBV-specific T cells might suggest that a proportion of effector cells attracted during the acute phase of infection locally differentiated to memory cells. In the mouse it has been documented that the transition of effector to memory $CD8^+$ T cells can indeed occur outside lymphoid tissues (40). While the infants of our control population had no signs of a respiratory infection at the time of sampling, $CD8^+$ T cells in BAL did not express CD127. However, the $CD8^+$ T cells of the controls had a significantly lower expression of activation markers (granzymeB, CD38, Ki-67 and HLA-DR) than the RSV-specific cells in patients that were positively identified by tetramer staining (Figure 2,4). The difference in CD127 expression on $CD8^+$ T cells in the airways of the young controls and on $CD8^+$ T cells in lung tissue of elderly patients described by the Bree might reflect the difference in age. Young infants are frequently exposed to infectious agents and the time elapsed after an earlier infection may not have been long enough for CD127 memory cells to develop. An alternative explanation might be that the airway environment, in contrast to lung tissue, does not allow for memory cell differentiation. Indeed it was recently shown in mice that T cells found in the airways after viral clearance are effector type cells, continuously replenished from blood. This process continues for months, as long as a reservoir of processed viral antigen is present in the draining lymphnodes (41).

Interestingly, in the RSV patients the percentage of RSV-specific $CD8^+$ T cells in the convalescence phase of the response seemed higher than at earlier time points, which might also reflect the continuous recruitment of effector cells. Some caution is required because we were unable to make this comparison longitudinally in the same patients. The opportunity to obtain material from the "lower" airways in the two tracheotomy patients that had recently recovered from a proven severe RSV infection were unique events. Therefore, we only had two samples that could be used to determine the level of the $CD8^+$ T cell response at a later time point. However, the observation that later after infection T cell numbers increased in the airways is similar to observations we made in mice (30).

It is not clear what percentage of $CD8^+$ T cells in BAL of patients reflected the RSV-specific response. The percentage of virus-specific cells detected with two different tetramers was low (<1.8%). However, there was a difference in the percentage of Ki-67 positive proliferating cells during acute infection in patients versus healthy controls (22.5% versus 4.1%) which points to recent activation of these cells in patients. In the $tetramer^+$ fraction the percentage of Ki-67 positive cells was even higher: about 47%. Because we found that in peripheral blood of patients the number of $CD8^+$ T cells responding to RSV-infected antigen presenting cells was much higher than the number of $tetramer^+$ cells it is likely that also in BAL the Ki-67^+ $CD8^+$ T cells reflected predominantly virus-specific cells. Unfortunately, for technical reasons we were unable to perform the experiments using RSV-infected DC with BAL cells.

Because the total number of virus-specific T cells was much higher than the numbers we detected with single tetramers it is likely that there are several yet undiscovered RSV epitopes restricted by different HLA-alleles (21). The tetramers used contained epitopes that were found to be immunodominant in memory responses of healthy adults. However, immunodominance during primary and secondary responses may not be the same as was previously shown for influenzavirus epitopes that were dominant during primary infection in mice, but less dominant during secondary infections (42;43). Also for RSV we have found in mice that the response during secondary infection is focussed on a single epitope derived from the Matrix protein while the primary response is more heterogeneous (30). During primary RSV infection there might thus be other epitopes with a significant contribution to the response that we missed because we based the choice for the epitopes contained in the tetramers on the specificity of adult memory T cell responses. Obviously, also T cells responding to HLA alleles for which we have not performed epitope searches might significantly contribute in the total antiviral response. In blood we found that the total RSV-specific $CD8^+$ T cell response was indeed much higher than the responses to single epitopes. Because we used partially HLA matched DC as antigen presenting cells and infected DC/T cell ratios might not have been optimal the total virus-specific response might approach the total activated T cell response even closer than is shown in Figure 7.

During primary RSV infections in mice there is a large lymphocytic influx into the lungs. It was shown that although depletion of $CD8^+$ T cells delayed viral clearance, it diminished illness severity (13). This observation led to the hypothesis that this delicate balance between viral clearance and immune pathology might account for the differences in severity of illness observed in infants during primary RSV infection. Because it is not easy for ethical reasons to obtain BAL samples from infants with mild infections, we have so far been unable to link levels of $CD8^+$ T cell responses in the airways to disease severity. Within the presented patient group there was no correlation between any of the clinical parameters and the level of the $CD8^+$ T cell response in BAL. However, in the group of seven patients from whom we obtained blood samples we found that the more severe patients

had higher numbers of activated $CD8^+$ T cells in blood. Obviously, these observations need to be extended in larger groups of patients.

In summary in the present report we present the first time evidence that RSV-specific $CD8^+$ T cells are present in NB-BAL during primary severe RSV infection. Although we found a low frequency of RSV-specific T cells by using HLA tetramers, these were highly activated effector cells. Comparisons with T cell responses in patients with mild disease could in the future contribute to discriminate between a role of viral load versus immune pathology as parameter affecting disease course.

Acknowledgements

We thank patients and parents for participation is this study, and the nurses, anesthetists and assistants for their help in obtaining BAL samples. We thank Prof. René A.W. van Lier and Dr. Debbie van Baarle and Michael V. Lukens for reading of the manuscript.

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Dynamics of human RSV-specific CD8$^+$ T cell responses in blood and airways during episodes of common cold

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Manuscript in preparation

Anna during the summer season

Summary

The dynamics of human T cell responses during ongoing infections in the airways is an unexplored field. Therefore, we determined secondary immune responses in blood and tracheostoma aspirates during the course of respiratory infections. We show that during localized respiratory infections the ratio of activated effector $CD8^+$ T cells to resting memory/naïve $CD8^+$ T cells in peripheral blood increases significantly. Furthermore, the number of memory T cells specific for respiratory viruses declines in blood and increases in the airways suggesting that these T cells redistribute from blood to airways. T cells specific for the infecting virus are maintained in the airways for longer periods at increased levels than non-specifically recruited bystander T cells. After clearance of the infection the ratio of resting memory and naïve $CD8^+$ T cells is normalized in peripheral blood and also memory T cell numbers specific for unrelated viruses that declined during the infection due to bystander recruitment are restored.

Together these results show a large systemic T cell response during a relatively mild secondary infection and extensive dynamics of virus-specific and non-specific antigen experienced T cells. During convalescence the balance of naïve and memory T cells in peripheral blood is restored to the pre-infection value as are levels of memory T cells to non-crossreactive heterologous virus epitopes.

Introduction

Antiviral CD8+ T cells are important for resolution of both primary and secondary viral infections. However, for respiratory infections like respiratory syncytial virus (RSV) and influenzavirus protective T cell mediated immunity decreases over time (1-3). Most insight into the dynamics of CD8+ T cell responses during respiratory infections has come from studies in mice. Until now lung CD8+ T cell responses during respiratory infections have not been studied in great detail in humans. In the mouse model, naive virus-specific CD8+ T cells differentiate and gain the capacity to traffic into inflamed tissue during primary infection. Typically, proliferating T cells are first detected in the draining lymph nodes (DLN) around day 4, from where they migrate to the lungs where they are found from day 6 onwards (4). Peak responses are found in all tissues around day 8-10, while viral load declines as soon as CTLs appear (5). After resolution of the infection the virus-specific T cell pool contracts and a small number of T cells survive and become long-lived memory T cells.

Upon secondary infection a three phase defense has been suggested (6). The first phase is represented by effector/memory CD8+ T cells that are already present in the lungs. Cytokines and chemokines are secreted by these cells to create a pro-inflammatory milieu that attracts other immune cells. During the second phase a wave of non-proliferating T cells is recruited to the lung around day 4 in a non-antigen specific manner. Memory T cells specific for the infecting virus as well as non-specific bystanders are recruited. Bystanders do not proliferate and are deleted by apoptosis or phagocytosis. The third phase exists of antigen specific effector cells that are derived from reactivated memory cells in the draining lymphnodes (7;8).

Few data have been published in the human host. A recent study has shown that CD8+ T cells specific for respiratory infections could be detected in lung tissue of elderly people that had no symptoms of infection at the time tissue biopsies were taken (9). There was a relative enrichment of CD8+ T cells specific for influenzavirus and RSV in the lung tissue compared to blood that was not found for EBV. These cells had progressed to a highly differentiated phenotype and were IL-7 receptor (CD127) positive reflecting the memory status of these cells. The relationship between airway and tissue resident CD8+ T cells and their relative contribution to viral clearance is currently unclear. In mice it was found that effector memory cells are predominantly recruited to the airways (10;11). These cells do not divide locally and virus-specific CD8+ T cell numbers decline in murine models around 6 months after infection (3).

Information obtained in murine models may not always reflect the human situation. Using human pathogens like influenzavirus and RSV in the mouse model and the difference in anatomy in the murine and human pulmonary circulation may lead to differences in the inflammatory milieu and in the in vivo trafficking

of immune cells (12;13). These differences may result in different populations of immune cells that contribute to the response. Therefore, we investigated the dynamics of human $CD8^+$ T cell responses in peripheral blood and tracheostoma aspirates of individuals during episodes of common cold

Participants, materials, and methods

Study population and sample collection

Twenty patients with a tracheostoma between 6 months and 18 years of age were included in the study during the winter season of 2005-2006. Exclusion criteria were immune disorders and the use of immune suppressive medication. Blood samples were taken prior or on day one of the first episode of common cold. In 3 patients with paralysis of the legs and absent pain perception, blood samples were drawn at all the time points when tracheostomal aspirate was collected. Parents were asked to collect morning samples of tracheostomal aspirate (TA) on day 2-5-8-15-28 and 56 after the start of a common cold. During RSV or influenzavirus infections a 7^{th} sample was collected on day 42. Samples were kept on ice during transport. All samples were processed within 4 hours after collection.

Healthy blood donors were followed during one winter season. Twenty ml of heparinized blood samples were drawn either once a week or at two time points before and after onset of common cold symptoms and at 1-4 time points during clinical signs of a common cold. Nose and throat swabs for viral PCR were taken at all time points. PBMC were isolated by Ficoll-Paque gradient centrifugation (Pharmacia Biotech) and used immediately or stored in liquid nitrogen. Healthy blood donors and parents of patients gave their informed written consent. The study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht.

Real-time PCR

TA samples or nose and throat swabs were evaluated for respiratory viruses by real time PCR. After spiking of samples with murine encephalomyocarditis virus and phocine herpes virus, nucleic acids were extracted using the total nucleic acid protocol with the MagNA pure LC nucleic acid isolation system (Roche Diagnostics, Basel, Switzerland). Each sample was eluted in 200 µl of buffer, which was sufficient for all real-time PCR analyses. cDNA was synthesized by using MultiScribe reverse transcriptase (RT) and random hexamers (both from Applied Biosystems, Foster City, CA, USA). Each 200 µl reaction mixture contained 80 µl of eluted RNA, 20 µl of 10 x RT buffer, 5.5 mM $MgCl_2$, 500 µM of each deoxynucleoside triphosphate, 2.5 µM random hexamer, and 0.4 U of RNase inhibitor per µl (all from Applied Biosystems, Foster City, CA, USA). After incubation for 10 min at 25°C,

RT was carried out for 30 min at 48°C, followed by RT inactivation for 5 min at 95°C.

Detection of viral and atypical pathogens was performed in parallel, using real-time PCR assays specific for: RSV A and B, influenzavirus A and B, parainfluenza virus 1 to 4, rhinoviruses, adenoviruses, human corona viruses OC43, NL63 and 229E, hMPV, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*. Real-time PCR procedures were performed as previously described (14). Briefly, samples were assayed in duplicate in a 25 µl reaction mixture containing 10 µl of cDNA, 12.5 µl 2xTaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and 300-900 nM of the forward and reverse primers and 75-200 nM of each of the probes (together 2.5 µl). Efficient extraction and amplification was monitored through the internal control viruses (murine encephalomyocarditis virus [RNA virus] and phocine herpes virus [DNA virus] (15).

HLA-mapping

To be able to perform HLA-tetramer staining in fresh TA samples, immediate limited HLA-mapping was required. Therefore, 50µl of whole blood was stained with a panel of HLA specific antibodies to be able to select the proper HLA-tetrameric complexes to be used for T cell identification (IHB-Hu-037 for HLA-A1/A36, BB7.2 for HLA-A2, and IHB-Hu-035 for HLA-B7 were kindly supplied by Dr. A. Mulder, LUMC Leiden, The Netherlands). After 30 min. of incubation at room temperature cells were labeled with fluorescein isothiocyanate (FITC)-labeled Fab fragments (IgG1 for IHB-Hu-035 and IgMκ for IHB-Hu-037, DAKO F0315 and F0317, IgG2b for HLA-A2) and incubated for another 30 minutes. Erythrocytes were lysed using lysis buffer (BD Bioscience). Cells were analyzed by FACS Calibur flow cytometer and CellQuest software (BD Biosciences). HLA-type of the patients was confirmed in a later stage by PCR-SSP using cultured EBV B cell lines of the patients. PCR-SSP was performed according to the instructions of the manufacturers (Biotest, Dreieich, Germany).

CD8+ T cell phenotyping

To maximize cell viability, TA samples were processed directly after sampling. Specimens were filtered through 70µM cell strainers (Falcon, BD) to remove mucus. Cells were washed in RPMI medium supplemented with penicillin and streptomycin and 10% FCS and blocked in FACS buffer containing 10% HPS for 30 minutes on ice. Cells were stained with the appropriate HLA-A1, -A2, -A3 or -B7 tetramer. The allophycocyanin (APC)-conjugated HLA-B7 tetramer loaded with the RSV peptide NPKASLLSL ($NP_{306-314}$), the HLA-A1 tetramer loaded with the RSV peptide YLEKESIYY ($M_{229-237}$), the HLA-A3 tetramer loaded with the RSV derived peptide RLPADVLKK ($M2_{151-159}$), the HLA-A2 tetramer loaded with the FLU peptide GILGFVFTL ($M1_{58-66}$) and the HLA-A2 tetramer loaded with the EBV peptide GLCTLVAML ($BMLF1_{300-308}$) were purchased from Sanquin (Amsterdam, The Netherlands). The HLA-A1 tetramer loaded with FLU NP-derived CTELKLSDY (NP_{44-52}) peptide was obtained from Proimmune (Oxford, UK)

After 20 minutes, cells were stained extra-cellular with different labeled mAbs for another twenty minutes at room temperature. FITC-labeled anti-CD8, -CD25, -CD27, -CD45RA, -CD45RO, -CCR5, -CD38, phycoerythrin (PE)-labeled anti-CD4, -CD8, -CD27, -CD28, -CD38, -CD45RO, -CD127, -HLA-DR, -CCR7, PerCP labeled anti-CD8,–HLA-DR and APC-labeled anti-CD3, -CD8, -CD28 were all purchased from BD-PharMingen (San Jose, California) except CD8-FITC (Sanquin, Amsterdam) and CD127-PE (Immunotech, Marseille, France). For intracellular staining cells were permeabilized and fixed using FACS permeabilizing/fixation solution (perm/Fix, BD-PharMingen). Cells were stained intra-cellular with FITC-labeled Ki-67, perforin (both BD-PharMingen), PE labeled GranzymeB (Sanquin, Amsterdam) or isotype control (BD-PharMingen) for 30 minutes at room temperature. Cells were washed twice in perm-wash and once in FACS buffer. For surface stained samples 7AAD (BD-PharMingen) was added just before FACS analysis to visualize cell viability. Cell staining was analyzed on a FACS-Calibur using CellQuest software (BD Bioscience, Mountain View, CA).

Statistics
Comparison of differences between patient groups (i.e. infected with RSV or rhinovirus) was made using the Mann Whitney U test. A P value < 0.05 was considered significant.

Curve fitting models were constructed by applying linear models as explained in the appendix.

Results

During respiratory viral infections bystander CD8$^+$ T cells specific for different respiratory viruses disappear from blood
Upon primary RSV infection both an antibody response and CD8$^+$ T cell immunity against well conserved epitopes develop. However, despite the memory B and T cell responses, re-infections in healthy adults occur every two to three years (1). To evaluate the dynamics of RSV-specific T cell memory during common respiratory infections, we followed a group of 10 healthy volunteers during the winter season. Each week blood was drawn and nose/throat swabs were taken to check for the presence of viruses. Eight out of ten individuals experienced at least one episode of common cold. In 5 individuals the virus was identified. Unfortunately, we did not find RSV (tabel IA). However, viral detection was not optimal, because sampling was performed only once a week.

After collection of all the blood samples the RSV-specific memory CD8$^+$ T cell response was investigated. Because we had previously found that RSV-specific CD8$^+$ T cells specific for single RSV epitopes were present at low frequencies(16),

we cultured CFSE-labeled PBMC with synthetic peptides for 6 days. On day 6 the presence of RSV-specific CD8+ T cells was determined by tetramer staining in the cell population with low CFSE, i.e. the cells that had divided in the cultures. In all patients with either clinical symptoms or asymptomatic infection we observed a reduction of RSV-specific cells during respiratory infection (Figure 1a-d).

Determination of exact frequencies of RSV-specific memory CD8+ T cells was impossible because of the in vitro expansion phase that was necessary to visualize RSV-specific T cells. To investigate the disappearance of virus-specific CD8+ T cells during infections with heterologous viruses without the need of in vitro expansion, we also determined the influenzavirus (FLU) specific T cell numbers that

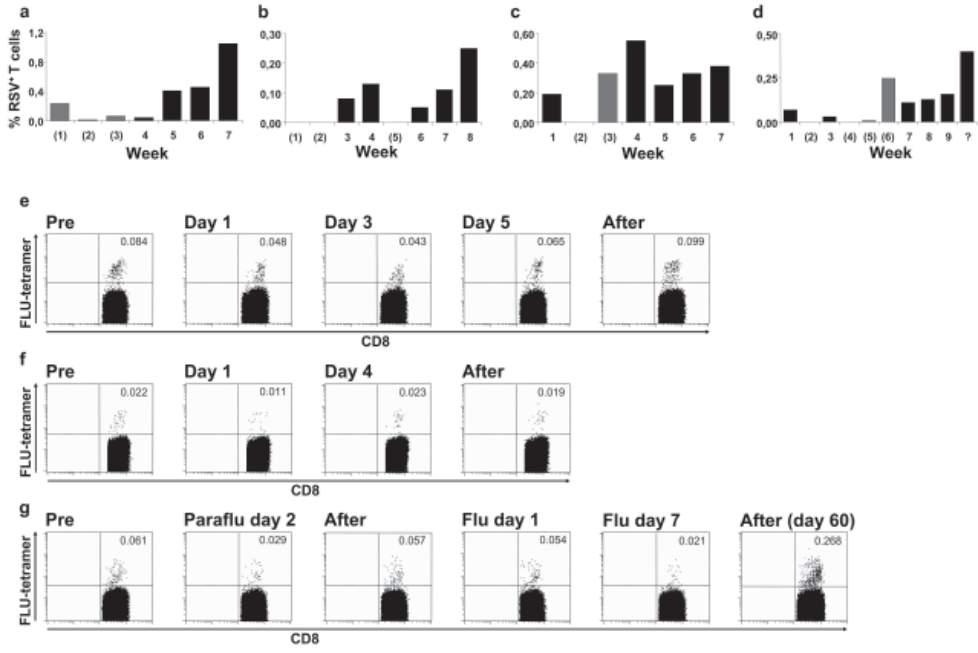

Figure 1a-d: *RSV-specific CD8+ T cell numbers in blood during episodes of common cold. CFSE labeled PBMC of healthy blood donors were stimulated for 6 days with peptide in the presence of IL-2. On day 6 cells were stained with the appropriate tetramer. Cells were gated based on negative 7AAD staining and positive CD8 staining. The percentage of tetramer+ CFSE negative cells of total CD8+ T cells is given for each time point. Comparable results were obtained in 8 patients.* ***a:*** *PBMC of an HLA-A1 positive individual were stimulated with the peptide YLEKESIYY (RSV-$M_{229-237}$). Nose/throat swabs showed a coronavirus infection in week 1 and an influenzavirus infection in week 2.* ***b:*** *PBMC of an HLA-A3 positive individual were stimulated with the peptide RLPADVLKK (RSV-$M2_{151-159}$). Nose/throat swabs were negative at all time points.* ***c,d:*** *Two HLA-A1 positive donors were stimulated with YLEKESIYY (RSV-$M_{229-237}$). For donor c influenzavirus was detected in week 2, donor d had a negative PCR in week 2, and rhinovirus in week 4. Gray colored bars and brackets around week numbers indicate that the individual had symptoms of a common cold on the day of specimen collection.* ***e-g:*** *Influenzavirus specific CD8+ T cells in PBMC during rhinovirus infection. PBMCs of three HLA-A2 positive donors were stained with the HLA-A2 tetramer loaded with the influenzavirus peptide $M1_{58-66}$: GILGFVFTL. Samples were taken 2 weeks before, on several time points after onset of clinical symptoms and after resolution of the infection. Cells were gated on a CD8+ gate and a live lymphocyte gate based on the forward/side scatter.*

Tabel I: Characteristics of blood donors

A: Characteristics of volunteers that were followed for RSV-specific CD8$^+$ T cell responses

Patient	Age	HLA type[a]	Symptoms[b]	virus
1	51	A1	yes	Influenza
2	27	A1,A3	yes	-
3	29	A1	no	Rhino
4	32	A1	yes	Influenza
			yes	Corona
5	34	A1	yes	Influenza
6	28	A3	yes	-
7	27	A1	yes	Rhino
8	26	A1	yes	-
9	28	A3	yes	-
10	30	A1	no	-

B: Characteristics of volunteers that were followed for influenzavirus-specific CD8$^+$ T cell responses

Patient	Age	HLA type	symptoms	virus
1	30	A2	yes	-
2	31	A2	yes	rhinovirus
3	34	A2	no	coronavirus
4	49	A2	yes	parainfluenzavirus
			yes	influenzavirus

[a]HLA molecule used for tetramer studies
[b]Symptoms of upper respirator tract infection i.e. runny nose, coughing, sore throat.

are present in higher frequencies in peripheral blood. In these experiments we performed direct staining with HLA tetrameric complexes. At several time points before, during and after the onset of clinical symptoms of respiratory infection, blood samples of HLA-A2 positive donors (Table Ib) were collected. One donor had an undetectable FLU response. In all other donors a reduction but not complete disappearance of FLU-specific CD8$^+$ T cells was found in peripheral blood during clinical symptoms caused by unrelated respiratory viruses (Figure 1e-g). In all donors the percentage of FLU-specific cells in peripheral blood was restored between day 4 and 7 after the onset of symptoms. One donor that experienced consecutive infections with parainfluenza and influenzavirus showed a disappearance of FLU-specific T cells during both episodes. However, during the influenzavirus infection, FLU-specific cells were still decreased in blood by day 7. Sixty days after infection, the percentage of FLU-specific CD8$^+$ T cells was almost 4 fold higher than before onset of symptoms. In most donors the number of PBMC recovered was lower during infection, and the percentage of CD8$^+$ T cells within the CD3$^+$ cell population decreased. Therefore, it appeared that the absolute number of FLU-specific cells also decreased. FLU-specific CD8$^+$ T cells were CCR7$^-$ at all time points, and partly CD45RO$^+$. No change in phenotype occurred during infection. These results

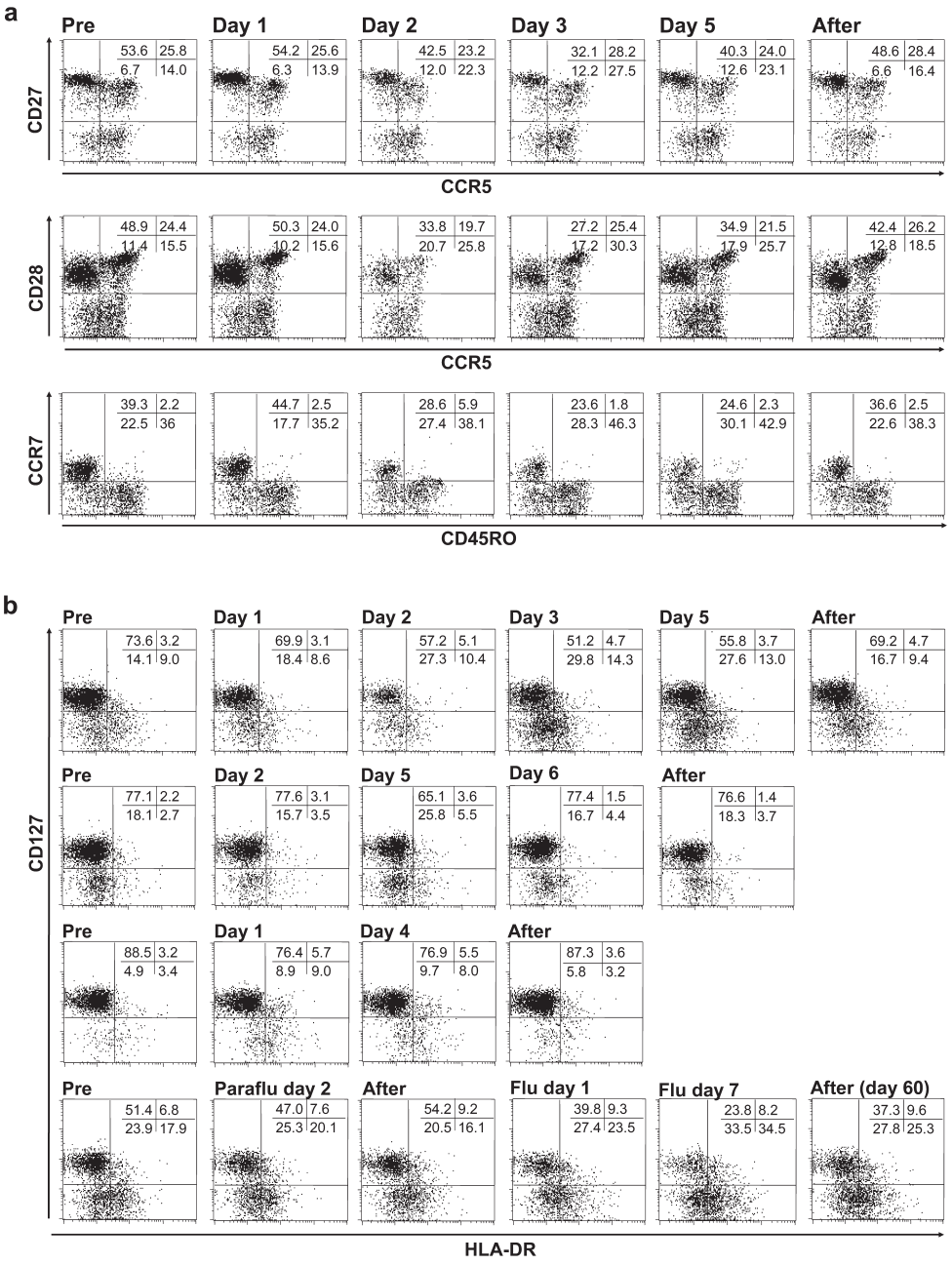

Figure 2a: *Dynamics of the CD8+ T cell population in blood during rhinovirus infection. PBMC were collected 2 weeks before, on several days after onset of symptoms and two months after resolution of the infection and stained for differentiation and activation markers. Similar patterns of population shifts were found in 4 donors.* ***b:*** *Decrease in naïve/memory CD8+ T cell populations (CD127+) and increase in effector population (CD127-) shown in four donors with respiratory infections. Cells were gated for live (FSC/SSC) CD8+ T cells.*

demonstrated the efflux from the blood of $CD8^+$ T cells directed against respiratory viruses during episodes of upper respiratory tract infection.

A significant increase in $CD8^+$ T cells with an effector phenotype is found in PBMC of adults with common colds

$CD8^+$ T cells directed against a single epitope of influenzavirus obviously represent only a minor part of the memory pool of T cells directed against respiratory viruses. Therefore, we next analyzed the dynamics of the entire $CD8^+$ T cell population in blood. During upper respiratory tract infection the percentage of activated effector cells i.e. $CD27^-$, $CD28^-$, $CD45RO^+$, $CCR7^-$ $CD127^-$, $HLA\text{-}DR^+$ cells increased (Figure 2). The peak of the response was found on day 3-7 after the onset of symptoms. The expression of chemokine receptors on these cells ($CCR5^+$, $CCR7^-$), indicated the capacity to migrate into inflamed tissue. The percentage of $CD8^+$ T cells with the effector phenotype varied among donors between 4 and 17% of total $CD8^+$ T cells.

During respiratory infections there is an influx of proliferating highly activated $CD8^+$ T cells into the airways

During infection with a respiratory virus the airway epithelium is the tissue where viral antigens are expressed and innate immune responses create an inflammatory environment attracting effector T cells. To test whether the activated effector

Tabel II: Characteristics of tracheostoma patients

Patient	Age	HLA-type	Underlying disorder	virus
1	2	A1	Subglottic stenosis	RSV
3	4,5	A1	Pierre Robin sequence	rhinovirus
4	3,5	A3	Spina bifida aperta	RSV
5	3	*	trisomy 11	RSV
6	6,5	A3	Congenital muscle dytrophy 1a	RSV
7	12,5	A2	Nemaline myopathy	unknown
11	16	A1	Spina bifida aperta	unknown
12	17	B7	Spina bifida aperta	rhinovirus
13	5	A2,B7	Mitochondrial complex 1 deficiency	rhinovirus->RSV->coronavirus -> rhinovirus
14	1	A1, B7	Longhypoplasia eci	coronavirus->rhinovirus and hMPV-> adenovirus and hMPV and rhinovirus-> influenzavirus
17	2,5	*	Unknown retardation	rhinovirus
18	2	A2	Spinal cord lesion	rhinovirus
19	14,5	A1	Spinal muscle dystrophy	rhinovirus
20	0.7	A1,A3,B7	Down Syndrome	unknown
22	1,2	A1	Crouzon Syndrome	RSV

**HLA type of these children did not include HLA-A1, -A2, -A3 or –B7, the HLA types for which tetramers were available.*

cells migrated from blood to the airways, we followed CD8+ T cell responses in the large airways during respiratory infection. Twenty children between 6 months and 18 years of age with a tracheostoma were followed during one winter season. On day 2,5,8,15,28 and 56 after the onset of cold symptoms the CD8+ T cell response in tracheal aspirate was studied. In 15 children 21 episodes of upper respiratory tract infection were studied (Table II). In most children new infections started before the study period of the previous infection was finished. While there was a higher influx of CD8+ T cells compared to CD4+ T cells early in the infection (day 2), the CD4/CD8 ratio increased from day 5 to reach initial levels (Figure 3a). During the first days of infection the number of HLA-DR+ cells increased (Figure 3b) to normalize to steady state levels between day 15 and 28. Expression of the activation marker CD38 was already high on a substantial fraction of CD8+ T cells in the uninfected airways and peak responses varied between day 2 and 5 of common colds. When the peak of the response in the first week was compared to the last time point measured on either day 28 or 56, there was a significant upregulation in the beginning of the response (p=0.011, data not shown). While in almost all patients >70% of CD8+ T cells expressed the cytotoxicity marker

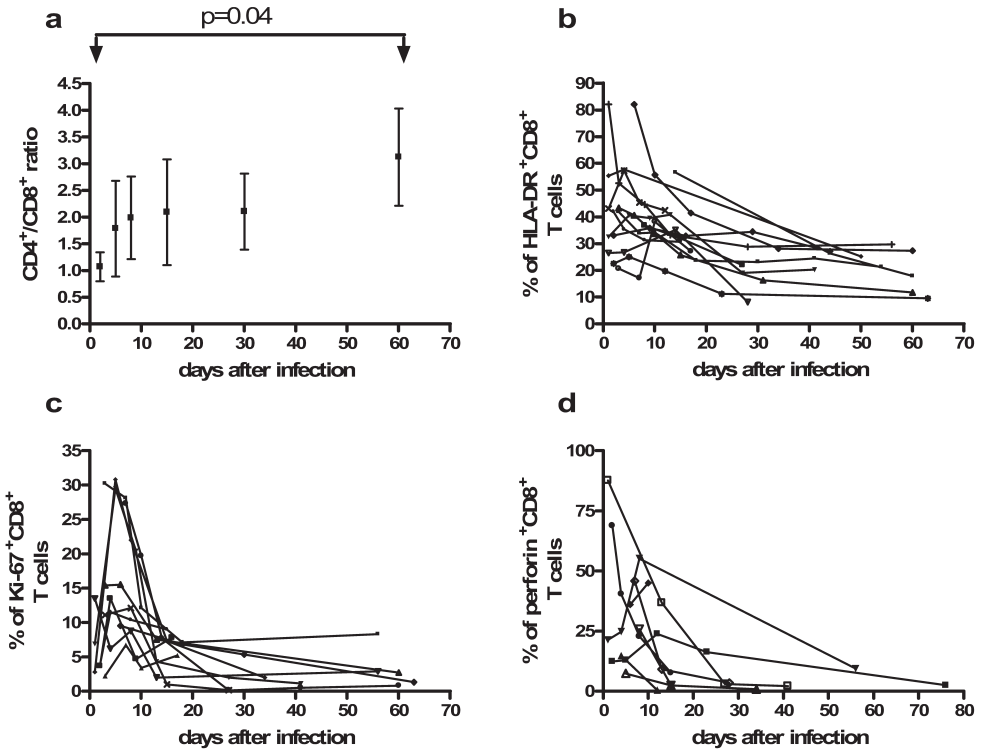

Figure 3: *Activation status of CD8+ T cells in tracheal aspirate during common colds. In 15 children TA was collected at 6 time points after onset of symptoms. TA cells were stained for several activation markers. Missing values are either due to interfering new infections or limited numbers of cells in the aspirate.*

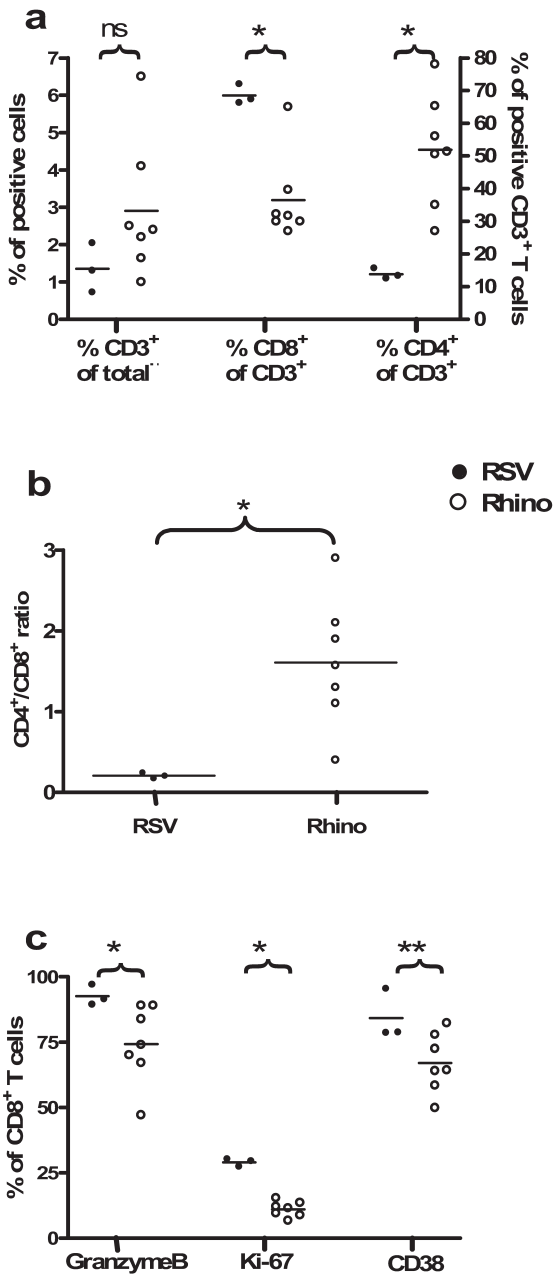

Figure 4: *Comparison of T lymphocyte populations in TA samples during RSV and rhinovirus infections.* ***a:*** *Percentage of $CD3^+$ lymphocytes of total cells in TA of patients infected with ● RSV, ○ rhinovirus. $CD4^+$ and $CD8^+$ T cells are depicted as the percentage of total live $CD3^+$ TA cells.* ***b:*** *Comparison of CD4/CD8 ratio in the same patients.* ***c:*** *Activation markers on $CD8^+$ T cells present in TA samples of RSV and rhinovirus infected patients. The values depicted are from the samples taken at the peak of the response usually day 2 or 5. Percentages of marker positive live $CD8^+$ T cells are shown, based on negative 7AAD staining and positive $CD8^+$ staining. * $p<0.02$, ** $p<0.05$, ns = not significant.*

GranzymeB during infection, the basic level of GranzymeB expression after 28 days varied tremendously among patients between 10 and 70% (data not shown). The number of T cells expressing perforin, another component of cytotoxic granules, declined much faster and less than 10% of CD8+ T cells was found positive on day 30 (Figure 3c). Proliferating CD8+ T cell numbers visualized by Ki-67 staining peaked between day 2 and 5 with up to 30% proliferating CD8+ T cells (Figure 3d). In all patients, CCR5, CD45RO and CD27 were expressed in more than 75% of CD8+ T cells at all time points. Twenty-five to fifty percent of CD8+ T cells expressed CD28. CD25 and CCR7 were expressed by less than 5% of CD8+ T cells. No variation in these values was found at the different time points of sampling (data not shown).

CD8+ T cells show higher rates of proliferation and activation during infections with RSV than during rhinovirus infection

To study the role of CD8+ T cells during infections with different viruses, we compared T cell responses in 3 children infected with RSV, and 7 episodes of rhinovirus infections. At the peak of the response RSV-infected children had a higher influx of CD8+ T cells into the airways, and significantly lower percentages of CD4+ T cells, resulting in lower CD4/8 ratios (Figure 4a and b) The number of proliferating (Ki-67+) CD8+ T cells and the number of cells expressing GranzymeB or CD38 were significantly higher during RSV infection (Figure 4c). In one child with four consecutive infections: rhinovirus, RSV, coronavirus and rhinovirus, we observed more proliferating CD8+ T cells during RSV- and coronavirus infection than during two rhinovirus infections, while the response was comparable between the two rhinovirus infections (Figure 5a-c).

Curve fitting modeling for CD8+ T cell activation upon respiratory infection

When using the Mann Whitney U test one can only compare differences between two groups of data, in this case the differences in marker expression between RSV and rhinovirus at one time point per test. However, we wanted to study

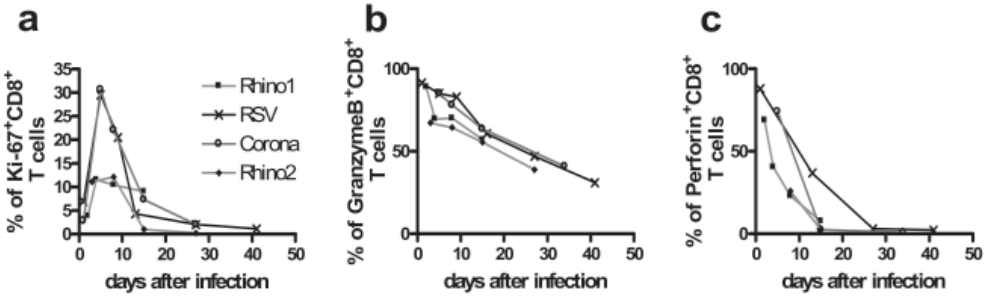

Figure 5: *Activation markers on CD8+ T cells in TA of a five year old patient (no 6, Table II) who suffered from 4 consecutive respiratory infections, respectively: rhinovirus, RSV, coronavirus and rhinovirus. Cells were gated on a CD8+ and live lymphocyte gate based on the forward/side scatter. Black line: RSV; dark grey line: coronavirus; light grey line: rhinovirus.*

the dynamics of marker expression through time. To investigate whether general patterns could be derived from the combined individual data including all time points, and to test whether different viral infections would lead to different marker expression in time, models were constructed. The curve-fitting method used is described in appendix 1. Figure 6 shows the results for each of the parameters. The data points given in the plots are corrected for the estimated starting value (log(time) = 0, or time = 1) of the individual infected child b_{0j} relative to the estimated mean starting value b_0, and were thus shifted by a factor ($b_0 - b_{0j}$) towards the regression line. These corrected data provide a better insight in the 'correctness' of the model, allowing us to study the dynamics through time without individual base line variability. By applying simple linear regression techniques we were able to fit curves that explained the data fairly well. The optimal regression functions have R^2 values between 0.73 (GranzymeB) and 0.85 (Ki-67), suggesting a reasonable to high correlation between the data and the regression curve. There were no different curves found for children with RSV or rhinovirus infections with regard to GranzymeB and HLA-DR, but marked differences were found for CD38, Ki-67 and Perforin. Perforin was the only marker where starting values for RSV and rhinovirus infections were notably different. The curves suggest an exponential decay (one compartment) for both GranzymeB and Perforin and an exponential increase followed by exponential decay (two compartments) for HLA-DR and Ki-67. The case of CD38 is apparently more complicated: after an initial rise, the CD38 expression starts to decay, but at a fairly slow rate, after which a form of stabilization seems to occur.

Bystander CD8$^+$ T cells are recruited to the airways during viral respiratory infections

We next tested the specificity of CD8$^+$ T cells attracted to the airways using HLA-tetrameric complexes. RSV and influenzavirus tetramers were applied in HLA-A1, -A2, -A3 or -B7 positive donors. Four patients were followed during 7 episodes of common cold. RSV-specific CD8$^+$ T cell responses were studied during 3 infections with rhinovirus and 1 infection with coronavirus. The FLU-specific CD8$^+$ T cell response was studied during 4 episodes of rhinovirus, 1 corona- and 1 RSV infection. During each episode influenzavirus- and/or RSV-specific CD8$^+$ T cells appeared in tracheal aspirate. T cell numbers peaked before day 10 after the onset of symptoms and disappeared soon after. In the child with 4 episodes of common cold, virus-specific T cells appeared with every episode and T cell numbers declined in symptom free periods (Figure 7a). Peak responses against single epitopes, measured by tetramer staining, varied between 0.1 and 0.83 of CD8$^+$ T cells. In one child we were able to compare the level of tetramer$^+$ cells simultaneously in TA and blood. Although the percentage of virus-specific cells is much lower in blood than in the airways, we found that influenzavirus-specific cells disappeared from blood and at the same time appeared in TA (Figure 7b). Thus CD8$^+$ T cells specific for respiratory viruses are attracted to the airways during unrelated respiratory infections which results in a temporary decline in peripheral blood.

Secondary virus-specific CD8+ T cell responses are of higher magnitude and of longer duration than bystander responses

During infection with RSV the number of RSV-specific CD8+ T cells in tracheal aspirate exceeded levels during infection with other viruses. When RSV-specific CD8+ T cell responses were compared between children with RSV infection and other viral infection, not only the magnitude was higher, but also the duration of the response was significantly extended in RSV-infected individuals (Figure 8). Similar observations were made for influenzavirus-specific responses (data not

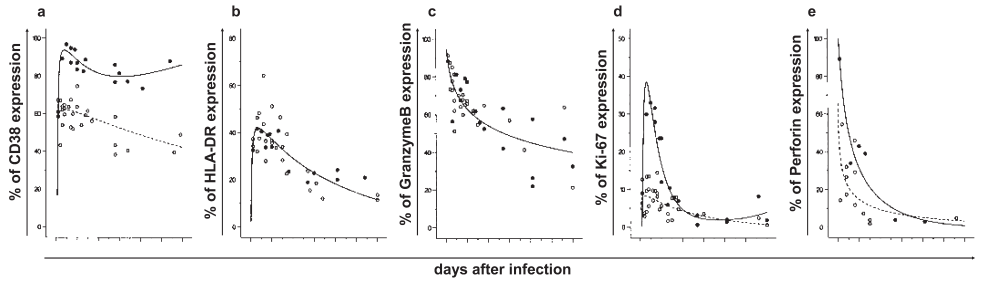

Figure 6: *Modelling of T cell responses during viral infection. Curve fitting models were constructed by applying a set of following linear models to each of the infectious parameters:*
τ*(parameter value)* $= \Sigma_i [\, b_i \times \{log(time)\}^i] + \Sigma_i [\, I \times c_i \times \{log(time)\}^i]$ *(i=0 to 3)*
where $\tau(pv) = \alpha = 2 \times arcsin(\sqrt{(pv/100)})$ *and I is an indicator function with values 0 for the RSV group and 1 for the rhinovirus group.*
For each parameter an optimal formula was chosen which was used for plotting the (backtransformed) regression functions. This backtransformation used the general formula $pv = 100 \times sin^2(\alpha/2)$. *The points in the Figure are corrected for initial differences between children. The solid dots refer to data from the RSV group, the open circles to the rhinovirus group. Where there are 2 lines given, the solid line refers to the RSV group, the dashed line to the rhinovirus group.*

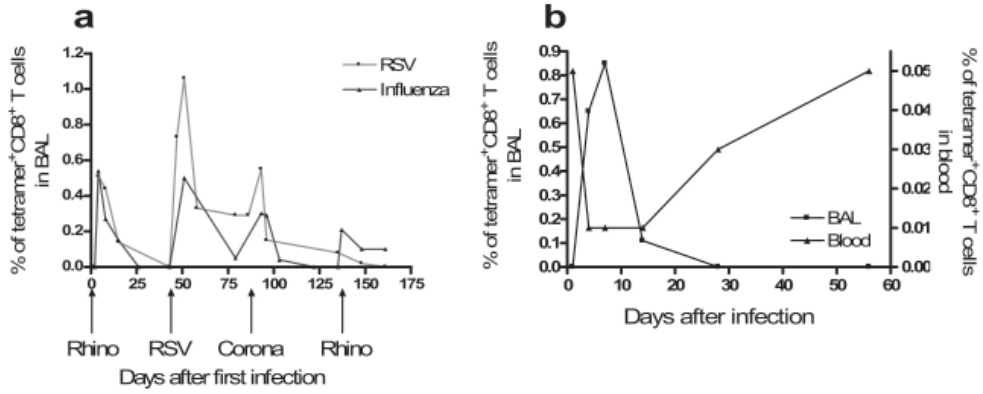

Figure 7: *Migration of bystander cells to the airways.* ***a:*** *In one patient with 4 consecutive infections, RSV and influenzavirus-specific CD8+ T cells were identified in TA by tetramer staining.* ***b:*** *Influenzavirus-specific cells appear in TA at the same time they disappear from blood. At 6 time points after onset of symptoms due to rhinovirus infection PBMC and TA of an HLA-A2 positive patient were stained with HLA-A2 specific tetrameric complexes loaded with the influenzavirus peptide* $M1_{58\text{-}66}$*. In both figures the percentage of tetramer+ cells of total live CD8+ T cells are shown.*

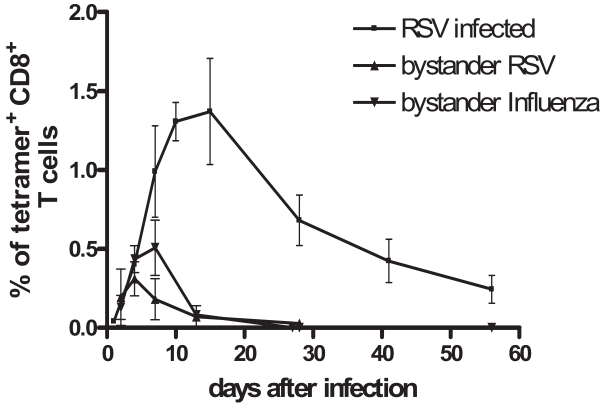

Figure 8: *RSV-specific CD8⁺ T cells in TA are present in higher quantities and are detectable for a longer time period after infection with RSV than after infection with an unrelated virus. Percentages of RSV-specific CD8⁺ T cells in TA of 5 patients with RSV were compared to the percentages of RSV-specific CD8⁺ T cells in 4 patients with respectively 3 rhinovirus and 1 coronavirus infection, and to the percentage of FLU-specific CD8⁺ T cells in 6 patients, 4 with rhinovirus-, 1 coronavirus and 1 RSV-infection. The percentage of tetramer⁺ cells of total live CD8⁺ T cells is shown.*

shown). Thus secondary anti-viral CD8⁺ T cell responses are of higher magnitude and longer duration than bystander responses during unrelated infections.

Discussion

The respiratory tract is one of the largest surface areas of the body and hence requires effective immune surveillance to prevent substantial damage caused by infectious agents and environmental antigens. Even in the absence of local infections the lung is the peripheral site that effectively attracts effector/memory T cells, a process that requires chemokine mediated recruitment from the vasculature into the tissue (17). This process of effector cell recruitment is enhanced during infection when local chemokine production is upregulated. When infections are cleared, substantial numbers of T cells remain detectible in the lung tissue and airways (18;19). In humans the dynamics of T cell recruitment and maintenance in the lung has poorly been addressed.

The role of different T cell populations that are recruited into lung tissue and the airways in the process of viral eradication is still unclear, as is the mechanism by which the balance between the different T cell populations is re-instilled in peripheral blood after viral clearance. The observations made in the present study in human patients showed that in the circulation large shifts in CD8⁺ T cell populations occur, closely resembling the processes described in mouse models. All of the three phases of the T cell responses observed during murine respiratory infec-

tions, reflecting three waves of different populations of memory cells contributing to the response in the airways, could be recognized in our experiments (20;21). The first phase is represented by effector/memory CD8+ T cells that are already present in the lungs. Indeed, long after clearance of a respiratory infection, cells present in tracheal aspirate are of the effector/memory type. The second phase of memory cells that are attracted to the inflammatory site in our experiments are identified as influenzavirus-specific cells and RSV-specific cells recruited during heterologous respiratory infections (22;23). Indeed, like it was described in the murine model, this nonspecific response composed of effector memory cells that originate from T cell pools present in the circulation, waned faster than the virus-specific response during homologous infections. While during heterologous infection RSV-specific cells disappeared from the airways within two weeks, during secondary RSV infection RSV-specific CD8+ T cells remained detectable up to 58 days. The sustained response of virus-specific CD8+ T cells during homologous infections is caused by the contribution of memory cells that encounter antigen-presenting cells in the lymphnodes draining the infection site. This induces T cell proliferation and effector function and the ability to migrate to the inflammatory site. These cells of the third wave are more apoptosis resistant than non-specific bystanders due to exposure to cognate antigen. Furthermore, a prolonged presence of antigen presenting cells that display viral antigens in the draining lymphnodes have been documented to be involved in a sustained specific anti-viral response (12). However, this prolonged antigen presentation might be less pronounced during secondary infections when infectious material is rapidly cleared and lower viral loads are reached. The observation that the influx of dividing i.e. Ki-67+ cells decreased in the airways before day ten while peak RSV-specific CD8+ T cell numbers did not decline until day 15 may indicate that prolonged survival times determined longer persistence of specific T cells during homologous infections.

It has been suggested that bystander recruitment early during respiratory viral infection is the result of the proinflammatory milieu at the site of infection created by the secretion of several chemokines (6). The rapid recruitment of all T cells to the site of inflammation provides a fast defense to previous experienced infections, and enables cross-reactive CD8+ T cells to contribute in heterologous infections. After recruitment to the airways, these bystander cells are presumably lost by apoptosis or phagocytosis, as in the mouse it has been shown that lung airway memory T cells are unable to migrate from the airways back into the circulation in contrast to T cells present in the lung interstitium (6;10;17;24). In our study we showed that virus-specific memory T cells re-appear in the blood after disappearance from the airways. Currently, we do not know whether in humans these CD8+ T cells migrated back from the airways, were redistributed from other sites or were replenished by proliferation.

Compared to children with Rhinovirus infections we found higher fractions of CD8+ T cells in the airways expressing GranzymeB, Ki-67 and CD38 in children infected

with RSV. Proliferation and cytotoxicity during RSV infection might be more intense by a higher viral load compared to rhinovirus infection and by the fact that rhinovirus infection is more limited to the upper respiratory tract than RSV. Indeed, all children with RSV infection were more severely ill than children with rhinovirus infection. When modeling these data using linear regression techniques, we found no difference for GranzymeB between the rhinovirus and RSV patient groups. This result deviates from the Mann Whitney U test which must be due to the different methods in testing. For the construction of our models, all data points were used. Although during the first few days the percentage of GranzymeB positive $CD8^+$ T cells seems to be smaller in the rhinovirus group, this does not lead to different models: the difference in slopes is apparently too small to be picked up by the regression technique. When applying the Mann Whitney U test, we can compare data from two groups at only one (time) point at a time. Since it is impossible to identify the exact time of actual infection, and because the first point of sampling differed between patients (mostly due to recognition of infection in a patient group with a continuous production of mucus due to irritation of their tracheostoma) we decided to compare peak responses in the two patient groups, rather than responses at a predetermined time point. Although the differences in percentages of GranzymeB expression between the two groups were small, they were every time in favor of the RSV group.

The possibility to obtain tracheal aspirate at different time points after infection provided a unique opportunity to study developing secondary immune responses at the local infection site. Using HLA tetrameric complexes we were able to study the dynamics of $CD8^+$ T cells with particular specificities. However, due to the fact that the single epitopes used in the HLA tetrameric complexes only represent a small fraction of virus-specific cells and the number of cells recovered from tracheostomal aspirates was low, it was impossible to obtain a full phenotypic characterization of the T cells that migrated to the lungs. Moreover, studies in patients will always have the disadvantage that the experimental setup cannot be controlled. Therefore, the exact time of virus inoculation was unknown, as was the viral load at the time of infection. Furthermore, the patient group was heterogeneous with respect to age and underlying disease condition and the history of unknown previous respiratory infections. We excluded children with immune disorders and children using immune suppressive medication to avoid immunological aspects of underlying diseases as much as possible. Due to the fact that the tracheostoma is an open connection between the outside environment and the trachea, the lower respiratory tract of tracheostoma patients is continuously exposed to bacterial infections. About 95% of tracheostoma openings in adults contain bacterial colonization, while in 30-46% of deeper brush cultures bacterial growth was found (25;26). It has previously been shown that this results in a persistent base line inflammation. Compared with healthy controls there is a larger influx of neutrophils in tracheostoma patients. Nevertheless, the tracheostoma patients provided us with unique material that is impossible to obtain from healthy individuals and the observations made provided valuable information on the dynamics of second-

ary T cell responses at the local infection site during the course of a respiratory infection. In summary these studies provided insight in human T cell dynamics upon viral infections. Studying human T cell responses in both peripheral and central sites will contribute to a broader understanding of the complex human T cell immunology necessary for future development of vaccines.

Acknowledgements

The authors would like to thank all patients, parents and blood donors for their cooperation. We would also like to thank Dr. Anne Schilder, Dr. Mike J Kampelmacher, Dr van Kesteren, and Prof. Adrianus J. van Vught for their help with inclusion of the patients. Mariska E.A van Dijk and Laura van Drie are thanked for technical assistance.

Appendix 1

Curve fitting models were constructed by applying the following linear models to each of the infectious parameters:

τ(parameter value) = Σ_i [$b_i \times \{\log(\text{time})\}^i$] + Σ_i [$I \times c_i \times \{\log(\text{time})\}^i$] (i=0 to 3) (Eq. 1)

where $\tau(pv) = \alpha = 2 \times arcsin(\sqrt{(pv/100)})$ and I is an indicator function with values 0 for the RSV group and 1 for the rhinovirus group. Consequently, the following set of regression formulas were fitted:

(a) $\tau(pv) = (b_0 + c_0)$

(b) $\tau(pv) = (b_0 + c_0) + b_1 \times \log(time)$

(c) $\tau(pv) = (b_0 + c_0) + b_1 \times \log(time) + b_2 \times [\log(time)]^2$

(d) $\tau(pv) = (b_0 + c_0) + b_1 \times \log(time) + b_2 \times [\log(time)]^2 + b_3 \times [\log(time)]^3$

(e) $\tau(pv) = (b_0 + c_0) + b_1 \times \log(time) + c_1 \times (group=rhino) \times \log(time)$

(f) $\tau(pv) = (b_0 + c_0) + b_1 \times \log(time) + b_2 \times [\log(time)]^2 + (group=rhino) \times \{ c_1 \times \log(time) + c_2 \times [\log(time)]^2 \}$

(g) $\tau(pv) = (b_0 + c_0) + b_1 \times \log(time) + b_2 \times [\log(time)]^2 + b_3 \times [\log(time)]^3 + (group=rhino) \times \{ c_1 \times \log(time) + c_2 \times [\log(time)]^2 + c_3 \times [\log(time)]^3 \}$

Formulae b-d form a set of increasing polynomials without interaction between group and time, formulae e-g form a similar set allowing for this interaction. The 'best' formula was chosen by considering the simplicity of the formula (no statistical improvement by adding interaction or higher-order terms) and a low residual mean square error. A different intercept was applied when ($c_0 > 0$).

The final formulae chosen can be summarised as depicted in Table III.

Log(time) as the time domain was used because the samples were taken approx. 2, 5, 8, 16, 30 and 60 days after the infection had occurred – this is close to a ^{2}log-scale. This allowed more appropriate curve fitting. The arcsine-transformation is known to stabilise the variance of fractions, and thus of percentages.

The data given in Table III were used to plot the (backtransformed) regression functions using the general formula $pv = 100 \times sin^2(\alpha/2)$.

Tabel III: Formulae used to plot regression function

Parameter	b_0	b_1	b_2	b_3	c_0	c_1	c_2	c_3	R_2
τ(CD38)	1,77	1,43	-0,725	0,100	-	1,39	-0,733	0,110	0.84
τ(Granzyme)	2,43	-0,259	-	-	-	-	-	-	0.74
τ(HLA-DR)	1,27	0,219	-0,0876	-	-	-	-	-	0.75
τ(Ki-67)	0,570	1,56	-0,929	0,131	-	1,52	-0,910	0,135	0.85
τ(Perforin)	2,51	-0,572	-	-	1,17	-0,332	-	-	0.73

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RSV-specific CD8$^+$ memory T cell responses in the elderly

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J. Infect. Dis. 2005;191:1710-1718

A life full of wisdom

Summary

Background
We investigated RSV-specific $CD8^+$ memory T cell responses in healthy control participants (*n*=31) and in patients with chronic obstructive pulmonary disease (COPD) (*n*=9) with respect to frequency, memory phenotype, and proliferative requirements.

Methods
The properties of RSV-specific $CD8^+$ T cells were analysed by use of RSV tetramers. The proliferative requirements of RSV-specific $CD8^+$ T cells were analyzed by culture of PBMC with RSV peptide in combination with distinct cytokines.

Results
RSV-specific $CD8^+$ memory T cells showed a high level of expression of CD27 and IL-7Rα, and a low level of expression of CCR7. In the healthy participants, the frequency of RSV $tetramer^+$ $CD8^+$ T cells was significantly lower than the frequency of influenzavirus A (FLU) $tetramer^+$ $CD8^+$ T cells (p=0.0001). In contrast to FLU $tetramer^+$ $CD8^+$ T cells, we could detect RSV $tetramer^+$ $CD8^+$ T cells in the subgroup of elderly healthy participants (age ≥55 years) and in the patients with COPD only after in vitro expansion. Expanded RSV-specific T cells produced IFN-γ and granzymeB.

Conclusion
We provide evidence that a pool of functional RSV-specific $CD8^+$ memory T cells persists in the peripheral blood of healthy individuals and patients with COPD. Low numbers of RSV-specific memory T cells in the elderly and in patients with COPD may explain the increased susceptibility for RSV infection in these populations.

Introduction

Respiratory syncytial Virus (RSV) circulates through communities during annual epidemics and causes serious infections in susceptible individuals. In young children, RSV can cause severe bronchiolitis, resulting in high morbidity. Although RSV infection is widespread and virtually everyone encounters RSV from an early age on, reinfections occur frequently during life (1). In healthy adults, reinfections lead to mild symptoms and often remain unrecognized. Recent studies have shown, however, that immunocompromised individuals, elderly individuals, and adults with underlying cardiopulmonary conditions (e.g. COPD), are at risk for severe RSV infection (2-5). These clinically severe manifestations of RSV infection indicate that immunity to RSV is not complete in these individuals.

Presently, most data on cellular RSV immunity are obtained from animal studies. Studies in mice have shown that cytotoxic T lymphocytes (CTLs) contribute to the clearance of RSV-infection (6). In addition, it has been shown that patients with defects in T cell immune responses do not effectively clear the virus, indicating that T cells do indeed play an important role in the control of RSV infection (7). Therefore, it may well be supposed that, analogous with other viral infections, CTL responses to RSV contribute to the control of infection and to the prevention of reinfection in humans. The establishment of a virus-specific $CD8^+$ memory T cell pool is essential to achieve long-term immunological protection. Advances in the characterization of virus-specific $CD8^+$ T cells have provided insight into the development of immunological memory for latent and cleared viral infections (8;9). The development of virus-specific $CD8^+$ memory T cells in humans has been shown to be related to the expression of different cell-surface molecules, such as CD27, CD28 and CCR7. Upon primary infection, virus-specific $CD8^+$ T cells are activated and differentiate into effector T cells ($CD27^+CD28^+CCR7^-$) that fight the virus. After clearance of the virus, the virus-specific T cell pool contracts, and a small memory T cell pool that can undergo rapid reactivation on reinfections persists. Virus-specific memory T cells specific for latent viruses may have distinct phenotypes, ranging from $CD27^+CD28^+CCR7^-$ to $CD27^-CD28^-CCR7^-$ (8). With respect to cleared viruses, He et al. have demonstrated that influenzavirus A (FLU)-specific T cells have a $CD27^+$ phenotype (10). Recently, in a murine model of lymphocytic choriomeningitis virus infection, it was found that memory T cells also express Interleukin (IL)-7Rα (11). The ability to respond to the homeostatic cytokine IL-7 may provide memory T cells with the ability to persist for prolonged periods in the absence of antigen. Until the present study, no data have been available on the expression of this cytokine receptor on virus-specific human $CD8^+$ T cells.

Little is known about the formation and characteristics of $CD8^+$ memory T cell responses to RSV in humans. Insight into the long-term maintenance of RSV-specific memory T cells in humans after natural RSV infection and into the characteristics of RSV-specific $CD8^+$ memory T cells is essential in the understanding of memory formation and may explain the susceptibility patterns for reinfection

in certain vulnerable groups, such as the elderly and patients with COPD. In the present study, we analyzed the size of the RSV-specific $CD8^+$ memory T cell pool in peripheral blood of young healthy control participants, elderly healthy control participants, and patients with COPD. We enumerated RSV-specific $CD8^+$ T cells by use of tetramers containing two recently discovered immunodominant HLA class I epitopes for RSV (12;13). The frequency of RSV-specific $CD8^+$ T cells was determined in relation to the frequency of FLU-specific $CD8^+$ T cells. In a subgroup of the healthy participants, RSV $tetramer^+$ $CD8^+$ T cells were characterized with respect to the expression of CD28, CD27, CCR7, IL-7Rα, and activation markers CD38 and HLA-DR. Furthermore, we determined the functional characteristics of RSV $tetramer^+$ $CD8^+$ T cells with respect to proliferative requirements.

Participants, materials, and methods

Study population

The healthy participants (total *n*=31; median age, 54 years; age range, 30-86 years), who were recruited via local advertisements, had no history of pulmonary disease and had normal lung function. The patients with COPD (*n*=9; median age, 69 years; age range, 50-76 years) were recruited from the outpatient clinic of the Academic Medical Center, Amsterdam. Inclusion criteria for patients with COPD were a smoking history of at least 15 pack-years and a forced expiratory volume in 1 s (FEV_1):vital capacity ratio<0.7. The reversibility of FEV_1 was ≤ 12% of baseline after inhalation of 400 microgram of salbutamol (Airomir, Pharma,). Spirometric evaluation of the healthy participants and the patients with COPD was performed as recommended elsewhere (14). All of the patients with COPD continued their medication, which consisted of bronchodilatator therapy and inhaled glucocorticosteroids. The healthy participants and the patients with COPD had no current symptoms of FLU or RSV infection. All of the healthy participants > 65 years old and all of the patients with COPD (*n*=12 and *n*= 9, respectively) had been immunized with influenzavirus vaccine 4-6 months before inclusion. All of the study participants provided written, informed consent, and the study was approved by the Medical Ethics Committee of the Academic Medical Center of the University of Amsterdam.

Peptides

The HLA-A1 RSV-$M_{229-237}$ peptide YLEKESIYY, HLA-B7 RSV-NL_9 peptide NPKASLLSL (13), HLA-A1 FLU-NP_{44-52} peptide CTELKLSDY (15) and the HLA-A2 FLU-$M1_{58-66}$ peptide GILGFVFTL (16) were purchased from the IHB-LUMC peptide synthesis library facility (Leiden, The Netherlands). The peptides were dissolved in dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) (5 mg/mL).

Peptide-major histocompatibility complex (MHC) class I tetramers
The following RSV and influenzavirus A (FLU) tetramers were used: allophycocanin (APC)-conjugated HLA-A1 tetramer loaded with the FLU-NP_{44-52} CTELKLSDY peptide (Proimmune); APC conjugated HLA-A1 tetramer loaded with the RSV-$M_{229-237}$ YLEKESIYY peptide; HLA-A2 tetramer loaded with the FLU-$M1_{58-66}$ GILGFVFTL peptide; and HLA-B7 tetramer loaded with the RSV-$NP_{306-314}$ NPKASLLSL peptide. The last 3 tetramers were provided by Dr D. van Baarle and Dr. K. Tesselaar (Sanquin (CLB), Amsterdam, The Netherlands).

Isolation of peripheral blood mononuclear cells (PBMC)
Heparinized venous-blood samples were obtained from HLA-A1-, HLA-A2-, and HLA-B7-positive healthy individuals and patients with COPD. PBMC were isolated by use of standard density gradient techniques and were cryopreserved until analysis.

Flow cytometric analyses
PBMC ($1x10^6$) were incubated with tetrameric complexes and different combinations of the following antibodies: CD27-FITC (home-made clone 3A12), CD28-FITC (BD Biosciences), HLA-DR-FITC (BD Biosciences), CD45RA-PE (Sanquin (CLB)), anti-CCR7-PE (BD Biosciences), anti-IL-7R-PE (Immunotech), CD38-PE (BD Biosciences), CD8-PerCP-Cy5.5 (BD Biosciences). PBMC were labeled in accordance with the manufacturers' instructions, washed with PBS containing 0.01% (wt/vol) NaN_3 and 0.5% (wt/vol) bovine serum albumin (PBA), and analysed by use of FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences).

Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling
PBMC were labeled with 0.5 µmol/L CFSE (Molecular Probes) in PBS by shaking for 10 min at 37°C. Cells were then washed and resuspended in IMDM (10% human pool serum (HPS)) with antibiotics.

Culture and stimulation of cells
CFSE-labeled cells were cultured in culture medium for 7 days in 24 wells plates at a concentration of $1x10^6$ cells/ml/well. RSV-A1, RSV-B7 or FLU-A1-derived peptides were added at a final concentration of 1.25 µg/ml. RSV and FLU antigens (10 ug/ml, Microbix Biosystems) were used to stimulate the cells. The FLU antigen contained total viral lysate, and the RSV antigen contained lysates of infected cells. For stimulation, IL-2 (50 U/ml; Biotest), IL-7 (10 ng/ml; Strahtmann), IL-15 (10 ng/ml; R&D Systems) or IL-21 (25 ng/ml; gift from Zymogenetics) was added. For blocking experiments anti-IL-2R α-chain (clone BG5; Sanquin) was used. Flow cytometric analysis was performed before culture and after 7 days of culture.

Cytokine-producing capacity was measured by restimulation of the cells after 7 days of culture by adding RSV-A1 or RSV-B7 peptides to the culture medium for 1 h at 37°C. Brefeldin A (1µM; Sigma Chemical) was added after 1 h, and cells were

incubated for another 5 h. Cells were treated with FACS Lysing Solution and FACS Permeabilizing Solution (BD Biosciences). The permeabilized cells were incubated with anti-IFNγ-PE (BD Biosciences), anti-GranzymeB-PE (BD Biosciences), or IgG1-PE (as isotype control) plus CD8-PerCP-Cy5.5 (BD Biosciences). Cells were washed with PBA and were analysed by use of a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences).

Generation of RSV-specific CD8$^+$ T cell line

RSV tetramer$^+$ CD8$^+$ T cells were sorted by FACSAria (BD Biosciences). Cells were resuspended in HPS, washed, and resuspended in IMDM (10% HPS). Autologous Epstein Barr virus (EBV)-transformed B cells, which were UV irradiated and loaded with RSV-A1 peptide, were used as antigen-presenting cells. RSV tetramer$^+$ CD8$^+$ T cells were plated in a 96-wells plate at a concentration of 1, 3, and 10 cells/well and were incubated with the RSV-A1 peptide-loaded EBV-transformed B cells ($1x10^4$/well), heterologous UV-irradiated PBMC from 3 different healthy participants ($1x10^4$/well) and recombinant IL-2 (25 U/ml). Cells were cultured at 37°C and were restimulated weekly by exchanging medium with RSV-A1 peptide (1.25 µg/ml) and recombinant IL-2 (25 U/ml).

Statistical analysis

Between-groups analysis was performed with the nonparametric Mann-Whitney *U* test. Within-group analysis was performed using the Wilcoxon signed rank test. For correlations, the Spearman nonparametric correlation test was used. Two-sided testing was perfomed; p-<.05 were considered to be statistically significant.

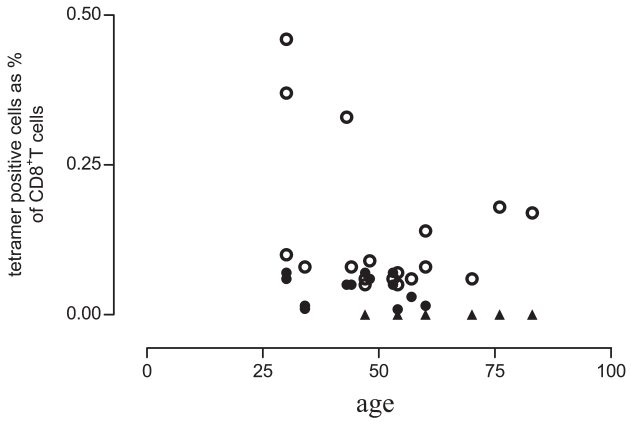

Figure 1: *Correlation between age (X-axis) and the frequency of RSV and FLU tetramer$^+$ CD8$^+$ T cells (Y-axis), in a subset of HLA-A1-positive healthy control participants (n= 21; median age, 53 years; age range 30 – 86 years). Black circles represent RSV-A1 tetramer$^+$ CD8$^+$ T cells, black triangles represent data for the healthy participants in whom RSV-A1 tetramer$^+$ CD8$^+$ T cells were not detectable before culture (Spearman rank correlation coefficient for total RSV-tetramer$^+$ CD8$^+$ T cells and age, r = -0.6; p=0.003), and white circles represent FLU-A1 tetramer$^+$ CD8$^+$ T cells (Spearman rank correlation coefficient for total FLU tetramer$^+$ CD8$^+$ T cells and age, r = -0.2; p=0.3) in the same donors. The frequencies of tetramer$^+$ T cells are expressed as percentages of total CD8$^+$ T cells.*

RESULTS

Decreasing frequency of RSV-specific CD8+ T cells with increasing age

The frequency of RSV (HLA-B7/NL_9 and HLAA1/$M_{229–237}$) tetramer+ CD8+ T cells in the peripheral blood of the healthy participants and the patients with COPD was analyzed. In parallel, the frequency of FLU (HLA-A1/$NP_{44–52}$ and HLA-A2/$M1_{58–66}$) tetramer+ CD8+ T cells was determined. In the healthy participants, there was a gradual decrease in the frequency of RSV tetramer+ CD8+ T cells with increasing age (r = -0.6; p = .003) (Figure 1), whereas there was no decrease in the frequency of FLU tetramer+ CD8+ T cells with increasing age (r = -0.2; p = .3). In the patients with COPD, we could not detect RSV tetramer+ CD8+ T cells, whereas we could detect FLU tetramer+ CD8+ T cells (median, 0.07%; range, 0.07%– 0.80%; expressed as a percentage of total CD8+ T cells). In those healthy participants in whom we could detect both RSV tetramer+ and FLU tetramer+ CD8+ T cells, the frequency of RSV tetramer+ CD8+ T cells (median, 0.015%; range, 0.003%–0.070%) was significantly lower than the frequency of FLU tetramer+ CD8+ T cells (median, 0.08; range, 0.03–0.46) (p = .0001) (Figure 2).

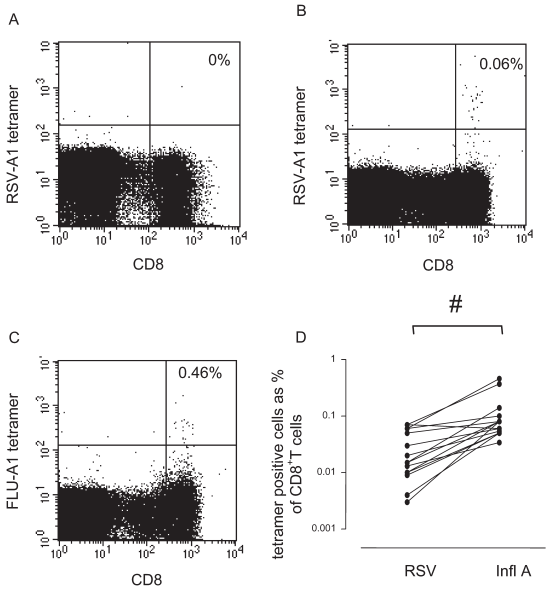

Figure 2: *Detection of low numbers of RSV tetramer+ CD8+ T cells in peripheral blood. Shown are representative dot plots for an HLA-A1-negative healthy participant and an HLA-A1-positive healthy participant.* ***a:*** *RSV-A1 tetramer staining for an HLA-A1-negative participant. Shown is a dot plot of anti-CD8-PerCP-Cy5.5 fluorescence versus RSV-A1 tetramer-APC of T cells gated on forward-scatter and side-scatters parameters.* ***b, c:*** *Dot plots showing anti-CD8-PerCP-Cy5.5 fluorescence versus RSV-A1 tetramer-APC and FLU-A1 tetramer-APC, respectively, of T cells gated on forward-scatter and side-scatters parameters. The dot plot showing the FLU-A1 tetramer staining contains fewer events than does the dot plot showing the RSV-A1 tetramer staining.* ***d:*** *Paired measurement of FLU HLA-A1 tetramer+ CD8+ T cells and RSV HLA-A1 tetramer+ CD8+ T cells in the peripheral blood of a subset of the healthy participants (n=15; median age, 44 years; age range, 30-60 years). The frequencies of tetramer+ T cells are expressed as percentages of total CD8+ T cells.* # *p <0.05 (Wilcoxon signed ranks test).*

To detect the presence of RSV-specific CD8$^+$ T cells in a more sensitive assay, we labeled total PBMC from the elderly healthy participants and the patients with COPD and cultured the cells for 7 days with RSV-A1 or RSV-B7 peptides in combination with IL-2. The fraction of tetramer$^+$ CFSE$_{low}$ cells, gated on total CD8$^+$ T cells, was used as a measure of the frequency of RSV-specific CD8$^+$ memory T cells. Culture in medium alone or in the presence of IL-2 alone did not result in the proliferation of tetramer$^+$ T cells. After culture with RSV peptide in the presence of IL-2, RSV tetramer$^+$ CFSE$_{low}$ cells, gated on CD8$^+$ T cells, were detected in the elderly healthy participants and in the patients with COPD at median frequencies of 0.2% (range, 0%–2.4%) and 0.06% (range, 0%–0.6%) (p= .1), respectively. The median frequencies of FLU tetramer$^+$ CFSE$_{low}$ cells, gated on CD8$^+$ T cells, in the elderly healthy participants and the patients with COPD were 0.4% (range, 0.07%–4.0%) and 0.3% (range, 0.1%–3.0%), respectively (Figure 3). The frequency of RSV tetramer$^+$ CD8$^+$ T cells tended to be lower than the frequency of FLU tetramer$^+$ CD8$^+$ T cells in both the elderly healthy participants and the patients with COPD, although the differences were not statistically significant (p = .08 and p = .2, respectively) (Figure 3).

Memory phenotype of circulating RSV-specific CD8$^+$ T cells

To characterize the memory phenotype of RSV-specific CD8$^+$ T cells, we analyzed the level of expression of CD28, CD27, CCR7, IL-7Rα, HLA-DR, and CD38 on RSV tetramer$^+$ CD8$^+$ T cells in 5 of the young healthy participants (median age, 34

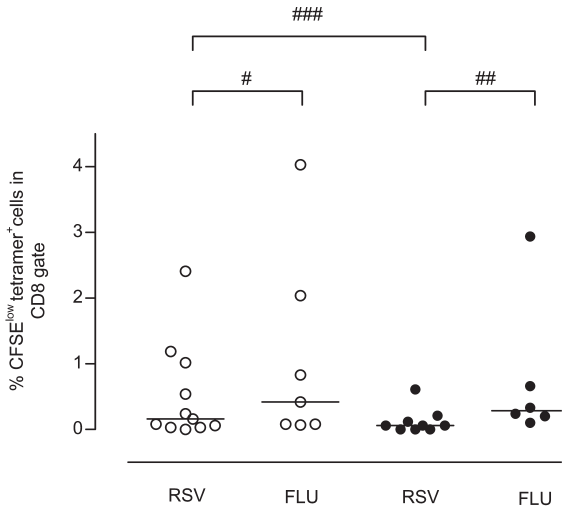

Figure 3: *Frequencies of RSV and FLU tetramer$^+$ CD8$^+$ T cells after culture in the presence of RSV or FLU peptide and IL-2 for 7 days. Cells are gated on CD8$^+$ lymphocytes. White symbols represent a subset of the healthy participants (n=11; median age, 72 years; age range, 54-86 years), and black symbols represent the patients with COPD (n=9; median age, 69 years; age range, 50-76 years) Cells from 5 healthy participants were stained with RSV HLA-A1 tetramer, and cells from 6 healthy participants were stained with HLA-B7 tetramer. For the patients with COPD these numbers were 5 and 4, respectively. # p = 0.08 (Wilcoxon signed rank test); ## p = 0.2 (Wilcoxon signed rank test), ### p = 0.2 (Mann-Whitney U test).*

years; age range, 30–54 years). RSV tetramer+ CD8+ T cells were uniformly characterized by a high level of expression of CD27 and CD28 and a low level of expression of CCR7. All RSV tetramer+ CD8+ T cells showed high level of expression of IL-7Rα (Figure 4a–4c). These findings demonstrate that RSV tetramer+ CD8+ T cells have a memory phenotype. In addition, most RSV tetramer+ CD8+ T cells were negative for HLA-DR and CD38, indicating that they were resting memory T cells (Figure 4c and Table I).

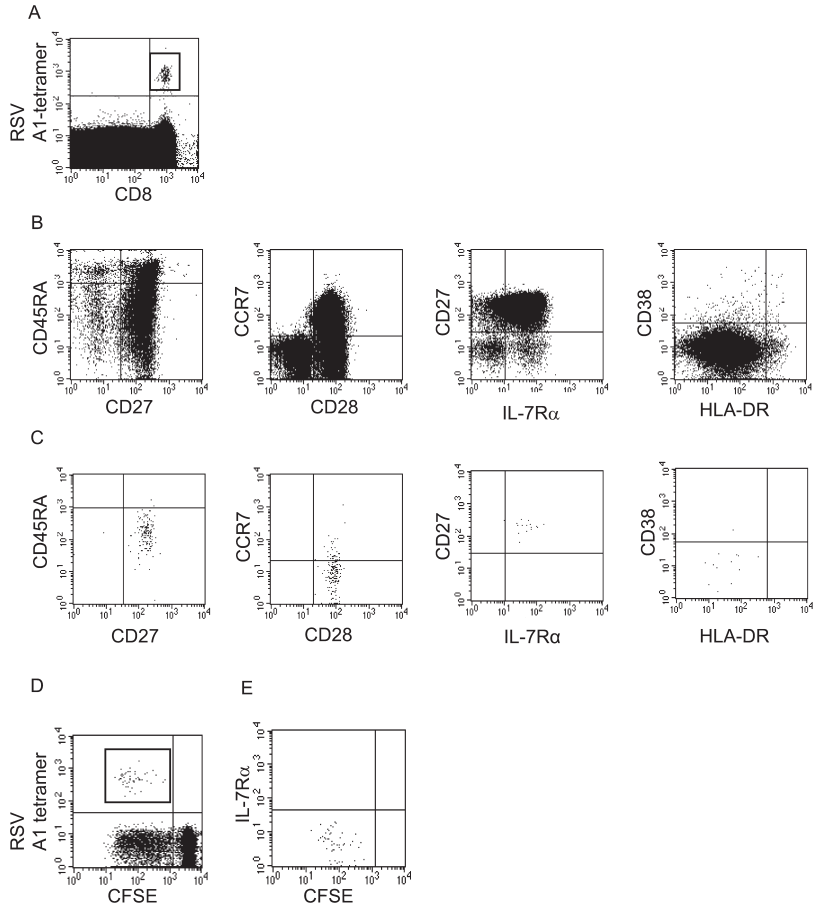

Figure 4: *Memory phenotype of RSV-specific CD8+ T cells. Shown is a representative dot plot from a healthy participant.* ***a:*** *Dot plot of anti-CD8-PerCP-Cy5.5 fluorescence versus RSV-A1-tetramer-APC of T cells gated on forward-scatter and side-scatter parameters.* ***b:*** *Dot plots gated on CD8bright T cells.* ***c:*** *Dot plots gated on RSV tetramer+ CD8+ T cells. The rectangle in panel A represents the gate for RSV tetramer+ CD8+ T cells. The stainings for CD27, IL-7Rα, HLA-DR and CD38 in panels* ***b*** *and* ***c*** *are obtained from a different experiment with the same PBMC sample from the same healthy participant.* ***d:*** *Dot plot of cells from the same participant cultured for 7 days in the presence of RSV-A1 peptide and IL-2. The rectangle in the dot plot in panel D represents the gate for proliferated RSV tetramer+ CFSElow T cells.* ***e:*** *Dot plot gated on proliferated RSV tetramer+ CFSElow T cells.*

Table I: Memory phenotype of RSV- and FLU-specific CD8$^+$ T cells

Marker	Total CD8$^+$ T cells	RSV tetramer$^+$ CD8$^+$ T cells[2]	FLU tetramer$^+$ CD8$^+$ T cells[2]
CD27	92 (60-98)[1]	99 (87-100)	94 (80-100)
IL-7Rα	85 (23-93)	91 (79-100)	84 (67-99)
CD28	86 (62-97)	93 (75-100)	93 (85-100)
CCR7	47 (27-77)	8 (0-71)	44 (0-57)
CD38	6 (3-49)	22 (0-44)	4 (0-8)
HLA-DR	5 (1-15)	12 (0-44)	8 (4-9)

[1]Values expressed are median (range) percentages of cells gated on total CD8$^+$ T cells.
[2]Values expressed are median (range) percentages of cells gated on tetramer CD8$^+$ T cells.

Functional capacity of RSV-specific CD8$^+$ memory T cells and need for antigen-induced factors to proliferate

An important feature of CD8$^+$ memory T cells is the capacity to expand in response to reinfection. To study whether RSV tetramer$^+$ CD8$^+$ T cells have this potential in vitro, we labeled, with CFSE, total PBMC from 3 HLA-A1–positive healthy participants and cultured the cells for 7 days either in the presence of RSV-A1 peptide alone or in combination with RSV antigen. After 7 days, the frequency of RSV tetramer$^+$ T cells was analyzed in the CFSE^{low}CD8$^+$ fraction. Culture with RSV peptide alone did not induce antigen-specific proliferation. Culture in combination with RSV antigen, however, did result in antigen-specific proliferation of RSV tetramer$^+$ CD8$^+$ T cells. This proliferation could be reduced to a large extent by addition of a blocking anti–IL-2R antibody to the culture medium (Figure 5a–5d). In summary, these data suggest that stimulation with RSV peptide alone is not sufficient to induce antigen-specific proliferation and that antigen-induced factors are needed to efficiently expand the RSV memory compartment.

Once a stable memory T cell pool is established in vivo, common γ-chain cytokines (IL-2, IL-7, IL-15, and IL-21) play a central role in the maintenance of memory T cells and can drive them to rapid expansion when the virus is reencountered (17). To investigate whether common γ-chain cytokines can induce RSV-specific memory T cells to proliferate, we incubated total PBMC in combination with peptide and either IL-2, IL-7, IL-15, or IL-21. As a control, we cultured total PBMC with cytokines alone. Coculture of peptide in combination with IL-15 and IL-2, and to a lesser extent with IL-7 and IL-21, induced a strong proliferation of RSV tetramer$^+$ CD8$^+$ T cells (Figure 5e–5h). When cytokines alone were cultured with PBMC, only a small percentage of RSV tetramer$^+$ CD8$^+$ T cells was detected (Figure 5i). After culture with RSV peptide in combination with IL-2 or RSV antigen, all RSV tetramer$^+$ CD8$^+$ T cells downregulated IL-7Rα (Figure 4d and 4e).

After restimulation, memory T cells have to develop into effector cells. Therefore, we tested, in 3 of the healthy participants, whether the expanded RSV tetramer$^+$ fraction was able to produce IFN-γ. We restimulated total PBMC, after culture in the

presence of RSV peptide and IL-2, with either RSV peptide or an HLA-mismatched peptide (FLU-HLA-A2/GILGFVFTL) as a negative control. After 6 h, we analyzed the intracellular expression of IFN-γ in the $CFSE^{low}CD8^+$ fraction. Stimulation with RSV peptide induced a subset of IFN-γ^+ cells (median, 0.8%; range, 0.4%–1.5%), gated on $CFSE^{low}CD8^+$ T cells, which correlated well with the number of tetramer$^+$ T cells after culture of PBMC from the same healthy participants (data not shown). Next, we generated an RSV-specific $CD8^+$ T cell line. After culture for 4 weeks and

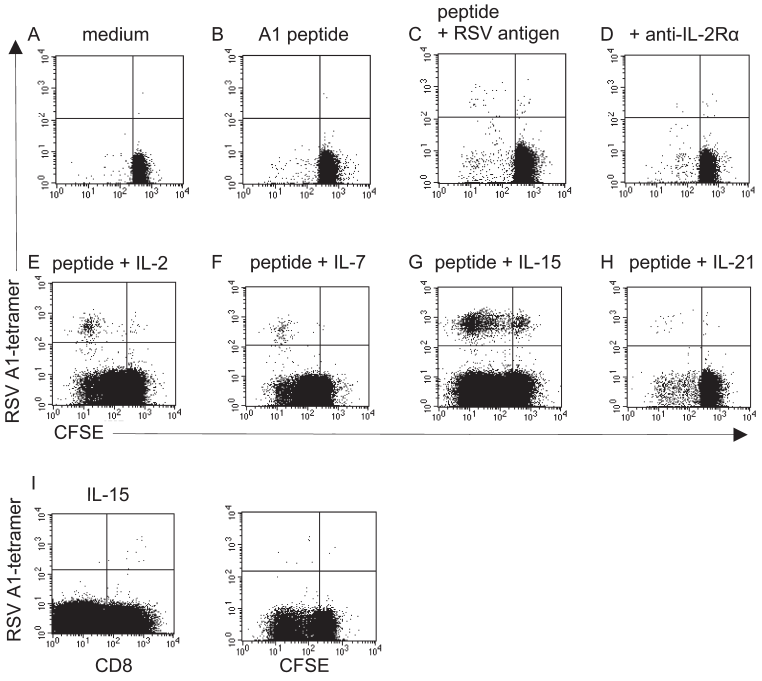

Figure 5: *Proliferative requirements of RSV-specific $CD8^+$ T cells. Dot plot of cells stimulated for 7 days with medium alone (**a**), RSV-A1 peptide (**b**), RSV-A1 peptide plus RSV antigen (**c**), RSV-A1 peptide plus RSV antigen in the presence of a blocking antibody against the IL-2R α-chain (**d**), RSV-A1 peptide plus IL-2 (**e**), RSV-A1 peptide plus IL-7 (**f**), RSV-A1 peptide plus IL-15 (**g**), RSV-A1 peptide plus IL-21 (H). I. Stimulation with IL-15 alone. The dot plot on the left is gated on total lymphocytes. The dot plot on the right is gated on total $CD8^+$ T cells. Flow cytometry shown is representative of 3 experiments.*

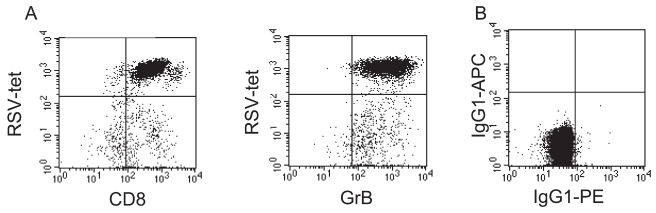

Figure 6: *Production of granzymeB by RSV tetramer$^+$ $CD8^+$ T cells after restimulation. Shown are dot plots of an RSV tetramer$^+$ $CD8^+$ T cell line (**a**) and isotype control (**b**).*

weekly restimulation with RSV peptide, all cells gained a high level of granzymeB expression (Figure 6). These findings indicated that the expanded RSV tetramer$^+$ CD8$^+$ T cells were indeed functionally competent memory T cells.

DISCUSSION

In the present study, we characterized RSV tetramer$^+$ CD8$^+$ T cells as resting memory T cells that circulated in low frequency in the peripheral blood of the young healthy participants and in even lower frequencies in the peripheral blood of the elderly healthy participants and the patients with COPD. These cells displayed the characteristics of fully functional memory T cells with respect to proliferative capacity, cytokine production, and expression of cytolytic mediators after reactivation in vitro. RSV is a ubiquitous virus that infects virtually everyone annually from an early age on. RSV infection causes severe bronchiolitis in young children. After primary infection, children generate a CTL response to RSV during the first year (18, 19). Once primary infection has been overcome, older children and healthy adults remain sufficiently protected against reinfection with RSV, which generally causes only mild symptoms. Several studies have shown that, in healthy adults, CTL responses to RSV are maintained and may contribute to the control of RSV infection. In these studies, the RSV nuclear protein and a fusion protein have been identified as targets for CTL responses (12, 13, 20, 21). Recent studies have shown, however, that elderly individuals and patients with underlying cardiopulmonary disease are at risk for the development of severe disease after reinfection with RSV. That RSV is associated with a high reinfection rate with accompanying high morbidity in susceptible individuals poses a challenge to the analysis of the development and maintenance of the CD8$^+$ T cell memory response to RSV. To achieve enduring immunological protection, it is essential that an RSV-specific CD8$^+$ memory T cell pool persist after primary infection. After clearance of the primary infection, a memory pool is established that is composed of different patterns of expression of cell-surface markers, depending on the viral specificity of the memory T cells (8, 22). In the present

study, we analyzed the persistence of RSV-specific CD8$^+$ memory T cells by tetramer staining for 2 immunodominant RSV epitopes (12, 13). In line with the findings of a study by Heidema et al. (12), we found a high level of expression of CD27 and CD28 on RSV tetramer$^+$ cells. We also performed a more extensive characterization of RSV-specific memory T cells and studied whether changes in these characteristics could be induced on in vitro reactivation of these cells. We observed a low level of expression of CD38 and HLA-DR on RSV tetramer$^+$ CD8$^+$ T cells; these cells can be characterized as resting memory T cells. This phenotype resembles that of FLU-specific CD8$^+$ T cells (10). On antigen-specific reactivation in vivo or after prolonged stimulation in vitro, CD27 and CD28 expression is

downregulated (23, 24). The decrease in the level of CD27 expression is associated with the acquisition of stable effector functions of $CD8^+$ T cells specific for persistent viruses, such as human cytomegalovirus (CMV) (24). In contrast to CMV-specific $CD8^+$ T cells, all RSV $tetramer^+$ T cells expressed high levels of CD27. This indicates that, at least in the circulation, RSV infection does not persist. A recent study by Kaech et al. (11) showed that, in mice, virus-specific $CD8^+$ T cells that express IL-7Rα have the properties of long-lived memory T cells. RSV-specific $CD8^+$ T cells uniformly expressed high levels of IL-7Rα which were down-modulated after stimulation in vitro. The high level of expression of IL-7Rα correlated with the expression of CD27 on RSV $tetramer^+$ T cells. The present data suggest that the expression of IL-7Rα on virus-specific $CD8^+$ T cells is related to functionally competent long-lived memory T cells in humans as well. It remains to be investigated, however, whether the kinetics of IL-7Rα expression in the formation of memory after primary infection in humans are the same as those that have been described in mice (11). In addition, we showed that expanded RSV $tetramer^+$ $CD8^+$ T cells produced IFN-γ and granzymeB when restimulated with their cognate peptide. These data indicate that RSV-specific $CD8^+$ T cells are able to regain effector functions on restimulation. The maintenance of a stable memory T cell pool is a dynamic process based on homeostatic proliferation of memory T cells and expansion on antigenic boosting. In this process, cytokines that use the common cytokine-receptor γ chain are involved. We showed that RSV-specific memory T cells adequately proliferate on stimulation with IL-2 and IL-15, and to a lesser extent on stimulation with IL-7 and IL-21, when combined with RSV peptide. These data indicate that RSV-specific memory T cells are able to reexpand in response to antigenic boosting and are responsive to stimulation with common γ -chain cytokines. In our experimental setup, we were not able to induce proliferation of RSV $tetramer^+$ $CD8^+$ T cells with RSV peptide alone, indicating that expansion of RSV-specific memory T cells is dependent on factors produced by helper cells. In contrast to the findings for the younger healthy participants, RSV-specific $CD8^+$ T cells were not directly detectable by tetramer staining in the peripheral blood of the elderly healthy participants and patients with COPD but were detectable only after culture. This was in contrast to FLU-specific $CD8^+$ T cells, which were detectable in all participants directly ex vivo. The use of tetramers containing immunodominant epitopes of RSV or FLU provides a valuable tool for the characterization of virus-specific T cells, although it must be kept in mind that a portion of virus-specific T cells may be missed because they do not recognize the epitopes contained in the tetramer. In the present study, we related the decrease in the frequency of RSV-specific T cells in the elderly healthy participants to the frequency of FLU-specific T cells in the same participants (Figure 2). Our findings indicate that the circulating RSV-specific $CD8^+$ memory T cell pool is considerably smaller than the FLU-specific $CD8^+$ memory T cell pool and that the RSV-specific $CD8^+$ memory T cell pool decreases in size with increasing age. These findings are consistent with those of the study by Looney et al. (25) showing, in elderly individuals, hampered RSV-induced IFN-γ production in total PBMC, a result that could be explained by a decrease in RSV memory response. We showed here that the frequency of

RSV-specific memory T cells in the patients with COPD was comparable to the frequency of RSV-specific memory T cells in the age-matched healthy participants. Our data indicate that the susceptibility to develop severe symptoms on infection with RSV in patients with COPD may be due to an age-related decrease in the size of the RSV-specific $CD8^+$ memory T cell pool. This susceptibility may be further increased by disease-associated local factors in the airways.

All of the healthy participants >65 years old and all of the patients with COPD in the present study received their annual influenzavirus vaccination 4–6 months before inclusion. Recent studies have shown that influenzavirus vaccination to some extent boosts the FLU-specific $CD8^+$ T cell compartment (26, 27). In addition, the natural reinfection rate for RSV is, apparently, lower than that for FLU (28). This frequent antigen-specific boosting may, at least in part, explain the higher frequency of FLU-specific $CD8^+$ T cells than of RSV-specific $CD8^+$ T cells observed here. We showed that RSV-specific T cells are fully competent memory T cells that are able to respond to antigen-specific stimulation in vitro but need help from CD4-derived factors. These findings provide starting points for the development of strategies for vaccination (and for the use of adjuvants) that may be useful in the prevention of severe RSV infection in vulnerable populations, such as the elderly.

Acknowledgements

We thank dr Joost C.Meijers, Viola van Riemsdijk and Renee van der Pal for their help with the inclusion of the healthy controls, and Wendy van Maren for the technical support.

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General discussion

Discussion

General discussion

In this thesis we investigated the role of $CD8^+$ T cells during RSV infections. In the general introduction the interplay between the virus and the immune response was discussed. However, many questions remained unanswered and prompted our studies into the contribution of T cells during human RSV infections. In this chapter the contribution of our studies in unravelling unknown aspects in the area of viral and immune pathology during infections in humans, as well as recently published views on these topics will be discussed.

The role of virus induced versus T cell induced pathology during primary RSV infection.

Although $CD8^+$ T cells are necessary for clearing viral infections, they can also cause immune pathology. RSV can induce a broad spectrum of diseases varying from mild colds to severe bronchiolitis. It is currently unknown whether inadequate immunity or immune mediated pathology determines a more severe disease course. Contradictory results have been reported about the viral load as determinant for disease severity. De Vincenzo et al. showed that a one log higher viral load caused a 0.8 day longer hospital stay (1), while others were unable to find any correlation between viral load and disease severity (2;3). Even if a higher initial viral load correlates with more severe disease it remains to be established that the damage is caused directly by the virus rather than a more intense immune response. Up to a certain threshold, increasing amounts of antigen lead to an increase in T cell activation (4). Beyond this threshold of T cell activation determined by viral load, type of antigen presenting cell (APC) and duration of antigen presentation, exhaustion and therefore loss of T cell function is observed. In several viral infections like LCMV in mice and hepatitis C virus in human it has been shown that during acute infection T cell function was impaired, a phenomenon called stunning, but was restored upon control of the infection (5;6). An influenzavirus mouse model showed that in the context of low viral load $CD8^+$ T cells have the potential to block viral replication without causing damage, while with high viral load they might exacerbate lung injury (7). Also for RSV a direct role for $CD8^+$ T cells in immune pathology has been demonstrated in the mouse model (8).

The most important argument against virus induced pathology during RSV infections is that peak severity of disease occurs at a time when the viral load has declined. Although in almost all infants that need hospitalization RSV can be detected 7 days after admission (3;9), the highest viral load is usually found early in infection (1). However, due to spreading of the virus from the upper respiratory tract to the lower airways, determination of viral load in nasal swabs may not be representative for the viral load in the lower airways, a parameter that may be more important for the pathogenic response at this local site.

There are several arguments that point towards a role for $CD8^+$ T cells in immune pathology. The average interval between the onset of symptoms and admission to the pediatric intensive care unit in our patient group was 5 days. As first symptoms occur approximately 3 days after infection, most infants experienced respiratory failure 8 days after infection. This coincides with peak $CD8^+$ T cell responses in lung, spleen and draining lymph nodes found in mice, where virus-specific $CD8^+$ T cells are found from day 6 onwards, and peak at day 8-10 (10;11). In addition, it has been shown in T cell depleted mice that both $CD4^+$ T cells and $CD8^+$ T cells were necessary for termination of the infection, and both contributed to illness severity (8). In a $CD8^+$ T cell transfer system in mice that expressed transgenic influenzavirus hemagglutinin in the alveoli it has been shown that, even in the absence of replicating virus, antigen specific $CD8^+$ T cell recognition and effector activities can induce lung pathology (12;13). For RSV the effect of $CD8^+$ T cells on both viral clearance and immune pathology seems to be related to IFN-γ expression rather than cytotoxic effector function (i.e. perforin(14). This relation of IFN-γ expression with both viral clearance and immune pathology is less profound in influenzavirus infection (15;16). Tumor necrosis factor (TNF-α) expressed by $CD8^+$ T cells plays a role in lung injury in both influenzavirus and RSV infection, while there is no role for TNF-α in viral clearance. This pathogenic effect of TNF-α is thought to be caused by TNF induced expression of chemokines by target epithelial cells and the recruitment of inflammatory cells by these chemokines (17). In our studies we found that upon antigen stimulation the proportion of $CD8^+$ T cell producing IFN-γ overlapped with the proportion of tetramer specific cells (chapter 5). When comparing TNF-α and IFN-γ levels in BAL of patients to healthy controls, we found a 24 fold higher level of TNF-α (1.4 pg/ml->33.1 pg.ml) in patients. In both patients and controls levels of IFN-γ were around the detection limit (data not shown). Thus, although these infants were severely ill, these levels of cytokines are very low and therefore do not suggest a prominent role in disease severity.

We also showed that during severe primary RSV infection all $CD8^+$ T cells found in the airways were highly activated effector cells, but less than 1.8% of these cells stained positive with single peptide containing tetrameric complexes (chapter 5). However, in PBMC of these patients the total responses against RSV reached up to 47% of the total activated $CD8^+$ T cell population. We therefore assumed that the responses found against single epitopes highly underestimated the total RSV-specific response. This was indeed confirmed by using RSV-infected dendritic cells, displaying the total repertoire of virus derived epitopes as antigen presenting cells, to stimulate PBMC of patients (chapter 5). We did not find a correlation between either the extent of $CD8^+$ T cell activation or the percentage of $tetramer^+$ T cells in the lung with markers of severity of disease such as days of hospitalization or ventilation pressure. This observation was similar to the group of van Benten et al. who did not show any difference in the total number of $CD8^+$ T cells in nasal washes of infants with RSV upper respiratory tract infections and infants with RSV lower respiratory tract infections (18). We did however find a significant correlation between the parameters of illness severity and the number

of activated T cells in blood of these same infants However, the peak of activated $CD8^+$ T cell numbers found in blood coincided with extubation of the patient, and therefore improvement of symptoms. This might suggest a role for $CD8^+$ T cells in clearance of disease. The increased levels of virus-specific T cells in blood late in the response might indicate that the inflammation in the lung diminishes and as a consequence also the migration of effector cells to the lung. It has been shown that viral antigen display on antigen presenting cells in draining lymph nodes is retained long after clearance of infecting virus which causes continuous activation of virus-specific $CD8^+$ T cells (19). Importantly, in our study all infants from which we obtained BAL samples needed oxygen support and were all seriously ill. For ethical reasons a control group with mild upper respiratory tract infection was lacking in our study. The work by van Benten et al was performed in small groups of patients, and nasal swabs might not fully represent the $CD8^+$ T cell response in the lungs of infants with lower respiratory tract infection. Moreover, due to the low numbers of cells recovered in nose swabs it will be impossible to study RSV-specific $CD8^+$ T cell responses by using tetramers in this easy accessible source of patient material. A better way of investigating the contribution of RSV-specific T cells to immune pathology would be to compare bronchoalveolar lavage (BAL) samples of infants on mechanical ventilation to BAL samples of infants that experienced mild upper respiratory tract infection. We have tried to initiate a study to access patient material in infants with mild disease. To be able to obtain BAL samples from infants with mild upper respiratory tract infections, we included all infants born after the RSV season of the previous year that needed surgery during the RSV season, hoping that by this method we could include infants that may have caught RSV just before surgery. To study peak $CD8^+$ T cell responses in BAL, the preferable time of sampling is at least one week after onset of symptoms. However, viral load might have declined to undetectable levels by then, especially in mild disease. To overcome this problem all infants were included two weeks prior to surgery. In case of symptoms of upper respiratory tract infection, nasal swabs were collected at home to diagnose RSV positive patients. At the time of surgery BAL was performed directly after intubation to study RSV-specific $CD8^+$ T cell responses. Unfortunately, this study failed to include an appropriate number of RSV-infected patients, although most parents agreed to participate in the study. This might be due to the fact that the peak of the RSV season lasts only several weeks, including the weeks around Christmas, when little elective surgery is performed. Another reason was that only a small number of infants undergoing surgical procedures below the age of nine months were intubated, as infants with short surgical procedures were ventilated through nasopharyngeal masks. Seventeen of the children in our study experienced upper respiratory tract infections within the two weeks prior to surgery. Only in two patients we were able to identify RSV as the cause of symptoms. As this is lower than expected, it is possible that in some infants viral load was diminished within a short time after infection and therefore patients undergoing RSV infections may have escaped detection. Obviously, this number of patients is far to low to draw any further conclusions.

Memory formation upon primary respiratory infections

A small number of surviving effector T cells is retained as long-lived memory T cells when the T cell pool contracts after resolution of a primary infection. Memory $CD8^+$ T cells rapidly respond to recall antigens. They depend less on co-stimulation, have an increased division rate and lower loss rate, and show more rapid and efficient differentiation into effector cells (20). About half of the memory $CD8^+$ T cell population against respiratory viruses persists in non-lymphoid tissue following clearance of the virus (21). There is no direct cytolitic activity of these cells, but upon re-stimulation rapid proliferation occurs and cytolitic activity is upregulated. The number of virus-specific cells declines several weeks after infection but small numbers remain detectable in the airways for months. In contrast, in lymphoid organs the number of virus-specific cells remains high for over one year after infection (22). Therefore, it is interesting to note that protective cellular immunity to influenzavirus is substantially reduced after 3 months (23), corresponding with declining Ag-specific cell numbers in the airways. Waning immunity is also seen after infections with RSV. Previously RSV-infected individuals and mice can be subsequently re-infected within months with an identical virus (24;25). On average re-infection with RSV occurs once every 3 years in adults, and 43% of children that were hospitalized due to RSV bronchiolitis became re-infected during the following year (26).

While RSV re-infections in children and adults usually cause mild symptoms, in the elderly severe morbidity and mortality has been observed. In chapter 7 we showed that RSV-specific $CD8^+$ T cell numbers decline with age. This decline in the number of virus-specific T cells possibly causes the loss in memory to RSV and could therefore explain the more severe course of disease in the elderly. To address this question of memory loss, insight in the homeostasis of the memory pool needs to be obtained. Also functional differences in primary and secondary memory $CD8^+$ T cell responses have been described. Moreover, a conversion of effector memory to central memory T cells, with different efficacy during recall responses, occurs gradually during the first year after infection (27). To evaluate the efficiency of established T cell immunity, the contribution of these different effector populations needs to be evaluated and potential differences between age groups established in order to design vaccines inducing long term protection.

Homeostasis of the memory T cell pool

The memory pool of $CD8^+$ T cells is relatively stable in resting state, as a result of a balanced IL-15 dependent cell division and apoptotic cell death. Human memory T cell populations have a half life of 8-15 years after vaccination with vaccinia virus (28). Maintenance of these memory cells does not require persistent antigen. However, during acute infections the fate of virus-specific $CD8^+$ T cells is dependent on several independent events. With every new viral infection, two waves of apoptotic events occur to create a new balance in the memory pool. The first apoptotic wave (29) creates a virus-induced lymphopenia that has been well described for many severe acute infections in human like influenzavirus, measles,

Ebola and SARS (30-34) and also RSV(35). Memory T cells express apoptotic markers including Annexin and TUNEL that might be caused by type 1 interferons. This lymphopenic state creates space in lymphoid organs for T cells to seed and develop, thus facilitating a vigorous virus-specific T cell response. The second apoptotic wave occurs during memory formation. After resolution of infection the large virus-specific T cell pool needs to decline to form a small stable memory pool. This fast contraction of the T cell pool upon clearance of the virus is still not completely understood. About half of the T cells express Annexin V, and apoptosis cannot be prevented by BCL-2. Survival correlates with upregulation of BCL-2, BCL-XL and IL-7R on surviving cells (29;36). There is more apoptosis in lymph nodes and spleen than in tissue, and some studies showed that the elimination during the contraction phase occurs in the liver, where significant numbers of virus-specific T cells can be identified (37;38). Antigen is not required for the maintenance of memory, but the presence of antigen highly influences the quantity of the memory $CD8^+$ T cells. In PBMC of healthy adults, about a hundred fold more $CD8^+$ T cells are specific for persistent viruses like EBV and CMV than for cleared viruses like influenzavirus and RSV.

Upon secondary infection, both bystander and cross-reactive T cells are distributed to the site of infection. Bystander recruitment is the phenomenon that upon heterologous infections with respiratory viruses T cells directed against other viruses are recruited to the lungs (chapter 6). Non-specific bystanders are deleted to create space for newly formed memory a process that does not influence immunodominance within the T cell memory pool. In contrast, activation of cross-reactive T cells can influence immunodominance during secondary responses. When a new virus is encountered, T cells specific for previously encountered viruses might recognize closely resembling epitopes of the heterologous virus and proliferate. The expanded T cell population might suppress responses to other, previously dominant epitopes, thus changing the hierarchy of immunodominance. This can result in protection, but also shift the response towards epitopes that cannot control the virus, therefore eliminating efficient memory to homologous infections. This could well be a cause for waning memory responses in the elderly. Cross-reactive T cells might even tip the scale towards immune pathology as seen in acute infectious mononucleosis after EBV infection, which is only seen in adults with large amounts of $CD8^+$ T cells directed against previously encountered influenzavirus (39). As most infants with severe RSV disease become infected in the first months of life, a role for heterologous infections in disease severity seems unlikely. However, there could be a role for cross-reactive RSV-specific cells in causing unexplained wheezing upon subsequent respiratory infections, as seen in 50% infants that previously experienced severe RSV infection.

Vaccination

In the 1960's a vaccination trial with formalin inactivated RSV was performed. However, all vaccinated infants developed a severe bronchiolitis upon natural infection that caused hospitalization in 80 percent of vaccinated infants and 2 in-

fants died. This disastrous event led to a huge draw back in vaccine development and extreme precaution need to be taken in new attempts to develop a vaccine. Presently, the only available preventive measure is the administration of the monoclonal antibody specific for the viral Fusion protein (Paluvizumab) a procedure that is protective in 55-70% of vaccinated individuals (40;41). Unfortunately, these antibodies only protect for 4 weeks, and Paluvizumab has to be administered several times at monthly intervals. Due to the extremely high costs, this treatment is nowadays only available for infants with risk factors for developing severe RSV infection i.e. premature born infants and infants with broncho-pulmonary dysplasia or congenital heart disease. Although these infants have a higher risk of developing severe disease, the major population in the intensive care due to RSV infection are previously healthy infants as these infants make up a much larger proportion of the population. Therefore, a vaccine that would induce long lasting protective immunity, available for all infants, would be of great benefit.

The major problem in developing a protective vaccine is the young age of the target group, i.e. infants in their neonatal period until the first year of life. Many infants with severe disease are infected before 44 weeks of gestational age (42). Therefore, a vaccine should preferably be administered at birth. However, during the first 12 months of life, the IgG and IgA antibody response to both viral and bacterial infection and also to vaccination remains weak (43-45). Vaccination immediately after birth hardly induces any antibody response (46). This might be partly due to maternal antibodies, but even in the absence of maternal antibodies the antibody response at birth is very poor and of short duration (47-49). This could be due in part from limiting bone-marrow homing of long-lived plasmocytes in early life (50).

Although neonates are competent to mount adult-level T cell responses in vivo, their responses to viral vaccines show reduced Th1-cell function as well as reduced memory responses after booster immunization (51;52). On the other hand, infants are capable of strong Th1 responses after BCG vaccination. Agents used to obtain mature vaccine responses, like DNA vaccination and addition of CpG, or IL-12 have been considered. However, great care should be taken before implementing these methods, as these agents might be toxic in the neonate as well as promote auto immune disease.

Our studies contributed in several ways to vaccine development. First of all, the identification of RSV-specific epitopes provides a method to study subsequent development of RSV-specific T cells upon vaccination. More importantly, before introducing a new vaccine to a virus that has been shown to be able to cause severe disease upon vaccination, more insight in immune responses upon naturally acquired RSV is necessary. As the natural course of disease shows a broad spectrum of severity, insight into factors that contribute to pathology will be helpful in designing protective vaccination methods. The role of $CD8^+$ T cells in immune pathology is an essential factor in this process. Although our studies in infants with

mild RSV infection failed to include sufficient numbers of patients, our study in severely ill patients provided initial information on the role of $CD8^+$ T cells during RSV infection. While the general opinion nowadays suggests a pathogenic role of $CD8^+$ T cells during infection, these opinions are build on observations in mouse studies, while natural RSV infection does not cause the same lung pathology in mice as observed in human. In our studies it was shown that infants with severe symptoms had low percentages of virus-specific $CD8^+$ T cells, although our results were limited by the use of single tetramers. More importantly we showed that the peak of RSV-specific $CD8^+$ T cell levels in both blood and BAL coincide with disease recovery. Last but not least there might be a role for vaccination in the elderly at risk for severe disease, as we showed that RSV-specific $CD8^+$ T cell wane in time. Boosting $CD8^+$ T cell immune responses in the elderly might prevent the morbidity and mortality seen in this population.

Concluding remarks

Although the precise mechanism of RSV induced disease remains to be unravelled, several items studied in this thesis contribute to a better understanding of the role of T cells during RSV infection. The identification of new MHC class I and class II epitopes supplied a method for studying RSV-specific T cell responses. Using HLA tetrameric complexes containing these epitopes, we were able to study RSV-specific immune responses in BAL and blood during severe primary RSV infection (chapter 5). This study showed that all $CD8^+$ T cells present in the BAL during RSV infection are highly activated effector cells. We found a 10 fold increase in RSV-specific cells during active infection and a further increase during convalescense. HLA-tetramer$^+$ cells amounted to only a small fraction of the total RSV-specific response in PBMC of the same infants which suggested a role for other possibly more dominant epitopes during primary infections.

We found a correlation between disease severity and the amount of activated T cells in blood but not the lungs. Future studies identifying the total $CD8^+$ T cell population directed against RSV in the airways and comparing these $CD8^+$ T cell responses to responses found infants with mild disease, will be difficult to design but would give a better insight in the role of immune pathology.

The secondary $CD8^+$ T cell response to respiratory infections in the airways and peripheral blood was studied in healthy adults and children with a tracheostoma. In these studies we showed that during respiratory infections there is activation and proliferation of a significant fraction of the $CD8^+$ T cell population, which normalizes after 15-30 days. The percentage of proliferating cells is higher during RSV infection when compared to the milder rhinovirus infections. In our studies we further showed that during respiratory infections with heterologous viruses,

RSV and influenzavirus specific $CD8^+$ T cells disappear from the blood and travel to the lungs. This bystander effect probably explains the lymphopenic state that is often found in human after viral infections. The fast recruitment of all effector/effector-memory T cells to the site of infection might contribute to faster clearance of the virus, as besides non-specific bystander cells, it will also attract both $CD8^+$ T cells specific to the infecting virus and cross reactive T cells. Compared to $CD8^+$ T cells directed against the homologous virus, this bystander response is less robust than the specific response during respectively RSV and influenzavirus infections and declines a few days after onset of symptoms. While $CD8^+$ T cells specific for the infecting virus proliferate to contribute to viral clearance, non specific $CD8^+$ T cells are redundant, and are therefore rapidly cleared from the site of infection.

In a study comparing RSV-specific T cells responses in healthy adults to more susceptible patient groups like the elderly and patients with COPD, it was shown that these more susceptible patient groups have diminished memory T cell numbers against RSV when compared to younger healthy adults. This suggests an essential role of $CD8^+$ T cells in protective immunity. Boosting RSV-specific $CD8^+$ T cell responses in the elderly might be an achievable method to reduce disease in this susceptible age group. Overall these studies contributed to deeper knowledge in the complex interplay between RSV and $CD8^+$ T cell responses.

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Human RSV-specific T cells

Summary

Summary

RSV is the most common cause of hospitalization due to lower respiratory tract infection in infants. However, the majority of infants experience symptoms of a normal common cold. It is currently appreciated that both $CD4^+$ T cells and $CD8^+$ T cells play an important role in clearing RSV infections. However, both T cell subsets might also augment disease (1). A role for $CD4^+$ T cells in severe pathogenesis was found during a vaccination trial with a formalin-inactivated RSV vaccine. Upon exposure to the natural virus enhanced pathology was observed in vaccinated children characterized by a strong proliferative T cell response and an influx of eosinophils into the lung. In addition, RSV bronchiolitis is associated with the development of wheezing during childhood, which is also a process that might involve a type 2 immune response characterized by the production of the cytokines IL-4, IL-5 and IL-13. Therefore, numerous studies investigated the role of Th2 cytokines versus Th1 cytokines in mouse models, whereby the G protein seemed to be implicated as target antigen for Th2 type cells (2;3). Studies in human patients on the role of $CD4^+$ T cells in severe disease have shown contradictory results and both type one and two cytokines have been found in PBMC and/or nasal aspirates from RSV-infected patients (4-7). In **chapter 3** we focused on human RSV-specific $CD4^+$ T cells specific for epitopes derived from the RSV G protein. We characterized a region within the G protein containing two overlapping peptides that were presented in the context of HLA-DPB1*0401 and *0402. These peptides elicited a different cytokine pattern when used to stimulate PBMC of healthy human blood donors. Furthermore, PBMC derived from two different donors homozygous for HLA-DPB1*0401 produced a different cytokine pattern upon stimulation with peptide G165-179. These findings suggested that both peptide length and the memory T cell repertoire of an individual could determine the type of response upon re-stimulation. Culturing of BAL cells in the presence of these G protein derived peptides showed mixed Th1/Th2 responses in 2 out of 5 infants with severe primary RSV infection. Therefore, it can be concluded that responses against the G protein are indeed elicited during primary RSV infection and different individuals might respond with a different cytokine signature.

Despite successful protection against several infectious diseases, inactivated vaccines that elicit strong antibody responses but poor T cell immunity do not seem to provide successful protection against all pathogens. Even natural RSV infections poorly protect against reinfections. For most viruses $CD8^+$ T cells play a role in the elimination of infected cells and thus prevention of virus propagation. In order to understand the contribution of virus-specific $CD8^+$ T cells to immune protection, their contribution during primary responses and dynamics during secondary infections needed to be addressed. Therefore, in **chapter 4** we focused on RSV-specific $CD8^+$ T cell responses. We identified a set of 5 new RSV-specific epitopes, that were presented in the context of commonly used HLA-types (HLA-A1, -A3, -B44 and -B51). We showed that these epitopes, derived from the matrix (M), nonstructural (NS1) and second matrix (M2) proteins of RSV, were processed

and presented by RSV-infected antigen presenting cells. Using HLA-A1 tetrameric complexes containing the peptide from the M protein, we found that 60% of healthy adult blood donors had $CD8^+$ memory T cells directed against this epitope in peripheral blood. The phenotype of $tetramer^+$ cells was $CD45RA^-$, expression of CD28 and CD27 was down regulated compared to the level of expression on naive T cells, and the chemokine receptor CCR7 was absent, which defines the cells as effector/effector-memory cells. The frequency of $tetramer^+$ cells varied between 0.4 and 3 per 10^4 $CD8^+$ T cells in PBMC of healthy a-symptomatic adult donors.

The newly identified epitopes and a previously described HLA-B7 restricted epitope were used to study the RSV-specific $CD8^+$ T cell responses during severe primary RSV infections in BAL and PBMC of infants (**chapter 5**). Compared to healthy controls, an influx of highly activated $CD8^+$ T cells was observed in BAL of infected infants. Furthermore, we were able to identify RSV-specific T cells in BAL in 22 out of 23 infants expressing the relevant HLA-type, using tetramers containing HLA-A1, -A3 or -B7 epitopes. All $tetramer^+$ cells were highly activated, showing high percentages of GranzymeB, CD38 and HLA-DR expression, and approximately half of them showed Ki-67 expression, indicating that they were recently activated proliferating cells. Compared to blood of healthy blood donors a 10 fold increase of RSV-specific cells was observed in BAL during primary RSV infection, with an average of 0.58% of $CD8^+$ T cells. There was no correlation between the percentage of RSV-specific $CD8^+$ T cells and time of sampling or disease severity. However, in PBMC of these infants the highest percentage of activated $CD8^+$ T cells and $tetramer^+$ cells was found at the time of extubation, and the level of the T cell response correlated with days on ventilation and maximum ventilation peak pressure required. Furthermore, using RSV-infected dendritic cells as antigen presenting cells it was shown that the number of RSV-specific cells in blood was much higher than the response visualized with tetramers containing single epitopes. These data showed that during primary RSV infection up to 3.5 % of total T cells in blood participated in the RSV-specific response.

In **chapter 6** we investigated $CD8^+$ T cell dynamics upon secondary respiratory infections in both blood and tracheostoma aspirate. PBMC of adults showed a 4-17% increase of effector type $CD8^+$ T cell that peaked on day 3-7 after clinical manifestation of upper respiratory tract infection. During the first few days of heterologous infections, RSV and influenzavirus-specific $CD8^+$ T cells disappeared from blood, to re-appear several days later. To investigate whether these cells were recruited to the lungs we followed the $CD8^+$ T cell dynamics in tracheal aspirate of children with a tracheostoma. After viral infection there was a large influx of activated cells into the tracheal aspirate. Indeed, we were able to show the influx of a small percentage of RSV or influenzavirus-specific cells upon heterologous infections, that disappeared within 14 days. Upon infection with homologous virus, ie RSV, both duration and magnitude of the RSV-specific response exceeded the RSV-specific responses seen after infection with heterologous viruses, i.e. rhinovirus. During secondary RSV infection RSV-specific $CD8^+$ T cells peaked after

two weeks, and frequencies were twice as high as responses found during primary infection (1.3% versus 0.58%).

Finally, in **chapter 7** we investigated the RSV-specific $CD8^+$ T cell responses in the elderly. While influenzavirus-specific cells remain at stable levels throughout age, RSV-specific $CD8^+$ T cell gradually decline, and were undetectable without expansion in both the elderly and in patients with COPD. Circulating RSV-specific $CD8^+$ T cell had a resting memory phenotype, but were able to regain effector cell function upon re-stimulation with RSV derived antigenic peptides in the presence of IL-2 or total RSV antigen. In chaper 8 we discussed the contribution of our studies to current knowledge about RSV induced disease.

In conclusion, the ability to study RSV-specific T cell responses in different patient groups and healthy individuals provides important knowledge on the presence of memory and effector T cell types during health and disease which might contribute to the rational development of vaccines that aim to elicit protective T memory responses. Moreover, the T cell epitopes will be valuable tools to monitor the efficacy of candidate vaccines.

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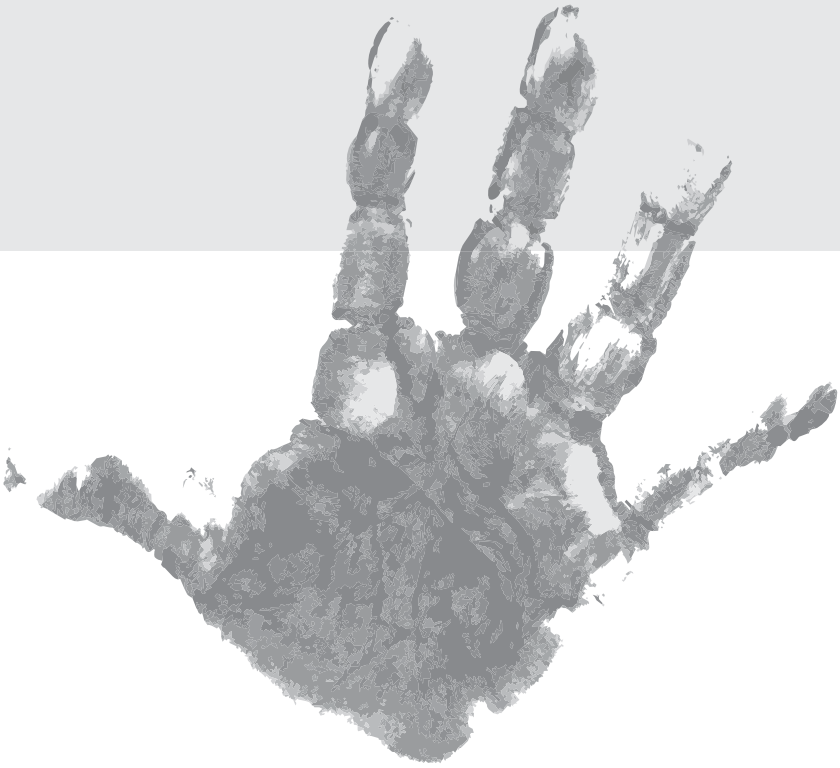

Nederlandse samenvatting, Dankwoord, Curriculum Vitae, List of publications

Nederlandse samenvatting

Respiratoir syncytieel virus (RSV, beter bekend als het RS-virus) is bij zuigelingen de meest voorkomende oorzaak van ernstige luchtweginfecties. Ongeveer 70% van alle kinderen wordt in het eerste levensjaar geïnfecteerd met RSV. Meestal veroorzaakt dit alleen de klachten van een normale verkoudheid, maar 22% van de kinderen ontwikkelt een ontsteking van de lagere luchtwegen, zoals een bronchiolitisbeeld, dat tot zuurstoftekort en voedingsproblemen kan leiden. Van alle kinderen in het eerste levensjaar wordt ruim 1% in het ziekenhuis opgenomen vanwege RSV-bronchiolitis, en 10-20% van hen heeft daarbij beademing nodig.

Waarom een virusinfectie een zo divers spectrum van ziekte-ernst kan veroorzaken, is tot op heden onbekend. Prematuriteit en hart- en longafwijkingen geven een verhoogd risico op ziekenhuisopname door RSV, hoewel de grootste groep opgenomen kinderen uit tot dan toe gezonde, voldragen kinderen bestaat. Waarschijnlijk speelt ook het afweersysteem een rol. Ons afweersysteem, ook wel immuunsysteem genoemd, beschermt ons tegen infecties met pathogenen: bacteriën, virussen en parasieten. Het bestaat uit een aspecifiek ofwel aangeboren deel en een specifiek ofwel adaptief deel. De aspecifieke afweercellen herkennen algemene structuren op pathogenen, zoals suiker- en eiwitpatronen. Ze kunnen snel reageren op een infectie, maar zijn niet erg specifiek voor de verwekker ervan en kunnen de afweer soms maar weinig in gang zetten. Ze helpen wel een infectie onder controle te brengen, maar om deze te klaren is vaak specifieke afweer nodig. De specifieke afweer is specifiek tegen het pathogeen gericht, maar heeft enige dagen nodig om op gang te komen. Dan is er meestal wel een sterke afweerrespons en een langdurige bescherming tegen reïnfecties.

Deze specifieke afweer bestaat o.a. uit B-cellen en T-cellen, ook wel lymfocyten genoemd. B-cellen danken hun naam aan hun uitrijpingsorgaan in vogels, de Bursa van Fabricius. In mensen rijpen B-cellen in het beenmerg uit en produceren antistoffen. Deze antistoffen kunnen binden aan het binnendringende pathogeen waarna dit complex kan worden herkend door andere afweercellen die de geïnfecteerde cel uit de weg kunnen ruimen. Bij virale infecties spelen T-cellen de grootste rol. Zij danken hun naam aan hun uitrijpingsorgaan in de mens, de thymus. Zij zijn onder te verdelen in $CD4^+$ T-cellen en $CD8^+$ T-cellen, zo genoemd naar bepaalde receptoren op het oppervlak. $CD4^+$ T-cellen worden ook wel T helpercellen genoemd en produceren verschillende cytokinen, die B-cellen en $CD8^+$ T-cellen ondersteunen. $CD8^+$ T-cellen, ofwel de cytotoxische T-cellen, kunnen geïnfecteerde cellen vernietigen door de uitscheiding van cytotoxische stoffen, zoals granzymeB en perforine. Hierdoor gaat de geïnfecteerde cel in apoptose: hij doodt zichzelf.

Hoewel het afweersysteem noodzakelijk is om infecties te bestrijden, brengen de stoffen die vrijkomen tijdens een afweerreactie ook schade toe aan de weefsels. Dat tijdens een RSV-infectie de rol voor T-cellen tweeërlei is, blijkt uit verschillende onderzoeken. Aan de ene kant kunnen kinderen zonder T-cellen het virus

niet klaren en zijn ze maandenlang ziek. Aan de andere kant vertonen met RSV-geïnfecteerde muizen zonder T-cellen minder ziekteverschijnselen dan muizen met T-cellen. Ook in mensen zijn er aanwijzingen voor deze zogenaamde immuunpathologie. Om RSV-bronchiolitis te voorkomen werden in de jaren '60 bij een vaccinatietrial honderden kinderen met RSV gevaccineerd dat door formaline was geïnactiveerd. Diegenen van hen die besmet werden met RSV, werden veel ernstiger ziek dan ongevaccineerde kinderen. Maar liefst 80% van de gevaccineerde kinderen belandde in het ziekenhuis, en ook zijn er enkele kinderen overleden. Post-mortem onderzoek liet een infiltraat van T-cellen in de longen zien. De ernstige gevolgen van deze trial hebben duidelijk gemaakt dat gedegen onderzoek naar het natuurlijke beloop van een RSV-infectie noodzakelijk is alvorens nieuwe vaccins kunnen worden ontwikkeld. Ook voor de ontwikkeling van andere medicijnen tegen RSV is het noodzakelijk om te weten waardoor het verschil in ziekteernst tussen kinderen wordt veroorzaakt.

Behalve dat primaire infecties dus serieuze klachten kunnen veroorzaken, is ook bekend dat ongeveer 50% van de kinderen die RSV-bronchiolitis hebben doorgemaakt opnieuw gaan piepen tijdens volgende virale infecties, een astma-achtig beeld dat meestal tussen het 8ste en 13de levensjaar verdwijnt. Hier ligt mogelijk een rol voor de CD4^{+} T-cel, ofwel de T helpercel. CD4^{+} T-cellen kunnen zich, afhankelijk van de eigenschappen van het pathogeen, in drie richtingen ontwikkelen: type 1 helper T-cel (Th1), type 2 helper T-cel (Th2) of regulatoire T-cel (Treg). In een gezond individu bestaat er een nauwkeurige balans tussen deze groepen cellen. Th1 cellen ondersteunen de CD8^{+} T-cel, die geïnfecteerde cellen kan doden. Een sterke Th1 afweer geeft verhoogde kans op auto-immuunziekten: ziekten waarbij het afweersysteem lichaamseigen weefsels aanvalt, zoals bij reuma of suikerziekte. Th2 cellen stimuleren de aanmaak van antistoffen door B-cellen. In allergie en astma wordt een sterke disbalans richting Th2 gevonden. Regulatoire T helpercellen houden het immuunsysteem onder controle. De verschillende typen helpercellen produceren verschillende cytokines: Th1 cellen produceren o.a. IL-2 en IFN-γ, cytokines die geassocieerd zijn met het aanzetten van CD8^{+} T-cellen. Th2 cellen produceren o.a. IL-4, IL-5 en IL-13, cytokinen die geassocieerd zijn met het aantrekken van eosinofielen en het maken van antistoffen. Het meten van deze cytokines geeft een idee over de aanwezige Th1/Th2-balans. Er bestaat al lange tijd een hypothese dat het ontstaan van piepen na RSV mogelijk veroorzaakt wordt door een te heftige Th2 afweer ten opzichte van de Th1 afweer. Diverse onderzoeksgroepen hebben dan ook cytokines in bloed en neusslijm van kinderen met RSV gemeten. De resultaten zijn echter niet eenduidig, waardoor tot op heden deze hypothese niet bevestigd is.

Een derde opmerkelijk aspect van RSV is het feit dat er geen goed immunologisch geheugen tegen het virus wordt opgebouwd. Gewoonlijk blijft na het doormaken van een infectie een deel van de T-cellen die specifiek gericht zijn tegen dat micro-organisme in leven, om ervoor te zorgen dat bij hernieuwde besmetting met dezelfde bacterie of virus de specifieke afweer heel snel op gang komt. Hierdoor

wordt een tweede ernstige infectie voorkomen (men kan bijvoorbeeld maar een keer waterpokken of mazelen doormaken). Sommige virussen hebben hiertegen een uitvlucht gevonden. Het influenzavirus verandert bijvoorbeeld continu, waardoor de stukjes van het virus die aan de B- en T-cellen gepresenteerd worden, bij iedere nieuwe infectie verschillen. Deze worden niet door de opgebouwde geheugencellen (de zogenaamde memory cellen) herkend, waardoor het virus opnieuw in staat is een infectie te veroorzaken. Van het RS-virus is echter bekend dat men van een geheel identiek virus opnieuw ziek kan worden. Na de eerste infectie, die meestal in het eerste levensjaar plaatsvindt, worden volwassenen gemiddeld één keer per 3 jaar ziek door RSV. Gelukkig beperken de symptomen zich in bijna alle gevallen tot een normale verkoudheid. Ouderen en patiënten met COPD kunnen echter opnieuw ernstig ziek worden van een RSV-infectie, en gemiddeld vier per 1000 ouderen overlijden eraan. Hoe het komt dat de afweer tegen RSV niet 100% effectief is, is tot op heden onbekend.

Hoofdstuk 1 en 2 van dit proefschrift geven een introductie en literatuurstudie naar de achtergronden van bovenstaande problematiek, leidend tot de vraagstelling van dit proefschrift: **welke rol spelen T-cellen tijdens infecties met RSV?**

Om T-cel gemedieerde afweer te kunnen bestuderen, is het noodzakelijk om de T-cellen die specifiek tegen RSV gericht zijn, te kunnen identificeren. In het eerste deel van mijn onderzoek (**hoofdstuk 3 en 4**) hebben we gekeken tegen welke delen van het virus de afweer gericht is. Als een virus een cel infecteert, wordt het virale antigeen in kleine stukjes geknipt en als peptides op de oppervlakte van de geïnfecteerde cel gepresenteerd. De cellen die dit presenteren, heten antigeenpresenterende cellen (APC). Zij hebben op hun oppervlak verschillende 'presenteerblaadjes', de zogenaamde MHC-moleculen. Deze MHC-moleculen worden bepaald door erfelijke factoren, het HLA gencomplex in de mens. Doordat men verschillende HLA moleculen van beide ouders erft, is ieders afweer anders. Dit kan verklaren waarom verschillende individuen anders op bepaalde infecties reageren.

In **hoofdstuk 3** hebben we de respons van de $CD4^+$ T-cel, ofwel de T helpercel bestudeerd. Allereerst hebben we twee peptides gevonden uit een deel van het eiwit dat voor aanhechting van RSV aan een cel zorgt, het G-eiwit. Deze twee peptides kwamen uit een overlappend deel van dit eiwit, en werden gepresenteerd door HLA-DPB1*0401 en HLA-DPB1*0402. Dit zijn HLA-moleculen die bij 75% van de blanke bevolking voorkomen. Een dominant peptide geeft dus de mogelijkheid de $CD4^+$ T-cel respons in een groot deel van de blanke populatie te bestuderen. Deze twee overlappende peptides gaven een verschillend cytokinen patroon wanneer witte bloedcellen (PBMC) van gezonde donoren met de peptides gestimuleerd werden. Tevens gaven PBMC's van twee homozygote donoren (die van beide ouders DPB1*0401 hadden geërfd) een verschillend cytokine patroon na stimulatie met peptide G165-179. Dit suggereert dat zowel de lengte van het

peptide als het immunologische geheugen van een individu een rol speelt bij de soort afweerreactie die na infectie ontstaat.

Vervolgens hebben we gekeken naar de afweerrespons tegen deze peptides bij kinderen aan de beademing ten gevolge van een ernstige RSV-infectie. Er werd bij deze kinderen op verschillende momenten een longspoeling gedaan, waarbij door het beademingsbuisje fysiologisch zout werd gespoten dat na enkele seconden weer werd opgezogen. Met deze methode, broncheo-alveolaire lavage (BAL), konden we een indruk krijgen van de cellen en cytokines die in lagere luchtwegen aanwezig waren. De door middel van BAL verkregen cellen werden gestimuleerd met de nieuw geïdentificeerde peptides. Na het kweken met deze peptides werd er in 2 van de 5 kinderen een gemengde Th1/Th2 respons gevonden. Hieruit hebben we geconcludeerd dat de afweer tijdens primaire RSV-infecties inderdaad tegen dit deel van het virus gericht is, en dat verschillende kinderen met een verschillend type afweerrespons kunnen reageren.

In **hoofdstuk 4** hebben we gekeken naar de delen van het RS-virus die door $CD8^+$ T-cellen worden herkend. Met behulp van het computerprogramma SYFPEITHI werd voorspeld welke peptides van het virus goed in de presenteerblaadjes (de HLA-moleculen) voor de $CD8^+$ T-cellen pasten. Door bloed van gezonde volwassenen te stimuleren met deze peptides werden 5 peptides gevonden die inderdaad herkend werden door $CD8^+$ T-cellen. Deze stukjes van het virus, de zogenaamde epitopen hebben we in HLA moleculen gezet, die met een fluorescente stof werden gelabeled. Met deze zogenaamde tetrameren konden we T-cellen die deze peptides herkennen identificeren. In deze studie hebben we gezien dat de specifieke afweerrespons tegen RSV in bloed van gezonde volwassenen heel laag is, tussen 0,01 en 0,1% van alle cytotoxische T-cellen.

Vervolgens hebben we in **hoofdstuk 5** naar de afweerrespons tijdens primaire RSV-infecties gekeken. Bij kinderen die vanwege hun RSV-infectie aan de beademing kwamen, werd in bloed en longspoelsel gekeken naar hun afweer. We zagen dat vergeleken met gezonde leeftijdsgenootjes hun totale T-cel populatie in bloed en longen veel meer geactiveerd was: de T-cellen deelden snel en brachten veel cytotoxische markers tot expressie. Met deze cytotoxische eigenschappen zijn T-cellen in staat geïnfecteerde cellen te doden. Ook vonden we bij bijna alle kinderen geactiveerde RSV-specifieke cellen. Deze kwamen in het longspoelsel ongeveer in een 10 keer zo hoog percentage voor als in het bloed. Doordat het onmogelijk is om van kinderen die alleen verkouden zijn longspoelsel te verzamelen (een kind heeft hiervoor een beademingsbuisje nodig) konden we deze afweerresponsen niet vergelijken met die van kinderen met milde RSV-infecties. Wel zagen we dat in het bloed het percentage van actieve T-cellen gecorreleerd was met de ziekteernst, namelijk met beademingsduur en de gebruikte druk voor de beademing. Dit zou kunnen betekenen dat de eigen afweerrespons van een kind inderdaad te maken heeft met het ontwikkelen van ernstige ziekte. Echter alleen een vergelijking met kinderen met milde symptomen kan hier definitief uitsluitsel over geven.

In **hoofdstuk 6** hebben we bij kinderen met een tracheostoma gekeken naar de afweer tijdens secundaire verkoudheden. Deze kinderen hebben om diverse redenen een permanente directe toegang tot de luchtwegen door een buisje bovenin de luchtpijp. Door dit buisje worden de kinderen regelmatig uitgezogen zodat wordt voorkomen dat slijm de ademweg blokkeert. Dit slijm werd op verschillende tijdstippen tijdens en na verkoudheden opgevangen, en gebruikt voor ons onderzoek. Ook hierin hebben we naar de RSV-specifieke afweer gekeken. Alle kinderen met een RSV-infectie lieten een goede RSV-specifieke afweer zien, die pas in de tweede week na infectie maximaal was. Ten opzichte van kinderen die verkouden waren door het rhinovirus, dat mildere symptomen geeft dan RSV, hadden kinderen met RSV veel meer delende en geactiveerde cellen. Dit zou opnieuw een aanwijzing kunnen zijn dat in kinderen ziekte-ernst juist gecorreleerd is met een heftige T-cel afweer. Een ander fenomeen dat we in deze kinderen onderzochten was het 'bystander effect'. We zagen dat tijdens infecties met andere virussen, bijvoorbeeld het rhinovirus, RSV- en Influenzaspecifieke cellen uit het bloed verdwenen, en in de longen verschenen. Dit wordt gezien als een methode van het lichaam om zo snel mogelijk op infecties te kunnen reageren: tijdens een willekeurige luchtweginfectie worden alle cellen aspecifiek naar de longen gedirigeerd om te kijken of er T-cellen bij zijn die deze infectie herkennen. Deze kunnen dan op snelle wijze de met virus geïnfecteerde cellen uit de weg ruimen.

Tenslotte bestudeerden we in **hoofdstuk 7** de virus specifieke $CD8^+$ T-cel afweer bij ouderen en bij patiënten met COPD. We vonden dat de $CD8^+$ T-cel afweer tegen influenzavirus stabiel blijft met het ouder worden, terwijl die tegen RSV afneemt. In zowel ouderen als patiënten met COPD was de RSV-specifieke afweer niet detecteerbaar zonder de cellen eerst te kweken. Dit zou de hoge morbiditeit en mortaliteit bij ouderen kunnen verklaren. Als de mortaliteit bij ouderen inderdaad komt door de verminderde T-cel afweer, zou een vaccinatie die deze T-cel respons verhoogt in deze patiëntenpopulatie een goede oplossing kunnen zijn.

In **hoofdstuk 8** van dit proefschrift wordt de huidige kennis bediscusseerd omtrent de rol van $CD8^+$ T-cellen tijdens RSV-infecties, en welke bijdrage ons onderzoek hieraan heeft geleverd.

We kunnen concluderen dat deze studies een beter inzicht hebben verschaft in de T-cel afweer tegen het RS-virus. Terwijl in ouderen de T-cel afweer tegen RSV vermindert, zijn er aanwijzingen dat in jonge kinderen deze T-cellen juist een bijdrage leveren aan de ziekte-ernst. De identificatie van dominante epitopen geeft een methode waarmee wij en anderen in de toekomst de effecten van vaccinatie en therapie kunnen bestuderen.

Dankwoord

Promoveren is leuk! Bijna altijd! Of in ieder geval meestal! Dat komt voornamelijk door de input van heel veel leuke mensen, die opweegt tegen het om zes uur 's ochtends in de file staan om een potje tracheostomaslijm op te halen, om vervolgens tot 's avonds laat achter de FACS te zitten… De motivatie van al deze mensen om met elkaar een mooi onderzoek uit te voeren heeft mij heel veel plezier in mijn werk gegeven.

Mijn grootste dank gaat uit naar alle kinderen, hun ouders en de gezonde vrijwilligers die aan dit onderzoek hebben meegewerkt: zonder jullie zou er geen onderzoek geweest zijn!
Ouders van de kinderen die met RSV op de intensive care lagen: ik vind het heel bijzonder dat jullie, ondanks moeilijke tijden en stress, allen bereid waren aan dit onderzoek mee te werken, met als enig oogmerk andere kinderen in de toekomst te helpen. Gelukkig weet ik van de meesten dat het inmiddels heel goed met jullie kinderen gaat.
Kinderen en ouders van de kinderen die meededen aan de tracheostomastudie: dank voor jullie actieve bijdrage! Door mijn vele bezoekjes bij jullie thuis en onze gesprekken onder de koffie heb ik nu een aardig beeld gekregen van jullie thuissituaties. Ik heb bewondering voor jullie dagelijkse liefdevolle zorg.
Collega's: dank voor de vele bloedafnames en neus- en keelswabs! Vanaf nu kunnen jullie weer ongestoord verkouden zijn ☺.
Ouders van de gezonde controlekinderen, en de verkoudheidsstudie: ook jullie dank ik hartelijk voor jullie medewerking aan dit project.

Ook gaat veel dank uit naar mijn copromoter en promotor, die mij begeleidden om dit onderzoek tot een goed einde te brengen.
Beste Grada, een dokter en een biologe: dat was even wennen. Jij hebt me wegwijs gemaakt in de wereld van de immunologie, en me een kritische denkwijze geleerd. Jouw begeleiding was intens, je kennis op het gebied van de T-celimmunologie inspirerend. Ik heb bewondering voor het perfectionisme waarmee je je taken aanpakt, en aldus een onderzoeksgroep en een huishouden weet te runnen. Het is geweldig te zien hoe je ogen gaan stralen als je het over de voetbal hebt: geniet van het leven!
Beste Jan, ik weet nog goed dat je me aansprak op de Boerhaave nascholingscursus infectieziekten, omdat je me herkende van de foto op mijn sollicitatiebrief. Of ik onderzoek wilde doen, of AGNIO wilde worden in het WKZ… En toen ik aangaf dat ik graag allebei zou willen, regelde je een AGIKO-constructie. Dat was kenmerkend voor hoe ik je ken: opmerkzaam en een prima regelaar en manager. Ik heb grote bewondering voor je veelzijdigheid als manager, onderzoeksbegeleider, opleider en clinicus, waarbij je aandacht houdt voor wat er om je heen gebeurt. Dank voor de kans die je me gaf om in het WKZ te mogen werken. Ik doe het met plezier!
Geachte leescommissie: dank voor jullie beoordeling van mijn manuscript.

Mijn rotsen in de branding: de studenten die mij hebben geholpen in die tijd van stress, chaos, en veel te veel werk voor één persoon: het RSV-seizoen. Wendy, mijn allereerste student: van jouw doorzettingsvermogen, nauwkeurigheid en bedrevenheid in het lab valt veel te leren. Jij kon al mijn zesduizend ideeën en proeven door elkaar tot een uitstekend einde brengen, en ik weet dan ook zeker dat je eigen promotie een succes gaat worden. Viola en Renee: dank voor jullie hulp bij de inclusie van de gezonde ouderen. Onze tochtjes naar de verzorgingstehuizen waren een gezellige afwisseling. Laura: jij werd een beetje in het diepe gegooid. Aan jou was de taak de studies tijdens mijn klinische stage toch door te laten gaan, een taak die je prima hebt volbracht. Mariette: jij hebt aan het begin gestaan van de tracheostomastudie. Dankzij jouw bezoekjes aan de mensen thuis wilde iedereen wel meedoen. Heel veel succes met het einde van je studie. Eva en Marianne, mijn studenten van het laatste uur: we hebben wat doorgemaakt in het laatste seizoen, want nu moest alles nauwkeurig en goed gaan, en dat bij vier grote studies tegelijk. Ik dank jullie niet alleen voor de enorme inzet om dit werk tot een goed einde te brengen, maar ook voor de gezelligheid van ons triootje ☺. Mede dankzij jullie ben ik still alive and kicking. Marianne, je bent een prima planner, een leuke dokter, en een geweldige levensgenieter. Kortom, je komt er wel! Eva, je bent een topper. Jouw interesse en inzicht in de mensen om je heen, en je enorme relativieringsvermogen en inzet, zijn ware gaven. Biertje?

De RSV-groep, aanvankelijk klein maar fijn, inmiddels groot en gezellig. Patricia: jij ging mij voor in het promotietraject, een traject dat niet altijd over rozen verliep. De manier waarop je telkens toch weer gedreven doorging, wat uiteindelijk leidde tot een prachtig proefschrift, is een waar voorbeeld voor mij. Dank voor al je hulp bij de laatste loodjes, en natuurlijk voor de gezelligheid van de afgelopen 4 jaar. Mariska: ook jij was er vanaf mijn allereerste dag, en leerde me de basislabtechnieken. Je hulp bij het inzetten van proeven en je leuke verhalen over het leven op de boerderie heb ik erg gewaardeerd. Michael, de enige student die het aandurfde bij ons terug te komen: je bent een leuke aanvulling op de groep. Dank voor al je hulp bij computerproblemen, en veel succes met je eigen proefschrift! Louis, Marieke, Michiel, Titia en Leontien: dank voor jullie actieve bijdragen aan de woensdag- en vrijdagdiscussies! Ook mijn overige collega's uit het lab van de eerste, tweede en derde: dank voor jullie gezelligheid en de leuke werkbesprekingen.

Flexkamergenootjes van de vierde, en Trinette, Pauline, Gerdien en Hannelie: ik heb met veel plezier kamers met jullie gedeeld. In de tijden dat promoveren even helemaal niet leuk was, stonden jullie altijd klaar met tissues en een goed gesprek, waarna het leven weer een stuk vrolijker werd. Hannelie, dank voor al je aandacht en hulp bij kleine dingen, je hebt een superhart. Ik wens je heel veel plezier in het lab en met de rest van je promotietraject.

Mensen met wie ik heb samengewerkt buiten de RS-groep: het enthousiasme voor ons onderzoek, en de leuke inspirerende samenwerking maakte dit onder-

zoek mogelijk en leuk.
Godelieve: het was leuk om je, nadat we elkaar enkele jaren uit het oog verloren waren, op deze manier weer tegen te komen. Ik vond onze brainstormsessies overal en nergens erg gezellig. Ernst en Cecile: onze samenwerking gaat nog even door, dat artikel komt er ooit nog wel. Barbara: succes met je genpolimorfismestudie; ik ben benieuwd naar de uitkomst. Ross and Malcolm: thank you very much for you hospitality to invite me to your hospital in Liverpool, and your willingness to teach me the correct method of performing BAL in infants.
Ook binnen het UMC stonden diverse mensen voor mij klaar: John: jouw aandeel reikte verder dan het diagnosticeren van alle virale verwekkers van de verkoudheden (wat op zich al een heel karwei was). Ik zal nooit vergeten hoe je op je vrije dag een sample in Brabant ging halen toen alle kinderen in het land tegelijk verkouden werden. Ik hoop dat je het naar je zin hebt in Tilburg, en dat we af en toe gezellig kunnen bijkletsen als je in de buurt bent. Henny: ik kon altijd terecht voor de HLA-typering van dat ene extra sample! Hans, Sjef en verpleegkundigen van de PICU: dank voor jullie hulp bij de organisatie en afnames van de BAL-samples bij de kinderen met RSV aan de beademing. Desiree en de anesthesisten van het WKZ (in het bijzonder Hannie en Ingrid): dank voor jullie hulp bij de BAL-afnames van de gezonde controlekinderen.

Collega's, bazen, verpleegkundigen en secretaresses uit het WKZ: dankzij jullie is werken in de kliniek leuk! Dank voor jullie geduld, aandacht, en collegialiteit die ik in mijn opleiding genoten heb. Belinda, dank voor je begeleiding in de nachtelijke uurtjes. Thanks to the doctors and nurses of St. Mary's Hospital, London: I truly enjoyed the lovely time at your hospital.

Vrienden, vriendinnen en familie: jullie hebben het af en toe met mij te stellen gehad als ik weer eens belde dat ik wat later kwam omdat het werk wat uitliep…Toch zijn het ook de leuke dingen buiten het werk die mij elke dag de energie geven met plezier naar mijn werk te gaan: de avondjes Cottonclub, het lesgeven met Arjan en Annette en de Haagstraat 34-avondjes. En niet te vergeten de gezelligheid en vriendschap met de Barendrechtclub (vanwie niemand meer in Barendrecht woont, en maar 25% er ooit gewoond heeft). Bijzondere dank aan Bjarne, Lotte, Jerome, Jude en Koen, bij wie ik altijd terecht kon. And thanks to all the people who make lindy hop the best dance in the world: I really love the atmosphere of the dance scene, the great people, the hospitality… Great that some of you took the effort to come her today to celebrate this special event.

Dank ook aan mijn paranimfen Caroline en Janneke. Caroline, er zijn zeker vier andere plaatsen in dit dankwoord waar je onbenoemd staat: als (bijna) kamergenoot, als RSV-onderzoeksmaatje, als collega en als vriendin, wat precies weergeeft waarom ik je heb gevraagd mijn paranimf te zijn. Ik ben blij dat je ondanks de drukte van je eigen promotie ja hebt gezegd; het weekendje brainstormen in Londen was erg gezellig. Janneke, al heel lang één van mijn beste vriendinnen: onze paden kruisten elkaar zo vaak, dat onze vriendschap onvermijdelijk was. Ik

vind het super dat je mijn paranimf wilt zijn, ook omdat dat inhield dat ik je de afgelopen tijd ondanks je verblijf in het buitenland heel veel heb kunnen zien. Ik denk dat ik dit elk jaar maar ga doen. Dank voor jouw bijdrage in de organisatie van deze promotie, voor je goede ideeën, je bereidheid overal over mee te denken, maar vooral voor je vriendschap.

De op-één-na-laatste plaats is voor Koen en Peter, voor wie mijn dank eeuwig is. Peter: dankzij jou is dit dankwoord niet de enige bladzijde van het proefschrift dat door iedereen bekeken wordt. Je kwam uit het niets en wilde wel wat foto's maken…: dat heb je geweten! ☺ Jouw creatieve inbreng in dit proefschrift maken het voor mij een mooi en speciaal boekje. Ook Lara, Anna, Marcia, Laura, Robin, Brianna, Thomas en al hun ouders wil graag voor hun creatieve bijdrage bedanken.
Koen: jouw aandeel in de lay-out is geweldig. Zonder jouw hulp zou ik dit boekje in het eindstadium vast geheel gefrustreerd in de prullenbak hebben gegooid. (Nu konden we tenminste samen lekker gefrustreerd zijn over mijn onkunde op computergebied). Jouw doorzettingsvermogen en het toch elke keer net iets beter willen maken, hebben tot een prachtige opmaak geleid. Dank, dank, dank.

Tenslotte mijn ouders: jullie hebben de taak op je genomen mij bij te staan op ieder mogelijk gebied als de stress toch wel wat hoog opliep, omdat mijn eigen plannen zelfs voor iemand met veel energie wel eens wat te groot kunnen zijn. Niet alleen dank ik jullie voor de manier waarop jullie mij hebben opgevoed en gestimuleerd kritisch te denken, maar ook ben ik jullie dankbaar voor het feit dat ik in alle mogelijke situaties altijd op jullie terug kan vallen. Papa, natuurlijk voor jou een extra woordje voor alle statistische hulp aan mij, mijn kamergenoten, de vrienden van mijn kamergenoten etc.: dank!

Curriculum Vitae

Jojanneke Heidema was born on January the 20th, 1974 in Zevenaar, The Netherlands. In 1992 she passed her secondary school exams (Gymnasium B) at the Monterssori Lyseum in Rotterdam. In her final years she studied the harp at the Music Academy of Rotterdam. She then started her medical degree at the University of Utrecht, combining her 3rd year of studies with an additional degree in school music at the Music Academy of Utrecht. During her final years of medical school, Jojanneke actively tutored medical students in statistics, physiology and epidemiology, and was a member of the board of the acrobatic rock and roll student society for whom she organized several events such as the Open Dutch Championships. In 1996 she conducted research on the identification of adhesins of the β-haemolytical *Streptococcus* Group B, supervised by Dr. R.G. Feldman and Dr. J Santangelo at the Hammersmith Hospital in London, for which she received the Thalma Eykman prize. After she was conferred her medical degree in 1999 she performed research on HIV in children, under the supervision of Prof. R.R. de Groot and Prof. A.D.M.E. Osterhaus at the Erasmus University of Rotterdam. In 2000 she worked as a senior house officer (AGNIO) in the Zuiderziekenhuis of Rotterdam (Prof. A. Oudensluys-Murphy). In April 2001 she started her pediatric specialist training at the Wilhelmina Childrens' Hospital in Utrecht under the supervision of Prof. J.L.L. Kimpen. She combined this training with a PhD that resulted in the work published in this thesis. Meanwhile she completed her training at the Eijkman Graduate School for Immunology and Infectious Diseases. From July to October 2006 she worked as a specialist registrar in pediatric infectious diseases at St Mary's hospital in London. In October 2008 she will complete her pediatric specialist training.

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