

## Chapter 3.1

### HLA class I-restricted cytotoxic T cell epitopes of the respiratory syncytial virus fusion protein.

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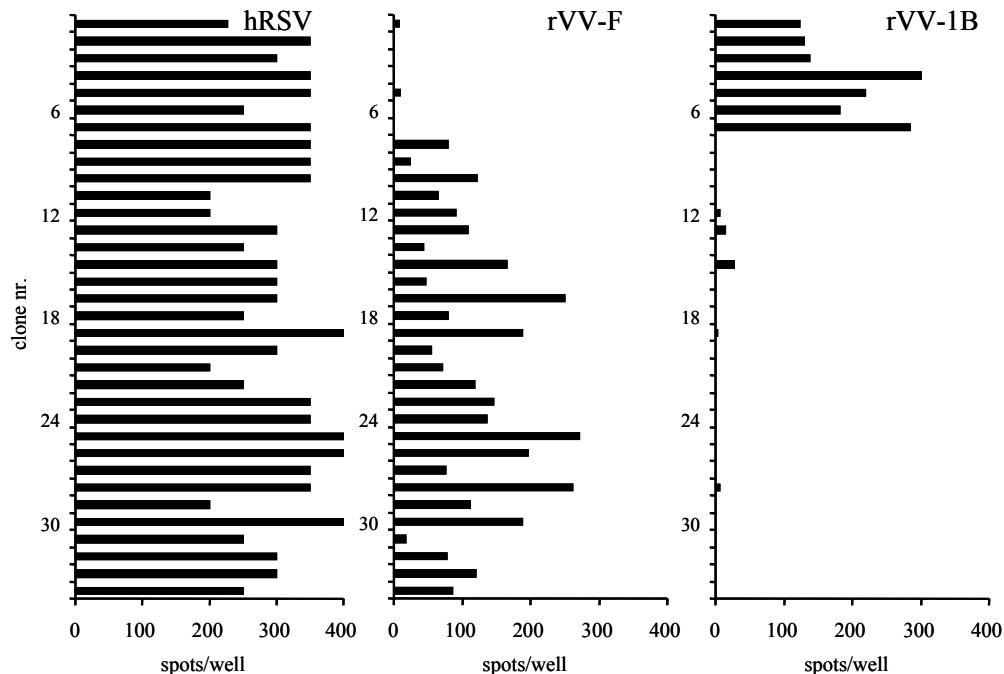
## Summary

Virus-specific cytotoxic T lymphocytes (CTL) play a major role in the clearance of human respiratory syncytial virus (hRSV) infection. We have generated cytotoxic T cell clones (TCC) from two infants who had just recovered from severe hRSV infection. These TCC were functionally characterized and used to identify HLA class I (B57 and C12)-restricted CTL epitopes of hRSV.

hRSV is a common cause of upper respiratory tract infections but may - especially in young infants, the elderly, or immunocompromised individuals - cause severe lower respiratory tract infections [1]. In rodent models, CD4<sup>+</sup>, as well as CD8<sup>+</sup>, hRSV-specific T lymphocytes proved to be involved in both recovery from and immunopathogenesis of the infection [125,192,209,210]. Therefore, a fine balance must exist in these models between protective and disease-enhancing effects of virus-specific T lymphocytes. hRSV-specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) against virtually all hRSV proteins have been demonstrated to circulate in humans after hRSV infection [211-213]. CTL may be expected to play a crucial role in the clearance of hRSV infections, but their role in protection and immunopathology remains unclear. Therefore, the identification of CTL epitopes of hRSV may contribute to future studies concerning the role of CTL in pathogenesis and protection from hRSV infection. Here we describe the functional characterization of CD8<sup>+</sup> cytotoxic TCC generated from two infants who had just recovered from severe hRSV infection. These TCC were also used to identify, for the first time, HLA class I-restricted CTL epitopes of hRSV.

Peripheral blood mononuclear cells (PBMC) were collected from two infants, 4 weeks after a severe, laboratory-confirmed hRSV infection. At the time of infection, they were 1 and 2 months old and had both been admitted to the intensive care unit. B-lymphoblastic cell lines (BLCL) were generated by Epstein-Barr virus transformation [198], infected with hRSV A2 (ATCC VR1322), and UV irradiated [214] to serve as autologous antigen-presenting cells (APC). PBMC (3 x 10<sup>4</sup>/well in 96-well round-bottom plates) were stimulated with APC (10<sup>4</sup>/well), and expanded in RPMI1640 medium supplemented with antibiotics, 10% pooled and heat-inactivated human serum, and recombinant human interleukin-2 (rhIL-2; 50 IU/ml). After 2 weeks, T cells were harvested and cloned by limiting dilution using phytohaemagglutinin stimulation as previously described [200]. TCC thus generated were expanded and tested for hRSV specificity by <sup>3</sup>H-thymidine incorporation assays as previously described [198]. All of the hRSV-specific TCC proved to be of the CD8<sup>+</sup> phenotype in fluorescence-activated cell scanner (FACScan) analysis. TCC whose specificity for hRSV was confirmed by a second proliferation assay were tested for protein specificity by an interferon-gamma (IFN- $\gamma$ ) ELISPOT assay (Mabtech AB, Stockholm, Sweden). In this test, paraformaldehyde-fixed autologous BLCL that had

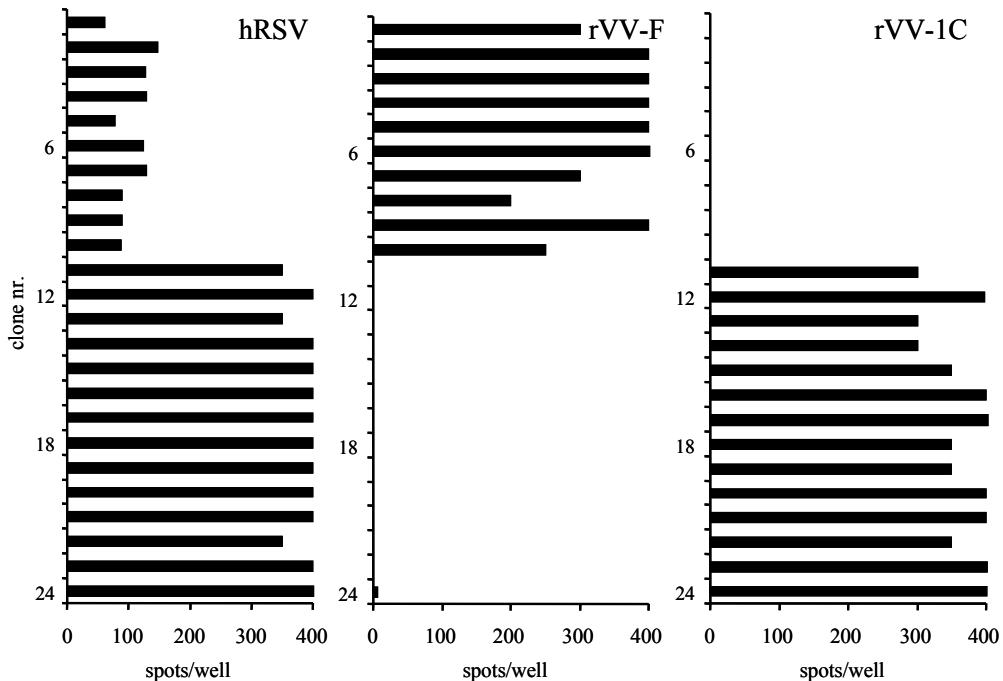
been infected with recombinant vaccinia viruses (rVV) expressing different hRSV proteins (F, G, N, P, M2, SH, M, 1B, or 1C) were used as APC. Briefly, TCC ( $5 \times 10^3$ /well) were incubated with APC ( $10^4$ /well) for 4 h, transferred to anti-human IFN- $\gamma$ -coated plates, and incubated for another 18 h. The ELISPOT assay was further performed in accordance to the kit manufacturer's instructions. The results are shown as numbers of IFN- $\gamma$  producing cells (spots) per well.



**Figure 1A:** Responses of hRSV-specific TCC from patient 1 measured by an IFN- $\gamma$  ELISPOT assay using autologous BLCL infected with hRSV A2 (left), rVV expressing the hRSV F (middle) or 1B (right) proteins. Results are indicated as numbers of spots per well. No responses against the BLCL infected with the other rVV were found (data not shown).

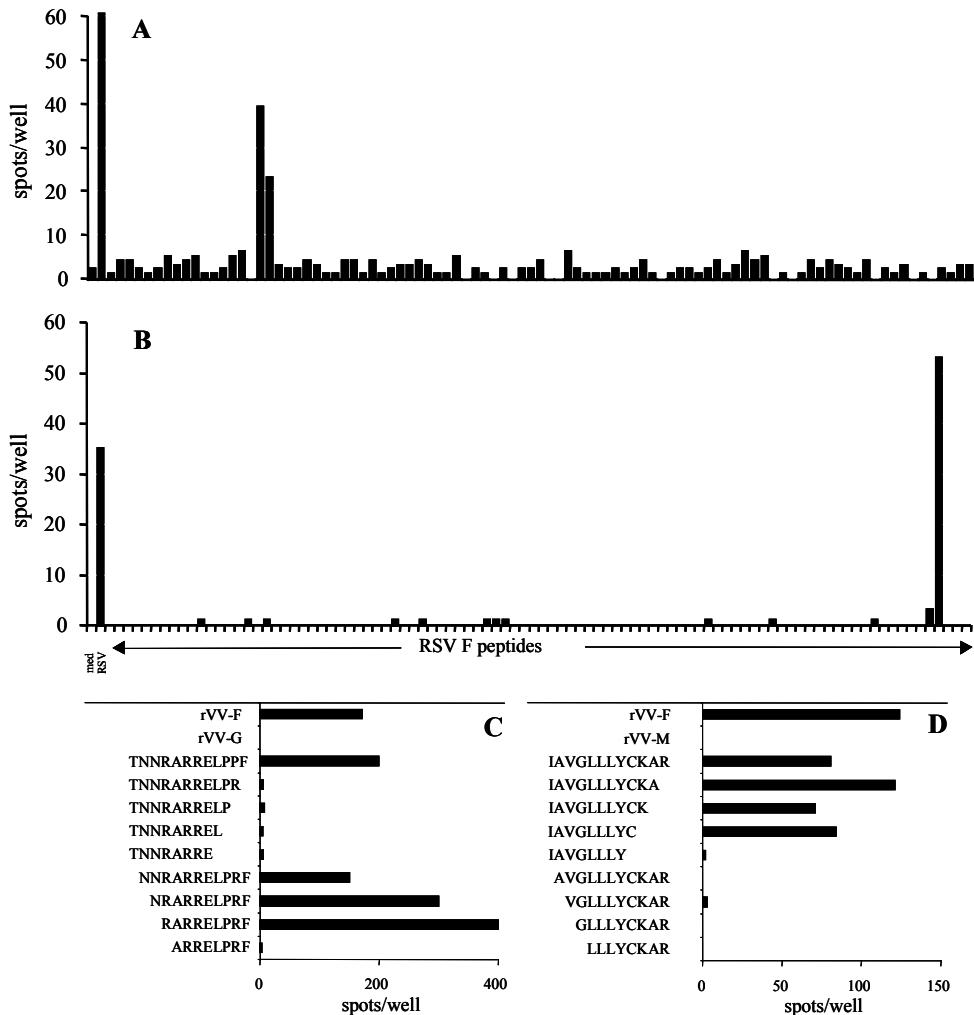
Thirty-four hRSV-specific TCC were generated from the PBMC of patient 1, as detected in a  $^3$ H-thymidine incorporation assay. Of these, 27 proved to be hRSV F specific and 7 were hRSV 1B specific in an IFN- $\gamma$  ELISPOT assay (figure 1A). Twenty-four hRSV-specific TCC were generated from the PBMC of patient 2 as detected in a  $^3$ H-thymidine incorporation assay. Of these, 10 were hRSV F specific and 14 proved to be hRSV 1C specific in an IFN- $\gamma$  ELISPOT assay (figure 1B). None of the clones detected by the  $^3$ H-thymidine incorporation assay was found to be negative by the IFN- $\gamma$  ELISPOT assay. Since the F protein is considered to be a major CTL target [211-213], further efforts were focused on the identification of CTL epitopes in the F protein. To this end, 18-mer peptide

amides overlapping by 12 amino acids, together spanning the entire F protein of hRSV A2 [215,216]( $n = 94$ ), were generated in an automated multiple-peptide synthesizer as previously described [217]. The purity of the peptides varied between 50 and 90%, as determined by analytical reverse-phase high-performance liquid chromatography (C<sub>8</sub> column; gradient of 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile). Autologous BLCL of patients 1 and 2 were pulsed overnight with 1 and 3  $\mu$ M



**Figure 1B:** Responses of hRSV-specific TCC from patient 2 measured by an IFN- $\gamma$  ELISPOT assay using autologous BLCL infected with hRSV A2 (left), or rVV expressing the hRSV F (middle) or 1C (right) proteins. Results are indicated as numbers of spots per well. No responses against the BLCL infected with the other rVV were found (data not shown).

peptide, respectively. HRSV F-specific TCC of patient 1 reacted with peptides 17 and 18 (figure 2A). All of the hRSV F TCC of patient 2 reacted with peptide 91 and marginally with peptide 90 (figure 2B). Subsequently, two additional sets of partially overlapping 8- to 12-mer peptides (80 to 95% purity) were generated to determine the respective minimal epitopes. Autologous BLCL of patients 1 and 2 were pulsed for 1 h with the different peptides at 1 and 3  $\mu$ M, respectively. All of the hRSV F-specific TCC of patient 1 reacted with one nine-mer peptide (RARRELPRF) spanning residues 118 to 126 of the F protein (figure 2C). The hRSV F TCC of patient 2 all reacted with one nine-mer peptide (IAVGLLLKYC) spanning residues 551 to 559 of the F protein (figure 2D).

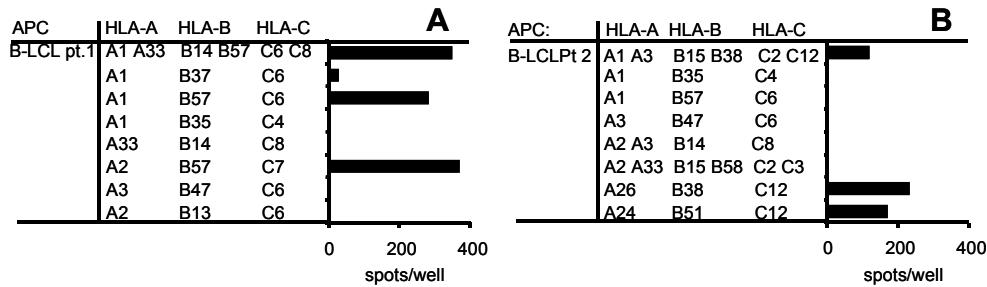


**Figure 2:** Fine mapping of T cell epitopes on the hRSV F protein. Responsiveness of TCC from patients 1 (A) and 2 (B) to partially overlapping 18-mer peptide amides ( $n=94$ ) spanning the F protein was measured by an IFN- $\gamma$  ELISPOT assay. Minimal peptide activation of TCC from patients 1 (C) and 2 (D) was measured in an IFN- $\gamma$  ELISPOT assay using APC pulsed with two additional sets of overlapping 8- to 12-mer peptides. med, medium.

The HLA restriction of the recognition by the hRSV F TCC was also determined by the IFN- $\gamma$  ELISPOT assay using a set of allogeneic BLCL with partially matched HLA types loaded with the respective minimal peptides, as shown in figure 3. Recognition of the hRSV F epitope of patient 1 proved to be HLA B57 restricted. Recognition of the epitope of patient 2 proved to be C12 restricted.

Well-growing TCC of both specificities from each of the patients were arbitrarily selected and tested for cytotoxic activity by a chromium release assay as previously described [218]. Briefly, autologous  $^{51}\text{Cr}$ -labeled, hRSV-

infected BLCL and control BLCL or, in the case of the F-specific TCC of patient 2, minimal-peptide-loaded BLCL or control BLCL were incubated with TCC for 4 h at 37°C at an effector-to-target cell ratio of 10:1, which was found to be the most discriminative ratio in preliminary experiments. Spontaneous  $^{51}\text{Cr}$  release (target cells plus medium) and maximum  $^{51}\text{Cr}$  release (target cells plus Triton X-100) were determined in 12 identical wells. Supernatants were harvested and analyzed in a gamma counter. Assay results were accepted only when the spontaneous-to-maximum release ratio was <25%. All of the TCC tested showed hRSV-specific lysis (figure 4A). Virus-specific production of cytokines in cell-free culture supernatant was measured as previously described [88]. TCC were stimulated with hRSV-infected BLCL or control BLCL or, in the case of the F-specific TCC of patient 2, minimal-peptide-loaded BLCL or control BLCL for 48 h. The concentrations of the following cytokines were measured using commercially available sandwich enzyme-linked immunosorbent assay systems in accordance with the manufacturers' instructions: IL-4 (CLB, Amsterdam, The Netherlands; detection limit, 7 pg/ml), IFN- $\gamma$  (Medgenix Diagnostics, Fleur, Belgium; detection limit, 25 pg/ml), IL-10 (Pharmingen, San Diego, CA, USA; detection limit, 20 pg/ml). All of the TCC produced predominantly IFN- $\gamma$ , indicating a type 1 phenotype *in vitro* (figure 4B).

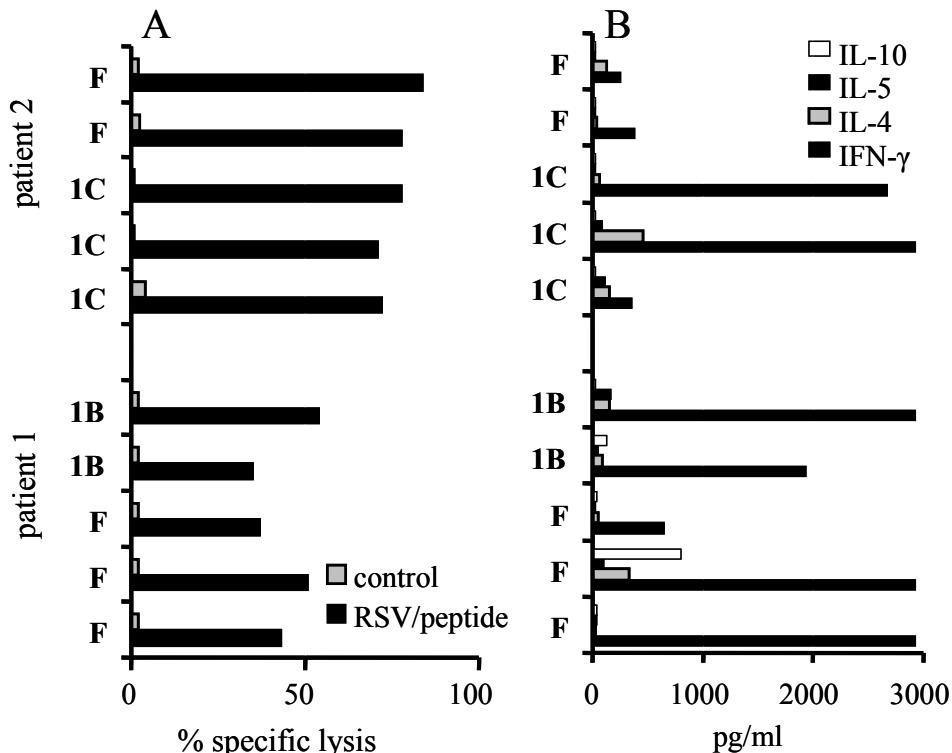


**Figure 3:** Determination of HLA restriction of hRSV F protein recognition by specific TCC using a set of autologous and partially HLA class I-matched BLCL for patients 1 (A) and 2 (B) by an IFN- $\gamma$  ELISPOT assay.

In the present study, we identified two nine-mer CTL epitopes on the hRSV fusion protein. To our knowledge, these are the first HLA class I-restricted CTL epitopes described for hRSV in humans. We found CTL epitopes with an HLA restriction with a low prevalence in the population. But using the techniques described here on samples of more children with a recent hRSV infection, CTL epitopes with more prevalent HLA restrictions may be identified.

The TCC found in the children studied were all CD8 $^{+}$  CTL with a type 1-like cytokine profile. No hRSV-specific CD4 $^{+}$  T cells were detected in the samples of these infants, while the use of similar stimulation protocols did result in the identification of such cells in other systems [198,219-221]. Virus-specific CTL responses play a major role in the clearance of hRSV

infections but may also be involved in pathogenesis [61,192,199,209,222]. In mice, enhanced lung pathology induced by priming with formalin inactivated hRSV has been associated with the absence of a CTL response [126,189]. In young children, a CTL response against hRSV has been described but poor responses were found in younger and more severely ill patients [223,224]. The poor CTL response in young children has been suggested to be one of the possible causes of more severe disease. In these two patients, it was possible to detect hRSV-specific cytotoxic TCC, showing that also in very young children with clinically severe infections, priming of a CTL response does occur, although we cannot say anything about the quantitative responses.



**Figure 4:** Cytotoxic responses (A) and cytokine production (B) of selected TCC with different protein specificities from patients 1 and 2. TCC were stimulated with autologous hRSV-infected or control BLCL and in the case of F-specific TCC of patient 2, minimal-peptide- or control (measles virus) peptide-loaded BLCL. Cytotoxic responses were measured in a chromium release assay using an effector-to-target cell ratio of 10. Cytokines in cell-free culture supernatant (IFN- $\gamma$ , IL-4, IL-5 and IL-10) were measured by enzyme-linked immunosorbent assay. Levels of cytokines in the supernatant of TCC stimulated with control BLCL were all below the detection limit and are not shown.

In conclusion, use of the IFN- $\gamma$  ELISPOT assay to determine epitope specificity proved to be sensitive and convenient, since only small numbers of T cells and APC were needed. This and similar studies may be important for future studies concerning the role of CTL in the pathogenesis of hRSV infection in children.

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## Chapter 3.2

### **Identification of a common HLA-DP4-restricted T cell epitope in the conserved region of the respiratory syncytial virus G protein.**

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## Abstract

The cellular immune response to hRSV is important in both protection and immunopathogenesis. In contrast to HLA class I, class II-restricted hRSV-specific T cell epitopes have not been identified. Here we describe the generation and characterization of two human hRSV-specific CD4<sup>+</sup> T cell clones (TCC) associated with type 0-like cytokine profiles. TCC 1 was specific for the matrix protein and restricted over HLA-DPB1\*1601, while TCC 2 was specific for the attachment protein G and restricted over either HLA-DPB1\*0401 or 0402. Interestingly, the latter epitope is conserved in both hRSV A and B viruses. Given the high allele frequencies of HLA-DPB1\*0401 and 0402 worldwide, this epitope could be widely recognized and boosted by recurrent hRSV infections. Indeed, peptide stimulation of peripheral blood mononuclear cells from healthy adults resulted in detection of specific responses in 8 of 13 donors. Additional G-specific TCCs were generated from three of these cultures, which recognized the identical ( $n=2$ ) or almost identical ( $n=1$ ) HLA-DP4-restricted epitope as TCC 2. No significant differences were found in the capacity of cell lines obtained from infants with a severe ( $n=41$ ) or mild ( $n=46$ ) hRSV lower respiratory tract infection to function as antigen presenting cells to the G-specific TCC, suggesting that severity of hRSV disease is not linked to the allelic frequency of HLA-DP4. In conclusion, we have identified an hRSV G-specific human T helper cell epitope restricted by the widely expressed HLA class II alleles DPB1\*0401 and 0402. Its putative role in protection and/or immunopathogenesis remains to be determined.

## Introduction

Human respiratory syncytial virus (hRSV), a member of the genus *Pneumovirus* of the family *Paramyxoviridae*, is a major cause of severe lower respiratory disease in infants, immunocompromised individuals and the elderly [17,18]. hRSV infections cause yearly epidemics in the winter season of moderate climate zones and are most often associated with relatively mild upper respiratory tract infections [17]. In general, specific immunity is insufficient for protection and hRSV infections continue to occur throughout life.

At present, no licensed hRSV vaccine is available. During vaccine trials in the 1960s, vaccination with a formalin-inactivated whole virus preparation (FI-hRSV) was found to predispose for enhanced clinical disease following natural infection with hRSV [85]. Although the exact mechanism of this apparently immunopathological phenomenon remains unclear, studies in both rodent and non-human primate models have suggested that a skewed hRSV-specific T helper type 2 (Th2) response was a key factor in this process [86,128]. Several studies have suggested that primary infections in young infants, resulting in severe hRSV bronchiolitis, are also associated with Th2

responses [87,91]. However, we have studied hRSV-specific cellular immune responses in two cohort studies of infants with either severe hRSV bronchiolitis or relatively mild hRSV upper respiratory tract infection, and were unable to confirm this observation [88,225].

Few studies have described the hRSV-specific T cell response at the epitope level. In rodents four T cell epitopes have been described of which three were MHC class I- and one MHC class II-restricted. The MHC class I-restricted epitopes were located in the hRSV F [72,73] and M2 [74] proteins, whereas the MHC class II-restricted epitope was located in the hRSV G protein [66]. In humans, HLA class I-restricted epitopes have been identified in the hRSV F [67,226] and NP [68,69] proteins. However, no HLA class II-restricted T cell epitopes have been described. Recently, van Bleek *et al.* [70] described the human CD4 response to the hRSV F protein. Using a set of overlapping peptides they were able to demonstrate *ex vivo* F-specific CD4 memory T cell responses. Similar hRSV F-specific CD4 responses were also described earlier by Levely *et al.* [71].

In the present study, we describe two hRSV-specific CD4<sup>+</sup> T cell clones (TCC) generated from clinical materials collected from infants during the acute or convalescent phase of a laboratory-confirmed hRSV infection. Interferon-gamma (IFN- $\gamma$ ) ELISPOT assays were used to determine protein specificity, minimal epitope and HLA restriction element.

## Materials & Methods

### *Antigen presenting cell lines*

Autologous B-lymphoblastic cell lines (BLCL) were established by transformation of peripheral blood mononuclear cells (PBMC) with Epstein-Barr virus as described previously [198]. For the generation of target and/or stimulator cells, BLCL were infected with hRSV A2 (ATCC-VR1322) at a multiplicity of infection (moi) of 100, resulting in persistently infected BLCL (BLCL-hRSV) as described previously [199]. The percentage of hRSV-positive cells was checked by fluorescence activated cell scanner (FACScan) with FITC-labeled hRSV-specific monoclonal antibodies (mAbs; Imagen, DAKO, Glostrup, Denmark). All BLCL were maintained in RPMI1640 medium (BioWhittaker, Verviers, Belgium) containing penicillin (100 U/ml; BioWhittaker), streptomycin (100  $\mu$ g/ml; BioWhittaker), L-glutamine (2mM; BioWhittaker),  $\beta$ -mercapto-ethanol ( $10^{-5}$ M; Merck KGaA, Darmstadt, Germany) [further referred to as culture medium, CM], supplemented with 10% heat-inactivated (HI; 30 min, 56°C) fetal bovine serum (FBS; Greiner, Frickenhausen, Germany). BLCL used for restriction element analysis were obtained from the European Collection of Cell Cultures (ECACC; cell lines represented by "I" followed by the IHW number), or were in-house generated (represented by "E" followed by an unique number). For autologous and in-house generated BLCL, molecular typing of the HLA-DRB1\*, DQB1\* and

DPB1\* loci was performed using a commercial typing system (GenoVision, Vienna, Austria).

#### *Generation of hRSV-specific T cell clones*

TCC 1 was generated from nasal brush cells collected from an infant (age 20 months) during the acute phase of a laboratory-confirmed hRSV-mediated upper respiratory tract infection. Nasal brush T cells were stimulated *in vitro* with autologous  $\gamma$ -irradiated (3000 rad) BLCL-hRSV, and TCCs were generated by limiting dilution as described before [227]. In short, T cells were seeded in 60-well Terasaki plates (Greiner Bio-One, Frickenhausen, Germany) at concentrations of 3, 1 and 0.3 cells per well and stimulated with allogeneic feeder cells and recombinant human IL-2 (rhIL-2; Red Swan, Utrecht, The Netherlands). After two weeks of expansion, positive cultures were restimulated specifically using  $\gamma$ -irradiated autologous BLCL-hRSV, expanded with rhIL-2 and tested for hRSV-specificity in an IFN- $\gamma$  ELISPOT assay (see below) after stimulation with BLCL or BLCL-hRSV. hRSV-specific TCCs were phenotyped by fluorescence using mAbs against CD3, CD4 and CD8 (DAKO).

TCC 2 was generated from PBMC collected from another infant (age 2 months) during the convalescent phase of an hRSV-mediated lower respiratory tract infection. PBMC were stimulated *in vitro* with  $\gamma$ -irradiated autologous BLCL-hRSV and TCCs were generated by limiting dilution as described above.

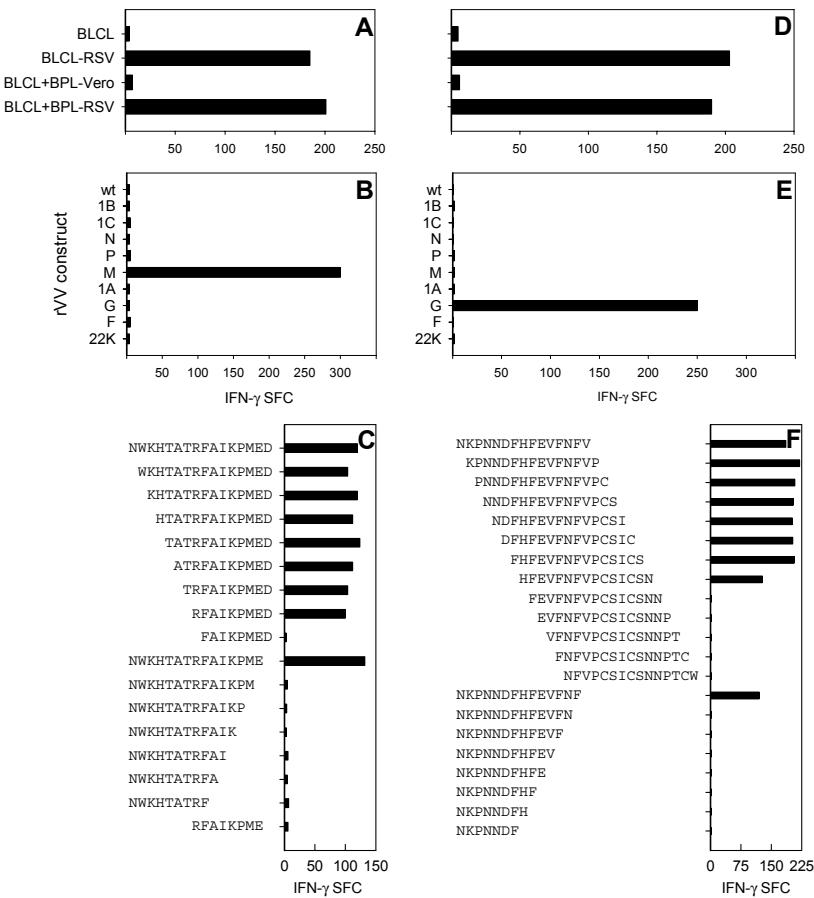
TCC P1, P2 and P3 were generated from PBMC collected from healthy adult donors. PBMC were stimulated *in vitro* with peptide G<sub>158-189</sub> (0.01  $\mu$ M) and TCC were generated by limiting dilution as described above.

T cells and T cell lines were maintained in CM supplemented with 10% HI human pooled serum (further referred to as R10H) and rhIL-2.

#### *ELISPOT assay*

Reactivity of TCC with antigen presenting cells (APC) was determined in IFN- $\gamma$  ELISPOT assays as described previously [225]. In short, TCC were seeded in a concentration ranging from 4,000 to 10,000 cells per well in a 96-well V-bottom plate (Greiner Bio-One), APC were added in an effector-to-target ratio (E:T) of 1:5 and incubated for one hr at 37°C. Cells were transferred to nylon bottom plates (Nalge Nunc, Rochester, NY, USA) pre-coated with a mAb against IFN- $\gamma$  (1-D1K; Mabtech AB, Stockholm, Sweden), incubated for four hr at 37°C, and subsequently washed with phosphate buffered saline containing 0.05% Tween-20 (Merck KGaA). Spots were visualized by incubation with a secondary biotinylated mAb against IFN- $\gamma$  (7-B6-1; Mabtech AB), streptavidine alkaline phosphatase (Mabtech AB) and NBT-BCIP (Kirkegaard & Perry Laboratories, Gaithersburg, MA, USA). Finally, the color reaction was stopped by washing the plates with distilled water and spots were counted using a stereomicroscope at a 25-fold

magnification. IFN- $\gamma$  ELISPOT results are shown in figures and tables as IFN- $\gamma$  spot forming cells (SFC) per well.



**Figure 1:** hRSV-specificity of TCC 1 (A) and TCC 2 (D) was determined in an IFN- $\gamma$  ELISPOT, using autologous BLCL either or not infected with hRSV (BLCL-RSV and BLCL) or pulsed with antigen (BLCL + BPL-Vero and BLCL + BPL-RSV). Protein specificity of TCC 1 (B) and TCC 2 (E) was determined using autologous BLCL infected with different rVV constructs. The minimal epitopes recognized by TCC 1 (C) and TCC 2 (F) were determined using autologous BLCL pulsed overnight with 0.1 (C) or 0.01  $\mu$ M (F) peptide. Results are shown as spot forming cells (SFC) per well.

#### RSV protein specificity

Recombinant vaccinia virus (rVV) constructs mediating the expression of the individual hRSV proteins (rVV-F, -G, -M, -P, -N, -1A, -1B, -1C, -L and -22K) and a wild-type vaccinia virus (VV-wt) were used to infect autologous BLCL at an moi of 10. rVV-infected BLCL were used as APC in IFN- $\gamma$  ELISPOT assays to determine protein specificity.

### Peptide-specific T cell responses

For evaluation of the responses to different peptides, BLCL were pulsed overnight with peptides at different concentrations (1 to 0.001μM) and subsequently used as APC in IFN-γ ELISPOT assays.

For TCC 1, 15-mer peptides (n=49) with 5 amino acids overlap spanning the M protein were tested (last peptide 16 amino acids). For fine T cell epitope mapping, peptides were constructed with one or more deletions on either the N-terminal or C-terminal side. Peptides with free N- and C-termini were synthesized as described before [228], dissolved in dimethyl sulfoxide at a concentration of 10 mg/ml and diluted to 100μM in RPMI1640 (BioWhittaker).

For TCC 2, a 101-mer peptide spanning the conserved region of the hRSV G protein (G2Na, amino acid 130-230, kindly provided by Dr. U.F. Power, Centre d'Immunologie Pierre-Fabre, Saint-Julien-en-Genevois, France) and smaller peptides spanning different regions of G2Na (G<sub>170-187</sub>, G<sub>187-223</sub>, G<sub>187-198</sub>, G<sub>174-189</sub> and G<sub>158-189</sub>) were tested. For fine epitope mapping overlapping 15-mers with 14 amino acids overlap or deletion mutants were used.

**Table I:** Determination of HLA-restriction elements of TCC 1 and 2

**A: TCC 1**

BLCL:	Aut. <sup>a</sup>	E-0002	I-9019	I-9023	I-9050	I-9095	I-9041	I-9063
DRB1*	0301	0102	0301	0301			1104	1302
DRB1*	0701	0701 <sup>b</sup>			0701	0701		
DQB1*	0201	0303	0201	0201		0201	0301	0604
DQB1*	0202	0501			0202	030302		
DPB1*	1101	0201	0402	0101	020102	0401	1101	
DPB1*	1601	0401				1301	0402	1601
IFN-γ SFC <sup>c</sup>	168	3	2	0	2	1	2	188

**B: TCC 2**

BLCL:	Aut.	E-0004	E-0001	E-7366	I-9003	I-9105	I-9026	E-0003	I-9013
DRB1*	0102	0102	0301	1501	0101	11041	0402	1301	1501
DRB1*	0701	0302	0701	0701				1501	
DQB1*	0303	0402	0201	0202		0603	0302	0602	0602
DQB1*	0501	0501	0202	0602	0501			0603	
DPB1*	0201	0101	1101	0301	1301	0201		0402	0402
DPB1*	0401	0301	1601	1001			0401	0401	
IFN-γ SFC <sup>d</sup>	126	1	0	3	1	3	145	130	122

<sup>a</sup>Aut. = autologous BLCL

<sup>b</sup> HLA class II phenotype that BLCL have in common with the autologous BLCL are shaded

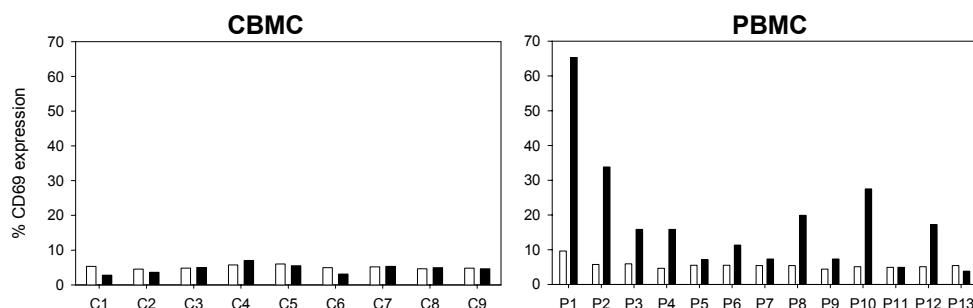
<sup>c</sup>IFN-γ spot forming cells (SFC) per well for TCC 1 were obtained by using BLCL pulsed with M<sub>241-256</sub>

<sup>d</sup>IFN-γ SFC per well for TCC 2 were obtained by using BLCL pulsed with G<sub>158-189</sub>

Protein preparations used to pulse BLCL overnight at a concentration of 1  $\mu$ g/ml were:  $\beta$ -propiolactone (Sigma Aldrich, St. Louis, MO) inactivated hRSV (BPL-RSV, virus cultured in Vero cells) and Vero cell antigen (BPL-Vero), as described by de Swart *et al.* [128].

#### *hRSV-specific T cell responses in PBMC or cord blood mononuclear cells*

PBMC collected from healthy adults or cord blood mononuclear cells (CBMC) were stimulated with peptide G<sub>158-189</sub> (0.1 or 0.01  $\mu$ M) in R10H, and expanded in the presence of rhIL-2. After 2.5 weeks of culture, outgrowth of specific cells was analyzed in a CD69 expression assay as described before [229]. In short, residual CD69 molecules were enzymatically removed (0.1% chymotrypsin type II [Sigma Aldrich] in PBS, 10 minutes, 37°C), and subsequently cells were stimulated with autologous BLCL pulsed with peptide G<sub>158-189</sub> (positive) or G<sub>174-189</sub> (negative). After six hours cells were washed, incubated with  $\alpha$ CD3-FITC (DAKO),  $\alpha$ CD69-PE (BD Pharmingen),  $\alpha$ CD8-PerCP (BD Pharmingen) and  $\alpha$ CD4-APC (BD Pharmingen) and analyzed in a FACSCalibur (BD Biosciences BV, Alphen a/d Rijn, The Netherlands).



**Figure 2:** Detection of G<sub>158-189</sub>-specific T cells in CBMC (C1-C9) or PBMC (P1-P13) stimulated for 2.5 weeks with 0.01  $\mu$ M peptide G<sub>158-189</sub>. The percentage of CD69<sup>+</sup> cells in the CD3<sup>+</sup>CD4<sup>+</sup> fraction of the expanded bulk cultures was determined after six hr of stimulation with autologous BLCL pulsed with G<sub>174-189</sub> (negative; open bars) or G<sub>158-189</sub> (positive; black bars). Responses were considered positive when the ratio of the percentage CD69<sup>+</sup> cells after stimulation with the positive and negative peptide was more than two.

#### *Cytokine ELISAs*

To determine cytokine profiles, TCCs (10<sup>5</sup> cells per well) were stimulated *in vitro* with autologous BLCL either or not infected with hRSV, or pulsed overnight with peptides (0.1  $\mu$ M) M<sub>245-256</sub> and M<sub>241-251</sub> (TCC 1 positive and negative peptide, respectively) or G<sub>161-175</sub> and G<sub>167-181</sub> (TCC P3 positive and negative peptide, respectively), at an E:T ratio of 2:1. After 5 days, culture supernatants were harvested and cytokine levels were determined according to the manufacturer's instructions for IFN- $\gamma$ , IL-2, IL-4 and IL-5 (Biosource, Fleurus, Belgium) and IL-13 (U-Cytech, Utrecht, The Netherlands).

**Table II:** Determination of HLA-restriction elements of TCC P1, P2 and P3**A: TCC P1**

BLCL:	Aut. <sup>a</sup>	I-9060	E-7366	I-9013	I-9105	I-9016	I-9002	I-9026
DRB1*	1301	1301 <sup>b</sup>	0701		11041	1602	0102	0402
DRB1*	1501		1501	1501				
DQB1*	0602		0602	0602		0301	0501	0302
DQB1*	0603	0603	0202		0603			
DPB1*	0401	1901	0301		0201		0401	0401
DPB1*	0402		1001	0402		0402		
IFN- $\gamma$ SFC <sup>c</sup>	200	0	1	140	10	126	190	180

**B: TCC P2**

BLCL:	Aut.	I-9032	I-9038	I-9016	I-9043	E-1519	I-9013	I-9002
DRB1*	0401	0401		1602	1101	0801		0102
DRB1*	1201		1201			1401	1501	
DQB1*	0301	0302	0301	0301	0301	0402	0602	0501
DQB1*						0503		
DPB1*	0301	02012	02012		1001	0301		0401
DPB1*	0402			0402		0501	0402	
IFN- $\gamma$ SFC	180	3	2	165	0	6	175	185

**C: TCC P3**

BLCL:	Aut.	I-9038	E-7366	I-9016	I-9043	E-0005	I-9105	I-9050	I-9002	I-9013
DRB1*	1201	1201	0701	1602	1101	1502	11041	0701	0102	
DRB1*	1501		1501							1501
DQB1*	0301	0301	0602	0301	0301			0202	0501	0602
DQB1*	0601		0202			0601	0603			
DPB1*	0201	02012	0301	0402	1001	0901	0201	02012		0402
DPB1*	0401		1001						0401	
IFN- $\gamma$ SFC <sup>c</sup>	125	80	1	104	2	1	130	80	124	112

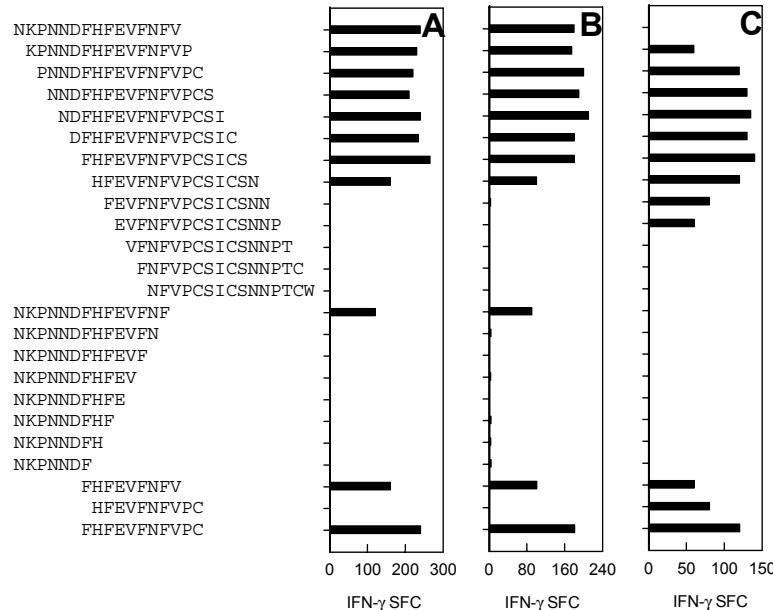
<sup>a</sup>Aut. = autologous BLCL<sup>b</sup>HLA class II phenotype that BLCL have in common with the autologous BLCL are shaded<sup>c</sup>IFN- $\gamma$  spot forming cells (SFC) per well for TCC P1, P2 and P3 were obtained by using BLCL pulsed with G<sub>158-189</sub>

## Results

*Generation and characterization of human hRSV-specific T cell clones*

TCC 1 was cloned from nasal brush cells collected from an infant during the acute phase of a laboratory-confirmed hRSV upper respiratory tract infection. It was hRSV-specific (figure 1A), of the CD3<sup>+</sup>CD4<sup>+</sup> phenotype and recognized the hRSV M protein (figure 1B). Of the overlapping peptides tested, peptide N<sub>241</sub>WKHTATRFAIKPMED<sub>256</sub> was recognized, and the minimal epitope was R<sub>248</sub>FAIKPM<sub>255</sub> with an additional amino acid on either side (figure 1C).

TCC 2 was cloned from PBMC collected from an infant during the convalescent phase of an hRSV lower respiratory tract infection. It was hRSV-specific (figure 1D), of the  $CD3^+CD4^+$  phenotype and recognized the hRSV G protein (figure 1E). The clone was also found to recognize BLCL pulsed with G2Na (G<sub>130-230</sub>) or with G<sub>158-189</sub> (data not shown). The minimal epitope was F<sub>163</sub>HFEVFNFV<sub>171</sub> (figure 1F).



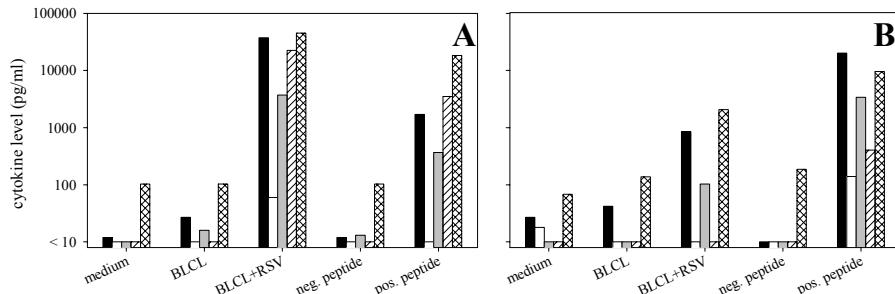
**Figure 3:** Determination of the minimal epitopes of TCC P1, P2 and P3 in an IFN- $\gamma$  ELISPOT assay using autologous BLCL pulsed with 0.01 $\mu$ M G<sub>158-189</sub> as APC. Results are shown as spot forming cells (SFC) per well.

Analysis of published sequences suggested that the M epitope is conserved in hRSV A but not in hRSV B viruses, whereas the G epitope is conserved in both subgroups (results not shown). This was confirmed in an IFN- $\gamma$  ELISPOT assay using autologous BLCL pulsed with hRSV A (L strain, ATCC-VR26) and hRSV B (B1 strain, ATCC-1400) antigen as APC. Where the G-specific TCC P2 was able to recognize both hRSV A and B pulsed APC, the M-specific TCC 1 was only capable of recognizing hRSV A pulsed APC (data not shown).

#### *Determination of HLA-restriction elements of TCC 1 and 2*

The class II genotype of the donor from whom TCC 1 originated was HLA-DRB1\*0301,0701; DQB1\*0201,0202; DPB1\*1101,1601; for the donor of TCC 2 this was HLA-DRB1\*0102,0701; DQB1\*0303,0501; DPB1\*0201,0401. By testing reactivity with peptide-pulsed BLCL matched or mismatched for HLA-DR or -DQ we were unable to determine the

restriction pattern for either of these TCC: for TCC 1 no responses were found, while for TCC 2 the majority of the APCs resulted in positive responses. When the HLA-DP alleles were included, it was found that TCC 1 was restricted by HLA-DPB1\*1601 (table IA), while TCC 2 recognized peptide in the context of either HLA-DPB1\*0401 or 0402 (table IB).



**Figure 4:** Cytokine levels in culture supernatants of TCC 1 (A) or TCC P3 (B) five days after stimulation with medium, autologous BLCL (BLCL), autologous BLCL-hRSV (BLCL-RSV), autologous BLCL pulsed with negative peptide (M<sub>241-251</sub> for TCC 1 and G<sub>167-181</sub> for TCC P3) or autologous BLCL pulsed with positive peptide (M<sub>245-256</sub> for TCC 1 and G<sub>161-175</sub> for TCC P3). Detection limits were 10 pg/ml for IFN- $\gamma$  (black bars), IL-2 (white bars), IL-4 (gray bars) and IL-5 (hatched bars) and 100 pg/ml for IL-13 (double-hatched bars).

#### *Peptide G<sub>158-189</sub> responses in the human population*

Since HLA-DPB1\*0401 and 0402 are the most frequent HLA class II alleles in the human population [230], we investigated the response to peptide G<sub>158-189</sub> in PBMC obtained from healthy adult donors and in CBMC. No specific T cells were found in the CBMC cultures, while G<sub>158-189</sub>-specific T cells were detected in 8 of 13 PBMC cultures (figure 2).

In order to confirm that the G<sub>158-189</sub>-expanded cells were indeed hRSV-specific T cells, TCC were generated from three of these cultures (P1, P2 and P3), and the minimal epitopes of these TCC were determined. As shown in figure 3, TCC P1 and P2 recognized the same minimal epitope as TCC 2, while TCC P3 recognized a 10-mer peptide shifted one amino acid in the C-terminal direction, H<sub>164</sub>FEVFNFVPC<sub>173</sub>.

The class II genotype of donor P1 was HLA-DRB1\*1301,1501; DQB1\*0602,0603; DPB1\*0401,0402, of donor P2 HLA-DRB1\*0401,1201; DQB1\*0301; DPB1\*0402,0301, and of donor P3 HLA-DRB1\*1201,1501; DQB1\*0301,0601; DPB1\*0401,0201. Similar to TCC 2, both TCC P1 and P2 recognized peptide in the context of HLA-DPB1\*0401 or 0402 (table IIA and IIB). Interestingly, TCC P3 was also able to recognize peptide in the context of HLA-DPB1\*0201, and 02012 (table IIC).

#### *All TCC produce both type 1 and 2 cytokines*

TCC 1 (two subclones), P2 and P3 were tested for their ability to produce cytokines after stimulation with different antigens. All TCCs showed

specific cytokine production after stimulation with hRSV-infected or peptide-pulsed autologous BLCL, predominated by IFN- $\gamma$  and IL-13. Interestingly, while TCC 1 (both subclones) produced higher levels of IL-5 than IL-4 upon stimulation, TCCs P2 and P3 produced more IL-4 than IL-5 (figure 4 and data not shown).

#### *HLA-DP4 phenotype in infants with hRSV infections of different clinical severity*

To determine if the T cell response to the HLA-DP4-restricted G epitope plays a role in the pathogenesis of severe hRSV disease, the allelic frequency of HLA-DP4 or the precursor frequency of the G-specific T cells could be compared between infants with hRSV disease of different severity. As alternative to the determination of HLA-DP4 allelic frequencies, specimens from a previous cohort study [88] allowed us to test the functional capacity of BLCL obtained from infants with a severe or mild hRSV lower respiratory tract infection to function as APC to the G-specific TCC. Positive responses of the TCC were found after stimulation by 35/41 (85%) and 50/64 (78%) peptide-pulsed BLCL, respectively (no significant difference,  $p>0.1$ , Fisher's Exact Test, 2-sided).

### **Discussion**

We have identified two HLA-DP-restricted T helper cell epitopes in the hRSV M and G protein, conserved in subgroup A or in both subgroup A and B, respectively. The M epitope was recognized by a TCC restricted over HLA-DPB1\*1601, while the G epitope was recognized in the context of either HLA-DPB1\*0401 or 0402. The ubiquitous distribution of the latter alleles and the results of our peptide PBMC bulk stimulations suggest that responses to the G epitope are generated in a large part of the human population.

The majority of studies on human T helper cell responses have focused on HLA-DR- and -DQ-restricted T cells, to a large extent because HLA-DP appeared less important in contributing to the risk of graft-versus-host disease (GVHD) [231]. However, several HLA-DP-restricted T cell epitopes have now been described [232], including viral epitopes [233,234]. To our knowledge, the hRSV M-specific TCC described here identifies the first HLA-DPB1\*1601-restricted epitope. Recently, a number of TCC have been described recognizing their epitope in the context of HLA-DPB1\*0401 and/or 0402, which both belong to the serologically defined HLA-DPw4 antigenic group [235]. HLA-DPw4 is the most prevalent HLA class II antigen, with an allelic frequency of 78% in the Caucasian population [236]. The hRSV G epitope identified in the present paper is consistent with the suggested motif for HLA-DP4-restricted epitopes as described previously [230].

HLA-DPB1\*0401 and 0402 differ by only three amino acids, and TCC raised in subjects with HLA-DPB1\*0401 were in some cases also able to recognize their antigen in the context of HLA-DPB1\*0402 [235]. We have found the same in the donor of TCC 2 and in donor P3, but have also raised a TCC in a donor who was HLA-DPB1\*0402 positive that could recognize its epitope in the context of HLA-DPB1\*0401 (donor P3). This last TCC, which recognized an epitope, which was shifted one amino acid in the C-terminal direction as compared to TCCs 2, P1 and P2, could also recognize its epitope in the context of HLA-DPB1\*0201 and 02012. Interestingly, HLA-DPB1\*0201 and HLA-DPB1\*0402 differ by only one amino acid in the P4 pocket of the peptide-binding groove, and HLA-DP2 and -DP4 were previously suggested to form a supertype of class II molecules on basis of the homology in the peptide-binding pockets P1 and P6 [230].

The functional studies using BLCL from infants with mild or severe hRSV-mediated lower respiratory tract disease as APC to the G-specific TCC suggested that severity of hRSV disease is not linked to the allelic frequency of HLA-DP4. However, frequency studies of epitope-specific T cells in infants with different disease severity will have to be performed to determine whether these play a role in hRSV pathogenesis. The fact that the conserved HLA-DP4-restricted epitope was found in the G protein brings another dimension to this question, since G-specific responses have often been suggested to be involved in natural or vaccine-mediated enhanced disease [64,191]. However, in our study similar Th0-like cytokine production profiles were found for both the M- and G-specific TCCs.

Castelli *et al.* [230] suggested that specific HLA-DP4-restricted epitopes could be used as peptide vaccines, because of the high frequency of HLA-DP4 worldwide. However, vaccination with non-replicating hRSV vaccines that induce only HLA class II-restricted T cell responses has been associated with immunopathology in humans [85], non-human primates [128,237] or rodents [86,172].

In conclusion, we have identified the first HLA-DPB1\*1601-restricted and a conserved HLA-DP4-restricted T cell epitope in the hRSV M and G protein, respectively. Whether immune responses to the latter epitope are involved in hRSV-mediated immunopathogenesis remains to be determined.

### Acknowledgments

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## Chapter 3.3

### **T cell responses to the respiratory syncytial virus fusion and attachment proteins in recombinant vaccinia virus MVA stimulated human bulk cultures.**

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*submitted*

## Abstract

We have studied memory T cell responses to the hRSV fusion (F) and attachment (G) proteins in peripheral blood mononuclear cells (PBMC) from healthy young adults. Stimulation with autologous cells infected with recombinant modified vaccinia virus Ankara expressing F (rMVA-F) yielded F-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell lines, while rMVA-G-stimulated PBMC only yielded G-specific CD4<sup>+</sup> T cell responses. Using a set of overlapping peptides spanning the F protein, previously undescribed epitope-containing regions were defined. This approach will be useful to define protein-specific T cell responses in different viral systems.

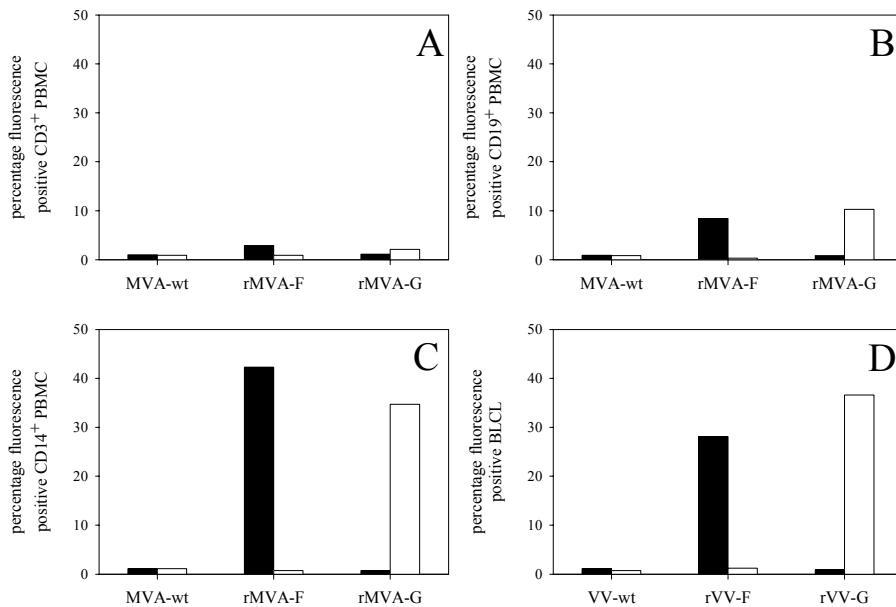
## Introduction

Infection with human respiratory syncytial virus (hRSV), a member of the genus *Pneumovirus* of the family *Paramyxoviridae*, is a major cause of severe respiratory disease in infants, immunocompromised individuals and the elderly. hRSV infections cause yearly epidemics in the winter season of moderate climate zones and are commonly associated with relatively mild upper respiratory tract infections [17]. Specific immunity induced by natural infection generally does not confer protection from re-infection, but does protect against the development of severe lower respiratory tract disease. No human hRSV vaccine is licensed to date, but a number of different vaccination approaches are currently being explored in preclinical and clinical studies [118]. However, vaccine development is still hampered by the results of clinical trials in the 1960s, when formalin-inactivated whole virus preparations were found to predispose for enhanced disease upon natural infection with hRSV [85]. Although the exact mechanism remains subject of debate, it is generally accepted that this enhanced disease had an immunopathological basis [86,129,172].

It has often been speculated that the hRSV G protein plays a role in the immunopathogenesis of vaccine-mediated or naturally occurring severe hRSV disease [64]. The G protein has several unique properties, including heavy glycosylation, the occurrence of mucin-like regions and the apparent inability to induce an MHC class I-restricted CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) response [64]. Studies characterizing the hRSV-specific cellular immune responses in humans remain limited. HLA class I- and/or class II-restricted T cell epitopes have been described in the nucleoprotein (N), fusion protein (F) and attachment protein (G). HLA class II-restricted T cell responses to the F protein were studied in relative detail by using a set of overlapping peptides, which resulted in the detection of multiple epitope-containing regions spanning the F protein [70,71].

The precursor frequencies of hRSV-specific human T cells present in peripheral blood have not been studied. However, to detect low frequent memory T cells a specific *in vitro* stimulation protocol is required. In contrast to the approach using *in vitro* stimulation with hRSV-infected cells [226] or

overlapping peptides [70], we have chosen to use recombinant modified vaccinia virus Ankara (rMVA) expressing the hRSV F (rMVA-F) or G (rMVA-G) genes to enrich for hRSV glycoprotein-specific T cells. MVA is replication-deficient in most mammalian cells, and was shown to be a highly effective and safe vaccine vector system, capable of inducing both MHC class I- and class II-restricted T cells [163,167]. The purpose of the present study was the further characterization of the epitope recognition in the F- and G-specific T cell response against hRSV, which may be of crucial importance for the rational design of safe and effective hRSV vaccines.

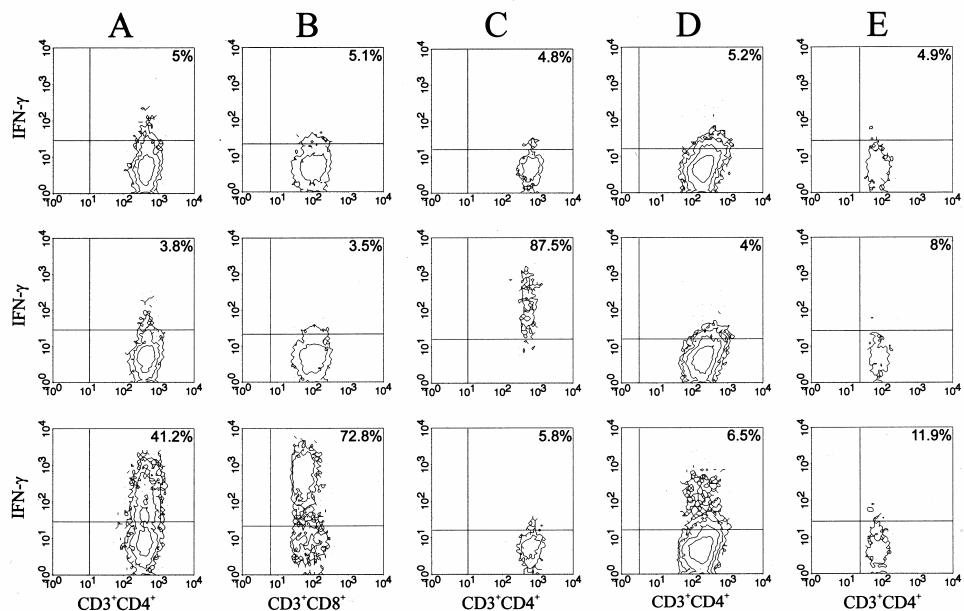


**Figure 1:** Detection of hRSV F or G in CD3<sup>+</sup> PBMC (A), CD19<sup>+</sup> PBMC (B), CD14<sup>+</sup> PBMC (C) or BLCL (D). PBMC were infected with MVA-wt, rMVA-F or rMVA-G, while BLCL were infected with VV-wt, rVV-F or rVV-G. Cells were stained using monoclonal antibodies to F (black bar) or G (white bar).

## Subjects, Materials & Methods

Peripheral blood mononuclear cells (PBMC) were collected from three healthy young adult blood donors with immunity to hRSV and without immunity to vaccinia virus (VV). The HLA class I and class II genotype of these donors was for donor 1: HLA-A\*11; B\*35,44; Cw\*04; DRB1\*11,13; DQB1\*03,06; DPB1\*0201,1301; donor 2: HLA-A\*01,03; B\*07,35; Cw\*04,07; DRB1\*12,14; DQB1\*03,05; DPB1\*0401,0402; and donor 3: HLA-A\*01,03; B\*08,15; Cw\*03,07; DRB1\*01,11; DQB1\*03,05; DPB1\*0201,1401.

PBMC of the first and second donor ( $10^5$  cells per well, 96-well round bottom plates) were stimulated with autologous monocytes (approximately  $10^4$  per well) infected (multiplicity of infection [moi] of 3) with rMVA-F or rMVA-G [156] or with wild-type MVA (MVA-wt). MVA viruses were grown in chicken embryo fibroblasts, purified by ultracentrifugation through sucrose and reconstituted in physiological saline. PBMC were cultured in RPMI1640 (BioWhittaker) supplemented with heat-inactivated human pooled serum, after 3 days recombinant human interleukin-2 (rhIL-2, Red Swan) was added and expansion of T cells was observed 3-5 days later in the F- and G-stimulated cultures. No expansion was found in the MVA-wt-stimulated cultures. After 2-4 weeks, individual wells were re-stimulated with autologous monocytes infected with the homologous rMVA construct and expanded with rhIL-2. In some cases these cultures were further expanded by stimulation with phytohaemagglutinin (Roche) and  $\gamma$ -irradiated (3000 rad) allogeneic feeder cells.



**Figure 2:** F-specific (A, B and D), G-specific (C) or false-positive (E) T cell responses in bulk cultures. Bulk cultures were stimulated in a six hr assay with autologous BLCL infected with VV-wt (top graphs), rVV-G (middle graphs) or rVV-F (bottom graphs) and subsequently stained for CD3, CD4, CD8 and IFN- $\gamma$ .

PBMC of the third donor ( $5 \times 10^4$  cells per well) were stimulated twice with  $\gamma$ -irradiated autologous rMVA infected (moi 3) PBMC ( $1.5 \times 10^5$  cells per well) and expanded using rhIL-2. Infection of PBMC resulted in surface expression of F or G in  $CD14^+$  (40% and 34%, respectively; figure 1C),

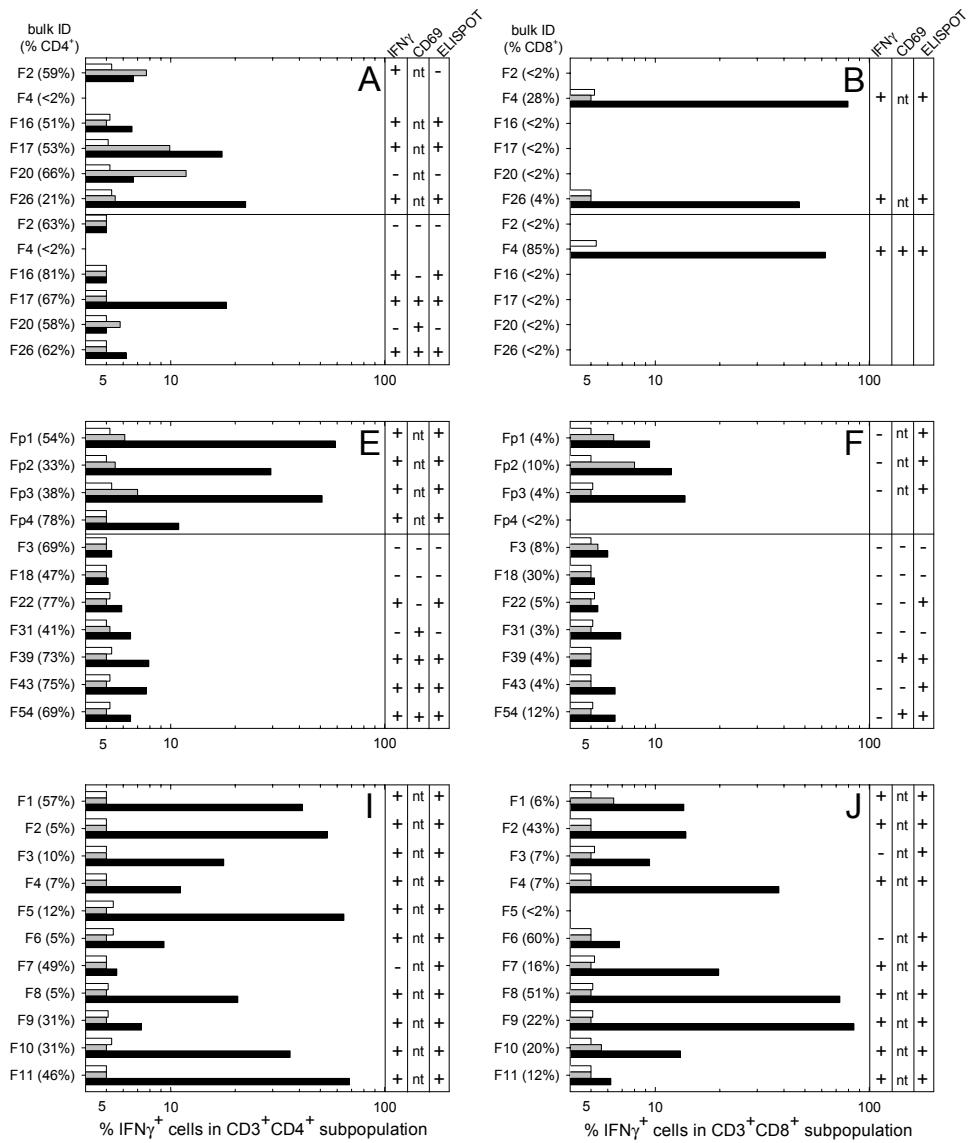
CD19<sup>+</sup> (8% and 10%, respectively; figure 1B) and CD3<sup>+</sup> (2% and 1%, respectively; figure 1A) cells as determined by FACS analysis.

For evaluation of specificity, autologous Epstein-Barr virus-transformed B lymphoblastic cell lines (BLCL) were infected with hRSV A2 (ATCC-VR1322) or recombinant VV (rVV) expressing F or G, or pulsed with peptides at a concentration of 1 $\mu$ M. These included overlapping peptides spanning the hRSV A2 F protein (NCBI accession number AAB86664; 18 amino acids in size with 12 overlap [226]) and five different hRSV A2 G peptides (NCBI accession number AAB86663; G<sub>130-230</sub>, G<sub>158-189</sub>, G<sub>174-189</sub>, G<sub>169-183</sub> or G<sub>162-175</sub>). Staining of rVV-F- or rVV-G-infected BLCL with F- and G-specific monoclonal antibodies showed a surface expression in 27% and 36% of the cells, respectively (figure 1D).

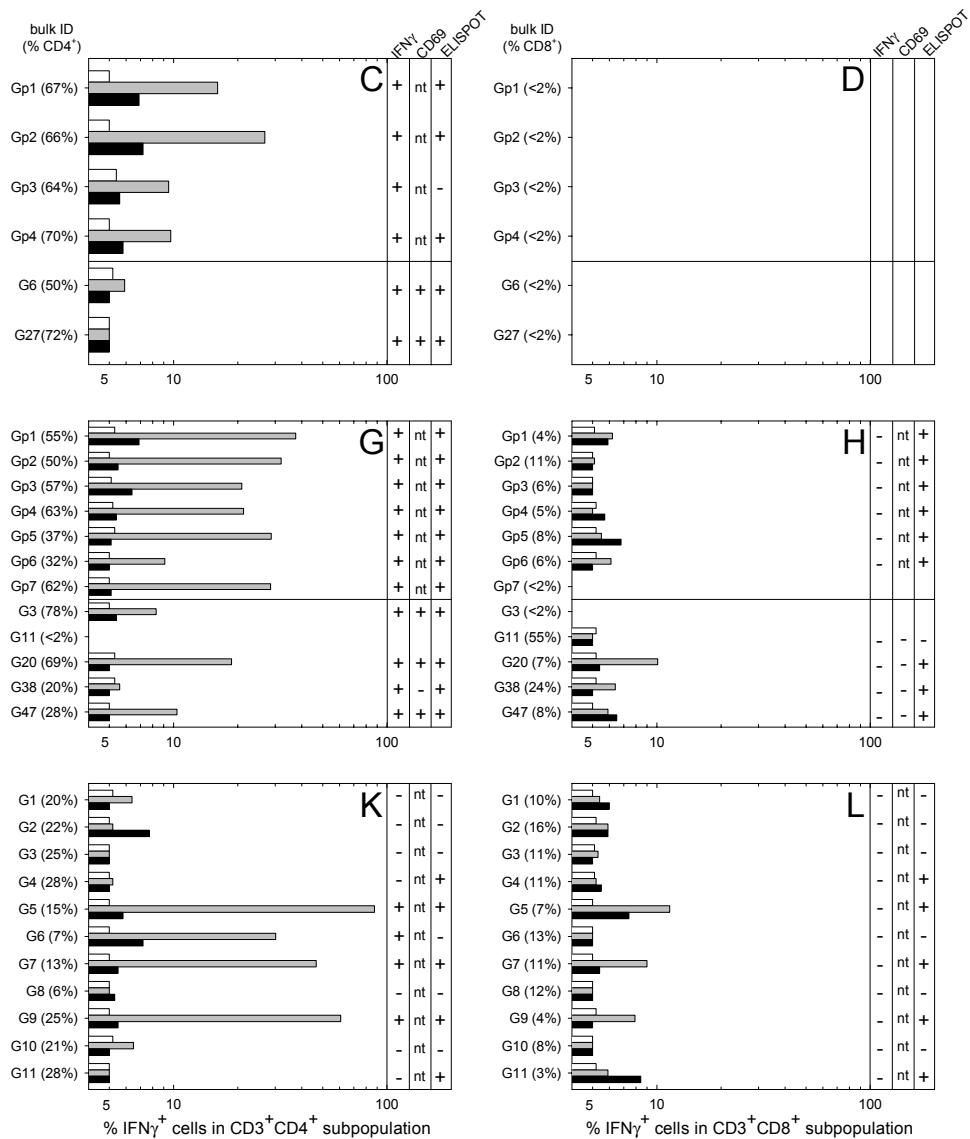
Reactivity of specific bulk cultures with antigen-presenting cells (APC) was determined in interferon-gamma (IFN- $\gamma$ ) ELISPOT, intracellular IFN- $\gamma$  FACS staining or induction of FACS-measured CD69 expression. IFN- $\gamma$  ELISPOT assays (Mabtech AB) were performed as described [225], and results were considered positive if the number of spots was more than three times the background. Intracellular IFN- $\gamma$  FACS staining was performed after six hr restimulation of bulk cultures in the presence of GolgiStop (Pharmingen), followed by surface staining with anti CD3, CD4 and CD8 and intracellular staining with anti IFN- $\gamma$ . CD69 induction assays were performed as described [229]: after enzymatic (0.1% chymotrypsin type II [Sigma Aldrich]) removal of residual CD69 molecules, cultures were restimulated and surface expression of CD3, CD4, CD8 and CD69 was analyzed by FACSCalibur (Becton Dickinson). For analysis, T cells were gated on basis of their FSC/SSC scatter and CD3, CD4 and CD8 expression. CD4<sup>+</sup> or CD8<sup>+</sup> T cell populations were analyzed if they consisted of more than 2% of the total CD3<sup>+</sup> population. For each bulk culture quadrants were set in the CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> T cell populations stimulated with uninfected or wild-type VV (VV-wt)-infected autologous BLCL at a level resulting in approximately 5% CD69 or IFN- $\gamma$  expressing cells.

## Results

rMVA-F- and -G-stimulated bulk cultures were first screened for the presence of hRSV-specific T cells in IFN- $\gamma$  ELISPOT and/or CD69 induction assay. Bulk cultures positive in either of these two assays were further analyzed in an intracellular IFN- $\gamma$  fluorescence assay using autologous BLCL infected with VV-wt, rVV-F or rVV-G as APC. In bulk cultures stimulated with rMVA-F, F-specific T cells could be detected of both the CD4<sup>+</sup> and CD8<sup>+</sup> phenotype, of which examples are shown in figure 2A and B. In bulk cultures stimulated with rMVA-G, G-specific CD4<sup>+</sup> T cells could be detected, of which an example is shown in figure 2C. The responses of all bulk cultures are shown in figure 3. Although some bulk cultures contained relatively high percentages of specific T cells similar to the examples shown in figure 2A-C, responses detected in other cultures were only slightly higher



**Figure 3<sup>1</sup>:** Overview of F-specific T cell responses in single (Fn) or pooled (Fpn) bulk cultures of donor 1 (A-B), 2 (E-F) or 3 (I-J). Bulk cultures had been expanded with rMVA-F and percentages of IFN- $\gamma$  positive cells were determined after a six hour stimulation with autologous BLCL infected with VV-wt (white bars), rVV-G (grey bars) or rVV-F (black bars) in the CD3<sup>+</sup>CD4<sup>+</sup> (A, E, I) or CD3<sup>+</sup>CD8<sup>+</sup> (B, F, J) population. Quadrants were set in the CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> T cell populations stimulated with VV-wt infected autologous BLCL at a level resulting in approximately 5% IFN- $\gamma$  expressing cells. The ID of the bulk culture is followed by the percentage of CD4<sup>+</sup> or CD8<sup>+</sup> cells in the total CD3<sup>+</sup> population (between brackets). The qualitative results of the intra-cellular IFN- $\gamma$  staining, CD69 assay and IFN- $\gamma$  ELISPOT assay are shown in columns on the right of each figure panel. nt = not tested.



**Figure 3<sup>2</sup>:** Overview of G-specific T cell responses in single (Gn) or pooled (Gpn) bulk cultures of donor 1 (C-D), 2 (G-H) or 3 (K-L). Bulk cultures had been expanded with rMVA-G and percentages of IFN- $\gamma$  positive cells were determined after a six hour stimulation with autologous BLCL infected with VV-wt (white bars), rVV-G (grey bars) or rVV-F (black bars) in the CD3<sup>+</sup>CD4<sup>+</sup> (C, G, K) or CD3<sup>+</sup>CD8<sup>+</sup> (D, H, L) population. Quadrants were set in the CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> T cell populations stimulated with VV-wt infected autologous BLCL at a level resulting in approximately 5% IFN- $\gamma$  expressing cells. The ID of the bulk culture is followed by the percentage of CD4<sup>+</sup> or CD8<sup>+</sup> cells in the total CD3<sup>+</sup> population (between brackets). The qualitative results of the intra-cellular IFN- $\gamma$  staining, CD69 assay and IFN- $\gamma$  ELISPOT assay are shown in columns on the right of each figure panel. nt = not tested.

than the background. Of the latter cultures, some were clearly positive (see example in figure 2D), while others seemed to be false-positives with increased background (see example in figure 2E). The low positive G-specific CD8<sup>+</sup> T cell responses (donor 2, bulk culture G20; donor 3, bulk cultures G5, G7 and G9) all belonged to this last category. For each bulk culture tested the qualitative results of the intracellular IFN- $\gamma$  staining, CD69 assay and IFN- $\gamma$  ELISPOT assay are shown in columns on the right of each figure panel.

Selected specific rMVA-F-stimulated bulk cultures were tested in IFN- $\gamma$  ELISPOT assays using autologous BLCL infected with hRSV or pulsed with overlapping peptides of the F protein. As shown in table I, separate bulk cultures of each donor were found to recognize different peptides. The positive peptides were subsequently tested in an intracellular IFN- $\gamma$  assay using peptide-pulsed autologous BLCL as APC to determine the phenotype of the specific T cells.

**Table I:** F-specific responses in IFN- $\gamma$  ELISPOT and IFN- $\gamma$  fluorescence

Donor	Bulk ID	IFN- $\gamma$ ELISPOT		IFN- $\gamma$ fluorescence	
		BLCL-RSV	F peptide (n=94)	CD4	CD8
1	F4 <sup>a</sup>	+	F <sub>109-126</sub>		?b
	F16	+	F <sub>139-156</sub>	F <sub>139-156</sub>	
2	Fp1 <sup>c</sup>	+	F <sub>331-348</sub> , F <sub>337-354</sub>	F <sub>331-348</sub> , F <sub>337-354</sub>	
	Fp2	+	F <sub>295-312</sub> , F <sub>337-354</sub> , F <sub>463-480</sub>	F <sub>295-312</sub> , F <sub>337-354</sub> , F <sub>463-480</sub>	
	Fp3	+	F <sub>265-282</sub> , F <sub>271-288</sub>	F <sub>265-282</sub> , F <sub>271-288</sub>	
3	F1	+	F <sub>337-354</sub>	F <sub>337-354</sub>	
	F2	+	F <sub>133-150</sub> , F <sub>139-156</sub> , F <sub>229-246</sub> , F <sub>235-252</sub>	F <sub>133-150</sub> , F <sub>139-156</sub> , F <sub>229-246</sub> , F <sub>235-252</sub>	
	F3	+	F <sub>139-156</sub> , F <sub>145-162</sub> , F <sub>223-240</sub> , F <sub>295-312</sub> , F <sub>301-318</sub> , F <sub>337-354</sub>	F <sub>139-156</sub> , F <sub>145-162</sub> , F <sub>223-240</sub> , F <sub>295-312</sub> , F <sub>301-318</sub> , F <sub>337-354</sub>	
	F4	+	F <sub>139-156</sub> , F <sub>289-306</sub> , F <sub>295-312</sub>	F <sub>139-156</sub>	F <sub>289-306</sub>
	F5	+	F <sub>433-450</sub> , F <sub>439-456</sub>	F <sub>439-456</sub>	
	F6	+	F <sub>229-246</sub> , F <sub>235-252</sub>	F <sub>229-246</sub> , F <sub>235-252</sub>	
	F7	+	F <sub>169-186</sub> , F <sub>235-252</sub>	F <sub>169-186</sub> , F <sub>235-252</sub>	
	F8	+	F <sub>271-288</sub> , F <sub>337-354</sub> , F <sub>343-360</sub>	F <sub>337-354</sub>	F <sub>271-288</sub>
	F9	+	F <sub>223-240</sub> , F <sub>229-246</sub> , F <sub>235-252</sub> , F <sub>271-288</sub>	F <sub>229-246</sub>	F <sub>271-288</sub>
	F10	+	F <sub>25-42</sub> , F <sub>31-48</sub> , F <sub>391-408</sub> , F <sub>463-480</sub>	F <sub>25-42</sub> , F <sub>31-48</sub> , F <sub>391-408</sub> , F <sub>463-480</sub>	
	F11	+	F <sub>163-180</sub> , F <sub>169-186</sub> , F <sub>241-258</sub> , F <sub>247-264</sub> , F <sub>391-408</sub> , F <sub>463-480</sub>	F <sub>163-180</sub> , F <sub>169-186</sub> , F <sub>241-258</sub> , F <sub>247-264</sub> , F <sub>391-408</sub> , F <sub>463-480</sub>	

<sup>a</sup> Fn = bulk culture originating from one well after stimulation with rMVA-F

<sup>c</sup> not confirmed in IFN- $\gamma$  fluorescence, but bulk contained almost exclusively CD8<sup>+</sup> T cells

<sup>b</sup> Fpn = pool of several bulk cultures after stimulation with rMVA-F

Selected specific rMVA-G-stimulated bulk cultures were tested in IFN- $\gamma$  ELISPOT assays using autologous BLCL infected with hRSV or pulsed with G<sub>130-230</sub>, G<sub>158-189</sub>, G<sub>187-223</sub>, G<sub>169-183</sub> or G<sub>162-175</sub>. The last peptide comprised a recently identified HLA-DP4-restricted T cell epitope [256]. The G-specific T cell lines tested ( $n=6$ ) from donor 2, who was of the HLA-DPB1\*0401 and 0402 genotype, were all specific for this peptide. The G-specific T cell lines of donors 1 and 3 mostly responded to the central conserved domain of the G protein (G<sub>130-230</sub>; 2 of 4 and 3 of 3 lines tested, respectively).

## Discussion

We generated hRSV F- and G-specific T cell lines by stimulation of human PBMC with rMVA-F- or rMVA-G-infected autologous cells. In previous studies with a similar objective, we had used rVV constructs for this purpose. Although this initially resulted in seemingly specific expansion of T cells, these cultures eventually all succumbed to VV infection. The replication deficiency of MVA ensures that cultures generated with the stimulation protocol used here stay free from infectious VV. An attractive property of using rMVA is that infected APC present peptides derived from the recombinant gene in the context of both HLA class I and class II.

In a recent study focusing on the characterization of the hRSV F-specific HLA class II-restricted T cell response, bulk cultures were generated after stimulation with pools of six consecutive overlapping peptides [70]. Comparison of the peptides recognized by specific T cells in both studies showed that new Th epitope-containing regions were identified in the present study: F<sub>133-168</sub>, F<sub>163-180</sub>, F<sub>247-264</sub> and F<sub>433-456</sub>. Combination of the results of both studies clearly shows that Th epitope containing regions are scattered over the entire F protein.

A clear difference between both studies is that we were able to detect F-specific CD8<sup>+</sup> T cells, while the responses upon bulk stimulation with peptides were mediated by CD4<sup>+</sup> T cells only [70]. It is interesting to note that peptides that did not result in expansion of F-specific CD8<sup>+</sup> T cells after stimulation of PBMC with relatively high (20 $\mu$ M) peptide concentrations, could successfully be used for detection of F-specific CD8<sup>+</sup> T cells in our rMVA-F expanded bulk cultures after stimulation with peptide-pulsed autologous BLCL.

One of the additional objectives of the present study was an attempt to identify HLA class I-restricted G-specific CTL responses. However, whereas stimulation with rMVA-F resulted in the detection of F-specific CTL responses in two out of three donors, upon rMVA-G stimulation only G-specific Th cells could be demonstrated. Although in some cultures low G-specific CTL responses were detected, these all proved to be false positives, since the results could not be reproduced and re-stimulation with rMVA-G infected APC did not result in outgrowth of specific CTL. Since only three donors were used in the present study, it cannot be concluded that HLA class

I-restricted G-specific CTL do not exist. We therefore intend to extend the current approach to a larger number of individuals to address this issue, after optimization of the restimulation protocol using rMVA-F infected PBMC, monocytes or dendritic cells.

In almost all cases G-specific CD4<sup>+</sup> T cell responses were directed to the central conserved region of the G protein. However, this does not exclude that the variable regions of the G protein induce Th cell responses. Due to the high variability in the G protein, the G sequences of the viruses that infected the adult donors during their lifetime were probably relatively distant from that of the A2 virus used to generate the rMVA-G virus. Therefore, if Th responses to variable regions of the G protein existed in these donors, they might simply not have been stimulated by the rMVA-G virus.

In conclusion, we have identified in three healthy young adults HLA class I- and class II-restricted T cell responses to multiple regions of the F protein, while T cell responses to the G protein were all restricted over HLA class II and mainly directed to the central conserved domain of the G protein. This study shows that stimulation of PBMC with a recombinant viral vector mediating the expression of a single protein and the subsequent use of overlapping peptides can result in the identification of multiple candidate epitopes in a single donor. This approach will allow the study of protein-specific T cell responses in several viral systems.

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