

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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The cellular immune response to respiratory syncytial virus (RSV) is important in both protection and immunopathogenesis. In contrast to HLA class I, HLA class II-restricted RSV-specific T-cell epitopes have not been identified. Here, we describe the generation and characterization of two human RSV-specific CD4⁺-T-cell clones (TCCs) 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 RSV type 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 RSV infections. Indeed, peptide stimulation of peripheral blood mononuclear cells from healthy adults resulted in the 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 between the capacities of cell lines obtained from infants with severe ($n = 41$) or mild ($n = 46$) RSV lower respiratory tract infections to function as antigen-presenting cells to the G-specific TCCs, suggesting that the severity of RSV disease is not linked to the allelic frequency of HLA-DP4. In conclusion, we have identified an RSV 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.

Respiratory syncytial virus (RSV), a member of the genus *Pneumovirus* of the family *Paramyxoviridae*, is a major cause of severe lower respiratory tract disease in infants, immunocompromised individuals, and the elderly (7, 29). RSV infections cause yearly epidemics in the winter season of moderate climate zones and are most often associated with relatively mild upper respiratory tract infections (29). In general, specific immunity is insufficient for protection, and RSV infections continue to occur throughout life.

At present, no licensed RSV vaccine is available. During vaccine trials in the 1960s, vaccination with a formalin-inactivated whole-virus preparation (FI-RSV) was found to predispose for enhanced clinical disease following natural infection with RSV (17). Although the exact mechanism of this apparently immunopathological phenomenon remains unclear, studies of both rodent and nonhuman primate models have suggested that a skewed RSV-specific T helper type 2 (Th2) response was a key factor in this process (11, 23). Several studies have suggested that primary infections in young infants resulting in severe RSV bronchiolitis are also associated with Th2 responses (24, 28). However, in two other cohort studies of infants with either severe RSV bronchiolitis or relatively

mild RSV upper respiratory tract infection, this observation was not confirmed (6, 12).

Few studies have described the RSV-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 was class II-restricted. The MHC class I-restricted epitopes were located in the RSV F protein (10, 16) and the RSV M2 protein (19), whereas the MHC class II-restricted epitope was located in the RSV G protein (33). In humans, class I-restricted epitopes have been identified in the RSV F (5, 27) and NP (14, 34) proteins. However, no human class II-restricted T-cell epitopes have been described. Recently, van Bleek et al. (32) described the human CD4 response to the RSV F protein. Using a set of overlapping peptides, they were able to demonstrate ex vivo F-specific CD4 memory T-cell responses. Similar RSV F-specific CD4 responses were also described earlier by Lively et al. (20).

In the present study, we describe two RSV-specific CD4⁺-T-cell clones (TCCs) generated from clinical materials collected from infants during the acute or convalescent phase of a laboratory-confirmed RSV infection. Gamma interferon (IFN- γ) enzyme-linked immunospot (ELISPOT) assays were used to determine the protein specificity, minimal epitope, and HLA restriction element.

MATERIALS AND METHODS

Antigen-presenting cell (APC) lines. Autologous B-lymphoblastic cell lines (BLCL) were established by transformation of peripheral blood mononuclear cells (PBMC) with Epstein-Barr virus as described previously (31). For the

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TABLE 1. Determination of HLA restriction elements of TCC 1

BLCL	Restriction elements ^c							
	Aut. ^a	E-0002	I-9019	I-9023	I-9050	I-9095	I-9041	I-9063
DRB1*	0301	0102	0301	0301			1104	1302
DRB1*	0701	0701			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 ^b	168	3	2	0	2	1	2	188

^a Aut., autologous BLCL.
^b IFN-γ SFC per well were obtained by using BLCL pulsed with M₂₄₁₋₂₅₆.
^c HLA class II phenotypes that BLCL have in common with the autologous BLCL are in boldface.

T cells and T-cell lines were maintained in culture medium supplemented with pooled 10% heat-inactivated human serum (referred to below as R10H) and rhIL-2.

ELISPOT assay. The reactivities of TCCs with APCs were determined in an IFN-γ ELISPOT assay as described previously (12). In short, TCCs were seeded in concentrations ranging from 4,000 to 10,000 per well in a 96-well V-bottom plate (Greiner Bio-One), and APCs were added in an effector-to-target ratio of 1:5 and incubated for 1 h at 37°C. The cells were transferred to nylon bottom plates (Nalge Nunc, Rochester, N.Y.) precoated with a MAb against IFN-γ (1-D1K; Mabtech AB, Stockholm, Sweden), incubated for 4 h at 37°C, and subsequently washed with phosphate-buffered saline containing 0.05% Tween 20 (Merck). The spots were visualized by incubation with a secondary biotinylated MAb against IFN-γ (7-B6-1; Mabtech AB), streptavidin alkaline phosphatase (Mabtech AB), and nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). Finally, the color reaction was stopped by washing the plates with distilled water, and the spots were counted with a stereomicroscope at 25-fold magnification. IFN-γ ELISPOT results are shown in figures and tables as IFN-γ spot-forming cells (SFC) per well.

RSV protein specificity. Recombinant vaccinia virus (rVV) constructs mediating the expression of the individual proteins of RSV (rVV-F, -G, -M, -P, -N, -1A, -1B, -1C, -L, and -22K) and a wild-type VV were used to infect autologous BLCL at a multiplicity of infection of 10. rVV-infected BLCL were used as APCs in IFN-γ ELISPOT assays to determine protein specificities.

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

For TCC 1, 15-mer peptides (n = 49) with 5-amino-acid overlaps spanning the M protein (GenBank accession no. P03419) 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 (13), dissolved in dimethyl sulfoxide at a concentration of 10 mg/ml, and diluted to 100 μM in RPMI 1640 (BioWhittaker).

For TCC 2, a 101-mer peptide spanning the conserved region of the RSV G protein (G2Na; amino acids 130 to 230; kindly provided by U. F. Power, Centre d'Immunologie Pierre-Fabre, Saint-Julien-en-Genevois, France) and smaller peptides spanning different regions of G2Na (G₁₇₀₋₁₈₇, G₁₈₇₋₂₂₃, G₁₈₇₋₁₉₈, G₁₇₄₋₁₈₉, and G₁₅₈₋₁₈₉) were tested. For fine epitope mapping, overlapping 15-mers with 14-amino-acid overlaps or deletion mutants were used.

The protein preparations used to pulse BLCL overnight at a concentration of 1 μg/ml were β-propiolactone (Sigma Aldrich, St. Louis, Mo.) inactivated RSV (BPL-RSV cultured in Vero cells) and Vero cell antigen (BPL-Vero), as described by De Swart et al. (11).

RSV-specific T-cell responses in PBMC or CBMC. PBMC collected from healthy adults or cord blood mononuclear cells (CBMC) were stimulated with peptide G₁₅₈₋₁₈₉ (0.1 or 0.01 μM) in R10H and expanded in the presence of rhIL-2. After 2.5 weeks of culture, the outgrowth of specific cells was analyzed in a CD69 expression assay as described before (30). In short, residual CD69 molecules were enzymatically removed (0.1% chymotrypsin type II [Sigma Aldrich] in PBS; 10 min; 37°C), and subsequently the cells were stimulated with autologous BLCL pulsed with peptide G₁₅₈₋₁₈₉ (positive) or G₁₇₄₋₁₈₉ (negative). After 6 h, the cells were washed; incubated with αCD3-FITC (DAKO), αCD69-PE (BD Pharmingen), αCD8-PerCP (BD Pharmingen), and αCD4-APC

(BD Pharmingen); and analyzed in a FACSCalibur (Becton-Dickinson, Erembodegem, Belgium).

Cytokine enzyme-linked immunosorbent assays. To determine cytokine profiles, TCCs (10⁵ per well) were stimulated in vitro with autologous BLCL either uninfected or infected with RSV or were pulsed overnight with peptides (0.1 μM) M₂₄₅₋₂₅₆ and M₂₄₁₋₂₅₁ (TCC 1 positive and negative peptides, respectively) or G₁₆₁₋₁₇₅ and G₁₆₇₋₁₈₁ (TCC P3 positive and negative peptides, respectively), at an effector-to-target ratio of 2:1. After 5 days, the culture supernatants were harvested, and cytokine levels were determined according to the manufacturer's instructions for IFN-γ, IL-2, IL-4, and IL-5 (Biosource, Fleurus, Belgium) and IL-13 (U-Cytech, Utrecht, The Netherlands).

RESULTS

Generation and characterization of human RSV-specific T-cell clones. TCC 1 was cloned from nasal-brush cells collected from an infant during the acute phase of a laboratory-confirmed RSV upper respiratory tract infection. It was RSV specific (Fig. 1A), of the CD3⁺ CD4⁺ phenotype, and it recognized the RSV M protein (Fig. 1B). Of the overlapping peptides tested, peptide N₂₄₁ WKHTATRFAIKPMED₂₅₆ was recognized, and the minimal epitope was R₂₄₈FAIKPME₂₅₅ with an additional amino acid on either side (Fig. 1C).

TCC 2 was cloned from PBMC collected from an infant during the convalescent phase of an RSV lower respiratory tract infection. It was RSV specific (Fig. 1D), of the CD3⁺ CD4⁺ phenotype, and it recognized the RSV G protein (Fig. 1E). The clone was also found to recognize BLCL pulsed with G2Na or with G₁₅₈₋₁₈₉ (data not shown). The minimal epitope was F₁₆₃HFEVFN₁₇₁ (Fig. 1F).

Analysis of published sequences suggested that the M epitope is conserved in RSV A but not in RSV B, whereas the G epitope is conserved in both subgroups (results not shown). This was confirmed in an IFN-γ ELISPOT assay using autologous BLCL pulsed with RSV A (Long strain; ATCC VR26) or RSV B (B1 strain; ATCC 1400) antigens as APCs. Whereas the G-specific TCC P2 was able to recognize both RSV A- and B-pulsed APCs, the M-specific TCC 1 was capable of recognizing only RSV A-pulsed APCs (data not shown).

Determination of HLA restriction elements of TCCs 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; that of the donor of TCC 2 was HLA-DRB1*0102,0701;DQB1*0303,0501;DPB1*0201,0401. By

TABLE 2. Determination of HLA restriction elements of TCC 2

BLCL	Restriction element ^c								
	Aut. ^a	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 ^b	126	1	0	3	1	3	145	130	122

^a Aut., autologous BLCL.
^b IFN-γ SFC per well were obtained by using BLCL pulsed with G₁₅₈₋₁₈₉.
^c HLA class II phenotypes that BLCL have in common with the autologous BLCL are in boldface.

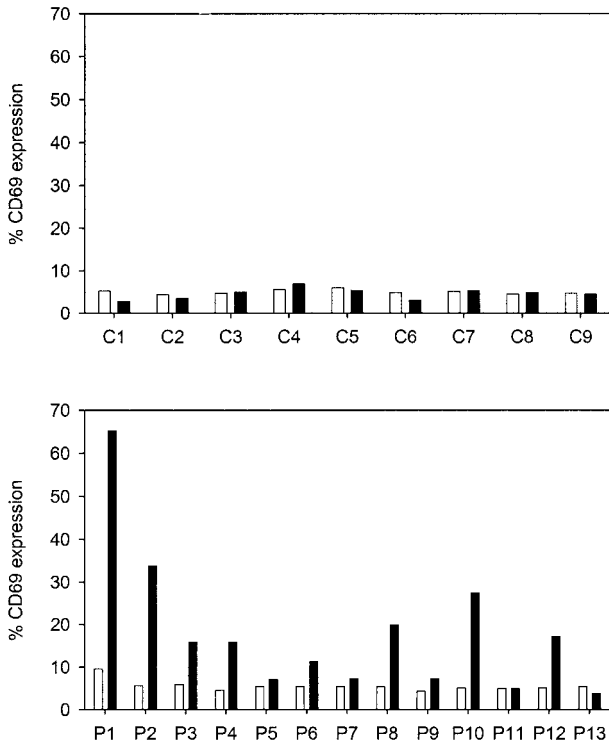


FIG. 2. Detection of G₁₅₈₋₁₈₉-specific T cells in CBMC (C1 to C9) or PBMC (P1 to P13) stimulated for 2.5 weeks with 0.01 μM peptide G₁₅₈₋₁₈₉. The percentage of CD69⁺ cells in the CD3⁺ CD4⁺ fraction of the expanded bulk cultures was determined after 6 h of stimulation with autologous BLCL pulsed with G₁₇₄₋₁₈₉ (negative; open bars) or G₁₅₈₋₁₈₉ (positive; black bars). Responses were considered positive when the ratio of the percentages of CD69⁺ cells after stimulation with the positive and negative peptides was >2.

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 TCCs: for TCC 1, no responses were found, while for TCC 2, the majority of the

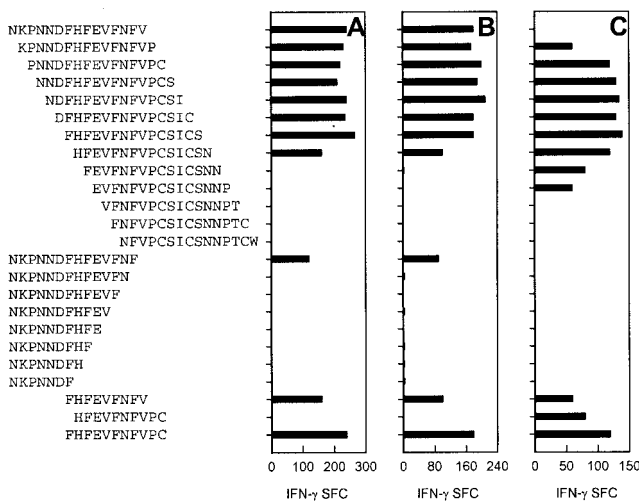


FIG. 3. Determination of the minimal epitopes of TCC-P1, -P2, and -P3 in an IFN-γ ELISPOT assay using autologous BLCL pulsed with 0.01 μM G₁₅₈₋₁₈₉ as APCs. The results are shown as SFC per well.

TABLE 3. Determination of HLA restriction elements of TCC P1

BLCL	Restriction element ^c							
	Aut. ^a	I-9060	E-7366	I-9013	I-9105	I-9016	I-9002	I-9026
DRB1*	1301	1301	0701		11041	1602	0102	0402
DRB1*	1501		1501	1501				
DQB1*	0602		0602	0602		0301	0501	0302
DQB1*	0603	0603	0202		0603			
DPB1*	0401		0301		0201		0401	0401
DPB1*	0402		1001	0402		0402		
IFN-γ SFC ^b	200	0	1	140	10	126	190	180

^a Aut., autologous BLCL.

^b IFN-γ SFC per well were obtained by using BLCL pulsed with G₁₅₈₋₁₈₉.

^c HLA class II phenotypes that BLCL have in common with the autologous BLCL are in boldface.

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 1), while TCC 2 recognized peptide in the context of either HLA-DPB1*0401 or -0402 (Table 2).

Peptide G₁₅₈₋₁₈₉ responses in the human population. Since HLA-DPB1*0401 and -0402 are the most frequent HLA class II alleles in the human population (8), we investigated the response to peptide G₁₅₈₋₁₈₉ in PBMC obtained from healthy adult donors and in CBMC. No specific T cells were found in the CBMC cultures, while G₁₅₈₋₁₈₉-specific T cells were detected in 8 of 13 PBMC cultures (Fig. 2).

In order to confirm that the G₁₅₈₋₁₈₉-expanded cells were indeed RSV-specific T cells, TCCs were generated from three of these cultures (P1, P2, and P3), and the minimal epitopes of these TCCs were determined. As shown in Fig. 3, TCC P1 and TCC P2 recognized the same minimal epitope as TCC 2, while TCC P3 recognized a 10-mer peptide shifted 1 amino acid in the C-terminal direction, H₁₆₂FEVFN₁₇₃.

The class II genotype of donor P1 was HLA-DRB1*1301, 1501;DQB1*0602,0603;DPB1*0401,0402, that of donor P2 was HLA-DRB1*0401,1201;DQB1*0301;DPB1*0301,0402, and that of donor P3 was HLA-DRB1*1201,1501;DQB1*0301, 0601;DPB1*0201,0401. Similarly to TCC 2, both TCCs P1 and P2 recognized peptide in the context of HLA-DPB1*0401 or -0402 (Tables 3 and 4). Interestingly, TCC P3 was also able to recognize peptide in the context of HLA-DPB1*0201 and -02012 (Table 5).

TABLE 4. Determination of HLA restriction elements of TCC P2

BLCL	Restriction element ^c							
	Aut. ^a	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-γ SFC ^b	180	3	2	165	0	6	175	185

^a Aut., autologous BLCL.

^b IFN-γ SFC per well were obtained by using BLCL pulsed with G₁₅₈₋₁₈₉.

^c HLA class II phenotypes that BLCL have in common with the autologous BLCL are in boldface.

TABLE 5. Determination of HLA restriction elements of TCC P3

BLCL	Restriction element ^c									
	Aut. ^a	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- γ SFC ^b	125	80	1	104	2	1	130	80	124	112

^a Aut., autologous BLCL.

^b IFN- γ SFC per well were obtained by using BLCL pulsed with G₁₅₈₋₁₈₉.

^c HLA class II phenotypes that BLCL have in common with the autologous BLCL are in boldface.

All TCCs produce both type 1 and 2 cytokines. TCCs 1 (two subclones), P2, and P3 were tested for the ability to produce cytokines after stimulation with different antigens. All of the TCCs showed specific cytokine production after stimulation

with RSV-infected or peptide-pulsed autologous BLCL, with IFN- γ and IL-13 predominating. 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 (Fig. 4 and data not shown).

HLA-DP4 phenotype in infants with RSV infections of different clinical severities. To determine if the T-cell response to the HLA-DP4-restricted G epitope plays a role in the pathogenesis of severe RSV disease, the allelic frequency of HLA-DP4 or the precursor frequency of the G-specific T cells could be compared among infants with RSV disease of different severities. As an alternative to the determination of HLA-DP4 allelic frequencies, specimens from a previous cohort study (6) allowed us to test the functional capacities of BLCL obtained from infants with severe or mild RSV lower respiratory tract infections to function as APCs to the G-specific TCCs. Positive responses of the TCCs were found after stimulation by 35 of 41 (85%) and 50 of 64 (78%) peptide-pulsed BLCL, respectively (no significant difference; $P > 0.1$; Fisher's exact test, two sided).

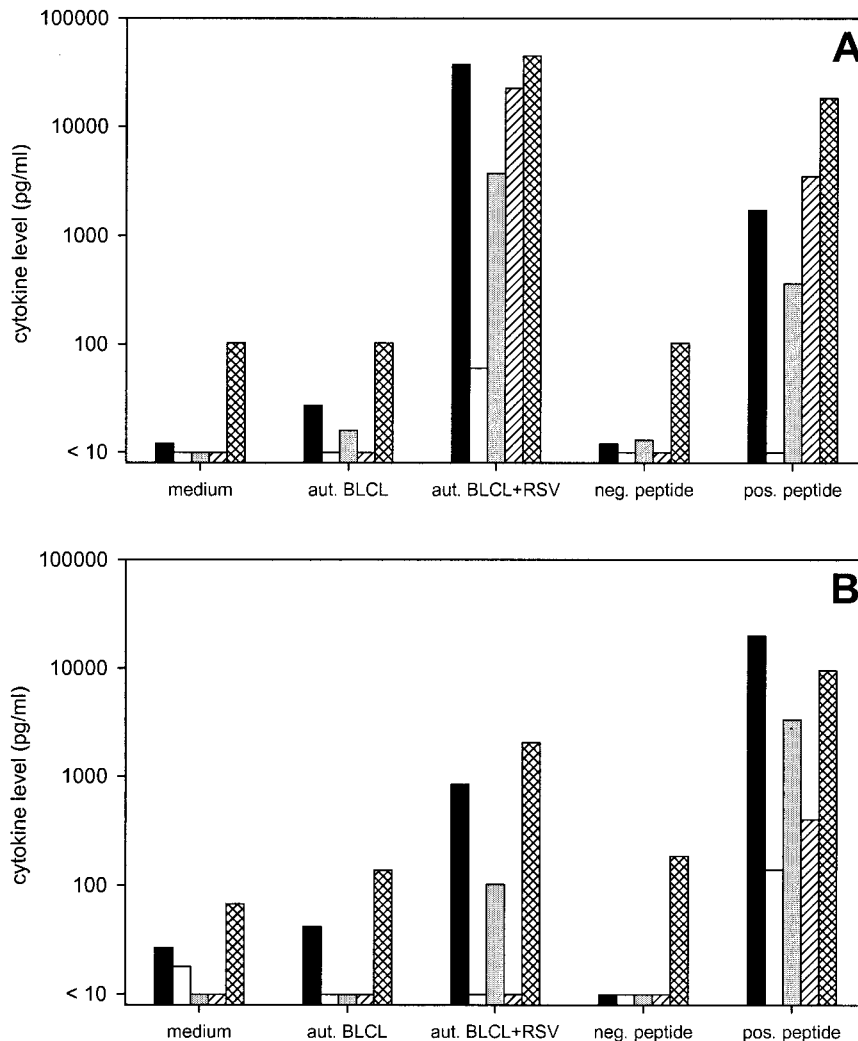


FIG. 4. Cytokine levels in culture supernatants of TCC 1 (A) and TCC P3 (B) 5 days after stimulation with medium, autologous (aut.) BLCL, autologous BLCL-RSV, autologous BLCL pulsed with negative (neg.) peptide (M₂₄₁₋₂₅₁ for TCC 1 and G₁₆₁₋₁₇₅ for TCC P3), or autologous BLCL pulsed with positive (pos.) peptide (M₂₄₅₋₂₅₆ for TCC 1 and G₁₆₁₋₁₇₅ for TCC P3). The detection limits were 10 pg/ml for IFN- γ (solid bars), IL-2 (open bars), IL-4 (shaded bars), and IL-5 (hatched bars) and 100 pg/ml for IL-13 (cross-hatched bars).

DISCUSSION

We have identified two HLA-DP-restricted T helper cell epitopes in the RSV M and G proteins, conserved in subgroup A and in both subgroups A and B, respectively. The M epitope was recognized by a TCC restricted by HLA-DPB1*1601, while the G epitope was recognized in the context of either HLA-DPB1*0401 or -0402. The ubiquitous distribution of the last 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 of 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 (21). However, several HLA-DP-restricted T-cell epitopes have now been described (2), including viral epitopes (9, 18). To our knowledge, the RSV M-specific TCC described here is the first HLA-DPB1*1601-restricted epitope identified. Recently, a number of TCCs have been described as recognizing their epitopes in the context of HLA-DPB1*0401 and/or -0402, which both belong to the serologically defined HLA-DPw4 antigenic group (4). HLA-DPw4 is the most prevalent HLA class II antigen, with an allelic frequency of 78% in the Caucasian population (1). The RSV G epitope identified in the present paper is consistent with the suggested motif for HLA-DP4-restricted epitopes as previously described (8).

HLA-DPB1*0401 and -0402 differ by only 3 amino acids, and TCCs raised in subjects with HLA-DPB1*0401 were in some cases also able to recognize their antigens in the context of HLA-DPB1*0402 (4). 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 (donor P3) that could recognize its epitope in the context of HLA-DPB1*0401. This TCC, which recognized an epitope that was shifted 1 amino acid in the C-terminal direction 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 1 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 the basis of homology in the peptide-binding pockets P1 and P6 (8).

The functional studies using BLCL from infants with mild or severe RSV-mediated lower respiratory tract disease as APCs to the G-specific TCCs suggested that the severity of RSV disease is not linked to the allelic frequency of HLA-DP4. However, frequency studies of epitope-specific T cells in infants with different disease severities will have to be performed to determine whether they play a role in RSV 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 (15, 22). However, in our study, similar Th0-like cytokine production profiles were found for both the M- and G-specific TCCs.

Castelli et al. suggested that specific HLA-DP4-restricted epitopes could be used as peptide vaccines, because of the high frequency of HLA-DP4 worldwide (8). However, vaccination

with nonreplicating RSV vaccines that induce only HLA class II-restricted T-cell responses has been associated with immunopathology in humans (17), nonhuman primates (11, 25), and rodents (23, 26).

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

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