Mapping of CD8 T Cell Epitopes in Human Respiratory Syncytial Virus L Protein

Yordanka Medina-Armenteros Luis E. Farinha-Arcieri Catarina J.M. Braga
Cassiano Carromeu Rodrigo E. Tamura Armando M. Ventura

Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil

Key Words

Human respiratory syncytial virus · L protein · Vaccine · CD8 T cell epitopes

Abstract

Objectives: Since it has been reported that in humans there is a relationship between human respiratory syncytial virus (hRSV)-specific cytotoxic T lymphocytes and symptom reduction, and that the polymerase (structural L protein) is highly conserved among different strains, this work aimed to identify the CD8 T cell epitopes H-2^d restricted within the L sequence for immunization purposes. **Methods:** We screened the hRSV strain A2 L protein sequence using two independent algorithms, SYFPEITHI and PRED^{BALB/c}, to predict CD8 T cell epitopes. The selected peptides were synthesized and used to immunize BALB/c mice for the evaluation of T cell response. The production of IFN- γ from splenocytes of hRSV-infected animals stimulated by these peptides was assayed by ELISPOT. **Results:** Nine peptides showing the best binding scores to the BALB/c MHC-I molecules (H-2K^d, L^d and D^d) were selected. Sequence homology analysis showed that these sequences are conserved among different hRSV strains. Two of these peptides induced significant IFN- γ production by ex vivo-stimulated T cells. **Conclusions:** Our results indicate that the hRSV L protein contains H-2^d-restricted epitopes.

© 2014 S. Karger AG, Basel

Introduction

Human respiratory syncytial virus (hRSV) is the major respiratory viral pathogen in infants and children worldwide. The infection causes mild symptoms like common cold, otitis or rhinitis, or more severe ones such as bronchiolitis, tracheobronchitis and pneumonia [1, 2]. hRSV is an enveloped virus classified in the Mononegavirales order, Paramyxoviridae family, Pneumovirinae subfamily and Pneumovirus genus. The genome is a single-stranded negative-sense RNA molecule of 15.2 Kb which codes for 10 genes. It produces three nonstructural proteins (NS1, NS2 and M2-2) and eight structural proteins (G, F, SH, M, N, P, M2-1 and L) [1, 3].

The viral particles are surrounded by a lipid bilayer derived from the host cell plasma membrane, in which three glycoproteins are inserted: the G glycoprotein, responsible for binding to the cellular receptor [4]; glycoprotein F, which mediates the fusion of viral and cellular membranes [5], and SH, a small hydrophobic protein with unknown function [3]. Just below the envelope there is a coating layer formed by the viral M (matrix) protein. Within the viral particle there is the nucleocapsid, which consists of the genomic RNA tightly bound to the nucleoprotein N, and other associated proteins: L, the main subunit of the viral polymerase; P, a phosphoprotein, and the M2-1, a transcription antitermination factor [1, 3].

Fig. 1. Localization of the selected peptides containing predictopes in the L protein. In the representation of the hRSV A2 L protein, the selected peptides positions are indicated by vertical bars (S1–S16). The predicted functional domains among the Mononegavi-

rales polymerases are indicated by filled boxes (reference for alignment was Sendai virus polymerase): domain I, interaction with protein P; domains II and III, polymerization; domain IV, polyadenylation activity; domains V and VI, capping.

The viral polymerase is composed of two subunits: the L protein, which contains the active site of polymerization and other enzymatic activities, and the P protein, which acts as a cofactor of the polymerase. We refer here to L as the polymerase. It comprises 2,165 amino acids (A2 strain), about 250 kDa, and is the least abundant of the structural proteins. The alignment of L protein sequences from Mononegavirales allowed the identification of six relatively conserved regions (domains I–VI; fig. 1). The highest degree of conservation is concentrated mainly in the N-terminal half (aas 422–938), including region III that contains four characteristic polymerase motifs. Of all hRSV proteins, L can be considered the least studied, and there is little information on its structural and immunogenic characteristics [1, 3, 6–8].

Currently, there is no licensed vaccine against hRSV infection. The first attempt to obtain a vaccine was carried out in the 1960s with a formalin-inactivated virus (FI-RSV). When this vaccine was used in a human clinical trial, the induction of a strong immune response was noted, but it was not protective [9]. Moreover, when children previously vaccinated with FI-RSV were exposed to wild hRSV they had a more severe manifestation of the respiratory disease than unvaccinated children [10]. This adverse reaction is associated with exacerbated pulmonary eosinophilia due to an excessive Th2 memory response. BALB/c infection by hRSV reproduces the exacerbated pulmonary eosinophilia observed in humans [11]. Immunization of these animals with either inactivated vaccine or the G glycoprotein alone induces an immunopathogenic response mediated by CD4 T cells, while the live attenuated vaccines are associated with Th1 responses [12, 13].

It is believed that an effective immunization against hRSV must induce Th1 immunity, involving the activation of cytotoxic T cells secreting IFN- γ . In adults, data suggest that the memory cytotoxic T lymphocytes (CTL)

of most individuals tested recognize the N protein. The SH, F, M, M2-1 and NS2 proteins are also recognized in some individuals, and there is no recognition of G, P or NS1 proteins by human CD8 T cells [14, 15].

In C57BL/6 mice, H-2^b-restricted T-CD8 epitopes were identified in the matrix protein M_{187–195} [16] as well as in F, G and N proteins [17]. In BALB/c mice, three major H-2^d-restricted T CD8 antigenic determinants were found. The M2-1 protein (second matrix protein) contains the immunodominant epitope M2_{82–90}, against which 30–50% of lung CD8 T cells respond at the peak of the cellular response [18, 19]. M2-1 also contains the subdominant epitope M2_{127–135} [20]. Another subdominant epitope was also found in the F protein between residues 85 and 93 [21]. All these epitopes are restricted by the H-2K^d allele. Some evidence suggests that the cytotoxic response is important in reducing hRSV pathology. CD8 T cells associated with immunity against hRSV are detected in the blood of previously infected adults, indicating a relationship between the CTL response and the reduction of clinical symptoms [22]. In the mouse model, CD8 T cells also mediate resistance to hRSV [23].

Immunization with the M2_{82–90} peptide, in association with the heat-labile toxins produced by some of enterotoxigenic *Escherichia coli* strains, elicited a mucosal CTL response able to accelerate the reduction of viral load. Additionally, M2_{82–90}-specific CD8 T cells reduce the Th2 response induced by immunization with rVV-G (a recombinant vaccinia virus expressing the G protein) followed by challenge with hRSV in an IFN- γ independent manner [24]. Immunization with FI-RSV does not induce a measurable CD8-specific response against hRSV. However, the establishment of a potent memory response of hRSV-specific CD8 T cells before immunization with FI-RSV annul the pulmonary eosinophilia after challenge with the wild-type virus [25]. These observations indicate that IFN- γ production inhibits the secretion of Th2 cyto-

kines and pulmonary eosinophilia, suggesting that the balance of T cell responses determines the outcome of the infection with hRSV [15]. In that sense, recombinant Sendai virus expressing the F protein from hRSV induced a protective response of neutralizing antibodies and F-specific CTL even though F contains only a subdominant CTL epitope. The protective CTL response was also observed in mice deficient in antibody production [26].

The most favorable vaccine strategy against hRSV for a seronegative population must consist of components which do not leave immunopathological sequelae, and at the same time achieve a balanced protective immune response mediated by CD8 T and CD4 Th1 cells. Little has been studied with respect to the immune response against the viral polymerase and the protective contribution of its potential T cell epitopes. This protein is highly conserved, an important aspect for a successful vaccine approach. The development of prediction programs allows the analysis of peptide sequences looking for epitopes that can be presented to both CD8 T cells (restricted by major histocompatibility complex type I, MHC-I) and CD4 T cells (restricted by MHC-II). This minimizes the number of experiments to be performed because it enables the systematic search of epitopes from large proteins [27–29]. In this work we identified epitopes (predictopes) of CD8 T cells within the L protein using prediction programs. The selected peptides were synthesized and their immunogenicity evaluated in cellular assays.

Materials and Methods

Epitope Prediction

The L protein sequence AAA47418.1-gi/1143829/gb (strain A2) was used to predict MHC-I-restricted T cell epitopes. Two independent online algorithms, SYFPEITHI (<http://www.syfpeithi.de/home.htm>) (H-2K^d and H-2L^d) and PRED^{BALB/c} (<http://antigen.i2r.astar.edu.sg/predBalbc/>) (H-2D^d, H-2K^d and H-2L^d) were used to predict MHC-I binding for MHC-I alleles from BALB/c. The following selection criteria were used. First, 9-mer sequences with high MHC binding scores were preselected from L protein. Next, sequences with the best MHC binding scores were selected from within the entire sequence and were ranked according to the MHC binding score for each online algorithm. Finally, the results from both algorithms were combined (consensus prediction). Sequences containing several 9-mer peptides with the best binding scores were selected and synthesized. The 9-mer peptides within these sequences were considered to be predicted epitopes or 'predictopes'.

Protein Sequence Alignment

L protein sequences from hRSV strains representative from subgroups A and B were obtained from NCBI (table 1). The alignments of the predictopes and these sequences were performed with the CLUSTAL W program.

Table 1. L protein sequences

Subgroup	Sequence No.	Observations
A	AAA47418.1-gi/1143829/gb	strain A2
	AAA84898.1-gi/333955	–
	AAC 57029.1-gi/1912297	strain S2
	AY911262-gi/60549163	ATCC VR-26
	AF254574-gi/7960297	strain detected in Spain
B	AF013254-gi/2582022	wild-strain B1
	NP_001781-gi/9629198	–
	AY353550-gi/38230482	strain 9320

Viral Stocks

Subconfluent Hep-2 cells were infected with the hRSV A2 strain; after 36–40 h the cells were collected, when the cytopathic effect had taken approximately 90% of the monolayer. The cells were centrifuged and the pellets were resuspended in PBSS (PBS, 2% of fetal calf serum and 30% of sucrose) and lysed in a dounce (tight) 15 times. The homogenate was centrifuged at 2,000 g for 15 min at 4°C and the supernatant containing the virus was collected. The viral suspension was diluted in NTE (NaCl 150 mM, Tris-HCl 50 mM, EDTA 1 mM) plus 30% sucrose and concentrated by ultracentrifugation in a Sorvall rotor AH-650 at 35,600 rpm for 3 h. The pellet containing the virus was resuspended in 1 ml of PBSS and stocked at –80°C. The virus was titrated by indirect immunofluorescence microscopy. Briefly, Hep-2 cells (ATCC) were seeded in a 96-well plate. When they reached 70–80% confluence they were infected with tenfold serial dilutions of virus (100 µl) and were incubated for 2 h at 37°C. The cells were then overlaid with 100 µl of fresh medium and incubated for 48 h. After that the cytopathic effect was observed by optic microscopy. To detect viral antigens in the infected monolayer, the C793 anti-G monoclonal antibody (kindly provided by Dr. Erling Norrby, Department of Virology, Karolinska Institute, Stockholm, Sweden) diluted 1:500 and anti-mouse IgG antibody conjugated with fluorescein isothiocyanate (KPL) diluted 1:50 were used. Cells were observed under a fluorescence microscope (Axiovert 200; Zeiss™). The viral titer was expressed as syncytia-forming units (SFU)/ml.

Immunization Assays and Infection of Mice

Six- to 8-week-old BALB/c mice were obtained from the Isogenic Mice Breeding Facility of the Institute of Biomedical Sciences, University of São Paulo. Animals were immunized with 4 doses of 20 µg of each peptide in 100 µl of inoculums at intervals of 1 week. The first dose was administered subcutaneously in complete Freund's adjuvant and the following doses were administered intraperitoneally in incomplete Freund's adjuvant. BALB/c mice were infected intranasally, with 1×10^6 SFU of the hRSV A2 strain. Control groups were inoculated with PBS in adjuvant or PBSS only, respectively. All animal handlings were carried out in accordance with the principles of the Brazilian code for the use of laboratory animals, and all protocols were approved by the Institute of Biomedical Sciences Committee on Ethical Use of Laboratory Animals, University of São Paulo.

Cell Recovery

Mice lungs and spleens were surgically removed and transferred into separate Petri dishes containing RPMI medium (supplemented with fetal calf serum 1%, gentamicin 100 µg/ml and tylosin 8 µg/ml) and washed. Spleens were macerated to obtain a single-cell suspension and the particulate matter was removed by centrifugation at 1,200 g at 4°C for 10 min. Lungs were sliced and digested with collagenase type IV (440 U/mg, Sigma) 1 mg/ml in the presence of CaCl₂ 5 mM at 37°C for 2 h with agitation (100 rpm). The enzyme was inactivated by adding 10 mM EDTA and incubating in ice for 5 min. The cell suspension was filtrated by gauze, centrifuged, and pulmonary cells were collected. Recovered cells from spleens and lungs were stained with trypan blue and counted in a Neubauer chamber.

Enzyme-Linked Immunospot Assay for IFN-γ Secreting Cells

Peptide-specific IFN-γ secreting cells were identified by an enzyme-linked immunospot (ELISPOT) assay essentially as described previously [30]. Microwell plates (MultiScreen-HA; Millipore) were coated with capture antibody (rat anti-mouse IFN-γ, clone RA-6A2; BD Pharmingen) 10 µg/ml, blocked with minimal essential modified Eagle's medium supplemented with 10% fetal calf serum and washed. Spleen and lung cells were added at 5×10^5 cell density per well and incubated ex vivo with the corresponding peptide (1.25 µg/ml) or media for 24–30 h at 37°C, in the presence or absence of an anti-CD8 monoclonal antibody (MCD0800; Invitrogen™). The plates were washed and incubated with the detection antibody (biotinylated anti-mouse IFN-γ, clone XMG 1.2; 2 µg/ml; BD Pharmingen) overnight at 4°C. After being further washed, plates were developed using streptavidin-alkaline phosphatase (BD Pharmingen) and DAB substrate (Sigma). IFN-γ spots were counted using the Nikon™ stereoscope SMZ645. Results were expressed as IFN-γ-positive cells, spot-forming cells (SFC)/10⁶ cells.

Detection of Virus Replication in Lungs

Lungs of infected mice were sliced and triturated in dounce (loose) in PBSS solution. The resulting homogenate was centrifuged in an angular rotor (Jouan 11174593) at 4,000 rpm for 15 min at 4°C. The supernatant was collected and the presence of the virus was determined by Western blot for matrix (M) protein using a polyclonal serum produced in our laboratory.

Statistics

A Mann-Whitney test was performed to compare the number of SFC and the percentage of reduction between groups. All tests were two-tailed and were performed with GraphPad Prism software, version 5. Differences were considered statistically significant with $p < 0.05$. Data are presented as means and SEM.

Results

Epitope Prediction

The L protein amino acid sequence from the hRSV A2 strain (AAA47418.1-gi/1143829) was scanned for 9-mer peptides with high binding scores to MHC-I H-2^d. Nine 9-mer peptides carrying predictopes with high binding

scores to BALB/c MHC-I were selected (table 2). Peptides were denominated S1, S5, S8, S9, S11, S12, S14, S15 and S16. Figure 1 shows the localization of the peptides along the protein. S5, S8 and S9 are located in domain III (polymerization), domain IV (polyadenylation activity) and domain V (capping), respectively. The remaining peptides are located outside of the predicted domains. These sequences are conserved among different hRSV A and B subgroup strains. In figure 2 the alignment for these L protein sequences is shown. The S8, S11 and S14 peptides are identical among strains from the different subgroups, while the rest of the peptides have only one amino acid change for some of the analyzed strains.

Immunogenicity of Peptides

To determine if the selected predictopes elicit a T cell response, we measured the IFN-γ production against the candidate peptides. Eight days after the last immunization with each peptide, the mice were sacrificed and the obtained splenocytes were stimulated ex vivo with the same peptide. IFN-γ-secreting splenocytes were quantified by ELISPOT. Figure 3a shows that peptides S8, S9, S11 and S16 were able to induce IFN-γ production with values of 100, 8, 23 and 7 SFC, respectively. These responses were specific for peptide since splenocytes obtained from mice immunized only with PBS and adjuvant did not produce IFN-γ when stimulated with each peptide (data not shown). S8 peptide was the most immunogenic showing the highest number of SFC. The other peptides tested were not able to induce IFN-γ-producing cells.

L Protein Contains CD8 T Cell Epitopes

The MHC-I molecule binds peptides of 8–15 amino acids in length within a single closed groove. However, the majority of the peptides that bind to MHC-I are 9 amino acids in length [31]. As can be observed in table 2, the majority of the predictopes selected are longer than 9 amino acids, except for S14 and S15. Moreover, ex vivo stimulation with peptides longer than 9 amino acids can also induce IFN-γ release by CD4 T cells [32]. To determine whether IFN-γ-producing cells were CD8+, the ELISPOT assay was performed in the presence or absence of an anti-CD8 antibody. Addition of an anti-CD8 mAb to ELISPOT wells abrogated spot formation after stimulation with peptides S8, S11 and M2 in 50, 84.6 and 60.6%, respectively (fig. 3b). This indicates that the responsive cells are, in part, CD8 T cells. The percentages of reduction for these peptides were not statistically different from positive control M2.

Table 2. Peptides selected from L protein

Peptides	Position	Peptide amino acid sequence	Predictopes	MHC-I restriction
S1	31–48	LGSYIFNGPYLKNDYTNL	GSYIFNGPY SYIFNGPYL NGPYLKNDY	D ^d D ^d , K ^d D ^d
S5	744–753	CTYRHAPPYI	CTYRHAPPY TYRHAPPYI	D ^d D ^d , K ^d
S8	1,114–1,129	IEPTYPHGLRVVYESL	IEPTYPHGL TYPHGLRVV GLRVVYESL	D ^d D ^d , K ^d K ^d
S9	1,224–1,238	IMYTMDIKYTTSTIS	IMYTMDIKY	D ^d
S11	1,513–1,531	IFEKDWGEGYITDHMFNL	EGYITDHMF GYITDHMFI	D ^d D ^d
S12	1,530–1,545	NLKVFFNAYKTYLLCF	VFFNAYKTY FFNAYKTYL AYKTYLLCF	K ^d D ^d , K ^d D ^d
S14	2,059–2,067	CYPITKKGI	CYPITKKGI	D ^d , K ^d
S15	2,113–2,121	VLNFRSTEL	VLNFRSTEL	K ^d
S16	2,125–2,140	HYLMVESTYPYLSELL	HYLMVESTY STYPYLSEL TYPYLSELL	D ^d D ^d D ^d , K ^d

Peptides were synthesized at 81% purity by BACHEM and analyzed by high-performance liquid chromatography. Peptides were dissolved in sterile water containing dimethyl sulfoxide (5–10%) at 1 mg/ml.

Immunogenicity of Peptides upon Viral Infection

Once it was demonstrated that the peptides were immunogenic, we evaluated peptide-specific cellular immune responses upon viral infection. To achieve that purpose, mice were infected twice with hRSV A2 strain with an 8-day interval. To confirm viral replication, 5 days after the first infection some animals were sacrificed and lung homogenates were obtained. These homogenates were used as samples to detect M protein by Western blot (fig. 4a). Viral presence was determined by detecting a 33 kDa-band (lane 2), corresponding to the M protein, indicating that the virus was actively replicating in the lungs. This band was absent in the homogenate of control mice immunized with PBSS (lane 1).

Ten days after the last infection, the animals were sacrificed and primary cell cultures from the lungs and spleens were obtained. The cells were stimulated *ex vivo* with the peptides and the immune response against them was evaluated. The production of IFN- γ upon viral infection was low for all the peptides tested either in

the spleen (fig. 4b) or in the lung (fig. 4c), except for the immunodominant epitope M2_{82–90} which had a higher response in lungs. S8 peptide was able to stimulate T cells to release IFN- γ in spleen and lung samples. The addition of an anti-CD8 antibody reduced the number of SFC in 50 and 60% in the spleen, and in 66.7 and 63.9% in the lungs for the S8 and M2 peptides, respectively.

Discussion

For a vaccine to be effective it must invoke a strong immune response of B and T cells, so epitope mapping is a key aspect in its design [27, 28]. Prediction methods *in silico* have the potential to accelerate the discovery of immunization epitopes considerably. Accordingly, scanning of pathogen genomes searching for potential epitopes using prediction algorithms has led to the characterization of real epitopes [28]. This work aimed at the

Fig. 2. Conservation of peptides in the L protein sequences. hRSV subgroups A and B L protein sequences (see table 1) alignment is shown. The selected peptides S1–S16 are indicated in boxes. The first sequence shown, A2 virus, was used for prediction.

Fig. 3. T cell immune response against the selected peptides. BALB/c mice were immunized with 4 doses (20 µg each) of the peptides indicated. Animals were sacrificed 8 days after the last dose, splenocytes were collected and stimulated with the same peptide, and IFN-γ-secreting cells were quantified by ELISPOT assay. **a** Assay with all selected L peptides. **b** Assay with selected L peptides and a peptide corresponding to immunodominant epitope M2₈₂₋₉₀ (used as a positive control) in the presence or the absence of anti-CD8 monoclonal antibody. The results are expressed as the mean of SFC per 10⁶ cells for each group (n = 3 mice) and deviation errors. Numbers above the bars indicate the percentage reduction of SFC number in the presence of anti-CD8 (* p < 0.05; ** p < 0.01).

identification of H2^d-restricted CD8 T cell epitopes within the hRSV L protein. To achieve this goal, CD8 T cell epitopes in the L protein were identified *in silico* and the cellular immune response against those peptides (9–19 amino acids in length), containing 9-mer predictopes, was evaluated in BALB/c mice. This protein was chosen because it is highly conserved among strains of hRSV, plays a crucial role in the infectious cycle of the virus and little has been studied with respect to the immune responses against it.

Peptide predictions were performed using the SYFPEITHI and PRED^{BALB/c} programs, which analyze the presence of binding motifs for MHC-I alleles H-2K^d/

Fig. 4. Characterization of the T cell response against the peptides upon hRSV infection. **a** Replication of hRSV in BALB/c mice. Five days after intranasal infection, lungs were harvested and their homogenates were analyzed for the presence of the matrix protein by Western blot. Lane 1: animal inoculated with PBSS; lane 2: animal infected with hRSV. **b, c** BALB/c mice (n = 5 mice per group) were infected with 1 × 10⁶ SFU/ml on days 1 and 9. The animals were sacrificed 10 days after the last infection. The IFN-γ release by the spleen (**b**) and lung (**c**) lymphocytes was evaluated by ELISPOT assay upon stimulation with the indicated peptides in the presence or absence of anti-CD8 monoclonal antibody. A peptide corresponding to the M2₈₂₋₉₀ immunodominant epitope was used as a positive control. The cells obtained from the infected animals were pooled for stimulation and the deviation error shown is between measurement replicates. The means and deviation errors for the experimental groups are shown. Numbers above the bars indicate the percentage reduction of SFC number in the presence of the anti-CD8 antibody.

L^d or H-2^d haplotypes of BALB/c mice, respectively [33]. The use of these programs allowed the selection of nine sequences distributed along the L protein (table 2; fig. 1) containing 9-mer predictopes with a high binding score to MHC-I molecules of H-2D^d and/or K^d. None of these

Table 3. Binding score to MHC-I of the CD8 T cell predictopes

Peptide	Sequence	Position	Score				
			PRED ^{BALB/c}			SYFPEITHI	
			H-2D ^d	H-2K ^d	H-2L ^d	H-2K ^d	H-2L ^d
S8	IEPTYPHGL	1,114–1,122	9.7	6.1	5.1	13	12
	TYPHGLRVV	1,117–1,125	9.52	9.06	4.66	20	5
	GLRVVYESL	1,121–1,129	7.58	9.1	4.06	16	11
S11	EGYITDHMF	1,520–1,528	10.0	0.62	4.36	0	12
	GYITDHMFI	1,521–1,529	9.4	8.5	3.8	23	6

PRED^{BALB/c} = Binding score to MHC-I 1–10; SYFPEITHI = binding score to MHC-I 1–31.

sequences contained epitopes responsive to H-2L^d molecule. This result is consistent with reports that in BALB/c mice only epitopes presented by H-2K^d molecule have been found in hRSV [18–21].

Although the majority of peptides that bind to MHC-I molecule are 9 amino acids in length [31], we synthesized longer peptides to analyze their immunogenicity since the efficient processing of an antigenic sequence for presentation by MHC class I molecules depends on its neighboring residues in the protein [34]. In the two prediction programs used, characterized peptide motifs are the reference for the analysis of the binding score to MHC-I. However, this does not take into account that flanking amino acids might influence the processing and presentation of peptides [34]. Therefore, not all potentially binding peptides are likely to be processed *in vivo*. Cellular immune responses against the selected peptides from L protein (S1, S5, S8, S9, S11, S12, S14, S15 and S16) was determined in BALB/c mice when they were used as immunogens or upon viral infection, with subsequent *ex vivo* stimulation in both cases. T cell responsive epitopes were found consistently in two of these peptides, S8 and S11 (fig. 3a, b). This indicates that in addition to the MHC-I binding probability, for an epitope to be functionally active, other events such as proteasome cleavage and linkage to the carrier and the membrane T cell receptor [35] are required.

The S8 and S11 peptides have 100% homology among the sequences of the viral strains tested (fig. 2), showing a high conservation degree and being present in the two subgroups of hRSV (A and B). This makes them an attractive target for directing the cellular immune response. The IFN- γ release after *ex vivo* stimulation with these peptides was reduced by addition of an anti-CD8 in the

ELISPOT assay, indicating that these sequences contain CD8 T cell epitopes (fig. 3b, 4).

The frequency of cells producing IFN- γ upon viral infection and subsequent stimulation with the M2_{82–90} peptide was high (fig. 4b, c). However, the amount of SFC upon viral infection and stimulation with the peptides S8 and S11 was low. In general, the immunization with peptides was able to induce a higher response of IFN- γ release than when they are presented to the immune system in the context of viral infection. This may be due to a masking effect of the immunodominant peptide, as reported by Mok et al. [36] showing that mutations in anchoring residues of the immunodominant epitope M2_{82–90} enhanced the immunogenicity of the subdominant epitope M2_{127–135}. These two peptides present in the M2-1 protein are presented by the H-2K^d molecule, so they can compete for binding to MHC-I. The addition of an anti-CD8 antibody inhibited IFN- γ production (fig. 3, 4) after stimulation with S8 or S11 peptides. Some predictopes identified in these peptides are H-2D^d restricted (table 3). The high score values for binding to MHC-I H-2D^d might facilitate their presentation, for not being subjected to competition with the immunodominant epitope H-2K^d restricted. One HLA-B27-restricted epitope in hRSV polymerase was identified previously using an interesting strategy of pull down the HLA-peptide complexes in an infected human cell line, followed by mass spectrometry analysis [37]. To our knowledge, however, data presented here is the first evidence suggesting the presence of H-2D^d-restricted epitopes for hRSV since only CTL epitopes restricted by H-2K^d have been reported earlier [18–21]. More detailed studies should be done in order to determine which MHC-I molecule actually presents these peptides.

The results reported here indicate that S8 and S11 are able to induce the release of IFN- γ specifically. The cellular immune response was observed either in the context of viral infection or when peptides were administered as immunogens, being higher in the latter case. Therefore, we conclude that the hRSV L protein (viral polymerase) presents responsive T cells epitopes and could be a good immunogen to be included in a vaccine candidate. This is the first time that T cell epitopes are reported within the hRSV L polymerase in the genetic background of BALB/c mice.

Acknowledgements

This study was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP (Sao Paulo State Research Support Foundation; proc. No. 2009/17587-9) and Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq (National Council of Scientific and Technological Development; proc. No. 477734/2009-0). We thank Dr. Erling Norrby (Department of Virology, Karolinska Institute, Stockholm, Sweden) for kindly providing the C793 anti-G monoclonal antibody. We also thank Dr. Luis Carlos de Souza Ferreira (São Paulo University, Brazil) for the facilities and instrumentation for immune response analysis.

References

- Collins PL, Crowe JE Jr: Respiratory syncytial virus and metapneumovirus; in Knipe DM, Howley PM (eds): *Fields Virology*. Philadelphia, Lippincott Williams & Wilkins, 2007, pp 1602–1646.
- Paramore LC, Ciuryla V, Ciesla G, Liu L: Economic impact of respiratory syncytial virus-related illness in the US: an analysis of national databases. *Pharmacoeconomics* 2004;22: 275–284.
- Melero JA: Molecular biology of human respiratory syncytial virus; in Cane P (ed): *Perspectives in Medical Virology 14*. Oxford, Elsevier, 2007, pp 1–42.
- Levine S, Klaiber-Franco R, Paradiso PR: Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. *J Gen Virol* 1987;68:2521–2524.
- Walsh EE, Hruska J: Monoclonal antibodies to respiratory syncytial virus proteins: identification of the fusion protein. *J Virol* 1983;47: 171–177.
- Carroumeu C, Simabuco FM, Tamura RE, Farinha-Arcieri LE, Ventura AM: Intracellular localization of human respiratory syncytial virus L protein. *Arch Virol* 2007;152: 2259–2263.
- Poch O, Blumberg BM, Bougueleret L, Tordo N: Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. *J Gen Virol* 1990;71:1153–1162.
- Poch O, Sauvaget I, Delarue M, Tordo N: Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J* 1989;8:3867–3874.
- Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH: Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *Am J Epidemiol* 1969;89:422–434.
- Openshaw PJ, Culley FJ, Olszewska W: Immunopathogenesis of vaccine-enhanced RSV disease. *Vaccine* 2001;20:27–31.
- Waris ME, Tsou C, Erdman DD, Zaki SR, Anderson LJ: Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. *J Virol* 1996;70:2852–2860.
- Connors M, Giese NA, Kulkarni AB, Firestone C-Y, Morse HC 3rd, Murphy BR: Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. *J Virol* 1994;68: 5321–5325.
- Hancock GE, Speelman DJ, Heers K, Bortell E, Smith J, Cosco C: Generation of atypical pulmonary inflammatory responses in BALB/c mice after immunization with the native attachment (G) glycoprotein of respiratory syncytial virus. *J Virol* 1996;70:7783–7791.
- Cherrie AH, Anderson K, Wertz GW, Openshaw PJ: Human cytotoxic T cells stimulated by antigen on dendritic cells recognize the N, SH, F, M, 22K, and 1b proteins of respiratory syncytial virus. *J Virol* 1992;66:2102–2110.
- Taylor G: Immunology of RSV; in Cane P (ed): *Perspectives in Medical Virology 14*. Oxford, Elsevier, 2007, pp 43–87.
- Rutigliano JA, Rock MT, Johnson AK, Crowe JE Jr, Graham BS: Identification of an H-2D(b)-restricted CD8 + cytotoxic T lymphocyte epitope in the matrix protein of respiratory syncytial virus. *Virology* 2005;337:335–343.
- Lukens MV, Claassen EA, de Graaff PM, van Dijk ME, Hoogerhout P, Toebes M, Schumacher TN, van der Most RG, Kimpen JL, van Bleek GM: Characterization of the CD8+ T cell responses directed against respiratory syncytial virus during primary and secondary infection in C57BL/6 mice. *Virology* 2006; 352:157–168.
- Chang J, Braciale TJ: Respiratory syncytial virus infection suppresses lung CD8+ T-cell effector activity and peripheral CD8+ T-cell memory in the respiratory tract. *Nat Med* 2002;8:54–60.
- Kulkarni AB, Collins PL, Bacik I, Yewdell JW, Bennink JR, Crowe JE Jr, Murphy BR: Cytotoxic T cells specific for a single peptide on the M2 protein of respiratory syncytial virus are the sole mediators of resistance induced by immunization with M2 encoded by a recombinant vaccinia virus. *J Virol* 1995;69:1261–1264.
- Lee S, Miller SA, Wright DW, Rock MT, Crowe JE Jr: Tissue-specific regulation of CD8+ T lymphocyte immunodominance in respiratory syncytial virus infection. *J Virol* 2006;81:2349–2358.
- Chang J, Srikiatkachorn A, Braciale TJ: Visualization and characterization of respiratory syncytial virus F-specific CD8+ T cells during experimental virus infection. *J Immunol* 2001;167:4254–4260.
- Isaacs D: Viral subunit vaccines. *Lancet* 1991; 337:1223–1224.
- Kulkarni AB, Morse HC 3rd, Bennink JR, Yewdell JW, Murphy BR: Immunization of mice with vaccinia virus-M2 recombinant induces epitope-specific and cross-reactive Kd-restricted CD8+ cytotoxic T cells. *J Virol* 1993;67:4086–4092.
- Simmons CP, Hussell T, Sparer T, Walzl G, Openshaw P, Dougan G: Mucosal delivery of respiratory syncytial virus CTL peptide with enterotoxin-based adjuvants elicits protective, immunopathogenic, and immunoregulatory antiviral CD8+ T cell responses. *J Immunol* 2001;166:1106–1113.
- Olson MR, Varga SM: CD8 T cells inhibit respiratory syncytial virus (RSV) vaccine-enhanced disease. *J Immunol* 2007;179:5415–5424.
- Voges B, Vallbracht S, Zimmer G, Bossow S, Neubert WJ, Richter K, Hobeika E, Herrler G, Ehl S: Recombinant Sendai virus induces T cell immunity against respiratory syncytial virus that is protective in the absence of antibodies. *Cell Immunol* 2007;247:85–94.

- 27 De Groot AS: Immunomics: discovering new targets for vaccines and therapeutics. *Drug Discov Today* 2006;11:203–209.
- 28 De Groot AS, Berzofsky JA: From genome to vaccine – new immunoinformatics tools for vaccine design. *Methods* 2004;34:425–428.
- 29 De Groot AS, Saint-Aubin C, Bosma A, Sbai H, Rayner J, Martin W: Rapid determination of HLA B*07 ligands from the West Nile virus NY99 genome. *Emerg Infect Dis* 2001;7:706–713.
- 30 Simmons CP, Dong T, Chau NV, Dung NT, Chau TN, Thao le TT, Dung NT, Hien TT, Rowland-Jones S, Farrar J: Early T-cell responses to dengue virus epitopes in Vietnamese adults with secondary dengue virus infections. *J Virol* 2005;79:5665–5675.
- 31 Rammensee HG, Falk K, Rötzschke O: Peptides naturally presented by MHC class I molecules. *Annu Rev Immunol* 1993;11:213–244.
- 32 Takita-Sonoda Y, Tsuji M, Kamboj K, Nussenzweig RS, Clavijo P, Zavala F: *Plasmodium yoelii*: peptide immunization induces protective CD4+ T cells against a previously unrecognized cryptic epitope of the circumsporozoite protein. *Exp Parasitol* 1996;84:223–230.
- 33 Zhang LG, Srinivasan KN, Veeramani A, August JT, Brusica V: PRED^{BALB/c}: a system for the prediction of peptide binding to H2^d molecules, a haplotype of the BALB/c mouse. *Nucleic Acid Res* 2005;33:180–183.
- 34 Del Val M, Schlicht HJ, Ruppert T, Reddehase MJ, Koszinowski UH: Efficient processing of an antigenic sequence for presentation by MHC class I molecules depends on its neighboring residues in the protein. *Cell* 1991;66:1145–1153.
- 35 Herd KA, Mahalingam S, Mackay IM, Nissen M, Sloots TP, Tindle RW: Cytotoxic T-lymphocyte epitope vaccination protects against human metapneumovirus infection and disease in mice. *J Virol* 2006;80:2034–2044.
- 36 Mok H, Lee S, Wright DW, Crowe JE Jr: Enhancement of the CD8+ T cell response to a subdominant epitope of respiratory syncytial virus by deletion of an immunodominant epitope. *Vaccine* 2008;26:4775–4782.
- 37 Infantes S, Lorente E, Barnea E, Beer I, Craggolini JJ, García R, Lasala F, Jiménez M, Admon A, López D: Multiple, non-conserved, internal viral ligands naturally presented by HLA-B27 in human respiratory syncytial virus-infected cells. *Mol Cell Proteomics* 2010;9:1533–1539.