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Article

Characterization of conserved and promiscuous Human Rhinovirus CD4 T cell epitopes

Marta Gomez-Perosanz [1], Tara Fiyouzi [1], Miguel Fernandez-Arquero [2], John Sidney [3], Alessandro Sette [3], Ellis L. Reinherz [4], Esther M. Lafuente [1], Pedro A. Reche [1]*

[1] Department of Immunology, School of Medicine, Complutense University of Madrid, Madrid, Spain
[2] Immunology Service, San Carlos University Hospital, Madrid, Spain
[3] La Jolla Institute for Immunology, Division of Vaccine Discovery, La Jolla, CA, USA
[4] Laboratory of Immunobiology, Dana-Farber Cancer Institute, Boston, MA, USA
* Correspondence: parecheg@med.ucm.es; Tel.: +34913947229

Abstract: Human Rhinovirus (HRV) is the most common cause of upper respiratory infections and exacerbations of asthma. In this work, we selected 14 peptides (6 from HRV A and 8 from HRV C) encompassing potential CD4 T cell epitopes. Peptides were selected for being highly conserved in HRV A and C serotypes and predicted to bind to multiple HLA II molecules. We found positive T cell recall responses by IFNγ-ELISPOT assays to 8 peptides, validating 7 of them (3 from HRV A and 4 from HRV C) as CD4 T cell epitopes through intracellular cytokine staining assays. Additionally, we verified their promiscuous binding to multiple HLA II molecules by quantitative binding assays. According to their experimental HLA II binding profile, the combination of all these 7 epitopes could be presented and recognized by > 95 % of the world population. We actually determined IFNγ responses to a pool encompassing these CD4 T cell epitopes by intracellular cytokine staining, finding positive responses in 29 out of 30 donors. The CD4 T cell epitopes identified in this study could be key to monitor HRV infections and to develop peptide-based vaccines against most HRV A and C serotypes.

Keywords: Human Rhinovirus; CD4 T cell; epitope; peptide.

1. Introduction

Human Rhinovirus (HRV) species A and C are the most frequent cause of viral respiratory tract infections worldwide [1]. In most individuals, HRV infections are relatively mild and self-limited to the upper respiratory tract, being the most common cause of the common cold. However, in those patients with chronic respiratory diseases, children and immunocompromised individuals, HRVs can infect the lower respiratory tract causing severe symptoms of bronchiolitis and pneumonia [2]. Likewise, it has been established that HRV C species are the main cause of acute exacerbations in individuals with underlying chronic lung diseases such as COPD (chronic obstructive pulmonary disease), cystic fibrosis or asthma [3]. In fact, it is estimated that between 70-90 % of asthma exacerbations requiring hospitalization in children are caused by HRV C [4, 5]. Currently, there is no vaccine for HRV and understating the immune response to HRV is a necessary step.

As in any viral infection, adaptive immunity against HRV is mediated by B and T cells, which recognize specific targets in antigens known as epitopes [6]. Identification of these HRV-specific epitopes is of great interest for many reasons, including the monitoring of HRV infections, understanding their immunopathology and the design of effective vaccines. Initial efforts to characterize the adaptive immune response to HRV were aimed to identify B cell epitopes on VP1, VP2 and VP3 capsid proteins which could be targeted by neutralizing antibodies [7]. Unfortunately, HRV genome is very plastic and surface capsid proteins are particularly variable in different HRV strains [8, 9]. In

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fact, attending specifically to the variability found in these capsid proteins, there has been described over 160-180 serologically distinct HRV serotypes [10]. As a result neutralizing antibodies can only recognize serotype-specific epitopes, exhibiting little or no cross-reactivity between different serotypes [11, 12].

Along with neutralizing antibodies, T cells also play a crucial role in HRV immunity [13], triggering both cellular and humoral immune responses. Interestingly, despite the low sequence identity among HRV exposed capsid proteins, it has been shown that HRV-specific CD4 and CD8 T cells can be reactive to multiple HRV serotypes [14-17], revealing the presence of HRV-specific conserved T cell epitopes. So far, only a few conserved HRV-specific T epitopes have been identified [14-18]. A recent study [17] identified the first HRV A and C conserved epitopes targeted by CD8 T cells, finding that they were distributed throughout the entire HRV proteome, including proteins of the capsid and non-structural proteins. Given link between antigen recognition by B and T cells, CD4 T cell epitope mapping has mainly been focused on external HRV capsid proteins VP1, VP2 and VP3 (reviewed in [19]), neglecting the remaining proteins.

In this work, we have validated 3 HRV A-specific CD4 T cell epitopes from VP4 capsid protein along with 4 HRV C-specific CD4 T cell epitopes mapping in various proteins of the capsid and in non-structural proteins. These epitopes are highly conserved in most HRV A and C serotypes and exhibit a promiscuous binding to multiple HLA II molecules highly frequent in the world population. In fact, a pool encompassing these epitopes was able to elicit IFNγ responses by CD4 T cells in 29 out of 30 donors. These identified CD4 T cell epitopes are clearly relevant for both monitoring HRV infections and for designing an epitope-based vaccine against HRV A and C serotypes with an ample population protection coverage.

2. Materials and Methods

2.1. Human subjects

Study was carried out in 33 healthy adults aged 24-58 years (13 female and 20 males). All donors previously signed the informed consent document for the use of blood samples for research purposes, following the legislation corresponding to the Royal Decree-Law 1088/2005 of September 16 (reference number: BOE-A-2005-15514).

2.2. PBMCs isolation and HLA typing

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples (20 ml) by a density gradient on FicollPaque™ PLUS (Amershan) following the manufacturer's instructions. Genomic DNA was extracted from peripheral blood samples from 3 donors (1 female and 2 males) by using MagNa pure Compact instrument (Roche) and HLA-DRB1, HLA-DQA1/B1, HLA-A and HLA-B typing were performed by PCR amplification and hybridization with allele-specific oligonucleotides as described elsewhere [20].

2.3. Peptide synthesis

Peptides were synthesized by ProteoGenix (Schiltigheim) at ≥ 95% purity as confirmed by reversed-phase high performance liquid chromatography (RP-HPLC). Mass of purchased synthetic peptides was verified by MALDI-TOF mass spectrometry (Research Assistance Center for Mass Spectroscopy at Complutense University of Madrid). Lyophilized peptides were dissolved in 40 % dimethyl sulfoxide, diluted in ultra-pure water to a peptide concentration of 5 mM and stored at −80 °C until use.

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2.4. CD4 T cell epitope prediction

VP4 protein from HRV A and whole consensus proteome from HRV C were chosen as targets for CD4 T cell epitope prediction. The HRV C consensus proteome was previously generated as described in [17] upon sequence variability analysis of 39 HRV C full proteomes using the Shannon Entropy (H) as variability metric. The HRV A peptides were 18-mer long, overlapping by 10 amino acids and covered the entire VP4 protein of HRV A with accession number NP_042288.1. Peptide binding affinity to 20 HLA-DRB1 and 20 HLA-DQA1/B1 molecules was predicted using RANKPEP [21] and NETMHCII 2.3 servers [22]. HLA-DRB1 and HLA-DQA1/B1 molecules targeted for binding prediction are listed in Table S1. A peptide was considered to bind to a particular HLA II molecule if it ranked among the top 10 % binding peptides with RANKPEP or NETMHCII. Prediction of peptide binding to HLA II molecules with NETMHCII 2.3 was in addition addressed considering a range of sizes between 12-18 amino acids.

2.5. IFNγ-ELISPOT assays

Peptide-specific IFNγ production by PBMCs from HLA II-typed donors was detected by standard IFNγ ELISPOT assays [23]. Briefly, 96-well PVDF plates (Millipore) were coated with anti-IFNγ (1-D1K mAb, Mabtech) and 1 x 10^5 PBMCs were pleated with RPMI 1640 (Gibco) supplemented with 10% of fetal bovine serum (Gibco), 100 µg/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine (Lonza). Individual HRV peptides were added at 10 µM. Plates were incubated at 37 °C and 5 % CO_2 and after 24 hours were processed according to the manufacturer's instructions [23]. PBMCs incubated with 10 µg/ml phytohemagglutinin (PHA) or without any stimuli were used as positive and background controls, respectively. The assay was run in triplicate for each individual peptide. The number of IFNγ-secreting cells (SFC, "spot forming cells") was determined with an ELISPOT reader (ImmunoSpot 5.0, CTL Analyzers).

2.6. Intracellular cytokine staining

HRV-specific T cells were expanded as described in [17]. Briefly, donor´s PBMCs were cultured at a density of 2 × 10^6 cells/ml in 24-well plates (BD Biosciences) with RPMI 1640 (Gibco) supplemented with 10% human serum (Gibco), 100 µg/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine (Lonza). PBMCs were stimulated with individual HRV peptides (10 µM) and 10 U/mL of IL-2 (Immunotools). Cells were kept at 37 °C in 5 % CO_2 for 6 days being fed and split as necessary with additional doses of peptide (10 µM) and IL-2 (10 U/mL) at day 3 of culture. In some experiments, the same PBMCs were also expanded and stimulated with 10 µM of $HRVC_{1974-1990}$ (GTSVFNTMINNIILRTL), $HRVA_{2029-2037}$ (YGDDVIFSY), CEFTA peptide pool (Mabtech), CEF peptide pool (Mabtech), HRV-specific peptide pool or an irrelevant peptide pool. $HRVC_{1974-1990}$ and $HRVA_{2029-2037}$ correspond to HRV-specific peptides that were not immunogenic in IFNγ ELISPOT assays. CEFTA peptide pool (Mabtech) contains 35 immunodominant HLA II-restricted epitopes from human Cytomegalovirus (CMV), Epstein-Barr (EBV) and Influenza viruses and Tetanus toxin. CEF peptide pool (Mabtech) contains 23 HLA I-restricted immunodominant epitopes from human CMV, EBV and Influenza viruses. HRV-specific peptide pool contains the 7 HRV-specific CD4 T cell epitopes identified in this study. The irrelevant peptide pool contains four 15-mer peptides (VHNSQTFGRELPMYW, WCRSGYHPVMLNAQF, LRVKGCFNITMQPYD and FNWLRSEMCHKPVAY) randomly generated using RandSeq [24] that were predicted not to bind to any of the 20 HLA-DR and 20 HLA-DQ molecules selected in this study. All peptide pools were added at a final concentration corresponding to 10 µM of each peptide contained in the pool.

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PBMCs expanded with the HRV peptides were stimulated for 14 hours with 10 μM of each stimuli in the presence of Brefeldin A (5 μg/ml) (ThermoFisher Scientific). Cells were washed with PBS and surface stained with APC-conjugated anti-CD4 (REA623 mAb, Miltenyi Biotec) and/or FITC-conjugated anti-CD8 (REA734 mAb, Miltenyi Biotec) antibodies. Cell surface was fixed and permeabilized using the FoxP3 staining buffer set (eBioscience, San Diego, CA) according to the manufacturer's instructions and then stained intracellularly with PE-conjugated anti–IFN-γ antibody (45-15 mAb, Miltenyi Biotec). Stained cells were detected by flow cytometry (FACScalibur, BD Biosciences).

2.7. Quantitative binding affinity assays

Quantitative binding affinity of HRV peptides to 8 different HLA-DRB1 molecules (DRB1*03:01, B1*07:01, B1*11:01, B1*11:04, B1*04:04, B1*08:02, B1*09:01 and B1*13:02) and 6 HLA-DQA1/B1 molecules (DQA1*01:01/B1*05:01, A1*01:02/B1*06:02, A1*02:01/B1*02:02, A1*03:01/B1*03:01, A1*05:01/B1*02:01 and A1*05:01/B1*03:01) was determined by classical competitive inhibition of binding assays following the protocol described in Sidney et al [25]. In brief, affinity-purified HLA II molecules (1-10 nM) were co-incubated at 37 ºC or room temperature with 0.1-1 nM of a high-affinity binding radiolabeled peptide and the individual HRV peptides in the presence of protease inhibitors. The respective high-affinity radiolabeled probe peptide used for each HLA II molecules is as listed in [25]. Each unlabeled competitor HRV peptide was tested at 6 different 10-fold concentrations (0.3 nM – 30 μM) in three or more independent assays. After 2-day incubation, HLA II-peptide complexes were captured on anti-HLA II mAb-coated plates (L243 mAb for anti-HLA-DR and SPVL-3 mAb for anti-HLA-DQ) and the percentage of radioactivity was determined using the TopCount microscintillation counter (Packard Instrument Company). Finally, the concentration of the unlabeled peptide that inhibits the binding of the high-affinity radiolabeled peptide by 50 % (IC_{50}) was calculated.

2.8. Other procedures

NetMHC 4.0 [26] was used for predicting peptides nested in $HRVA_{65\text{-}82}$ (IPTLQSPTVEACGYSDRI) binding to HLA I molecules. Epitope population protection coverage (PPC) was computed with IEDB PPC tool [27] considering the allelic frequency of 21 different ethnicities around the world. Statistical analyses were performed using the statistical packages on GraphPad Prism 8 (GraphPad Software Inc., La Jolla, USA). The non-parametrical Kruskal-Wallis test was applied to assess statistical significance between means. Post-hoc analysis was performed using Dunn´s correction and a P value < 0.05 was considered statistically significant.

3. Results

3.1. Computational selection of conserved HRV peptides with potential CD4 T cell epitopes

T cell epitope mapping is costly and time-consuming, therefore, in order to reduce the experimental load we used two different approaches to select the candidate CD4 T cell epitopes. For HRV A we targeted the internal capsid protein VP4, as it is highly conserved among different HRV A serotypes [8, 9] [17]. However, since HRV C is highly variable [17], we targeted all HRV C proteins using the consensus HRV C proteome with variable residues masked generated previously in [17] (see Materials and Methods). Considering that CD4 T cells can only recognize peptides presented by HLA II molecules, we predicted CD4 T cell epitopes through HLA II binding predictions using RANKPEP and NetMHCII. Since HLA II molecules are highly polymorphic and there are hundreds of allelic variants, we selected 20 HLA-DR and 20 HLA-DQ molecules that are common in the world population; they cover 99.8 % of the population (listed in Table S1).

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Following the criteria described in Materials and Methods we finally selected for experimental scrutiny 14 peptides (6 from HRV A and 8 from HRV C) that contained potential CD4 T cell epitopes (Table 1). All of them were predicted to bind to at least one HLA-DRB1 and/or HLA-DQA1/B1 allele highly frequent in the world population and were 12-18-mer long. We synthesized the peptides listed on Table 1 for experimental scrutiny and subjected them to functional assays.

Table 1. Predicted HLA II-binding profile of the HRV A and C peptides selected in this study.

Virus	Peptide	Sequence	Protein [1]	Position [2]	Predicted HLA II binding profile [3]	
					HLA-DRB1	HLA-DQA1/B1
HRV A	$HRVA_{17-34}$	NSVSNGSSLNYFNINYFK	VP4	17-34	DRB1*12:01	DQA1*01:01/B1*05:01
	$HRVA_{23-40}$	LNYFNINYFKDAASSGAS	VP4	23-40	DRB1*04:01 DRB1*04:04 DRB1*08:02 DRB1*12:01	DQA1*01:01/B1*05:01 DQA1*01:01/B1*05:02 DQA1*01:04/B1*05:03
	$HRVA_{57-74}$	VKDVLEKGIPTLQSPTVE	VP4	57-74	DRB1*11:01	-
	$HRVA_{65-82}$	IPTLQSPTVEACGYSDRI	VP4	65-82	-	DQA1*05:01/B1*03:02
	$HRVA_{89-106}$	DSTITSQDVANAVVGYGV	VP4	89-106	-	DQA1*01:02/B1*06:02 DQA1*02:01/B1*02:02 DQA1*03:01/B1*03:01 DQA1*05:01/B1*03:01
	$HRVA_{97-114}$	VANAVVGYGVWPHYLTPE	VP4	97-114	DRB1*04:04	DQA1*01:01/B1*05:01 DQA1*03:01/B1*03:01 DQA1*05:01/B1*03:01 DQA1*05:01/B1*04:02 DQA1*06:01/B1*04:02
HRV C	$HRVC_{24-36}$	VVKYFNINYYKDA	VP4	24-36	DRB1*12:01 DRB1*15:01	DQA1*01:01/B1*05:01 DQA1*01:02/B1*05:02
	$HRVC_{61-75}$	LTNPALMSPSVEACG	VP4	61-75	-	DQA1*01:03/B1*06:03 DQA1*02:01/B1*03:03 DQA1*05:01/B1*03:02 DQA1*05:01/B1*03:03
	$HRVC_{258-274}$	INLRTNNSSTIVVPYIN	VP2	258-274	DRB1*13:02	DQA1*01:02/B1*05:01 DQA1*01:02/B1*06:02 DQA1*01:03/B1*06:03 DQA1*02:01/B1*03:03 DQA1*05:01/B1*03:03
	$HRVC_{945-959}$	YEIQESEYYPKHIQY	2A	945-959	-	DQA1*01:04/B1*05:03
	$HRVC_{1582-1592}$	KEKFRDIRRFIP	3A	1582-1592	DRB1*08:02 DRB1*11:01	-
	$HRVC_{1791-1806}$	GLEPLDLNTSAGFPYV	3D	1791-1806	DRB1*07:01 DRB1*09:01 DRB1*13:02	-
	$HRVC_{1835-1847}$	DLPYVTYLKDELR	3D	1835-1847	-	DQA1*02:01/B1*02:02 DQA1*05:01/B1*02:01
	$HRVC_{1974-1990}$	GTSVFNTMINNIILRTL	3D	1974-1990	DRB1*01:01 DRB1*01:03 DRB1*04:01 DRB1*04:03 DRB1*04:04 DRB1*04:05 DRB1*07:01 DRB1*13:02	DQA1*01:02/B1*05:01

[1] Protein of HRV that contain the peptide sequence [2] Position of the peptide in the selected reference HRV polyproteins. [3] HLA II molecules predicted to bind the corresponding peptides.

3.2. Screening of CD4 T cell epitope candidates by IFNγ-ELISPOT assays

We first screened for immunogenicity of the HRV peptides by IFNγ-ELISPOT assays using PBMCs from three HLA II-typed donors stimulated with the individual HRV peptides as described in Materials and Methods. We considered a response to a peptide as positive if, after subtracting the mean ± standard deviation of the background control, the mean of detected IFNγ-SFC was at least 60 SFC / 10^6 PBMCs. As shown in Figure 1, 8 out of the 14 peptides (4 of HRV A and 4 of HRV C) were able to trigger positive IFNγ recall responses (> 60 SFC / 10^6 PBMCs) in at least one donor. Among HRV A peptides (Figure 1A), $HRVA_{89-106}$, $HRVA_{57-74}$ and $HRVA_{97-114}$ prompted IFNγ recall response in all three donors, being $HRVA_{89-106}$ the peptide that promoted a greater IFNγ release. $HRVA_{65-82}$

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only obtained a positive result in Donor #2. Regarding HRV C peptides (Figure 1B), all three donors responded to peptides HRVC$_{1582-1592}$ and HRVC$_{1791-1805}$, with a mean of 200-300 IFNγ-SFC per million PBMCs. The rest of the HRV C peptides with a positive result (HRVC$_{945-959}$, HRVC$_{1835-1847}$) were able to stimulate IFNγ production by T cells in at least one donor. After IFNγ-ELISPOT assays we discarded 2 HRV A peptides and 4 HRV C peptides that did not elicit enough IFNγ recall responses, selecting 8 peptides to confirm peptide-specific IFNγ production by CD4 T cells.

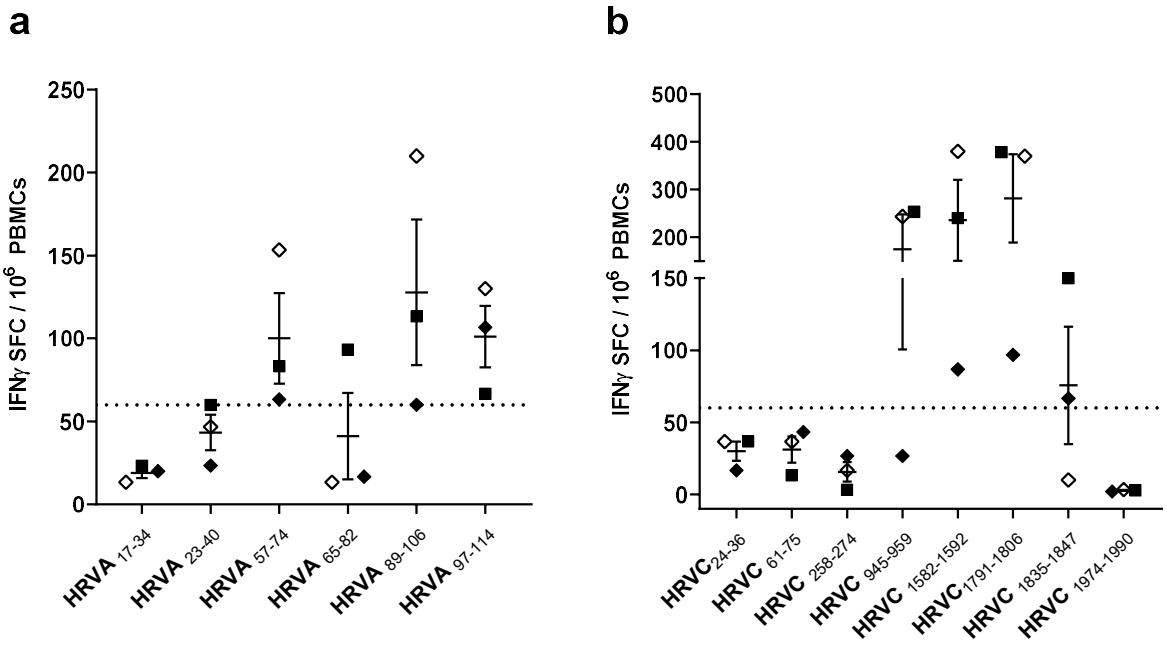

Figure 1. T cell responses to conserved HRV A and C peptides **(a)** Recall T cell responses to 18-mer overlapping peptides contained in VP4 protein from HRV A. **(b)** Recall T cell responses to conserved 12-17-mer HRV C peptides. Recall T cell responses were measured by IFNγ-ELISPOT assays in PBMCs from 3 HLA-II typed donors as described in Materials and Methods. Results for each peptide are expressed as the mean of IFNγ spot forming cells (SFC) / 10^6 PBMCs in each subject after subtracting the mean ± standard deviation of the background control. Error bars represent mean ± SEM of all donors tested. The horizontal line represents the threshold used for positive responses (> 60 SFC / 10^6 PBMCs). We found that 8 out of 14 peptides were able to elicit a positive IFNγ recall response in at least one donor.

3.3. Characterization of CD4 T cell epitopes

In order to validate the selected peptides as CD4 T cell epitopes, we first carried out intracellular cytokine staining assays to confirm that peptide-specific IFNγ production is mediated by CD4 T cells (details in Materials and Methods). Briefly, we first expanded PBMCs from each responding donor with the corresponding HRV peptides (those producing a positive response in the previous section) and stained intracellularly to detect IFNγ positive CD4 T cells by flow cytometry.

As shown in Figure 2, IFNγ responses to HRV peptides varied widely among different donors. We found peptide-specific CD4 T cells producing IFNγ in all responding donors to 7 out of 8 peptides (all but HRVA$_{65-82}$). The percentage of IFNγ-producing CD4 T cells actually increased 5-15 fold in all donors in response in any of those 7 peptides. The strongest recall response was elicited by HRVA$_{89-106}$ (8-18 fold increase independently of the donor), followed by peptides HRVA$_{57-74}$, HRVC$_{258-274}$, HRVC$_{1791-1806}$, HRVA$_{97-114}$, HRVA$_{1582-1592}$ and HRVC$_{1835-1847}$.

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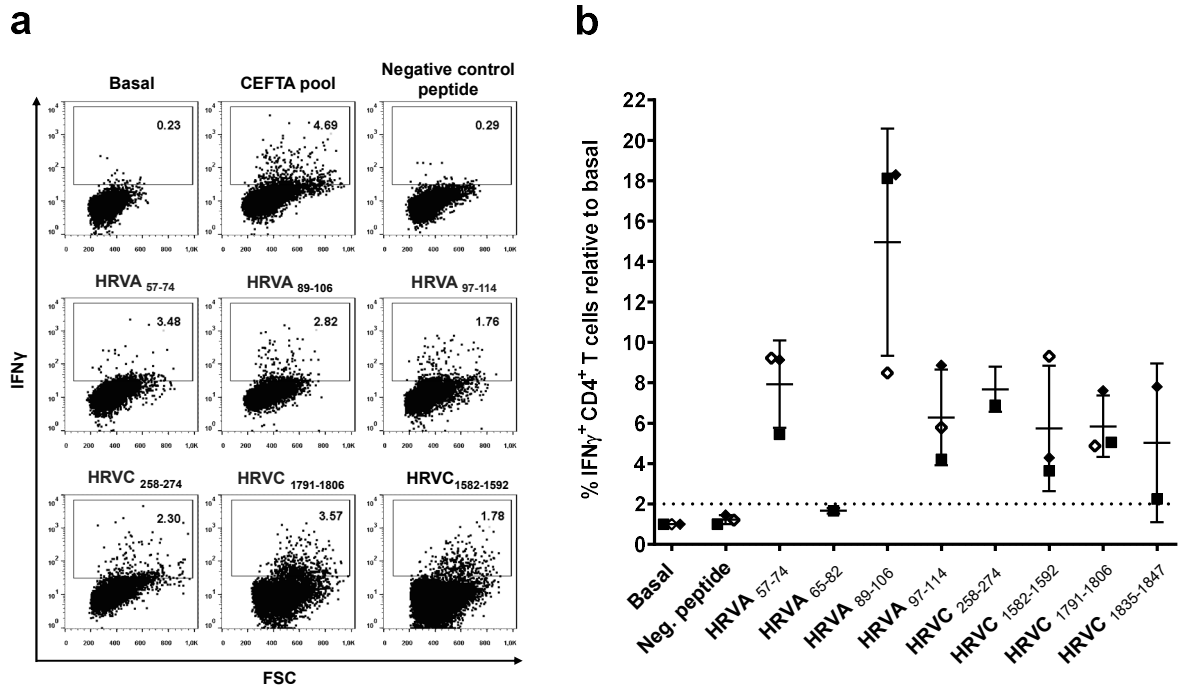

Figure 2. Peptide-specific IFNγ production by CD4 T cells in response to HRV A and C conserved peptides. Donors PBMCs expanded with the HRV peptides were stimulated with individual peptides in the presence of Brefeldin A and stained intracellularly for detection of IFNγ production as described in Materials and Methods. **(a)** Panel shows a representative experiment resulting from intracellular cytokine staining of PBMCs from Donor #2. Data is expressed as the percentage of peptide-specific IFNγ-producing CD4 T cells within the total of gated-CD4 T cells. **(b)** Plot depicting the percentage of peptide-specific IFNγ-producing CD4 T cells in responding donors. Represented values are relative to basal IFNγ-producing CD4 T cell in the absence of peptides. Individual symbols represent the mean of two independent experiments for each donor. Error bars represent mean ± SD of IFNγ-producing CD4 T cells of all donors tested. The horizontal line represents the threshold used for positive responses. Positive and negative peptide controls were obtained by expanding and stimulating the same PBMCs with CEFTA peptide pool and the peptide HRVC$_{1974-1990}$ (GTSVFNTMINNIILRTL), respectively (See Materials and Methods). Peptide HRVC$_{1974-1990}$ was not immunogenic in IFNγ-ELISPOT assays.

Peptide HRVA$_{65-82}$ failed to stimulate IFNγ production by CD4 T cells from Donor #2 (< 1.6 % of IFNγ$^+$ CD4$^+$ T cells relative to basal), despite giving a positive response in ELISPOT assays in this donor. Thereby, we discarded this peptide as CD4 T cell epitope. HRVA$_{65-82}$–specific IFNγ production detected by ELISPOT likely stems from shorter peptides released after processing of HRVA$_{65-82}$ that can be recognized by CD8 T cells. In fact, we could detect by flow cytometry peptide-specific CD8 T cells producing IFNγ when stimulated with HRVA$_{65-82}$ (Figure S1). Moreover, we found two potential CD8 T cell epitopes in HRVA$_{65-82}$, peptides IPTLQSPTV and SPTVEACGY, that are predicted to bind to HLA-B*35:03 (rank 1.5 and 0.40 %, respectively), which is expressed by Donor #2. No other potential CD8 T cell epitope was predicted in HRVA$_{65-82}$ (IPTLQSPTVEACGYSDRI) judging by their ability to bind to the HLA I molecules expressed by Donor #2 (HLA-A*02:01, HLA-A*11:01, HLA-B*35:03 and HLA-B*44:03). The predicted binding of all 9-mer peptides in HRVA$_{65-82}$ to the HLA I molecules expressed by Donor #2 is provided in Table S2.

To further characterize the 7 HRV-specific CD4 T cell epitopes identified here, we determined the binding affinity of each HRV peptide to 14 different HLA-DRB1 and HLA-DQA1/B1 molecules highly frequent in the world population (details in Materials and Methods). In Figure 3 we show the binding affinity of each individual HRV peptide to all those alleles that gave a detectable binding affinity (< 40000 nM in terms of IC$_{50}$). The vast majority of experimentally verified CD4 T cell epitopes have an affinity for their

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corresponding HLA II restricting element of 1000 nM or better [28-30]. Therefore, we considered that peptides binding to a particular HLA II molecule with an IC_{50} was < 1000 nM IC_{50} could be potential CD4 T cell epitopes restricted by that HLA II element. Following this approach, we confirmed peptide binding to at least one HLA-DRB1 and/or HLA-DQA1/B1 molecule for all 7 HRV CD4 T cell epitopes. Peptide $HRVA_{89-106}$ (DSTITSQDVANAVVGYGV) was highly promiscuous, binding with high affinity (IC_{50} < 900 nM) to 7 different HLA II molecules. Altogether, the 7 HRV CD4 T cell epitopes could bind to 7 different HLA-DR (HLA-B1*04:04, B1 DRB1*07:01, B1*08:02, B1*09:01, B1*11:01, B1*11:04, and B1*13:02) and 6 HLA-DQ molecules (HLA-DQA1*01:01/B1*05:01, A1*01:02/B1*06:02, A1*02:01/B1*02:02, A1*03:01/B1*03:01, A1*05:01/B1*02:01 and A1*05:01/B1*03:01).

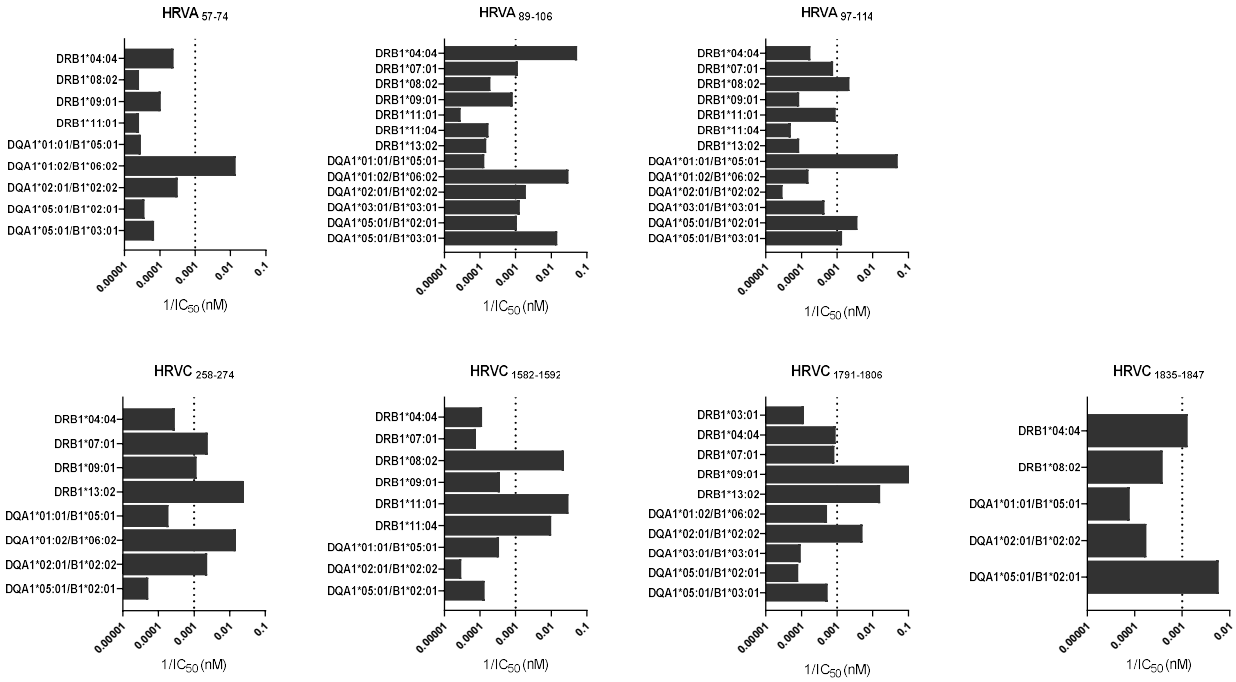

Figure 3. Binding affinity of HRV peptides to HLA II molecules. Figure depicts the HLA-DR and -DQ molecules with detectable binding affinity for each of HRV-specific CD4 T cell epitopes (IC_{50} < 40000 nM). Binding affinity is given as $1/IC_{50}$ value (nM) as determined by competitive inhibition of binding assays (details in Materials and Methods). The vertical line represents the threshold used as selection criteria for positive binding (IC_{50} < 1000 nM).

3.4. Population Coverage of HRV-specific T cell responses

According to their HLA II binding profile, the 7 identified in this work could be expected to elicit responses in up to 98 % of the population, regardless of their ethnicity [27] (see Materials and Methods). This estimation is computed after the assumption that CD4 T cell epitopes shown to be immunogenic under a particular HLA II context will also be immunogenic in any subject expressing at least one of the HLA II molecules confirmed to bind the epitopes. There is considerable evidence for this assumption [31-33] and we here tested its extend. To that end, we determined recall CD4 T cell responses to a pool encompassing all 7 CD4 T cell epitopes in 30 donors through intracellular IFNγ staining as described earlier. We confirmed positive CD4 T cell responses in 29 out of 30 subjects, 96.6 % of the cohort (Figure 4).

The magnitude of the response depended of the donor, varying from a 1.71 to a 30.03 fold increase of IFNγ-producing CD4 T cells in response to the peptides but, on average, increased a 7.17 fold (Figure 4a). Interestingly, the CD4 T cell recall response to the

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HRV-specific peptide pool was greater than that to the commercial CEFTA peptide pool (% IFNγ+ CD4+ T cells average fold increase of 7.17 and 5.62, respectively), although not in a statistically significant manner ($p = 0.291$). Moreover, more subjects responded to the HRV-specific peptide pool (96.6 %) than to the CEFTA peptide pool (73.3 %). We consider a donor responding to the HRV-specific peptide pool if the percentage of IFNγ+ CD4+ T cells was at least 2.5 times greater than the basal IFNγ response of the donor.

Given that CD4 T cell epitopes can bear nested CD8 T cell epitopes [17], which are key for antiviral responses, we also tested CD8 T cell responses to the HRV-specific peptide pool. We could detect an increase of IFNγ-producing CD8 T cells in response to the HRV peptide pool in 24 out of 30 donors (80.0 %) (Figure 4b). Considering exclusively the responses in the responding subjects, the average CD8 T cell response was lower than that determined for CD4 T cells (% IFNγ+ CD4+ or CD8+ T cells average fold increase of 6.47 and 7.38, respectively).

Together, these results reveal the potential of the 7 conserved HRV-specific CD4 T cell epitopes identified to activate both HRV-specific CD4 and CD8 T cells, providing a broad population coverage.

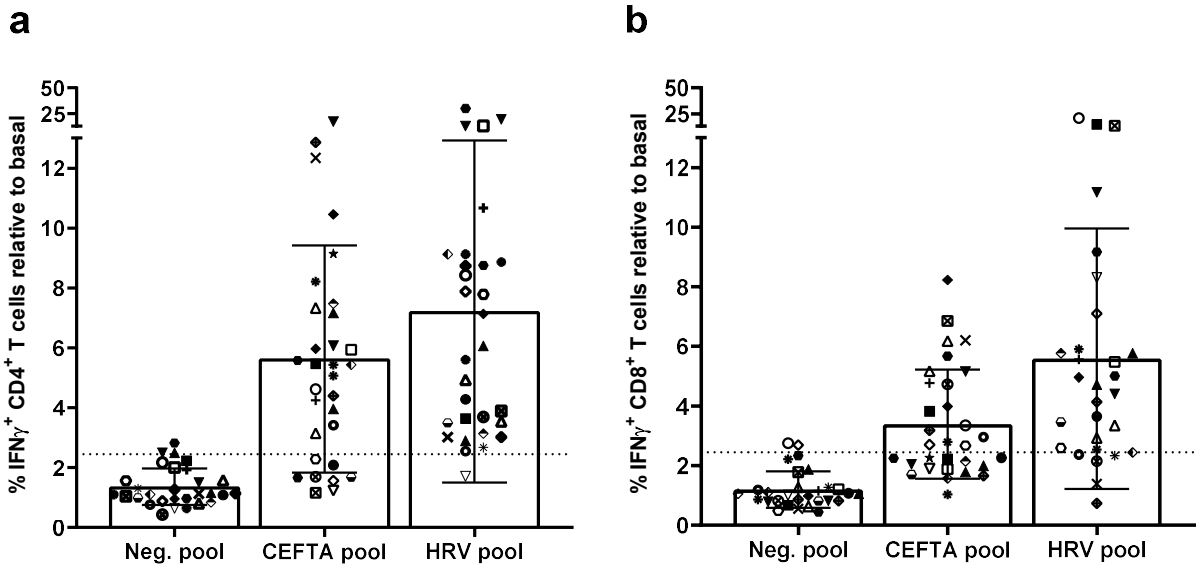

Figure 4. T cell responses to HRV-specific CD4 T cell epitope pool. IFNγ-production by CD4 **(a)** and CD8 **(b)** T cells in response to different peptide pools. PMBCs from 30 donors were expanded and stimulated with a peptide pool containing 10 μM of each HRV-specific CD4 T cell epitope identified in this study in the presence of Brefeldin A, labeled with anti-CD4 and anti-CD8 antibodies and stained intracellularly for IFNγ (see Materials and Methods). Results are expressed as the percentage of peptide-specific IFNγ-producing CD4 or CD8 T cells within the total of gated-CD4 or CD8 T cells as relative to the basal IFNγ production of each donor. Basal IFNγ production was obtained by incubating donor PBMCs without the addition of exogenous peptide. CEFTA peptide pool was used as positive control and as negative control we used a peptide pool containing 4 irrelevant peptides (see Materials and Methods). Mean and standard deviation of all donors tested are plotted The vertical line represents the threshold used as selection criteria for positive responses (> 2.5 % IFNγ+ CD4+ or CD8+ T cells fold increase as relative to basal).

4. Discussion

Human Rhinoviruses (HRVs) are considered as mild pathogens and HRV infections are often disregarded. However, HRV infections are a leading cause behind severe bronchiolitis and are also linked to acute exacerbations of chronic pulmonary diseases [2]. Moreover, HRV infections have a large economic impact worldwide in terms of healthcare costs and work absenteeism [34].

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HRVs are small RNA viruses (~7500 bp) belonging to the family Picornaviridae and the genus Enterovirus. HRVs exhibit highly variable genomes and have been classified in three species: HRV A, B and C. HRV A and C are the more relevant species in the clinic as they are responsible for more than 90 % of HRV infections [1, 10]. HRVs are known to induce potent humoral and cellular immune responses [7]. The humoral response includes IgG and IgA neutralizing antibodies directed against viral surface proteins, which can provide protective immunity to secondary infections with related species [11]. HRV-specific T cell responses are particularly relevant to contain HRV infection and clear viral particles. In healthy individuals HRV infections induce a Th1 polarization of CD4 T cells which contribute to activation of cytotoxic CD8 T cells capable of killing infected cells. However, in asthmatic individuals HRV infections have been shown to induce increased levels of IL-4 and IL-13, along with an increase infiltration of macrophages and neutrophils on the respiratory tract, which is linked to the ability of HRV to enhance asthma exacerbations [35, 36]. Most studies of HRV immunity have focused on the characterization of antigen targets for antibodies, which have allowed to classify HRVs into different serotypes; so far, more than 180 distinct HRV serotypes have been identified [10]. However, the study of the targets of HRV-specific T cell responses has received less attention. In fact, we recently described the first HRV-specific CD8 T cell epitopes reported up to date [17]. Since antigen recognition by CD4 T cells and B cells is linked, there are more known CD4 T cell antigen targets. Thus, some HRV-specific CD4 T cell epitopes have been mainly identified on capsid proteins targeted also by antibody responses [14-16, 18]. In this study, we contributed to identify novel HRV-specific CD4 T cell epitopes through a computer-aided approach.

Table 2. Summary of the HRV-specific CD4 T cell epitopes identified in this study.

Peptide	Sequence	Protein	Confirmed HLA II binding profile [1]		PPC [2]
			HLA-DRB1	HLA-DQA1/B1	
HRVA$_{57-74}$	VKDVLEKGIPTLQSPTVE	VP4	-	DQA1*01:02/B1*06:02	34.55
HRVA$_{89-106}$	DSTITSQDVANAVVGYGV	VP4	DRB1*04:04 DRB1*07:01	DQA1*01:02/B1*06:02 DQA1*02:01/B1*02:02 DQA1*03:01/B1*03:01 DQA1*05:01/B1*02:01 DQA1*05:01/B1*03:01	95.09
HRVA$_{97-114}$	VANAVVGYGVWPHYLTPE	VP4	DRB1*08:02	DQA1*01:01/B1*05:01 DQA1*05:01/B1*02:01 DQA1*05:01/B1*03:01	81.58
HRVC$_{258-274}$	INLRTNNSSTIVVPYIN	VP2	DRB1*07:01 DRB1*09:01 DRB1*13:02	DQA1*01:02/B1*06:02 DQA1*02:01/B1*02:02	67.68
HRVC$_{1582-1592}$	KEKFRDIRRFIP	3A	DRB1*08:02 DRB1*11:01 DRB1*11:04	-	17.37
HRVC$_{1791-1806}$	GLEPLDLNTSAGFPYV	3D	DRB1*09:01 DRB1*13:02	DQA1*02:01/B1*02:02	36.28
HRVC$_{1835-1847}$	DLPYVTYLKDELR	3D	DRB1*04:04	DQA1*05:01/B1*02:01	52.04

[1] HLA II molecule binding the peptide with a high binding affinity (IC_{50} < 1000 nM) as determined by competitive inhibition binding assays. [2] Population protection coverage (PPC), meaning the percentage of the world population that express at least one of the HLA II alleles. PPC was computed using the IEDB PPC tool [27] considering the allelic frequency of 21 different ethnicities around the world. The PPC of all 7 epitopes reaches 98 %.

Identification of CD4 T cell epitopes generally begins with the selection of peptides suitable for presentation by HLA II molecules [37]. However, HLA II molecules are extremely polymorphic and allelic variants bind and present distinct sets of peptides [38]. Thus, we targeted for peptide binding predictions 20 HLA-DR and 20 HLA-DQ molecules which have a combined phenotypic frequency of 99.80 % in the world population [27]. We selected 14 peptides for experimental scrutiny after HLA II binding predictions (Table 1). In the end, we could validate trough IFNγ-ELISPOT assays (Figure 1) and in-

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tracellular cytokine staining (Figure 2) 3 HRV A-specific and 4 HRV C-specific CD4 T cell epitopes mapping in various proteins of the capsid and in non-structural proteins (Summarized in Table 2). These CD4 T cell epitopes are highly conserved, can bind to various HLA II molecules (Figure 3) and none of them has been previously reported. All 3 HRV A-specific epitopes ($HRVA_{57-74}$, $HRVA_{89-106}$ and $HRVA_{97-114}$) are located on the internal capsid protein VP4. VP4 is located on the amino terminal extreme of the HRV polyprotein and this location has been shown to favor antigen processing and presentation to T cells [39]. Of the 4 HRV C-specific epitopes, one ($HRVC_{258-274}$) is located in VP2 capsid protein. The other 3 HRV C-specific epitopes ($HRVC_{1582-1592}$, $HRVC_{1791-1806}$ and $HRVC_{1835-1847}$) are distributed on viral proteins 3A and 3D, which are both implicated in the replication and assembly of the viral genome [8]. To our knowledge, these are the first CD4 T cell epitopes identified in these HRV proteins.

The CD4 T cell epitopes identified in this work were capable of inducing strong IFNγ recall responses (Figure 2) and could bind to various HLA II molecules (Figure 3). By comparing the HLA II-typing of the responding donors with the experimental HLA II binding profile of the epitopes (Table S3) we could anticipate the potential restriction elements of some epitopes. Thus, $HRVC_{1582-1592}$ could be restricted by HLA-DRB1*11:01 since it could bind $HRVC_{1582-1592}$ with high affinity and the relevant allele is expressed by the responding donors (Donors #2 and #3). In a similar way, HLA-DRB1*07:01 could be restricting the response of peptide $HRVC_{258-274}$ and DQA1*05:01/B1*03:01 of peptide $HRVA_{97-114}$. However, we did not verify formally that these were the restrictions elements responsible for the response. For some of the epitopes ($HRVA_{57-74}$ and $HRVC_{1791-1806}$), the HLA II-typing of the responding donors does not match any of the HLA II molecules that can bind the epitopes. These epitopes must surely be presented to CD4 T cells by other HLA II molecules expressed by the donor that were not tested in the binding assays. It is worth noting that predicted peptide binding to HLA II alleles could be confirmed experimentally for 94 % of peptide-HLA II pairs. However, many of the HLA II molecules that experimentally did bind peptides with an IC_{50} < 1000 nM (12 of 25) could not be predicted.

Epitopes shown to be immunogenic in the HLA II context of a particular subject are also expected to be immunogenic in other subjects provided that they express any of the HLA II molecules that such epitope can bind [31-33]. Under this assumption, and taking into consideration the HLA II binding profiles, our peptides were expected to elicit responses in up to 98 % of the population, regardless of their ethnicity [27]. A pool encompassing these epitopes was indeed able to elicit IFNγ recall responses by CD4 T cells in 29 out of 30 donors (96.6 %) (Figure 4a). We realize that all donors were Caucasians but nonetheless we detected more responses to this pool than to a commercial peptide pool including 35 peptides (CEFTA pool), often used as positive control for ELISPOT assays. Detection of peptide-specific IFNγ recall responses by CD4 T cells reveals that these peptides are *bona fide* CD4 HRV-specific T cell epitopes, which are processed and targeted during HRV infections. Interestingly, we could likewise detect strong IFNγ recall responses by CD8 T cells in 80 % of the donors (Figure 4b). CD8 T cell responses to CD4 T cell epitope are somewhat expected, since CD4 T cell epitopes can bear shorter peptides (9-11-mer) capable of binding to HLA I molecules and be recognized by CD8 T cells. In fact, in a previous work, we identified CD8 T cell epitopes within some of the CD4 T cell epitopes reported here [17]. Moreover, we also described that $HRVC_{1791-1806}$ (GLEPLDLNTSAGFPYV) is an unusually long HLA-A*02:01-restricted CD8 T cell epitope [17].

Given the extent of the responses, the 3 HRV A- and 4 HRV C-specific CD4 T cell epitopes identified are of particular interest for monitoring HRV infections. More importantly, these epitopes represent excellent candidates to develop an epitope-based vaccine against HRV as they can also induce CD8 T cell responses. Moreover, they could be combined with additional CD8 T cell epitopes to enhance such responses. It is noteworthy that CD4 T cell epitopes alone or in conjunction with CD8 T cell epitopes could be

readily incorporated into RNA vaccine technologies for parenteral or intranasal forms of administration in the future.

Supplementary Materials: Table S1: HLA II molecules used for predicting peptide binding, Table S2: HLA I binding predictions of all 9-mer peptides contained in $HRVA_{65-82}$ to HLA I molecules expressed by Donor #2. Table S3: IFNγ-responses from HLA II-typed donors to HRV-specific CD4 T cell epitopes. Figure S1: Peptide-specific IFNγ production by CD8 T cells

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