Human rhinovirus-specific CD8 T cell responses target conserved and unusual epitopes

Marta Gomez-Perosanz1 | Jose L. Sanchez-Trincado1 | Miguel Fernandez-Arquero2 | John Sidney3 | Alessandro Sette3 | Esther M. Lafuente1 | Pedro A. Reche1

1Department of Immunology, School of Medicine, Complutense University of Madrid, Madrid, Spain
2Immunology Service, San Carlos University Hospital, Madrid, Spain
3Division of Vaccine Discovery, La Jolla Institute for Immunology, La Jolla, CA, USA

Correspondence
Pedro A. Reche, Department of Immunology, School of Medicine, Complutense University of Madrid, Pza. Ramón y Cajal, s/n, Madrid 28040, Spain. Email: parecheg@med.ucm.es

Funding information
Ministerio de Economía y Competitividad (MINECO), Grant/Award Number: BIO2014:54164-R

Abstract
Human Rhinovirus (HRV) is a major cause of common cold, bronchiolitis, and exacerbations of chronic pulmonary diseases such as asthma. CD8 T cell responses likely play an important role in the control of HRV infection but, surprisingly, HRV-specific CD8 T cell epitopes remain yet to be identified. Here, we approached the discovery and characterization of conserved HRV-specific CD8 T cell epitopes from species A (HRV A) and C (HRV C), the most frequent subtypes in the clinics of various pulmonary diseases. We found IFNγ-ELISPOT positive responses to 23 conserved HRV-specific peptides on peripheral blood mononuclear cells (PBMCs) from 14 HLA I typed subjects. Peptide-specific IFNγ production by CD8 T cells and binding to the relevant HLA I were confirmed for six HRV A-specific and three HRV C-specific CD8 T cell epitopes. In addition, we validated A*02:01-restricted epitopes by DimerX staining and found out that these peptides mediated cytotoxicity. All these A*02:01-restricted epitopes were 9-mers but, interestingly, we also identified and validated an unusually long 16-mer epitope peptide restricted by A*02:01, HRVC1791-1806 (GLEPLDLNTSAGFYV). HRV-specific CD8 T cell epitopes described here are expected to elicit CD8 T cell responses in up to 87% of the population and could be key for developing an HRV vaccine.

KEYWORDS
CD8-positive T-lymphocytes, epitopes, peptides, rhinovirus

Abbreviations: CDHR3, Cadherin-related family member 3; ELISPOT, enzyme-linked immunospot assay; H, Shannon entropy; HLA I, human leukocyte antigen class I; HRV, human rhinovirus; HTLV, human T cell leukemia virus type 1; IC50, half maximal inhibitory concentration; ICAM-1, intercellular adhesion molecule 1; IFNγ, interferon gamma; IgG, immunoglobulin G; IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-6, interleukin 6; LDH, lactate dehydrogenase; LDL, low density lipoprotein; mAb, monoclonal antibody; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MHC I, major histocompatibility complex class I; MSA, multiple sequence alignment; NCBI, national center for biotechnology information; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PDB, protein data bank; PHA, phytohemagglutinin; PPC, population protection coverage; RP-HPLC, reversed-phase high-performance liquid chromatography; RPMI, Roswell Park Memorial Institute medium; SFC, spot forming cells; TAP, transporter-associated with antigen processing; TNFα, tumor necrosis factor alpha; β2m, beta 2 microglobulin.

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INTRODUCTION

Human Rhinovirus (HRV) respiratory tract infections are the most frequent cause of the common cold.\(^1\) In most individuals, HRV infection does not lead to serious illness and is generally perceived as benign. However, HRV is a leading cause behind severe bronchiolitis needing hospitalization in infants and exacerbations of chronic pulmonary diseases, such as asthma or chronic obstructive pulmonary disease.\(^2\) It has been found that HRV infections during early life are associated with a subsequent development of asthma during childhood.\(^3\) Besides, it is estimated that HRV infections represent a large economic burden annually in terms of medical costs and work absenteeism.\(^4\) In this context, finding a good candidate for a vaccine or unravel possible therapeutic targets for this virus could minimize many of the health and economic problems associated with HRV infections, especially in those individuals with underlying chronic pulmonary diseases.

So far, over 150 antigenically distinct serotypes of HRV have been described. Molecular advances have allowed to classify them into three genetically distinct groups known as HRV A (74 serotypes), HRV B (25 serotypes), and the novel species HRV C (50 serotypes).\(^5\) It has been estimated that HRV respiratory infections are mainly due to HRV A and HRV C species.\(^6\) Despite both HRV A and C species having similar prevalence and rate of infection, it is now established that HRV C is a major cause of asthma and chronic obstructive pulmonary disease exacerbations, compared to HRV A.\(^2,7\) However, the pathogenic mechanisms of HRV C and HRV A infections leading to such complications are still poorly understood.

Like other Picornaviruses, HRVs are small viruses that contain a positive-sense single-stranded RNA genome.\(^1\) The viral genome consists of a single gene that is translated into a single polyprotein. The entire viral proteome is produced upon proteolytic processing of the polyprotein. Specifically, the polyprotein is cut by virally encoded proteases to produce 11 proteins: four (VP1, VP2, VP3, and VP4) form the capsid, while the remaining nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) are involved in the replication and assembly of the viral genome.\(^8\) The external structural proteins VP1, VP2, and VP3 are responsible for most of the antigenic diversity of the virus, while VP4, found on the inner side of the capsid, has little variability.\(^8,9\)

HRV infects upper and lower respiratory tract epithelial cells. Viral entry starts with attachment of VP1 capsid protein to ICAM-1, LDL, or CDHR3 cell-surface receptors.\(^10,11\) Typically, the entrance of the virus into the host epithelial cells is associated with a release of inflammatory mediators and neutralizing antibodies.\(^12\) Neutralizing antibodies directed against the VP1, VP2, and VP3 surface-exposed areas of the capsid are considered as the main protective mechanism against HRV infection.\(^13\) However, these regions of the capsid display high variability between different serotypes. As a result, antibodies induced by past exposures to particular HRV serotypes cannot recognize other serotypes; hence, there is a lack of effective cross-protective antibody-mediated immunity among HRV serotypes.\(^14\)

It has been shown that both CD4 and CD8 memory T cells capable of recognizing HRV-specific antigens are present in the circulation of healthy subjects.\(^15\) However, in contrast to the serotype-specific antibody response, virus-specific CD4 T cells have been shown to respond to multiple serotypes,\(^16–18\) indicating the existence of conserved epitopes within the large antigenic heterogeneity between HRV serotypes. CD4 T cell responses against HRV have a typical Th1 cell helper type 1 (Th1) pro-inflammatory cytokine profile, including IFNγ, IL-1β, TNFα, and IL-6.\(^12\) In contrast, CD8 T cell responses against HRV remain largely unexplored but, judging from other respiratory viral infections, are likely critical for HRV clearance.\(^19\)

CD8 T cells combat viral infections thanks to their ability to detect and kill infected cells. To that end, CD8 T cells recognize viral peptides displayed on the surface of infected cells bound to class I major histocompatibility complex (MHC I) molecules; in humans known as HLA I molecules (human leukocyte antigens).\(^20\) Currently, many of these peptides (CD8 T cell epitopes) have been identified for most common viruses but, to the best of our knowledge, not a single one for HRV. However, the identification of HRV-specific epitopes will be of great interest, as they will enable the induction of virus-specific CD8 T cell responses, thereby providing an alternative approach to that of eliciting neutralizing antibodies for vaccine development. Attempts to produce conventional vaccine against HRV have failed due to the large number of antigenically distinct serotypes.\(^14,21\)

In this work, we successfully tackled the identification of CD8 T cell epitopes from HRV A and C species, the most frequent subtypes in the clinics of various pulmonary diseases. T cell epitope mapping is costly and time-consuming as it involves testing numerous epitope candidates. To reduce the experimental load, we used a computer-assisted method to select non-variable CD8 T cell epitope candidates for experimental scrutiny. We used Shannon Entropy to assess HRV sequence variability\(^22\) and predicted CD8 T cell epitopes within non-variable regions. Since CD8 T cells can only recognize peptides presented by MHC I molecules, we predicted CD8 T cell epitopes through peptide-MHC I binding predictions using methods based on profile matrices\(^23\) and artificial neural networks.\(^24\) Thus, we selected 31 peptides for screening by IFNγ-ELISPOT assays, finding positives responses to 23 peptides and validating 9 of them as CD8 T cell epitopes through additional molecular and cellular assays. Interestingly, among the characterized HRV-specific CD8 T cell epitopes, we report a 16-mer epitope restricted by A*02:01 (HRVC\(_{1791-1806}\), GLEPLDLNTSAGFPYV).
2 | MATERIALS AND METHODS

2.1 | Donors and HLA I typing

Blood samples were collected from 14 healthy donors (7 females and 7 males) who had 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). Genomic DNA was extracted from the peripheral blood samples of 14 healthy donors using MagNa pure Compact instrument (Roche, UK) and preserved at −70°C. HLA-A and -B typing was performed using the LIFECODES HLA-A and -B SSO typing kit (Immucor, Stamford, CT, USA).

2.2 | Synthetic peptides

Peptides used in this study were synthesized by ProteoGenix (Schiltigheim, France) at ≥95% purity as confirmed by reversed-phase high-performance liquid chromatography (RP-HPLC). Mass of purchased synthetic peptides was verified in house 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.

2.3 | HRV sequence variability analysis and generation of consensus proteomes

Consensus HRV A and C proteomes with variable residues masked were generated upon sequence variability analysis as described elsewhere. Briefly, from all HRV A and C proteins available at NCBI, we selected 87 HRV A and 39 HRV C protein sequences corresponding to entire polyproteins (sequence length ≥ 3000 amino acids) and generated a multiple sequence alignment (MSA) for both HRV A and C species using MUSCLE. Subsequently, we used the Shannon Entropy \( H = - \sum_{i} M P_i \log_2(P_i) \) (Equation 1) to compute the sequence variability per site/position in the MSA. A and C polyproteins was assigned to HRV A and HRV C polyproteins with accession numbers NP_042288.1 and YP_001552411.1, respectively. Subsequently we masked any residue site with \( H \geq 1.0 \), thus generating reference consensus polyproteins for HRV A and HRV C. It should be noted that sites with \( H < 1 \) consist almost entirely of sites with no more than two amino acids per site.

2.4 | Prediction of peptide binding to HLA I molecules

Peptide-HLA binding predictions were used as the main basis to anticipate CD8 T cell epitopes. Prediction of peptide binding to the 55 most common HLA class I molecules was assessed using RANKPEP. RANKPEP uses profile motifs to compute peptide-HLA I binding scores telling the proximity in sequence space of test peptides to peptides that are known to bind to a given HLA I molecule. Since the range of binding scores provided by different profile motifs varies widely, here a given peptide was considered to bind to a particular HLA I molecule if its binding score ranked among the 2% percentile of scores computed for 1000 random peptides using the same relevant HLA I-specific profile. Profile motifs used in this study were only suited for peptides of nine residues in length (9-mer), the most common size of peptides found to bind HLA I molecules. For longer peptides, HLA I binding was obtained upon evaluating the binding of all 9-mer peptides within the longer peptide. Binding affinity of 9-mer peptides to selected HLA I molecules was also determined using NetMHC 4.0. NetMHC uses a neural network method that returns IC50 values of peptides to HLA I molecules.

2.5 | Isolation of Peripheral blood mononuclear cells and in vitro expansion of HRV-specific T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from donor’s blood samples (30 mL) by a density gradient on FicollPaque PLUS (Amershan). For in vitro expansions, PBMCs were cultured in RPMI 1640 (Gibco, NY, USA) supplemented with 10% human serum (Gibco NY, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine (Lonza, Walkersville, USA) at a density of \( 2 \times 10^5 \) cells/mL in 24-well plates (BD Biosciences) and stimulated with individual HRV peptides (10 µM final concentration) and IL-2 (10 U/mL; Immunotools). Cells were kept at 37°C in 5% CO2 for 6 days being split and fed as necessary with an additional doses of IL-2 (10 U/mL; Immunotools) and individual HRV peptides (10 µM) at day 3 of culture. Expanded cells were washed twice with...
PBS (Gibco, NY, USA) and let rest for 4 hours in RPMI 1640 without both, human serum and stimulation, prior to any functional assay.

2.6 | IFNγ-ELISPOT assays

Production of IFNγ by PBMCs from HLA I typed donors in response to stimulation with the corresponding HRV peptides was detected by ELISPOT assays, following standard procedures.

2.6.1 | Production of IFNγ by PBMCs from HLA I typed donors in response to stimulation with the corresponding HRV peptides was detected by ELISPOT assays, following standard procedures. Briefly, 1 x 10^5 cells in RPMI 1640 (Gibco, NY, USA) supplemented with 100 U/ml penicillin, 100 μg/mL streptomycin, and 2 mM l-glutamine (Lonza, Walkersville, USA) were plated in 96-well PVDF (Millipore, Germany) coated with an anti-IFNγ capture mAb 1-D1K (Mabtech) and individual HRV peptides were added at each well at 10 μM final concentration. Plates were incubated at 37°C and 5% CO2 for 24 hours and processed as described.

2.7 | Intracellular cytokine staining

PBMCs from matching donors were cultured for 14 hours in RPMI 1640 (Gibco, NY, USA) supplemented with 10% human serum (Gibco NY, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM l-glutamine (Lonza, Walkersville, USA) at 37°C and 5% CO2 with the HRV peptides (10 μM) in the presence of Brefeldin A (5 μg/mL) (Thermo Fisher Scientific). The same PBMCs cultured with media alone were used as negative control. CEF peptide pool was used as a positive control. After culture, cells were washed with PBS and surface stained with APC-conjugated anti-CD8 REA734 mAb (Miltenyi Biotec) followed by intracellular staining with FITC-conjugated anti-IFN-γ 45-15 mAb (Miltenyi Biotec) according to the manufacturer's instructions. Stained cells were detected by flow cytometry (FACScalibur, BD Biosciences).

2.8 | T2 binding assay

Binding stability and presentation of some of the A*02:01-restricted HRV peptides were tested in cellular assays using A*02:01+ TAP-deficient T2 hybridoma cells as follows. T2 cells in AIM-V free serum medium (Gibco) were plated at a density of 10^5 cells/ml in 96-well plates and pulsed overnight at 37°C and 5% CO2 with the tested peptides at six concentrations varying from 1 to 100 μM and 5 μg/mL of β2m (BD biosciences). A*02:01-restricted HTLV-TAX peptide (LFGYPVV) was used as a positive control. A*02:01-surface expression was determined by flow cytometry (FACScalibur, BD Biosciences) using FITC-conjugated HLA-A2 mAb BB7.2 (BD biosciences).

2.9 | Intracellular cytokine staining

PBMCs from matching donors were cultured for 14 hours in RPMI 1640 (Gibco, NY, USA) supplemented with 10% human serum (Gibco NY, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM l-glutamine (Lonza, Walkersville, USA) at 37°C and 5% CO2 with the HRV peptides (10 μM) in the presence of Brefeldin A (5 μg/mL) (Thermo Fisher Scientific). The same PBMCs cultured with media alone were used as negative control. CEF peptide pool (Mabtech) was used as a positive control. After culture, cells were washed with PBS and surface stained with APC-conjugated anti-CD8 REA734 mAb (Miltenyi Biotec) followed by intracellular staining with FITC-conjugated anti-IFN-γ 45-15 mAb (Miltenyi Biotec) according to the manufacturer's instructions. Stained cells were detected by flow cytometry (FACScalibur, BD Biosciences).

2.10 | DimerX staining

A chimeric protein consisting of a mouse Immunoglobulin G (IgG) fused with two A*02:01 molecules commercialized under the name of DimerX (BD Biosciences) was used to detect CD8 T cells recognizing A*02:01-restricted epitopes as described. Briefly, DimerX was passively loaded with individual A*02:01-restricted HRV peptides at 640 molar excess in PBS pH 7.2 at 37°C overnight. Peptide-expanded PBMCs were incubated with DimerX for 24 hours at 37°C and 5% CO2. Stained cells were detected by flow cytometry (FACScalibur, BD Biosciences).
from A*02:01-donors were then stained with peptide-loaded DimerX for 1 hour at room temperature (RT) and cognate CD8 T cells were detected by flow cytometry (FACScalibur, BD Biosciences) using PE-conjugated anti-mouse IgG1 A85-1 mAb (BD biosciences) and APC-conjugated anti-CD8 REA734 mAb (Miltenyi Biotec). The same PBMCs stained with unloaded DimerX or with DimerX loaded with the A*01:01-restricted peptide HRVA2029-2037 (YGDDVIFSY) were used as negative controls.

2.11 Cytotoxicity assays

Cytotoxic activity was determined by quantification of lactate dehydrogenase (LDH) release using the Pierce LDH non-radioactive cytotoxicity assay kit (Thermo Fisher Scientific) following manufacturer’s instructions. Target (T) cells consisted of T2 cells pulsed overnight at 37°C and 5% CO2 with 10 µM of test peptides and 5 µg/mL β2 microglobulin in AIM-V-free serum medium (Gibco). T2 cells pulsed with the A*01:01-restricted peptide HRVA2029-2037 (YGDDVIFSY) were used as a negative control. Peptide-expanded PBMCs from A*02:01 donors were used as effector cells (E). Target cells (5 × 10^5) were plated in 96-well plates and co-cultured with effectors cells at E:T ratios 1:1, 5:1, 10:1, and 30:1 at 37°C and 5% CO2 for 8 hours. The percentage of specific lysis of the target cells was determined with the formula: 100%([experimental release − effector spontaneous release − target spontaneous release]/[target maximum release − target spontaneous release]), where target maximum release was determined by adding 10X Lysis Buffer (provided by the manufacturer) to target cells 45 minutes prior to harvest the supernatants for LDH quantification.

2.12 Other procedures

The tertiary structure of A*02:01 complex with a 16-mer A*02:01-restricted HRV peptide identified in this work was generated by homology modeling after the known tertiary structures of three A*02:01-peptide complexes (PDB IDs: 1I1F, 2V2X, 4U6Y), using a standalone version of MODELLER. Tertiary structure models were subjected to MODELLER energy optimization methods the best model was chosen based on the discrete optimized potential energy (DOPE) scores. Superimposition of tertiary structures and molecular graphic representations were obtained using PyMol Molecular Graphics System, Version 2.0 Schrödinger, LLC. Population protection coverage (PPC) and identification of optimal epitope combination with the largest PPC were obtained using the method by Molero et al implemented in EPISOPT.

3 RESULTS

3.1 Computational selection of conserved HRV peptides with potential CD8 T cell epitopes

We sought to select HRV A and C conserved peptides that were predicted to bind to different HLA I molecules. To that end, we first carried out sequence variability analysis of HRV A and C proteomes generating reference consensus proteomes with variable sites (H ≥ 1) masked (details in Materials and Methods). Our variability analysis revealed that HRV C contains 34.1% of variable residues, whereas these residues represent 27.9% in HRV A. We next targeted HRV A and C consensus proteomes for peptide-HLA I binding predictions (as described in the Materials and Methods) to identify potential CD8 T cell epitopes. For HRV A, we selected 22 conserved peptides with the optimal size (9 residues) for HLA I binding. However, for HRV C we just sought for conserved peptides, selecting 9 conserved peptides with a length ranging from 11 to 17 residues. These peptides encompass various 9-mer peptides predicted to bind to different HLA I molecules and can be tested on a higher number of HLA I typed donors (Table 1). All these peptides were synthesized and subjected to functional assays.

3.2 Identification of conserved HRV-specific CD8 T cell epitopes

We tested the immunogenicity of HRV peptides by IFNγ-ELISPsOT assays using PBMCs from 14 HLA-I typed subjects. We tested each peptide in HLA-I matched subjects; therefore, peptides were only tested on subjects expressing at least one HLA I molecule included on the predicted peptide-HLA I binding profiles. Subject-specific responses for individual peptides are summarized in Figure 1 and additional information regarding HLA I typing and peptide-specific responses of the donors is provided in Table S1. We found that 23 out of the 31 conserved peptides were able to elicit recall T cell responses in at least one of the subjects that were tested. The peptides with the broader responses were HRVA60-80 (VLEKGIPTL) and HRVC24-36 (VVKYFNINYYKDA), four out of six and six out of eight donors, respectively, responded.

Of the 22 HRV A peptides, 16 gave a positive response in one or more subjects with HLA I typing matching at least one of the HLA I molecules included in the predicted peptide-HLA I binding profile. To identify the most likely HLA I restriction element of the immunogenic HRV A peptides, we compared the number of subjects with matching HLA I
| Virus | Peptide | Sequence | Protein<sup>a</sup> | Position<sup>b</sup> | Predicted HLA I binding<sup>c</sup> |
|-------|---------|----------|--------------------|-----------------|----------------------------------|
| HRV A | HRVA<sub>19-27</sub> | VSNGLNLNY | VP4 | 19-27 | A*11:01, B*15:08, B*15:16, B*57:02 |
| HRV A | HRVA<sub>24-32</sub> | SLNYFNINYY | VP4 | 24-32 | A*02:01, A*03:01, A*11:01, B*15:08 |
| HRV A | HRVA<sub>26-34</sub> | NYFNINYYK | VP4 | 26-34 | A*03:01, A*31:01, A*33:01, A*68:01 |
| HRV A | HRVA<sub>49-57</sub> | DPSKFTDPV | VP4 | 49-57 | B*07:02, B*35:01, B*51:01, B*51:02, B*51:03, B*54:01 |
| HRV A | HRVA<sub>60-68</sub> | VLEKGIPTL | VP4 | 60-68 | A*02:01, A*02:02, A*02:03, A*02:04, A*02:05, A*02:06, A*02:09, A*02:14, B*15:10 |
| HRV A | HRVA<sub>70-78</sub> | SPTVEACGY | VP4 | 70-78 | B*15:08, B*35:01, B*44:02, B*53:01 |
| HRV A | HRVA<sub>96-104</sub> | DVANAVGNY | VP4 | 96-104 | A*11:01, B*15:08 |
| HRV A | HRVA<sub>286-294</sub> | RHNNWLSLVI | VP2 | 286-294 | B*38:01, B*48:01 |
| HRV A | HRVA<sub>503-511</sub> | VPWVSASHF | VP3 | 503-511 | B*07:02 |
| HRV A | HRVA<sub>588-594</sub> | NEVLVVPNI | VP1 | 586-594 | B*44:02 |
| HRV A | HRVA<sub>764-772</sub> | SIASAYYM | VP1 | 764-772 | A*24:02 |
| HRV A | HRVA<sub>877-885</sub> | NLIYRNHLI | 2A | 877-885 | A*02:01, B*08:01 |
| HRV A | HRVA<sub>909-916</sub> | YSSDLVIYR | 2A | 898-906 | A*31:01, A*68:01, A*11:01 |
| HRV A | HRVA<sub>954-962</sub> | KHIQYNLLI | 2A | 954-962 | B*38:01 |
| HRV A | HRVA<sub>1080-1088</sub> | SGSPWRLFK | 2B | 1080-1088 | A*11:01 |
| HRV A | HRVA<sub>1288-1296</sub> | QMVSSVTFLI | 3D | 1288-1296 | A*02:01 |
| HRV A | HRVA<sub>1377-1385</sub> | CPFICGKAV | 3D | 1377-1385 | B*07:02 |
| HRV A | HRVA<sub>1665-1663</sub> | RMLKYNPTY | 2C | 1655-1663 | A*02:01, B*48:01 |
| HRV A | HRVA<sub>1859-1867</sub> | VTLKDEL | 3D | 1859-1867 | A*31:01, A*33:01, A*68:01 |
| HRV A | HRVA<sub>2009-2017</sub> | RTLVLDAYK | 3D | 2009-2017 | A*30:01, A*31:01, A*11:01 |
| HRV A | HRVA<sub>2029-2037</sub> | YGDDVIFSY | 3D | 2029-2037 | A*01:01, A*66:01, B*35:01 |
| HRV A | HRVA<sub>2147-2155</sub> | ALYPPYEL | 3D | 2147-2155 | A*02:01, A*02:02, A*02:03, A*02:04, A*02:05, A*02:06, A*02:09 |

| HRV C | HRVC<sub>24-36</sub> | VVKYFNNYKDA | VP4 | 24-36 | A*03:01, A*11:01, A*30:01, A*31:01, A*33:01, A*68:01, B*15:01 |
|-------|---------|----------|--------------------|-----------------|----------------------------------|
| HRV C | HRVC<sub>61-75</sub> | LTPALMSPSVEACG | VP4 | 61-75 | A*02:01, B*07:02, B*35:01, B*51:01, |
| HRV C | HRVC<sub>256-274</sub> | INLRTNSSSTIVPYIN | VP2 | 258-274 | A*01:01, A*30:01, A*68:01, B*15:01, B*35:01 |
| HRV C | HRVC<sub>630-640</sub> | IENFLGSRALW | VP1 | 630-640 | A*24:02, B*14:02, B*40:02 |
| HRV C | HRVC<sub>666-696</sub> | GLMQMYVPAG | VP1 | 686-696 | A*30:01, B*15:01 |
| HRV C | HRVC<sub>1582-1592</sub> | KEKFRDIFRIFP | 3D | 1582-1592 | A*30:01, A*31:01 |
| HRV C | HRVC<sub>1791-1806</sub> | GLEPLDLNTSAGFYY | 3D | 1791-1806 | A*01:01, A*02:01, A*11:01, A*30:01, A*66:01, A*68:01, B*15:01, B*35:01 |
| HRV C | HRVC<sub>1835-1847</sub> | DLPYVYTLKDEL | 3D | 1835-1847 | A*31:01, A*33:01, A*68:01 |
| HRV C | HRVC<sub>1974-1990</sub> | GTSVFNTMINNII | 3D | 1974-1990 | A*11:01, A*24:02, A*31:01, A*33:01 |

<sup>a</sup>Protein of HRV that contain the peptide sequence.  
<sup>b</sup>Position of the peptide in the selected reference HRV polyproteins  
<sup>c</sup>HLA I molecules predicted to bind the corresponding peptides or nested 9-mer peptides.
alleles that responded vs those that did not respond (Table S2). As a result, we selected for further evaluation six HRV A peptides; four potentially restricted by A*02:01 (HRVA60-68, HRVA877-885, HRVA1288-1296, and HRVA2147-2155), one by A*01:01 (HRVA 2029-2037) and one by A*30:01 (HRVA2009-2017). All these peptides were found to be immunogenic in at least three subjects and non-immunogenic in zero or only one subject matching the corresponding HLA I allele (Table S2).

All nine HRV C peptides but one elicited recall T cell responses in at least one donor. These peptides have a length (> 11 residues) that likely precludes direct binding to HLA I molecules, without some processing in the culture. Thereby, we predicted which 9-mer nested peptides could potentially be presented by HLA I molecules expressed by the responding donors. Following this approach, we anticipated potential HRV C CD8 T cell epitopes that are likely responsible for the observed IFNγ-responses along with their HLA I restriction element (Table S3). After this analysis, we synthesized the peptide KYFNINYYK (HRVC26-34), which is a potential A*11:01-restricted CD8 T cell epitope included in peptide HRVC24-36, and confirmed strong positive responses by IFNγ-ELISPOT in A*11:01 donors (data not shown). This peptide was also selected for further evaluation.

Overall, the combination of computational and IFNγ-ELISPOT assays allowed us to identify six immunogenic peptides from HRV A virus that are potentially restricted by A*02:01 (HRVA60-68, HRVA877-885, HRVA1288-1296, and HRVA2147-2155), A*01:01 (HRVA2029-2037) and A*30:01 (HRVA2009-2017). Additionally, we identified an immunogenic peptide from HRV C virus, HRVC26-34, which is potentially restricted by A*11:01. All these peptides have nine residues, the optimal for binding and presentation by HLA I molecules, and likely correspond to bona fide CD8 T cell epitopes. In fact, we detected by intracellular cytokine staining peptide-specific production of IFNγ by CD8 T cells in PBMCs from matching donors (Figure 2). We found out that the percentage of peptide-specific IFNγ-producing CD8 T cells when stimulated with the HRV peptides were: 1.27% for the A*11:01-peptide, 1.12% for the A*01:01-peptide, 1.16% for the A*30:01-peptide, and ranged between 0.37% and 1.25% for the A*02:01-peptides.

3.3 Validation of selected CD8 T cell epitopes

We carried out further experiments to validate the seven HRV-specific CD8 T cell epitopes identified in the previous section by intracellular staining assays. We carried out quantitative competitive inhibition assays to confirm binding of these CD8 T cell epitopes to the relevant purified HLA I molecules (details in Materials and Methods). As shown in Figure 3A, HRVA60-68 (VLEKGIPTL), HRVA877-885 (NLIYRNLHL), and HRVA1288-1296 (QMVSSVTFI) bound with high affinity (IC\textsubscript{50} < 200 nM) to A*02:01, while...
HRVA_{2147-2155} (ALYIPYPYEL) bound with intermediate affinity (586 nM) to this same HLA I molecule. In these experiments, we also assayed the binding of HRVC_{1791-1806} (GLEPLDLNTSAGFPYV) to A*02:01. This peptide was included in the binding experiments as a negative control since its large length should prevent binding to HLA I molecules. Interestingly, HRVC_{1791-1806} bound to A*02:01 with high affinity (91 nM). Therefore, we selected this peptide for additional analysis (see below). Moreover, quantitative competitive inhibition assays allowed us to confirm that peptides HRVC_{26-34} (KYFNINYK), HRVA_{2029-2037} (YGDDVIFSY), and HRVA_{2009-2017} (RTLVDAYK) bound to A*11:01, A*01:01, and A*30:01, respectively, with high affinity (IC_{50} < 200 nM).

We selected all four A*02:01-restricted CD8-T cell epitopes (HRVA_{60-68}, HRVA_{877-885}, HRVA_{1288-1296}, and HRVA_{2147-2155}) for additional validation and carried out DimerX staining and killing assays (details in Materials and Methods). In these experiments, we used PBMCs from A*02:01 subjects that were previously stimulated with individual A*02:01-peptides. DimerX staining experiments, shown in Figure 3B, revealed the existence of CD8 T cells that were specific for each of the A*02:01-peptides. The percentage of CD8 T cells that recognized the A*02:01-peptides in the representative subject shown in Figure 3, varied from 0.91% for HRVA_{877-885} to 3.08% for HRVA_{1288-1296}. Moreover, killing assays (Figure 3C), showed that all A*02:01-peptides mediated cytototoxic activity. In the representative assay shown in Figure 3C, up to 20% to 40% of T2 cells loaded with HRV A*02:01-peptides underwent peptide-specific lysis when incubated with effector cells consisting of PBMCs from a responding A*02:01 subject. Note that T2 cells were not lysed when pulsed with the A*01:01-peptide HRVA_{2029-2037} (YGDDVIFSY).
3.4 Validation of an HRV C-specific A*02:01-restricted 16-mer CD8 T cell epitope

As noted earlier, we detected strong binding of the peptide HRVC1791-1806 (GLEEPDLNTSAGFPYV) to A*02:01. Since most A*02:01 subjects responded to HRVC1791-1806 (Figure 1 and Table S1), we aimed to determine if the responses were directed against this peptide or if smaller nested peptides were responsible for the observed responses in A*02:01 subjects. We actually found 4 peptides within HRVC1791-1806 with a length between 9 and 11 residues that were predicted to bind to A*02:01 (NTSAGFPYV, LNTSAGFPYV, DLNTSAGFPYV, and GLEEPDLNT). We synthesized these peptides and carried out further analyses.

We first determined the binding capacity of the nested peptides to A*02:01, and compared it with the binding of the 16-mer peptide HRVC1791-1806. Quantitative competitive inhibition assays (Figure 4A) demonstrated that the A*02:01 peptides nested in HRVC1791-1806 could bind to A*02:01 (IC50 range 100-500 nM), but with lower affinity than HRVC1791-1806 (IC50 < 100 nM). We also tested binding and presentation by A*02:01 of HRVC1791-1806 and the nested 9-mer peptide that bound to A*02:01 with the highest affinity (HRVC1798-201) using T2 binding assays (Figure 4B). T2 cells are A*02:01 + TAP-deficient cells with no or little detectable A*02:01 in the cell surface unless exogenous peptides capable of binding to A*02:01 are provided. As positive control we used T2 cells loaded with the A*01:01 peptide HRVA2029-2037 (YGDDVIFSY). Open squares represent T2 spontaneous lysis during the 8 hours of the assay. Results depicted in panels B and C correspond to a representative experiment obtained using PBMCs from A*02:01-subject #14.
We next studied the production of IFNγ by CD8 T cells induced by GLEPLDLNTSAGFPYV and NTSAGFPYV using PBMCs from responding A*02:01 subjects. As shown in Figure 4C, the percentage of peptide-specific IFNγ-producing CD8 T cells was higher when PBMCs were stimulated with the 16-mer peptide than with the 9-mer nested peptide. Thus, IFNγ-producing CD8 T cells induced by GLEPLDLNTSAGFPYV represented a 2.03%, while those induced by NTSAGFPYV represented a 0.43%.

Finally, to complete the validation of the 16-mer peptide as a bona fide A*02:01-restricted CD8 T cell epitope, we carried out DimerX staining and cytotoxicity assays with PBMCs from responding A*02:01 subjects previously stimulated with the peptides. DimerX staining results (Figure 5A) showed that there is a considerable population of CD8 T cells recognizing the 16-mer peptide (2.38% of CD8 T cells) that doubles that of the 9-mer peptide (1.12% of CD8 T cells). In addition, cytotoxicity assays (Figure 5B) demonstrated that GLEPLDLNTSAGFPYV mediated cytotoxic activity; over 30% of T2 cells pulsed with this peptide were lysed by PBMCs from A*02:01 subjects. The cytotoxicity mediated by GLEPLDLNTSAGFPYV was comparable to that of HRVA1288-1296, an A*02:01-restricted CD8 T cell epitope described previously (Figure 3C). Likewise, the peptide NTSAGFPYV also mediated cytotoxic activity, less than HRVC1791-1806, but yet in range with other A*02:01-restricted CD8 T cell epitopes identified in this study. Altogether, our data reveals a dominant CD8 T cell response toward HRVC1791-1806 peptide, shadowing that to other canonical A*02:01-restricted epitopes nested within.

**DISCUSSION**

HRV causes respiratory tract infections that are associated with acute exacerbations of chronic pulmonary diseases such as asthma.1,2 A major characteristic of HRV is the high number of antigenically distinct serotypes (more than 150 serotypes have been described) which have been classified in HRV A, B, and C species.5 HRV A and HRV C are the most infective species and HRV C is the most pathogenic, being a major cause of asthma exacerbations.2,7 In addition to a direct effect on respiratory epithelial cells, the innate and adaptive host responses also have a role in the pathogenesis of HRV infection by promoting inflammatory mediators.12 It has been shown that HRV induce potent humoral and T cell responses. The humoral response includes neutralizing antibodies but they are serotype-specific, exhibiting little
cross-reactivity. CD4 T cells contribute to antiviral immunity through the recognition of viral antigens, triggering both cellular and antibody-mediated immune responses. Unlike HRV-specific antibodies, HRV-specific CD4 T cells can be cross-reactive and recognize shared epitopes between serotypes. Interestingly, in healthy individuals, HRV-specific CD4 T cells polarize to Th1 facilitating HRV clearance, while in asthmatic individuals polarize to Th2, fueling asthma exacerbations. CD8 T cells are also expected to play a key role facilitating viral clearance by killing infected cells. However, CD8 T cell responses against HRV are still poorly studied and, in fact, to our knowledge HRV-specific CD8 T cell epitopes remain to be identified. In the present study, we targeted HRV A and C species, which together are responsible for almost 90% of HRV infections, for CD8 T cell epitope identification. To that end, we followed a computer-aided approach summarized in Figure 6. Briefly, for HRV A, we selected for experimental validation potential 9-mer CD8 T cell epitopes predicted to bind to common HLA I molecules. However, since HRV C is much more variable than HRV A, we first selected conserved peptides (11-17-mers) predicted to encompass various 9-mer CD8 T cell epitopes, screened them for immunogenicity using IFNγ-ELISPOT assays and subsequently proceed to validate the CD8 T cell epitopes nested within the immunogenic peptides.

We selected 31 conserved HRV peptides (22 for HRV A and 9 for HRV C) encompassing potential CD8 T cell epitopes upon sequence variability analysis and HLA I binding predictions. In IFNγ-ELISPOT assays, 23 out of 31 peptides (74%) were able to elicit a detectable T cell recall responses (Figure 1) in HLA I matched donors. IFNγ responses induced by peptides of both HRV A and C were of similar magnitude to those found in similar assays of PBMCs to papillomavirus, norovirus, and coronavirus. Since the immunogenicity of the peptides was tested on a small cohort including 14 HLA I typed subjects, we cannot discard that other peptides might also be immunogenic in other backgrounds and/ or individuals. Intracellular cytokine staining allowed us to confirm that the observed responses were owed to CD8 T cells (Figure 2) and we verified HLA I-restriction elements for 7 of the 23 immunogenic peptides: HRVA26-34 (A*11:01), HRVA2029-2037 (A*01:01), HRVA209-2037 (A*30:01), and
As shown in Figure 7, immunogenic peptides are distributed uniformly in HRV proteome but the density is greater in VP4 protein, which is highly conserved. VP4 is located on the amino terminal extreme of the polyprotein and a previous study revealed that this location favors presentation by HLA I molecules and consequent recognition by CD8 T cells.

In contrast to HRV-specific CD4 T cell epitope identification, which have been mainly focused on capsid proteins, here we report CD8 T cell epitopes that are distributed throughout the whole HRV proteome.

The responses to A*02:01-restricted CD8 T cell epitopes were further characterized using DimerX staining and cytotoxicity assays. As noted earlier and depicted in Figure 2 all A*02:01-restricted CD8 T cell epitopes were able to induce significant populations of IFNγ-producing CD8 T cells that were similar to those of peptide-specific CD8 T cells with a TCR capable of recognizing the epitopes (Figure 3B). An exception was HRVA1288-1296, which was recognized by a 3.08% of CD8 T cells, but only a 0.37% of the CD8 T cells produced IFNγ after peptide-stimulation (Figures 2 and 3B). It could be that some of the CD8 T cells recognizing HRVA1288-1296 in this representative subject are non-functional or exhausted, but we found similar responses to this peptide in other A*02:01 subjects (data not shown). In any case, poor T cell reactivity to HRVA1288-1296 does not appear to be related with weak binding to A*02:01, as the IC₅₀ is similar to that of other A*02:01
epitopes (Figure 3A). The reactivity of CD8 T cell epitopes depends on several factors,\textsuperscript{19} and perhaps most of the CD8 T cells recognizing HRVA\textsubscript{1288-1296} do not have enough affinity for A*02:01 to trigger IFNγ responses.

All the CD8 T cell epitopes with verified HLA I restriction described earlier were 9-mers, however, we also identified a 16-mer epitope restricted by A*02:01 (HRVC\textsubscript{1791-1806}, GLEPLDLNTSAGFPYV). This 16-mer epitope contained a 9-mer epitope nested in its sequence (HRVC\textsubscript{1798-1806}, NTSAGFPYV) that could also bind to A*02:01 and induced CD8 T cell recall responses (Figure 4). However, the 16-mer epitope bound with greater affinity to A*02:01 and exhibited a dominant response. Thus, we found larger numbers of peptide-specific (Figure 5A) and IFNγ-producing (Figure 4C) CD8 T cells for GLEPLDLNTSAGFPYV than for NTSAGFPYV. Likewise, GLEPLDLNTSAGFPYV mediated a greater cytotoxic activity than NTSAGFPYV (Figure 5B). In sum, there is no doubt that HRVC\textsubscript{1791-1806} is a \textit{bona fide} HRV C-specific CD8 T cell epitope targeted during HRV C infection. To the best of our knowledge GLEPLDLNTSAGFPYV is the longest A*02:01-restricted epitope reported so far.
Typically HLA I molecules bind and present 8-11-mer peptides but there are reports of longer peptides that can be presented by HLA I molecules and readily recognized by T cells. Nonetheless, it may seem difficult that a peptide like GLEPLDLNTSAGFPYV can fit into A*02:01 and be recognized by a TCR. To visualize how that could happen, we modeled the tertiary structure of HRVC1791-1806 in complex with A*02:01 and superimposed it with the solved 3D-structure of an A*02:01-peptide-TCR complex (Figure 8). We found that the central residues of GLEPLDLNTSAGFPYV form a super-bulged structure which protrudes from A*02:01 and fits within the Vα and Vβ of the TCR without interfering with the recognition.

The development of broadly protective vaccines against HRV represents a significant clinical challenge since there are more than 150 HRV serotypes currently described. The CD8 T cell epitopes identified here are conserved—they were selected after sequence variability analysis—and they are likely subjected to structural and functional restraints that limit their variation. In fact, we noticed that HRVA877-885 bear residue 884, which is dispensable for the correct activity of protease 2A, and HRVA2009-2017 epitope contains residue 2013, essential for RNA-dependent RNA polymerase. In addition, the HRVC26-34 (KYFNINYYK) epitope is conserved not only in HRV C species, but also in many HRV A strains, and could be a source of cross-reactive immunity between HRV A and HRV C. Thus, the elicitation of CD8 T cell responses against these conserved epitopes could serve as a basis for developing a cross-serotype vaccine against HRV. Unfortunately, the population protection coverage (PPC) of this type of vaccine would be limited to those individuals expressing any of the HLA I molecules restricting the T cell responses, which we computed to be about 58% of the population. However, we predicted that these epitopes can also be presented by other HLA I molecules and could elicit CD8 T cell responses in up to 87% of the population (Table 2). Moreover, we found that as few as 3 epitopes (VLEKGIPTL, NLIYRNLHL, and GLEPLDLNTSAGFPYV) provide a PPC ≥ 84%, regardless of the ethnicity of the population, reaching a PPC of 95% and 91% in Caucasians and Asians, respectively.

To conclude, it should be noted that HRV-specific CD8 T cell epitopes here identified might not be the dominant epitopes; those focusing the immune response. However, dominant epitopes are not necessarily protective, and virus can escape immune response to them through exhaustion or variation. Therefore, sub-dominant but yet conserved epitopes are of particular interest for epitope-vaccine design, regardless of their immunodominance.

5 | CONCLUSIONS AND LIMITATIONS

We identified and validated six HRV A-specific and three HRV C-specific CD8 T cell epitopes, respectively, including a 16-mer A*02:01-restricted epitope from HRV C. These epitopes are conserved in the relevant HRV species and are expected to elicit CD8 T cell responses in up to 87% of the population. A much-needed HRV vaccine is

| Peptide | Sequence | Protein | Validated HLA I | Extended HLA I binding profilea | PPC (%)b |
|---------|----------|---------|----------------|---------------------------------|----------|
| HRVA60-68 | VLEKGIPTL | VP4     | A*02:01        | A*02:01, A*02:02, A*02:03, A*02:04, A*02:05, A*02:06, A*02:09, A*02:14, B*15:10 | 43.82    |
| HRVA877-885 | NLIYRNLHL | 2A      | A*02:01        | A*02:01, B*08:01 | 40.06    |
| HRVA1288-1296 | QMVSSVTI | 3D      | A*02:01        | A*02:01 | 33.46    |
| HRVA2009-2017 | RTLVLDAYK | 3D      | A*30:01        | A*11:01, A*30:01, A*31:01 | 26.62    |
| HRVA2029-2037 | YGDDVIFSY | 3D      | A*01:01        | A*01:01, A*66:01, B*35:01 | 26.88    |
| HRVA2147-2155 | ALYIPPYLE | 3D      | A*02:01        | A*02:01, A*02:02, A*02:03, A*02:04, A*02:05, A*02:06, A*02:09 | 43.24    |
| HRVC26-34 | KYFNINYYK | VP4     | A*11:01        | A*11:01, A*31:01 | 25.75    |
| HRVC1791-1806 | GLEPLDLNTSAGFPYV | 3D | A*02:01 | A*01:01, A*20:01, A*11:01, A*30:01, A*66:01, A*68:01, B*15:01, B*35:01 | 75.09    |
| HRVC1798-1806 | NTSAGFPYV | 3D      | A*02:01        | A*02:01 | 33.46    |

aHLA I molecules predicted to bind the corresponding peptides.
bPopulation protection coverage (PPC), meaning the percentage of the population that exhibits at least one of the HLA I alleles in which the epitope could elicit an immune response. PPC was computed independently for five ethnic groups and here we report the average PPC.
not available due to the variability of the virus and these epitopes represent excellent candidates to develop an effective HRV vaccine. However, this vaccine will need additional components to engage CD4 T cells and B cells. Interestingly, the 16-mer peptide is predicted to bind to various different HLA II molecules and we actually detected intracellular IFNγ production by CD4 T cells after HRVC1791-1806 stimulation in different donors (not shown). Stimulation of HRV-specific CD8 T cell responses alone may also have therapeutic utility, helping infected individuals to clear the infection by increasing local cytokine production and killing of infected cells.

ACKNOWLEDGMENTS
This work was supported by the Spanish Department of Science at MINECO through grant BIO2014:54164-R to PAR. MGP and JLS were supported by Complutense University of Madrid through Grant CT17/17 - CT18/17. We wish to thank the Complutense University of Madrid for special grant action B AE21/20-23164 to PAR. MGP and JLS were supported by Complutene Science at MINECO through grant BIO2014:54164-R.

CONFLICT OF INTEREST
All the authors declare that they have no competing interest.

AUTHOR CONTRIBUTIONS
P.A. Reche, conceptualization; M. Gomez-Perosanz and J.L. Sanchez-Trincado, methodology; J. Sidney and A. Sette, HLA I typing: MFA, quantitative competitive inhibition assays; M. Gomez-Perosanz and P.A. Reche, writing-original draft; M. Gomez-Perosanz, P.A. Reche, and E.M. Lafuente, final writing and editing. All authors have read and approved the final manuscript.

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