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Abbreviations: DCs, dendritic cells; HCV, hepatitis C virus; ICS, immunogenic consensus sequences; NS, nonstructural; PBMC, peripheral blood mononuclear cell; SVR, sustained virologic response

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Abstract

Hepatitis C virus (HCV) is a major cause of liver disease. Spontaneous resolution of infection is associated with broad, MHC class I- ($CD8^+$) and class II-restricted ($CD4^+$) T cell responses to multiple viral epitopes. Only 20% of patients clear infection spontaneously, however, most develop chronic disease. The response to chemotherapy varies; therapeutic vaccination offers an additional treatment strategy. To date, therapeutic vaccines have demonstrated only limited success in clinical trials. Vector-mediated vaccination with multi-epitope-expressing DNA constructs provides an improved approach. Highly-conserved, HLA-A2-restricted HCV epitopes and HLA-DRB1-restricted immunogenic consensus sequences (ICS, each composed of multiple overlapping and highly conserved epitopes) were predicted using bioinformatics tools and synthesized as peptides. HLA binding activity was determined in competitive binding assays. Immunogenicity and the ability of each peptide to stimulate naïve human T cell recognition and IFN-γ production were assessed in cultures of total PBMCs and in co-cultures composed of peptide-pulsed dendritic cells (DCs) and purified T lymphocytes, cell populations derived from normal blood donors. Essentially all predicted HLA-A2-restricted epitopes and HLA-DRB1-restricted ICS exhibited HLA binding activity and the ability to elicit immune recognition and IFN-γ production by naïve human T cells. The ability of DCs pulsed with these highly-conserved HLA-A2- and –DRB1-restricted peptides to induce naïve human T cell reactivity and IFN-γ production *ex vivo* demonstrates the potential efficacy of a multi-epitope-based HCV vaccine targeted to dendritic cells.

Keywords. dendritic cell; EpiMatrix; epitope; HCV; vaccine; T cell

Introduction

Hepatitis C virus (HCV), a small single-stranded RNA virus, constitutes a major cause of liver disease [1]. The positive-sense genome encodes an ~3,000 amino acid poly-protein precursor, which is cleaved by cellular and viral proteases to yield three structural [core, envelope 1 (E1) and E2], and seven nonstructural (p7, NS2, NS3, NS4a, NS4b, NS5a and NS5b) proteins [2]. Spontaneous resolution of HCV infections is associated with broad, MHC class I- ($CD8^+$) and class II-restricted ($CD4^+$) T cell responses to multiple viral epitopes derived from these proteins [3,4]. Unfortunately, only 20% of patients clear infection spontaneously, most develop chronic disease [5]. Seventy to eighty percent of patients infected with HCV genotype 1 (the principal causative agent of hepatitis C in the U.S.) experience a sustained virologic response (SVR) following treatment that includes protease inhibitors, i.e., telaprevir or boceprevir, administered in conjunction with PEGylated interferon and ribavirin. A significant number of those treated remains infected, however, the cost of treatment is high, and the risk and severity of side effects are considerable [6,7]. New approaches to treating chronic HCV infections are urgently needed.

Therapeutic vaccination concurrent with or without drug therapy offers an additional approach to treating chronic hepatitis C. Indeed, the capacity of a significant percentage of patients to resolve acute infections spontaneously suggests that an effective therapeutic vaccine is a realistic goal. A safe and effective vaccine must elicit broad, vigorous $CD4^+$ and $CD8^+$ T cells responses to conserved viral epitopes, which culminate in the elimination of HCV without causing liver pathology. Development of such a vaccine has proven problematic, however, due primarily to: infidelity of the viral RNA polymerase (NS5b), genetic diversity and the rapid emergence of viral variants [8]. To date, a number of vaccine strategies have demonstrated negligible or only limited success in clinical trials [9,10].

Vaccination with HCV epitope expressing dendritic cells (DCs) offers a vector-mediated approach to treating chronic, HCV infected patients. DCs play a central role in $CD4^+$ and $CD8^+$ T cell

activation and the induction of immunity [11]. The potential effectiveness of DC-based vaccines in treating chronic hepatitis C has been demonstrated in animal models [12-14]. Moreover, in a recent Phase I clinical trial, chronically-infected patients vaccinated with monocyte-derived DCs pulsed with 6 HCV-specific, HLA-class I-restricted peptides exhibited peptide-specific CD8 T cell responses [15]. These responses were not sustained, however, and there was no effect on viral load suggesting that HCV clearance might require vaccination with DCs that expressed a broader range of viral epitopes. Toward this end, immunoinformatics tools were used to predict 21 HLA-A*0201-restricted epitopes and 19 HLA-DRB1-restricted immunogenic consensus sequences (ICS, each composed of multiple epitopes), which were highly-conserved and encoded by HCV genotype 1. These predicted epitopes/ICS were synthesized as peptides and their capacities to bind HLA molecules were determined. Subsequently, their immunogenicity and ability to elicit the peptide-specific responses of naïve human T cells were validated in an *in vitro* peripheral blood mononuclear cell (PBMC) immunogenicity assay. Similarly, monocyte-derived DCs pulsed with these same peptides induced the epitope-specific responses of naïve $CD4^+$ and $CD8^+$ T cells in culture demonstrating the potential efficacy of a multi-epitope-based HCV vaccine that targets dendritic cells.

Materials and Methods

Genome Collection

Hepatitis C viral sequences marked complete, representing a total of 871 genotype 1a and genotype 1b polyproteins, were acquired from the Los Alamos sequence and immunology database [16,17].

Conserved 9-mer search

Nine-mer amino acid sequences, constituting the length of the peptide chain that fits into the binding groove of the HLA class I and class II molecules, were parsed out of the polyproteins and compared for identical parsed 9-mers in matching open reading fames of other genotype 1a or 1b isolates using the Conservatrix algorithm [18]. The potential immunogenicity of these identical 9-mer sequences was predicted using the computational method described below.

Epitope mapping

Approximately 50% of the population residing in the U.S. expresses cell-surface HLA-A2; essentially the entire population expresses one or more HLA-DRB1 alleles [19,20]. Consequently, each 9-mer was scored for its predicted potential to bind a panel of eight HLA-DRB1 alleles (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301 and DRB1*1501) using EpiMatrix, a matrix-based algorithm for mapping T cell epitopes [21,22]. Additionally, all parsed 9-mers were scored for the potential to bind HLA-A2. Putative HLA-A2 epitopes were selected based on conservation in genotype 1a and b, EpiMatrix HLA A2-matrix predicted binding score, and reports of the *ex vivo* response of PBMCs obtained from HCV-infected patients.

Immunogenic consensus sequences

HLA-DRB1-restricted ICS were constructed using EpiAssembler, an algorithm that maximizes epitope density by assembling potentially immunogenic 9-mers (identified using EpiMatrix) into 18-25 amino acid stretches [23]. To avoid potential cross-reactivity with the human proteome, any peptide that shared more than 7 identities per 9-mer frame was eliminated from further consideration

[24]. The final HLA-A2- and –DRB1-restricted peptide sequences were synthesized using 9-fluoronylmethoxycarbonyl chemistry and purified >85% by 21st Century Biochemicals (Marlboro, MA).

HLA Binding Assay

The capacity of predicted epitopes (peptides) to bind multiple HLA-DRB1 alleles was assessed using a competitive, HLA class II binding assay as we described previously [24,25], using HLA molecules obtained from Bill Kwok, Benaroya Research Institute, Seattle, WA). Assays were performed for HLA-DRB1*0101, -DRB1*0301, -DRB1*0401, -DRB1*0701, -DRB1*1101 and -DRB1*1501, alleles that provide broad representation of HLA class II that are prevalent in human populations [20]. IC_{50} values were estimated and the predicted peptides were classified as exhibiting very high (< 1 μM), high (1 μM - 10 μM), moderate (10 μM - 100 μM) or low (>100 μM) affinity. Peptides that exhibited very high, high or moderate affinity were considered binders (a more detailed classification is provided in the results section).

The ability of predicted epitopes to bind HLA-A*0201 was assessed using a fluorescence polarization-based competitive peptide-binding assay according to published methods [26]. The concentration of experimental peptide that inhibited 50% binding of the FITC-labeled reference peptide (IC_{50}) was determined. Experimental peptides were considered: high (IC_{50}<5 μM), moderate (5 μM<IC_{50}<50 μM) and low (IC_{50} = 50 - 100 μM) affinity binders. Peptides that failed to demonstrate dose-dependent inhibition or exhibited an IC_{50}>100 μM were considered non-binders.

Human subjects

Whole-blood leukocyte reduction filters (blood filters; Sepacell RZ-2000, Baxter Healthcare Corporation, Irvine CA) were obtained from the Rhode Island Blood Center (Providence, RI). These used, de-identified filters contain white cells derived from blood donated with informed consent by

healthy volunteers. The Lifespan Institutional Review Board (Rhode Island Hospital) approved this study.

Peripheral blood mononuclear cell (PBMC) recovery and purification

PBMCs were recovered from blood filters according to the methods of Meyer *et al.* [27]. Filters obtained within a 4-hour period following the leukocyte depletion step were back-flushed at room temperature with Ca- and Mg-free Hank's basic salt solution containing sodium-EDTA and sucrose. The recovered leucocytes were purified by centrifugation on Ficoll-Paque Plus (1.077; Pharmacia, Uppsala, Sweden) gradient. All donors expressed HLA-A*0201 and HLA-DRB1.

Naïve PBMC cultures

The peptide-specific responses of naïve human T cells were induced by culturing purified PBMCs under conditions described by others and modified in our laboratory [28]. PBMCs, 2.5 x 10^5/200 µl serum-free X-VIVO 15 medium (Lonza, Walkerville, MD) supplemented with glutamine, penicillin, streptomycin, and 50 U/ml recombinant human IL-2 (Roche Applied Science, Indianapolis, IN), were transferred to 96-well round bottom tissue culture plates and incubated 14 days with single (10 µg/ml) HLA-A2- or -DRB1-restricted peptide. Half the spent medium was replaced with fresh medium containing IL-2, but no additional peptide, on days 3, 7 and 10 of the incubation period.

ELISpot assays

Cells collected after 14 days incubation under the culture conditions described were washed and resuspended in X-VIVO 15 medium supplemented with glutamine and antibiotics. The frequency of epitope-specific T lymphocytes was quantified using human IFN-γ ELISpot assay kits purchased from eBioscience, Inc. (San Diego, CA) and the protocol provided. Triplicate wells were inoculated with 50,000 cells/200 µl X-VIVO 15 medium and 10 µg/ml of the same peptide present during the pre-stimulation period. Positive (phytohemagglutinin) and negative (0.1% DMSO) controls were included.

DC priming of naïve T cell *ex vivo*

Monocyte-derived DCs were generated *in vitro* in accordance with methods described by others [29]. $CD14^+$ monocytes were purified from PBMCs back-flushed from blood filters using anti-CD14-coated magnetic beads (Miltenyi Biotec; Auburn, CA). T lymphocytes were purified by negative selection using the human pan T cell isolation kit II also purchased from Miltenyi. Biotin-conjugated anti-mouse CD25 (Miltenyi) was added to the biotin-conjugated antibody cocktail supplied with the kit to eliminate $CD25^+$ regulatory T cells. Purified T lymphocytes were frozen and stored in liquid nitrogen for later use. The purified $CD14^+$ monocytes were suspended in X-VIVO 15 medium that contained L-glutamine, penicillin, streptomycin, 100 ng/ml GM-CSF (PreproTech, Rocky Hill, NJ) and 25 ng/nl IL-4 (PeproTech), and cultured in 24-well, ultra-low attachment tissue culture plates (Corning Inc., Lowell, MA). Half the medium was replenished with fresh medium containing GM-CSF and IL-4 on day 3. The resultant DC population was collected on day 5; the cells were suspended in fresh X-VIVO medium, transferred to individual wells of a 96-well, round bottom plate (1×10^4 cells/200 µl medium) and pulsed with single (10 µg/ml) peptides. On the following day, 25 ng/ml TNF-α (PeproTech) was added to promote DC maturation. After another 48 hours incubation, 100 µl of medium was removed, 6×10^5 naïve T lymphocytes (derived from the population purified and frozen above)/100 µl X-VIVO medium were added to each well; and the cells were co-cultured.

To quantify the epitope-specific responses of purified T cells co-cultured with peptide-pulsed DCs, the cells were collected after 14 days incubation, centrifuged and resuspended in fresh X-VIVO 15 medium. The cells (50,000/100 µl) were transferred to IFN-γ capture antibody-coated ELISpot plates that contained 5,000 mature DCs/100 µl X-VIVO 15/well generated as described in the previous section and pulsed with the same peptide present during the initial co-culture period. The remainder of the assay was conducted according to the supplier's protocol referenced in the preceding section.

Results

Epitope/ICS predictions

A set of 21, HLA-A*0201-restricted epitopes were selected for synthesis from a set of more than 100 highly conserved, high-scoring peptides predicted using the EpiMatrix and Conservatrix algorithms (Table I). Twenty of these epitopes scored in the top 1% (Z score ≥2.32) and one (peptide ID# 13) scored in the top 5% (Z score ≥1.64) of predicted binders, indicating a very high probability of HLA-A*0201 binding. The amino acid sequence, A*0201 EpiMatrix Z Scores and relative conservation of these peptides among HCV genotypes 1a and 1b isolates (calculated using Conservatrix) are shown in Table II.

Twenty-five HLA-DRB1-restricted ICS from the input HCV genotype 1a and 1b sequences were selected from a list of 79 that were predicted using the EpiAssembler algorithm; these varied from 15 to 27 amino acids in length. Each was composed of multiple, overlapping 9-mers (epitopes) capable of binding more than one HLA-DRB1 allele. The number of epitopes contained within each ICS predicted to bind across all 8 alleles was determined and ranged from 5 to 28 epitopes/ICS. Among the original 25 ICS, three exhibited significant homology to sequences found in the human genome and three overlapped protein junctions located within the HCV poly-protein; these six ICS were excluded from further study. The amino acid sequence, HLA-DRB1 EpiMatrix Z scores, number of predicted epitopes and conservation of the 19 remaining ICS among HCV genotypes 1a and 1b isolates are shown in Table II.

Binding analyses

Each predicted epitope/ICS was synthesized. HLA binding affinity and concurrence with computational predictions were determined. Positive predictions were defined as epitope scores ≥1.64 on the EpiMatrix Z-scale and HLA binding of IC50<100μM. Overall, the proportion of predictions that concurred with binding was ~90% (19/21) and 84% (96/114) for the HLA-A*0201-

restricted peptides (Table III) and HLA–DRB1-restricted ICS (Table IV), respectively. We expect no more than three to five percent of randomly selected peptides to bind to any given HLA. In the current case, approximately 85% of all predictions were confirmed, consistent with our previous experience. The small number of negative predictions present in this dataset makes statistical validation of the correlation between predicted binding and observed results difficult. We suggest that the correlation between prediction and observed outcome is self-evident.

Epitope/ICS validation.

All predicted A2-restricted epitopes and DRB1-restricted ICS that exhibited HLA binding affinity induced naïve T cell reactivity and IFN-γ production *ex vivo*, validating their immunogenicity. PBMCs obtained from healthy blood donors and cultured 14 days in the presence of single peptides subsequently exhibited peptide-specific IFN-γ production in ELISpot assays (Figure 1). Similar results were obtained when purified, naïve human T lymphocytes were co-cultured with $CD14^+$ monocyte-derived DCs pulsed with peptides. DCs pulsed with single HLA-A2-restricted or –DRB1-restricted peptides induced marked increases in IFN-γ production by naïve human T cells in ELISpot assays (Figure 2). Notably, all peptide sequences induced IFN-γ production, but not to the same extent in co-cultures derived from different blood donors.

Discussion

Triple drug therapy (telaprevir or boceprevir, combined with PEGylated interferon and ribavirin) is recommended by the American Association for the Study of Liver Diseases as the standard treatment for unresolved HCV genotype 1 infections [30]. The SVR rate is improved significantly in patients who receive triple therapy compared to those treated with PEGylated interferon and ribavirin alone. While the outcome is improved, the overall success of triple drug therapy is limited by a variety of factors that include: general access to health care, cost of therapy, patient compliance, drug-drug interactions, emergence of anti-viral resistant variants, adverse side effects, and host factors that affect relative effectiveness [30,31]. Indeed, logistics and expense are major deterrents in treating chronically infected patients with antiviral drugs worldwide. As such, the development of a safe, effective and affordable vaccine represents the best hope for bringing the global hepatitis C epidemic under control, a stated objective in the US Department of Health and Human Services' Viral Hepatitis Action plan [32].

A safe and effective therapeutic vaccine for chronic hepatitis C must elicit broad, vigorous $CD4^+$ and $CD8^+$ T cell responses to conserved viral epitopes, which result in viral elimination in the absence of significant liver injury. To date, four distinct vaccine strategies have demonstrated only limited success in clinical trials: recombinant protein, peptide, genetic or DNA-based and vector-mediated [9,10,33]. Recombinant protein vaccines are safe and well tolerated, but generally ineffective owing to their inability to induce a $CD8^+$ T cells. Peptide-based HCV vaccines are similarly ineffective despite their ability to elicit weak, epitope-specific T cell responses; only a transient reduction in viral load was determined in a minority of chronically-infected patients [34,35]. Similarly, patients vaccinated with a codon-optimized HCV NS3/4A DNA construct exhibited only a transient reduction in serum viral load [36]. Finally, just half of the chronically-infected patients vaccinated in a Phase I clinical trial with modified vaccinia virus Ankara [MVA] engineered to express HCV NS3-5B proteins exhibited a temporary reduction in viral load [37].

Therapeutic vaccination with HCV epitope-expressing DCs concurrent with or without chemotherapy offers an additional vector-mediated approach to treating chronic, HCV-infected patients. The utility of this approach is supported by clinical trials demonstrating tumor regression and long-term survival in a subset of cancer patients administered antigen-pulsed DCs [38]. Indeed, FDA approval of Dendreon's prostate cancer vaccine, Sipuleucel-T (Provenge), demonstrates the feasibility of developing a DC-vectored therapeutic vaccine for chronic hepatitis. The potential efficacy of DC-based vaccines in treating chronic hepatitis C was demonstrated in a recent Phase I clinical trial in which patients vaccinated with monocyte-derived DCs pulsed with 6 HCV-specific, HLA-class I-restricted peptides exhibited peptide-specific CD8 T cell responses [15]. These responses were not sustained, however, nor was an effect on viral load discerned. The failed response of patients vaccinated with peptide-pulsed DCs in this clinical trial was attributed to the dearth of viral epitopes presented [15]. Immunization with DCs that express a broad array of HLA class I- and class II-restricted viral epitopes offers a means of significantly enhancing vaccine efficacy.

The data presented herein support the EpiMatrix algorithm as an approach to high-volume genomic screening for vaccine candidates. Nineteen of 21 predicted, HLA class I-restricted peptides (>90%) were bound by HLA A*0201 *in vitro*, in accordance with previously published data [39]. All predicted HLA-class II-restricted peptides were bound by at least three HLA-DRB1 alleles, substantiating their promiscuity and potential recognition by a large, diverse human population [20]. A large, retrospective comparison conducted previously found EpiMatrix was >75% accurate across the HLA-DRB1 alleles studied here, and more accurate than all other epitope mapping algorithms in the public domain [22]. In the present study, 96 of the 114 HLA-DRB1-peptide pairs exhibited binding as predicted. Conceivably, a number of factors (e.g., peptide folding or aggregation under assay conditions, and the accuracy of immunoinformatic algorithms) contribute to the lack of a strict correlation between predicted and actual binding.

Conclusion

IFN-γ is a principal mediator of anti-HCV-specific T cell responses [40,41]. All the predicted, HLA-A2-restricted and –DRB1-restricted peptides that exhibited HLA binding activity also induced IFN-γ production in cultures of naïve human PBMCs, thus validating their immunogenicity and the prognostic accuracy of the EpiMatrix algorithm. Similarly, monocyte-derived DCs pulsed with the same peptides and co-cultured with naïve $CD4^+$ and $CD8^+$ human T cells induced epitope-specific IFN-γ production. This latter observation supports the therapeutic potential of DCs pulsed *ex vivo* with a broad array of HLA-A2- and –DRB1-restricted epitopes in treating chronic, HCV-infected patients. This approach suffers, however, from a number of practical limitations, foremost of which is the estimated high cost of treatment. Instead, we are currently exploring alternative strategies that include delivering the epitopes validated herein to DCs *in situ*. Regardless, epitope-driven immunotherapy alone or in combination with chemotherapy offers an additional means of treating the expanding patient population affected by chronic hepatitis C.

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Authors' contributions

SM, PTL and SHG: experimental design and performance, data analyses, manuscript preparation; AS and RT: experimental performance and data acquisition; FT, MA, WM and ADG: EpiMatrix analysis, interpretation and discussion. All authors have read and approve of this article.

Disclosures

Anne De Groot and William Martin are senior officers and majority shareholders at EpiVax, Inc. These authors acknowledge a potential conflict of interest and attest that the work contained in this report is free of any bias that might be associated with the commercial goals of the company. None of the remaining co-authors has any potential financial conflict of interest related to the manuscript to disclose.

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Table I. Predicted, HLA-A*0201-restricted HCV epitopes

Peptide ID#	Name	Sequence	A*0201 Z Score	Conservation 1a	Conservation 1b
1	**HCV_G1_NS4b_1917**	WMNRLIAFA	2.48	100%	99%
2	**HCV_G1a_NS5b_2734**	MLVCGDDLV	2.44	100%	ns[a]
3	**HCV_G1_NS4b_1765**	HMWNFISGI	2.66	98%	94%
4	**HCV_G1_NS3_1451**	SVIDCNTCV	2.60	98%	99%
5	**HCV_G1b_NS5b_2829**	WLGNIIMYA	2.80	ns	97%
6	**HCV_G1_E2_615**	RLWHYPCTV	3.24	35%	86%
7	**HCV_NS3_1586**	YLVAYQATV	3.20	99%	86%
8	**HCV_G1_E2_685**	ALSTGLIHL	2.68	74%	97%
9	**HCV_G1_core_133**	DLMGYIPLV	2.91	95%	97%
10	**HCV_G1_NS3_1074**	CINGVCWTV	2.73	92%	34%
11	**HCV_G1_E1_323**	MMMNWSPTT	2.54	70%	61%
12	**HCV_G1_E1_364**	SMVGNWAKV	2.42	90%	80%
13	**HCV_G1_NS3_1274**	GIDPNIRTGV	2.07	56%	16%
14	**HCV_G1_NS4b_1808**	LLFNILGGWV	2.95	93%	91%
15	**HCV_G1_NS3_1607**	QMWKCLIRL	2.76	72%	84%
16	**HCV_G1_NS5b_2559**	IMAKNEVFCV	2.63	98%	75%
17	**HCV_G1_E1_277**	YVGDLCGSV	2.62	91%	92%
18	**HCV_G1_E1_281**	DLCGSVFLV	2.59	84%	74%
19	**HCV_G1_NS5b_2945**	YLFNWAVRT	2.57	78%	61%
20	**HCV_G1_NS4b_1769**	FISGIQYLA	2.51	98%	95%
21	**HCV_G1_NS3_1326**	SILGIGTVL	2.32	90%	31%

[a]ns = not significant.

Table II. Predicted, HLA-DRB1-restricted HCV ICS

Peptide ID#	Name	Sequence	DRB1*0101 Z Score	Predicted # Epitopes	Conservation 1a	Conservation 1b
1	**HCV_G1_NS3_1246**	AQGYKVLVLNPSVAATLGFG	2.15	20	>90%	>90%
2	**HCV_G1_NS4b_1876**	VDLLVNLLPAILSPGA	2.75	16	>90%	>90%
3	**HCV_G1_NS5b_2879**	LGNIIQRLHGLSAFSLHSY	2.85	15	>90%	>90%
4	**HCV_G1_NS4b_1769**	ISGIQYLAGLSTLPGNPA	2.54	11	>90%	>90%
5	**HCV_G1_NS4b_1941**	AARVTQILSSLTITQLLKRLHQWI	2.33	23	>90%	>80%
6	**HCV_G1_NS5b_2440**	KLPINALSNSLLRHH	3.00	8	>90%	>80%
7	**HCV_G1_NS4b_1725**	AEQFKQKALGLLQTASRQAE	1.76	12	>90%	>80%
8	**HCV_G1a_NS5b_2485**	LQVLKEVKAAASKVKANL	2.14	11	>90%	ns[a]
9	**HCV_G1_NS4b_1790**	LMAFTAAVTSPLTTS	2.55	18	>80%	>80%
10	**HCV_G1_NS5b_2840**	WARMILMTHFFSVLIARDQLEQ	1.96	14	>80%	>80%
11	**HCV_G1_E2_732**	AYCLWMMLLISQAEAALELIT	1.87	16	>80%	>70%
12	**HCV_G1a_E1_255**	AAILRRHIDLLVGSATLCSALY	2.20	13	>80%	ns
13	**HCV_G1_NS3_1605**	DQMWKCLIRLKPTLHGPTP	2.30	15	>70%	>80%
14	**HCV_G1_NS5b_2941**	CGKYLFNWAVRTKLKLT	2.61	11	>70%	>60%
15	**HCV_G1a_E1_359**	GIAYFSMVGNWAKVL	2.75	5	>70%	ns
16	**HCV_G1a_NS2_909**	VPYFVRVQGLLRICALARKAV	2.58	24	>60%	ns
17	**HCV_G1b_NS5b_2898**	PGEINRVASCLRKLGVPPLRAY	2.31	12	ns	>80%
18	**HCV_G1b_NS5b_2913**	VPPLRVWRHRARSVRAKLLSQGGRA	1.90	16	ns	>70%
19	**HCV_G1b_NS2_748**	LENLVVLNAASVAGAHW	2.54	17	ns	>60%

[a]ns = not significant.

Table III. Predicted, HCV class-I restricted epitopes bind HLA-A*0201

Peptide ID#	Sequence	IC_{50}
1	HCV_G1_NS4b_1917	
2	HCV_G1a_NS5b_2734	
3	HCV_G1_NS4b_1765	
4	HCV_G1_NS3_1451	
5	HCV_G1b_NS5b_2829	
6	HCV_G1_E2_615	
7	HCV_NS3_1586	
8	HCV_G1_E2_685	
9	HCV_G1_core_133	
10	HCV_G1_NS3_1074	
11	HCV_G1_E1_323	
12	HCV_G1_E1_364	
13	HCV_G1_NS3_1274	
14	HCV_G1_NS4b_1808	
15	HCV_G1_NS3_1607	
16	HCV_G1_NS5b_2559	
17	HCV_G1_E1_277	
18	HCV_G1_E1_281	
19	HCV_G1_NS5b_2945	
20	HCV_G1_NS4b_1769	
21	HCV_G1_NS3_1326	

The peptides listed exhibited: IC_{50} high (IC_{50}<5 μM, black bar); moderate (5 μM<IC_{50}<50 μM, gray bar) or no (IC_{50}>100 μM; white bar) affinity for HLA-A*0201 in a competitive binding assay.

Table IV. Promiscuous, class II-restricted HCV ICS bind multiple HLA-DRB1 alleles

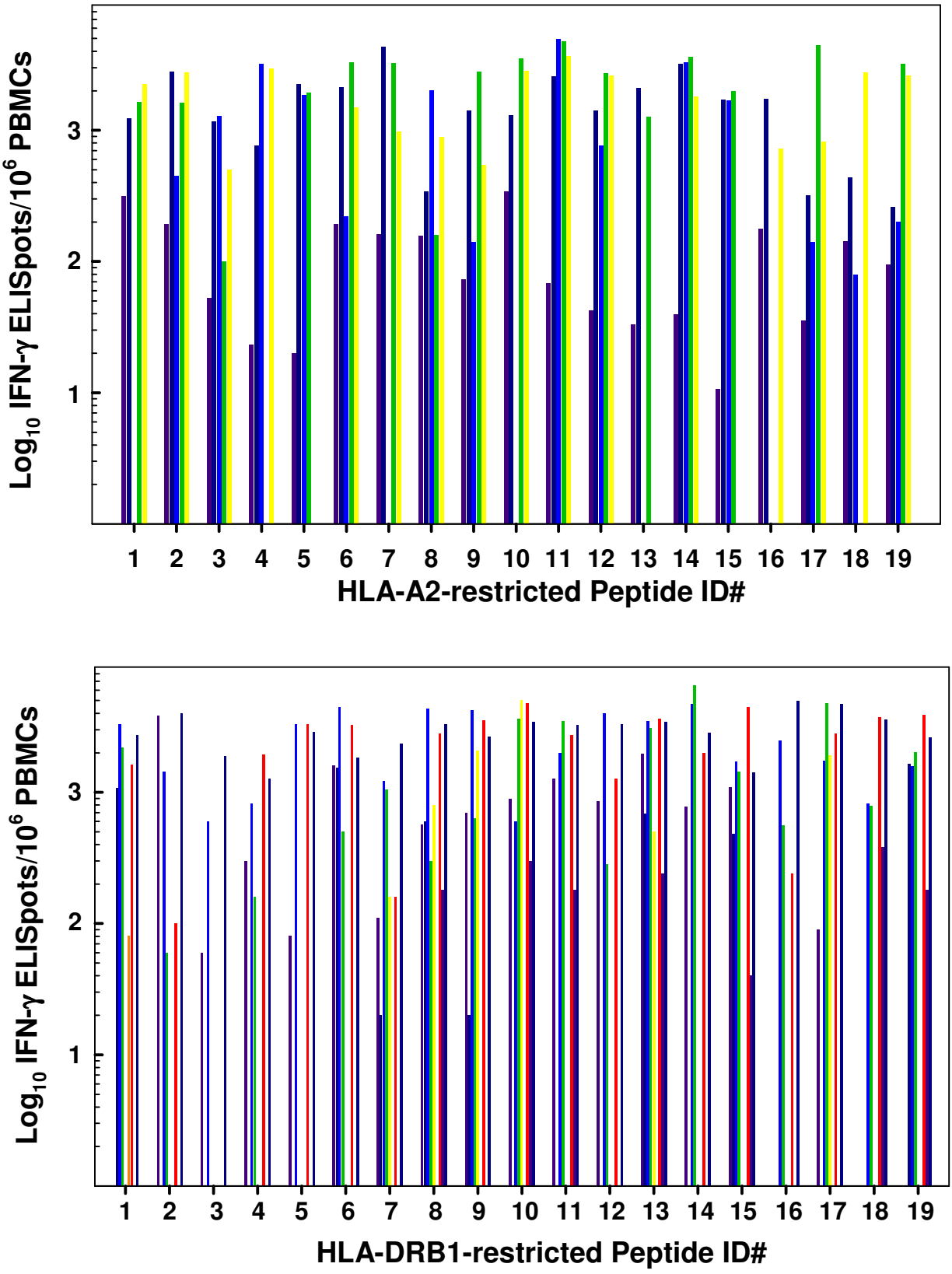
Peptide ID#	Name	*0101	*0301	*0401	*0701	*1101	*1501
1	HCV_G1_NS3_1246						
2	HCV_G1_NS4b_1876						
3	HCV_G1_NS5b_2879						
4	HCV_G1_NS4b_1769						
5	HCV_G1_NS4b_1941						
6	HCV_G1_NS5b_2440						
7	HCV_G1_NS4b_1725						
8	HCV_G1_NS5b_2485						
9	HCV_G1_NS5b_2840						
10	HCV_G1_NS4b_1790						
11	HCV_G1_E2_732						
12	HCV_G1_E1_255						
13	HCV_G1_NS3_1605						
14	HCV_G1_NS5b_2941						
15	HCV_G1_E1_359						
16	HCV_G1_NS2_909						
17	HCV_G1_NS5b_2898						
18	HCV_G1_NS5b_2913						
19	HCV_G1_NS2_748						

The peptides listed exhibited: IC_{50} high (IC_{50}<10 μM, black bar); moderate (10 μM<IC_{50}<100 μM, gray bar) or low (IC_{50}>100 μM; white bar) affinity for the alleles denoted in a competitive binding assay.

Figure Legends

Figure 1. Predicted HLA-A*0201- and HLA-DRB1-restricted HCV peptides induce epitope-specific IFN-γ production by naïve human PBMCs. PBMCs back-flushed from whole-blood leukocyte reduction filters were cultured with the HLA-A*0201-restricted (top) or HLA-DRB1-restricted (bottom) peptides listed. The cells were collected after 14 days incubation and IFN-γ ELISpot assays were performed. Each bar represents the average of triplicate wells minus the average negative control (0.1% DMSO + 2 SD) and the analysis of cells obtained from 5 and 9 healthy donors are shown in the top and bottom panels, respectively.

Figure 2. Peptide-pulsed DCs induce HCV epitope-specific IFN-γ production by naïve human T cells. Purified T cells were co-cultured with monocyte-derived DCs pulsed with the HLA-A2-restricted (top) and HLA-DRB1-restricted (bottom) peptides listed. The sensitized T cells were collected after 14 days and mixed with fresh, peptide-pulsed DCs and IFN-γ ELISpot assays were performed. Each bar represents the average of triplicate wells minus the average negative control (0.1% DMSO + 2 SD) and the analysis of cells obtained from 6 and 8 healthy donors shown in the top and bottom panels, respectively.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1
2
3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
HLA-A2-restricted Peptide ID#
Log10 IFN-γ ELISpots/10^6 PBMCs
1
2
3
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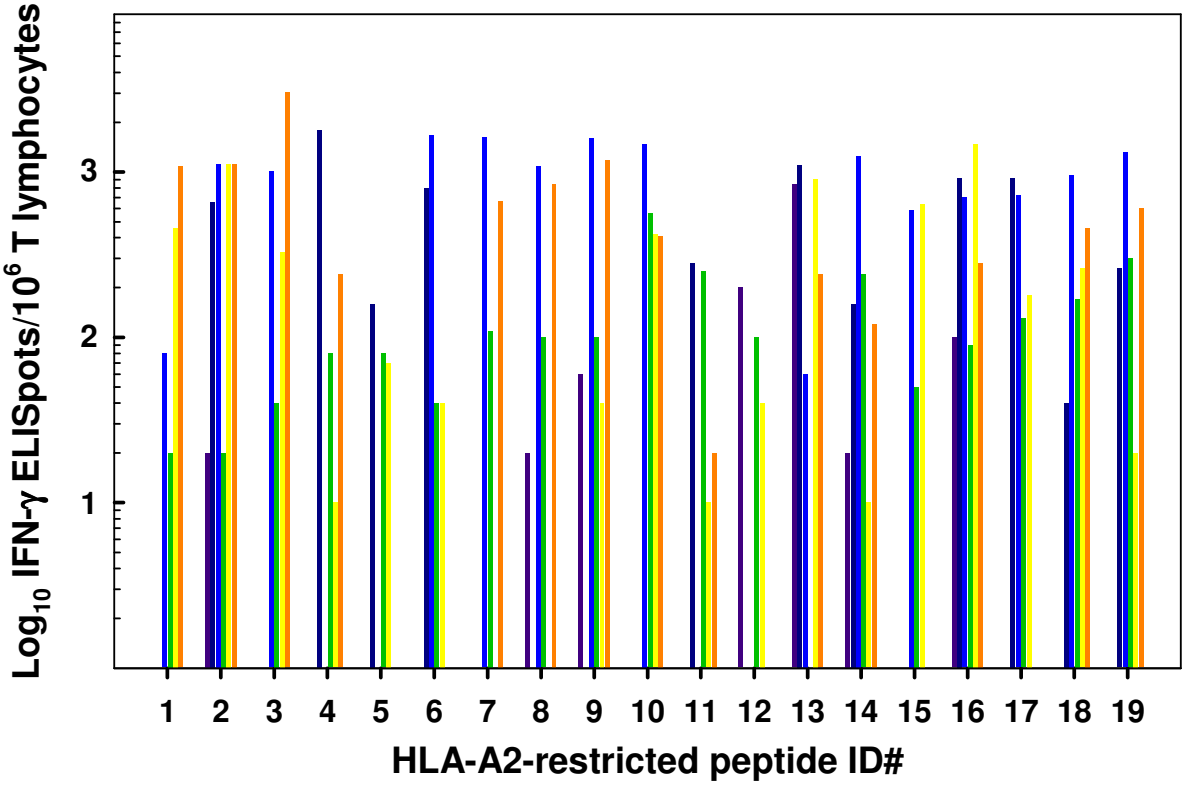
HLA-DRB1-restricted Peptide ID#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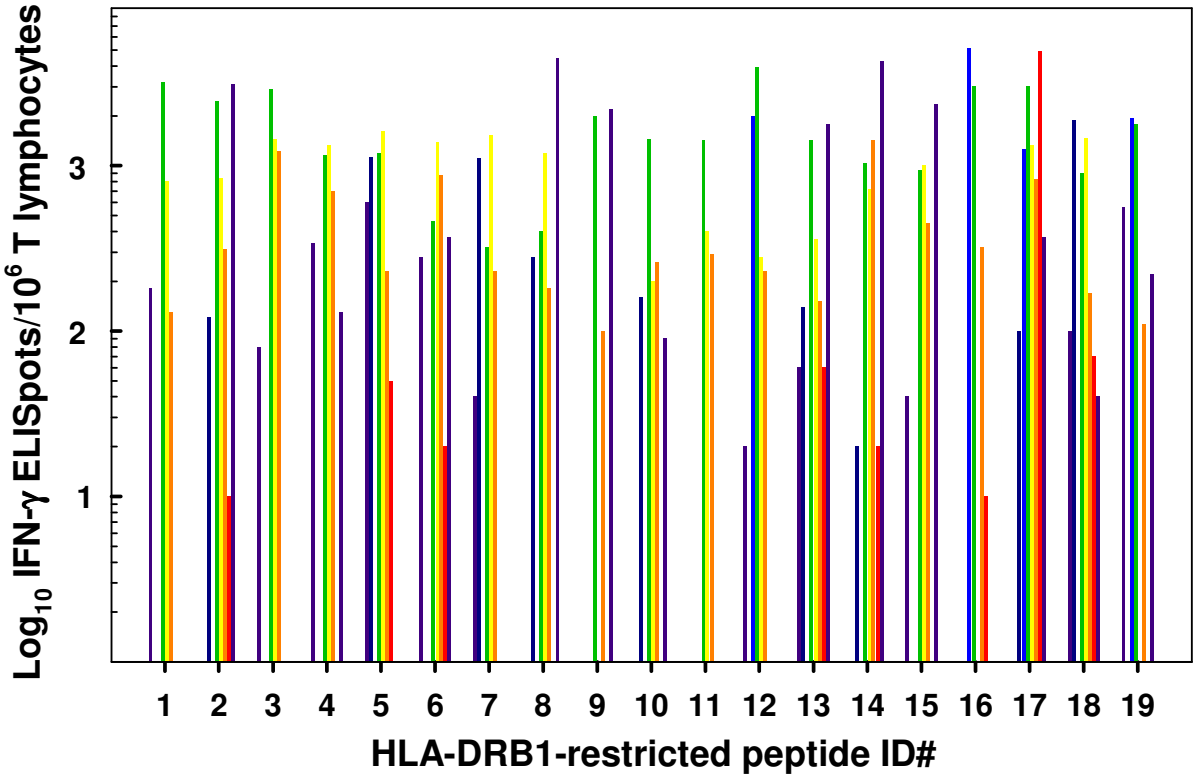
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2
3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
HLA-DRB1-restricted peptide ID#