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J Immunol 2001; 166:5300-5308; ;
doi: 10.4049/jimmunol.166.8.5300
http://www.jimmunol.org/content/166/8/5300

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

T Cell Responses to HLA-A*0201-Restricted Peptides Derived from Human α Fetoprotein[1]

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α fetoprotein (AFP)-derived peptide epitopes can be recognized by human T cells in the context of MHC class I. We determined the identity of AFP-derived peptides, presented in the context of HLA-A*0201, that could be recognized by the human (h) T cell repertoire. We screened 74 peptides and identified 3 new AFP epitopes, $hAFP_{137–145}$, $hAFP_{158–166}$, and $hAFP_{325–334}$, in addition to the previously reported $hAFP_{542–550}$. Each possesses two anchor residues and stabilized HLA-A*0201 on T2 cells in a concentration-dependent class I binding assay. The peptides were stable for 2–4 h in an off-kinetics assay. Each peptide induced peptide-specific T cells in vitro from several normal HLA-A*0201 donors. Importantly, these hAFP peptide-specific T cells also were capable of recognizing HLA-A*0201^+/AFP^+ tumor cells in both cytotoxicity assays and IFN-γ enzyme-linked immunospot assays. The immunogenicity of each peptide was tested in vivo with HLA-A*0201/K^b-transgenic mice. After immunization with each peptide emulsified in CFA, draining lymph node cells produced IFN-γ on recognition of cells stably transfected with hAFP. Furthermore, AFP peptide-specific T cells could be identified in the spleens of mice immunized with dendritic cells transduced with an AFP-expressing adenovirus (AdVhAFP). Three of four AFP peptides could be identified by mass spectrometric analysis of surface peptides from an HLA-A*0201 human hepatocellular carcinoma (HCC) cell line. Thus, compelling immunological and physiochemical evidence is presented that at least four hAFP-derived epitopes are naturally processed and presented in the context of class I, are immunogenic, and represent potential targets for hepatocellular carcinoma immunotherapy. ***The Journal of Immunology,*** **2001, 166: 5300–5308.**

Hepatocellular carcinoma (HCC)[3] has almost the lowest reported overall 5-year survival rate of all malignancies in the United States. The global annual incidence of this disease is 1.2 million. Current treatment options are largely surgical and of potential benefit to only a small percentage of patients. Because of the global pandemic of hepatitis B and C infections, the incidence of HCC is rapidly rising in both Asian and Western countries (1), and this trend is expected to continue for the next 50 years because of the long latency between infection and development of HCC. Therefore, novel treatment strategies are urgently needed for this disease.

Approximately 80% of HCC reactivate α fetoprotein (AFP) expression (2). The murine and human T cell repertoires can recognize AFP-derived peptide epitopes in the context of MHC class I (3, 4). Despite being exposed to high plasma levels of this oncofetal protein during embryonic development, clearly some AFP-specific T cells have not been deleted during the ontogeny of the immune system. Activation of these T cell clones can be achieved by presenting these epitopes in an immunostimulatory context, such as by professional Ag-presenting dendritic cells (DC). DC, when transduced with a recombinant adenovirus (AdV) vector encoding AFP, will process and present peptide epitopes in the context of MHC and will induce AFP-specific protection in mice (4).

We previously reported that $hAFP_{542–550}$ (GVALQTMKQ) is naturally processed and presented in the context of HLA-A*0201, is recognized by the human T cell repertoire, and can be used to generate AFP-specific CTL in human T cell cultures and in HLA-A*0201/K^b-transgenic mice (3). Herein, we report a complete analysis of 74 AFP-derived peptides and identify 3 additional HLA-A*0201-restricted immunodominant epitopes as well as 10 potentially subdominant epitopes. These peptides have been characterized by both immunological and physicochemical methods. Our finding suggests that the expression of AFP by HCC cells can be exploited for immunotherapy strategies.

Materials and Methods

Sequence analysis and computer screening

The University of Wisconsin (Madison, WI) Genetics Computer Group program "find patterns" was used to screen the hAFP sequences (GenBank accession numbers: J00077, J00076, and V01514) and identify 9- and 10-mer peptides, as described previously (3). The general HLA-A2.1 motif used was I, L, M, or V in P2 and V, I, or L in P9 (5).

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Received for publication June 12, 2000. Accepted for publication February 15, 2001.

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[1] This work was supported by National Institutes of Health/National Cancer Institute Grants ROI CA 77623, RO1 CA 79976, T32 CA75956, and K12 CA 79605, the Monkarsh Fund, Naify Fund, and the Stacy and Evelyn Kesselman Research Fund (all to J.S.E.), Tumor Immunology Training Grant CA 09120 (to C.M.V.), and a Jonsson Cancer Center Fellowship (to W.S.M.).

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[3] Abbreviations used in this paper: HCC, hepatocellular carcinoma; AFP, α fetoprotein; h, human; DC, dendritic cells; AdV, adenovirus; ELISPOT, enzyme-linked immunospot; LCL, lymphoblastoid cell lines; KLH, keyhole limpet hemocyanin; TFA, trifluoroacetic acid; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight.

0022-1767/01/$02.00

Cells, Abs, cytokines, and viruses

HLA-A*0201 donors and cell lines were screened with the BB7.2 (HLA-A2) Ab and subtyped by PCR analysis by the University of California Los Angeles (UCLA) Tissue Typing Laboratory. HepG2, Hep3B, K562, B95-8, BB7.2, and W6/32 cell lines were obtained from American Type Culture Collection (Manassas, VA). The amount of AFP produced by HepG2 (606 ng/ml/10^6 cells/24 h) and Hep3B (100ng/ml/10^6 cells/24 h) was determined by the UCLA Clinical Labs. The M202 human melanoma cell line has been described previously (6). T2 cells were provided by Peter Cresswell (Yale University School of Medicine, New Haven, CT). JY cells (HLA-A*0201 homozygous) were provided by Martin Kast (Loyola University Cancer Center, Chicago, IL). Jurkat/A2K^b cells were provided by Linda Sherman (Scripps Research Institute, La Jolla, CA). EBV-transformed lymphoblastoid cell lines (LCL) were generated by incubating PBMC with supernatant from B95-8 cells. Cell lines were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD) or IMDM (JY cells; Life Technologies) with 10% FBS (Omega Scientific, Tarzana, CA) and PSF (penicillin/streptomycin/fungizone; Life Technologies). Anti-β_2-microglobulin and the anti-CD4, CD14, CD19, and CD56 NA/LE Abs were obtained from BD PharMingen (San Diego, CA), anti-pan class I Ab was prepared from concentrated supernatant of W6/32 hybridoma, and CD4-FITC, CD8-PE, and CD16-PE Abs were obtained from Caltag (Burlingame, CA).

The stable transfectant cell lines LCL/hAFP, M202/hAFP, JurkatA2/K^b/hAFP (Jurkat/AFP), and JurkatA2/K^b/MART1 (Jurkat/MART) have been described previously (3). IL-2 was provided by Hoffman-LaRoche (Nutley, NJ), IL-7 was obtained from Biosource (Camarillo, CA), and keyhole limpet hemocyanin (KLH) and β_2-microglobulin were obtained from Sigma (St. Louis, MO).

The AdVhAFP contains the 1.9-kb hAFP cDNA and is driven by the CMV promoter/enhancer in a pAC-CMVpLpA AdV type 5 backbone (3). The empty AdV vector, AdVRR5, has been described previously (7) and served as a control.

Peptide synthesis

Peptides were synthesized by Chiron (Victoria, Australia) and at the UCLA Peptide Synthesis Facility (Dr. Joseph Reeve, Jr., Director) with standard F-moc technology.

T2 binding assay

Each peptide was tested for concentration-dependent binding to T2 cells in an HLA-A*0201 stabilization assay (8, 9). T2 (TAP deficient) cells, that had been incubated at room temperature the previous night to increase cell surface MHC class I molecule expression, were then incubated overnight with each peptide over a range of peptide concentrations from 0.1–100 μM. Stability of HLA-A*0201 was assayed by flow cytometry after staining the cells with anti-HLA-A2 Ab (BB7.2) and goat anti-mouse-FITC. The HLA-A*0201 strongly binding Flu matrix peptide (aa 58–66; Flu) was used as a positive control.

MHC-peptide complex stability

The off-kinetics assay was performed as described (3, 10). HLA-A*0201 LCL were stripped with a mild pH 3.2 citrate-phosphate acid buffer. Each peptide was immediately pulsed onto cells at 200 μM for 1 h in the presence of β_2-microglobulin at 3μg/ml at room temperature. Excess peptide was washed off and the cells were incubated at 37°C for 0, 2, 4, and 6 h. Cells were washed at the end of each time point and stained for cell surface HLA-A2 expression and then analyzed by flow cytometry. The peptide-MHC class I complex was considered stable if the mean fluorescence intensity increased at least 1.5-fold from cells that were stripped but not pulsed with peptide.

Human CTL generation from peptide-pulsed PBMC

Peptide-specific CTL were generated as described previously (3, 11, 12) to various peptides. Briefly, normal donor HLA-A*0201 PBMC were pulsed with peptide, washed, and cultured on day 0 with IL-7 (10–25 ng/ml) and KLH (5 μg/ml) in RPMI 1640/10% autologous serum at 3×10^6 cells/1.5 ml/well. Cells were restimulated weekly by removing the nonadherent cells from the culture and adding them to fresh, autologous, peptide-pulsed, washed, and irradiated PBMC at a 1:1 ratio. IL-2 was added twice weekly at 10 U/ml. After 3–4 wk of culture, the cultures were tested for cytotoxicity and/or cytokine release.

Human CTL generation from AdV-transduced DC

DC (prepared as described from PBMC incubated with GM-CSF and IL-4; Refs. 13 and 14) were transduced with AdVhAFP or AdVMART1 at a multiplicity of infection of 1000 for 2 h. Transduced DC were washed, irradiated, and plated at $1–2 \times 10^5$ cells/ml to serve as stimulators for CTL generation. Autologous nonadherent cells were depleted of CD4, CD14, CD19, and CD56$^+$ cells by magnetic bead depletion (Dynal, Lake Success, NY) to prepare CD8$^+$-enriched responder cells (population generally 80% CD8$^+$, not shown). The CD8$^+$ cells were plated with the transduced DC at 2×10^6 cells/ml in 5% autologous medium plus IL-7 at 10–25 ng/ml. Cultures were supplemented with IL-2 at 10 U/ml every 3–4 days. The CD8$^+$ CTL were restimulated weekly with fresh, autologous AdV-transduced DC at a ratio of 1 DC to 10–20 CD8$^+$ CTL. Most cultures were phenotyped for CD4$^+$ and CD8$^+$ cells on a weekly basis.

Human and murine cytotoxicity assay

Chromium release assays were performed as described previously after 6 days of in vitro restimulation of primed splenocytes (3). Briefly, target cells were chromated (and T2 cells were peptide pulsed at 50μg/ml) for 1.5–2 h and plated. Target cells were washed three times, diluted to 5×10^4 (or 1×10^5 for murine assay) cells/ml, and plated with CTL. To control for nonspecific lysis, a 10–50 fold excess of unchromated K562 were added to target populations before adding to CTL (in human assay). After 4–5 h, supernatants were harvested and counted in a γ counter. Triplicate wells were averaged and the percentage of specific lysis was calculated as follows: (sample − spontaneous release)/(maximum release − spontaneous release).

Human enzyme-linked immunospot (ELISPOT) assay

To determine the frequency of Ag-specific cytokine-producing cells, the ELISPOT technique was used as described previously (3, 15, 16). T cell restimulation was performed with $2–5 \times 10^6$ CTL incubated with 1×10^5 autologous LCL or JY cells pulsed with specific or nonspecific peptides or with tumor cell lines. Restimulator cells without CTL served as a negative control. The capture Ab (BD PharMingen)-coated plates (Millipore, Bedford, MA) were incubated with restimulated cells (in duplicate at three dilutions) at 37°C for 24–48 h. The colored spots, representing cytokine-producing cells, were counted under a dissecting microscope.

*HLA-A*0201/K^b-transgenic mice*

HLA-A*0201/K^b-transgenic female mice (created by Dr. Linda Sherman, Scripps Research Institute) were originally purchased from Harlan Sprague-Dawley (Indianapolis, IN), and currently are bred by the animal facility of the Dept. of Radiation Oncology at UCLA and handled in accordance with the animal care policy of the UCLA. For peptide immunizations, mice received 100 μg of AFP or control peptide emulsified 1:1 in CFA (Difco, Detroit, MI) s.c..

Preparation of murine DC and adenoviral transduction

DC were differentiated from murine bone marrow progenitor cells following the Inaba method (17) with modifications (3, 18). In vitro cultured DC were transduced in RPMI 1640/2% FCS at an multiplicity of infection of 100. Transduction was conducted for 2 h at 37°C, the DC then were washed and resuspended at 5×10^5 DC per 0.2 ml of PBS per animal for injection. In all cases, viability exceeded 95%.

Murine CTL generation

Two weeks after priming (by AdV/DC or peptides), splenocytes (3×10^6/well) were activated ex vivo with irradiated, mitomycin C-treated Jurkat/hAFP or Jurkat/MART (5×10^5/well) in 2 ml RPMI 1640/10% FBS and 50 U/ml IL-2 in 24-well plates for 6 days.

Murine ELISPOT assay

Groups of HLA-A*0201/K^b mice were primed by AdV-transduced or peptide-pulsed DC immunizations, and 2 wk later, splenocytes (4×10^6 cells/ml) were activated ex vivo with an optimal concentration of peptide in complete medium (RPMI/10%FBS) for 48 h, or 5×10^6 cells/ml were cultured in T-25 flasks in an equal volume with irradiated Jurkat-MART or -AFP cells (1×10^5 cells/ml) plus 50 U/ml IL-2. After restimulation for 48 h, the ELISPOT assay was performed as described. The frequency of Ag-specific cells was determined from the difference between the number of spots seen with and without Ag during restimulation.

HPLC separation of naturally processed peptides from viable cells

To elute peptides, HepG2 and Hep3B cells were washed three times with PBS before being incubated with 5 ml of citrate-phosphate buffer at pH 3.2 (19) for 1 min. The suspension was centrifuged (800 × *g* for 5 min) and a total of 500 ml of cell-free supernatant was collected for each cell line. The materials were lyophilized to dryness and stored at −20°C.

Lyophilized materials were redissolved in 30 ml of water/acetonitrile/triflouroacetic acid (W/A/TFA, 95/5/0.1 all by vol.). This solution was pumped onto an analytical reverse-phase HPLC column (C_{18} Betasil, 250 mm × 2 mm, 5-μm particle size, 100-Å pore size; Keystone Scientific, Bellefonte, PA) equilibrated in W/A/TFA, at a flow rate of 0.2 ml/min. The column was eluted by using an increasing linear gradient of 0.1% TFA in acetonitrile (time/% acetonitrile = 0/5, 5/5, 55/100, 60/100). Column eluate absorbance was monitored at 215 and 280 nm, and 1-min fractions were collected. The retention times of the synthetic peptides with amino acid sequences corresponding to the immunostimulatory peptides were obtained by using the same separation gradient on a separate column.

Mass analysis

A Voyager-RP (PerSeptive Biosystems, Framingham, MA) matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) instrument was used to acquire the mass spectra. The instrument uses a stainless-steel target on which the samples are deposited and dried. All spectra were externally calibrated with insulin, resulting in mass accuracy typically within ± 0.1%. Lyophilized HPLC fractions were resuspended in 10 μl of 70% acetonitrile with 0.1% TFA. A total of 1μl of this material was spotted along with 1 μl of the matrix α-cyano-4-hydroxycinnamic acid (Sigma, 10 mg/ml in 70% ACN/0.1% TFA). Spectra were obtained by scanning from *m/z* 500-7000.

Results

Identification of immunogenic AFP peptides

We identified potential HLA-A*0201-restricted hAFP peptide epitopes by using a computer-based analysis of the human AFP amino acid sequence. A total of 74 9- and 10-mer peptides, the sequences of which conformed to the well characterized HLA-A*0201 binding motif (3, 20–22), were synthesized. Several immunological strategies were used to determine whether any of these peptides were immunogenic and naturally processed and presented in the context of HLA-A*0201. Fourteen of these AFP peptides were reproducibly positive in one or more of these assays and this subset of candidate epitopes is presented in Table I (and for comparison, 10 consistently negative hAFP peptides, and positive controls Flu $M1_{58-66}$ and MART-1_{27-35}). Their structural characteristics also are shown in this table and include length, number of anchor residues in P2 and P9/10 positions, concentration required

Table I. *Properties of peptides*

AA[a]	Length[b]	Sequence[c]	Anchor[d]	Binding[e]	Off-Kinetics[f]	Tg Mouse AdVhAFP/DC[g]	AdVAFP Ratio[h]	Human Peptide CTL[i]	AFP$^+$ Tumor[j]	AdVhAFP/ huDC[k]	Tg Mouse pep/CFA[l]
Positive peptides											
1	9	MKWVESIFL	1	0.5	2	++	2.0	Yes	Yes	Yes	Yes
137	**9**	**PLFQVPEPV**	**2**	**2.5**	**4**	**+++**	**2.5**	**Yes**	**Yes**	**Yes**	**Yes**
158	**9**	**FMNKFIYEI**	**2**	**0.5**	**4**	**+++**	**3.2**	**Yes**	**Yes**	**Yes**	**Yes**
178	9	ILLWAARYD	1	100	>6	+	1.4	No	No	Yes	Yes?
218	9	LLNQHACAV	2	0.5	n.s.	+	1.9	Yes	No	No	ND
235	9	FQAITVTKL	1	>100	>6	++	2.35	Yes	Yes	ND	ND
306	10	TTLERGQCII	1	>100	<2	+	1.7	ND	ND	ND	ND
325	**10**	**GLSPNLNRFL**	**2**	**10**	**2**	**+++**	**4.4**	**Yes**	**Yes**	**Yes**	**Yes**
485	9	CIRHEMTPV	2	25	6	++	2.1	ND	ND	ND	ND
492	9	PVNPGVGQC	1	100	4	++	2.67	Yes	?	ND	Yes
507	10	NRRPCFSSLV	1	>100	<2	+	1.7	ND	ND	ND	ND
542	**9**	**GVALQTMKQ**	**1**	**>100**	**6**	**+++**	**3.0**	**Yes**	**Yes**	**Yes**	**Yes**
547	10	TMKQEFLINL	2	>100	4	+	1.5	Yes	Yes?	Yes?	Yes?
555	9	NLVKQKPQI	2	>100	6	++	2.35	Yes	Yes?	Yes?	Yes
Negative peptides											
121	9	RHNCFLAHK	0	>50	<2	0	0.7	ND	ND	ND	ND
211	9	KELRESSLL	1	>100	2	0	1.0	No	ND	ND	ND
242	9	KLSQKFTKV	2	50	n.s.	0	1.1	ND	ND	ND	ND
257	9	KLVLDVAHV	2	0.5	n.s.	0	1.0	Yes	No	?	ND
287	9	YICSQQDTL	1	50	2	0	1.3	No	No	ND	ND
294	9	TLSNKITEC	1	50	n.s.	0	1.2	No	ND	ND	ND
404	9	YIQESQALA	1	10	2	0	0.75	No	ND	No?	ND
441	10	QLTSSELMAI	2	50	<2	0	1.0	No	ND	ND	ND
549	9	KQEFLINLV	1	>100	<2	0	1	Yes	No	No	No
599	9	LISKTRAAL	2	25	4	0	0.8	ND	ND	No	ND
Control peptides											
58	9	GILGFVFTL (FLUM1)	2	0.25	6	ND	ND	Yes	ND	ND	ND
27	9	AAGIGILTV (MART-1)	1	25	2	0	1	Yes	No	No	No

[a] Starting amino acid.
[b] Length of peptide in amino acids.
[c] Peptide sequence.
[d] Number of anchor residues for HLA-A2.1.
[e] Concentration of detectable binding to T2 cells in μM.
[f] Length of time bound to JY cells. n.s., Not stable; <2, detected at time 0 only; 2, 4, 6, >6, length detected.
[g] Recognition of peptide by AdVhAFP/DC-immunized HLA-A2.1/K^b-transgenic (tg) mouse splenocytes by IFN-γ ELISPOT; +++, highly reproducible and high frequency; ++, reproducible and frequency ratio 2.0 or greater; +, reproducible and somewhat weaker response; 0, no reproducible response.
[h] Ratio of AFP peptide/control peptide IFN-γ spots from column 7.
[i] Peptide-specific human CTL generated.
[j] Recognition of AFP-expressing tumor target by peptide-specific human CTL.
[k] Recognition of peptide by AdVhAFP/DC-stimulated human T cells by IFN-γ ELISPOT.
[l] Recognition of AFP-expressing target by peptide/CFA-immunized tg mouse splenocytes by IFN-γ ELISPOT. ?, Weak response, not always reproducible.

for detectable binding to TAP-deficient T2 cells, and duration of binding to acid-stripped JY cells in an off-kinetics assay.

HLA-A*0201/K^b-transgenic mice proved to be a convenient and sensitive means to screen these 74 potential peptide epitopes. DC were prepared from bone marrow progenitors by differentiation in GM-CSF and IL-4 and were transduced with a recombinant AdV vector that encoded hAFP (AdVhAFP). Splenocytes from HLA-A*0201/K^b mice immunized with AdVhAFP/DC recognized AFP-transfected targets in IFN-γ ELISPOT and cytotoxicity assays (Fig. 1, *A* and *B*, and data not shown). Over a dozen of the 74 AFP peptides also could be recognized by these primed splenocytes. Three peptides ($hAFP_{137–145}$ (PLFQVPEPV), $hAFP_{158–166}$ (FMNKFIYEI), and $hAFP_{325–334}$ (GLSPNLNRFL)) and the previously reported $hAFP_{542–550}$ (GVALQTMKQ; Ref. 3) consistently elicited cytokine production and cytotoxicity, and therefore are "immunodominant" (23). The remaining peptides had either weak or less reproducible responses but were positive in more than one type of assay and therefore are potential "subdominant" epitopes. These experiments indicated that the DC from these HLA-A*0201/K^b mice were capable of processing and presenting peptide epitopes in the context of HLA-A*0201, which elicited responses from the available murine T cell repertoire.

These four putative hAFP epitopes then were subjected to additional immunological and physicochemical studies. These studies included: (1) repetitive peptide stimulation of naive HLA-A*0201 human T cell cultures, which would demonstrate peptide immunogenicity in the context of the human T cell repertoire and the ability of peptide-specific T cells to recognize AFP-transfected targets; (2) generation of a human T cell response in vitro by AdVhAFP transduced DC, which provides information about the spectrum of human T cell specificities to DC-processed AFP epitopes; (3) peptide/CFA immunization of HLA-A*0201/K^b mice, which is essentially an in vivo counterpart to peptide/human T cell cultures; and (4) HPLC and mass spectrometric identification of AFP peptides eluted from an HLA-A*0201 human HCC line. All of these lines of evidence, summarized in Table I, are formally presented for the four immunodominant epitopes we have identified.

*Screening for AFP peptide-specific responses in HLA-A*0201/K^b-transgenic mice immunized with AdVhAFP/DC*

HLA-A*0201/K^b mice were immunized with AdVhAFP/DC, and their splenocytes were restimulated in vitro with each AFP peptide separately and assayed for both cytotoxicity and IFN-γ production by ELISPOT assays. AdVhAFP/DC immunization induced AFP peptide-specific responses compared with a control A*0201-restricted melanoma Ag $MART\text{-}1_{27–35}$ peptide (Fig. 1*C*). Conversely, AdVMART1/DC-immunized mice specifically recognized the immunodominant $MART\text{-}1_{27–35}$ peptide but not AFP-pulsed targets (not shown). Naive splenocytes showed no cytotoxicity and had few IFN-γ-producing cells (not shown). Responses to the previously identified peptide $hAFP_{542–550}$ were reproducibly stronger than responses to the three new epitopes.

hAFP peptide-specific human T cell cultures

Synthetic peptides then were used to stimulate human T cells in vitro. Bulk T cell cultures were generated from PBMC pulsed with each AFP-derived peptide (supplemented with KLH, IL-7, and IL-2) and were tested between weeks 3 and 7 of expansion for the ability to recognize both peptide-pulsed and AFP-expressing targets (Fig. 2). These cultures expanded peptide-specific T cells (Fig. 2*A*), as evidenced by the ability to secrete IFN-γ on recognition of specific peptide-pulsed JY cells and not control $MART\text{-}1_{27–35}$-pulsed JY in the ELISPOT assay. The AFP peptide-specific bulk T cells also recognized both AFP-stably transfected and AdVhAFP-transduced HLA-

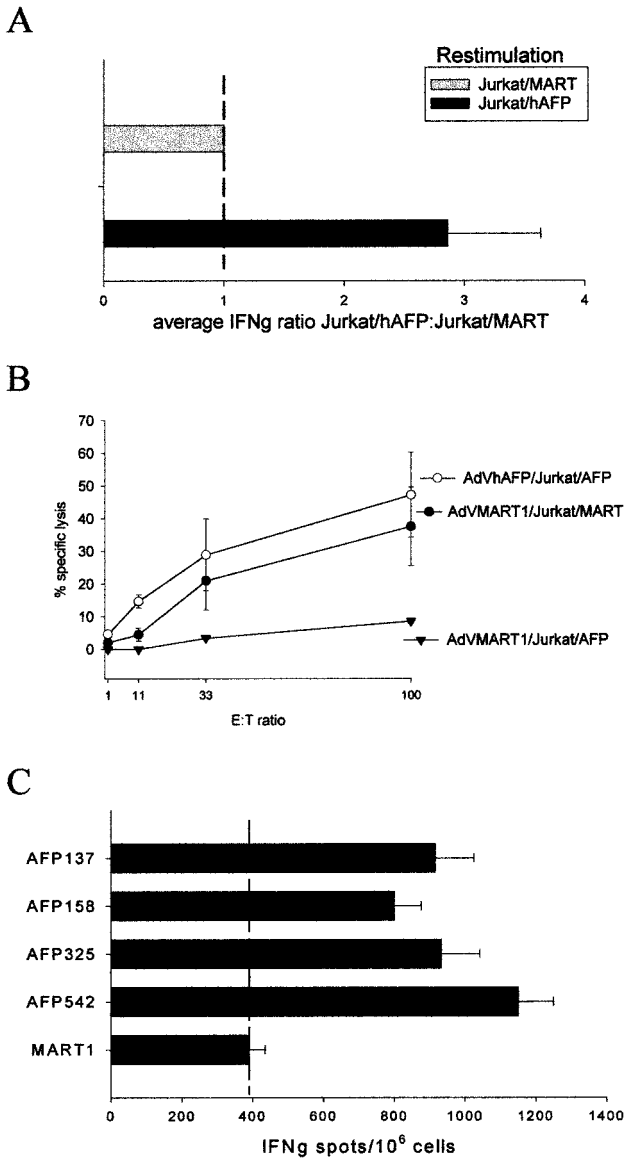

FIGURE 1. AFP-specific CTL activity and cytokine release from AdVhAFP/DC or AdVMART1/DC immunization in A*0201/K^b-transgenic mice. *A* and *B*, Two weeks after immunization, splenocytes from mice were restimulated with Jurkat cells stably transfected with hAFP (Jurkat/AFP) or with MART-1 (Jurkat/MART). *A*, Frequency of AFP-specific vs MART-1-specific IFN-γ-release was determined by ELISPOT, $p < 0.02$, averages of three independent experiments. *B*, Cytotoxicity was assayed against Jurkat/AFP and Jurkat/MART in a 5-h ^{51}Cr release assay. AdVhAFP/DC-immunized vs AdVMART1-immunized splenocyte recognition of Jurkat/AFP: E:T 1:1, $p < 0.01$; 11:1, $p < 0.07$; 33:1, $p < 0.3$; 100:1, $p < 0.2$. AdVMART1/DC recognition of Jurkat/MART vs Jurkat/AFP: E:T 1:1, $p < 0.4$; 11:1, $p < 0.4$; 33:1, $p < 0.3$; 100:1, $p < 0.3$. *C*, Two weeks after immunization, splenocytes from mice were restimulated with 30 μg of hAFP peptide, $MART\text{-}1_{27–35}$, or no peptide for 48 h before assaying cytokine secretion in an ELISPOT assay. Lower frequency of spots was observed in splenocytes from AdVMART-1/DC-immunized mice to the same hAFP peptides in the same experiments (not shown). Three similar experiments averaged is shown AFP_{137}, AFP_{158}, AFP_{325}, and AFP_{542} vs $MART\text{-}1_{27}$ recognition $p < 0.01$, 0.01, 0.01, and 0.002, respectively. Replicate ELISPOT assays confirmed recognition of each AFP-derived peptide: AFP_{137}, 8/8 experiments; AFP_{158}, 4/4 experiments; AFP_{325}, 6/6 experiments; AFP_{542}, 18/18 experiments.

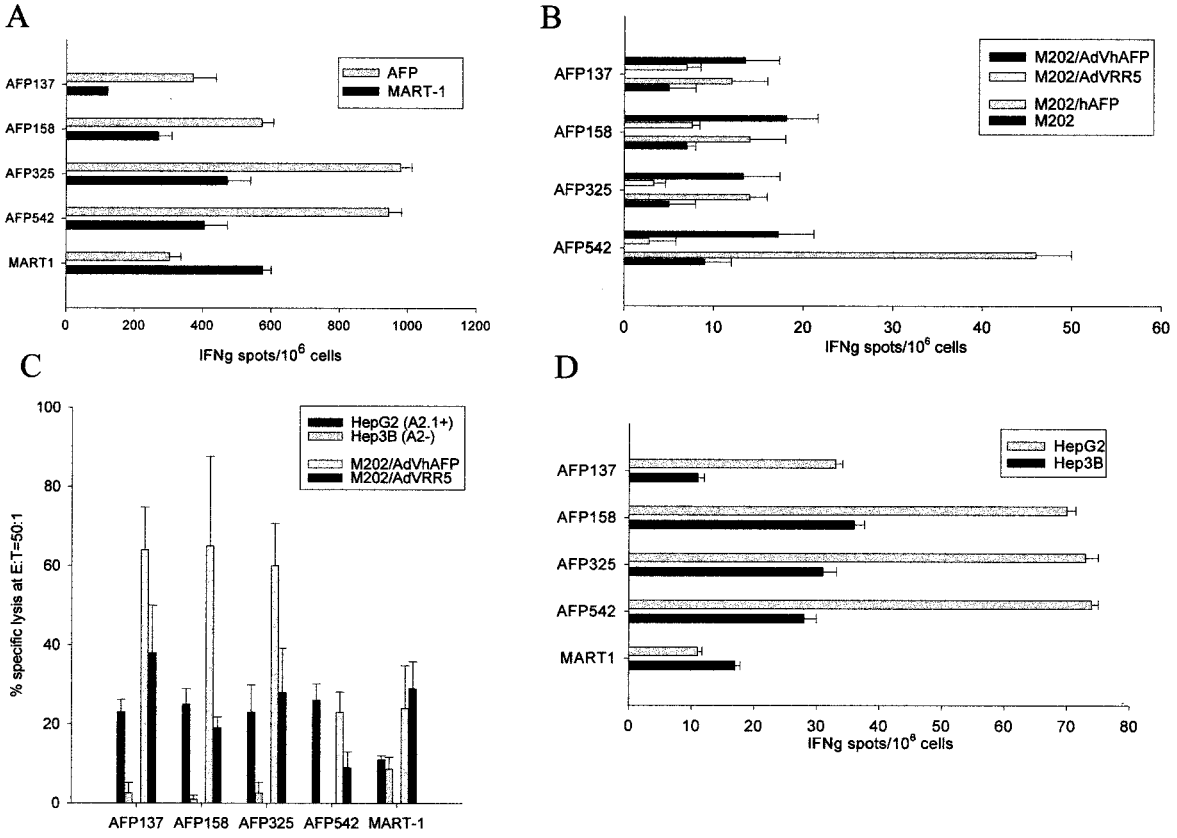

FIGURE 2. Peptide and AFP-specificity of hAFP peptide lymphocyte cultures. *A*, PBMC were stimulated weekly with different hAFP peptides and assayed (after 4–5 wk of expansion) for the frequency of IFN-γ-producing T cells after restimulation with peptide-pulsed JY cells to confirm peptide-specificity. AFP_{137}, AFP_{158}, AFP_{325}, and AFP_{542} recognition of AFP vs MART-1, $p < 0.04$, 0.05, 0.02, and 0.01, respectively. *B*, hAFP peptide-stimulated bulk T cell cultures were assayed for frequency of AFP-specific IFN-γ-secreting cells by ELISPOT after restimulation with AFP-stable transfectant (M202/hAFPhyg), AdVhAFP-transduced (M202AdVhAFP), control AdVRR5-transduced (M202RR5), or untransfected parental HLA-A*0201$^+$M202 melanoma cells. AFP_{542} recognition of M202hAFP vs parental cells, $p < 0.01$; M202AdVhAFP vs M202AdVRR5, $p < 0.003$. These trends for all four of the peptides were reproducible in three additional experiments. *C*, Cytotoxicity of hAFP peptide cell cultures against HepG2 (AFP$^+$/A*0201$^+$) and Hep3B cells (AFP$^+$/A*0201$^-$) or M202AdVhAFP- and M202AdVRR5-transduced cells. AFP_{137}, AFP_{158}, AFP_{325}, AFP_{542}, and MART-1_{27} T cell recognition of HepG2 vs Hep3B targets at E:T = 50:1; $p < 0.008$, 0.001, 0.04, 0.003, and 0.57, respectively. AFP_{137}, AFP_{158}, AFP_{325}, AFP_{542}, and MART-1_{27} T cell recognition of or M202AdVhAFP vs M202AdVRR5 targets at E:T = 50:1; $p < 0.17$, 0.08, 0.10, 0.06, and 0.73, respectively. *D*, Frequency of HepG2 and Hep3B-reactive IFN-γ-producing cells in peptide-stimulated cultures AFP_{137}, AFP_{158}, AFP_{325}, AFP_{542}, and MART-1_{27} T cell recognition of HepG2 vs Hep3B cells: $p < 0.01$, 0.0001, 0.04, 0.006, and 0.34, respectively. Overall, AFP peptide-stimulated T cell cultures recognized AFP-expressing, HLA-A*0201$^+$ targets in eight IFN-γ ELISPOT assays and five cytotoxicity assays.

A*0201 melanoma cells (M202) compared with unmodified or empty AdVRR5-transduced parental cells (Fig. 2*B*) as shown by an increased frequency of IFN-γ-producing AFP-specific T cells. In cytotoxicity assays, these bulk AFP peptide-stimulated T cells were able to lyse the AdVhAFP-transduced melanoma cells (Fig. 2*C*). To assess the ability to recognize the HLA-A*0201$^+$, naturally AFP-expressing HCC cell line HepG2 (compared with the HLA-A2-/AFP$^+$ HCC line Hep3B), both cytotoxicity (Fig. 2*C*) and ELISPOT assays (Fig. 2*D*) were preformed. Levels of cytotoxicity were lower than for AFP-transduced melanoma cells, but in general, frequencies of IFN-γ-producing T cells were similar for the HCC and transfected/transduced melanoma cells.

AdVhAFP/DC-stimulated T cell cultures recognize AFP-derived peptides

Because AdVhAFP/DC in vitro stimulated human T cells specifically recognized hAFP-transfected targets in both CTL and ELISPOT assays (3), we determined whether these four AFP peptides were specifically recognized by the AdVhAFP/DC stimulated T cells. After 7–21 days of culture, CD8-enriched T cells stimulated weekly with AdVhAFP/DC were tested for both cytotoxicity (Fig. 3*A*) and the frequency of hAFP peptide-specific IFN-γ cytokine-producing cells (Fig. 3*B*). AdVhAFP/DC T cell cultures were cytotoxic for JY cells pulsed with each of the four AFP peptides, indicating that CTL to these peptides could be expanded from peripheral blood of normal donors. After restimulation with autologous peptide-pulsed LCL or JY cells, these bulk cultures also contained a much higher frequency of IFN-γ-secreting cells specific for AFP peptides compared with MART-$1_{27–35}$, indicating that, in addition to $hAFP_{542–550}$, these three peptides also are naturally processed and presented by AdVhAFP-transduced DC.

The AdVhAFP/DC-stimulated T cell cultures also had a low frequency of cytokine-producing cells that recognized the A*0201$^+$/AFP$^+$ HCC line HepG2 but not the A*0201$^-$/AFP$^+$ HCC line Hep3B, as shown in the ELISPOT in Fig. 3*C*. T lymphocytes synthesizing the Th1 cytokines IFN-γ and TNF-α were detected, whereas the Th2 cytokine IL-4 was not detected.

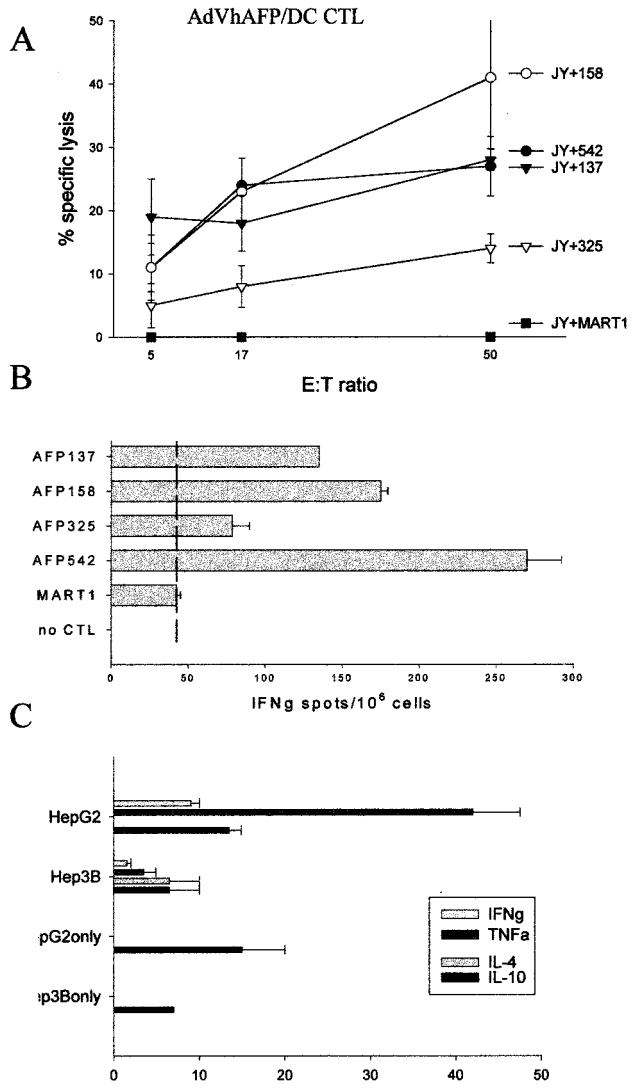

FIGURE 3. Cytotoxicity and cytokine release by AdVhAFP-transduced human DC/T cell cultures. *A*, $CD8^+$-enriched lymphocyte cultures were stimulated with AdVhAFP/DC for 2 wk and assessed for peptide-specific cytotoxicity against AFP peptides or MART-$1_{27–35}$-pulsed JY cell targets. AFP_{137}, AFP_{158}, AFP_{325}, and AFP_{542} vs MART-1_{27} T cell recognition $p < 0.001$, 0.02, 0.004, and 0.005, respectively, at E:T = 50:1; $p < 0.01$, 0.01, 0.07, and 0.0001, respectively, at E:T = 17:1; and $p < 0.03$, 0.04, 0.2, and 0.1, respectively, at E:T = 5:1. *B*, AdVhAFP/DC T cell cultures were assayed for frequency of IFN-γ-secreting cells by ELISPOT at 21 days of culture after restimulation with peptide-pulsed autologous LCL cells. AFP_{137}, AFP_{158}, AFP_{325}, and AFP_{542} recognition vs MART-1, $p < 0.0007$, 0.001, 0.08, and 0.009, respectively. Overall, these AdVhAFP/DC-stimulated T cell cultures were tested for reactivity to these four AFP peptides and controls in six IFN-γ ELISPOT assays and four cytotoxicity assays with similar results. *C*, AdVhAFP/DC T cell cultures were assayed for frequency of IFN-γ-, TNF-α-, IL-4-, and IL-10-secreting cells by ELISPOT at 14 days of culture after restimulation with the human HCC lines HepG2 (HLA-A*0201$^+$/AFP$^+$) and Hep3B (HLA-A*0201$^-$/AFP$^+$). AFP-specific T cells secreting IFN-γ to HepG2 vs Hep3B cells, $p < 0.02$; T cells secreting TNF-α to HepG2 vs Hep3B cells, $p < 0.002$; to IL-4, $p < 0.2$; and to IL-10, $p < 0.09$. IL-10 synthesis by HepG2 or Hep3B cells alone vs by HCC cells with AFP-specific T cells, $p < 0.6$ and 0.8, respectively.

IL-10 also was detected when the HCC lines were plated without T cells, indicating that production of this cytokine was tumor cell derived.

Cytotoxicity against $hAFP_{137–145}$- and $hAFP_{325–334}$-pulsed targets was generally more difficult to detect than against $hAFP_{158–166}$- and $hAFP_{542–550}$-pulsed targets by chromium release assay. Similarly, a lower frequency of IFN-γ-producing cells were obtained for these two peptides.

*AFP-specific responses in HLA-A*0201/K^b mice immunized with peptide in CFA*

To confirm the in vivo immunogenicity of these four hAFP peptides, HLA-A*0201/K^b mice were immunized with each AFP peptide pulsed onto syngeneic DC. IFN-γ-specific ELISPOT assays were performed with splenocytes restimulated in vitro with either the immunizing AFP peptide (or MART-1 peptide, Fig. 4*A*) or with Jurkat/AFP or Jurkat/MART transfected cell lines (Fig. 4*B*). Immunization with each hAFP peptide and subsequent restimulation with either peptide or Jurkat/AFP induced large numbers of AFP-specific IFN-γ-producing cells. Lymphocytes from PBS-injected mice showed neither cytotoxicity nor IFN-γ production regardless of restimulation (Fig. 4 and data not shown). Mice immunized with MART-$1_{27–35}$ peptide produced MART-1-specific responses but no AFP peptide responses.

Mass spectrometry analysis

We then sought physicochemical evidence for their presence on the surface of a human HCC line. We performed MALDI-TOF

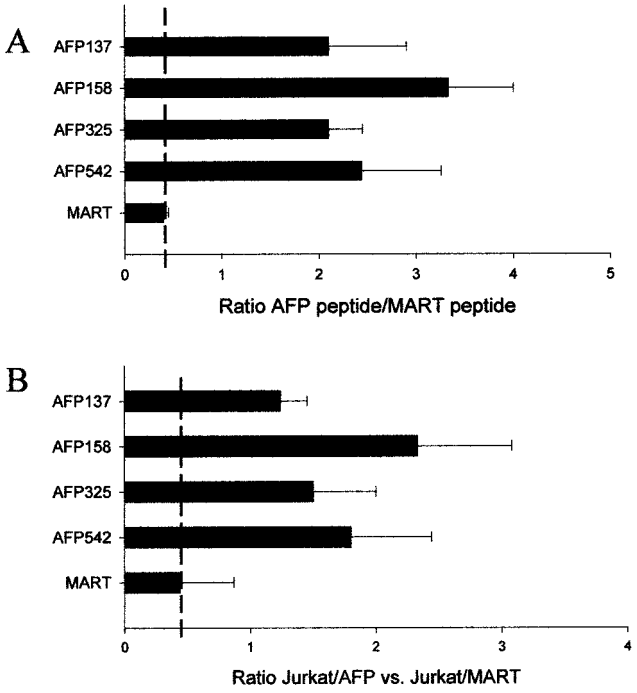

FIGURE 4. AFP-specific cytokine release from hAFP peptide-pulsed DC-immunized transgenic mice. Increased frequency of (*A*) AFP peptide-specific and (*B*) AFP Ag-specific IFN-γ production was detected in splenocytes from A*0201/K^b-transgenic mice immunized with each hAFP peptide (or MART-1_{27}) on DC. Two weeks after immunization, splenocytes from mice were restimulated with either the immunizing peptide (*A*) or J-AFP or J-MART (*B*) for 48 h before assaying cytokine secretion in an ELISPOT assay. Two similar experiments averaged is shown. Splenocyte recognition of immunizing AFP_{137}, AFP_{158}, AFP_{325}, and AFP_{542} vs MART-1_{27}, $p < 0.005$, 0.0001, 0.001, and 0.001, respectively. Splenocyte recognition of Jurkat/AFP vs Jurkat/MART $p < 0.004$, 0.003, 0.0002, and 0.008, respectively. MART-1_{27} immunized splenocyte recognition of MART-1_{27} vs AFP_{542}, $p < 0.001$; Jurkat/MART vs Jurkat/AFP, $p < 0.002$.

Table II. *Summary of the MALDI-TOF mass analysis*

Peptide[a]	HPLC Retention Time of Synthetic Peptide (min)[b]	Calculated $(M+H)^{+}$[c]	Observed $(M+H)^{+}$ in HepG2[d]	Observed $(M+H)^{+}$ in Hep3B[e]	$(M+H)^{+}$ Identified in HPLC Fraction[f]	Immunologically Reactive Fractions (IFNγ ELISPOT)[g]	
						HepG2	Hep3B
$hAFP_{542–550}$	21.2	975.5	975.6	None	21	20, 21	0
$hAFP_{158–166}$	28.9	1204.6	1204.9	None	28	27, 28, 29	0
$hAFP_{137–145}$	28.1	1025.6	None	None	–	27, 28, 29	0
$hAFP_{325–334}$	27.7	1130.6	1130.1	None	28	27, 28, 29	0

[a] Peptide identification.
[b] Typical HPLC retention time of control synthetic peptide.
[c] Expected mass/charge measurement.
[d] Observed mass/charge measurement in acid-eluted peptides from HepG2.
[e] Observed mass/charge measurement in acid-eluted peptides from Hep3B.
[f] Fraction (min) of observed hAFP peptide mass/charge measurement in acid-eluted peptides from HepG2.
[g] Fractions containing peptides capable of restimulating AdVhAFP/DC primed splenocytes by IFN-γ ELISPOT.

mass spectrometry to analyze HPLC-fractionated peptides acid-eluted from the AFP-producing HCC cell lines HepG2 (HLA-$A2^{+}$) and Hep3B (HLA-$A2^{-}$). We identified potential mass candidates for three of the four peptides in the HPLC-fractionated peptide pool eluted from the HLA-$A*0201^{+}$ HepG2 cells but not from the HLA-$A*0201^{-}$ Hep3B cells.

The MALDI-TOF analysis of the HPLC fractions established that almost all fractions contained up to 20 different peptides in the mass range from 700 to 1500 Da, although frequently with a few dominating signals. Out of this complex mixture, we identified peaks with *m/z* values corresponding to the calculated monoisotropic protonated molecules ($(M+H)^{+}$) of $hAFP_{542–550}$, $hAFP_{158–166}$, and $hAFP_{325–334}$ in the peptide pool eluted from HepG2 cells (Table II and Fig. 5). A peptide of *m/z* 975.6 was identified in HPLC fraction 21 from the HepG2 peptide pool (Fig. 5*A*). The calculated $(M+H)^{+}$ of $hAFP_{542–550}$ was 975.5, and the retention time of the synthetic peptide with amino acids corresponding to $hAFP_{542–550}$ was 21.2 min. Furthermore, no signal at *m/z* of 975.5 ± 1 was observed in samples with matrix alone (data not shown) and in HPLC fractions 18–22 from the Hep3B elution (fraction 21 shown in Fig. 5*A*). Similarly, peaks with *m/z* corresponding to the calculated $(M+H)^{+}$ of $hAFP_{158–166}$ and $hAFP_{325–334}$ also were found in the appropriate fraction (28) derived from HepG2 predicted from the behavior of the standard peptides (Table II and Fig. 5, *B* and *D*). These peaks were absent in fractions 26–32 in the peptide pool eluted from Hep3B (fraction 28 shown in Fig. 5). A peak at 1152.2 *m/z* was observed in fraction 28, suggesting the presence of the sodium adduct of $hAFP_{325–334}$ (Fig. 5*D*).

In the three peptides that were identified, the peaks were observed in repeated scanning of the spotted samples. A board peak at *m/z* 1020.9 was observed in fraction 29 from the HepG2 peptide pool (Fig. 5*C*). Because this peak was beyond the margin of error tolerated by this physicochemical analysis, we concluded that we could not document the presence of $hAFP_{137–145}$ on the surface of HepG2 cells.

To confirm the presence of AFP peptides in these fractions immunologically, 1 μl of each HPLC fraction from either HepG2 or Hep3B cells was used to restimulate AdVhAFP/DC-immunized murine splenocytes in an ELISPOT assay (Table II). A total of 200–250 spots/10^6 cells were observed from fractions containing AFP immunodominant peptides, whereas 100–130 spots/10^6 cells were observed from the other fractions, and a maximum of 50 spots/10^6 cells were observed from Hep3B fractions. This further supports the mass spectrometry identification of these AFP peptides.

Discussion

The oncofetal protein AFP is transcriptionally derepressed in HCC. We have demonstrated previously that neither the murine nor human T cell repertoire are tolerant of AFP-reactive T cells despite having been exposed to high serum concentrations of this protein during embryogenesis. Rather, the immune system appears to be clonally ignorant of AFP unless it is presented in an immunostimulatory context. We report herein a comprehensive screening of the human AFP protein sequence for immunogenic epitopes and specifically characterize three additional HLA-A*0201-restricted peptide epitopes recognized by AFP-specific T cells, the 9-mer $hAFP_{137–145}$ (PLFQVPEPV), and $hAFP_{158–166}$ (FMNK FIYEI) and the 10-mer $hAFP_{325–334}$ (GLSPNLNRFL). These results were obtained by using immunological and physicochemical approaches. DC genetically engineered to express hAFP were able to stimulate AFP peptide-specific T cell responses in CD8-enriched human lymphocyte cultures. Similarly, immunized HLA-$A*0201/K^{b}$ mice also recognized AFP peptide-pulsed cells in cytokine release assays. We also showed that AFP peptide-stimulated human and HLA-$A*0201/K^{b}$ mouse T cell responses recognized hAFP-engineered targets and, to a lesser extent, naturally AFP-expressing human HCC cells. Finally, mass spectrometry was used

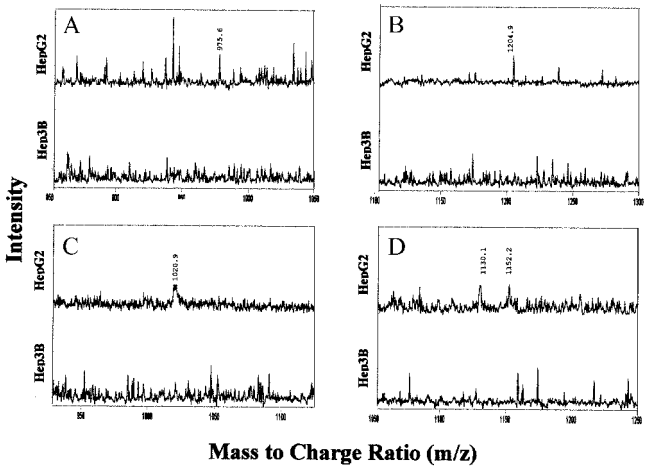

FIGURE 5. Mass spectra of HPLC-fractionated peptide pools acid eluted from HepG2 and Hep3B cells showing the *m/z* regions ($(M+H)^{+}$ ± 200) of (*A*) $hAFP_{542–550}$, (*B*) $hAFP_{158–166}$, (*C*) $hAFP_{137–145}$, and (*D*) $hAFP_{325–334}$. The spectra were normalized to approximately the same level of background noise.

to identify at least three AFP epitopes from complex mixtures of peptides eluted from HLA-A*0201$^+$ HCC cells. Thus, we provide multiple lines of evidence that each of these four AFP peptides is immunogenic and naturally processed and presented in the context of HLA-A*0201.

The human AFP protein has 65% amino acid homology to murine AFP. Therefore, the T cells in these transgenic mice recognize hAFP as a xenoantigen in the context of the human HLA-A*0201. For the four hAFP immunodominant peptides, there are 3, 2, 4, and 1 amino acid differences, respectively, between the human and murine 137, 158, 325, and 542 peptide sequences. Although studies by others have demonstrated that A*0201 mice respond to largely the same set of HLA-A2.1-binding peptides as humans (24, 25), we performed the human T cell culture assays to support the human immunogenicity of each peptide. Moreover, we have demonstrated previously that mAFP is immunogenic in normal inbred mice if presented in the immunogenic context of engineered DC (3).

Each of these three newly reported peptides has two anchor residues and strong binding affinity to HLA-A*0201. Molecular modeling of $hAFP_{137–145}$ (PLFQVPEPV), $hAFP_{158–166}$ (FMNK FIYEI), and $hAFP_{325–334}$ (GLSPNLNRFL) in the HLA-A2 binding groove show that the peptides adopt the general backbone conformation seen in the x-ray structures of HLA-A2 (26, 27): the NH2 and COOH termini of peptides are accommodated by conserved pockets at both ends of the binding groove, whereas the backbone in the central portion of the peptides bulges upward toward the TCR. It should be noted that the proline residues at positions 6 and 8 in $hAFP_{137–145}$ (PLFQVPEPV) impose a conformation of the peptide that is more rigid than the other peptides in this study. As expected, the side chains of the anchor residues at positions 2 and 9 (10 in $hAFP_{325–334}$) of the peptides are buried in the deep polymorphic pockets of HLA-A2 (pockets B and F, respectively). Side chains in the central part of the peptide, both hydrophilic and hydrophobic, tend to project upward toward the TCR (K4, F5, Y7, and E8 in $hAFP_{158–166}$ (FMNKFIYEI); Q4, V5, and E7 in $hAFP_{137–145}$ (PLFQVPEPV); N5, L6, N7, R8, and F9 in $hAFP_{325–334}$ (GLSPNLNRFL)). These contrast with $hAFP_{542–550}$, which lacks a hydrophobic side chain for pocket F.

It proved to be important to screen candidate peptides with fewer than two anchor residues, as 8 of 14 immunogenic peptides had one anchor residue. Although strong binding, or "on-rate" (10 μM or less) was a characteristic of 3 of 4 immunodominant peptides, the fourth immunodominant peptide ($hAFP_{542–550}$) and 8 of 14 immunogenic peptides overall showed weak (>50 μM) binding. Of the physical characteristics assessed, off-kinetics proved to correlate best with immunogenicity with 9 of 14 immunogenic peptides having off-kinetics greater than 2 h and 5 of 14 greater than 4 h. Of the five peptides with 6 h or longer kinetics, all were immunogenic. A sufficiently long half-life is likely to facilitate the stimulation of many TCRs on a given T cell, which is thought to be important for strong T cell activation (28, 29). Thus, it is possible that additional immunogenic epitopes exist from Ags for which candidate determinants were screened based on only two anchor residues or strong binding affinity (30–32).

The three new immunodominant and the 10 potentially subdominant peptides described in this report may serve as important tools for direct peptide-based immunotherapy as well as analysis of the diversity of the response to whole AFP Ag-based immunotherapy. We have initiated a phase I/II clinical immunotherapy trial in which A*0201/AFP-positive patients with unresectable HCC are immunized with all four of these AFP immunodominant peptides emulsified in IFA. Our early observations are that these patients are capable of generating peripheral T cell responses to all four peptides as measured by tetramer and ELISPOT assays (data not shown). Together, this data confirms that AFP can serve as a tumor-specific Ag in HCC and may be a suitable target for T cell-mediated immunotherapy strategies.

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