T-Cell Responses to HLA-A*0201 Immunodominant Peptides Derived from α-Fetoprotein in Patients with Hepatocellular Cancer

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ABSTRACT

Purpose: **An existing immunological paradigm is that high concentrations of soluble protein contribute to the maintenance of peripheral tolerance/ignorance to self protein. We tested this hypothesis in a clinical immunotherapy trial using class I-restricted peptide epitopes derived from α-fetoprotein (AFP). AFP is a self protein expressed by fetal liver at high levels, but transcriptionally repressed at birth. AFP is de-repressed in a majority of hepatocellular carcinomas (HCCs) and patients with active disease can have plasma levels in the mg/ml range. We previously identified four immunodominant HLA-A*0201-restricted peptides derived from human AFP that could stimulate specific T-cell responses in normal volunteer peripheral blood lymphocytes cultures. We wished to test the hypothesis that AFP peptide-reactive T cells could be expanded *in vivo* in HCC patients immunized with these four AFP peptides.**

Experimental Design: **We undertook a pilot Phase I clinical trial in which HLA-A*0201 patients with AFP-positive HCC were immunized with three biweekly intradermal vaccinations of the four AFP peptides (100 μg or 500 μg each) emulsified in incomplete Freund's adjuvant.**

Results: **All of the patients ($n = 6$) generated T-cell responses to most or all of the peptides as measured by direct IFNγ enzyme-linked immunospot (ELISPOT) and MHC class I tetramer assays.**

Conclusions: **We conclude that the human T-cell repertoire is capable of recognizing AFP in the context of MHC class I even in an environment of high circulating levels of this oncofetal protein.**

INTRODUCTION

The mammalian T-cell repertoire can recognize many "self" proteins in the context of MHC molecules; that there is not a much higher incidence of autoimmune disease can be explained by the peripheral ignorance of T cells to these antigens that are not ordinarily presented in an immunostimulatory context. The recognition that many tumor rejection antigens in melanoma are melanocyte-lineage self proteins underscores the close relationship between tumor immunity and autoimmunity. This important immunological concept has spawned an intensive search for, and testing of, other normal proteins overexpressed in tumors that might serve as potential rejection antigens. Among the proteins undergoing preclinical and clinical study are melanoma antigen recognized by T cells, melanoma antigen, tyrosinase, tyrosinase-related protein-1, carcinoembryonic antigen, prostate specific antigen, Her2/neu, telomerase, G250, p53, and others (1–3).

We originally reported that AFP[5] could be recognized by both murine and human T cells and serve as a tumor rejection antigen in a murine tumor model (4, 5). AFP is produced by 80% of HCCs; its measurement in serum has played an important role in diagnosis and in monitoring responses to treatment for the last several decades (6). AFP is expressed by the fetal liver with serum levels of 1 mg/ml but is transcriptionally repressed shortly after birth (7–9). Our ability to generate potent AFP-specific T-cell immunity in mice clearly indicates that, despite being exposed to high plasma levels of this protein during embryonic development, some AFP-specific T-cell clones are not deleted during ontogeny.

Using a combination of strategies (*A*0201/K*^{b} transgenic mice, human T-cell cultures, mass spectrometric analysis), we identified four AFP-derived peptides that are naturally processed and presented in the context of HLA-A*0201 (5, 10–12). At least three of these peptides could be isolated from the

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[5] The abbreviations used are: AFP, α-fetoprotein; HCC, hepatocellular cancer; PBMC, peripheral blood mononuclear cell; UCLA, University of California-Los Angeles; ELISPOT, enzyme-linked immunospot.

Table 1 Patient characteristics

Patient no.	Dose of peptides	Stage	Toxicity[a]	Pre-AFP[b] (ng/ml)	Post-AFP[c] (ng/ml)	Clinical response	OS[d] (mo)	Current status[e]
A1	100 μg	IVa	Skin II	501	2,816 (day 35)	Progr.	2	D
A2	100 μg	IVa	Skin II	105,617	199,500 (day 56)	Progr.	6	D
A3	100 μg	IVb	Skin II	1,475	2,070 (day 56)	Progr.	10	D
B1	500 μg	IVb	Skin II	162	271 (day 56)	Progr.	17	D
B2	500 μg	IVb	Skin II	3,131	5,003 (day 56)	Progr.	16	D
B3	500 μg	IVb	Skin II	42,218	65,034 (day 56)	Progr.	5	D

[a] Treatment-related toxicity.
[b] Pretreatment serum AFP level.
[c] Posttreatment AFP level (and day of trial when tested).
[d] OS, overall survival; Progr., progressive disease; D, deceased.
[e] As of April 2003.

surface of an *A*0201/AFP*-positive human HCC cell line. These peptides can stimulate T-cell responses, both cytotoxic and cytokine production, in bulk T-lymphocyte cultures from normal and AFP-positive HCC patients. These peptide-specific T cells recognize both peptide-pulsed targets as well as AFP-expressing tumor lines.

The next step was to determine whether these human AFP peptides were immunogenic *in vivo*. We designed a Phase I pilot trial in which *HLA-A*0201*-positive patients with AFP-positive HCC were immunized with all four AFP peptides emulsified in incomplete Freund's adjuvant (Montanide ISA-51). This small dose-escalation trial demonstrated that these patients could generate AFP-specific T-cell responses and that AFP immunization was safe.

MATERIALS AND METHODS

Clinical Trial Design

Six patients were enrolled in this first-in-human Phase I trial of AFP-based immunotherapy for HCC. All of the patients were required to express the *HLA-A*0201* allele, have an AFP-producing HCC, have adequate renal and hepatic function (Child-Pugh class A or B), and demonstrate immune competence by a positive skin delayed-hypersensitivity test to at least one recall antigen (candida, tetanus toxoid, or mumps). All of the subjects provided signed informed consent. This trial underwent review and approval by the Institutional Review Board (IRB no. 99-05-003) and the Internal Scientific Peer Review Committee at UCLA, and the Food and Drug Administration (BB IND no. 8746). Clinical-grade peptides were subjected to identity, purity, and potency testing, including sequencing, quantification of possible adventitious agents, and preclinical testing in mice and human T-cell cultures to demonstrate their ability to generate AFP-specific cellular responses.

Vaccine Preparation and Administration

Patients received three biweekly intradermal vaccinations. The first three patients received 100 μg of each peptide, and the second cohort of three patients received 500 μg of each peptide. Each peptide was emulsified separately in 0.5 ml of Montanide ISA-51 (Seppic Inc., Fairfield, NJ) by vortexing for 15 min, was pooled into one syringe, and was administered at four sites (0.5 ml/injection), bilaterally under the arms and in the lower trunk. Patients were premedicated with acetaminophen 650 mg and diphenhydramine 50 mg and were monitored for 2 h postimmunization at the UCLA General Clinical Research Center. The principal compound of Montanide ISA-51 is the mineral oil mannide oleate, which is also the main ingredient in incomplete Freund's adjuvant.

Peptides

The AFP-derived peptides $hAFP_{137–145}$ (PLFQVPEPV), $hAFP_{158–166}$ (FMNKFIYEI), $hAFP_{325–334}$ (GLSPNLNRFL), and $hAFP_{542–550}$ (GVALQTMKQ) were synthesized at the UCLA Peptide Synthesis Facility (Dr. Joseph Reeve, Jr., Director). Peptides were synthesized on an Advanced ChemTech 396 Multiple Peptide Synthesizer using industry FMOC [*N*-(9-fluorenyl)methoxycarbonyl] procedures and were purified by reverse-phase high-performance liquid chromatography on C-18, C-4, and phenyl columns until above 95% purity. Peptides were characterized by mass spectral analysis and microsequence analysis on an Applied Biosystems Sequencer and High Performance Capillary Electrophoresis.

Patient Characteristics

The characteristics of each patient are shown in Table 1. All were stage IV and heavily pretreated, with an average age of 51 years (range, 22 to 76 years).

Preclinical Toxicology

Because there was no previous human experience attempting to generate AFP-specific cellular responses, preclinical toxicology studies aimed at detecting possible autoimmune phenomena or liver-targeted toxicity were performed. *HLA-A*0201/K^b* transgenic mice present the same antigenic epitopes as *HLA-A*0201* human subjects bound to their major histocompatibility class I molecules. Therefore, these mice were an excellent animal model to test *in vivo* the safety and toxic effects of immunotherapy approaches aimed at generating responses in *HLA-A*0201* subjects (13). Additionally, these mice were generated in a C57BL/6 background, a mouse strain known to

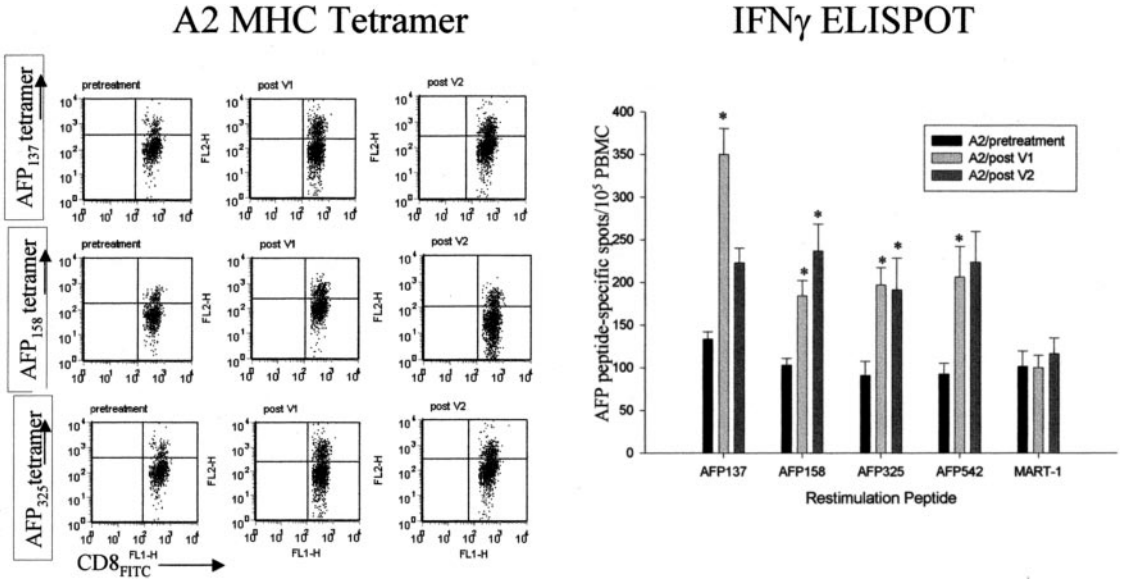

Fig. 1 Tetramer and ELISPOT analyses of patient A2 PBMC samples. For MHC tetramer binding (*left*) lymphocytes were gated on by forward and side scatter; CD4-, CD13-, and CD19-positive cells were gated out; and the percentage of CD8+/tetramer+ lymphocytes was analyzed. Cells shown were stained for the AFP_{137}, AFP_{158}, and AFP_{325} peptide tetramers over time. Nonspecific tetramer binding to healthy donor PBMC was subtracted. The IFNγ ELISPOT data (*right*), is shown in spots/total cells plated, with the MART-$1_{27–35}$ peptide control data. *, significant differences in ELISPOT values ($P \leq 0.05$). *post V*, postvaccination.

produce AFP in fetal and adult life (mean serum level, 222 ng/ml; Ref. 14). Intradermal immunization of *HLA-A*0201/K^b* transgenic mice with the four AFP peptides in Montanide ISA-51 produced no local macroscopic or microscopic inflammatory changes at the injection sites and, in careful evaluation of the liver, produced no evidence of adverse effects on liver function tests in peripheral blood or on organ weight or of other pathological changes.

Toxicity

The immunizations were generally well tolerated, with local pain and itching at the time of AFP peptides/Montanide ISA-51 injection. All of the subjects developed Common Toxicity Criteria (CTC) grade II skin toxicity at the injection site, consistent with durable nodular, erythematous, and aseptic lesions of 0.5–3 cm in maximum diameter. None required surgical intervention. Subject B3 developed CTC grade I hepatic toxicity demonstrated by the elevation of serum glutamic oxalacetic transaminase (SGOT) after the second vaccination, which was previously within normal limits. This elevation resolved after the third vaccination and was not accompanied by elevations of other liver function tests; hence; it is not thought to be related to the vaccine. Two other subjects (A1 and A2) had concomitant elevations of bilirubin, transaminases, and alkaline phosphatase during the vaccination period that were within a context of gross evidence of disease progression within the liver and that were, thus, attributed to progressive HCC.

Immunological Monitoring

Tetramer Analysis. Tetramers were obtained from the Tetramer Facility sponsored by the National Institutes of Allergy and Infectious Diseases. AFP_{542} would not fold properly into the A2.1 tetramer, nor would an anchor-substituted version (12). Patient PBMCs from each time point were thawed simultaneously and treated with DNase (0.002%); then 10^6 cells were stained with each individual tetramer plus CD8-FITC (Caltag) and antibodies used to gate out non-CD8+ lymphocytes [tricolor-conjugated CD4, CD13, and CD19 (Caltag)]. Staining was performed at room temperature for 30 min in the dark. The cells were then washed and analyzed immediately. The lymphocytes were gated on by forward and side scatter, and cells positive for CD4/13/19 were gated out. The AFP peptide-specific cells were a distinct population of CD8-FITC+/tetramer-phycoerythrin+ cells [Figs. 1 (*A2*), 2 (*B1*), and 3 (cumulative data)]. When available (patients A1, B1, B2, and B3),' MART-$1_{27–35}$ tetramers (NIAID) were used as negative controls (average MART-1 tetramer staining of normal donor PBMCs = 0.54%; average MART-1 tetramer staining of HCC patient PBMCs = 0.63%). Background tetramer staining was detected for each time, and for each tetramer with healthy donor PBMCs (from a single healthy donor leukapheresis), the background staining was subtracted.

IFNγ ELISPOT. The ELISPOT technique was used as described previously (5, 15, 16). PBMCs were thawed as above, then T-cell restimulation was performed overnight with 1–2 × 10^6 PBMCs incubated with 1 × 10^5 JY cells previously pulsed with specific AFP or nonspecific (MART-$1_{27–35}$) peptides. Because of variability in the viability and activity of PBMCs cryopreserved from HCC patients at different time points, the ELISPOT is performed by restimulating the PBMCs overnight with both the immunizing AFP-derived peptides and another control peptide that is physically similar (MART-$1_{27–35}$) but that should not vary between vaccine immunizations. This peptide, derived from the melanocyte self antigen MART-1, is also

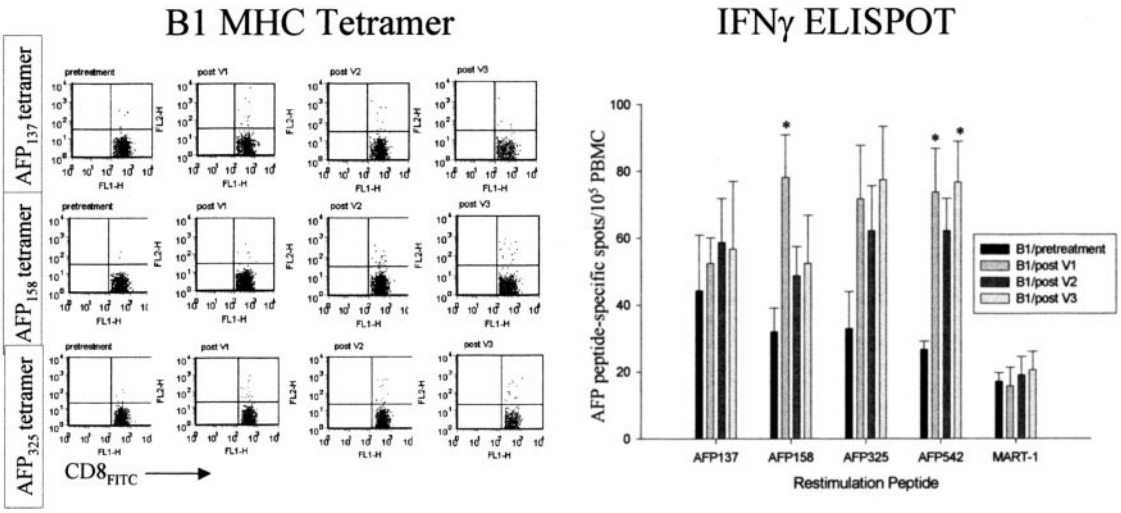

Fig. 2 Tetramer and ELISPOT analyses of patient B1 PBMC samples. For MHC tetramer binding (*left*) lymphocytes were gated on by forward and side scatter; CD4-, CD13-, and CD19-positive cells were gated out; and the percentage of CD8+/tetramer+ lymphocytes was analyzed. Cells shown were stained for the AFP_{137}, AFP_{158}, and AFP_{325} peptide tetramers over time. Nonspecific tetramer binding to healthy donor PBMC was subtracted. The IFNγ ELISPOT data (*right*), is shown in spots/total cells plated, with the MART-$1_{27–35}$ peptide control data. ∗, significant differences in ELISPOT values ($P \leq 0.05$). *post V*, postvaccination.

a 9-amino-acid-long peptide with one anchor residue for HLA-A2.1, which has been shown to bind weakly to HLA-A2.1 and to be immunogenic. Testing the different PBMC samples at the same time eliminates the interassay variability, and the ratio of AFP:control spots corrects for any difference in overall viability and activity of the specific sample. JY cells without CTLs also served as a negative control. The IFNγ antibody (PharMingen)-coated plates (Millipore, Bedford, MA) were incubated with restimulated T cells (in duplicate at three dilutions) at 37°C. The colored spots, representing cytokine-producing cells, were counted under a dissecting microscope.

Statistical Analysis

A paired *t* test was performed to compare the differences between pretreatment and posttreatment values of immunological assays. All *P*s are two-tailed. Significant differences in ELISPOT values ($P \leq 0.05$) are marked with an asterisk in Figs. 1 and 2.

RESULTS

Patients and Vaccination. Six patients were enrolled in this pilot peptide trial (described in detail in "Materials and

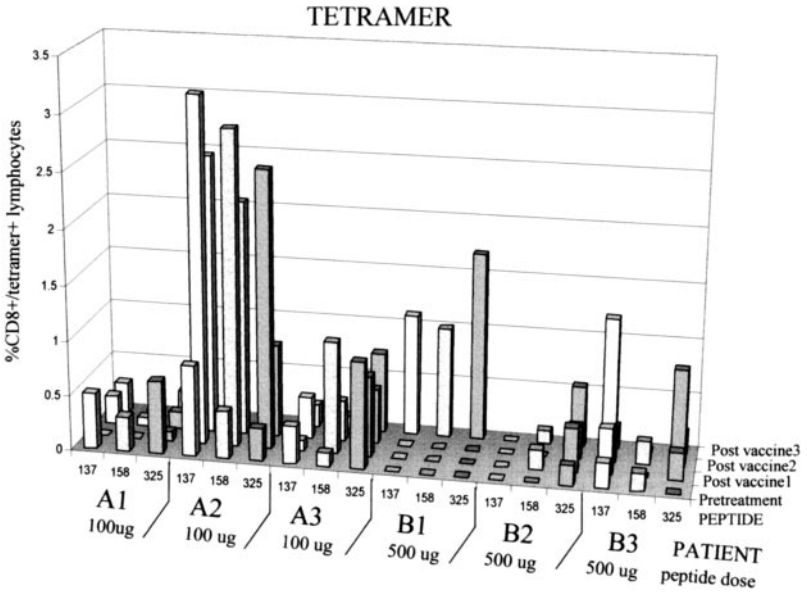

Fig. 3 Tetramer analysis of patient PBMC samples. Patient identifiers and peptide doses are shown on the *X* axis, along with individual AFP peptide responses analyzed. The time point of blood samples is shown on the *Z* axis. Lymphocytes were gated on by forward and side scatter; CD4-, CD13-, and CD19-positive cells were gated out; and the percentage of CD8+/tetramer+ lymphocytes was analyzed. Cells were stained separately for the three AFP peptide tetramers, and the %CD8+/tetramer+ lymphocytes over time is shown on the *Y* axis.

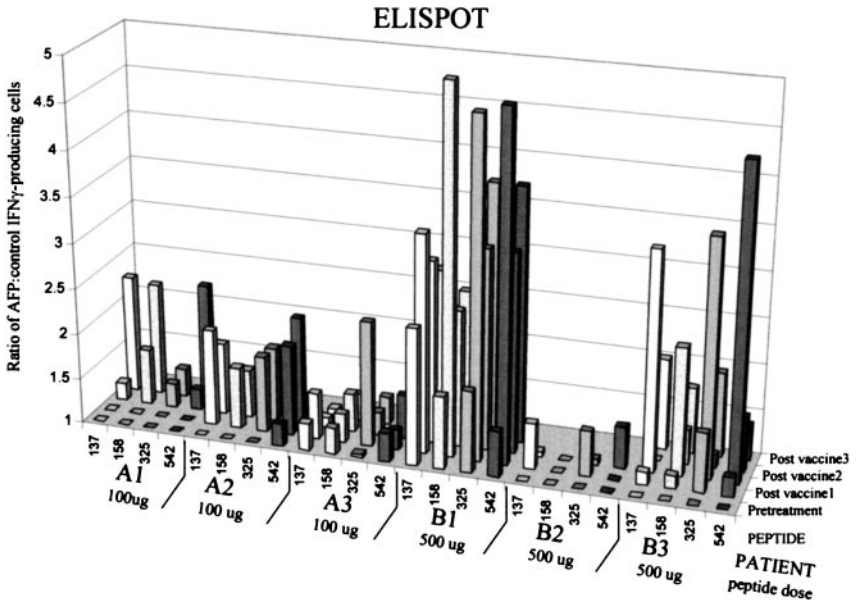

Fig. 4 IFNγ ELISPOT analysis of patient PBMC samples. Patient identifiers and peptide doses are shown on the *X* axis, along with individual AFP peptide responses analyzed. The time point of blood samples is shown on the *Z* axis. Each sample was restimulated with each AFP peptide or MART-$1_{27–35}$ overnight to detect the frequency of peptide-specific IFNγ producing cells. The ratio of AFP-peptide-specific:control-MART-1 spots is shown on the *Y* axis. Overall, significance differences were found between pre- and posttreatment values for peptides $AFP_{158–166}$ (mean difference, 70.5; SE 19.2; $P = 0.01$), $AFP_{325–334}$ (mean difference, 101; SE 19.3; $P = 0.003$), and $AFP_{542–550}$ (mean difference, 98.8; SE, 21.7; $P = 0.006$).

Methods"). All of them had biopsy-proven HCC that expressed AFP and that expressed the *HLA-A*0201* class I allele (Table 1). Most of the patients were heavily pretreated with surgery and/or chemotherapy and had unresectable or metastatic disease. Patients received three biweekly injections of a pool of four AFP peptides [$hAFP_{137–145}$ (PLFQVPEPV), $hAFP_{158–166}$ (FMNKFIYEI), $hAFP_{325–334}$ (GLSPNLNRFL) and $hAFP_{542–550}$ (GVALQTMKQ)] emulsified in 2.0 ml of Montanide ISA-51 injected into four divided intradermal sites. Patients A1, A2, and A3 received 100 μg of each peptide per dose, and patients B1, B2, and B3 received 500 μg of each peptide per dose.

Immunological Responses. AFP-specific T-cell responses were measured by tetramer and ELISPOT analysis. The former assay allows the enumeration of CD8 T cells capable of binding class I-restricted peptide epitopes complexed to a fluorochrome-labeled synthetic HLA-A*0201 tetramer and, thus, represents binding to a peptide-specific T-cell receptor. The ELISPOT assay is functional in that it measures the frequency of T cells that produce a specific cytokine, in this case the Th1 cytokine IFNγ, when specifically stimulated by peptide in the context of MHC. Thus, these assays represent both physicochemical and functional measures of peptide-specific T-cell responses.

Peripheral blood lymphocytes were isolated from these patients before, during, and after AFP peptide immunizations and were cryopreserved. All of the samples collected from each patient were then thawed simultaneously and subjected to tetramer (Fig. 1, *data from patient A2*; Fig. 2, *data from patient B1*; Fig. 3, *all data*) and ELISPOT analysis (Figs. 1, 2, and 4) without *in vitro* restimulation culture. All of the patients generated T-cell responses to most of the peptides. By tetramer analysis, expansion of CD8+ T cells capable of binding AFP peptides 137, 158, and 325 was observed in five of six patients for one to three peptides. Some striking responses were seen (Figs. 1 and 3, *patient A2*) in which almost 3% of circulating CD8 T cells had T-cell receptors capable of binding these peptides after immunization.

Likewise, in the ELISPOT analysis, all four peptides could stimulate and expand peripheral T cells capable of IFNγ production when restimulated overnight with peptide (Figs. 1, 2, and 4). In most patients, responses to all four peptides were generated. Both tetramer and ELISPOT assays included the HLA-A*0201-restricted melanoma antigen peptide MART-$1_{27–35}$ as a control, there were no nonspecific alterations in patient MART-1 reactivity.

DISCUSSION

We report here for the first time that T-cell responses to AFP can be detected and expanded in patients with advanced HCC with very high circulation levels of AFP protein, which indicates that the human T-cell repertoire can recognize AFP peptide epitopes in the context of MHC class I.

HCC has a dismal prognosis. Small tumors are potentially curable with surgical or ablative approaches, including liver transplantation (17, 18). However, the great majority of cases are detected at a more advanced stage, which, even if adequately treated locally, relapse systemically. There are no effective systemic therapies for this disease (19, 20). This leads to an overall 6% probability of being alive at 5 years after diagnosis in the United States, the lowest survival rate for any type of cancer listed by the Surveillance, Epidemiology and End Results, National Cancer Institute.[6] Thus, novel therapies for this disease are needed.

AFP-specific T cells expanded in all of the patients in response to the majority of immunizing peptides. There were too

[6] Internet address: /raterisk/rates28.html,h.r.n.n.g.N.P.I.

few patients in this pilot study to draw meaningful conclusions with regard to a dose-response effect, response kinetics, or immunodominance. It is interesting that superior tetramer (patient A2) and ELISPOT (patients B1 and B3) were not necessarily concordant. Because the two assays measure either total frequency (based on T-cell receptor binding) or frequency of IFNγ producing cells (regardless of total number), this lack of concordance is not unexpected. For example, low frequencies of tetramer-positive cells may be capable of enhanced cytokine production postvaccination, and the lower sensitivity of the tetramer assay may make these ELISPOT-positive cells harder to detect. In another example, patients A1, A2, and B3 by ELISPOT showed no IFNγ production in response to peptides pretreatment, but each had tetramer-positive circulating cells to at least one peptide. This indicates that the AFP-specific circulating cells were nonfunctional or at least unable to make IFNγ on recognition of a peptide before peptide immunization. Although a number of peptide-based clinical trials have performed only a single immunological assay to monitor patient responses, some have used more than one assay and attempted to correlate immunological responses between these assays as well as with clinical response. In some cases of multiple immunological assays, the low frequency of responses makes correlation difficult or a definite lack of correlation is found (21). Many studies (22–26) have found partial correlation between assays like tetramer and FastImmune or cytotoxicity or ELISPOT, like ourselves. There are also studies (27, 28) that have found good correlations between tetramer and functional assays, in particular one that isolated tetramer-positive cells and found additional markers that correlated with the functionality of the cells (29).

The small separation between CD8+/tetramer+ T cells and CD8+/tetramer− cells was consistent in all of the patients [and in many healthy donor *in vitro* assays (10) and unpublished observations].[7] Some reports have suggested that this may correlate with the low avidity of these cells (30). This might be expected for these self-antigen-specific cells, in particular in advanced cancer patients with high levels of circulating antigen. A functional analysis of the avidity of these cells is under way. Future analyses also include testing recognition of HLA-matched AFP+ HCC cells, which may also provide important insights into the activity of these AFP peptide-specific cells before and after immunization.

Many of the immunological responses were transiently detected in the peripheral blood. This is in agreement with a study that we recently completed with immunological monitoring of melanoma patients who were immunized with MART-$1_{27–35}$ peptide-pulsed autologous dendritic cells (31), as well as with other studies (22). The fate of the expanded peptide-specific cells is unknown; they may traffic to tumor deposits or exhibit apoptosis in the periphery. As previously mentioned, several patients had detectable tetramer-positive cells pretreatment, but in most cases, these PBMC samples did not contain active, IFNγ-producing cells. Patient B1 is an exception, having clear evidence of active, AFP peptide-specific T cells (in particular, AFP_{137}) before immunization.

There was a wide range of serum AFP levels in these subjects (162–105,617 ng/ml; Table 1). Among these six patients, there is no correlation between serum AFP level and T-cell response. The patient with the highest serum AFP (patient A2) had the highest tetramer levels detected (Figs. 1 and 3) as well as positive IFNγ ELISPOT response. By comparison, patient B1 (with the lowest serum AFP) had the strongest IFNγ ELISPOT response. Although not statistically significant, these data indicate that high-serum AFP protein does not preclude immune response to this antigen.

Treatment-related toxicity was limited to local reactions at the vaccination sites. Specifically, there was no clinical evidence of associated hepatotoxicity (see "Materials and Methods"). Because the goal of this strategy was to generate an autoimmune response to a self protein, this was an important trial end point. The absence of hepatotoxicity was in support of preclinical pharmacology/toxicology studies performed in *HLA-A*0201/K^b* transgenic mice (see "Materials and Methods") and in our previously described murine AFP genetic immunotherapy tumor model (4).

In summary, strong T-cell responses could be generated in patients with high circulating levels of AFP. It is clear that patients with HCC, even those with high circulating levels of AFP and significant tumor burdens, are not tolerant to AFP. This is the first reported clinical trial that establishes the proof of principle that a human T-cell response can be generated to this tumor antigen *in vivo*. We are currently testing the hypothesis that robust and clinically beneficial immune responses can be generated if AFP is presented in a more immunostimulatory context such as on AFP-engineered dendritic cells or AFP-based DNA vaccines.

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