A Phase I/II Trial Testing Immunization of Hepatocellular Carcinoma Patients with Dendritic Cells Pulsed with Four α-Fetoprotein Peptides

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Abstract α-Fetoprotein (AFP) is a self protein expressed by fetal liver at high levels, but is transcriptionally repressed at birth. AFP is up-regulated in hepatocellular carcinomas, and patients with active disease could have plasma levels as high as 1 mg/mL. We previously identified four immunodominant HLA-A*0201-restricted peptides [$hAFP_{137-145}$ (PLFQVPEPV), $hAFP_{158-166}$ (FMNKFIYEI), $hAFP_{325-334}$ (GLSPNLNRFL), and $hAFP_{542-550}$ (GVALQTMKQ)] derived from human AFP that could stimulate specific T cell responses in healthy donor peripheral blood lymphocytes *in vitro*. We conducted a phase I/II clinical trial in which HLA-A*0201 patients with AFP-positive hepatocellular carcinoma were immunized with three biweekly intradermal vaccinations of the four AFP peptides pulsed onto autologous dendritic cells (DC). DCs were prepared from adherent peripheral blood mononuclear cells cultured with granulocyte-macrophage colony-stimulating factor and interleukin-4 for 7 days. Sixteen subjects were enrolled and 10 were treated. Peripheral blood lymphocytes were isolated from these patients before, during, and after AFP peptide/DC immunization and were tested *ex vivo* with MHC tetramer and IFNγ ELISPOT analysis. Six of 10 subjects expanded statistically significant levels of AFP-specific T cells postvaccine to at least one peptide by MHC tetramer. Also, 6 of 10 subjects increased IFNγ producing AFP-specific T cell responses to at least one of the peptides postvaccination, by ELISPOT. We conclude that the human T cell repertoire is capable of responding to the AFP self antigen after the administration of AFP peptide-pulsed DC even in an environment of high circulating levels of this oncofetal antigen.

We originally reported that the self antigen α-fetoprotein (AFP) could be recognized by both murine and human T cells and serve as a tumor rejection antigen in a murine tumor model (1, 2). AFP is produced by 50% to 80% of hepatocellular carcinomas (HCC), and its measurement in serum has played an important role in diagnosis and monitoring responses to treatment for the last several decades (3). AFP is expressed by the fetal liver with serum levels of 1 mg/mL, but is transcriptionally repressed shortly after birth (4–6). Our ability to generate potent AFP-specific T cell immunity to murine AFP in mice and to human AFP in *in vitro* human T cell cultures clearly indicates that, despite being exposed to high plasma levels of this protein during embryonic development, some AFP-specific T cells are not deleted during ontogeny.

Using a combination of strategies (HLA-A*0201/K^b transgenic mice, human T cell cultures, and mass spectrometric analysis), we identified four immunodominant AFP-derived peptides that are naturally processed and presented in the context of HLA-A*0201 (1, 7–9). At least three of these peptides could be isolated from the surface of an HLA-A*0201/AFP-positive human HCC cell line, HepG2. These peptides can stimulate T cell responses, both cytotoxic and cytokine production, in bulk T lymphocyte cultures from healthy donors and patients with AFP-positive HCC. 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 conducted a phase I pilot clinical trial in which six HLA-A*0201-positive patients with AFP-positive HCC were immunized with all four AFP peptides emulsified in incomplete Freund's adjuvant at two different dose levels (Montanide ISA-51). This small dose

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Table 1. Patient characteristics

Patient*	DC dose†	Age‡	Sex§	Race‖	ECOG¶	Risk factor**	Stage††	Previous treatments‡‡
A1	1×10^6	36	F	Caucasian	0	Unknown	IVb	Chemoembo, CDDP, Adriamycin, 5-Fluorouracil, Xeloda, Thalidomide
A2	1×10^6	66	M	Asian	1	HBV	IVa	Chemoembo
B2	1×10^6	60	F	Hispanic	1	Unknown	IVa	Surgery
B3	5×10^6	55	M	Caucasian	0	HCV	IVa	Chemoembo RFA
B5	5×10^6	72	F	Caucasian	1	HBV	IVa	Chemoembo, Carbo, Taxol, Xeloda
B6	5×10^6	53	M	Hispanic	0	HCV	IVb	X-ray therapy
B8	5×10^6	60	M	Caucasian	1	HCV	IVb	Chemoembo
B9	1×10^7	55	M	Caucasian	1	HCV	IVa	None
B11	1×10^7	60	M	Caucasian	1	Porphyria	IVa	Chemoembo
B12	1×10^7	52	M	Caucasian	0	Unknown	III	Chemoembo RFA

*Patient designation.
†Number of DC per injection.
‡Patient age.
§Patient gender.
‖Patient race.
¶Eastern Cooperative Oncology Group status at enrollment.
**Etiology of HCC.
††Stage of disease.
‡‡Previous treatments received (Chemoembo, chemoembolization; CDDP, cisplatin; RFA, radiofrequency ablation; Carbo, carboplatin).
§§Sites of HCC at enrollment.
‖‖Baseline serum AFP level on day of first vaccine, ng/mL.
¶¶Last tested serum AFP level (day listed), ng/mL.
***Clinical response.
†††Progression-free survival duration.
‡‡‡Overall survival duration. At final follow up (June, 2005), all patients were deceased due to disease progression.
§§§Sites of tumor progression.

escalation trial showed that these patients could generate AFP-specific T cell responses by MHC tetramer enumeration and IFNγ ELISPOT functional analysis, and that AFP immunization was safe (10).

Considerable data from murine tumor models supports the use of immunostimulatory dendritic cells (DC) as an optimal vehicle for vaccination. We have conducted two clinical trials with DC in melanoma and observed both immunologic and clinical activity in both the phase I and phase II trials using granulocyte-macrophage colony-stimulating factor/interleukin-4 (IL-4) DC vaccines (11, 12). Therefore, we elected to test this same DC protocol for AFP peptide-based immunization in HCC. Here, we describe our phase I/II dose escalation trial testing immunization of HLA-A*0201+ HCC patients with AFP-expressing tumors with a vaccine consisting of autologous DC pulsed with four immunodominant AFP-derived peptides. We find that this vaccine was a potent immunologic stimulus, but did not result in objective clinical responses in this group of HCC patients.

Materials and Methods

Clinical trial design. Sixteen patients were enrolled and 10 were fully treated in this phase I/II, dose escalation, single-site study to evaluate the safety and immunologic effects of AFP peptide-pulsed autologous DC in HLA-A*0201 subjects with AFP-expressing HCC. Increasing doses of AFP peptide-pulsed DC (1×10^6, 5×10^6, and 1×10^7) were given to groups of three to four patients intradermally. Patients received three biweekly vaccinations. All 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 show immune competence by a positive skin delayed hypersensitivity test to at least one recall antigen (candida, tetanus toxoid, or mumps). All subjects provided signed informed consent. This trial underwent review and approval by the Institutional Review Board (No. 00-01-026) and the Internal Scientific Peer Review Committee at University of California at Los Angeles, and the Food and Drug Administration (BB IND No. 9395).

Patient characteristics. The characteristics of each patient are shown in Table 1. All were stage III (1), IVa (6), or IVb (3), and 9 of 10 were heavily pretreated, with an average age of 56.9 (range, 36-72). Pretreatment serum AFP averaged 2,469.1 ng/mL (range, 96-6,310). Risk factors for HCC were HCV (4), HBV (2), porphyria (1), or unknown (3). There were seven males and three females. All had liver disease, three patients (A1, B6, and B8) also had additional metastases.

Six subjects (A3, A4, B1, B4, B7, and B10) were enrolled but not fully treated. Two subjects (A3 and B7) could not generate acceptable DC by this protocol and A3 could not be treated, whereas B7 had early progression and could not have additional vaccinations attempted. Three subjects (A4, B1, and B10) progressed quickly after leukapheresis, two without receiving a vaccination (A4 and B10), one (B1) after the

Table 1. Patient characteristics (Cont'd)

Sites of disease[§§]	Pre-AFP[‖‖]	Post-AFP[¶¶]	Response***	PFS (mo.) [†††]	OS (mo.) [‡‡‡]	Progression site[§§§]
Liver, Bone, Lung	2,811	2,748 (d28)	Progressive	0	4	Liver, lung
Liver	4,740	7,053 (d35)	Progressive	0	20	Liver
Liver	5,100	9,750 (d56)	Progressive	0	4	Liver
Liver	102	61 (d35)	No evidence of disease	0	35	
Liver	1,630	2,515 (d112)	Progressive	0	11	Liver
Liver, bone	712	5,980 (d112)	Progressive	0	10	Liver
Liver nodes	96	134 (d112)	Progressive	0	6	Liver
Liver	2,963	3,909 (d28)	Progressive	0	2	Liver
Liver	6,310	10,558 (d56)	Progressive	0	3	Liver
Liver	227	2,170 (d112)	Progressive	0	9	Liver

first DC vaccination. One subject (B4) did not complete leukapheresis and could not be vaccinated due to early progression.

Toxicity and clinical response. Toxicity and response assessments were followed by blood chemistry tests including albumin, bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, and AFP. Clinical response, although not a primary goal of this study, was assessed by Response Evaluation Criteria in Solid Tumors standards, and patients were followed by CT scans and MRI. As of the last follow-up date, all patients had expired due to progressive disease. No toxicities were observed which were attributed to vaccine administration.

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 University of California at Los Angeles Peptide Synthesis Facility (Dr. Joseph Reeve, Jr.). Peptides were synthesized on an Advanced Chem Tech 396 Multiple Peptide Synthesizer using industry *N*-(9-fluorenyl)methoxycarbonyl procedures, and purified by reversed phase high-pressure liquid chromatography on C-18, C-4, and phenyl columns until >95% purity was reached. Peptides were characterized by mass spectral analysis, and microsequence analysis on an Applied Biosystems (Foster City, CA) sequencer and high-performance capillary electrophoresis. 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 show their ability to generate AFP-specific cellular responses. These peptides were approved for clinical use under IND 9395.

AFP peptide-pulsed DCs. A single unmobilized leukapheresis processing one plasma volume was done to obtain peripheral blood mononuclear cells (PBMC), which were isolated by Ficoll-Hypaque (Amersham-Pharmacia, Piscataway, NJ) gradient centrifugation and cryopreserved in RPMI 1640 (Life Technologies, Gaithersburg, MD) plus 20% heat-inactivated autologous serum, and 10% DMSO (Sigma, St. Louis, MO). DCs were generated from adherent PBMC using granulocyte-macrophage colony-stimulating factor (800 units/mL, Immunex, Seattle, WA) and IL-4 (500 units/mL, Schering-Plough, Kenilworth, NJ) as previously described (11). On the day of immunization, DC were harvested, washed, and pulsed separately with 10 μg/mL each of AFP peptide in serum-free RPMI 1640 for 1 to 2 hours, then washed and pooled. After confirming sterility results and DC phenotype lot release criteria [highly positive for CD86 (B7-2) and HLA-DR], DC were prepared for immunization by washing and resuspending in saline. Patients received pretreatment with 50 mg diphenhydramine and 650 mg of acetaminophen, both orally, and an intradermal test dose consisting on a $1/10^4$ dilution of the final vaccine. After 30 minutes of observation for possible allergic reactions, the full dose was injected intradermally in the lower abdominal region above the groin.

Vaccine lot release criteria. For release testing of each DC vaccine, the clinical grade peptides, the leukapheresis cell product, all reagents used for vaccine preparation, and each vaccine preparation were tested for *Mycoplasma*, endotoxin, bacterial, and fungal sterility, and followed for 14 days of culture to detect contaminants. Viability acceptance criterion was >70% viable by trypan blue exclusion. For vaccine phenotype, fresh samples were stained immediately for cell surface markers CD86, HLA-DR, and CD14 (all from Caltag, Burlingame, CA). Large granular lymphocytes were gated on by forward and side scatter and mean fluorescence intensity was determined for the entire DC population (Fig. 1A). The percentage of DC in the total cell population was determined by the percentage of CD86+/HLA-DR+ large granular lymphocytes of the total events (over the threshold size). Based on these criteria, the mean DC content of the vaccines was 24.7% (range, 3-50%). This is similar to the 32% DC in the melanoma DC vaccine trial we observed following a closely related protocol (11).

For additional phenotypic characterization of the DC vaccines, many vaccines were stained with a panel of antibodies: CCR6, CCR7 (BD-PharMingen, San Diego, CA), CD40, CD80, and CD83 (Caltag) in addition to CD14, CD86, and HLA-DR. Analyses were done on an LSR, Caliber, or FACScan II FACS machine (BD Biosciences, San Jose, CA). Samples of healthy donor DC were stained in parallel, and 10,000 large granular lymphocyte events were collected.

ELISA. On day 7 of each DC vaccine, three 1-mL cell-free supernatant samples were removed after pelleting the DC, and frozen at −80°C until use. Several batched DC culture supernatant samples were assayed by ELISA for IL-1β (3.9 pg/mL sensitivity, PharMingen), IL-6 (2.3 pg/mL sensitivity, BD Biosciences), IL-10 (62.5 pg/mL sensitivity, PharMingen), IL-12 p70 (7.8 pg/mL sensitivity, PharMingen), IL-15 (1.9 pg/mL sensitivity, PharMingen), and tumor necrosis factor-α (3.9 pg/mL sensitivity, PharMingen). Healthy donor DC culture supernatants were assayed in parallel.

Immunologic monitoring

Tetramer analysis. Tetramers were obtained from the Tetramer Facility sponsored by the NIAID and from Immunomics (Beckman-Coulter, San Diego, CA). AFP_{542} would not fold properly into the A2.1 tetramer, nor would an anchor-substituted version (8). Patient PBMC from each time point were thawed simultaneously and treated with DNase (0.002%), then 10^6 PBMC (or 3×10^5 CD8+ purified cells, Miltenyi Biotec, Auburn, CA) were stained with each individual tetramer plus CD8 (Caltag) and antibodies used to gate out non-CD8+ lymphocytes (CD4, CD13, and CD19; Caltag). In some cases, additional antibodies were used as described to obtain additional phenotyping data from tetramer-stained cells. Staining was done at room temperature for 30 minutes in the dark. The cells were then washed and analyzed immediately. The range of lymphocyte events acquired was 33,000 to 136,000, and the range of total CD8+ lymphocyte events collected was 11,000 to 81,000. The lymphocytes were gated on by forward and side scatter, and cells positive for CD4, CD13, and CD19 were gated out. The AFP peptide-specific cells were a distinct population of CD8+/tetramer+ cells (Fig. 2). When sufficient cells were available (patients A1, A2, B2, B3, B5, B6, B8, and B9), MART-1_{27-35} tetramers (NIAID) or negative tetramers (Coulter/Immunomics) were used as negative controls which should not change over time (not subtracted). Background tetramer staining was detected with staining of healthy donor PBMC (from a single healthy donor leukapheresis) and was subtracted from all patient sample frequencies (as a control for different lots and different manufacturers of AFP tetramers). When sufficient cells remained after the initial analysis (consistently for prevaccine leukapheresis, rarely for postvaccine time points), tetramer and ELISPOT assays were repeated with similar results.

Criterion for positivity was >0.02% CD8+ lymphocytes, and change of 2-fold postvaccination with the additional limitation that at least two time points must be positive. A patient with only one time point positive for only one peptide would not be considered a true positive immune response by tetramer.

IFNγ ELISPOT. The ELISPOT technique was used as previously described (1, 13, 14). PBMC were thawed as above, then T cell restimulation was done overnight in the ELISPOT plate with titrated PBMC (or purified CD8+ cells) in duplicates, at $2 \times 10^5/10^5/5 \times 10^4$ patients cells per well (six wells total per condition) incubated with 10^5 JY or K562/A2.1 cells per well pulsed with peptides. In cases of limited cells, patient cells were used at reduced concentrations. Due to variability in the viability and activity of PBMC cryopreserved from HCC patients at different time points, the ELISPOT is done in batch by stimulating PBMC with the immunizing AFP-derived peptides, no peptide or control peptide (MART-1_{27-35}, which should not vary between vaccine immunizations). JY or K562/A2.1 cells without CTL also served as a negative control for any cytokines potentially produced by these cells (always negligible). The IFNγ antibody (PharMingen)

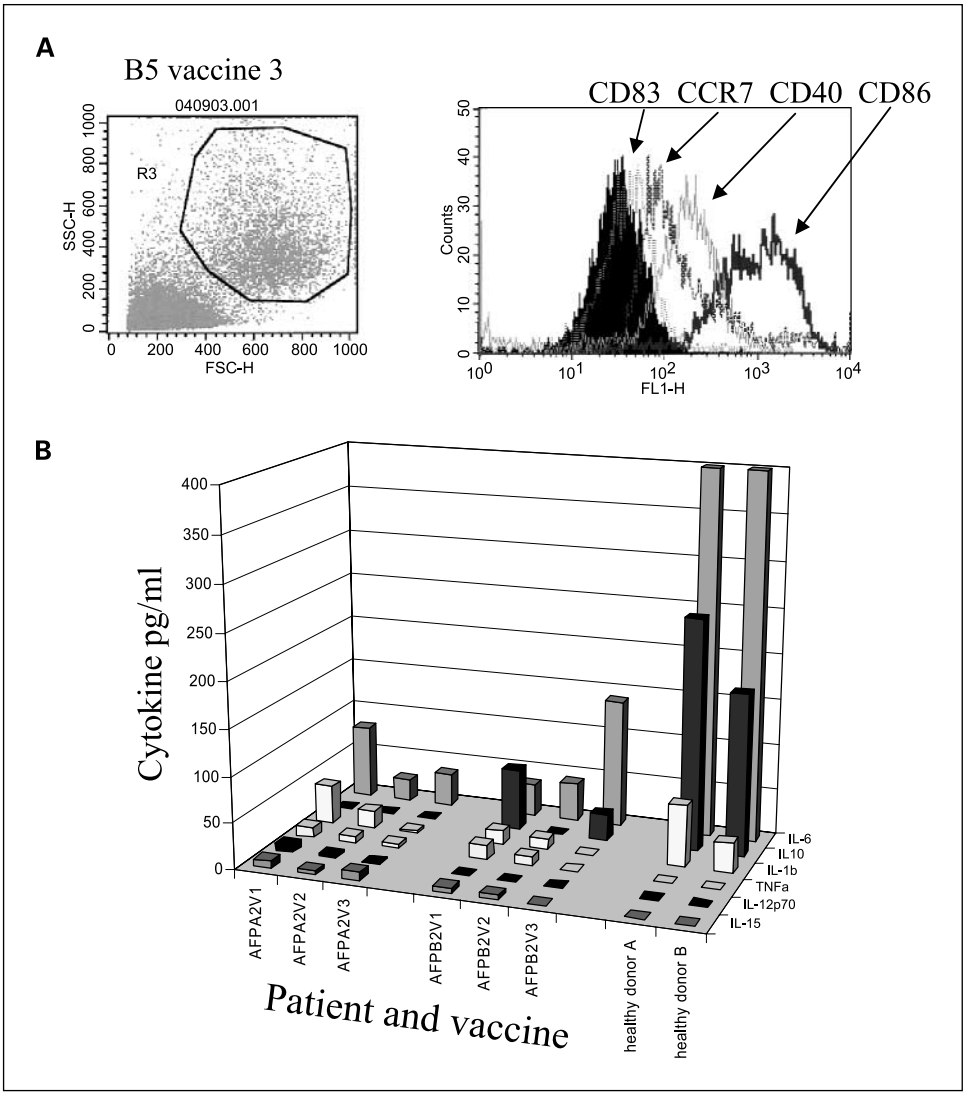

Fig. 1. HCC patient DC vaccine phenotype and culture cytokine milieu. *A*, an example of the DC flow cytometric analysis. DC were identified by forward and side scatter as large granular lymphocytes. They were 90%-100% HLA-DR+ (data not shown) and 90%-100% CD86+, indicating that they were DC. In many cases, they were further characterized by expression of the costimulatory molecule CD40, the lymph node trafficking marker CCR7 and maturation marker CD83. The third vaccine from patient B5 is shown. *B*, ELISA assays were performed on day 7 culture supernatants on the day of DC vaccine harvest. The patient and healthy donor samples were thawed and tested in parallel for the six cytokines shown. Averaged yields of duplicate wells are shown.

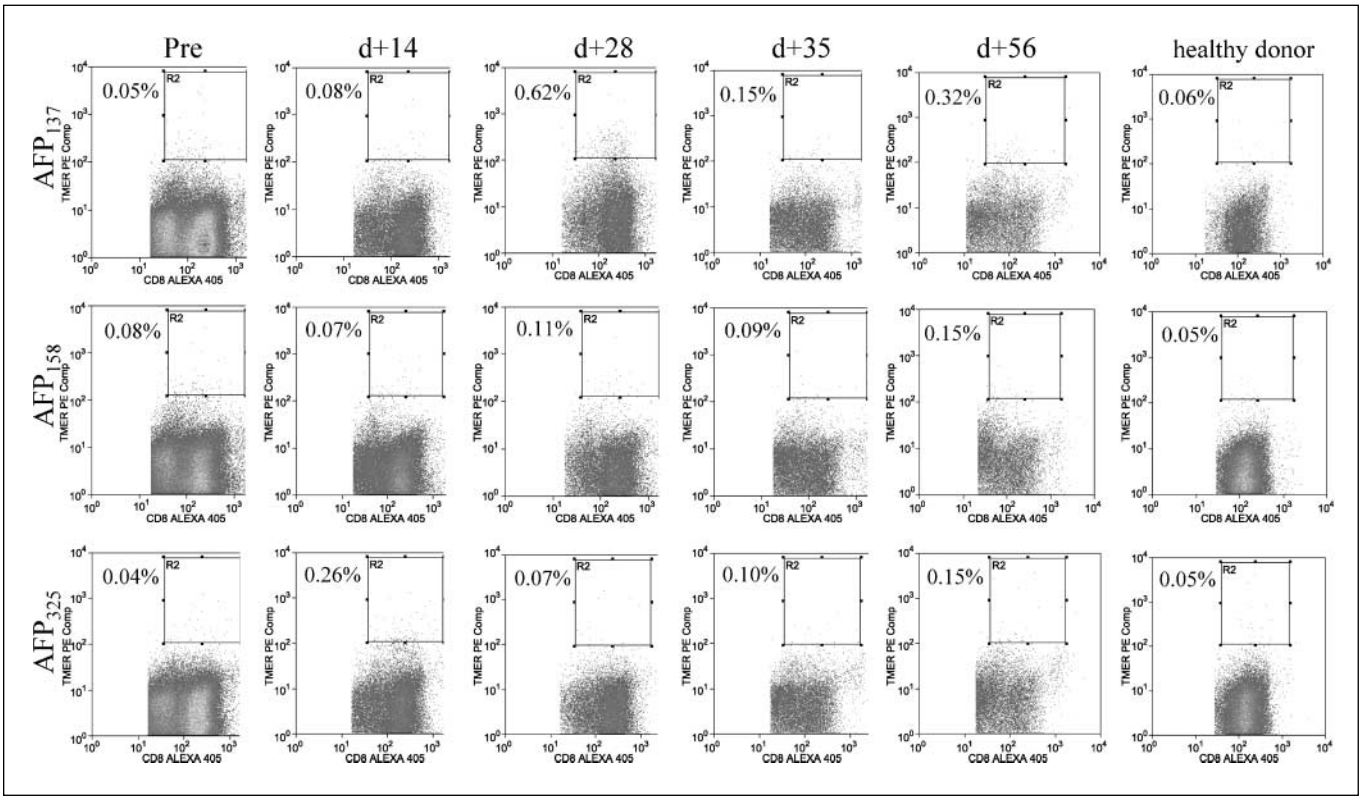

Fig. 2. Example of patient B11 MHC tetramer analysis. The raw tetramer data for each AFP tetramer at each time point (pre, d+14, etc) is shown, as well as healthy donor control staining (background). The CD8+ lymphocytes were gated on (CD8 alexa 405), and tetramer-positive cells (TMER PE) were gated on as shown by the boxed regions (R2).

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 and counts confirmed in an automated ELISPOT counter (Zeiss, Thornwood, NY or CTL Technologies, Cleveland, OH). Any background spots from patient cells plated with the restimulator cells (JY, K562/A2.1) without peptide were subtracted.

Criterion for positivity was >10 spots and a change of 2-fold postvaccination with the additional limitation that at least two time points must be positive. A patient with only one time point positive for only one peptide would not be considered a true positive immune response by ELISPOT.

Statistical analysis. Statistical tests were Wilcoxon rank sum, except for analysis of immune response and overall survival, which is log-rank. All *P* values are exact. Immune response criteria are that a positive response is at least two consecutive responses for any peptide; one time point responses to individual peptides are not considered positive.

Results

Patients and vaccination. Ten patients were treated in this peptide-pulsed DC trial (described in detail in Materials and Methods). All had biopsy-proven HCC which expressed AFP, and expressed the HLA-A*0201 class I allele (Table 1). Most patients were heavily pretreated with surgery and/or chemotherapy, and had unresectable or metastatic disease. Patients received three biweekly injections of DC pulsed separately with each of four AFP peptides injected into intradermal sites. There were three doses delivered (1×10^6, 5×10^6, and 1×10^7) in three cohorts. An additional subject (B8) received the 5×10^6 dose due to altered timing of the vaccines delivered to the previous subject (B6, vaccine no. 2, delayed 1 week).

Toxicity. Thirty-three doses of DC vaccines were given, two subjects (A4 and B1) received two and one vaccines (respectively) but did not complete three vaccinations due to worsening of liver function (A4), and worsening of performance status (B1). Several patients had increases in some or all liver function tests over time (B2, B3, B5, B8, B11, and B12) or transient increases (A2 and B6). There were no adverse events attributable to vaccination. Nine of 10 vaccinated patients progressed during the enrollment period, and there was no evidence of objective clinical responses by Response Evaluation Criteria in Solid Tumors standards. As of the final follow up (June, 2005), all patients were deceased due to disease progression.

DC vaccine phenotype. The phenotype of these DC was studied first, to confirm the expression of critical antigen-presenting cell markers (CD86 and HLA-DR) for vaccine release criterion, and second, to determine maturation and trafficking markers for more detailed analysis. A representative phenotype for these DC is shown in Fig. 1A. The DC expressed high levels of CD86 (100%) and HLA-DR (100% of DC vaccines), they also expressed CD40 (96%), and some vaccines expressed CD80 (31%). A similar percentage of DC vaccines expressed a low level of maturation marker CD83 (31%) and lymph node trafficking marker CCR7 (41%). These DC retained expression of CD14 (100%), and were, therefore, not mature.

DC culture cytokine milieu. The culture supernatant from the DC was saved to test for the presence of Th1 or Th2-biasing cytokines or growth factors for T cells. Supernatants were tested

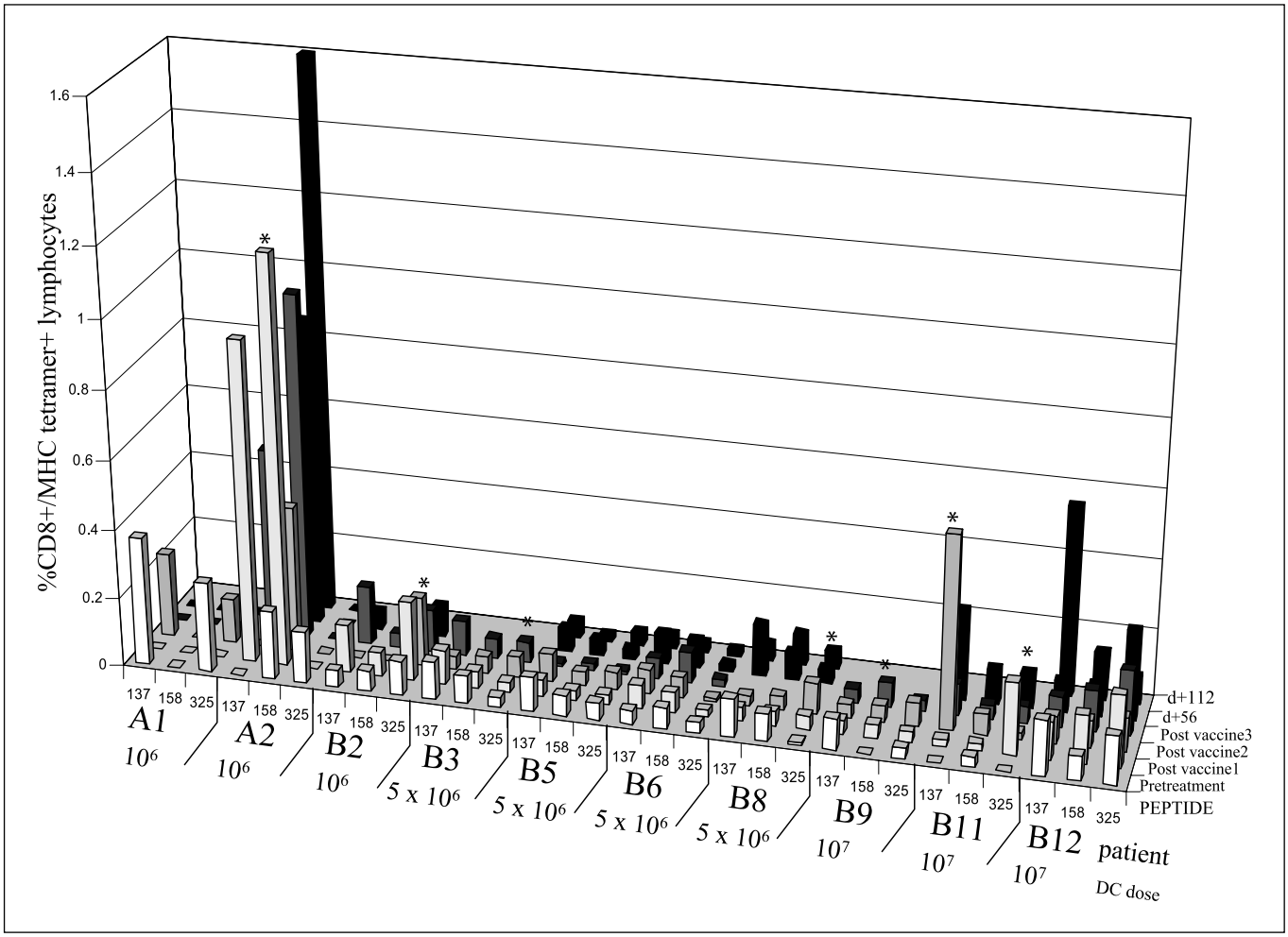

Fig. 3. Tetramer analysis summary of patient PBMC samples. Cumulative data or the percentage of CD8+ lymphocytes staining positive for AFP MHC tetramers is shown. The *z*-axis shows patient identifiers, dose of vaccine received, and the peptide MHC tetramer tested. Significant changes in frequencies are marked with an asterisk.

for IL-1β, IL-6, IL-10, IL-12p70, IL-15, and tumor necrosis factor-α. An example of this analysis is shown in Fig. 1B, comparing healthy donor DC culture with HCC patient samples. For IL-6, IL-10, and IL-1β, patient samples had reduced levels.

The autologous serum from these HCC subjects contains AFP which has recently been shown to interfere with DC function at concentrations >10 μg/mL (15). Although none of the fully treated patients had serum AFP at that level, two enrolled patients (A4 and B1) with rapidly progressive disease prior to AFP/DC administration, had AFP levels of 10,800 and 77,000 ng/mL, respectively. The percentage of DC in culture, DC cell surface phenotype, and DC culture cytokine milieu were similar in those high-serum AFP subjects (data not shown) when compared with the treated patients (highest AFP, 6,310 ng/mL at enrollment; Fig. 1A and B).

Immunologic 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 flourochrome-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. Peripheral blood lymphocytes were isolated from these patients before, during, and after AFP peptide immunizations and cryopreserved. All of the samples collected from each patient were then thawed simultaneously and subjected to tetramer (Fig. 2, data from patient B11; Fig. 3, summary of all data) and ELISPOT analysis (Figs. 4 and 5) without *in vitro* restimulation.

MHC tetramer analysis. By tetramer analysis, statistically significant expansion of CD8+ T cells capable of binding AFP peptides 137, 158, and 325 was observed in 6 of 10 patients for one to three peptides (no increases in patients A1 and B5 postvaccination; single time point increase only for peptide 137 in B6 and nonconsecutive increases for B12). Some striking responses were seen (Fig. 2, patient B11, day +28, AFP_{137}, and day +14 AFP_{325}; Fig. 3, patients A2 and B11) in which >0.5% of circulating CD8 T cells had T cell receptors capable of binding these peptides after immunization.

The majority of these tetramer responses were modest, with prevaccine frequencies averaging 0.08% (range, 0-0.37%) and postvaccine frequencies averaging 0.12% (range, 0-1.63%). We noted that the tetramer staining intensity was low (*γ*-mean), compared to staining with foreign viral peptide tetramers

(flu, cytomegalovirus, EBV, and data not shown). Although this could indicate low-affinity T cells, it is similar to the staining intensity we observed in the previous AFP peptide trial (10), as well as the staining in healthy donor *in vitro* expanded AFP peptide–specific T cells (data not shown); hence, this is not an HCC patient–specific phenomenon. We next wished to determine the functional state of these cells.

IFNγ ELISPOT analysis. In the IFNγ ELISPOT analysis, all four peptides could stimulate and expand peripheral T cells capable of cytokine production when restimulated overnight with peptide (Figs. 4 and 5). In six patients, statistically significant, consecutive responses to multiple peptides were generated (A1, B2, B3, B6, B8, and B9). In patient A2, increases were nonconsecutive for each peptide, for B5, the only response was at a single time point (d112) to AFP_{325}, for B11 and B12, responses were never greater than the 10 spot minimum at consecutive time points.

Examples of average spot counts over background (with controls), are shown in Fig. 4. In Fig. 4A, the first patient treated, A1, is shown. This patient had low levels of IFNγ-producing AFP peptide-specific T cells prevaccine, only slightly higher than nonspecific peptide (MART-1_{27-35}) control. These levels increased 2 weeks after the second vaccine (day +28) and remained elevated. Patient B3 (Fig. 4B) had no detectable AFP-specific IFNγ production prevaccine. These preexisting T cells (detected by MHC tetramer; Fig. 3) became activated to produce IFNγ 2 weeks after the first vaccine (day +14) and remained elevated until at least a week after the third vaccine (day +35). The data from each subject is shown in Fig. 5. One striking response was observed in cells from patient B6. This subject had functional IFNγ-producing AFP-specific cells prevaccine, in particular, for peptide AFP_{158}. Overall, when comparing this functional data to the MHC tetramer data, in five of six positive patients, the assays were concordant.

Serum AFP. Each patient was followed serially for serum AFP. In most cases, as patients progressed, the serum AFP levels increased at different rates (Table 1). In two patients (A1 and B3), there were decreases in serum AFP, although for A1, the level of decrease is within the expected variability of the assay. Whereas some patients experienced transient decreases in serum AFP during the immunization period (B5 and B8), they were elevated over the baseline at the last time point measured. One patient, B3, had no evidence of disease for 35 months postvaccination and had overall reduced serum AFP (Table 1). We do not attribute this response to the DC vaccination because B3 responded to chemoembolization and radiofrequency ablation, and then received the AFP/DC vaccines in the early postchemoembolization period, whereas his AFP was still in a downward trend, which could explain his decreasing AFP. He later relapsed and passed away 35 months after enrollment.

Discussion

We report here that AFP-specific CD8 T cell responses can be expanded in patients with advanced HCC with very high circulation levels of AFP protein after immunization with AFP peptide-pulsed autologous DC. The toxicity of this vaccination was limited to decreased performance status and liver function, which is expected in progressing HCC.

Considerable controversy exists in the literature regarding the optimal protocol and cytokine combination for the generation of immune-stimulatory clinical grade DC, and the optimal phenotype of these cells (16–19). It has been suggested that DC cultures that have not undergone a final dedicated maturation step, or DC cultures preferentially producing type 2 (or Th2)-biasing cytokines like IL-10 may induce antigen-specific tolerance rather than activation. We began the trial enrollment in January 2001, and prepared autologous DC by the same protocol used in our related trial of MART-1_{27-35} peptide-pulsed DC in melanoma patients (11, 12) in which loosely adherent PBMC are cultured in RPMI 1640/5% autologous serum supplemented with 800 units/mL of granulocyte-macrophage colony-stimulating factor and 500 units/mL of IL-4 for 7 days, with no subsequent maturation step. This protocol had successfully generated DC which had clinical efficacy in the melanoma subjects. Although this protocol was not successful in every enrolled subject (A3), there was evidence for successful immune activation in the majority of immunized subjects, indicating that the DC vaccines were not tolerogenic. We compared the serum AFP levels and phenotype of DC generated and did not observe any correlation between the ability to make DC or DC phenotype and serum AFP concentration.

The majority (6 of 10) of patients expanded statistically significant levels of AFP-specific T cells to the majority of immunizing peptides using the strict criteria of multiple responses, eliminating single responses to individual peptides.

Many of the immunologic responses were only transiently detected in the peripheral blood. This may reflect the trafficking

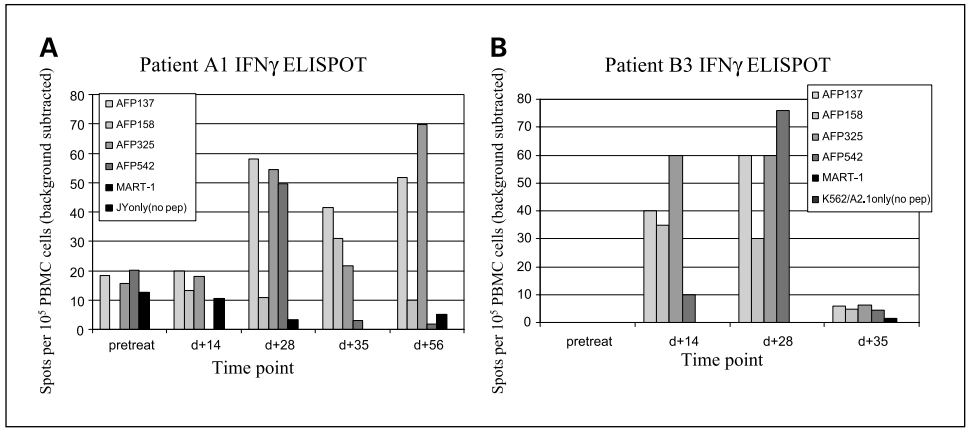

Fig. 4. Examples of IFNγ ELISPOT analyses of patient PBMC showing patients A1 and B3. The average number of INFγ spots observed per 10^5 PBMC plated is shown. The time points tested are shown with the AFP peptides and MART-1_{27-35} negative peptide tested. Any spot detected using T cells with restimulator cells alone ("no peptide") (*A*, JY; *B*, K562/A2.1) is background and is subtracted.

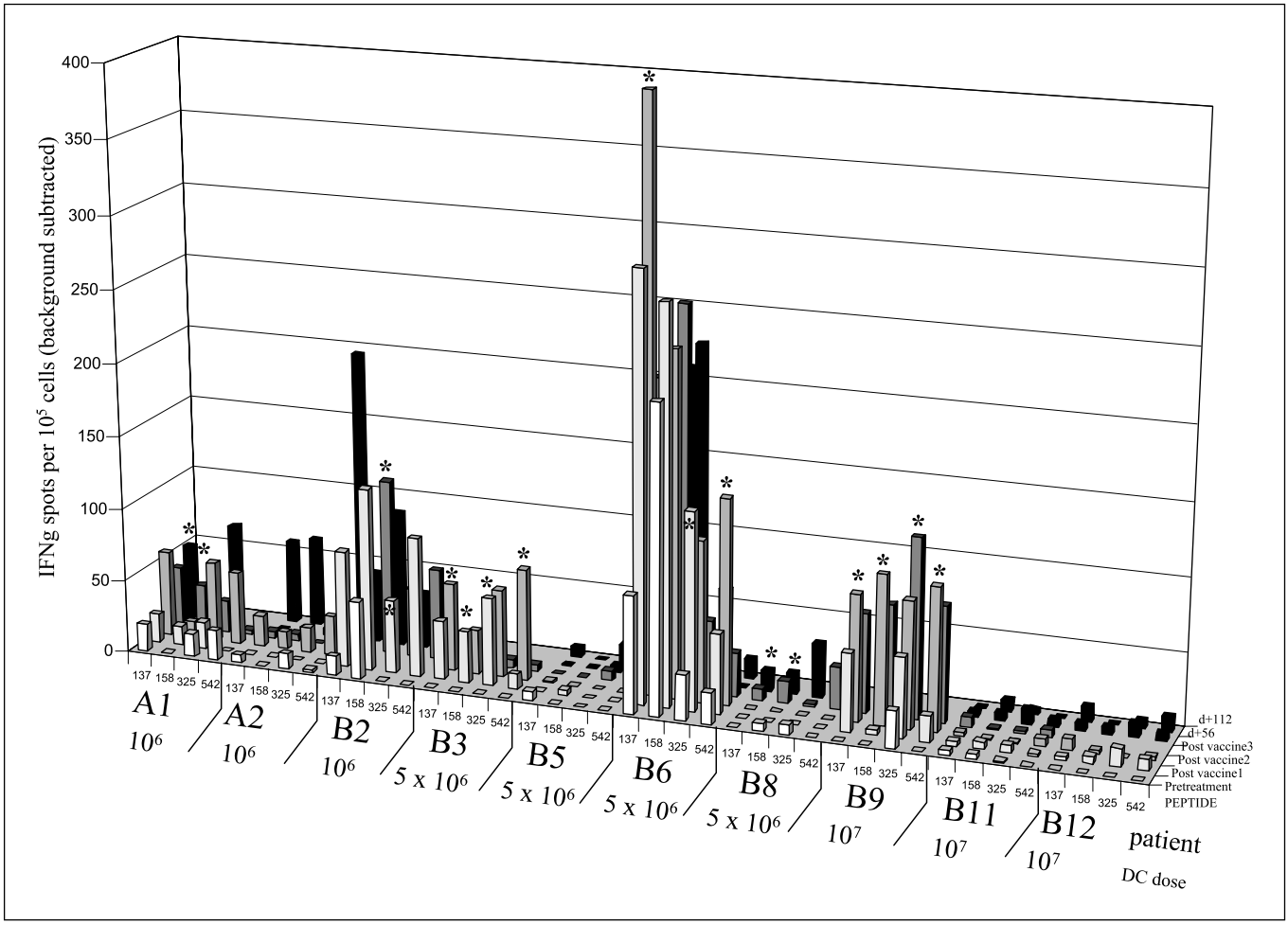

Fig. 5. Summary of IFNγ ELISPOT analysis of patient PBMC. Cumulative data for the number of PBMC secreting INFγ in response to AFP peptides is shown. The *z*-axis shows patient identifiers, dose of vaccine received, and the peptides tested. Significant changes in frequencies are marked with an asterisk.

of activated cells to sites of antigen expression by tumor (difficult to assess in HCC) or the short life span of these cells. Recent data, in particular, in murine models, has addressed the critical role of CD4 helper T cells in optimal function and proliferation of CD8 T cells. Our own data in murine models and in human *in vitro* T cell cultures has found DC expressing the entire tumor antigen (engineered with adenoviral vectors) to be superior to DC pulsed with MHC class I-restricted peptides (20). Of the possible explanations for limited, transient CD8 T cell responses, lack of CD4 help leading to "helpless" CD8 T cells of limited function may be important.

There were too few patients in this pilot study to draw meaningful conclusions with regard to a dose-response effect, response kinetics, or such patient characteristics as HBV/HCV status, stage of disease, or serum AFP level. None of these comparisons with the immune response data yielded statistically significant correlation. As before, we found instances of patients with high levels of AFP tetramer staining and little IFNγ ELISPOT responses (B11 and B12). In these cases, the tetramer-binding cells may be anergic, or they may produce another cytokine. Conversely, B6 did not show strong increases in tetramer staining with vaccination, yet had baseline AFP-specific IFNγ-producing cells by ELISPOT which did increase in frequency over the vaccination time. This may indicate that a high percentage of the tetramer-binding cells were capable of IFNγ production and this fraction increased with vaccination, without much proliferation by these cells.

Several small pilot studies have been done testing antigen-presenting cell-based immunizations in HCC. In a trial with activated B cells from tonsils fused (via polyethylene glycol) to tumor cells (Wu et al., 1995 AACR abstract), 11 patients were treated and three partial tumor responses were reported. Three publications have tested DC pulsed with tumor or tumor lysate. First, two patients with metastatic HCC were treated with immature DC (21), and one had slowed tumor growth compared their pretreatment status. Second, DC loaded with tumor lysate, stimulated with tumor necrosis factor-α, and mixed with keyhole limpet hemocyanin before injection were tested (22). Ten subjects with unresectable HCC were treated, seven developed positive delayed-type hypersensitivity responses to the keyhole limpet hemocyanin (indicating successful vaccination), and one subject had a mixed tumor response. Third, tumor lysate loaded DC matured with tumor necrosis factor-α, and mixed with IL-2, were tested in a mixed population of subjects, two of whom had HCC (23). There were no tumor responses reported. Utilizing tumor without DC, a randomized phase II trial was published in which tumor was mixed with granulocyte-macrophage

colony-stimulating factor, IL-2, and Bacillus Calmette-Guerin (24). Forty-one stage I-IIIa subjects, postcurative resection, were enrolled and randomized; 19 received vaccine. Overall, treated patients had statistically significant improvements in risk of recurrence, time to recurrence as well as recurrence-free survival. In this trial, overall survival was also improved at $P = 0.01$. Lastly, 17 patients with metastatic gastrointestinal carcinomas (nine had primary liver tumors) were treated with AdVIL-12 transduced DC intratumorally (25). Treatment induced a marked increase of infiltrating CD8+ T lymphocytes in 3 of 11 tumor biopsies analyzed. A partial response was observed in one patient with pancreatic carcinoma. Together, the trials thus far indicate that immunotherapy strategies for HCC are safe and have biological effects, although to date, clinical benefit is limited.

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. We are currently testing the hypothesis that robust and clinically beneficial immune responses can be generated if AFP is presented to both CD8 and CD4 T cells by more mature DC on AFP-engineered DCs as well as by AFP-based DNA vaccines.

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