Published in final edited form as:
Cancer Immunol Immunother. 2010 October ; 59(10): 1467–1479. doi:10.1007/s00262-010-0871-8.

WT1 PEPTIDE VACCINATIONS INDUCE CD4 AND CD8 T CELL IMMUNE RESPONSES IN PATIENTS WITH MESOTHELIOMA AND NON-SMALL CELL LUNG CANCER

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Abstract

BACKGROUND—The transcription factor, WT1, is highly overexpressed in malignant pleural mesothelioma (MPM) and immunohistochemical stains for WT1 are used routinely to aid in its diagnosis. Using computer prediction analysis we designed analog peptides derived from WT1 sequences by substituting amino acids at key HLA-A0201 binding positions. We tested the safety and immunogenicity of a WT1 vaccine comprised of four class 1 and class 2 peptides in patients with thoracic neoplasms expressing WT1.

METHODS—Therapy consisted of six subcutaneous vaccinations administered with Montanide adjuvant on weeks 0, 4, 6, 8, 10, and 12, with 6 additional monthly injections for responding patients. Injection sites were pre-stimulated with GM-CSF (70mcg). Immune responses were evaluated by DTH, CD4 T-cell proliferation, CD8 T-cell interferon gamma release, intracellular cytokine staining, WT1 peptide MHC-tetramer staining, and cytotoxicity against WT1 positive tumor cells.

RESULTS—Nine patients with MPM and 3 with NSCLC were vaccinated, with 8 patients receiving at least 6 vaccinations; in total, 10 patients were evaluable for immune response. Six out of nine patients tested demonstrated CD4 T-cell proliferation to WT1 specific peptides, and five of the six HLA-A0201 patients tested mounted a CD8 T-cell response. Stimulated T cells were

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capable of cytotoxicity against WT-1 positive cells. Vaccination also induced polyfunctional CD8 T cell responses.

CONCLUSIONS—This multivalent WT1 peptide analog vaccine induces immune responses in a high proportion of patients with thoracic malignancies with minimal toxicity. A randomized trial testing this vaccine as adjuvant therapy in MPM is planned.

Keywords

WT1; vaccine; mesothelioma; non-small cell lung cancer

INTRODUCTION

The Wilms' tumor suppressor gene, WT1, was first identified in childhood renal tumors, but it is also highly expressed in multiple other hematologic and solid tumors including mesothelioma [1–5]. WT1 was originally identified by cDNA mapping to a region of chromosome 11p13. The WT1 cDNA encodes a protein containing four Kruppel zinc fingers and contains a complex pattern of alternative splicing resulting in four different transcription factors. Each WT1 isoform has different DNA binding and transcriptional activities [6], and can positively or negatively regulate the expression of various genes involved in cellular proliferation, differentiation, apoptosis, organ development and sex determination. WT1 is normally expressed in tissues of the mesodermal origin during embryogenesis including the kidney, gonads, heart, mesothelium and spleen [7]. Although originally described as a tumor suppressor gene, the WT1 proteins appear to be involved in tumorigenesis.

The strong expression of WT1 protein in mesothelioma makes it a rational therapeutic target in this disease. The expression is so common that pathologists use immunohistochemical stains for WT1 to help distinguish epithelial mesothelioma from pulmonary adenocarcinoma. In three different pathology series, the rate of WT1 nuclear staining ranged from 72% to 93% [8–10]. Although WT1 is a nuclear protein, it is processed and presented on the cell surface in the context of MHC molecules [1]. For this reason, WT1 could be targeted using a T cell based immunotherapeutic approach [1]. Indeed, at a National Cancer Institute immunotherapy workshop, WT1 achieved the top priority ranking among potential antigens [11]. Several groups have pursued vaccine strategies against WT1, though these studies have been conducted primarily in patients with myeloid malignancies and none have enrolled patients with mesothelioma [12–13].

WT1 protein is a self-antigen, and, as a result, breaking tolerance is necessary for effective vaccination. One strategy to circumvent the poor immunogenicity of tumor-associated peptides is to design synthetic analog peptides that will be more immunogenic. Such “heteroclitic” peptides could generate an immune response that recognizes the immunizing epitopes and also cross-reacts with the original native peptides. By using computer prediction analysis, we designed a large number of synthetic peptides derived from WT1 protein sequences in which single or double amino acid substitutions were introduced into the peptides at key HLA A0201 binding positions. Peptides predicted to bind with high affinity to HLA A0201 molecules were directly assayed for their ability to stabilize MHC

molecules on the surface of TAP-negative T2 cell line. The new peptides could stabilize MHC molecules better than native sequences. Avidly binding peptides were then assayed in vitro for their ability to elicit HLA-restricted, peptide-specific CTL responses using purified T cells from healthy donors. In addition, CD8+ T cells stimulated with the new synthetic peptides displayed heteroclitic features and cross-reacted with the native WT1 peptides and also were able to mediate peptide specific cytotoxicity. Importantly, T cells stimulated with the new synthetic peptides were able to kill WT1 expressing, HLA matched CML blasts [14]. In addition, we modified previously identified WT1 peptide segments by adding flanking amino acid segments in order to stimulate the CD4+ response necessary for inducing CD8 T-cell memory [15]. These lengthened peptides are recognized in multiple HLA-DRB1 settings. Using cross priming experiments, it was shown that the WT1 peptides are presented on the surface of mesothelioma tumor cells and could be recognized by the T cells stimulated by the individual WT1DR peptides. Human T cells stimulated with the analog WT1-A1 can kill WT1+ mesothelioma cell lines [14–15].

In order to broaden immunogenicity over a range of HLA subtypes, we selected four WT1 peptides to combine into a vaccine. The vaccine contains one WT1 heteroclitic peptide to stimulate CD8 responses (WT1-A1), two longer WT1 native peptides to stimulate CD4 responses (WT1-427 long and WT1-331 long) and one longer heteroclitic peptide which could stimulate both CD4 and CD8 cells (WT1-122A1 long). The peptides were combined with Montanide adjuvant before injection, and the injection sites were primed with GM-CSF. We conducted a pilot trial to determine the safety and immunogenicity of the WT1 peptide vaccine in humans. We tested the vaccine in patients with MPM, and despite the much lower expression rate, also in patients with NSCLC because responses have been reported in a prior WT1 vaccine study [12].

PATIENTS, MATERIALS & METHODS

Eligibility criteria

The trial was conducted on an FDA-approved IND for a protocol approved by the Institutional Review Board at Memorial Sloan-Kettering Cancer Center. Patients were eligible if they had 1) MPM with unresectable or relapsed disease and had received no more than one prior pemetrexed-containing chemotherapy regimen, or 2) NSCLC, either stage III or IV and completed all initial treatment with surgery and/or chemotherapy and/or radiation therapy. In either case, patients must have had tumors that stained positively for WT1 (greater than 10% of cells). Patients were required to be age ≥18 years of age and have a Karnofsky Performance Status (KPS) of ≥70%. Patients were also required to have adequate organ and bone marrow function, including: absolute neutrophil count > 1000/mcL, platelets > 50 K/mcL, total bilirubin ≤2.0 mg/dl, AST and ALT ≤2.5×upper limits of normal, creatinine ≤2.0 mg/dl. At least 4 weeks must have elapsed between the patient's last chemotherapy or radiation treatment and the first vaccination. Exclusion criteria included pregnant or lactating women, patients with an active infection requiring systemic antibiotics, patients with a serious and unstable medical illness, and patients actively taking corticosteroids. Patients were eligible regardless of HLA haplotype. Patients signed a two-

part informed consent, first to allow testing of their tumor sample for WT1 expression, and second to proceed with the WT1 vaccine treatment.

Peptides vaccine preparation and administration

Peptides used in this study were synthesized by the American Peptide Company (Sunnyvale, CA). The peptides were previously characterized for their binding to MHC molecules and ability to generate human T cell responses in vitro [14–15]. The amino acid sequences are:

Name of Peptide	Sequence
WT1-A1	YMFPNAPYL
WT1-122A1 long	SGQAYMFPNAPYLPSCLES
WT1-427 long	RSDELVRHHNMHQRNMTKL
WT1-331 long	PGCNKRYFKLSHLQMHSRKHTG

The peptides were 98% pure, sterile and endotoxin free. Each dose of vaccine was prepared by mixing 200 μg of each of the 4 peptides with equal volume of adjuvant Montanide 51. 200 μg was chosen as the dose because it is within the range of safe and active doses used in other peptide vaccines. Vaccinations were administered subcutaneously with vaccination sites rotated between extremities. Injection sites were pre-stimulated with Sargramostim (GM-CSF) (70mcg) injected subcutaneously on days −2 and 0.

Treatment Plan

Therapy consisted of six vaccinations of the WT1 peptides (1.0 mL of emulsion) administered weeks 0, 4, 6, 8, 10, and 12. Patients who had a clinical response, or who had a molecular or immunologic response without disease progression, were able to continue with up to 6 more vaccinations administered monthly.

At baseline, all patients had routine blood work, urinalysis, HLA typing, and a CT scan of the chest and other relevant disease sites. Prior to each vaccination and also on week 2, toxicities were recorded, a physical examination was performed, and blood work including a CBC and comprehensive panel was obtained. A follow up CT scan was performed at week 14 (or sooner if deemed medically necessary). Immune responses were measured at baseline, after 3 and 6 vaccinations and at the end of study, using delayed type hypersensitivity (DTH) skin testing and T-cell proliferative response assays (see below).

Evaluation of immunologic responses

Cell lines—The WT1 positive, ALL-derived cell line 697 was kindly provided by Hans J. Stauss (University College London, UK). The WT1 negative B lymphoma cell line SKLY-16 was obtained from the American Type Culture Collection. Both cell lines were HLA-A0201 positive. The cell lines were cultured in RPMI 1640 medium supplemented with 5% FCS, penicillin, streptomycin, 2 mmol/L glutamine, and 2-mercaptoethanol at 37°C, 5% CO_2. All cells were HLA typed by the Department of Cellular Immunology at

Memorial Sloan-Kettering Cancer Center. Expression of WT1 transcripts were determined by quantitative reverse transcription PCR as described earlier [14].

DTH response—DTH was performed by injecting the peptides (15mcg per peptide in 70 mL of PBS) intradermally into a marked area the forearm of the patient. A positive control (candida) was injected into a separate site and marked as well. The candida control was only performed at baseline. A positive result was read as redness and induration of >0.5 cm at 24–72 hours.

CD4 T cell response—WT1-specific CD4 T cell response was measured by proliferation of the unprimed CD4 T cells against the peptides. In brief, CD4+ T cells were purified from PBMC by standard magnetic beads isolation using anti-CD4 mAb (Miltenyi Biotec). The population contains both CD4+T cells and CD14+ antigen-presenting cells (APC), because CD14+ monocytes/macrophages also express low level of CD4. The cells (1×10^5/well) were incubated in 200 ul/well of RPMI 1640 supplemented with 5% pooled autologous plasma (AP) in 96-well round-bottomed microtiter plates for 5 days, in the presence or absence of peptides. One μCi [^{3}H]-thymidine was added to each well and 20 hours later, the cells were harvested with a Harvester Mach IIIM (Tomtec, Hamden, CT) and counted in a 1450 MicroBeta TriLux (Wallac, Turku, Finland). The measured counts/min represented mean values of quadruplicate microwell cultures. A response was called positive for reactivity with the test peptides if the result was at least 2 fold higher for the test peptides as compared to the control peptides and statistically significant.

CD8 T cell response

In vitro stimulation: To reliably detect CD8 T cell responses, we performed two rounds of stimulations of CD3 T cells, *in vitro*. Peripheral blood mononuclear cells (PBMC) from patients were obtained by Ficoll density centrifugation and frozen at −180 C until the samples from all time points were obtained and were tested at the same time. CD14+ monocytes were isolated by positive selection using mAb to human CD14 coupled with magnetic beads (Miltenyi Biotec) and part of the cells were used for the first stimulation of T cells at a ratio of 10: 1 (T: APC). The CD14 negative fraction of PBMC was used for isolation of CD3 T cells, by negative immunomagnetic cell separation using a pan T cell isolation kit (Miltenyi Biotec). Purified CD3 T cells were stimulated with immunizing peptides WT1A1, 122A1 or with their native peptides WTA1 and 122A respectively (20 ug/ml) to expand the WT1A-specific CD8 T cells. The cell cultures were carried out in RPMI 1640 supplemented with 5% autologous plasma, 1 ug/mL β2-microglobulin (β2-M; Sigma), and 10 ng/mL IL-15 (R&D Systems) for 7 days. Monocyte-derived dendritic cells (DCs) were generated from remaining CD14+ cells, by culturing the cells in RPMI 1640 medium supplemented with 1% AP, 500 units/mL recombinant IL-4, and 1,000 units/mL GM-CSF. On days 2 and 4 of incubation, fresh medium with IL-4 and GM-CSF was either added or replaced half of the culture medium. For the 122A or 122A1 cultures, 20 ug/mL 122A or 122A1 peptides were added to the immature DCs on day 5, to allow the processing of the long peptides. Maturation cytokine cocktail (IL-4, GM-CSF, 500 IU/mL IL-1, 1,000 IU/mL IL-6, 10 ng/ml TNF-α, and 1 ug/mL PGE-2), was added to all DC cultures on day 6. On day 7, the mature DCs were used for secondary stimulation of CD3+T cells at a ratio of

1: 30, with the same condition for the first stimulation. Seven days later, IFN-γ secretion of the cells was examined by enzyme-linked immunospot (ELISPOT) assay and tetramer staining.

IFN-γ ELISPOT: Autologous CD14+monocytes (10^4/well) were incubated with CD3T cells (10^5 cells) for 24 hrs, in the presence or absence of the testing peptides. All conditions were done in quadruplicate. Phytohemagglutinin (PHA, Sigma) at a concentration of 20ug/ml was used as a positive control for the assay. Spot numbers were automatically determined with the use of a computer-assisted video image analyzer with KS ELISPOT 4.0 software (Carl Zeiss Vision), as described previously[14].

Tetramer staining: HLA-A0201-PE labeled tetramer loaded with WT1 peptide (RMFPNAPYL) was constructed by the Sloan Kettering Institute tetramer core facility. CD3+T cells after two rounds of stimulations were stained with WT1A/HLA-A0201 tetramer (1:50 dilution) and mAbs against CD3, CD4, CD8 and other T cell markers (CD27, CD28, CD45RA and CCR7), followed by flow cytometry acquisition. Cells were considered positive for tetramer staining when they formed a clear population with mean fluorescence intensity that was at least 1 log above the MHC Class I negative tetramer control (Beckmann Coulter). Events (10^5) were collected after live gating on lymphocytes by forward and side scatter. 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA) was used to gate out dead cells for tetramer staining. Cells were analyzed using a CYAN-ADP flow cytometer with Summit software (Dako Cytomation California Inc., Carpinteria, CA). Analysis was performed using FlowJo software (version 8.1; TreeStar, Inc.).

Chromium-51 cytotoxicity assay: The presence of specific CTLs was measured in a standard 4-hour chromium release assay as described [14]. Briefly, target cells are pulsed with 20 μg/mL of synthetic peptides for 2 hours at 37°C, after which they are labeled with 50 μCi of $Na_2\ ^{51}CrO_4$ (NEN Life Science Products, Inc.) per one million cells. After extensive washing, target cells are incubated with T cells at various E:T ratios. All conditions were done in triplicate. Plates were incubated for 4 h at 37°C in 5% CO_2. Supernatant fluids were harvested and radioactivity was measured in a gamma counter. Percentage specific lysis was determined from the following formula: 100 × [(experimental release − spontaneous release) / (maximum release − spontaneous release)]. Maximum release was determined by lysis of radiolabeled targets in 1% SDS.

Intracellular polyfunctional cytokine staining: CD3+T cells from the same cultures as above were resuspended in 1ml 10% PHS RPMI medium at a density of 2 million cells/ml. The cells were then stimulated with the addition of peptides for the first 2 hours at 37°C and then in the presence of 5 μg/ml each of Brefeldin A and monensin (BD Bioscience) for 4 hours. PE-Cy5-CD107a (5 ul/ml BD Pharmingen) was added prior to stimulation. The cells were harvested and washed with 2 ml FACS buffer once. A total of 106 cells were resuspended in 50 μl FACS buffer and stained with the following cell surface markers for 30 min at 4°C: Pacific blue-CD3, APC-AF750-CD8 (eBioscience) and ECDCD4 (Beckman Coulter). After another wash with FACS buffer, the cells were fixed and permeabilized with 100 μl BD Cytofix/Cytoperm solution (BD Bioscience) for 20 min at 4°C, followed by two

washes with 1× BD Perm/Wash (BD Bioscience) solution. Finally, the cells were stained with the following cytokine antibodies: PE-MIP-1b, PE-Cy7-TNF-α and FITC-IFN-γ (BD Pharmingen). Samples were then acquired on a CYAN flow cytometer. Cell doublets were excluded using forward scatter versus pulsing with parameters. Gating for each cytokine or chemokine was based on a positive control sample stimulated with staphylococcal enterotoxin B (Sigma-Aldrich, St. Louis, MO) and on a negative control sample that was unstimulated. The data analysis program Simplified Presentation of Incredibly Complex Evaluations (SPICE software, version 4.1.6) was used to analyze and generate graphical representations of T cell responses detected by multi-color flow cytometry. All values used for analyzing proportionate representation of responses are background subtracted.

Statistical considerations

The primary endpoints of this pilot study were to evaluate the safety and immunogenicity of the peptide vaccine. We planned to enroll ten evaluable patients with MPM or NSCLC. Patients who completed eight weeks of the study were considered evaluable. A parallel cohort of patients with myeloid malignancies was also studied in this protocol, and those results will be reported separately.

An immunologic response in six or more out of ten patients would classify this vaccine promising for future study [16]. Clinical and molecular responses were also recorded. Anti-tumor responses were determined based on CT scans using standard RECIST criteria for NSCLC and using modified RECIST criteria [17] for the patients with mesothelioma. Toxicities were tabulated according to the NCI Common Toxicity (version 3.0). If three patients developed ≥grade 3 toxicity felt possibly, likely, or definitely related to the study drug in the opinion of the treating physician, the study was to be closed.

RESULTS

1. Patient characteristics

39 patients consented to have tumor samples analyzed for WT1 expression by immunohistochemistry, and 32 had evaluable specimens. 8/9 (89%) of the screened MPM tumors had positive staining. Five patients with mesothelioma were enrolled on the treatment portion of the study based on positive WT1 staining evaluated at the time of diagnosis; those samples were not retested. 4/23 (17%) of the NSCLC tumors had positive staining, but it was generally less intense than the MPM samples.

Nine patients with mesothelioma were treated with the WT1 vaccine, and 8 were evaluable for immune response (Table 1). This included 5 patients who relapsed after multimodality therapy 14–29 months after completion of therapy; 3 who had unresectable disease and were previously treated with chemotherapy 4–10 months previously; and one patient who was untreated. All had epithelioid histology. One patient had disease progression after just two injections and was not evaluable for immune response. Three patients with NSCLC were treated, all of whom had stage III disease treated with combined modality therapy which completed 4–7 months before enrollment. One patient withdrew consent after one vaccination, so two patients were evaluable for immune response.

2. Safety and toxicity

Seven of the 12 patients experienced grade 1 injection site reactions which included swelling, redness or pruritus. No other toxicities attributable to the vaccine were noted, and specifically no pulmonary, renal, or hematologic toxicities.

3. Induction of DTH responses

2 out of 7 patients with MPM that were tested for DTH developed a response. The 3 other patients with MPM were unable to be tested. One out of 2 NSCLC patients tested had a DTH positive reaction.

4. Vaccination induces WT1-specific CD4 T cell response

CD4 T cell response to immunizing WT1 peptides 331, 427 and 122A1 and the native peptide 122A was directly assessed by unprimed CD4+T cell proliferation. A total of nine patients were tested after 3 or 6 vaccinations: seven patients were tested at both timepoints, and patients 9 and 11 were tested only after 3 vaccinations, as summarized in Figure 1A . Prior to vaccination, none of patients showed any peptide-specific responses. The small response to 331, 427 or 122A1 seen in patient 1 on time 0 was not significant statistically ($p = 0.24$, 0.28 or 0.16, respectively). Following vaccinations, six patients (# 4, 6, 8, 10, 11 and 12) showed significantly increased proliferation to the immunizing peptides (defined as an increase of more than twice the stimulation index SI: counts per minute in the test sample divided by counts per minute in the control and a $p<.05$). Three patients (# 1, 3 and 9) did not have measurable responses to any of the peptides tested. Four patients responded to peptide 331, five patients responded to 427, and 4 patients responded to 122A1 peptides. (There were not enough cells for patient 11 to test all the peptides, and therefore, peptide 122A1 was not tested). Among the peptides tested, WT1-331 seemed to be the most immunogenic, as shown by 4 out of 6 patients who had strong responses to the peptide. Two of five patients who responded to 122A1 also showed a weak response to its native peptide 122A (data not shown). As an example of a typical raw data set, detailed data of the CD4 T cell proliferation from patient 10 are shown in Figure1 B. After 3 vaccinations, at concentrations of 20 or 50ug/ml peptides tested, CD4 T cell proliferation increased 47.5 and 39.4-fold to 331; 37.4 and 10.8- fold increase to 427 and 2.7 -fold to 122A1, respectively (middle panel). Similar responses were also detected after 6 vaccinations (lower panel).

5. Vaccination induces WT1-specific CD8 T cell responses

CD8 T cell response specific for the epitope WT1-A was measured by IFN-γ secretion, frequency of the WT1-A/HLA-A0201 tetramer positive cells and cytotoxicity against the WT1+ tumor cells. Evaluable patients who were HLA-A0201 positive were tested for their CD8 T cell response to HLA-A0201- restricted peptide WT1-A1 and WT1-A by IFN-γ ELISPOT assay (n=5) and WT1-A-specific tetramer staining (n=6). To reliably detect the peptide-specific response, CD3+T cells were stimulated with immunizing peptides and their native sequences *in vitro* for two rounds to expand the frequency of the cells. A positive response to the vaccine was defined as a 2-fold increase in IFN-γ-secreting cells and in frequencies of CD8+WT1-A tetramer+ cells, over the controls (irrelevant peptides), with at least 30 spots and $p< 0.05$.

The IFN-γ secretion from the five patients was summarized as the fold-increase over the control peptide for the time point before and after 3 and 6 vaccinations (Figure 2A) and in more detail for patient #1 for the post 9 and 12 vaccination time points (Figure 2B). CD3 T cells were stimulated with analogue peptide WT1-A1 and were tested against its native peptide WT1-A. Prior to vaccination, only one patient (#4) showed significant IFN-γ secretion by CD3 T cells specific for WT1-A peptide (50 times over the control peptide EW). Four other patients had no WT1-A specific responses before vaccination. Following vaccination, all 5 patients showed significant increases in the numbers of IFN-γ-secreting cells against WT1 peptide over the irrelevant peptide EW, though at different time points.

There was no response in patient #1 after 3 and 6 vaccinations; however, after 9 and 12 vaccinations, the response to the WT1-A native sequence increased 3 fold after WT1-A and WT1-A1 stimulation (Figure 2B).

IFN-γ secretion increased two fold for patient #3 after 6 vaccinations (p: 0.014); however, the increase was not significant compared to the pre-vaccination 1.4 fold-increase over the control peptide.

Patient #4 had very strong pre-existing WT1-A-specific ELISPOT response and no further increase in IFN-γ secretion was induced after 3 or 6 vaccinations, although there were 29 and 47 fold-increases over the control peptide at the above time points.

A strong response was seen for patient #8 after 3 vaccinations, with a 43-fold increase in IFN-γ secretion over the control. Unfortunately, there were not enough cells after 6 vaccinations to test the duration of response.

The strongest response was observed in patient 12, with a 262-fold increase in IFN-γ secretion specific for WT1-A peptide over the control, after 6 vaccinations. A similar response was also seen in the cultures when T cells were stimulated with the native WT1-A peptide (data not shown).

The frequency of WT1-A/HLA-A0201 tetramer positive CD8 T cells increased as early as after 3 vaccinations in 3 patients (#4, #8, #12) who also had increased IFN-γ secretion (Figure 3A). No increase in tetramer positive populations was detected in patients 1, 3 and 11 (not shown.) A detailed analysis on WT1-A/HLA-A0201 tetramer staining from patient #4 is illustrated (Figure 3B.) Stimulating CD3 T cells with the analogue peptide WT1-A1 would demonstrate the cross-reactivity between the analogue and its native peptides; stimulating T cells with longer peptides 122A or 122A1 would address the question of the processing and the presentation of the WT1-A epitope. Percentages of WT1-A/HLA-A0201 tetramer positive CD8 T cells from pre-, and after 3 and 6 vaccinations are shown as T0, T3 and T6. Prior to vaccination, WT1-specifc T cells comprised 1.17% of CD8 T cells, in response to WT1-A peptide stimulation. The percentage of the tetramer positive cells in this culture is much higher than the stimulation with other peptides WT1-A1, 122A and 122A1, suggesting that the WT1-specifc precursor CD8 T cells existed and expanded efficiently when the native peptide was used for the stimulation. Following vaccination, a robust increase in WT1-A specific CD8 T cells was seen in all cultures with indicated peptide stimulations. Of note is that with WT1-A1 analog peptide stimulation, the percentage of

WT1-A-specifc CD8 T cells steadily increased from 0.11% to 2.47% and 4.36%, after 3 and 6 vaccinations. This result demonstrated vaccination of patients with analog peptide WT1-A1 elicited an efficient CD8 T cell response against the native sequence (i.e. a heteroclitic response). Interestingly, stimulating cells with 122A1 class II analog peptide induced WT1-specific CD8 T cell responses, in a similar magnitude as did class I analog peptide WT1-A1, demonstrating efficient processing, presentation of, and cross reactivity to the WT1-A epitope *in vitro*.

In addition, we measured the cytotoxicity in patients who had sufficient CD3 T cells. T cells were defrosted and re-stimulated with autologous DCs, CD14+ cells, or CD40L-activated B cells (20) and cytotoxicity was measured against the WT1 positive cell line, 697, or WT1-negative cell line, SKLY-16. Both cell lines were HLA-A0201-positive and have been well defined in our previous studies as suitable to test HLA-A0201-restricted, WT-1-specific killing by stimulated T cells (13, 14) Cytotoxicity was not observed with cells obtained before vaccination, but after 3 vaccinations, CD3 T cells stimulated with WT1-A peptide were able to kill 697 cells, but not SKLY-16 cells (Figure 4A, patient #8). We also detected the similar WT1-specific cytotoxicity in patient #3 after 3 and 6 vaccinations and patient #4 after 9 vaccinations (cells from other time points were not available) (Figure 4B). These results demonstrated that the vaccinations were able to elicit a WT1-specific, HLA-A0201-restricted cytotoxic CD8 T cell response.

6. WT1-specific CD8 T cell responses are polyfunctional

The quality of T cell response is a crucial factor in defining a protective T cell response. The frequency of IFN-γ-producing T cells has been the most widely used parameter to assess vaccine-induced T cell response. However, the magnitude of a T cell response measured by a single parameter does not always reflect its full functional potential. Recent studies in infectious diseases have used multiparameter flow cytometry to characterize the quality of the T cell response. These studies provided compelling evidence that cytokine-producing profiles could define distinct populations of T cells, and T cells that are able to produce multiple cytokines (polyfunctional T cell response) correlate with improved protection against viral infection [18]. Similarly, an effective cancer vaccine should also be able to elicit this type of response. For example, a phase I/II trial of human GM-CSF DNA in conjunction with a multipeptide vaccine (gp100 and tyrosinase) in stage III/IV melanoma patients showed polyfunctional CD8+ T cell response to the gp100 peptide [19].

We evaluated if the WT1 peptide vaccination could induce such polyfunctional T cell responses. CD3 T cell were challenged with CD8 epitope WT1-A , its analogue WT1-A1 or irrelevant peptide EW. The expression of the surface CD107a (marker for mobilization of degranulation), and the production of IFN-γ, IL-2 macrophage inflammatory protein (MIP)1-β, and tumor necrosis factor (TNF)-α were measured by intracellular cytokine staining, in conjunction with other T cells markers. Representative data from patient 12 show the cytokine production profile from CD3+ CD8+ T cells (Fig. 5). Before vaccination (T0), most of the T cells produced only one to two cytokines out of the 4 cytokines measured (yellow and green bars in the X-axis). After 6 vaccinations (T6), the percentage of the cells capable of producing 2 to 4 cytokines significantly increased in response to the

challenge with either WT1-A or WT1-A1 peptides, but not to the irrelevant EW peptide These data further demonstrated that the CD8 T cell polyfunctional response was WT1-specific. Challenging with either WT1-A or WT1-A1 induced a similar cytokine-producing profile, thus indicating that cross reactivity between the two peptides was effectively achieved. Similar results were also observed in patient 8 (data not shown). These results clearly demonstrated that an effective poly-functional T cell response could be elicited by the WT1 peptide vaccination.

7. Clinical outcomes

Among the nine patients with mesothelioma, 8 developed disease progression ranging from 1 to 6 months after starting the vaccinations most received palliative chemotherapy, and all subsequently died from disease. However, one patient (patient 1) remains without progression 36 months after the start of the study. The median survival for the patients with mesothelioma (measured from the date of the first vaccinations) is 14 months.

Both of the evaluable patients with NSCLC relapsed. Patient 4 recurred 8 months after starting vaccinations died 22 months later. Patient 7 recurred in an adrenal gland 20 months after starting vaccinations. He underwent adrenalectomy and remains alive without evidence of disease.

DISCUSSION

WT1 is a nuclear protein that regulates the expression of several genes involved in tumor growth. Because it is processed and presented in the context of MHC molecules on the cell surface, WT1 has potential as a target for T cell therapies. Several groups are pursuing this strategy. Oka and colleagues in Japan conducted a Phase I clinical study of immunotherapy targeting a different WT1 class 1 peptide in patients with leukemia, MDS, lung cancer, or breast cancer.11 Patients were intradermally injected with an HLA-A*2402-restricted, native, or modified 9-mer WT1 peptide emulsified with Montanide ISA51 VG UFCH adjuvant at 0.3, 1.0, or 3.0 mg per body at 2-week intervals, with toxicity and clinical and immunological responses as the principal endpoints. Twenty-six patients received one or more WT1 vaccinations, and 18 of the 26 patients completed WT1 vaccination protocol with three or more injections of WT1 peptides. Twelve of the 20 patients for whom the efficacy of WT1 vaccination could be assessed showed clinical responses such as reduction in leukemic blast cells or tumor sizes and/or tumor markers. A clear correlation was observed between an increase in the frequencies of WT1-specific cytotoxic T lymphocytes after WT1 vaccination and clinical responses. Keilholz and colleagues in Germany reported the findings from their study vaccinating 19 patients with AML or MDS with an HLA-A201 restricted WT1 class 1 peptide 126–134 (the native sequence to the WT1-A1 analog used in the trial here) with KLH and GM-GSF adjuvants [13]. Interestingly, they detected IgM and IgG responses to WT1 in about 15% of patients before treatment. The vaccine augmented IgM responses in 6 of 6 patients at week 26 or 30, though no CD4 T-cell or IgG responses were noted [20]. WT1 tetramer+ T-cells increased in the blood or bone marrow in 45% of cases. Anti-tumor activity was noted in several patients, primarily with prolonged stable disease and some hematologic improvement, but remarkably one patient with secondary

AML achieved a complete cytogenetic response. A three-fold decrease in WT1 mRNA levels was noted in 35% of patients. In both the Japanese and German studies, toxicity was essentially limited to injection site reactions. At the NIH, a combined vaccine of two leukemia-associated antigenic peptides, PR1 and WT1 was tested in eight patients with myeloid neoplasms.[21] After vaccination, PR1 or WT1(+)CD8(+) T cell responses were associated with drops in WT1 mRNA expression, suggesting an antileukemia effect of vaccination. We have also observed robust CD4 and CD8 T-cell immune responses to WT1 peptides in patients with AML vaccinated in the companion part of this trial (Maslak et al, submitted.) Ongoing studies of WT1 immune-directed therapy are being conducted by Moffit Cancer Center, Duke University, and Glaxo-Smith Kline (www.clinicaltrials.gov).

In comparison to these other studies, ours has several unique aspects. Foremost, this is the first study that included patients with mesothelioma, a disease known to have high expression of WT1 and therefore an optimal target population. Furthermore, we are the first to target multiple epitopes and both CD4 and CD8 WT1 epitopes simultaneously. We utilized a multivalent vaccine that included four different WT1 peptides that were designed to elicit both CD4 and CD8 T cells, across a broad range of HLA subtypes [15]. In addition, heteroclitic analogs of the CD8 epitope were used to enhance the immune response to the WT1 self -protein, to which many patients might be tolerant. By using an altered amino acid sequence not found in the native sequence, but which bound with higher affinity to the HLA-A0201, we postulated that a stronger immune response could be generated and that this response would cross react with the native sequence.

This response was observed after vaccination in the trial. We observed a high rate of both CD8 and CD4 immune responses to the vaccine. CD4 T cell responses did not correlate with particular HLA-DR types, suggesting that such promiscuous peptides [15] could prime CD4 T cell responses in a broader range of patients, compared to the WT1 peptides that have been reported to date. The vaccination also induced polyfunctional CD8 T cell responses in the patients tested, suggesting a broader repertoire of T cell response could be elicited. Other studies have suggested that CD8+ T cells that secrete both IFN-γ and TNF-α have enhanced cytolytic activity compared with CD8+ T cells that secret IFN-γ alone [22–23]. Therefore, analyzing such polyfunctional T cell responses might be insightful for understanding the mechanisms underlying vaccine efficacy for the future cancer vaccine studies. We also examined whether a long peptide designed to elicit CD4 responses (122-A1) containing a cryptic CD8 analog epitope buried within it (WT1-A1) could generate an appropriate responses in vitro to the CD8 native epitope. CD8 ELISPOT and tetramer staining studies confirmed that this was the case.

Conclusions about therapeutic activity of this vaccine are difficult to surmise, particularly in patients with MPM. This is a heterogenous patient population—half had relapsed disease after prior multimodality therapy including surgery, and half had unresectable disease, most of whom had received chemotherapy—making predictions of expected outcomes difficult. Two prior studies provide some guidance: 1) In a multicenter trial of trimodality therapy for MPM, the median time to progression was 10 months and the median survival was 16 months[24], and 2) In the phase III trial of pemetrexed and cisplatin, the median survival from the *onset of chemotherapy* was 12 months [25]. Thus, the median survival of 14

months in this study, which is measured from the *initiation of vaccination*, is encouraging. Of particular interest is the one patient who has not demonstrated disease progression three years after initiating the vaccine program. Nonetheless, this is a highly selected patient population and a small sample size. Similarly encouraging outcomes were noted in a parallel trial in which patients with acute myelogenous leukemia were vaccinated with the same WT1 peptides (Maslak, manuscript in press). As in the other WT1 peptide vaccine studies, toxicity was mostly grade one and limited to local injection site reactions. The lack of significant toxicity becomes important for this group of patients who are generally older and frail due to their disease. Based on these findings we have designed a randomized phase II trial in patients comparing treatment with our WT1 vaccine to treatment with adjuvant alone in patients with resected MPM to determine if clinical benefit can be measured. This overall approach of a pilot trial followed by a randomized phase II study conforms with a paradigm established by Cancer Vaccine Clinical Trial Working Group [26]. Incorporation of the WT1 vaccine in conjunction with chemotherapy for patients with advanced disease may be another valid approach to pursue.

Acknowledgments

We thank Dr. Bo Dupont and Alice Yeh at Memorial Sloan-Kettering Cancer Center for HLA genotyping and Dr. M. Roederer for providing the SPICE software.

Supported by NIH PO1 23766, Innovive Pharmaceuticals, the Mesothelioma Applied Research Foundation, Tudor and Glades Funds and the Experimental Therapeutics Center of MSKCC

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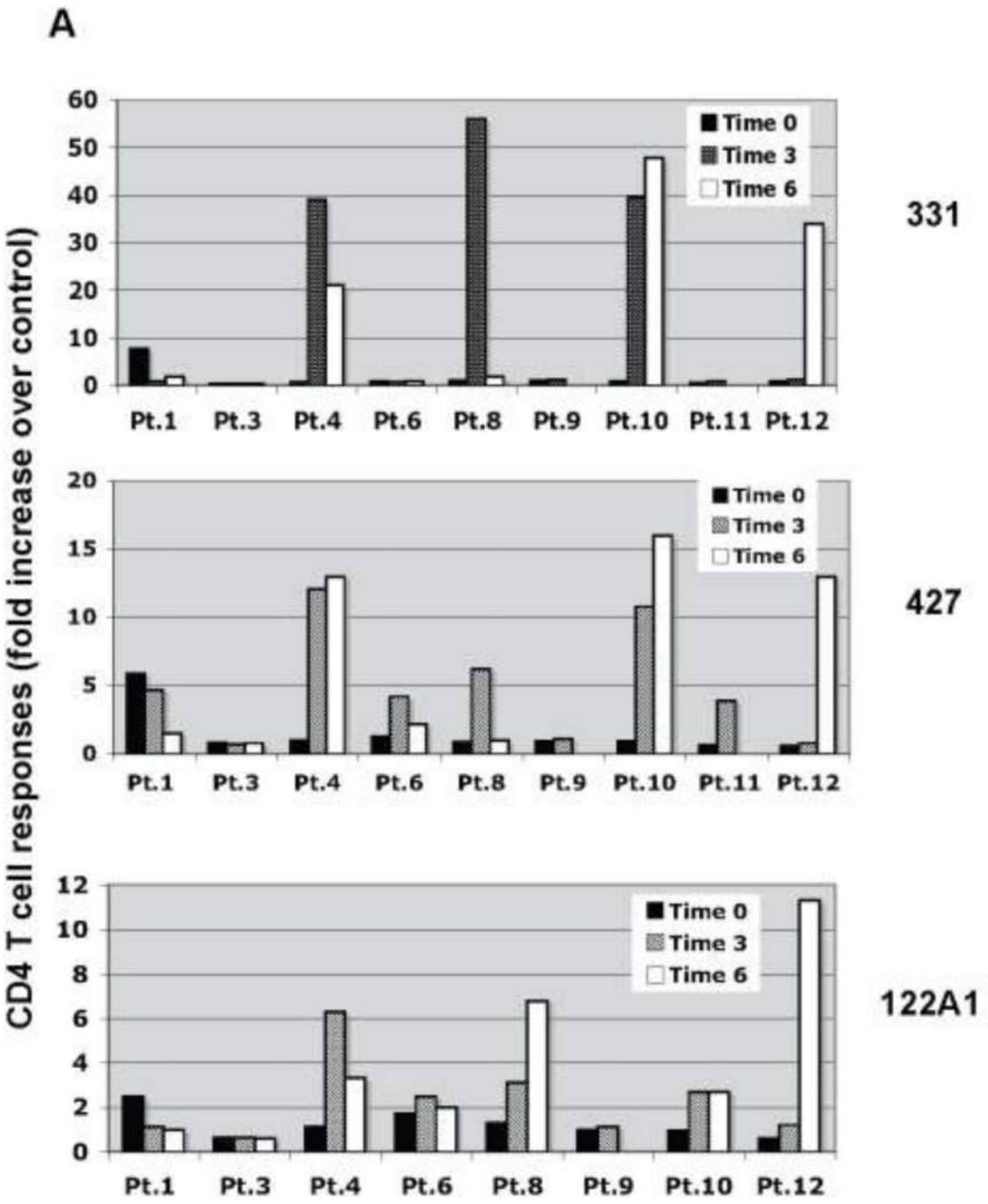


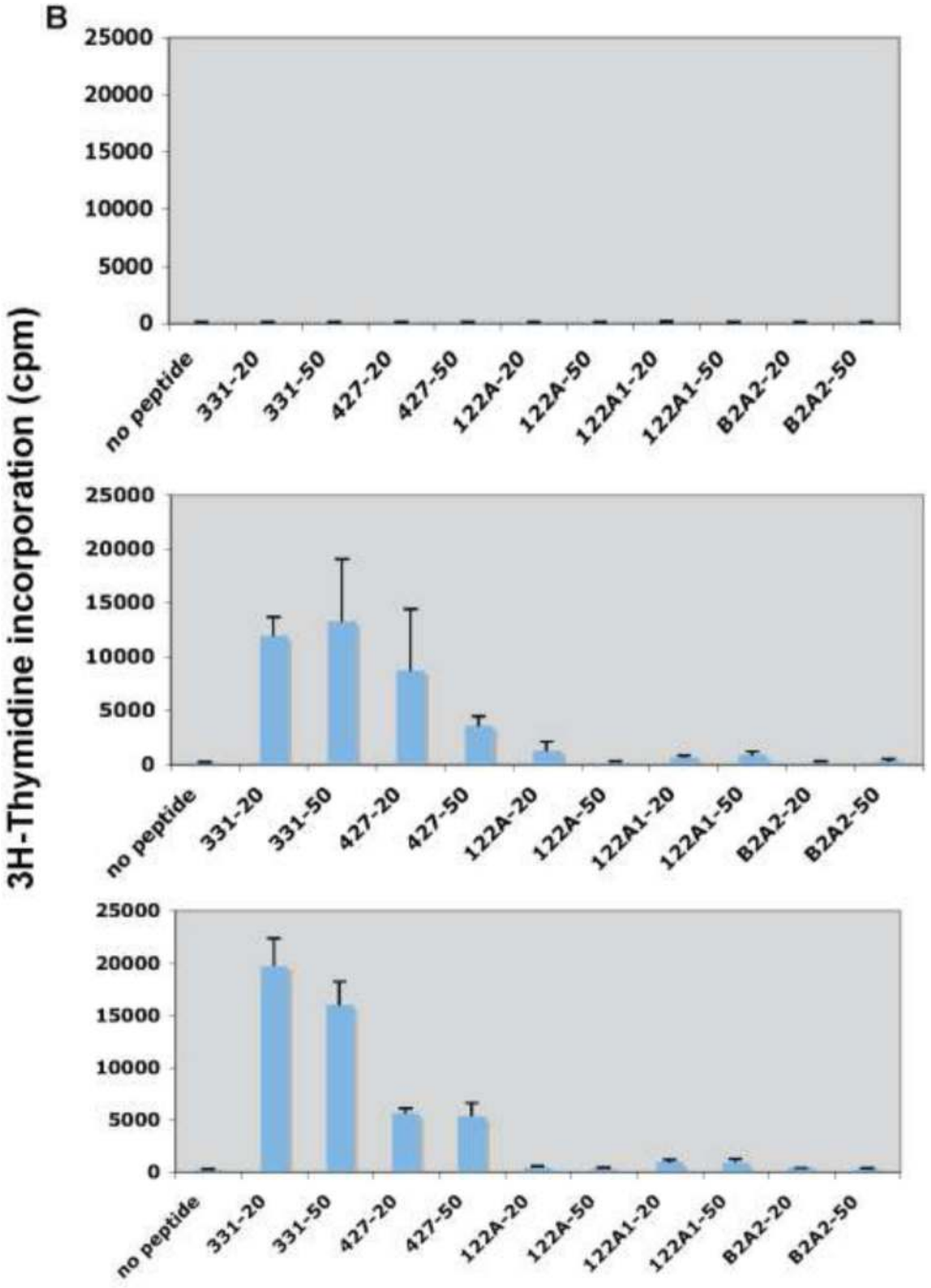


Fig. 1.

Proliferation of CD4 T cells. (A) CD4 T cell responses were calculated by the fold-increase of CD4 T cell proliferation against 331, 427 and 122A1 peptides over the irrelevant peptide B2A2 long. All the peptides were used at a concentration of 50 ug/ml. (B) CD4 T cells from pre- (upper panel), 3 (middle panel) and 6 (lower panel) vaccinations from patient 10, were incubated with indicated peptides at concentrations of 20 ug/ml or 50 ug/ml, for 5 days and 1 uCi 3H-thymidine/well was added to the cultures for 20 hours. The cell proliferation was determined by 3H-thymidine incorporation. Data represent mean +/− standard deviation (SD) from quadruplicate cultures.

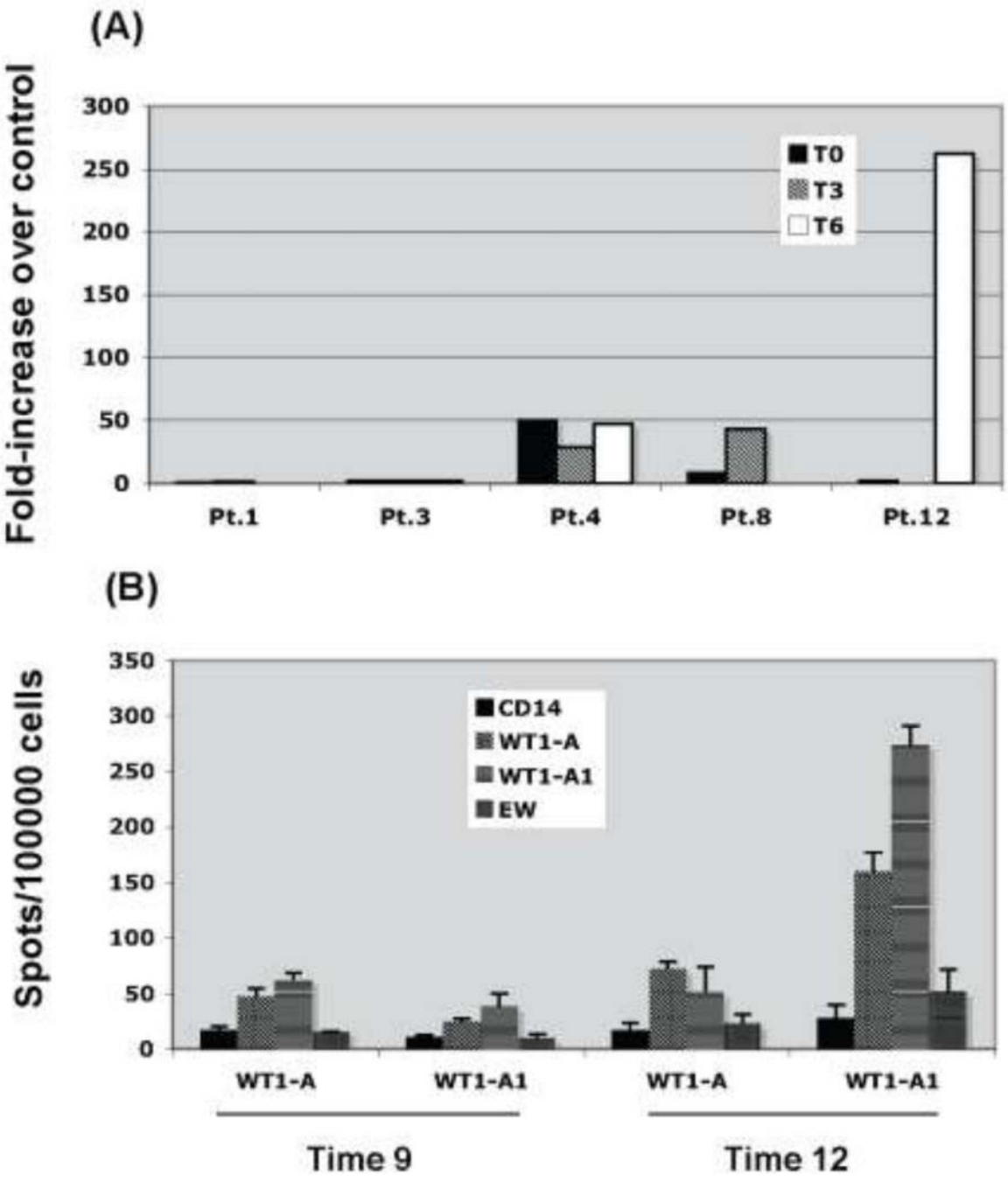
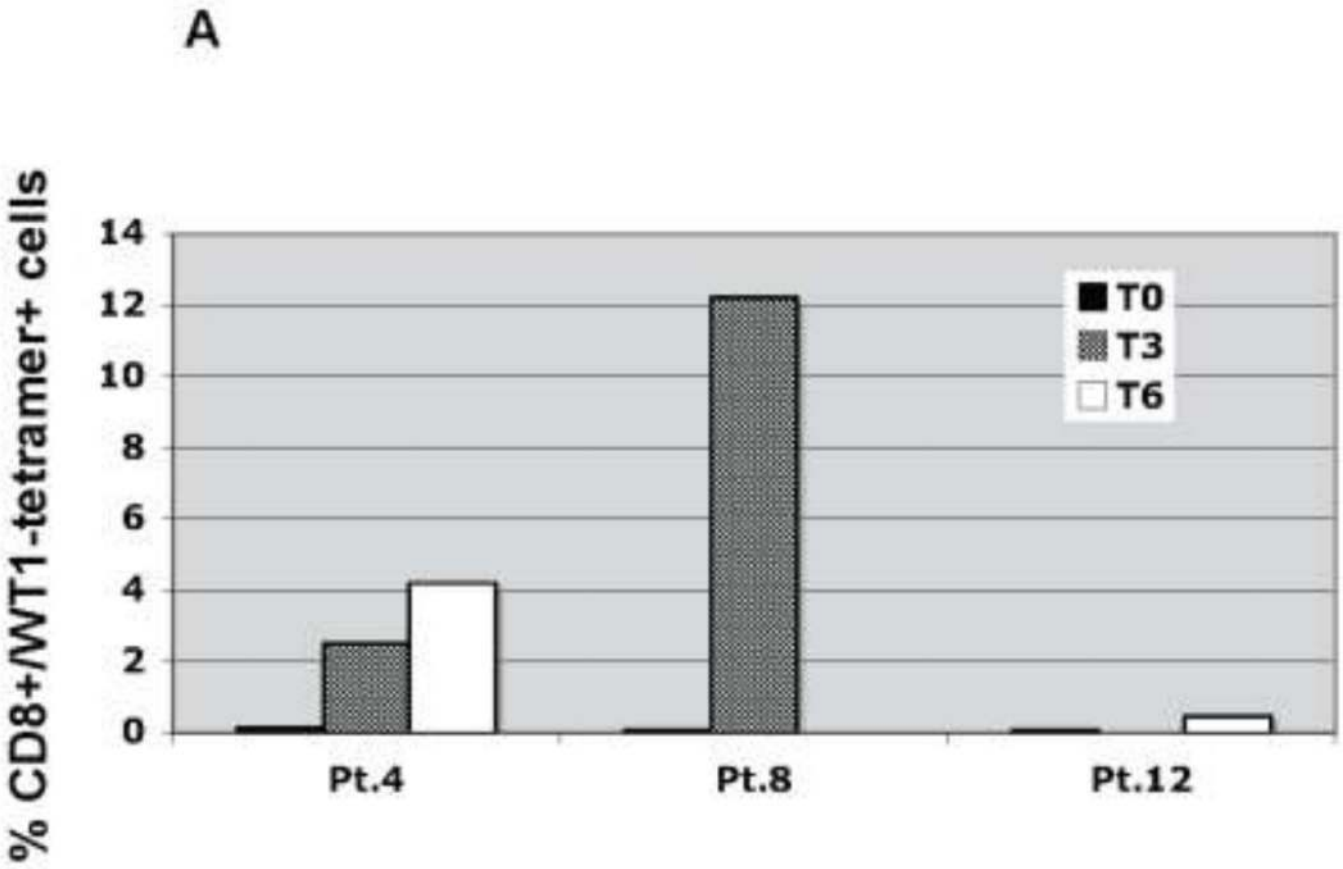


Fig. 2.

IFN-gamma secretion by CD3 T cells in the HLA-A0201+ patients. (A) CD3 T cells were stimulated in vitro with analog peptide WT1-A1 and the responses were tested against its native peptide WT1-A. Data represent the fold-increase of the IFN-gamma secretion by CD3 T cells against WT1 peptide, over the irrelevant peptide EW (pt.11 was not tested due to lack of cells). (B) Representative data from patient 1, after 9 and 12 vaccinations. CD3 T cells were stimulated with either WT1-A or WT1-A1 and the peptide-specific response was tested against both WT1-A and WT1-A1. CD14 positive cells alone or pulsed with irrelevant peptide EW are negative controls. The data are representative of quadruplicate cultures +/− SD.



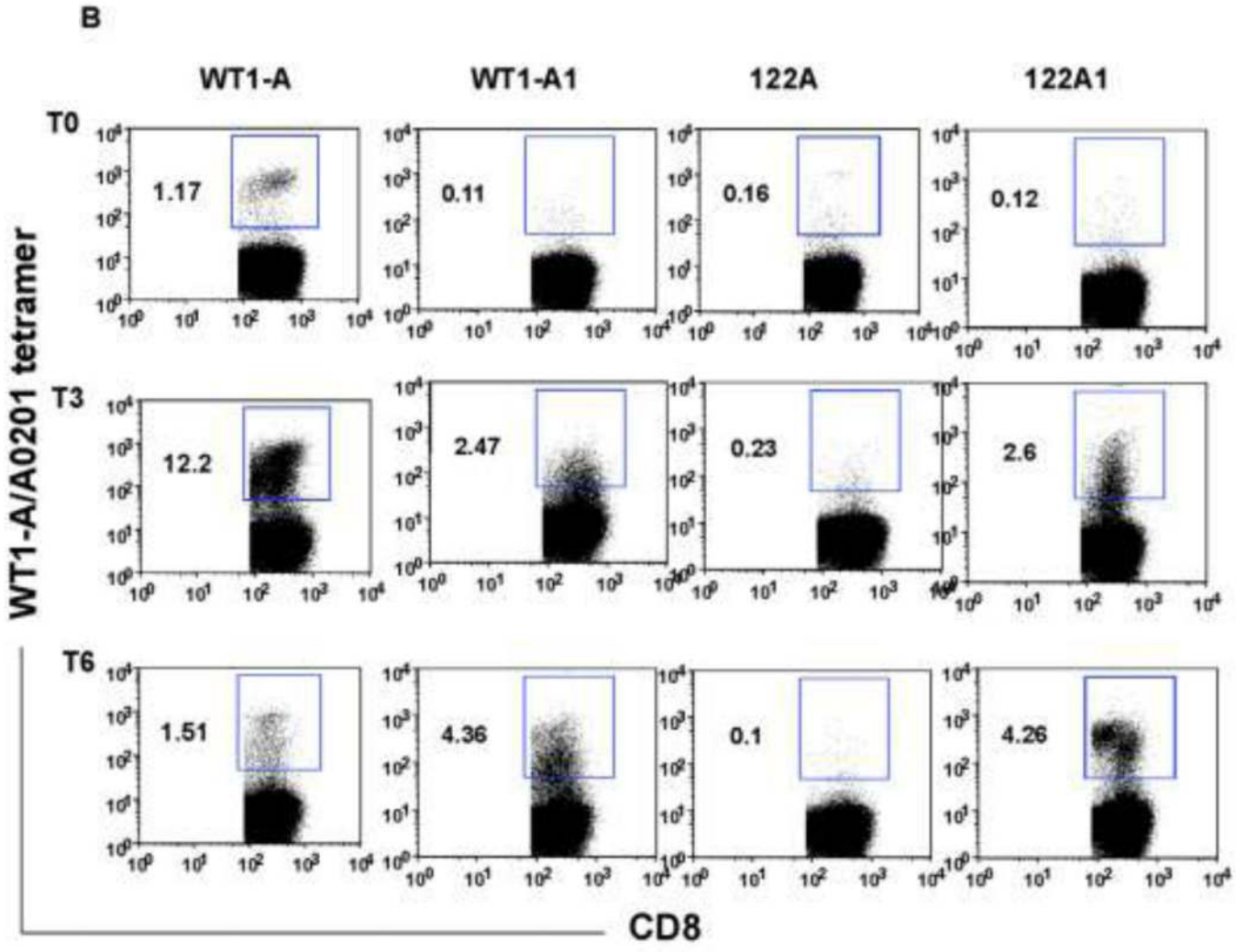
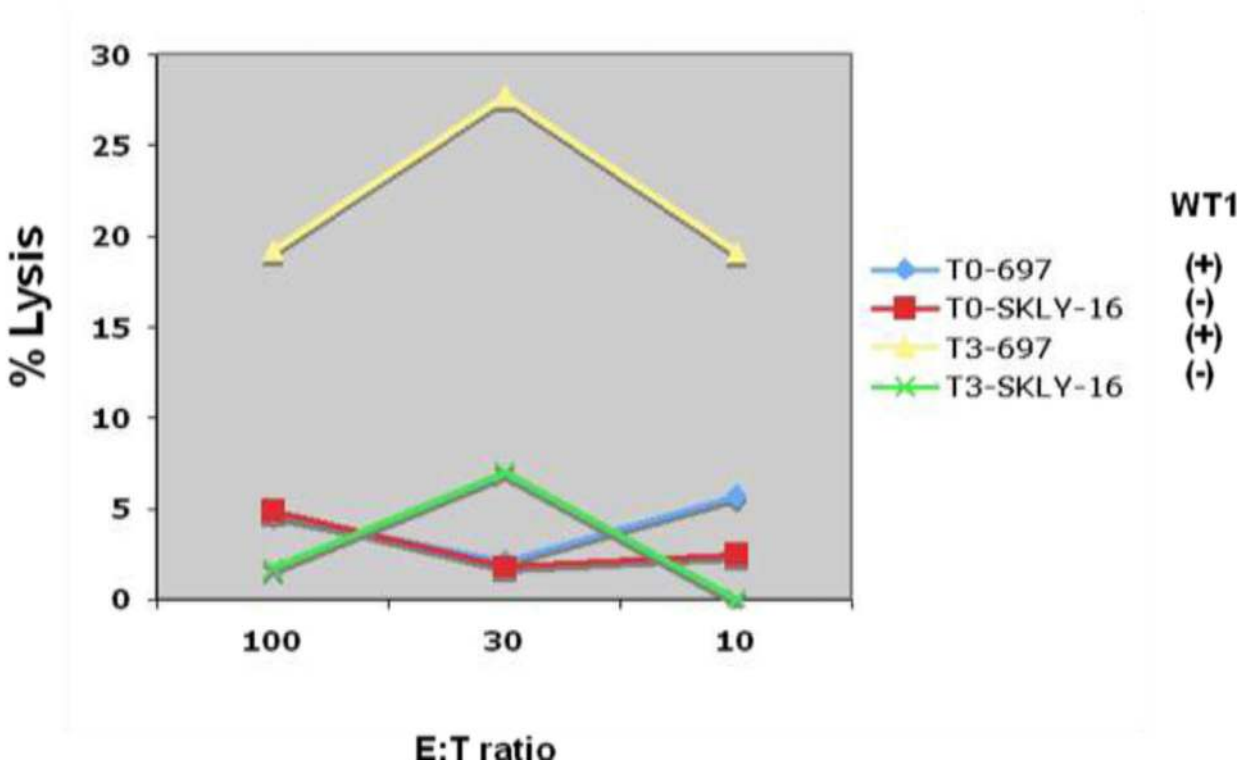


Fig. 3.

WT1-A/HLA-A0201 tetramer staining. (A) CD3 T cells were stimulated in vitro with analog peptide WT1-A1 and the percentage of CD8+/WT1-A tetramer+ cells was shown as fold-increase over the control gp100/HLA-A0201 tetramer staining. (B) CD3 T cells from patient 4 were stimulated twice with WT1-A (native), WT1-A1 (analog), 122A (native) or 122A1 (analog) peptides and were stained with WT1-A/HLA-A0201 tetramer with mAbs to CD8 and other T cell markers. Percentage of tetramer positive CD8 T cells is shown after gating on CD3+ events. Pre-vaccination, and after 3 and 6 vaccinations were shown as T0, T3 and T6. The inset number is the percentage of dual positive cells. The data are representative of triplicate staining.

A



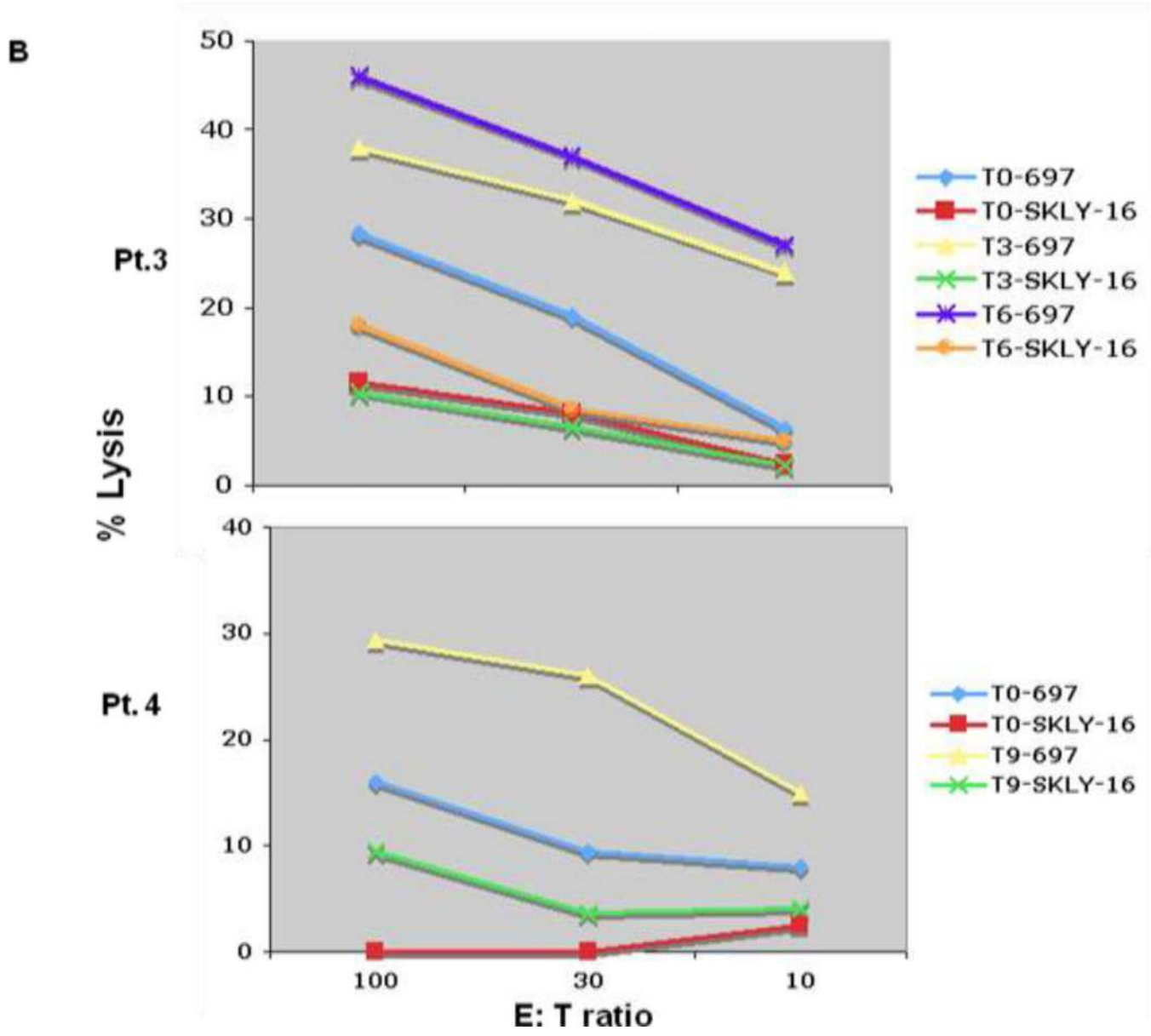


Fig. 4.

Cytotoxicity assay. (A) CD3 T cells from patient 8 that were previously stimulated with WT1-A peptide twice were stimulated with autologous DCs for 5 days in the presence of WT1 peptide and IL-15 as described in the Materials and methods. The cytotoxicity of the cells was measured by standard 51Cr-releasea assay against WT1-positive 697 cells or WT1-negative cell line SKLY-16 at the effector: target ratios indicated in X-axis. The SKLY-16 cells pulsed with WT1-A or irrelevant peptide EW serve as positive or negative control. (B) Similarly, CD3 T cells that had been stimulated with WT1-A1 peptide were stimulated again with the peptide and activated B cells (B, patient 3 and 4) for a week and the cytotoxicity was tested against the 697 and SKLY-16 cells. Due to the limited numbers of the cells, the peptide-pulsed controls could not be included. All data represent mean of triplicate micro-well cultures.

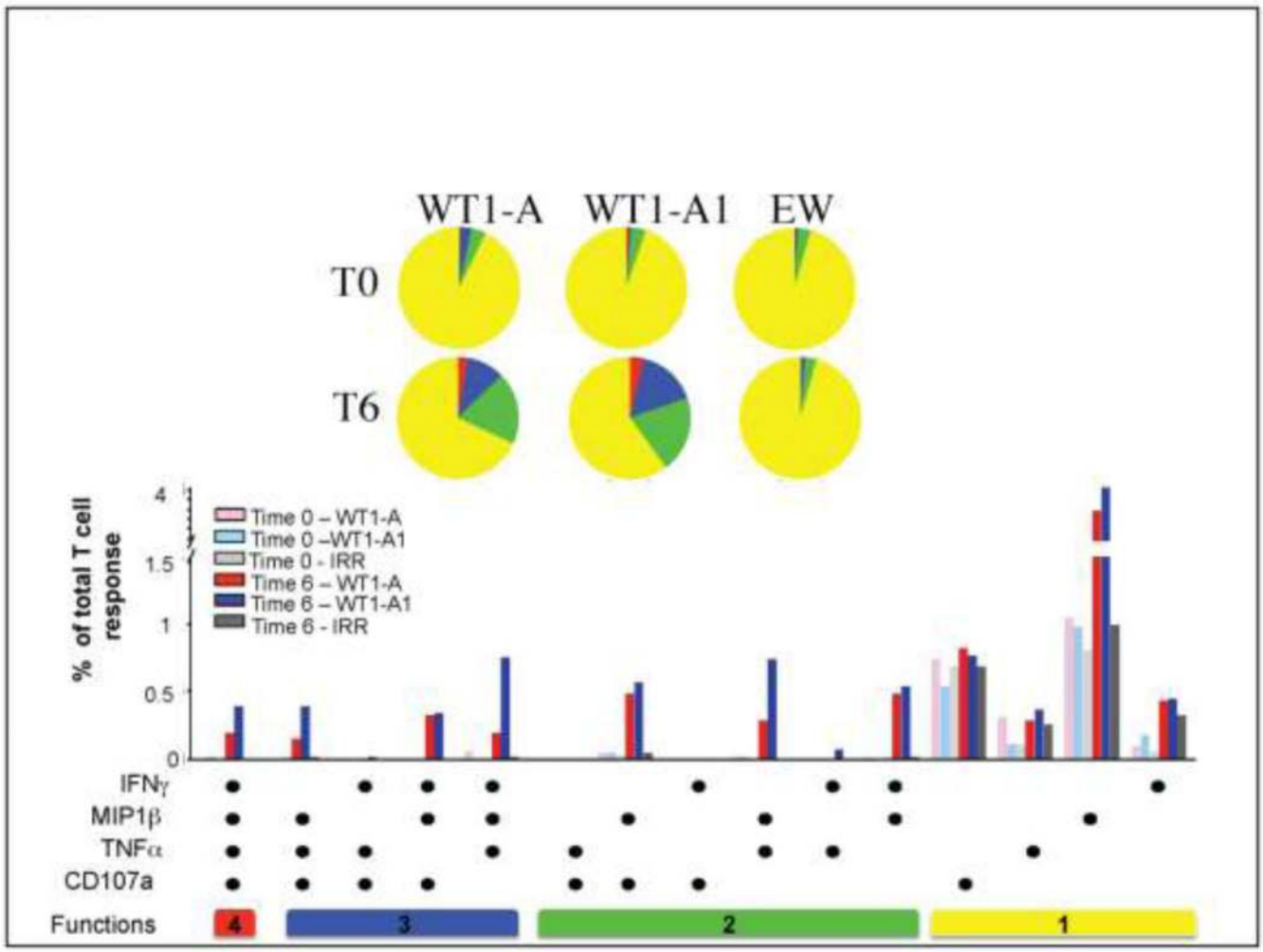


Fig. 5.

Polyfunctional CD8 T cell responses. CD3+T cells were challenged with WT1-A, WT1-A1 or irrelevant control peptide EW, and intracellular cytokine staining was performed to measure the secretion of IFN-γ, MIP-1β, TNF-α or CD107a, as described in the Materials and Methods. The data show the cytokine production on the gates of CD3+CD8+ cells from patient 12 , before vaccination (T0) and after 6 vaccinations (T6). All possible combinations of 4 cytokine production are shown on the X-axis. Bars indicate the percentage of total response by CD8+ T cells (Y-axis). Responses are grouped and color-coded: red= 4 cytokines, blue = 3 cytokines, green = 2 cytokines, yellow = 1 cytokine. Each slice on the pie charts represents the fraction of the total responses that is CD8 T cell positive for a given number of functions.

Table 1

Patient characteristics, clinical outcomes, and summary of immune responses

Patient #	Age, Gender	HLA	Dx (Histology)	Stage	Prior rx	# vaccinations	TTP (mo)	Survival (mo)	DTH Skin test	CD4 response[1]	CD8 response[2]
1	62 M	A0101/0201; B0702/0702 C0701/0702: DRb1*0401/1501 DQb1*0301/0602	MPM (epithelioid)	IV (Recurrence)	Chemo, EPP, RT	12	N/A	36+	Neg	Neg	Pos
2	74 M	A0205/2601; B3801/4101 C1203/0701: DRb*0402/1305 DQb*0302/0301	MPM (epithelioid)	IV (Recurrence)	Chemo, EPP, RT	3	4	9	Neg	Not eval	Not eval
3	76 M	A0201/2301; B2705/4403 C0102/0401: DRb1*0101/1501 DQb1*0501/0602	MPM (epithelioid)	IV (Recurrence)	EPP, RT	6	4	17	Pos	Neg	Pos
4	59 F	A0201/0205; B1402/1501 C0802/0304; DRb1*0401/1301 DQb1*0302/0603	NSCLC (adeno)	IIIA	Chemo, surgery	10	8	30	Pos	Pos	Pos
5	77 F	A1101/3303; B3701/5401 C0602/0102; DRb1*1001/1202 DQb1*0501/0301	MPM (epithelioid)	III	Chemo	2	1	3	Not eval	Not eval	Not eval
6	84 M	A2402/2601; B3501/3801 C0401/1203; DRb1*0102/1104 DQb1*0501/0301	MPM (epithelioid)	III	Chemo	6	3	26	Neg	Pos	Non-HLAA02
7	55 F	A0201/6802; B1501/2703 C0304/0202; DRb1*0302/1301 DQb1*0402/0603	NSCLC (NOS)	IIIB	Chemo/RT	1	N/A	29+	Not eval	Not eval	Not eval
8	65 M	A0101/0201; B0801/0801 C07/07; DRb1*0301/0803	MPM (epithelioid)	II	None	8	6	8	Pos	Pos	Pos
9	75 M	A3101/3002; B1402/4001 C0802/0304; DRb1*0101/102 DQb1*0501/0609	MPM (epithelioid)	IV (Recurrence)	Chemo, EPP, RT	5	3	15	Neg	Neg	Non-HLAA02
10	85 M	A1101/2902; B1402/4403 C0802/1601; DRb1*0301/0701 DQb1*0201/0202	MPM (epithelioid)	IV	Chemo	8	6	13	Neg	Pos	Non-HLAA02
11	53 M	A0101/0201; B0802/4402 C07/0501; DRb1*0401/1501 DQb1*0301/0602	NSCLC (squamous)	IIIB	Chemo/RT	12	20	22+	Neg	Pos	Neg

Patient #	Age, Gender	HLA	Dx (Histology)	Stage	Prior rx	# vaccinations	TTP (mo)	Survival (mo)	DTH Skin test	CD4 response[1]	CD8 response[2]
12	45 M	A0101/0201; B0801/1501 C0701/0304; DRb1*0301/0401 DQb1*0201/0302	MPM (epithelioid)	IV (Recurrence)	Chemo, EPP, RT	6	3	14	Not eval	Pos	Pos

MPM = malignant pleural mesothelioma
NSCLC = non-small cell lung cancer
EPP = extrapleural pneumonectomy
RT = radiation therapy
TTP = time to progression (from initiation of vaccines)

+ indicates that patient remains alive at the time of this manuscript

[1] CD4 T cell response was measured by the proliferation of unprimed CD4 T cells as described in the Materials and Methods. Positive response is defined as an increased CD4 T cell proliferation against one or more HLA-DR.B1 peptides, at one or more time points after vaccinations.

[2] CD8 T cell response to HLA-A0201 peptide was measured by: A) IFN-γ ELISPOT of CD3 T cells against HLA class I peptides WT1A and WT1-A1; B) WT1A/HLA-A0201 tetramer staining vs CD3/CD8 T cell staining. Positive response is defined as an increased IFN-γ secretion, or WT1-A/A2 tetramer positive cells, or both, at one or more time points after vaccinations.