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Immunization with analogue peptide in combination with CpG and Montanide expands tumor antigen-specific CD8⁺ T cells in melanoma patients

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Abstract

Analogue peptides represent a promising tool to further optimize peptide-based vaccines in promoting the expansion of tumor antigen-specific CTLs. Here, we report the results of a pilot trial designed to study the immunogenicity of the analogue peptide NY-ESO-1 157-165V in combination with CpG 7909/PF3512676 and Montanide ISA 720 in patients with stage III/IV NY-ESO-1-expressing melanoma. Eight Patients were immunized either with Montanide and CpG (arm 1, three patients), Montanide and peptide NY-ESO-1 157-165V (arm 2, two patients) or with Montanide, CpG and peptide NY-ESO-1 157-165V (arm 3, three patients). Only the three patients immunized with Montanide, CpG and peptide NY-ESO-1 157-165V in arm 3, developed a rapid increase of effector-memory NY-ESO-1-specific CD8⁺ T cells, detectable *ex vivo*. The majority of these cells exhibited an intermediate/late-stage differentiated phenotype (CD28[−]). Our study further demonstrated that our vaccine approach stimulated spontaneous tumor-reactive NY-ESO-1-specific CD8⁺ T cells in two patients with advanced disease but failed to prime tumor-reactive NY-ESO-1-specific T cells in one patient with no spontaneously tumor-induced CD8⁺ T cell responses to NY-ESO-1. Collectively, our data support the capability of the analogue peptide NY-ESO-1 157-165V in combination with CpG and Montanide to promote the expansion of NY-ESO-1-specific CD8⁺ T cells in patients with advanced cancer. They also suggest that the presence of tumor-induced NY-ESO-1-specific T cells of well-defined clonotypes is critical for the expansion of tumor-reactive NY-ESO-1-specific CD8⁺ T cells following peptide-based vaccine strategies.

Keywords

Cancer vaccines; analogue peptides; CpG; Melanoma

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INTRODUCTION

There is now ample evidence that T cells recognize epitopes presented in the context of MHC molecules by tumor cells (1). Although peptide-based vaccines have successfully induced tumor rejection in animal models, to date their capability to promote objective clinical responses in a significant number of patients with cancer, remains elusive (2). The failure of peptide-based vaccines to induce significant clinical responses in patients contrasts with their capability to consistently stimulate tumor-antigen-specific responses. This observation has nourished skepticism regarding peptide-based vaccine approaches, as well as the use of immune responses as a surrogate marker of clinical response (1). Strikingly, the majority of research based upon peptide-based vaccine approaches in humans has used *in vitro* stimulation (IVS) to detect tumor antigen-specific T cells, suggesting that these responses were either present at extremely low levels or impaired by tumor-induced immunosuppression (3). The need for IVS represents a major obstacle to the study of the quantitative and qualitative (differentiation/activation status and repertoire) properties of vaccine-induced T cells. Very few studies using specific tumor-shared antigens have demonstrated their capability to stimulate *ex vivo* detectable tumor antigen-specific CD8⁺ T cell responses in patients with advanced disease. To the best of our knowledge, only two recent studies have shown that peptide NY-ESO-1 157-165A in combination with Montanide (4) or NY-ESO-1 protein in combination with Montanide and CpG (5) could stimulate *ex vivo* NY-ESO-1-specific CD8⁺ T cells in cancer patients. NY-ESO-1 is a cancer-germline antigen expressed by a wide range of tumors of different histological types but not by normal tissues except testis (6). NY-ESO-1 is strongly immunogenic and gives rise to CD8⁺ T cell and antibody responses in patients with active NY-ESO-1-expressing tumors (7, 8). One HLA-A2 epitope, NY-ESO-1 157-165 has been previously identified (7). Because cysteinylolation reduces the antigenicity of synthetic peptides binding to MHC class I molecules (9), modifications of cysteine 165 in the NY-ESO-1 157-165 peptide have been proposed to explain its increased immunogenicity (10, 11). Substitution of the cysteine for a valine at position 165, which gives rise to the analogue peptide NY-ESO-1 157-167V, has been shown to further increase the expansion of NY-ESO-1-specific T cells *in vitro* (11). Further studies have demonstrated that the cysteine-to-valine substitution at position 9 improves not only peptide binding to MHC but also the interactions between the analogue peptide and the TCR (12). The analogue peptide NY-ESO-1 157-165V stimulated faster polarization of lytic granules to the immunological synapse, reduced the dependence on CD8 binding and induced greater numbers of cross-reactive CTLs, when compared to the original peptide NY-ESO-1 157-165. On the basis of the promising *in vitro* data, we have initiated a pilot trial to study the immunogenicity of the analogue peptide NY-ESO-1 157-165V in patients with active NY-ESO-1-expressing tumors. We have chosen CpG as a potent adjuvant in combination with Montanide ISA 720 (Montanide). In humans, CpG activates plasmacytoid dendritic cells and B cells through TLR9 triggering and indirectly activates myeloid dendritic cells, promoting Th-1 polarization (13, 14). Two recent studies in humans have confirmed that CpG acts as a very potent adjuvant in combination with Montanide and one of the HLA-A2-restricted Melan-A/MART-1 epitopes (15) or the recombinant NY-ESO-1 protein (5), respectively. Our data demonstrate the capability of the analogue peptide NY-ESO-1 157-165V in combination with CpG and Montanide to stimulate tumor-reactive NY-ESO-1-specific CD8⁺ T cell responses detectable *ex vivo* in patients with advanced NY-ESO-1-expressing melanoma. We have further studied the differentiation/activation status and the repertoire of the vaccine-elicited T cells, thus providing insights on the quality of the T cell responses induced by the analogue peptide NY-ESO-1 157-165V in combination with adjuvants.

MATERIALS AND METHODS

Patients and study protocol

Eight HLA-A2⁺ patients with refractory metastatic stage III/IV melanoma and circulating anti-NY-ESO-1 antibodies were included after informed consent in this pilot phase I study approved by the University of Pittsburgh Institutional Review Board (Table 1A). The trial was conducted under an investigator new drug application IND 11216. All patients had measurable disease as defined by the Response Evaluation Criteria in Solid Tumors (RECIST) and evaluated with magnetic resonance imaging or computed tomographic scan of the head, chest, abdomen and pelvis within 4 weeks of therapy. Eligibility criteria included age ≥ 18 , tumor expressing NY-ESO-1 as determined by RT-PCR or immunohistochemistry and/or serum positive for anti-NY-ESO-1 antibodies, serologic or genotypic HLA-A0201 positive typing, negative serology tests for HIV 1 and 2, HTLV-1, Hepatitis B and C, adequate hematologic, renal, and liver function (as evidenced by the following: WBC $\geq 3,000/\text{mm}^3$, Lymphocytes $\geq 1,000/\text{mm}^3$, Platelets $\geq 100,000/\text{mm}^3$, Serum Creatinine $\leq 1.5 \times$ upper limit of normal (ULN), Serum Bilirubin $\leq 2.5 \times$ ULN, Serum AST/ALT $\leq 2.5 \times$ ULN). Melanoma patients (MP) were immunized with a stable emulsion composed of 3 ml Montanide in combination with either 2 mg CpG (MP 1 to 3 in arm 1), 400 μg analogue peptide NY-ESO-1 157-165V (SLLMWITQV) (MP 4 and 5 in arm 2), or CpG and peptide NY-ESO-1 157-165V (MP 6 to 8 in arm 3). The final volume of immunization was 4 ml and it was administered as 4 separate subcutaneous injections. MP received at least six biweekly immunizations before being evaluated for clinical and immune responses. One patient (MP 6) who had stable disease after 4 months of treatment continued to receive vaccinations monthly until disease progression. Peptide was provided by MPS (San Diego, CA, USA), CPG 7909/PF3512676 (CpG) by Coley Pharmaceuticals Group (Wellesley, MA, USA) and Montanide ISA 720 (Montanide) by Seppic (Paris, France).

Ex vivo frequency and phenotype analysis of HLA-A2/NY-ESO-1-specific CD8⁺ T cells

PBMCs from melanoma patients were collected using a Ficoll gradient and were frozen in Iscove's DMEM medium (Mediatech Inc., Herndon, VA, USA) containing 10% human serum (Sigma-Aldrich, St. Louis, MO, USA) and 10% DMSO (FisherBiotech, Fair Lawn, NJ, USA) in liquid nitrogen until further processing. CD8⁺ lymphocytes were purified from PBMCs by magnetic cell separation using MACS Column Technology (Miltenyi Biotech, Auburn, CA, USA). CD8⁺ T cells were then incubated with pre-titrated PE-labeled HLA-A2/NY-ESO-1 157-165 (SLLMWITQC) or HLA-A2/HIV-POL 476-484 (ILKEPVHGV) tetramers for 30 min at room temperature. Tetramers were provided by the Ludwig Institute for Cancer Research, Lausanne branch. After washing, cells were surface stained with the following monoclonal antibodies (mAbs): anti-CD8^{FITC}, anti-CD45RA^{ECD} (Beckman Coulter, Fullerton, CA, USA), anti-CCR7^{PE-Cy7}, anti-CD28^{PE-Cy5} (BD Pharmingen, San Diego, CA, USA), anti-CD27^{APC-Cy7} (eBioscience, San Diego, CA, USA) and anti-CD127^{APC} (R&D Systems, Minneapolis, MN, USA). A violet amine reactive dye (Live/Dead Cell Stain Kit, Invitrogen, Eugene, OR, USA) was used to exclude dead cells from analysis. Approximately 1×10^6 cells per sample were acquired with a BD FACSAria machine (BD Biosciences, San Jose, CA, USA). Alternatively, CD8⁺ T cells were either surface-stained with PE-labeled tetramers, anti-CD3^{PE-Cy5}, anti-CD8^{ECD} and anti-PD-1^{FITC} (BD Pharmingen) mAbs, or permeabilized with a 0.5% saponin solution (Sigma-Aldrich) in staining buffer and stained intracellularly with anti-granzyme A^{FITC} or anti-granzyme B^{FITC} or anti-perforin^{FITC} mAbs (BD Pharmingen). Flow cytometry data were analyzed with FlowJo version 8.2 software (Tree Star, Inc., Ashland, OR, USA).

IFN- γ ELISPOT assays

NY-ESO-1-specific CD8⁺ T cell responses before and after vaccinations were assessed *ex vivo* by IFN- γ ELISPOT assays as previously described (16, 17). Briefly, 5×10^4 peptide-pulsed (10 μ g/ml) T2 cells were added to 1×10^5 CD8⁺ T cells isolated from PBMCs of patients in wells of 96 flat-bottomed nitrocellulose plates coated with anti-human IFN- γ mAbs (Mabtech, Inc., Cincinnati, OH, USA) and incubated at 37°C for 24 hours in AIM V medium (Invitrogen Corporation, Carlsbad, CA). Alternatively, 1×10^3 FACS sorted HLA-A2/NY-ESO-1 157-165 tetramer⁺ CD8⁺ T cells or CD8⁺ T cell clones were used as effector cells. As target cells, we used the HLA-A2⁺ NY-ESO-1⁺ melanoma cell line UPCI-MEL 285 and the HLA-A2⁺ NY-ESO-1⁻ melanoma cell line UPCI-MEL 136. In blocking experiments, 3 μ g of W6/32 (anti-HLA-A, -B and -C) or L243 (anti-HLA-DR) antibodies were added in wells. Spot numbers and spot sizes were determined with computer-assisted video image analysis (Cellular Technologies, Cleveland, OH). Assays were performed in triplicates. For statistical evaluation, a *t* test for unpaired samples was used. A value of *p* < 0.05 was considered significant.

CD8⁺ T cell cloning

HLA-A2/NY-ESO-1 157-165 tetramer⁺ CD8⁺ T cells were sorted by flow cytometry into 96-well plates (one cell per well) and expanded with allogenic feeder cells in culture medium containing PHA (1 μ g/ml) (Sigma-Aldrich) and rhIL-2 (200 IU/ml) (PeproTech, Rocky Hill, NJ, USA).

⁵¹ Chromium (⁵¹Cr)-release assay

Cytolytic activity of NY-ESO-1-specific CD8⁺ T cell clones was measured as described (16). Melanoma cells were treated for 48 hours with 50 units/ml of rhIFN- γ (PeproTech) before ⁵¹Cr-release assay. Target cells were the melanoma cell lines UPCI-MEL 285 and UPCI-MEL 136. In blocking experiments, 3 μ g of W6/32 or L243 antibodies were added in wells. Assays were performed in triplicates.

NY-ESO-1-specific CD8⁺ T cells TCR V β usage analysis

TCR V β repertoire analysis of NY-ESO-1-specific CD8⁺ T cells was performed *ex vivo* by flow cytometry. CD8⁺ T cells were isolated from PBMCs by magnetic separation and incubated with APC-labeled HLA-A2/NY-ESO-1 157-165 tetramers. Cells were then surface stained with anti-CD3^{ECF}, anti-CD8^{PE-Cy7} and a panel of PE- and FITC-labeled anti-V β mAbs (IOtest Beta Mark, Beckman Coulter, France).

TCR V β gene usage

Total RNA was isolated from 1×10^6 CD8⁺ T cell clones using the RNeasy Mini kit (Qiagen, Valencia, CA) and reverse transcription was conducted as previously reported (16). A panel of 24 specific primers complementary to TCRV β and a primer for the constant region were used for PCR amplification (18). The PCR products were run on 1.5% agarose gel and purified using the QIAquick gel extraction kit (Qiagen). The purified PCR products were sequenced using the ABI 3100 automated DNA sequencer.

RESULTS

Ex vivo detectable CD8⁺ T cells directed against NY-ESO-1 157-165

All three patients (MP 6, 7 and 8) with stage III/IV active disease included in arm 3, received at least six biweekly immunizations with an emulsified preparation containing the analogue peptide NY-ESO-1 157-165V, CpG and Montanide as previously described in section MATERIALS AND METHODS. PBMCs were collected at different time points and

used for *ex vivo* flow cytometry-based tetramer studies. As shown in Fig. 1A, all three patients exhibited an increase of NY-ESO-1 157-165-specific CD8⁺ T cells. MP 7 and 8 had *ex vivo* detectable NY-ESO-1-specific CD8⁺ T cells (4.17 % and 0.021%, respectively) that increased after six to eight immunizations (7.76 % and 0.17% for MP 7 and 8, respectively). Furthermore, MP 6 who had no *ex vivo* detectable NY-ESO-1-specific T cells pre-immunization (i.e. < 0.001%) developed *ex vivo* detectable NY-ESO-1 157-165-specific CD8⁺ T cells with a frequency of 0.12%. The number of NY-ESO-1-specific CD8⁺ T cells increased for all patients in arm 3 (Fig. 1B). In contrast, none of the patients (MP 1, 2 and 3) immunized with CpG and Montanide without NY-ESO-1 157-165V peptide (arm 1) and none of the patients (MP 4 and MP5) immunized with peptide NY-ESO-1 157-165V in Montanide without CpG (arm 2), exhibited a significant increase in the number of NY-ESO-1-specific CD8⁺ T cells (Fig. 1C).

Altogether, our findings support the capability of immunization with the analogue peptide NY-ESO-1 157-165V, CpG and Montanide to stimulate NY-ESO-1-specific CD8⁺ T cells that are detectable *ex vivo* in patients with active NY-ESO-1-expressing tumors.

Differentiation status of NY-ESO-1-specific CD8⁺ T cells

We next assessed the state of differentiation/activation of the NY-ESO-1-specific CD8⁺ T cells that increased following immunizations in the three patients included in arm 3, using multiparameter flow cytometry-based assays combining HLA-A2/NY-ESO-1 tetramers and antibodies against CD45RA, CCR7, CD28, CD27, CD127, granzyme A, granzyme B, perforin and PD-1. In all three patients, the majority of the NY-ESO-1-specific CD8⁺ T cells detectable *ex vivo* expressed low levels of CD45RA and CCR7, thus exhibiting an effector-memory phenotype (Fig. 2A) (19). These cells were predominantly CD27⁺CD28⁻ for two patients (MP 6 and 8), corresponding to an intermediate antigen-experienced phenotype (20). In contrast, the NY-ESO-1-specific CD8⁺ T cells were predominantly CD27⁻CD28⁻ for the third patient (MP 7), exhibiting a late-antigen-experienced phenotype (Figure 2B). In two patients with spontaneous NY-ESO-1-specific T cells detectable *ex vivo* that increased after immunization (MP 7 and 8), the proportion of effector-memory CD8⁺ T cells (i.e., CD45RA⁻CCR7⁻) did not change significantly (Figure 2A). However, for MP 7, we observed a shift towards a late-experienced phenotype as the CD27⁻CD28⁻ cell subset increased (from 58.9% to 73.3%) while the CD27⁺CD28⁻ decreased (from 39.4% to 24.9%) (Fig. 2B).

As shown in Fig. 2C, in MP 6, a significant fraction of the vaccine-induced HLA-A2/NY-ESO-1 tetramer⁺ CD8⁺ T cells, which were predominantly CD27⁺CD28⁻, expressed CD127/IL-7R α (47.6%). In contrast, only a small fraction of the HLA-A2/NY-ESO-1 tetramer⁺ CD8⁺ T cells detectable *ex vivo* pre- and post-immunization expressed CD127, whether they were predominantly CD27⁺CD28⁻ (MP 8), or CD27⁻CD28⁻ (MP 7). In agreement with their late-stage differentiated status, the HLA-A2/NY-ESO-1 tetramer⁺ CD8⁺ T cells in MP 7 expressed granzyme A, granzyme B and perforin pre- and post-immunization. Interestingly, the large majority of these cells expressed the programmed death-1 receptor (PD-1) pre- and post-immunization (Figure 2D).

NY-ESO-1-specific CD8⁺ T cells produce IFN- γ and are lytic in the presence of peptide-pulsed APCs and NY-ESO-1-expressing tumor cells

To assess the capability of the NY-ESO-1-specific CD8⁺ T cells pre- and post-immunization to produce IFN- γ , we have isolated CD8⁺ T cells from PBMCs of the patients in this study and tested their capability to produce IFN- γ in the presence of APCs pulsed with peptides in *ex vivo* IFN- γ ELISPOT assays. None of the patients included in arm 1 (Montanide and CpG) or arm 2 (Montanide and peptide NY-ESO-1 157-165V) showed a

significant increase in the number of IFN- γ -producing NY-ESO-1-specific CD8⁺ T cells (Fig. 3A and 3B). In contrast, all three patients (MP 6, 7 and 8) who were immunized with the peptide analogue NY-ESO-1 157-165V in combination with CpG and Montanide (arm 3) exhibited an increased number of *ex vivo* detectable IFN- γ -producing NY-ESO-1-specific CD8⁺ T cells that recognized both the wild-type peptide NY-ESO-1 157-165 and the analogue peptide NY-ESO-1 157-165V (Fig. 3C).

In order to test the capability of the NY-ESO-1-specific CD8⁺ T cells to recognize the normally processed and presented epitopes, we sorted the HLA-A2/NY-ESO-1 tetramer⁺ CD8⁺ T cells from MP 6 and 7 pre- and/or post-immunizations and tested their capability to recognize not only peptide-pulsed APCs but also HLA-A2-matched NY-ESO-1⁺ (UPCI-Mel 285) and NY-ESO-1⁻ (UPCI 136) melanoma cells in IFN- γ ELISPOT assays. As shown in Figure 3D, NY-ESO-1-specific CD8⁺ T cells from MP 6 recognized only T2 cells pulsed with the analogue peptide and the wild-type peptide (10 μ g/ml). In contrast, NY-ESO-1-specific CD8⁺ T cells from MP 7 recognized not only T2 cells pulsed either with the analogue or the wild-type peptide but also HLA-A2⁺, NY-ESO-1⁺ tumor cells UPCI 285 (Fig. 3D). Clones were generated with limiting dilution from tetramer⁺ sorted NY-ESO-1-specific CD8⁺ T cells. We obtained fifteen, twenty-three and thirty-two NY-ESO-1-specific CD8⁺ T cell clones from MP 6, 7 and 8, respectively. Two representative clones, 93/13 and 95/3, isolated from PBMCs of MP 7 and 8, respectively, after at least six immunizations, produced IFN- γ not only in the presence of T2 cells pulsed with peptides but also HLA-A2⁺ NY-ESO-1⁺ melanoma cell line UPCI-MEL 285. (Fig. 4A). The tumor recognition was completely abolished in the presence of anti-MHC class I antibody (W6/32) but not anti-HLA-DR antibody (L243). The lytic activity of these two clones was also evaluated in conventional ⁵¹Cr assays. As shown in Fig. 4B, the two representative clones derived from MP 7 and 8 lysed the HLA-A2⁺, NY-ESO-1⁺ melanoma cell line UPCI-MEL 285 but not the HLA-A2⁺, NY-ESO-1⁻ melanoma cell line UPCI-MEL 136. The tumor lysis by the CTL clones was completely abolished in the presence of anti-MHC class I antibody (W6/32) but not anti-HLA-DR antibody (L243). In contrast and as shown for one representative clone, 80/1, the vaccine-elicited CD8⁺ T cell clones isolated from PBMCs of MP 6 post-immunization recognized NY-ESO-1 157-165 peptide-pulsed T2 cells but not HLA-A2⁺, NY-ESO-1⁺ melanoma cells in ELISPOT and ⁵¹Cr assays.

We have further studied the capability of the three clones derived from each of the three melanoma patients to recognize titrated doses of peptide NY-ESO-1 157-165 and peptide NY-ESO-1 157-165V (Fig. 4C). We have found that the peptide concentration corresponding to the half maximal stimulation of the tumor-reactive clone 93/13 was ten times lower in the presence of the original peptide NY-ESO-1 157-165 (20 nM) than the analogue peptide NY-ESO-1 157-165V (200 nM). The other tumor reactive clone, 95/3, better recognized the analogue peptide NY-ESO-1 157-165V, yet still exhibited a half-maximal stimulation of 200 nM in the presence of the original peptide sequence NY-ESO-1 157-165. In contrast, the non tumor-reactive clone, 80/1, recognized well the analogue peptide (half-maximal stimulation peptide concentration of 20 nM), but exhibited only a very poor recognition of the original peptide NY-ESO-1 157-165 (half-maximal stimulation peptide concentration of 4 μ M).

Collectively, our data confirmed the capability of immunization with the analogue peptide NY-ESO-1 157-165V in combination with CpG and Montanide ISA 720 to expand spontaneously tumor-induced NY-ESO-1-specific CD8⁺ T cells in two patients but failed to prime tumor-reactive NY-ESO-1-specific T cells in the third patient.

Repertoire of spontaneous and vaccine-induced NY-ESO-1-specific T cells

We then studied TCR V β chain usage by the HLA-A2/NY-ESO-1 tetramer⁺ CD8⁺ T cells pre- and post-immunization using a panel of anti-V β antibodies against the main V β chains in combination with HLA-A2/NY-ESO-1 tetramer staining. As shown in Table 2A, the majority of NY-ESO-1-specific CD8⁺ T cells detectable *ex vivo* before immunization from PBMCs of MP 7 and 8, expressed V β clonotypes previously known to be preferentially expressed spontaneously (i.e., V β 8, V β 13.1 and V β 1). Of interest, we have also found that nearly 19% of the naturally occurring NY-ESO-1-specific CD8⁺ T cells expressed V β 3 in MP 8, thus introducing another spontaneously tumor-induced clonotype. Following immunization, MP 6, who pre-immunization had no spontaneous NY-ESO-1-specific CD8⁺ T cells detectable *ex vivo*, developed NY-ESO-1 specific CD8⁺ T cells expressing the V β 4 and V β 14 chains.

We have sequenced the TCR β CDR3 gene region of the three NY-ESO-1-specific CD8⁺ T cell clones isolated from MP 6, 7 and 8, respectively. Two of the three clones exhibited the V β chain expressed by the large majority of the HLA-A2/NY-ESO-1 tetramer⁺ T cells (Table 2B).

Altogether, our data confirmed the capability of the analogue peptide NY-ESO-1 157-165V in combination with CpG and Montanide ISA 720 to expand naturally-occurring NY-ESO-1-specific clonotypes and to prime non-tumor-reactive CD8⁺ T cells exhibiting a novel clonotype in the one patient with no spontaneous CD8⁺ T cell response to NY-ESO-1.

Side effects and clinical outcomes

Our pilot study was also designed to study side effects and clinical responses. We observed grade I/II skin toxicity at the injection sites with redness and pain in all patients, as well as flu-like symptoms and fatigue in a minority of patients (Table 1B). None of the patients developed objective clinical responses. All patients in arms 1 and 2 progressed and went out of study after receiving eight immunizations. MP 6 with stage IIIc disease (melanoma in transit) remained stable for six months and then progressed. The two other patients in arm 3 with stage IV disease progressed after six and eight immunizations although one exhibited very high NY-ESO-1-specific CD8⁺ T cells.

DISCUSSION

In the present study, we have investigated the expansion of tumor-reactive NY-ESO-1-specific CD8⁺ T cells in patients with advanced NY-ESO-1-expressing tumors following immunizations. We observed an increase of *ex vivo* detectable NY-ESO-1-specific CD8⁺ T cells only in the three patients immunized with the peptide analogue NY-ESO-1 157-165V in combination with CpG and Montanide. Such levels of CD8⁺ T cell responses to a cancer-germline antigen-derived epitope have been only rarely observed in patients with advanced disease. In particular, one previous study of immunization of melanoma patients with the analogue peptide NY-ESO-1 157-165V (1 mg) in incomplete Freund's adjuvant was successful in stimulating NY-ESO-1-specific CD8⁺ T cells detectable in a 12-day IVS assay in the majority of the vaccinated patients (21). These CD8⁺ T cells recognized recognized HLA-A2⁺ NY-ESO-1⁺ melanoma cells in three out of twenty-nine patients. To the best of our knowledge, only one peptide-based trial of immunization with the analogue peptide NY-ESO-1 157-165A (500 μ g) in combination with Montanide ISA 51 has successfully stimulated NY-ESO-1-specific CD8⁺ T cells detectable *ex vivo*, in four melanoma patients with advanced NY-ESO-1-expressing tumors (4). Notably, our vaccine strategy with CpG appears to induce consistently higher levels of NY-ESO-1-specific CD8⁺ T cells (i.e. > 0.12%) than those observed in patients immunized with NY-ESO-1 157-165A and

Montanide (4). Furthermore, our vaccine strategy of biweekly immunizations led to an increase of NY-ESO-1-specific CD8⁺ T cells that were already detectable after just one month (i.e., two immunizations). Therefore, and in contrast with the majority of published cancer vaccine studies with cancer-germline-derived epitopes, our vaccine strategy stimulated one of the most rapid increases of vaccine-induced T cells to date.

We observed that vaccine emulsions with CpG and Montanide without peptide (arm 1) failed to stimulate the expansion of NY-ESO-1-specific CD8⁺ T cells, underlining the role of the analogue peptide in promoting the expansion of NY-ESO-1-specific CD8⁺ T cells. We have also noted that neither of the two stage IV melanoma patients treated with peptide NY-ESO-1 157-165V and Montanide without CpG developed *ex vivo* detectable NY-ESO-1-specific CD8⁺ T cells (arm 2). Our findings are in line with one previous vaccine study of immunization with Montanide and Melan-A/MART-1 peptide with or without CpG, suggesting the role of CpG as a potent vaccine adjuvant capable of promoting a rapid and strong expansion of Melan-A/MART-1-specific CD8⁺ T cells in patients with melanoma (15).

Although analogue peptides are superior in activating and recruiting a larger number of antigen-specific T cells *in vitro* (11, 22, 23), the vaccine-induced T cells *in vivo* appeared to represent a heterogeneous group comprised of both tumor-reactive and non-tumor-reactive CTLs. In particular, immunizations with the analogue peptide NY-ESO-1 157-165A stimulated both tumor-reactive and non-tumor-reactive CD8⁺ T cells (24). In contrast, tumor-antigen-specific CD8⁺ T cells spontaneously induced by tumors appeared to be high-affinity and tumor-reactive (2, 3). Although it may be tempting to speculate that immunization with the original peptide sequence is superior in terms of eliciting tumor-reactive T cells, one recent study of immunization with wild-type peptide NY-ESO-1 157-165 in combination with CpG and Montanide ISA 51 also found a heterogeneous composition among the vaccine-induced T cells, including both tumor-reactive and non-tumor-reactive T cells in two melanoma patients (25). In this study, the detection of NY-ESO-1-specific T cells from PBMCs of patients pre- and post-vaccination was observed only after IVS, suggesting that the wild-type peptide stimulated only weak antigen-specific T cell responses *in vivo*. Collectively, these data, together with our findings, suggest that although analogue peptides may allow for the strongest expansion of vaccine-induced T cells, other factors may play a critical role in promoting tumor-reactivity. In particular, several studies in mice and humans have suggested that antigen density plays a critical role in selecting high-avidity T cells capable of tumor recognition (26, 27).

The vaccine-elicited NY-ESO-1-specific CD8⁺ T cells exhibited tumor-reactivity in the two patients with spontaneous NY-ESO-1-specific CD8⁺ T cells. In these two patients, we observed an expansion of the clonotypes previously described as being preferentially expressed by spontaneous tumor-reactive NY-ESO-1-specific T cells (i.e. V β 1, V β 8.1 and V β 13.1) (28). We found that a significant fraction of spontaneous NY-ESO-1 specific CD8⁺ T cells from MP 8 expressed V β 3. Our studies have further demonstrated that the V β 3⁺ CD8⁺ T cell clone, 95/3, produced IFN- γ and lysed HLA-A2⁺ NY-ESO-1⁺ tumor cells, thus defining a novel clonotype associated with naturally-occurring and tumor-reactive NY-ESO-1-specific CD8⁺ T cells. In the one patient with no spontaneous CD8⁺ T cells (MP 6), we observed the selective expansion of the clonotype V β 4 that is not usually expressed by spontaneous CD8⁺ T cells and which did not exhibit tumor reactivity. This V β 4 clonotype was previously found in vaccine-induced and non-tumor-reactive NY-ESO-1-specific T cells following immunization with peptide NY-ESO-1 157-165A (28). Collectively, our findings show that the analogue peptide NY-ESO-1 157-167V in combination with adjuvants successfully promotes the expansion of tumor-reactive preexisting clonotypes but fails to prime tumor-reactive CD8⁺ T cells in patients with NY-ESO-1-expressing tumors

with no evidence of spontaneous CD8⁺ T cells. They further suggest that the expansion of tumor-reactive NY-ESO-1-specific CD8⁺ T cells may depend on the existence of a T cell repertoire present in patients with spontaneous tumor-induced CD8⁺ T cells. Whether the existence of such a repertoire may predict the successful amplification of tumor-reactive NY-ESO-1-specific T cells will need to be carefully investigated.

One particularly important finding from our study is that our vaccine strategy expanded CD28⁺ NY-ESO-1 157-165-specific effector-memory T cells in all three patients immunized with peptide analogue NY-ESO-1 157-165V, CpG and Montanide. In the one patient with extremely high spontaneous NY-ESO-1-specific CD8⁺ T cells (MP 7), we observed a shift towards a late-experienced phenotype (CD27⁺ CD28⁺) following immunizations. These findings contrast with one study reporting the *ex vivo* expansion of CD28⁺ NY-ESO-1-specific CD8⁺ T cells in three patients with NY-ESO-1-expressing tumors following immunizations with the peptide analogue NY-ESO-1 157-165A and Montanide without CpG (4). Therefore, considered alongside data from this previous report, our findings suggest that CpG plays a role both for the expansion of antigen-specific T cells and also for their differentiation towards a more mature/activated phenotype (with a high level of effector mediators such as granzyme B and perforin). Although proposed pathways of memory and effector T cell differentiation remain poorly defined in humans, a number of studies have supported a linear model of T cell differentiation with progressive loss of CCR7, CD28 and CD27 cell surface expression (20) and have proposed the existence of four functionally distinct populations of effector-memory CD8⁺ T cells (29). Our observation of vaccine-induced CD27⁺ CD28⁺ NY-ESO-1-specific CD8⁺ T cells that either predominantly expressed or not IL7- α /CD127, in MP 6 and 8, respectively, further adds to the heterogeneity of the CD27⁺ CD28⁺ CD8⁺ T cell compartment. The lack of tumor-reactivity by the CTL clones isolated from the CD27⁺ CD28⁺ CD127⁺ T cells (MP 6) in contrast to CTLs derived from CD27⁺ CD28⁺ CD127⁺ T cells (MP 8) suggests that these two T cell compartments are functionally different.

The existence of *ex vivo* detectable tumor-reactive NY-ESO-1-specific CD8⁺ T cells was not associated with evidence of objective clinical responses as the two patients with tumor-reactive CD8⁺ T cells (MP 7 and 8) progressed after three and four months, respectively. Strikingly, one of these two patients (MP 7) had nearly 8% of NY-ESO-1-specific and tumor-reactive CD8⁺ T cells from circulating PBLs. We also observed that the large majority of these cells expressed PD-1 pre- and post-immunization. Therefore, our findings stress the need to further dissect the role of the PD-1/Programmed death ligand 1 (PD-L1) pathway in promoting NY-ESO-1 specific CD8⁺ T cell dysfunction at the tumor site where IFN- γ -producing T cells may induce PDL-1 expression by melanoma cells (30, 31). This may possibly contribute to the discrepancy between the evidence of *ex vivo* tumor antigen-specific immune responses in patients and the lack of objective clinical responses. In summary, our data showed that immunizations with the analogue peptide NY-ESO-1 157-165V in combination with CpG and Montanide successfully stimulated the expansion of NY-ESO-1-specific CD8⁺ T cells that are detectable *ex vivo* in patients with advanced NY-ESO-1-expressing melanoma. They suggest the role of tumor-induced NY-ESO-1-specific T cells of well-defined clonotypes in promoting the expansion of tumor-reactive NY-ESO-1-specific CD8⁺ T cells following peptide-based vaccine strategies. The successful expansion of antigen-specific CD8⁺ T cells detectable *ex vivo* in patients with advanced melanoma represents a critical step to further study the differentiated/activated status of the vaccine-elicited CD8⁺ T cells and the expression of molecules involved in tumor-induced immunosuppression in the goal of optimizing our proposed vaccine approach with combinatorial therapeutic interventions. The immunological and clinical implications of our findings will need to be further studied in the context of a larger clinical trial in patients with NY-ESO-1-expressing tumors.

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FIGURE 1. Expansion of *ex vivo* detectable NY-ESO-1-specific CD8+ T cells following immunizations with the analogue peptide NY-ESO-1 157-165V, CpG and Montanide CD8+ T cells were isolated from PBMCs of patients obtained at different time points during vaccination. Numbers of NY-ESO-1-specific CD8+ T cells were assessed by *ex vivo* flow cytometry using PE-conjugated HLA-A2/NY-ESO-1 157-165 tetramers. (A) Dot plots from total CD8+ T cells of MP 6, 7 and 8 before and after vaccination. Numbers indicate percentages of *ex vivo* detectable NY-ESO-1 157-165-specific CD8+ T cells. As negative control, we evaluated the number of HIV-pol specific CD8+ T cells using HLA-A2/HIV-pol 476-484 tetramers. HIV-pol-specific CD8+ T cells were undetectable in all three patients. (B) Kinetics of HLA-A2/NY-ESO-1 tetramer⁺ (tet⁺) CD8+ T cells following immunizations. All three patients immunized with peptide NY-ESO-1 157-165V, CpG and Montanide (arm 3) exhibited an increase in the frequency of NY-ESO-1-specific CD8+ T cells detectable already after one month of treatment. (C) As control, kinetics of HLA-A2/NY-ESO-1 tetramer⁺ (tet⁺) CD8+ T cells is also shown in patients who were not immunized with the analogue peptide NY-ESO-1 157-165V (arms 1 and 2). No significant increase in NY-ESO-1-specific CD8+ T cells could be detected following immunizations. *Vertical lines*, time points of vaccinations.

FIGURE 2. Maturation status of spontaneous and vaccine-elicited NY-ESO-1-specific CD8+ T cells

CD8+ T cells from the three patients in arm 3 were isolated from PBMCs pre- and post-immunizations and used in *ex vivo* multiparameter flow cytometry assays combining HLA-A2/NY-ESO-1 157-165 tetramers and antibodies against multiple cell surface markers as reported in MATERIAL AND METHODS. Numbers in each quadrant represent percentages of tetramers⁺ cells for each phenotypically distinct CD8+ T cell subset defined by the expression of the following surface markers: (A) CD45RA/CCR7 (A), (B) CD28/CD27; and (C) CD127. (D) Dot plots showing *ex vivo* expression of granzyme B, granzyme A, perforin and PD-1 on HLA-A2/NY-ESO-1 157-165 tetramer⁺ CD8+ T cells before and after vaccination for MP 7.

FIGURE 3. Ex vivo assessment of NY-ESO-1-specific CD8+ T cells following immunizations
 CD8+ T cells were isolated pre- (solid bars) and post-vaccination (white bars) from PBMCs of MP 1, 2 and 3 in arm 1 (A), MP 4 and 5 in arm 2 (B) and MP 6, 7 and 8 in arm 3 (C), and incubated in the presence of T2 cells pulsed either with peptide NY-ESO-1 157-165 or peptide NY-ESO-1 157-165V *in vivo* IFN- γ ELISPOT assays. (D) *Ex vivo* recognition of peptide-pulsed T2 cells and HLA-A2⁺ NY-ESO-1⁺ melanoma cells by NY-ESO-1-specific CD8+ T cells from two melanoma patients in arm 3, pre- and post-vaccination. Briefly, HLA-A2/NY-ESO-1 tetramer⁺ (tet⁺) CD8+ T cells were sorted by flow cytometry from PBMCs of MP 6 and 7 (arm 3) as described in MATERIAL AND METHODS. One thousand CD8+ T cells were incubated in a 24h IFN- γ ELISPOT assay in the presence of T2 cells pulsed with either peptide NY-ESO-1 157-165 (10 μ g/ml) or peptide NY-ESO-1 157-165V (10 μ g/ml). The CD8+ T cells were also incubated in the presence of the HLA-0201⁺, NY-ESO-1⁺, melanoma cell line UPCI-MEL 285.1 cells +/- anti-HLA-A,-B,-C antibodies (W6/32). The HLA-0201⁺, NY-ESO-1⁻, melanoma cell line, UPCI-MEL 136.1 was used as control. IFN- γ spots were developed and counted by computer-assisted video image analysis. Each column represents the mean spot number of triplicates $\pm$ SD (bars). *P* values < 0.05 were considered significant and are indicated with asterisks.

FIGURE 4. Functional studies of NY-ESO-1-specific CD8+ T cell clones derived from patients immunized with peptide NY-ESO-1 157-165V, CpG and Montanide
(A) Recognition of peptide-pulsed-T2 cells and the HLA-A2⁺, NY-ESO-1⁺, melanoma cell line UPCI-MEL 285 by CD8+ T cell clones 80/1, 93/13 and 95/3 isolated from PBMCs of MP 6, 7 and 8, respectively. One thousand CD8+ T cell clones were incubated in a 24-h IFN-γ ELISPOT assay in the presence of T2 cells pulsed either with the wild-type peptide NY-ESO-1 157-165 or the analogue peptide NY-ESO-1 157-165V (10 μg/ml), or UPCI-MEL 285 cells ± anti-HLA-DR antibodies (L243) or ± anti-HLA-A, -B, -C antibodies (W6/32). The HLA-A2⁺, NY-ESO-1⁻, melanoma cell line UPCI-MEL 136 was used as a negative control. **(B)** Lysis of the HLA-A2⁺ NY-ESO-1⁺ melanoma cell line UPCI-MEL 285 by clones 80/1, 93/13 and 95/3 at indicated effector-to-target cell ratios +/- L243 and W6/32 antibodies. ⁵¹Cr-release was measured after 4 h of incubation. **(C)** Reactivity of CD8+ T cell clones 80/1, 93/13 and 95/3 to titrated doses of peptides NY-ESO-1 157-165 and NY-ESO-1 157-165V assessed in a 24hr IFN-γ ELISPOT assay. Each column represents the mean spot number of triplicates ± SD (bars). *P* values < 0.05 were considered significant and are indicated by asterisks.

TABLE 1

Patient demographics and toxicities (N=8)

A. Patient demographics						
Patient	Sex	Age	Stage of Disease	Time since Diagnosis	Prior Therapy *	Time to Progression
Arm 1						
MP1	F	78 y	Stage IV	53 mo	S	4 mo
MP2	M	69 y	Stage IV	77 mo	I	6 mo
MP3	M	79 y	Stage IV	61 mo	C,I	4 mo
Arm 2						
MP4	F	82 y	Stage IV	75 mo	S,C	6 mo
MP5	M	52 y	Stage IV	85 mo	S,C,I	4 mo
Arm 3						
MP6	F	75 y	Stage IIIc	72 mo	S,R,I	6 mo
MP7	M	81 y	Stage IV	47 mo	S	4 mo
MP8	F	55 y	Stage IV	68 mo	S,C	3 mo

B. Toxicities		
Toxicity/grade (N = 8)	1/2	3/4
Fatigue	3/0	0/0
Skin toxicity at the site injection	8/0	0/0
Fever	2/0	0/0
Rigor, Chills	2/0	0/0
Flu symptoms	2/0	0/0

* S, Surgery; I, Immunologic; C, Chemotherapy; R, Radiation.

TABLE 2

TCR V β chain usage of NY-ESO-1 157-165-specific CD8⁺ T cells assessed before and after vaccination in patients from arm 3

A. <i>Ex vivo</i> TCR Vβ repertoire analysis of HLA-A2/NY-ESO-1 157-165 tetramer⁺ CD8⁺ T cells				
Patients	Before vaccination		After vaccination	
MP 6	No detectable response		V β 4	91.8 %
			V β 14	5.2 %
			others	3.0 %
			undefined	0.0 %
MP 7	V β 8	81.9 %	V β 8	76.7 %
	V β 13.1	17.8 %	V β 13.1	22.7 %
	others	0.3 %	others	0.6 %
	undefined	0.0 %	undefined	0.0 %
MP 8	V β 1	45.5 %	V β 3 *	
	V β 3	19.2 %		
	V β 14	3.4 %		
	V β 13.1	3.0 %		
	V β 17	3.0 %		
	V β 21.3	2.5 %		
	V β 8	1.0 %		
	others	2.1 %		
	undefined	20.3 %		

B. TCR V β usage of HLA-A2/NY-ESO-1 157-165-specific CD8⁺ T cell clones

Patients	Clones	Vβ	CDR3β		Jβ
MP 6	80/1	4	CSV	VGAGGGDTQ	YFG 2.3
MP 7	93/13	8	CAS	SLGGTEA	FFG 1.1
MP8	95/3	3	CAS	SPGGFKEL	FFG 2.1

NOTE: (A) Numbers represent frequency of NY-ESO-1-specific CD8⁺ T cells stained by the indicated anti-V β mAbs among NY-ESO-1 tetramer⁺ CD8⁺ T cells, assessed by flow cytometry.

* TCR V β chain usage of NY-ESO-1 157-165-specific CD8⁺ T cell clones generated from PBMCs of MP 8 following immunizations. (B) The TCR β CDR3 gene region is given for three representative clones, 80/1, 93/13 and 95/3, isolated from PBMCs of MP 6, 7 and 8, respectively, after at least six immunizations.