Programmed death-1 blockade enhances expansion and functional capacity of human melanoma antigen-specific CTLs

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Abstract

Negative co-stimulatory signaling mediated via cell surface programmed death (PD)-1 expression modulates T and B cell activation and is involved in maintaining peripheral tolerance. In this study, we examined the effects of a fully human PD-1-abrogating antibody on the in vitro expansion and function of human vaccine-induced CD8+ T cells (CTLs) specific for the melanoma-associated antigens glycoprotein 100 (gp100) and melanoma antigen recognized by T cells (MART)-1. PD-1 blockade during peptide stimulation augmented the absolute numbers of CD3+, CD4+, CD8+ and gp100/MART-1 MHC:peptide tetramer+ CTLs. This correlated with increased frequencies of IFN-γ-secreting antigen-specific cells and augmented lysis of gp100+/MART-1+ melanoma targets. PD-1 blockade also increased the fraction of antigen-specific CTLs that recognized melanoma targets by degranulation, suggesting increased recognition efficiency for cognate peptide. The increased frequencies and absolute numbers of antigen-specific CTLs by PD-1 blockade resulted from augmented proliferation, not decreased apoptosis. Kinetic analysis of cytokine secretion demonstrated that PD-1 blockade increased both type-1 and type-2 cytokine accumulation in culture without any apparent skewing of the cytokine repertoire. These findings have implications for developing new cancer immunotherapy strategies.

Introduction

Programmed death (PD)-1 is a recently defined Ig superfamily member related to CD28 and cytotoxic T lymphocyte antigen-4 (CTLA-4). It is a 50-55-kDa transmembrane protein that was originally identified in a mouse T cell hybridoma undergoing activation-induced cell death (1). PD-1 expression has been found on CD4+/CD8+ thymocytes, mature T and B cells following activation and occasionally macrophages (2–4). Two natural ligands for PD-1 have been described: programmed death ligand (PD-L)-1 (also known as B7-H1) and PD-L2 (also known as B7-DC), both being members of the Ig superfamily (5, 6). PD-L1 is expressed on resting and up-regulated on activated B, T, myeloid and dendritic cells (DCs) (7). PD-L2 is found primarily on macrophages and DCs, but low levels are occasionally seen on activated T cells (7, 8).

Experimental evidence strongly suggests that the PD-1 pathway impacts negatively on T cell activation (for a detailed review, see ref. 9). The PD-1 pathway is known to downregulate immune responses to self-antigens, T cell proliferation, T cell cytokine secretion and inhibit T cell-mediated lysis of tumor cells (9). Recent work has demonstrated that antibody-mediated inhibition of PD-L1 and/or PD-L2 augments the proliferation of functional murine and human antigen-specific T cells (10–15). It was also recently shown that
plate-bound agonistic anti-PD-1 antibody induces apoptosis of human PD-1-expressing HIV-specific CTLs in the absence of antigen stimulation (12). It is currently unclear if signaling through the PD-1 receptor has similar effects on CTL apoptosis during antigen-driven expansion. PD-1-mediated inhibition of T cell activation appears to be dependent on TCR signaling. Ligation of the PD-1 molecule on T cells induces recruitment of Src-homology phosphatase 1 and Src-homology phosphatase 2 to the immunoreceptor tyrosine-based switch motif of the PD-1 cytoplasmic domain (16). These phosphatases likely inhibit proximal signaling kinases of the TCR pathway, thereby blocking TCR signal transduction and leading to attenuated T cell activation. Previously published and ongoing cancer vaccine trials have demonstrated that CD4+ and CD8+ T cell responses to tumor antigens are often induced in patients after vaccination (17–19). As measured by enzyme-linked immunospot (ELISPOT) assay and MHC:peptide tetramer labeling from the peripheral blood (and occasionally sentinel immunized nodes and tumors), the induction of immune responses to immunizing tumor antigens does not clearly confer clinical benefit (17–19). It has been suggested that the majority of vaccine-induced T cells have low recognition efficiency (RE; also known as ‘functional avidity’) for cognate peptide presented endogenously by tumor cells, which may account for their lack of clinical effect (20–22). Furthermore, exposure to high antigen levels—which is likely the case with cancer vaccines that employ repetitive immunizations—can lead to functional exhaustion that is associated with elevated PD-1 expression on cognate T cells (10, 13, 14). Hence, obstacles such as preferential induction of low RE T cells and T cell exhaustion likely need to be overcome to achieve optimal in vivo anti-tumor activity by vaccination. Cancer vaccine strategies may thus be improved not only by increasing the absolute numbers of induced T cells but also by increasing T cell RE and reversing T cell exhaustion. It has been suggested that modulation of co-stimulatory signals as an adjunct to vaccination in mice can selectively induce high RE T cells that are specific for self- and non-self antigens (23). To this end, we examined the effects of PD-1 blockade using a fully human antibody on in vitro stimulated melanoma antigen-specific (i.e. self-antigens) CTLs derived from the peripheral blood of vaccinated melanoma patients.

Methods

Generation of a fully human PD-1-abrogating mAb
Antibodies to human PD-1 and a matching IgG4 isotype control were kindly provided by Medarex, Incorporated (Milpitas, CA, USA). Anti-PD-1 antibodies were generated in mice transgenic for human Ig loci by immunization with CHO transfectants expressing human PD-1, followed by boosting with PD-1/human IgG1 Fc fusion protein. Antibodies were screened for binding to PD-1 transfectants and activated human T cells and the ability to enhance T cell proliferation and IFN-γ secretion in allogeneic DC:T cell mixed lymphocyte reactions (MLRs). The antibodies were also tested for lack of reactivity to the PD-1 homologs CD28, CTLA-4 and inducible co-stimulator (ICOS). Anti-PD-1 antibody (clone anti-hPD-1.5), which demonstrated high affinity and specificity for PD-1, was selected and expressed in CHO cells. This antibody inhibited binding of soluble PD-L1/PD-L2-Ig to plate-bound PD-1 and to PD-1-expressing CHO cells, confirmed by ELISA and flow cytometry, respectively (data not shown). A corresponding monovalent F(ab’) fragment was also tested, which displayed similar activity to the full-length anti-hPD-1.5 antibody in allogeneic DC:T cell MLRs and antigen-specific stimulations described herein (data not shown). To reduce binding to Fc receptors, the anti-hPD-1.5 antibody was converted to an IgG4 antibody with a hinge mutation (S228P) (data not shown). An IgG4 (S228P) isotype control antibody specific for Diphtheria toxin—which demonstrated no effect on T cell proliferation—was generated and used in this study.

Preparation of patient PBMC specimens
Apheresis specimens were collected from HIV-seronegative stage III/IV resected melanoma patients (all HLA-A*0201+) who were vaccinated with the glycoprotein 100 (gp100)209–217/217(1A4) and/or melanoma antigen recognized by T cells (MART)-126–36(27L) heteroclitic peptide analogs. All patients were participants in clinical melanoma vaccine trials—including those published previously (24, 25)—conducted at the University of Southern California/Norris Comprehensive Cancer Center (Los Angeles, CA, USA). All patients were required to comprehend and sign an informed consent form approved by the Los Angeles County/University of Southern California Institutional Review Board prior to treatment. Pre- and post-vaccination apheresis specimens were processed to purify PBMCs by sedimentation on Lymphoprep (Greiner Bio-One, Longwood, FL, USA) and extensive washing in HBSS (Mediatech, Herndon, VA, USA). Cells were frozen in 40% human AB serum (HS; Omega Scientific, Tarzana, CA, USA), 50% RPMI 1640 medium (Mediatech) and 10% dimethyl sulfoxide (Sigma, St Louis, MO, USA). All PBMCs were stored in a secure liquid nitrogen freezer at −168°C until use. T cells from post-vaccination PBMC samples were used in this study.

Generation of DCs and CTL stimulation
DCs found in lymphoid tissue demonstrate varying maturational states (26). As such, we used monocyte-derived DCs with an intermediate immature/mature phenotype as antigen-presenting cells for our in vitro stimulation model. Plastic adherent monocytes from PBMC specimens were cultured in X-VIVO 15 (Cambrex, East Rutherford, NJ, USA) supplemented with 1000 U ml−1 each of rhGM-CSF (Berlex, Richmond, CA, USA) and rhIL-4 (R&D, Minneapolis, MN, USA). On day 6 of culture, rhGM-CSF and rhIL-4 were replenished (1000 U ml−1 each). The following day, DCs were pulsed (minimum 2 h) with gp100-2M, MART-27L or control HLA-A2*0201-binding peptide HIV RT46–484 (HIV) (10 μg ml−1 each). All DCs were then harvested with warm PBS (Mediatech), washed and re-suspended in AIM V medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% HS. As described previously, these DCs possess the ability to capture soluble antigen and also express CD80, CD86, HLA-DR and PD-L1 (8, 27). We confirmed surface expression of these molecules by flow cytometry (data not
shown). All DCs were added (2 × 10^4/100 μl per well) to 96-well round-bottom cluster plates (Corning, Corning, NY, USA).

Because PD-1-mediated interactions may occur naturally between different T cell subsets (i.e. CD4+ and CD8+) (28), bulk CD3+ T cells were used as responders for peptide stimulation. Autologous CD3+ cells were purified from thawed PBMCs using the MACS® (Miltenyi Biotec, Auburn, CA, USA) technique for positive selection. The CD3- PBMC population was refrozen in 90% HS/10% dimethyl sulfoxide for later use as antigen-presenting cells in ELISPOT assays. The purified CD3+ cells were washed and re-suspended in AIM V 5% HS, anti-PD-1 antibody or matching IgG isotype control (10 μg ml^-1 final each), and then added to peptide-pulsed DCs. All cultures were incubated for 11 days at 37°C/5% CO₂. For 11-day stimulations (one cycle), exogenous cytokines were not added at any point.

**MHC-peptide tetramer labeling**

Aliquots of fresh PBMCs and peptide-stimulated (one cycle) CD3+ effector cells were stained according to the manufacturers' protocols with gp100-2M and/or MART-27L HLA-A*0201 tetramer-PE or tetramer-allophycocyanin (APC) (both from Beckman-Coulter, San Diego, CA, USA), CD3-PE-Cy7, CD4-PE-Cy5 (both from PharMingen) and CD8-ECD (Beckman-Couler). PD-1 expression was measured using an anti-PD-1-FITC antibody and matching IgG1 isotype control (both from PharMingen).

**IFN-γ ELISPOT assay**

Cultured CD3+ effector cells were harvested following 11-day in vitro peptide stimulation and added to ELISPOT IP plates (Millipore, Bedford, MA, USA) in concentrations of 10^5 and 3.3 × 10^4 per well (triplicates of 50 μl per well). Autologous CD3-negative flow-through populations (2 × 10^5/50 μl per well) pulsed with gp100 or MART-1 melanoma peptides (heteroclitic or native) or control HIV peptide (10 μg ml^-1 final each) were used as antigen-presenting cells. The plates were incubated (20 h) at 37°C/5% CO₂. Color development was performed with 3-amino-9-ethyl-carbazole (Sigma). Spot numbers were determined with the aid of computer-assisted video imaging analysis (Carl Zeiss ELISPOT Reader System, Oberkochen, Germany) and normalized to 10^5 input cells if needed.

**Chromium-release assay**

CD3+ effector cells were stimulated for two consecutive cycles (11 days then 7 days) using peptide-pulsed DCs. On day 3 of the second cycle of stimulation, IL-2 (100 U ml^-1) was added to all conditions to maintain culture viability. CD8+ cells were MACS-purified from the total effector population prior to assay. The HLA-A*0201+ melanoma lines 526mel, 624mel and A-375 were used as targets. Each cell line was negative for PD-L1 and PD-L2 surface expression (data not shown). Both 526mel and 624mel endogenously express the gp100 and MART-1 antigens (29) and encode the gp100_{289-297} and MART-1_{196-205} immunodominant epitopes restricted by HLA-A*0201 (30, 31). Melanoma line A-375 does not express gp100 or MART-1 (29), and was used as a negative control. All targets were labeled with ^{51}Cr (PerkinElmer, Wellesley, MA, USA). Supernatants were collected and assayed for radioactivity using a γ-counter (Packard Cobra-II; PerkinElmer). Percent specific lysis was calculated as follows: [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100. Lytic units (LU) per 10^5 effector cells were calculated from the LUₜ₀ values of each specific lysis curve (determined using XLfit™), as previously described (32).

**CD107a mobilization assay**

Incubations were done in 96-well U-bottom microtiter plates. To each well, the following were added in order: melanoma peptide-stimulated (one cycle) CD3+ effector cells (1 × 10^6 per well), melanoma targets 526mel, 624mel or A-375 (2 × 10^6 per well) and anti-CD107a–APC antibody (10 μl per well; Southern Biotech, Birmingham, AL, USA). The plates were centrifuged (300 × g for 1 min) and incubated for 5 h at 37°C/5% CO₂. Thereafter, each sample was stained according to the manufacturers’ protocols with gp100-2M or MART-27L HLA-A*0201 tetramer–PE, CD3–PE–Cy7, CD8–ECD and CD4–FITC.

**Cytokine multiplex array**

Culture supernatants from melanoma peptide stimulations were collected on days 2, 4, 6, 8 and 11 of stimulation and immediately frozen at −80°C. Samples were assayed in duplicates for cytokine content using a Th1/Th2 suspension array kit (Bio-Rad, Hercules, CA, USA).

**Detection of proliferation and apoptosis of antigen-specific CTLs**

PD-1-blocked and IgG4 control-treated CD3+ cells were stimulated with autologous melanoma peptide-pulsed DCs for 11 days as described above. To measure intracellular Ki67 expression, effector cells were harvested at days 6 and 11 of culture and stained according to the manufacturers’ protocols with gp100-2M or MART-27L HLA-A*0201 tetramer–PE, CD3–PE–Cy7, CD8–ECD and CD4–FITC. Antibodies to Ki67–FITC and matching IgG1 isotype control were obtained from PharMingen.

Carboxyfluorescein succinimidyl ester (CFSE) dilution in antigen-specific CTLs was measured by labeling CD3+ cells with 0.5 μM CFSE (Invitrogen) for 10 min at 37°C. Labeling was stopped with 100% fetal bovine serum and subsequent washings with AIM V 5% HS. CFSE-labeled cells were then stimulated with melanoma peptide-pulsed DCs and analyzed by flow cytometry 11 days thereafter.

Apoptosis was measured by Annexin V labeling. Effector cells were harvested at days 6 and 11 of culture and stained with gp100-2M or MART-27L HLA-A*0201 tetramer–PE, CD3–PE–Cy7, CD8–ECD and CD4–PE-Cy5. All cells were washed with Annexin V buffer (PharMingen) and stained with Annexin V–FITC (PharMingen).

**Statistical analyses**

Probability (P) values were analyzed using the two-tailed Student's t-test. Values < 0.05 were considered statistically significant.
Results

PD-1 is expressed on post-vaccination melanoma antigen-specific CTLs

We analyzed PD-1 expression on post-vaccination gp100-and MART-1-specific CTLs from 19 melanoma patients. Antigen-specific CTLs from freshly thawed, uncultured PBMC specimens were detected with HLA-A*0201 tetramer-PE and/or tetramer–APC and co-labeled with an anti-PD-1–FITC antibody. Tetramer+ CTLs from all specimens expressed PD-1. PD-1 expression on tetramer+ CTLs varied between patients, ranging from approximately 30–85% positive, averaging 59.8% ± 17.5 (Fig. 1 and Table 1). In comparison, PD-1 expression on the total tetramer– CTL populations were significantly lower, averaging 22.9% ± 13.4 positive (Table 1). Of note, we did not find any significant correlation between percent PD-1 expression on tetramer+ CTLs and the particular vaccine regimens the corresponding patients received or the degree of augmentation in functional assays after in vitro PD-1 blockade.

PD-1 blockade increases the frequencies and absolute numbers of melanoma antigen-specific CTLs

PD-1 blockade during peptide stimulation increased the frequencies of gp100- and MART-1-specific CTLs (leftward column in Fig. 2), compared with IgG4 control-treated cells (center column in Fig. 2). Augmented MHC:tetramer binding was seen in 11/11 tested patient specimens (patients 2, 3, 6, 10–13, 16–19) that were selected based on inventory availability. Immune responses to both gp100 and MART-1 were simultaneously enhanced in 4/4 tested patient specimens (patients 16–19) with sufficient CTL precursor frequency, and thus were not analyzed for PD-1 expression. Immune responses to both gp100 and MART-1 peptides and their corresponding heteroclitic peptide analogs was observed. HIV-specific reactivity was negligible (<10 spots) for all culture conditions (data not shown) and was subtracted from experimental values. Furthermore, PD-1-blocked CTLs from 3/3 tested specimens demonstrated increased specific lysis of gp100+MART-1+ melanoma cells (526mel and 624mel) after two stimulation cycles, (Fig. 4). PD-1-blocked effector cells (6/6 specimens) also demonstrated increased specific lysis of T2 targets pulsed with heteroclitic melanoma peptide analogs and their corresponding native peptides after one stimulation cycle (data not shown). Taken together, these data show that PD-1 blockade during melanoma peptide stimulation increased the total quantities

Table 1. PD-1 expression on fresh post-vaccination gp100- and MART-1-specific CTLs

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Peptide specificity</th>
<th>Percent tetramer+ cells</th>
<th>PD-1 expression</th>
<th>Percent tetramer+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>gp100-2M</td>
<td>1.2</td>
<td>30.8</td>
<td>8.4</td>
</tr>
<tr>
<td>2</td>
<td>gp100-2M</td>
<td>1.4</td>
<td>84.6</td>
<td>1.9</td>
</tr>
<tr>
<td>3</td>
<td>gp100-2M</td>
<td>12.0</td>
<td>83.3</td>
<td>37.9</td>
</tr>
<tr>
<td>4</td>
<td>gp100-2M</td>
<td>0.6</td>
<td>40.0</td>
<td>6.1</td>
</tr>
<tr>
<td>5</td>
<td>gp100-2M</td>
<td>0.2</td>
<td>50.0</td>
<td>9.0</td>
</tr>
<tr>
<td>6</td>
<td>MART-27L</td>
<td>0.6</td>
<td>83.3</td>
<td>40.8</td>
</tr>
<tr>
<td>7</td>
<td>gp100-2M</td>
<td>0.3</td>
<td>66.6</td>
<td>17.0</td>
</tr>
<tr>
<td>8</td>
<td>gp100-2M</td>
<td>0.2</td>
<td>50.0</td>
<td>12.8</td>
</tr>
<tr>
<td>9</td>
<td>MART-27L</td>
<td>0.3</td>
<td>33.3</td>
<td>27.7</td>
</tr>
<tr>
<td>10</td>
<td>gp100-2M</td>
<td>1.1</td>
<td>54.5</td>
<td>46.9</td>
</tr>
<tr>
<td>11</td>
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<td>0.8</td>
<td>60.0</td>
<td>30.9</td>
</tr>
<tr>
<td>12</td>
<td>gp100-2M</td>
<td>2.6</td>
<td>76.9</td>
<td>27.5</td>
</tr>
<tr>
<td>13</td>
<td>gp100-2M</td>
<td>0.2</td>
<td>50.0</td>
<td>31.7</td>
</tr>
<tr>
<td>14</td>
<td>gp100-2M</td>
<td>0.2</td>
<td>50.0</td>
<td>9.7</td>
</tr>
<tr>
<td>15</td>
<td>MART-27L</td>
<td>0.8</td>
<td>75.0</td>
<td>20.2</td>
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<tr>
<td>16</td>
<td>gp100-2M</td>
<td>0.6</td>
<td>66.6</td>
<td>16.9</td>
</tr>
<tr>
<td>17</td>
<td>gp100-2M</td>
<td>0.3</td>
<td>66.6</td>
<td>16.9</td>
</tr>
<tr>
<td>18</td>
<td>MART-27L</td>
<td>0.7</td>
<td>85.7</td>
<td>18.9</td>
</tr>
<tr>
<td>19</td>
<td>MART-27L</td>
<td>0.2</td>
<td>50.0</td>
<td>18.9</td>
</tr>
<tr>
<td>20</td>
<td>gp100-2M</td>
<td>1.0</td>
<td>60.0</td>
<td>43.6</td>
</tr>
<tr>
<td>21</td>
<td>MART-27L</td>
<td>0.4</td>
<td>75.0</td>
<td>43.6</td>
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<tr>
<td>22</td>
<td>gp100-2M</td>
<td>0.3</td>
<td>33.3</td>
<td>27.0</td>
</tr>
<tr>
<td>23</td>
<td>MART-27L</td>
<td>0.4</td>
<td>50.0</td>
<td>27.0</td>
</tr>
</tbody>
</table>

Average *59.8 ± 17.5 22.9 ± 13.4

Tetramer+ CTLs were acquired by gating on the CD3+/CD8+ population. [*P < 0.001 versus (fraction PD-1+ (tetramer– cells).]
of melanoma antigen-specific, IFN-γ-secreting cells and augmented the generation of tumor-cytolytic effector cells.

**PD-1 blockade augments CTL clones that degranulate against tumor targets**

It is increasingly acknowledged that antigen-specific T cells can have diverse requirements for cognate peptide (the epitope that is recognized by a T cell) concentration on targets (20–22, 33–38). T cell RE (also known as functional avidity) refers to a T cell's sensitivity to different stimulatory peptide concentrations on antigen-presenting cells or targets (20, 36). As defined previously, we use the term RE (as opposed to functional avidity) in our study to describe the functional interaction between effector T cells and cognate peptide-bearing cells (20). In a tumor setting, low RE CTLs can be defined by their requirement for high peptide concentration on cells for activation, and therefore are typically not activated by antigen-expressing tumor targets (20, 38). Such low RE CTLs are expanded during in vitro peptide stimulation (38), and also appear to predominate in the peripheral blood of melanoma patients vaccinated with peptides (20–22). It has been suggested that high densities of relevant peptides on professional antigen-presenting cells, such as DCs, paradoxically drive the preferential expansion of low RE T cells that are not efficiently activated by tumor cells in vitro (21).

We used MHC:peptide tetramers to directly enumerate functional, high RE CTLs on the basis of CD107a (also called lysosomal-associated membrane protein-1) externalization upon exposure to antigen-expressing melanoma targets in vitro. During the process of target killing, specialized secretory lysosomes (also called lytic granules) fuse with the T cell membrane and release cytotoxic mediators including perforin and granzymes (39). CD107a is a secretory lysosome membrane protein that is transiently externalized on the cell membrane during this degranulation process. CD107a mobilization against antigen-expressing melanoma targets by cloned gp100-specific CTL lines is closely associated with high RE for cognate peptide (20–22). Thus, the externalization of CD107a can be a surrogate marker to identify individual antigen-specific CTLs with high RE (among a population with diverse RE) for cognate peptide presented endogenously by tumor cells.

The data in Fig. 5 and Table 3 show that after one cycle of peptide stimulation, PD-1 blockade enriched for gp100- and MART-1-specific CTLs (tetramer+) that degranulated upon exposure to HLA-A2+, gp100+/MART-1+ melanoma cell lines (526mel and 624mel). These results were consistent for 6/6 tested patient specimens. Comparatively, low background CD107a staining of tetramer+ CTLs was seen against the HLA-A*0201+, gp100−/MART-1− melanoma cell line A-375, thus demonstrating that reactivity was dependent on expression
of the gp100 and MART-1 antigens by targets. Not only was there an increase in the total frequencies of tetramer+CD107a+ cells in PD-1-blocked cultures but also a modest consistent increase in the fraction of tetramer+ cells that externalized CD107a. Hence, these data suggest that PD-1 blockade altered the functional repertoire of melanoma peptide-stimulated CTL populations in our model, enriching for clones that are efficiently activated.

### Table 2. IFN-γ ELISPOT assay of peptide-stimulated CTLs

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Peptide specificity (Ana. no. of spots)</th>
<th>IgG4 + mel peptides (Ana. no. of spots)</th>
<th>αPD-1 + mel peptides (Ana. no. of spots)</th>
<th>αPD-1 + HIV peptide (Ana. no. of spots)</th>
<th>αPD-1 + mel peptide(s) (Nat. no. of spots)</th>
<th>IgG4 + mel peptides (Nat. no. of spots)</th>
<th>αPD-1 + mel peptide(s) (Nat. no. of spots)</th>
<th>αPD-1 + HIV peptide (Nat. no. of spots)</th>
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<tbody>
<tr>
<td>2</td>
<td>gp100-2M</td>
<td></td>
<td>*123 ± 7</td>
<td></td>
<td>*56 ± 11</td>
<td>9 ± 3</td>
<td>2 ± 1</td>
<td>13 ± 8</td>
</tr>
<tr>
<td>3</td>
<td>gp100-2M</td>
<td></td>
<td>*1308 ± 31</td>
<td></td>
<td>*911 ± 13</td>
<td>336 ± 3</td>
<td>283 ± 16</td>
<td>363 ± 13</td>
</tr>
<tr>
<td>10</td>
<td>gp100-2M</td>
<td></td>
<td>*616 ± 17</td>
<td></td>
<td>*432 ± 13</td>
<td>109 ± 12</td>
<td>60 ± 16</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>15</td>
<td>gp100-2M</td>
<td></td>
<td>*188 ± 4</td>
<td></td>
<td>*120 ± 20</td>
<td>5.5 ± 2</td>
<td>2 ± 3</td>
<td>1 ± 1</td>
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<tr>
<td>12</td>
<td>gp100-2M</td>
<td></td>
<td>*413 ± 13</td>
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<td>*269 ± 17</td>
<td>182 ± 16</td>
<td>101 ± 6</td>
<td>27 ± 5</td>
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<td>13</td>
<td>gp100-2M</td>
<td></td>
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<td>*9 ± 2</td>
<td>1 ± 2</td>
<td>3 ± 3</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>16</td>
<td>gp100-2M</td>
<td></td>
<td>*2424 ± 181</td>
<td></td>
<td>*1590 ± 23</td>
<td>1045 ± 38</td>
<td>744 ± 31</td>
<td>80 ± 18</td>
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<tr>
<td>17</td>
<td>gp100-2M</td>
<td></td>
<td>*1287 ± 166</td>
<td></td>
<td>*869 ± 42</td>
<td>615 ± 35</td>
<td>268 ± 49</td>
<td>57 ± 8</td>
</tr>
<tr>
<td>18</td>
<td>gp100-2M</td>
<td></td>
<td>*478 ± 65</td>
<td></td>
<td>*346 ± 58</td>
<td>3 ± 1</td>
<td>0 ± 0</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>19</td>
<td>gp100-2M</td>
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<td>*114 ± 14</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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<td>20</td>
<td>gp100-2M</td>
<td></td>
<td>*416 ± 34</td>
<td></td>
<td>*350 ± 68</td>
<td>58 ± 1</td>
<td>45 ± 1</td>
<td>5 ± 5</td>
</tr>
<tr>
<td>21</td>
<td>gp100-2M</td>
<td></td>
<td>*167 ± 3</td>
<td></td>
<td>*138 ± 11</td>
<td>54 ± 2</td>
<td>24 ± 9</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>22</td>
<td>gp100-2M</td>
<td></td>
<td>*212 ± 47</td>
<td></td>
<td>*173 ± 3</td>
<td>142 ± 15</td>
<td>116 ± 4</td>
<td>28 ± 9</td>
</tr>
<tr>
<td>23</td>
<td>gp100-2M</td>
<td></td>
<td>*230 ± 6</td>
<td></td>
<td>*182 ± 17</td>
<td>120 ± 8</td>
<td>87 ± 7</td>
<td>18 ± 7</td>
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</table>

CD3+ cells were stimulated with autologous peptide-pulsed DCs for 11 days prior to assay. Reactivity was assayed against autologous CD3–PBMCs pulsed with melanoma peptide analogs [gp100-209–217 (G2M) or MART-1-26–35 (M27L)] or their corresponding native peptides. Data represent number of IFN-γ spots per 10⁶ effector cells. Background reactivity against a control HIV peptide was <10 spots for all conditions (data not shown) and subtracted from experimental conditions. (mel, melanoma; ana, analog peptide; nat, native peptide; *P < 0.02 versus [IgG4 + mel peptides].)
by endogenous levels of antigen presented by melanoma cells.

**Effect of PD-1 blockade on type-1 versus type-2 cytokine repertoire and secretion kinetics**

Type-1 versus type-2 polarization is associated with clinical prognosis in melanoma and renal cell carcinoma (40). Specifically, type-1- or mixed type-1/type-2-polarized cellular immune responses are associated with more favorable prognosis compared with type-2-polarized responses. To our knowledge, an in-depth analysis of the cytokine repertoire and secretion kinetics of PD-1-blocked human CD3+ T cells during *in vitro* peptide stimulation has not been previously reported.

We analyzed T cell culture supernatants for cytokine content on days 2, 4, 6, 8 and 11 of melanoma peptide stimulation, using a Th1/Th2 cytokine multiplex array method. In PD-1-blocked cultures, we observed increased accumulation of IL-5, IL-13, granulocyte macrophage colony stimulating factor (GM-CSF) and IFN-γ on day 11 of stimulation (Fig. 6).

Increases in IL-2, IL-10 and tumor necrosis factor (TNF)-α were seen on earlier days of stimulation, with each diminishing by day 11 (Fig. 6). No significant amount of IL-4 or IL-12 was detected at any time point. These data suggest that the use of a PD-1-abrogating antibody *in vitro* resulted in increased cytokine accumulation in human CD3+ T cell cultures without skewing the type-1 (IL-2, IL-12, GM-CSF, IFN-γ...
and TNF-α) versus type-2 (IL-4, IL-5, IL-10 and IL-13) balance. The increase in type-1 cytokines, in particular IL-2 and TNF-α, may be a contributing factor toward the enhanced generation of melanoma antigen-specific CTLs by PD-1 blockade in our model.

**PD-1 blockade modulates proliferation but not apoptosis of melanoma antigen-specific CTLs**

Tetramer labeling (Fig. 2) and ELISPOT assays (Table 2) showed that PD-1 blockade during in vitro peptide stimulation enhanced the generation of functional gp100- and MART-1-specific CTLs. To elucidate the mechanisms by which increased frequencies and absolute numbers of antigen-specific CTLs were observed in our model, we sought to determine if PD-1 blockade induced a larger fraction of antigen-specific CTLs to proliferate or if it decreased CTL apoptosis. Figure 7(A) shows that both PD-1-blocked and IgG4 control-treated tetramer+ CTLs proliferated in response to melanoma peptide stimulation, with peak expression of the proliferation-associated nuclear protein Ki67 observed

**Table 3. CD107a mobilization by peptide-stimulated CTLs**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Peptide specificity</th>
<th>αPD-1 + melanoma peptide</th>
<th>IgG4 + melanoma peptide</th>
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<tr>
<td></td>
<td></td>
<td>526mel (%)</td>
<td>624mel (%)</td>
</tr>
<tr>
<td>2</td>
<td>gp100-2M</td>
<td>5.5</td>
<td>5.3</td>
</tr>
<tr>
<td>3</td>
<td>gp100-2M</td>
<td>10.6</td>
<td>5.3</td>
</tr>
<tr>
<td>6</td>
<td>MART-27L</td>
<td>57.7</td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td>gp100-2M</td>
<td>18.9</td>
<td>11.8</td>
</tr>
<tr>
<td>11</td>
<td>gp100-2M</td>
<td>27</td>
<td>19.4</td>
</tr>
<tr>
<td>12</td>
<td>gp100-2M</td>
<td>16.8</td>
<td>15.8</td>
</tr>
<tr>
<td>Average fold increase</td>
<td>*2.2 ± 0.3</td>
<td>*1.8 ± 0.5</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

Data shown represent the fraction of gp100 and MART-1 tetramer+ CTLs that externalized CD107a during incubation with HLA-A*0201+/gp100+/MART-1+ melanoma targets (526mel and 624mel). A-375 (HLA-A*0201+/gp100−/MART-1−) was used as a negative control. Also shown is average fold increase in the fraction of CD107a+/tetramer+ CTLs from PD-1-blocked cultures compared with IgG4 control-treated cultures (excluding patient 2, where fold increase is infinite). (*P < 0.02.)
on day 6 of culture. Notably, a higher fraction of PD-1-blocked tetramer+ CTLs expressed Ki67. Augmented Ki67 expression by PD-1 blockade was also seen in tetramer− CTLs, which may represent reactivity to endogenous DC antigens or processed culture media proteins. This induction of proliferation was not sustained, however, as Ki67 expression in both PD-1-blocked and IgG4 control-treated cells returned to background levels by day 11 of stimulation. These data show that PD-1 blockade induced a higher fraction of antigen-specific CTLs to exhibit a burst of proliferation in response to peptide stimulation. Analysis of CFSE dilution in antigen-specific CTLs corroborated these findings, as a higher fraction of PD-1-blocked tetramer+ CTLs underwent more than six rounds of division (Fig. 7B). However, we found that PD-1 blockade did not have any significant effect on the fraction of tetramer+ CTLs undergoing apoptosis, as measured by Annexin V staining (Fig. 8). These findings suggest that the increased frequencies and absolute numbers of melanoma antigen-specific CTLs observed in our model resulted primarily from augmented proliferation and not decreased apoptosis.

**Discussion**

The CD28 family of co-stimulatory molecules is involved in fine-tuning immune responses in both normal and pathological settings. These molecules provide critical positive and negative signals that initiate, sustain, attenuate and/or terminate lymphocyte responses. Two major groups of co-stimulatory receptors that modulate T cells have been described: (i) the Ig superfamily that includes CD28, PD-1, CTLA-4, B- and T-lymphocyte attenuator-4, B7-H3 and ICOS and (ii) the TNF superfamily that includes OX40, CD27, 4-1BB, CD30 and herpes virus entry mediator (41). Negative co-stimulatory members of the Ig superfamily, CTLA-4 and PD-1, are known to restrict immune responses against self-antigens. As such, these
molecules have emerged as potential therapeutic targets for treating autoimmune disease and cancer. If expression of CTLA-4 and/or PD-1 can alter the expansion of tumor antigen-specific T cells and thus diminish their functional recognition of tumor targets, then abrogation of those immunoregulatory molecules might have clinical utility, especially in strengthening recognition of known self-antigens used in many cancer vaccines.

Recent studies in murine tumor model systems and in human cancer vaccine trials have suggested that antibody-mediated blockade of the CTLA-4 pathway enhances T cell activity and correlates with clinical benefit (23, 42–44). Treatment with a human CTLA-4-abrogating antibody alone or as an adjunct to a peptide vaccine caused significant evidence of autoimmunity as well as anti-tumor responses in melanoma patients (42–44). Interestingly, clinical response and time to relapse appeared to correlate with autoimmunity associated with CTLA-4 abrogation (43, 44). The PD-1 pathway in T cells, which has a distinct mechanism of action from that of CTLA-4 (45), is known to attenuate T cell activation both in vivo and in vitro (9). PD-1 knockout mice, like CTLA-4 knockout mice, exhibit autoimmune phenotypes (9). This suggests that PD-1 blockade, like CTLA-4 blockade, may overcome tolerance mechanisms in vivo. Recent studies have shown that antibody-mediated blockade of PD-L1 and/or PD-L2 has enhancing effects on the proliferation and function of antigen-specific T cells, including HIV-specific CTLs from unimmunized patients (10–15). To date, no study has examined the effects of antibody-mediated PD-1 blockade on tumor antigen-specific CTLs from vaccinated cancer patients.

In this work, we used a human in vitro system to characterize how PD-1 abrogation by a fully human antibody alters the expansion and functional capacity of human melanoma antigen-specific CTLs. We found that PD-1 blockade during peptide stimulation increased the absolute numbers of total CD3+, CD4+ and CD8+ cells and preferentially augmented the expansion of gp100- and MART-1-specific CTLs. These results were determined by MHC:peptide tetramer labeling (Fig. 2) and ELISPOT assays (Table 2). Augmented generation of effector cells that lysed gp100+/MART-1+ melanoma targets was also observed (Fig. 4). There also appeared to be an increase in the fraction of gp100 and MART-1 tetramer+ CTLs that degranulated upon exposure to gp100+/MART-1+ melanoma targets, suggesting an increase in CTL RE (Fig. 5). Furthermore, PD-1 blockade increased total cytokine accumulation in our model, but did not selectively enhance either type-1 or type-2 cytokines (Fig. 6). Taken together, these data suggest that PD-1 blockade augments human T cell activation and enhances the expansion of functional effector CTLs derived from melanoma patient peripheral blood.

The overall phenotypes of PD-1-blocked and IgG4 control-treated gp100/MART-1 tetramer+ CTLs were similar, both demonstrating an activated effector memory phenotype characterized by a CD45RA(low), CD45RO(high), CCR7(low), CD62L(low), and CD44(high) expression profile on day 11 of peptide stimulation (data not shown). They were also similar in expression of PD-1, PD-L1, PD-L2, CTLA-4, CD44, CD69, CD25, CD125, CD28, CD137, Lymphotoxin-1 and Very late antigen (VLA)-4 (data not shown). Of significance, PD-1 blockade during peptide stimulation did not inhibit the expression of PD-1 on cognate tetramer+ CTLs. This suggests that sustained expansion of PD-1-expressing CTLs in vitro and in vivo may require continuous treatment with anti-PD-1 antibody.

The exact mechanisms of augmented CTL generation by PD-1 blockade in our model are currently unclear. We observed increased absolute numbers of CD4+ cells in PD-1-blocked cultures, leaving the possibility that the augmented frequencies and absolute numbers of gp100- and MART-1-specific CTLs is, in part, due to the augmented provision of CD4+ Tn. Furthermore, CD4+ Tn may contribute to augmentation of CTL RE (46). In our unpublished observations, we have found that in vitro peptide-driven expansion of post-vaccination human gp100- and MART-1-specific CTLs is markedly impaired by CD4+ depletion. Hence, the enhancing effects of PD-1 blockade in our model may be directly due to PD-1 abrogation in melanoma antigen-specific CTLs and indirectly due to augmented expansion and activity of CD4+ Tn.

It was intriguing that PD-1 blockade did not significantly affect apoptosis of melanoma antigen-specific CTLs in our in vitro stimulation model. It was recently shown that plate-bound agonistic anti-PD-1 antibody induces apoptosis of PD-1-expressing HIV-specific CTLs after 12 h of treatment in the absence of antigen stimulation (12). However, PD-1-negative HIV-specific CTLs also demonstrated increased susceptibility to apoptosis, thus complicating the interpretation of these findings (12). Nevertheless, it is possible that the PD-1 pathway directly induces CTL apoptosis under conditions whereby antigen stimulation is not concomitantly occurring.

In our in vitro peptide stimulation model, the effect of PD-1 blockade on antigen-specific CTL apoptosis was insignificant as measured by Annexin V labeling on days 6 and 11 of...
culture (Fig. 8). Due to TCR down-regulation resulting from stimulation with high concentrations of cognate peptide (22), we were unable to detect significant levels of gp100 and MART-1 tetramer+ CTLs until day 6 of culture. However, if protection against apoptosis by PD-1 blockade was occurring on days 1–5, we would expect percent tetramer staining on day 6 to be significantly lower in IgG4 control-treated cultures. This was not observed in KI67 and Annexin V-labeling assays, however (Figs 7A and 8). Rather, percent tetramer staining was not significantly different between PD-1-blocked and IgG4 control-treated cultures until after day 6. During days 6–11, it was clear that proliferation was enhanced by PD-1 blockade but without any clear evidence of reduced apoptosis (Figs 7A, B and 8). Furthermore, we did not see any significant effect of PD-1 blockade on Annexin V labeling of total CD8+ and CD4+ cells at any time point (data not shown). Taken together, our data therefore suggest that the most significant effect of PD-1 blockade in our model was enhancing T cell proliferation and not protecting against apoptosis. In support of this, previous studies have suggested that undefined PD-L1/PD-L2-binding receptors—distinct from PD-1—may transduce an apoptotic signal in CTLs (47, 48). Nonetheless, the apparent differential effects of PD-1 ligation on CTL apoptosis under varying experimental conditions requires further investigation.

In conclusion, our study suggests that PD-1 blockade by a fully human antibody may be a useful strategy to augment the generation of functional, melanoma antigen-specific CTLs that efficiently recognize tumor targets. The use of a PD-1-abrogating antibody in vitro may also improve the growth and functional profile of expanded human T cells used in current anti-cancer-adaptive transfer strategies (49, 50). Based in part on the work herein, the clinical utility of a PD-1-abrogating antibody in vivo should be tested alone and in a phase I cancer vaccine trial. Its combination with a CTLA-4-abrogating antibody should also be a consideration, given the different modes of signal transduction triggered by ligation of these molecules (45).

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Abbreviations

ACPE allophycocyanin

CFSE carboxyfluorescein succinimidyl ester

CTLA-4 cytotoxic T lymphocyte antigen-4

DC dendritic cell

ELISPOT enzyme-linked immunospot

GM-CSF granulocyte macrophage colony stimulating factor

gp100 glycoprotein 100

HS human AB serum

ICOS inducible co-stimulator

LFA Lymphocyte function-associated antigen

LU lytic units

MART melanoma antigen recognized by T cell

MLR mixed lymphocyte reaction

PD programmed death

PD-L programmed death ligand

RE recognition efficiency

TNF tumor necrosis factor

VLA Very late antigen

References


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