IL10 and PD-1 Cooperate to Limit the Activity of Tumor-Specific CD8<sup>+</sup> T Cells
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Abstract

Immune checkpoint inhibitors show great promise as therapy for advanced melanoma, heightening the need to determine the most effective use of these agents. Here, we report that programmed death-1<sup>high</sup> (PD-1<sup>high</sup>) tumor antigen (TA)-specific CD8<sup>+</sup> T cells present at periphery and at tumor sites in patients with advanced melanoma upregulate IL10 receptor (IL10R) expression. Multiple subsets of peripheral blood mononuclear cells from melanoma patients produce IL10, which acts directly on IL10R<sup>+</sup> TA-specific CD8<sup>+</sup> T cells to limit their proliferation and survival. PD-1 blockade augments expression of IL10R by TA-specific CD8<sup>+</sup> T cells, thereby increasing their sensitivity to the immunosuppressive effects of endogenous IL10. Conversely, IL10 blockade strengthened the effects of PD-1 blockade in expanding TA-specific CD8<sup>+</sup> T cells and reinforcing their function. Collectively, our findings offer a rationale to block both IL10 and PD-1 to strengthen the counteraction of T-cell immunosuppression and to enhance the activity of TA-specific CD8<sup>+</sup> T cell in advanced melanoma patients. Cancer Res; 75(8); 1–10. ©2015 AACR.

Introduction

T cells recognize tumor antigens (TA) expressed by melanoma cells and can induce tumor regression in animals and in humans (1). However, in the presence of high antigen load in chronic viral infections and in cancer, antigen-specific CD8<sup>+</sup> T cells become dysfunctional/exhausted and lose their capacities to proliferate, produce cytokines, and lyse tumor cells (2, 3). These dysfunctional antigen-specific CD8<sup>+</sup> T cells upregulate a number of inhibitory receptors, including programmed death-1 (PD-1), and PD-1 blockade augments their expansion and functions in vitro (4–8). The capability of PD-1 blockade to provide persistent clinical benefit to approximately 30% to 40% of patients with advanced melanoma has now been demonstrated in multiple clinical trials (9, 10). To further improve the clinical efficacy of PD-1 blockade, it appears critical to identify additional strategies to counteract the major negative immunoregulatory pathways impairing TA-specific CD8<sup>+</sup> T cells in the tumor microenvironment (TME).

IL10 is a potent anti-inflammatory molecule produced by innate and adaptive immune cells, including T cells, natural killer cells, antigen-presenting cells (APC) as well as tumor cells, including melanoma (11–15). The immunosuppressive role of endogenous IL10 in impeding APCs is supported by the demonstration that neutralizing IL10 with anti-IL10R antibodies is required for the stimulation of potent Th1 OVA-specific and TA-specific T-cell responses in mice treated with Toll-like receptor ligands (16, 17). The role of IL10 in cancer immunology remains controversial. In experimental tumor models, IL10 appears to either promote or facilitate tumor rejections (18–26). The effects of IL10 and IL10 blockade on human TA-specific CD8<sup>+</sup> T cells have not been thoroughly evaluated yet. In chronic viral infections, IL10 and PD-1 pathways act synergistically through distinct pathways to suppress T-cell functions, and dual IL10 and PD-1 blockade appears more effective in restoring antiviral CD8<sup>+</sup> and CD4<sup>+</sup> T-cell responses and viral clearance than either single blockade alone (27, 28). Whether IL10 added to PD-1 blockade further enhances TA-specific CD8<sup>+</sup> T-cell functions in melanoma patients remains unknown.

Here, we report for the first time that PD-1<sup>high</sup> CD8<sup>+</sup> T cells directed against the cancer-germline antigen NY-ESO-1 and PD-1<sup>high</sup> CD8<sup>+</sup> tumor-infiltrating lymphocytes (TIL) isolated from patients with advanced melanoma, upregulate IL10 receptor (IL10R). Although PD-1 blockade in the presence of cognate antigen increases the expansion and functions of NY-ESO-1-specific CD8<sup>+</sup> T cells, it also augments IL10R expression by TA-specific CD8<sup>+</sup> T cells. We show that IL10 blockade adds to PD-1 blockade to increase the expansion and functions of NY-ESO-1–specific CD8<sup>+</sup> T cells, supporting the role of dual IL10 and PD-1 blockade to enhance TA-specific CTL responses to melanoma.

Materials and Methods

Subjects

Blood samples and tumor specimen were obtained under the University of Pittsburgh Cancer Institute Institutional Review Board–approved protocols 00-079 and 05-140 from 12 HLA-A2<sup>+</sup> patients with NY-ESO-1<sup>+</sup> stage IV melanoma and spontaneous NY-ESO-1–specific CD8<sup>+</sup> T cells (Supplementary Table S1). The peripheral blood mononuclear cells (PBMC) used in this...
study were obtained from melanoma patients with no prior immunotherapy. The same patients were used across all assays.

Phenotypic analysis

CD8\(^+\) T lymphocytes were purified from PBMCs of patients using MACS Column Technology (Miltenyi Biotec). Alternatively, PBMCs were incubated for 6 days in culture medium containing 50 IU/mL rhIL2 (PeproTech) with peptide NY-ESO-1 157–165 or medium alone in the presence of 10 ng/mL anti-IL10R (clone 2F6; BioLegend) or anti–PD-L1 (clone MIH1; eBioscience) or isotype control antibodies and/or 20 ng/mL rhIL10 (PeproTech). Cells were incubated either with HLA-A2/ NY-ESO-1 157–165, HLA-A2/CMV 495–503, HLA-A2/EBV-BMLF-1 280–288, HLA-A2/Flu-M 58–66, or HLA-A2/MART-1 26–35 tetramers (TC metric Ltd.) before staining with PD-1-PerCP-Cy5.5, IL10R-PE (BioLegend), and CD8-PECy7, CD14-ECD, CD19-APC (BioLegend), and streptavidin-ECG (Invirogen)–conjugated antibodies or reagent. Alternatively, after tetramer labeling, cells were stained with PD-1-PECy7 (BioLegend), CD8-V500, CD69-FITC, or CD57-FITC, CD38-PerCPCy5.5 (BD Biosciences), HLA-A2/CMV 495–503, HLA-A2/EBV-BMLF-1 280–288, HLA-A2/Flu-M 58–66, or HLA-A2/MART-1 26–35 tetramers (TC metric Ltd.) before staining with PD-1-PerCP-Cy5.5, IL10R-PE (BioLegend), and CD8-PECy7, CD14-ECD, CD19-APC (BD Biosciences), CD8-PECy7, CD4-PerCP-Cy5.5 (BioLegend), CD14-ECD, and IL10R-PE. A violet amine reactive dye (Invirogen) was used to assess the viability of the cells. pSTAT3-Alexa 488 (BD Biosciences) was used to identify the phosphorylated form of STAT3 (Ser272). A total of 2.5 × 10^6 events were collected on a FACSaria machine (BD Biosciences) and analyzed with FlowJo software (Tree Star).

IL10 detection

The concentrations of IL10 in supernatant or sera were determined using BD OptEIA Human IL10 ELISA Set (BD Biosciences). To test IL10 production, CD8\(^+\) T cells were purified from PBMCs (MACS Column Technology), and labeled with tet-APC, CD8-PECy7, CD4-PE, and violet. A total of 6 × 10^4 FACS-sorted cells were distributed into 96 wells with 200 μL medium containing 50 IU/mL rhIL2, T2 cells (2:1 ratio) pulsed with peptide NY-ESO-1 157–165 or control peptide HIVpol 476–484 (10 μg/mL). Supernatant was collected for ELISA assay after 48-hour incubation. Alternatively, CD4\(^+\) and CD8\(^+\) T cells were separated by MACS Column Technology, the rest of the cells were labeled with CD14-ECD, CD11c-Alexa700, CD3-PerCP-Cy5.5, CD56-PE, and CD19-FITC, and sorted. Total RNA was extracted from each cell subset, and IL10 mRNA was detected using RT-QPCR as previously described (29).

Carboxyfluorescein diacetate succinimimidyl ester proliferation assay

Proliferation assay was performed as described previously (8). CD8\(^+\) TILs were separated by MACS Column Technology and cultured in medium containing 50 IU/mL rhIL2 for 2 days followed by carboxyfluorescein diacetate succinimimidyl ester (CFSE) labeling and stimulation with anti-CD3 antibodies and autologous non-CD3 cells obtained from the same tumor for 6 days. Cells were stained and analyzed by flow cytometry.

Intracellular cytokine staining assay and apoptosis studies

In vitro stimulation assays were performed as described previously (8). Briefly, PBMCs were stimulated for 6 days in the presence of 10 μg/mL anti-IL10R and/or anti–PD-1 (clone EH12.2H7; BioLegend) blocking mAb or isotype control antibodies before intracellular cytokine staining. Alternatively, cells were harvested and then surface stained with APC-labeled A2/NY-ESO-1 tetramers, and subsequently with CD8-PECy7 and Annexin V-FITC (ApoScreen Annexin V-FITC Apoptosis Kit; Beckman Coulter).

Statistical analysis

Statistical hypotheses were tested with the Wilcoxon signed rank test (for paired results from the same patient) and unpaired t test (for IL10 concentration) with GraphPad Prism statistical analysis program. Tests were two-sided and considered significant for a P value of <0.05.

Results

IL10R is upregulated by PD-1\(^{\text{high}}\) NY-ESO-1–specific CD8\(^+\) T cells

Using HLA-A2 (A2) tetramers (tet), we first investigated the expression of IL10R and PD-1 on NY-ESO-1–specific, MART-1–specific, virus-specific [cytomegalovirus (CMV), Epstein-Barr virus (EBV), and influenza virus (Flu)] and total CD8\(^+\) T cells that are detectable ex vivo in PBMCs of nine HLA-A\(^*\) 0201 (HLA-A\(^2\)) stage IV melanoma patients. The percentages of IL10R\(^+\) cells among NY-ESO-1–specific CD8\(^+\) T cells (mean 8.8% ± SD 2.8%) were significantly higher than those of MART-1–specific (2.4% ± 1.5%), Flu-specific (1.6% ± 1.3%), EBV-specific (3.4% ± 2.7%), and total CD8\(^+\) T cells (Supplementary Fig. S1A and S1B, left), albeit not significantly different from the percentages of IL10R\(^+\) CMV-specific CD8\(^+\) T cells (5.1% ± 4.2%). Similar observations were made in terms of mean fluorescence intensity (MFI; Supplementary Fig. S1B, right). In agreement with previous findings (11, 30), IL10R was also constitutively expressed by multiple cell subsets of PBMCs with higher expression detected on myeloid dendritic cells (DC) and B cells (P < 0.01, Supplementary Fig. S1C).

As previously shown, NY-ESO-1–specific and CD8\(^+\) T cells expressed higher PD-1 levels (frequencies and MFI) than MART-1–specific and virus-specific CD8\(^+\) T cells present in PBMCs of patients with advanced melanoma (6). Notably, PD-1\(^{\text{high}}\) CD8\(^+\) T cells were detected in NY-ESO-1–specific CD8\(^+\) T cells but not in MART-1–specific, virus-specific and total CD8\(^+\) T cells (Fig. 1A and Supplementary Fig. S1D). The percentages of IL10R\(^+\) cells within PD-1\(^{\text{high}}\) NY-ESO-1–specific CD8\(^+\) T cells (mean 23.6% ± SD 10.2%) were significantly higher than within PD-1\(^{\text{low}}\) (4.2% ± 1.7%) and PD-1\(^{\text{low}}\) (1.3% ± 0.9%) NY-ESO-1–specific CD8\(^+\) T cells. Similar observations were made in terms of MFI (Fig. 1B, right).

In agreement with previous studies (13, 31, 32), we detected higher levels of IL10 in sera of advanced melanoma patients than in healthy donors (Supplementary Fig. S2A). Although multiple cellular subsets produced IL10, monocytes and DCs expressed the highest IL10 levels in both melanoma patients and healthy donors (Supplementary Fig. S2B). Interestingly, in 1 patient with high NY-ESO-1–specific CD8\(^+\) T-cell frequency, ex vivo sorted PD-1\(^{\text{high}}\) NY-ESO-1–tet\(^+\) CD8\(^+\) T cells produced IL10 in the presence of cognate peptide-pulsed APCs (Supplementary Fig. S2C), supporting that dysfunctional PD-1\(^{\text{high}}\) antigen-specific CD8\(^+\) T cells can produce IL10 upon T-cell receptor (TCR) activation (33).

Collectively, our results show that circulating PD-1\(^{\text{high}}\) NY-ESO-1–specific CD8\(^+\) T cells upregulate IL10R expression, and
that IL10 is produced at low levels by multiple cells in PBMCs of patients with advanced melanoma.

NY-ESO-1–specific CD8+ T cells upregulate IL10R along with PD-1 upon TCR activation

To determine the activation status of IL10R+ PD-1high NY-ESO-1–specific CD8+ T cells, we evaluated their expression of HLA-DR, CD38, and CD57. IL10R+ PD-1high cells expressed higher levels of HLA-DR, CD38, and CD57 than IL10R+ PD-1int, IL10R+ PD-1low, and IL10R- PD-1low NY-ESO-1–specific CD8+ T cells, suggesting that they represent a highly activated NY-ESO-1–specific CD8+ T-cell subset (Fig. 2A).

To investigate whether IL10R upregulation occurs upon TCR activation, we next assessed the sequential expression of IL10R and PD-1 by spontaneous NY-ESO-1–specific CD8+ T cells present in PBMCs of patients with advanced melanoma over a 6-day in vitro stimulation in the presence of cognate or irrelevant peptide. We observed a significant increase in IL10R expression (percentage and MFI) by a fraction of NY-ESO-1–specific CD8+ T cells following cognate antigen stimulation from day 4 to day 6 of in vitro stimulation (Fig. 2B). As previously reported, NY-ESO-1–specific CD8+ T cells also upregulated PD-1 expression upon stimulation with cognate peptide (8), with a majority of the T cells displaying a PD-1high phenotype starting at day 4 (Supplementary Fig. 3A and 3B). The percentages of IL10R+ PD-1high among NY-ESO-1–specific CD8+ T cells as well as the frequencies of IL10R+ PD-1high NY-ESO-1–specific CD8+ T cells among total CD8+ T cells increased upon stimulation with cognate peptide (Fig. 2C).

Figure 1.

IL10R is upregulated by PD-1high NY-ESO-1–specific CD8+ T cells. A and B, dot plots from one representative patient (A) and summary data for all 9 patients with advanced melanoma (B) showing ex vivo IL10R expression by PD-1high and/or PD-1int and PD-1low subsets of A2/NY-ESO-1 157–165, A2/MART-1 26–35, A2/CMV 495–503, A2/Flu-M 58–66, A2/EBV BMLF1 280–288 tet+, and total tet- CD8+ T cells; *P < 0.05 and **P < 0.01. Horizontal bars depict the mean percentage or MFI of IL10R expression. Data, representative of two independent experiments performed in duplicate.
NY-ESO-1–specific CD8⁺ T cells upregulate IL10R and PD-1 upon TCR activation. A, pooled data from melanoma patients (n = 8) showing the percentage (left) and MFI (right) of ex vivo expression of HLA-DR, CD38, and CD57 on IL10R⁺ and IL10R⁻ subsets in PD-1high and on IL10R⁻ subsets in PD-1int and PD-1low NY-ESO-1–specific CD8⁺ T cells. B, IL10R expression on NY-ESO-1 tet⁺ CD8⁺ T cells assessed ex vivo and after indicated hours following in vitro stimulation with cognate peptide (NY-ESO-1 peptide) or irrelevant peptide (HIV peptide; n = 6). C, the percentage of IL10R⁺PD-1high in NY-ESO-1 tet⁺ CD8⁺ T cells (left) and in total CD8⁺ T cells (right) tested in B. Data, representative of at least two independent experiments; *, P < 0.05 and **, P < 0.01. Horizontal bars depict the mean percentage or MFI of cells that express the corresponding molecule.
Collectively, our findings show that IL10R<sup>hi</sup> PD-1<sup>hi</sup> NY-ESO-1–specific CD8<sup>+</sup> T cells represent a highly activated T-cell subset that expands upon prolonged TCR activation.

**NY-ESO-1–specific CD8<sup>+</sup> T cells upregulate IL10R expression upon PD-1 blockade**

To investigate the effects of PD-1 blockade on IL10R expression by total NY-ESO-1–specific CD8<sup>+</sup> T cells, PBMCs of melanoma patients were stimulated with NY-ESO-1 peptide for 6 days in the presence of blocking mAbs against PD-L1, IL10R, or isotype control antibodies, before tetramer and IL10R or PD-1 labeling. We observed an increase in IL10R expression by total NY-ESO-1 tet<sup>+</sup>, but not tet<sup>-</sup> CD8<sup>+</sup> T cells after incubation in the presence of cognate peptide and anti–PD-L1 antibodies, as compared with cognate peptide and IgG control antibodies (mean percentage of IL10R<sup>+</sup>: 35.3% ± SD 12.7% vs. 25.0% ± 9.8% and mean MFI IL10R: 537 ± SD 220 vs. 394 ± 156, respectively; Fig. 3A and B). In contrast, IL10R blockade did not increase PD-1 expression by NY-ESO-1–specific CD8<sup>+</sup> T cells (Supplementary Fig. S4A and S4B).

Collectively, our findings show that NY-ESO-1–specific CD8<sup>+</sup> T cells upregulate IL10R upon PD-1 blockade and prolonged antigen stimulation, whereas IL10R blockade has no effect on PD-1 expression.

**IL10 impedes the expansion of NY-ESO-1–specific CD8<sup>+</sup> T cells upon antigen stimulation**

Because we have shown that IL10R expression is upregulated by PD-1<sup>hi</sup> NY-ESO-1–specific CD8<sup>+</sup> T cells and increases following TCR activation, we next investigated the effect of IL10 on the expansion and survival of NY-ESO-1–specific CD8<sup>+</sup> T cells upon stimulation with cognate antigen. CFSE-labeled PBMCs from melanoma patients were stimulated for 6 days with NY-ESO-1 157–165 peptide in the presence or absence of rhIL10 and/or anti-IL10R mAbs. The frequencies of proliferating CFSE<sup>hi</sup> NY-ESO-1–specific CD8<sup>+</sup> T cells significantly decreased after in vitro stimulation in the presence of IL10 as compared with stimulation with peptide only (0.5-fold change) and were restored in the presence of blocking anti-IL10R mAbs (Fig. 4A–C). The percentages of Annexin V<sup>-</sup> NY-ESO-1–specific CD8<sup>+</sup> T cells increased after a 6-day in vitro stimulation with cognate peptide in the presence of rhIL10 (1.3-fold change), but not rhIL10 in combination with anti-IL10R mAbs, as compared with in vitro stimulation with peptide only (Fig. 4D). Notably, in the presence of rhIL10, the expression of phosphorylated STAT3 (p-STAT3) in NY-ESO-1–specific CD8<sup>+</sup> T cells was significantly higher than in EBV-specific CD8<sup>+</sup> T cells, which express lower levels of IL10R, as compared with untreated cells (3- and 1.6-fold increase, respectively). The effect of IL10 on p-STAT3 expression was abrogated in the presence of blocking anti-IL10R mAbs, suggesting that IL10 acts directly on T cells through IL10R activation (Fig. 4E).

Collectively, our findings show that IL10 impedes the expansion of NY-ESO-1–specific CD8<sup>+</sup> T cells by decreasing their proliferative capacity and promoting their apoptosis. We also observed that IL10 acts directly on IL10R<sup>hi</sup> NY-ESO-1–specific CD8<sup>+</sup> T cells.

**IL10R blockade adds to PD-1 blockade to increase the expansion and functions of NY-ESO-1–specific CD8<sup>+</sup> T cells**

We then investigated whether IL10R blockade alone or in combination with PD-1 blockade increased NY-ESO-1–specific CD8<sup>+</sup> T-cell expansion and functions in response to cognate antigen. We observed a modest increase in the frequencies of proliferating (CFSE<sup>hi</sup>) and total NY-ESO-1 tet<sup>+</sup> CD8<sup>+</sup> T cells in the presence of cognate peptide and anti-IL10R mAbs as compared with cognate peptide and IgG control antibodies (1.5- and 1.3-fold changes, respectively; Fig. 5A and B). In line with previous findings (6, 7), PD-1 blockade significantly increased the frequencies of CFSE<sup>hi</sup> and total NY-ESO-1–specific CD8<sup>+</sup> T cells (1.7- and 1.4-fold changes, respectively). There was no significant difference between single IL10R blockade and single PD-1 blockade on NY-ESO-1–specific CD8<sup>+</sup> T cells proliferation. Interestingly, dual IL10R and PD-1 blockade further increased the frequencies of proliferating and total NY-ESO-1–specific CD8<sup>+</sup> T cells as compared with incubation with IgG control antibodies, anti-IL10R mAbs alone or anti–PD-1 mAbs alone, resulting in the highest fold changes in the frequencies of CFSE<sup>hi</sup> and total NY-ESO-1 tet<sup>+</sup> CD8<sup>+</sup> T cells (2.5- and 1.9-fold changes, respectively; Fig. 5A and B). The frequencies of NY-ESO-1–specific CD8<sup>+</sup> T cells that produced IFNγ, but not TNF, increased after a 6-day in vitro stimulation in the presence of cognate peptide and anti-IL10R mAbs as compared with incubation with IgG control antibodies (1.3-fold change; Fig. 5C and D). The frequencies of IFNγ- and TNF-producing NY-ESO-1–specific CD8<sup>+</sup> T cells (1.6- and 1.5-fold changes, respectively). Dual IL10 and PD-1 blockade further...
increased the frequencies of IFNy- and TNF-producing NY-ESO-1-specific CD8+ T cells (2.1- and 1.9-fold changes, respectively; Fig. 5C and D, top and Supplementary Fig. S5C and S5D). In addition, we observed an increase in the frequencies of IFNy+ TNF+ NY-ESO-1-specific CD8+ T cells (1.9-fold change, Fig. 5C and D, bottom), suggesting that dual IL10R and PD-1 blockade expands polyfunctional TA-specific CD8+ T cells.

Collectively, our findings show that IL10 blockade adds to PD-1 blockade to enhance the expansion and functions of NY-ESO-1-specific CD8+ T cells.

CD8+ TILs present in metastatic melanoma coprepare IL10R and PD-1

To determine whether our findings on circulating TA-specific CD8+ T cells were relevant to CD8+ TILs present in the TME, we assessed IL10R and PD-1 expression by CD8+ TILs obtained from metastatic lesions of nine melanoma patients. The percentages of IL10R+ cells were significantly higher in CD8+ TILs (mean 30.0% ± SD 19.6%) than in total NY-ESO-1 tet+ (8.8% ± 2.8%) and tet- (1.7% ± 1.0%) CD8+ T cells present in PBMCs of melanoma patients (Fig. 6A and B left). Similar observations were made in terms of MFI (Fig. 6B right). CD8+ TILs also upregulated PD-1 expression and the percentages of IL10R+ cells within PD-1high CD8+ TILs (mean 39.8% ± SD 24.4%) were significantly higher than within PD-1int (29.2% ± 27.6%) and PD-1low (20.7% ± 23.4%) CD8+ TILs. Similar observations were made in terms of MFI (Fig. 6C and D).

To investigate whether IL10 blockade alone or in combination with PD-1 blockade increases the expansion of CD8+ TILs, CD8+ T cells isolated from one metastatic tumor single cell suspension were incubated in vitro for 6 days in the presence of anti–CD3 antibodies and autologous non-CD3 cells obtained from the same tumor. We observed an increase in the proliferation of CD8+ TILs in the presence of anti-IL10R or anti–PD-1 antibodies as compared with IgG control antibodies. Dual IL10R and PD-1 blockade further increased the frequencies of proliferating CD8+ TILs (Fig. 6E).

Collectively, our findings show that CD8+ TILs present in metastatic melanoma coprepare IL10R and PD-1.

Discussion

In this article, we report that PD-1high TA-specific CD8+ T cells in PBMCs and TILs of patients with advanced melanoma upregulate IL10R expression. Among PD-1+ antigen-specific CD8+ T
cells, it is now well established that PD-1\textsuperscript{high} CD\textsuperscript{8} T cells represent a dysfunctional T-cell subset occurring upon persistent antigen stimulation in chronic infections and in cancer (4, 5, 8, 34, 35). TA-specific dysfunctional CD\textsuperscript{8} T cells present at periphery and at tumor sites upregulate a number of inhibitory receptors, including PD-1, BTLA, and Tim-3, that bind to their respective ligands expressed by APCs and tumor cells in the TME (7, 8, 36). Here, we show that TA-specific CD\textsuperscript{8} T cells increase IL10R expression upon TCR activation and PD-1\textsuperscript{high}IL10R\textsuperscript{+} cells upregulate HLA-DR and CD38, supporting that IL10R is a T-cell activation marker expressed by chronically activated TA-specific CD\textsuperscript{8} T cells in patients with advanced melanoma.

IL10 is an immunoregulatory cytokine, which is produced by multiple innate and adaptive immune cells as well as melanoma cells (11). IL10 inhibits antigen-specific T-cell responses by impeding antigen presentation and costimulation, inducing human CD\textsuperscript{4} T-cell anergy (37, 38). A number of observations have also suggested that IL10 may exert immunostimulatory effects on CD\textsuperscript{8} T cells, depending on their state of activation (39). Elevated IL10 levels have been reported in patients with HCV, HBV, and HIV infections, and correlate with T-cell dysfunction and uncontrolled viral replication (17, 40–42).

The role of IL10 in cancer immunology remains controversial. IL10 transgenic mice, which produce moderate and transient

**Figure 5.**

IL10R blockade adds to PD-1 blockade to increase the expansion and functions of NY-ESO-1–specific CD\textsuperscript{8} T cells. A, representative flow-cytometry analysis from one melanoma patient showing the percentages of CFSE\textsuperscript{lo} NY-ESO-1–specific CD\textsuperscript{8} T cells among total CD\textsuperscript{8} T cells in CFSE-based proliferation assay (n = 9). B, fold change of the frequencies of CFSE\textsuperscript{lo} (left) and total (right) NY-ESO-1–specific CD\textsuperscript{8} T cells after \textit{in vitro} stimulation with cognate peptide and aPD-1 and/or aIL10R. The ratio of the percentages of CFSE\textsuperscript{lo} and total NY-ESO-1–specific CD\textsuperscript{8} T cells in the presence of indicated antibody treatment and isotype control antibody is shown. C, representative flow-cytometry analysis from one melanoma patient showing the percentages of IFN\textgamma\textsuperscript{+} and TNF-producing NY-ESO-1–specific CD\textsuperscript{8} T cells among total CD\textsuperscript{8} T cells (top) and the percentages of IFN\textgamma\textsuperscript{+}TNF\textsuperscript{+} NY-ESO-1–specific CD\textsuperscript{8} T cells among NY-ESO-1–specific CD\textsuperscript{8} T cells (bottom). PBMCs were incubated with NY-ESO-1 157–165 peptide or with HIVpol 476–484 peptide and aPD-1 and/or aIL10R or an isotype control antibody (IgG) before evaluating intracellular cytokine production of NY-ESO-1–specific CD\textsuperscript{8} T cells upon stimulation with cognate peptide (n = 8). D, fold change of the frequency of cytokine-producing NY-ESO-1–specific T cells from melanoma patients in the presence of indicated antibody treatment and isotype control antibody is shown. *, P < 0.05 and **, P < 0.01. Data, representative of two independent experiments performed in duplicate. IVS, \textit{in vitro} stimulation.
levels of IL10 upon TCR activation, are unable to control tumor growth (18, 19), supporting the immunosuppressive and protumoral effects of IL10. Paradoxically, the administration of high-dose IL10 and pegylated IL10 appears to promote tumor regression in animals, and enhances the expansion and functions of CD8⁺ T cells that express elevated levels of IL10R, resulting in tumor regression (20, 25, 26). In addition, chemically induced transplanted tumors appear to grow better in IL10⁻/⁻ mice (24). In combination with cancer vaccines, and depending on the schedule of injection, high-dose IL10 either promotes T-cell–mediated tumor rejection or tumor progression, illustrating the two opposite effects of IL10 on T cells in vivo depending on their cell activation state (23). In sharp contrast with the experimental models supporting the antitumoral activity of high-dose IL10, we measured low-level circulating IL10 in melanoma patients’ sera. Although multiple immune cells in PBMCs of patients with advanced melanoma produce low-level IL10, we found that the highest IL10 levels are produced by circulating CD11c⁺ and CD14⁺ cells in agreement with a previous study (41). In one patient with a high frequency of NY-ESO-1–specific CD8⁺ T cells that upregulate PD-1, we observed that TA-specific CD8⁺ T cells produce IL10. These findings are in line with previous reports of IL10 production by exhausted/dysfunctional LCMV-specific CD8⁺ T cells that upregulate inhibitory receptors in chronic viral infections (33, 42).

In the present study, we show that IL10 decreases TA-specific CD8⁺ T-cell proliferation upon prolonged stimulation with cognate antigen and increases TA-specific CD8⁺ T-cell apoptosis. Furthermore, upon IL10 exposure, p-STAT3 expression in NY-ESO-1–specific CD8⁺ T cells was significantly higher than in EBV-specific CD8⁺ T cells that do not upregulate PD-1 and IL10R, suggesting that IL10 exerts its immunosuppressive effects by acting directly on IL10R⁺ TA-specific CD8⁺ T cells. Collectively, our data support that endogenous IL10 produced at low levels by multiple innate and adaptive immune cells impedes the proliferation and survival of chronically activated TA-specific CD8⁺ T cells in patients with advanced melanoma. Notably, IL10 production by circulating CD14⁺ cells of patients with advanced melanoma appears to correlate with poor clinical outcome (41).

The immunosuppressive role of endogenous IL10 on T cells has served as a rationale for neutralizing IL10 to stimulate potent antigen–specific T-cell responses in experimental models. Neutralization of IL10 with anti-IL10 mAbs in combination with LPS renders soluble antigen capable of stimulating potent Th1 type T-cell responses in vivo (16). IL10 blockade improves virus-specific T-cell function in mice chronically infected by LCMV virus (43), and dual IL10 and PD-1 blockade appears more effective in restoring virus-specific T-cell function and controlling persistent viral infections (27). Monocytes isolated from HIV patients upregulate PD-1 expression, produce IL10 upon PD-1 ligation in vitro to impair CD4⁺ T-cell activation, and dual IL10 and PD-1 blockade restores T-cell responses to HIV in vivo (28). In cancer immunology, IL10 blockade in combination with intratumoral injection of CPG reverses tumor-infiltrating DC dysfunction to prime potent antitumor T-cell responses leading to tumor regression in mice (17). One novel finding in this article is that IL10 blockade adds to PD-1 blockade to increase the proliferation and
functions of TA-specific CD8+ T cells isolated from PBMCs and THLs. Most interestingly, we observed that TA-specific CD8+ T cells further upregulate IL10R upon PD-1 blockade. It is, therefore, tempting to speculate that upon PD-1 blockade, activated TA-specific CD8+ T cells may be rendered more sensitive to the immunosuppressive effects of endogenous IL10. Therefore, IL10 blockade may represent a potent therapeutic strategy to counteract the direct negative regulatory effects of IL10 on T cells in the TME, which could further increase antitumor T-cell responses in combination with PD-1 blockade.

In summary, our findings demonstrate the upregulation of IL10R by PD-1high TA-specific CD8+ T cells at periphery and at tumor sites in patients with advanced melanoma. They also show that IL10 blockade adds to PD-1 blockade to further enhance the expansion and functions of TA-specific CD8+ T cells. Such findings may have significant implications in terms of potent immunotherapy of melanoma to further improve the clinical efficacy of PD-1 blockade in the clinic.

Disclosure of Potential Conflicts of Interest

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References


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