TIGIT and PD-1 impair tumor antigen–specific CD8+ T cells in melanoma patients

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T cell Ig and ITIM domain (TIGIT) is an inhibitory receptor expressed by activated T cells, Tregs, and NK cells. Here, we determined that TIGIT is upregulated on tumor antigen–specific (TA-specific) CD8+ T cells and CD8+ tumor-infiltrating lymphocytes (TILs) from patients with melanoma, and these TIGIT-expressing CD8+ T cells often coexpress the inhibitory receptor PD-1. Moreover, CD8+ TILs from patients exhibited downregulation of the costimulatory molecule CD226, which competes with TIGIT for the same ligand, supporting a TIGIT/CD226 imbalance in metastatic melanoma. TIGIT marked early T cell activation and was further upregulated by T cells upon PD-1 blockade and in dysfunctional PD-1– TIM-3– TA-specific CD8+ T cells. PD-1–TIGIT+, PD-1–TIGIT−, and PD-1+TIGIT+ CD8+ TILs had similar functional capacities ex vivo, suggesting that TIGIT alone, or together with PD-1, is not indicative of T cell dysfunction. However, in the presence of TIGIT ligand–expressing cells, TIGIT and PD-1 blockade additively increased proliferation, cytokine production, and degranulation of both TA-specific CD8+ T cells and CD8+ TILs. Collectively, our results show that TIGIT and PD-1 regulate the expansion and function of TA-specific CD8+ T cells and CD8+ TILs in melanoma patients and suggest that dual TIGIT and PD-1 blockade should be further explored to elicit potent antitumor CD8+ T cell responses in patients with advanced melanoma.

Introduction

T cells recognize tumor antigens expressed by melanoma cells but often fail to promote tumor regression in humans (1). There is now ample evidence that tumor antigen–specific (TA-specific) CD8+ T cells become dysfunctional and exhausted upon chronic antigen exposure, losing their capacity to proliferate, produce cytokines, and lyse tumor cells (2, 3). Dysfunctional TA-specific CD8+ T cells upregulate a number of inhibitory receptors including PD-1, TIM-3, and BTLA, which bind to their respective ligands expressed by antigen-presenting cells (APCs) and tumor cells, impeding T cell survival and functions in the tumor microenvironment (TME) (4–8). Blocking Abs targeting these inhibitory receptors successfully improve T cell responses in vitro and promote tumor regression in vivo in animals (5–7, 9, 10). Immune checkpoint blockade with anti–PD-1 Abs has provided persistent clinical benefits for approximately 30% to 40% of patients with advanced melanoma in multiple clinical trials (11, 12). In addition, dual PD-1 and CTLA-4 blockade appears to further improve clinical outcome in patients (13). It is therefore expected that targeting multiple inhibitory pathways in the TME will prove useful for the majority of patients with advanced cancers, including melanoma.

T cell Ig and ITIM domain (TIGIT) is an inhibitory receptor that is expressed by activated T cells, Tregs, and NK cells and binds the adhesion molecules CD155 (Nect-5, also known as PVR) and CD112 (nectin-2, also known as PRR2 or PVR2) with high and low affinity, respectively (14–17). CD155 and CD112 also bind to other ligands including the costimulatory counterpart to TIGIT, CD226 (DNAM-1), which associates with LFA-1 to positively regulate T cell responses (18, 19), and CD96 (20). CD155 and CD112 play a role in T cell– and NK cell–mediated cytotoxicity against tumors (21, 22). CD155 is expressed by neural tissues (23), endothelial cells, epithelial cells, platelets, CD14+ cells, and DCs, as well as by activated T cells and TLR-activated B cells (16, 23–27). CD112 is expressed by endothelial cells, hematopoietic cells, and immune cells including activated T cells and B cells, CD14+ cells, and DCs (28–30). Notably, CD155 and CD112 are also expressed by various human tumors, including melanoma (31–34).

Initial studies suggested that TIGIT exerts its immunosuppressive effects by enhancing IL-10 production by DCs through CD155, impeding CD4+ T cell proliferation and function (16). However, it was later demonstrated that TIGIT also exerts CD4+ T cell–intrinsic inhibitory effects via recruitment of SHP phosphatases that suppress cytokine production and proliferation (35, 36) and competes with CD226 for PVR binding (37). The TIGIT locus is demethylated in Tregs and may potentially bind to FOXP3 (38). TIGIT+ Tregs are highly activated, secrete the soluble effector molecule fibrinogen-like protein 2, and selectively inhibit Th1 and Th17 responses (39).

Most recently, TIGIT expression by CD8+ tumor-infiltrating lymphocytes (TILs) has been reported using gene expression analyses in a number of mouse and human solid tumors including lung, colon, breast, uterine, and renal cancers. Elevated TIGIT expression appears to correlate with CD8 and PD-1 expression. TIGIT expression on CD8+ TILs was observed in...
mouse tumors and in 3 human tumor samples, including non-small-cell lung and colon cancers (40). Interestingly, TIGIT blockade synergized with PD-L1 blockade to enhance CD8+ TIL functions in mice and promoted the rejection of transplanted tumors, while single-agent blockade had no effect (40). Whether TIGIT is upregulated by TA-specific CD8+ T cells in the periphery and at tumor sites in patients with advanced melanoma remains unknown. In addition, its role in regulating the expansion and function of TA-specific CD8+ T cells in melanoma patients has not yet been investigated. Here, we show that TIGIT is upregulated and coexpressed with PD-1 on the majority of circulating TA-specific CD8+ T cells directed against the cancer germline antigen NY-ESO-1 and on the majority of CD8+ TILs isolated from metastatic melanoma. CD8+ TILs downregulated CD226, supporting an imbalance of TIGIT/CD226 expression in metastatic melanoma. The TIGIT ligands CD155 and CD112 were upregulated by the majority of APCs and melanoma cells in the TME. TIGIT blockade added to PD-1 blockade to increase the expansion and functions of circulating TA-specific CD8+ T cells and CD8+ TILs. Altogether, our findings support the use of TIGIT blockade in combination with PD-1 blockade to enhance CD8+ T cell responses to melanoma and improve the clinical efficacy of PD-1 blockade for patients with advanced melanoma.

Results

TIGIT and PD-1 are coexpressed on NY-ESO-1–specific CD8+ T cells. Using HLA-A2 (A2) tetramers, TIGIT expression on the surface of NY-ESO-1–, influenza- (Flu), and CMV-specific CD8+ T cells isolated from PBMCs from 8 HLA-A0201+ stage IV melanoma patients was assessed by flow cytometry ex vivo. In melanoma patients, the frequencies of TIGIT+ cells among A2/NY-ESO-1–specific CD8+ T cells (mean frequency, 90.7% ± SD 4.1%) were significantly higher than those among Flu-specific, CMV-specific, effector (CD45RA+CCR7+), and effector memory (CD45RO–CCR7–) CD8+ T cells (40.1% ± 12.2%, 45.4% ± 26.7%, 57.7% ± 20.33%, and 15.9% ± 15.9%, respectively; Figure 1, A and B). Similar results were observed in terms of mean fluorescence intensity (MFI) (Figure 1B).

We next assessed the coexpression of PD-1 and TIGIT ex vivo on NY-ESO-1–, Flu–, and CMV-specific CD8+ T cells. The large majority of NY-ESO-1–specific CD8+ T cells coexpressed TIGIT and PD-1, with mean frequencies of TIGIT+PD-1+ NY-ESO-1–specific CD8+ T cells (83% ± SD 7.8%) being significantly higher than those of TIGIT+PD-1+, PD-1+ TIGIT+, and PD-1–TIGIT– cells (6.8% ± 3.3%, 5% ± 2.8% and 5.3% ± 3.9% respectively; Figure 1, C and D). TIGIT and PD-1 coexpression on NY-ESO-1–specific CD8+ T cells was positively correlated in terms of frequencies and MFI (τ = 0.77, P = 0.025 and r = 0.092, P = 0.0012, respectively; Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI80445DS1). In sharp contrast to NY-ESO-1–specific CD8+ T cells, Flu- and CMV-specific CD8+ T cells, as well as effector and effector memory tet CD8+ T cells, were predominantly TIGIT+PD-1+ (mean frequency of 56.5% ± SD 17%, 50.2% ± 23.7%, 37.6% ± 17.7%, and 51.7% ± 13.1%, respectively), while TIGIT+PD-1+ cells (5.4% ± 3.9%, 7.4% ± 7.7%, and 14.9% ± 7.5%, respectively) and TIGIT+PD-1– cells (9.2% ± 8.1%, 4.2% ± 5.5%, 4.7% ± 4.3%, and 7.5% ± 6.3%, respectively) represented small subsets of cells. In contrast to NY-ESO-1–specific CD8+ T cells, TIGIT and PD-1 were rarely coexpressed by Flu- or CMV-specific CD8+ T cells (Figure 1, C and D).

We have also evaluated TIGIT expression on different subsets of mononuclear cells including CD8+ T cells, CD4+ T cells, NK cells (CD56+), B cells (CD19+), monocytes (CD14+), and myeloid DCs (mDCs) (CD11c+) isolated from PBMCs from melanoma patients and healthy donors. TIGIT was expressed on subsets of CD8+ T cells, CD4+ T cells, and NK cells, with no significant differences observed between melanoma patients and healthy donors (Supplemental Figure 1, C and D).

Collectively, our results demonstrate that TIGIT expression is upregulated on tumor-induced NY-ESO-1–specific CD8+ T cells in patients with advanced melanoma. The vast majority of NY-ESO-1–specific CD8+ T cells coexpress TIGIT and PD-1, unlike Flu-specific, CMV-specific, tet effector, or tet effector memory CD8+ T cells in the same melanoma patients.

TIGIT+PD-1+ NY-ESO-1–specific CD8+ T cells exhibit high levels of T cell activation. We next assessed the differentiation and activation status of NY-ESO-1–specific and tet CD8+ T cells according to TIGIT and/or PD-1 expression in patients with advanced melanoma. To this end, in 8 stage IV melanoma patients, we compared the percentages of CD8+ T cells, which express the following markers ex vivo: CCR7, CD45RA, HLA-DR, and CD38 among TIGIT+PD-1+, TIGIT+PD-1–, TIGIT–PD-1+, and TIGIT–PD-1– tet CD8+ T cells. Because of the low frequencies of PD-1–TIGIT+, PD–1TIGIT+, and PD–1 TIGIT–NY-ESO-1–specific CD8+ T cells, we compared the phenotype of TIGIT+PD-1+ tet CD8+ T cells with that of PD–1TIGIT+, PD–1TIGIT–, and PD–1 TIGIT– tet CD8+ T cells (Figure 2, A and B). The percentages of HLA-DR+ cells were higher among PD–1TIGIT+ tet CD8+ T cells than among PD–1 TIGIT+ and PD–1 TIGIT+ tet CD8+ T cells (mean percentage, 73.1% ± SD 12.4% vs. 55.6% ± 26.6% and 27.4% ± 22.4%, respectively). The frequencies of CD38+ cells were significantly higher among PD–1TIGIT+ NY-ESO-1–specific CD8+ T cells (68.6% ± 18.5%) than among PD–1 TIGIT+ tet CD8+ T cells (22.4% ± 14.4%) and PD–1 TIGIT+ tet CD8+ T cells (31.5% ± 27.1%), but not PD–1 TIGIT+ tet CD8+ T cells (38% ± 28.2%). Notably, HLA-DR expression on PD–1 TIGIT+ NY-ESO-1–specific CD8+ T cells was significantly higher than on PD–1 TIGIT+ tet CD8+ T cells (83.1% ± 9.4% vs. 55.6% ± 26.6%, Figure 2B).

The percentages of CCR7+ and CD45RA+ TIGIT+PD–1+ tet CD8+ T cells were lower than those of TIGIT+PD–1+ and TIGIT–PD–1+ tet CD8+ T cells (20% ± 14.6% vs. 31.9% ± 27.5% and 49.8% ± 29.8%, respectively, for CCR7 and 36.1% ± 14.8% vs. 57.8% ± 23.4% and 56.8% ± 16.9%, respectively, for CD45RA). In addition, the percentages of CD45RA+ cells were lower among TIGIT+PD–1 NY-ESO-1–specific CD8+ T cells than among TIGIT–PD–1+ tet CD8+ T cells (14.4% ± 11.6% vs. 36.1% ± 27.5%; Figure 2B), while the percentages of CCR7+ cells were similar in these 2 cell subsets, supporting our finding that TIGIT+PD–1+ CD8+ T cells are effector memory cells.

We observed that TIGIT expression (MFI) was higher on dysfunctional PD–1TIM–3+ than on PD–1TIM–3 NY-ESO-1–specific CD8+ T cells, suggesting that TIGIT expression is upregulated upon chronic antigen exposure (Figure 2C). To investigate further, we isolated NY-ESO-1–specific CD8+ T cells from the peripheral
Figure 1. TIGIT is upregulated and coexpressed with PD-1 on NY-ESO-1–specific CD8+ T cells. (A) Representative dot plots for 1 melanoma patient showing ex vivo TIGIT expression on A2/NY-ESO-1 157-165, A2/Flu-M 58-66, and A2/CMV 495-503 tet+ CD8+ T cells. CD8+ T cells stained with A2/HIV pol 476-484 tetramers or PE-labeled IgG control mAbs were used to establish the threshold for identifying tet+ and TIGIT+ cells, respectively. (B) Pooled data showing the percentage and MFI of TIGIT expression on NY-ESO-1–, Flu–, and CMV–specific CD8+ T cells as well as on total effector (CD45RA+CCR7–) and effector memory (CD45RO+CCR7–) CD8+ T cells from melanoma patients (n = 8). P values were obtained by repeated-measures ANOVA, followed by Tukey’s multiple comparisons test. (C) Dot plots for 1 representative melanoma patient showing ex vivo TIGIT and PD-1 expression on A2/NY-ESO-1 157-165, A2/Flu-M 58-66, and A2/CMV 495-503 tet+ CD8+ T cells. (D) Pooled data showing the distribution of NY-ESO-1–, Flu–, and CMV–specific CD8+ T cells, as well as of total effector and effector memory CD8+ T cells according to TIGIT and PD-1 expression in cells from melanoma patients (n = 8). P values were obtained by Friedman’s test, followed by Dunn’s multiple comparisons test. Horizontal bars depict the mean percentage or MFI. *P < 0.05; **P < 0.01; ***P < 0.001. Data shown are representative of 3 independent experiments.
TCR activation and is further upregulated by highly dysfunctional PD-1+TIM-3+ TA-specific CD8+ T cells in patients with advanced melanoma.

TIGIT blockade adds to PD-1 blockade to increase proliferation of NY-ESO-1–specific CD8+ T cells upon antigen stimulation. We next evaluated the effect of TIGIT blockade alone or in combination with PD-1 blockade on the proliferation of NY-ESO-1–specific CD8+ T cells upon cognate antigen stimulation. CFSE-labeled PBMCs from 9 patients with advanced melanoma were incubated in blood of melanoma patients and assessed the expression of TIGIT over the 6 days of in vitro stimulation (IVS) with cognate or irrelevant peptide (Supplemental Figure 2, A and B). TIGIT expression (MFI) was increased after 24 hours of stimulation with cognate peptide but did not significantly increase thereafter. This differs considerably from PD-1 expression (5), which was not significantly increased until day 4 of stimulation.

Altogether, our findings demonstrate that TIGIT is an early activation marker expressed by TA-specific CD8+ T cells upon TCR activation and is further upregulated by highly dysfunctional PD-1+TIM-3+ TA-specific CD8+ T cells in patients with advanced melanoma.

**Figure 2. TIGIT-PD-1+ NY-ESO-1–specific CD8+ T cells exhibit an effector memory and activated T cell phenotype.** (A and B) Representative dot plots for 1 melanoma patient (A) and summary data for 8 melanoma patients (B) indicating the frequencies (%) of CD38, HLA-DR, CD45RA, and CCR7 expressed on A2/NY-ESO-1 tet+ CD8+ T cells and tet− CD8+ T cells expressing TIGIT and/or PD-1. P values were obtained by Friedman’s test, followed by Dunn’s multiple comparisons test (top left panel) and by repeated-measures ANOVA, followed by Tukey’s multiple comparisons test (all other panels). (C) Ex vivo expression of TIGIT (MFI) on A2/NY-ESO-1–specific CD8+ T cells according to PD-1 and TIM-3 coexpression. P values were obtained by repeated-measures ANOVA, followed by Tukey’s multiple comparisons test. Horizontal bars depict the mean percentage or MFI. *P < 0.05; **P < 0.01; ***P < 0.001.
bated for 6 days with cognate peptide in the presence of blocking mAbs against TIGIT (aTIGIT) and/or PD-1 (aPD-1) or of an irrelevant control mAb (Figure 3). As shown for 1 representative patient (Figure 3A) and for 9 patients, TIGIT or PD-1 blockade alone resulted in modest increases in the frequencies of CFSE<sup>+</sup> (Figure 3B) and total (Figure 3C) NY-ESO-1–specific CD8<sup>+</sup> T cells as compared with frequencies observed in the presence of IgG control mAbs (fold-change of 1.4 and 1.3, respectively, for TIGIT blockade; Figure 3D and E). Dual TIGIT and PD-1 blockade further increased CFSE<sup>+</sup> and total NY-ESO-1–specific CD8<sup>+</sup> T cell frequencies (Figure 3, B and C), resulting in a fold-change of 2.5 and 2.3, respectively, as compared with IgG control, aTIGIT, or aPD-1 mAbs alone (Figure 3, D and E).

Collectively, our findings show that upon stimulation with cognate antigen, dual TIGIT and PD-1 blockade stimulates stronger NY-ESO-1–specific CD8<sup>+</sup> T cell proliferation than does either blockade alone.

**TIGIT blockade alone or in combination with PD-1 increases the frequencies of cytokine-producing NY-ESO-1–specific CD8<sup>+</sup> T cells upon stimulation with cognate antigen.** We next assessed whether TIGIT blockade increases cytokine production by NY-ESO-1–specific CD8<sup>+</sup> T cells. To this end, we isolated PBMCs from 9 melanoma patients and stimulated the cells with NY-ESO-1 157-165 peptide for 6 days in the presence of anti-TIGIT and/or anti-PD-1–blocking or IgG control mAbs. On day 6, cells were restimulated with cognate peptide for a 6-hour intracellular cytokine assay prior to flow cytometry (Figure 4). As shown for 1 representative patient (Figure 4A) and for 9 patients (Figure 4B), TIGIT or PD-1 blockade alone increased the frequencies of IFN-γ and TNF-producing NY-ESO-1–specific CD8<sup>+</sup> T cells as compared with IgG control mAbs, resulting in increases of 1.6- and 2.2-fold, respectively, for TIGIT blockade and increases of 1.2- and 1.2-fold, respectively, for PD-1 blockade (Figure 4C). Dual blockade of TIGIT and PD-1 further increased the frequency of IFN-γ and TNF-producing NY-ESO-1–specific CD8<sup>+</sup> T cells, resulting in increases of 2.7- and 4.3-fold, respectively (Figure 4C). Dual PD-1 and TIGIT blockade increased the frequency of IFN-γ and TNF-producing cells among total NY-ESO-1–specific CD8<sup>+</sup> T cells as compared with IgG control mAbs, suggesting that it augmented NY-ESO-1–specific CD8<sup>+</sup> T cell functions on a cell-by-cell basis (Supplemental Figure 3, A and B).

We next investigated the mechanisms supporting the inhibitory effects of TIGIT blockade on TA-specific CD8<sup>+</sup> T cells and CD8<sup>+</sup> TILs. A number of experimental studies have suggested that the TIGIT pathway impedes T cell function by promoting IL-10 production by APCs through CD155 engagement (16), exerting direct T cell–intrinsic effects, competing with CD226 and galectin-9 (mean percentage 45.2% ± 27.2%, 8.3% ± 11.8% and 68.9 ± 30.3, respectively). Strikingly, CD155 and CD112 expression levels on melanoma cells were higher than those of PD-L1, HVEM, and galec tin-9 (mean percentage 45.2% ± 27.2%, 8.3% ± 11.8% and 5.1% ± 6.1%, respectively; Figure 6B and C). Additionally, monocytes and DCs isolated from tumors expressed elevated CD155 when compared with those isolated from the peripheral blood of patients and healthy donors (mean percentage 64% ± 23% vs. 23% ± 25% and 19.9% ± 18.5%, respectively, for monocytes and 68% ± 23.7% vs. 26.8 ± 10.3% and 32.5% ± 14.3%, respectively, for
DCs; Figure 6, B and D). Similar observations were made in terms of MFI (925 ± 240 vs. 304 ± 138 and 271 ± 110, respectively, for monocytes and 1,242 ± 800 vs. 246 ± 63 and 439 ± 259, respectively, for DCs). In addition, monocytes isolated from melanoma patients exhibited higher levels of CD112 expression in both melanoma tumors and PBMCs as compared with levels in peripheral monocytes from healthy donors (mean percentage, 82.2% ± SD 11% and 82.1% ± 16.7% vs. 42.7% ± 30.9%, respectively; Figure 6, B and D). Similar observations were made with regard to MFI (1,376 ± 1,171 and 1,538 ± 526 vs. 252 ± 96, respectively).

The high expression levels of both TIGIT ligands by melanoma cells and melanoma-infiltrating APCs suggest that the TIGIT immunoregulatory pathway plays a critical role in regulating the expansion and functions of TA-specific CD8+ T cells within metastatic melanoma.

**CD8+ TILs upregulate TIGIT and PD-1, and TIGIT blockade adds to PD-1 blockade to further increase the expansion of functional TILs after stimulation.** We next investigated whether CD8+ T cells expressing TIGIT and PD-1 were present at the tumor site. In CD8+ TILs from metastatic melanoma patients (Figure 7, A and B, and Supplemental Figure 5A), we observed upregulation of TIGIT expression both in terms of frequency and MFI as compared with circulating CD8+ T cells from both melanoma patients and healthy donors (mean frequency, 67% ± SD 17.9% vs. 43% ± 13.5% and 30.8% ± 18.5%, respectively, and MFI, 1,317 ± SD 748 vs. 493 ± 352 and 676 ± 766, respectively; Figure 7A). Notably, the majority of TIGIT+ CD8+ TILs coexpressed PD-1 (mean frequency of TIGIT+PD-1+CD8+ TILs, 47.6% ± SD 19.7%), while PD-1+TIGIT+ CD8+ TILs, and PD-1+TIGIT+CD8+ TILs represented much smaller T cell subsets (25.7 ± 18.5%, 19.8 ± 10.9%, and 6.8
± 6.3%, respectively; Figure 7, A and B). We also noticed that the majority of CD8+ TILs were CD27+CD28– and that TIGIT+PD-1+CD8+ TILs expressed more CD27 and CD57 than did TIGIT–PD-1−, TIGIT–PD-1+, or TIGIT+PD-1− CD8+ T cell subsets (Supplemental Figure 5B). Similar to what we observed in the PBMCs from melanoma patients, and in sharp contrast to the TIGIT–PD-1–, TIGIT– PD-1+, and TIGIT+PD-1– CD8+ T cell subsets, the majority of TIGIT+PD-1+ CD8+ TILs were CD45RA−CCR7− and expressed high percentages of HLA-DR and CD38 (Supplemental Figure 5B), suggesting that these TILs are more differentiated and activated. Notably, we detected no significant difference between TIGIT–PD-1+ and TIGIT–PD-1– CD8+ TILs in terms of CD45RA, CCR7, CD38, HLA-DR, and CD57 expression (Supplemental Figure 5B), suggesting that these 2 cell subsets exhibit similar states of differentiation and activation.

To investigate whether the upregulation of TIGIT expression by CD8+ TILs correlates with T cell dysfunction, CD8+ TILs were stimulated with PMA and ionomycin in vitro prior to flow cytometric analysis of cytokine production (Figure 7C and Supplemental Figure 6A). We observed that PD-1+TIGIT+ and PD-1–TIGIT+ CD8+ TILs produced less TNF (mean percentage, 4.5% ± SD 7.4%) and IL-2 (1.6% ± 2.3%) than did PD-1–TIGIT−, PD-1+TIGIT−, PD-1–TIGIT+, or PD-1+TIGIT+ CD8+ TILs (22.2% ± 23.2, 51.6% ± 36.8%, 43.2% ± 40.3%, and 35.3% ± 35.9%, respectively, for TNF; 11.4% ± 15.2%, 27.1% ± 24.7%, 18.2% ± 17.5%, and 14.7% ± 14.7%, respectively, for IL-2; Figure 7C).

We next evaluated the effects of TIGIT and PD-1 blockade on the capacity for proliferation and degranulation (CD107a expres-
CD8+ TILs exhibit an imbalance of TIGIT/CD226 expression in metastatic melanoma. We next evaluated the expression of CD226 on total CD8+ T cells from healthy donors and patients with advanced melanoma, as well as on circulating NY-ESO-1–specific CD8+ T cells and CD8+ TILs from melanoma patients (Figure 8). CD226 was upregulated on circulating NY-ESO-1–specific CD8+ T cells and total CD8+ T cells in PBMCs from healthy donors and melanoma patients in terms of both frequency (mean 99.8% ± SD 0.4%, 89.35 ± 6.7%, and 91.3% ± 4.1%, respectively; Figure 8A) and MFI (5,390 ± 1,395, 4,322 ± 1,148, and 4,193 ± 1,482, respectively; Figure 8B). In sharp contrast, we detected lower CD226+ CD8+ T cell frequencies in metastatic melanoma cells (69.9% ± 16.5%; Figure 8A) and CD226 expression levels were strongly downregulated (MFI, 5,390 ± 1,395, 4,322 ± 1,148, and 4,193 ± 1,482, respectively; Figure 8B). In sharp contrast, we detected lower CD226+ CD8+ T cell frequencies in metastatic melanoma cells (69.9% ± 16.5%; Figure 8A), and CD226 expression levels were strongly downregulated (MFI, 5,390 ± 1,395, 4,322 ± 1,148, and 4,193 ± 1,482, respectively; Figure 8B), suggesting an imbalance of TIGIT/CD226 expression in metastatic melanoma.

Collectively, our findings show an imbalance of TIGIT/CD226 expression by CD8+ TILs in metastatic melanoma that may enhance the negative immunoregulatory effects of TIGIT.

Discussion
In the present study, we report that TIGIT is upregulated by the majority of TA-specific CD8+ T cells in the periphery and within metastatic tumors of patients with advanced melanoma. In con-
The TIGIT ligands CD155 and CD112 are upregulated on metastatic melanoma cells and APCs in the TME. (A) Frequencies of APCs and tumor cells within the CD3− cells isolated from metastatic melanoma single-cell suspensions (n = 11). (B) Representative flow cytometric analysis of CD155 and CD112 expression on CD11c+CD14− SSC−CSPG4+ melanoma cells, CD11c−CD14+ monocytes, and CD11c+CD14+ DCs from CD3− cells from PBMCs from 1 healthy donor and 1 melanoma patient and from 1 metastatic melanoma single-cell suspension. (C) Pooled data showing CD155 (n = 16), CD112 (n = 10), PD-L1 (n = 20), HVEM (n = 17), and galectin-9 (n = 5) expression by CSPG4+ cells from melanoma single-cell suspensions. P values were obtained by Friedman’s, test followed by Dunn’s multiple comparisons test. (D) Pooled data showing CD155 and CD112 expression (percentage and MFI) on monocytes and DCs from PBMCs from healthy donors (n = 12) and melanoma patients (n = 8) and from metastatic melanoma single-cell suspensions (n = 15). P values were obtained by a 1-way ANOVA, followed by Tukey’s multiple comparisons test (bottom row, second panel from the left) and by a Kruskal-Wallis test, followed by Dunn’s multiple comparisons test (all other panels). Data are representative of 3 independent experiments. Horizontal bars depict the mean. *P < 0.05; **P < 0.01; ***P < 0.001.
We provide several lines of evidence supporting the notion that TIGIT is an early T cell activation marker that is further upregulated by dysfunctional TA-specific CD8+ T cells upon chronic stimulation. First, in agreement with previous studies in mice and healthy donors (16, 35–37), TA-specific CD8+ T cells upregulated TIGIT expression after 1 day of IVS with cognate antigen, while PD-1 upregulation has been shown to occur only after 4 days (5). Second, TA-specific CD8+ T cells upregulated TIGIT expression upon PD-1 blockade in the presence of cognate antigen. Finally, highly dysfunctional PD-1+TIM-3+ TA-specific CD8+ T cells (6, 41) expressed higher TIGIT levels than did PD-1+TIM-3- and PD-1-TIM-3- TA-specific CD8+ T cells.

Unlike the NY-ESO-1–specific CD8+ T cells that were present in the PBMCs of patients with advanced melanoma, a significant number of CD8+ TILs upregulated TIGIT expression upon PD-1 blockade in the presence of cognate antigen. Finally, highly dysfunctional PD-1-TIM-3- TA-specific CD8+ T cells (6, 41) expressed higher TIGIT levels than did PD-1-TIM-3- and PD-1-TIM-3- TA-specific CD8+ T cells.

and TIGIT PD-1+ CD8+ T cell subsets. However, there was no significant difference in activation or differentiation between TIGIT PD-1- and TIGIT-PD-1 CD8+ TILs, although those subsets were more differentiated and activated than TIGIT PD-1- CD8+ TILs.
Our findings in patients with advanced melanoma are in line with 1 immunostaining study showing that CD155 is expressed by melanomas and correlates with melanoma progression (31). In addition, the frequencies of melanoma cells expressing CD155 and CD122 were higher than those expressing PD-L1, HVEM, and galectin-9, suggesting that the interaction of CD155 and CD112 with their ligands plays an important role in regulating TA-specific T cell responses at tumor sites. Although PD-L1 expression was clearly induced by inflammatory cytokines including IFN-γ, we observed no upregulation of CD155 or CD112 by melanoma cells upon exposure to IFN-γ or TNF (Chauvin et al., unpublished observations) and found no correlation between the percentages of PD-L1+ melanoma cells and the frequencies of CD155+ or CD122+ melanoma cells ex vivo, suggesting that CD155 and CD112 upregulation by melanoma cells is supported by mechanisms other than PD-L1 expression.

In summary, the findings in this study demonstrate that TIGIT is coexpressed with PD-1 by the large majority of TA-specific CD8+ T cells in the periphery and at tumor sites in patients with advanced melanoma. In addition, CD226 was downregulated by CD8+ TILs, while the TIGIT ligands CD155 and CD112 were upregulated by the majority of melanoma cells, monocytes, and DCs in metastatic melanoma, suggesting that the TIGIT pathway plays a major immunoregulatory role in metastatic melanoma. Importantly, TIGIT blockade adds to PD-1 blockade to enhance TA-specific CD8+ T cell function and proliferation in the periphery and at tumor sites. Therefore, the present findings strongly support the use of dual TIGIT and PD-1 blockade to stimulate potent antitumor CD8+ T cell responses in patients with advanced melanoma.

Methods

**TA-specific CD8+ T cell phenotypic analysis.** Using MACS Column Technology (Miltenyi Biotec), CD8+ T lymphocytes from PBMCs obtained from patients were purified to greater than 95% and incubated for 30 minutes at room temperature with APC-labeled HLA-A2/NY-ESO-1 157-165, HLA-A2/CMV 495-503, HLA-A2/Flu-M 58-66, or (as a negative control) HLA-A2/HIVpol 476-484 tetramers (TCellmix). The mean percentage of antigen-specific CD8+ T cells detected in patients using these tetramers was 0.01% of total CD8+ T cells. Next, for membrane staining, cells were incubated for 20 minutes at 4°C with a combination of the following conjugated Abs and reagents: CD8-V500, CD38-PerCp-Cy5.5, and CD69-FITC (from BD);
Using a FACSAria flow cytometer, CD8^+ TILs and non-CD3 cells were analyzed. Two million events were collected during flow cytometric analysis on a FACSAria flow cytometer (BD) and analyzed using FlowJo software (Tree Star Inc.).

**Generation of anti-TIGIT Abs.** Anti-human TIGIT Abs were generated in HuMab mice (42, 43) immunized with a TIGIT-Fc fusion protein. Abs, which bound to full-length TIGIT expressed on CHO transfectants, were tested for their ability to block the binding of TIGIT-Fc to CHO transfectants expressing CD155 (PVR). The anti-TIGIT mAb 10D7.G8 (IgG4) was selected and found to bind well to activated human T cells. 10D7.G8 was also shown to enhance NF-κB reporter gene expression in full-length TIGIT transfectants of Jurkat cells incubated with CHO cells expressing membrane-bound, single-chain anti-CD3 and full-length human PVR.

**CFSE proliferation assay.** For PBMCs from melanoma patients, 4.5 million CFSE-labeled cells per condition and per well of a 24-well plate were incubated for 6 days in 10% human serum Iscove’s DMEM containing 50 IU/ml recombinant human IL-2 (rIL-2; Peprotech) with NY-ESO-1 157-165 or HIVpol 476-484 (2 μg/ml) peptide in the presence of 10 μg/ml fully human anti-PD-1 (BMS-936558, IgG4, BMS) and/or anti-TIGIT (10D7.G8, IgG4, BMS) blocking mAbs or IgG4 isotype control Ab (DT-1D12-g4P, BMS). On day 6, cells were stained with APC-labeled HLA-A2/NY-ESO-1 157-165 tetramers, then CD14-APC-Cy7, CD19-APC-Cy7 (BD), CD8-PerCP-Cy5.5 (BioLegend), TIGIT-PE, (eBioscience), and PD-1-PE-Cy7 (Beckman Coulter), followed by incubation with a violet amine–reactive dye. Two million events were collected during flow cytometric analysis using a FACSAria flow cytometer. CD8^+ TILs and non-CD3 cells were isolated from tumor single-cell suspensions using MACS Column Technology. CD8^+ TILs were rested for 2 days in Iscove’s DMEM and 100 IU/ml rIL-2, while non-CD3 cells were treated with 100 ng/ml IFN-γ. CD8 cells were labeled with CFSE and coincubated with non-CD3 for 5 days at a 1:1 ratio in the presence or absence of IgG, anti-PD-1, and/or anti-TIGIT mAbs in medium containing 100 IU/ml rIL-2 and 0.1 μg/ml OKT3 (eBioscience) to provide stimulation signals. On day 5, cells were stained with CD14-ECD, CD4-PeCy7 (Beckman Coulter), CD19 Pacific Blue, CD107a-PerCP-Cy5.5, and CD8-APC (BD), followed by incubation with a violet amine–reactive dye. Flow cytometry was performed using an LSR-II flow cytometer (BD) and the data analyzed using FlowJo software.

**Intracellular cytokine staining assay.** For PBMCs from melanoma patients, 4.5 million cells were incubated for 6 days in Iscove’s DMEM containing 50 IU/ml rIL-2 with NY-ESO-1 157-165 or HIVpol 476-484 (2 μg/ml) peptide in the presence of anti-PD-1 and/or anti-TIGIT blocking mAbs, isotype control Abs, or TIGIT-Fc fusion protein. Supernatants was determined using a BD OptEIA Human IL-10 ELISA detection after 6 days of incubation. The concentration of IL-10 in supernatants was determined using a BD OptEIA Human IL-10 ELISA Set (BD Biosciences).

**Ex vivo phenotypic analysis of subsets from tumor single-cell suspensions.** Cells from tumor single-cell suspensions were divided and placed into several tubes for analysis. Tumor cells were detected using a mouse anti-human CSPG4 Ab (763.74; gift of S. Ferrone, Massachusetts General Hospital, Boston, Massachusetts, USA) targeted with FITC goat anti-mouse Abs (Beckman Coulter). Cells were washed with PBS-based buffer containing mouse serum, then labeled with CD155-PE (eBioscience) or CD112-PE (BioLegend) and a mix of CD3-PerCP-Cy5.5 (BioLegend), CD19-APC-Cy7 (BD), CD14-ECD, CD56-PeCy7 (Beckman Coulter), and CD11c Alexa Fluor 700 (eBioscience) Abs. In another tube, cells were labeled with CD226-biotin (Abcam), then streptavidin-FITC (Invitrogen), TIGIT-PE, CD11c Alexa Fluor 700 (eBioscience), CD56-FLTC (Beckman Coulter), CD4-PerCP-Cy5.5 (BioLegend), CD14-APC-Cy7, CD19-APC-Cy7, and CD8-V500 (BD). All samples were also incubated with the violet amine–reactive dye to exclude dead cells. Flow cytometry was performed using an LSR-II or a Fortessa (BD) flow cytometer, and data analysis was performed using FlowJo software. MFI was compared between experiments analyzed on the LSR-II flow cytometer.

**Statistics.** The normality of each variable was evaluated using the Shapiro-Wilk test. In cases of normally distributed data, the comparison of variables was performed using a 1-way ANOVA or a repeated-measures ANOVA for unpaired and paired data, respectively, followed by a Tukey’s multiple comparisons test. When the data were not normally distributed, the comparison of variables was performed with a Kruskal-Wallis test or a Friedman test for unpaired and paired data, respectively, followed by a Dunn’s multiple comparisons test. In these tests, P values of less than 0.05 were considered significant.

These analyses were performed with SAS software, version 9.1 (SAS Institute), and GraphPad Prism 6 (GraphPad Software).

**Study approval.** Blood samples were obtained from 11 healthy volunteers or from 11 HLA-A2^+ patients with NY-ESO-1–expressing stage IV melanoma under the University of Pittsburgh Cancer Institute’s IRB-approved protocols 00-079, 05-140, or 96-099. NY-ESO-1 expression in tumors from melanoma patients was assessed by RT-PCR and IHC. Serum NY-ESO-1–specific Abs from all selected patients were detected by ELISA. PBMCs from 9 patients who exhibited responses against NY-ESO-1 157-165 were used. NY-ESO-1–specific CD8^+ T cells were detected by flow cytometry using APC-labeled HLA-A2/NY-ESO-1 157-165 tetramers. The percentages of detectable NY-ESO-1 157-165–specific CD8^+ T cells isolated from patients’
PBMCs ranged from 0.01% to 5.7% of total CD8+ T cells (median, 0.03%). Tumor and TIL samples were collected under the University of Pittsburgh Cancer Institute’s IRB-approved protocol 96-099.

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