Reversal of NK-Cell Exhaustion in Advanced Melanoma by Tim-3 Blockade

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Abstract

The immunoregulatory protein T-cell immunoglobulin- and mucin-domain–containing molecule-3 (Tim-3) mediates T-cell exhaustion and contributes to the suppression of immune responses in both viral infections and tumors. Tim-3 blockade reverses the exhausted phenotype of CD4\(^+\) and CD8\(^+\) T cells in several chronic diseases, including melanoma. Interestingly, natural killer (NK) cells constitutively express Tim-3; however, the role of Tim-3 in modulating the function of these innate effector cells remains unclear, particularly in human diseases. In this study, we compared the function of Tim-3 in NK cells from healthy donors and patients with metastatic melanoma. NK cells from the latter were functionally impaired/exhausted, and Tim-3 blockade reversed this exhausted phenotype. Moreover, Tim-3 expression levels were correlated with the stage of the disease and poor prognostic factors. These data indicate that Tim-3 can function as an NK-cell exhaustion marker in advanced melanoma and support the development of Tim-3–targeted therapies to restore antitumor immunity.

Introduction

Malignant melanoma is the fifth most common cancer in men, with incidence increasing more rapidly than that of any other malignancy (1). It is the most aggressive type of skin cancer, accounting for more than 70% of skin cancer–related deaths (2). The median survival of patients with advanced melanoma (stage IV) was until recently less than 1 year (3), but new therapeutic options are improving response rates and overall survival (4, 5).

One such intervention is ipilimumab (5), a monoclonal antibody against CTL antigen-4 (CTLA-4), a negative regulator that is upregulated on exhausted T cells in cancers and chronic infections (6). The exhaustion phenotype has been described for T cells as a state of cellular dysfunction that arises as a consequence of continuous and chronic stimulation by viral or tumor antigens, as well as by immunosuppressive cytokines. It is characterized by an early loss of proliferative capacity, cytotoxic potential, and the ability to produce cytokines, as well as sustained expression of inhibitory receptors (7). Besides CTLA-4 (8, 9), program death-1 (PD-1; ref. 10) and T-cell immunoglobulin- and mucin-domain–containing molecule-3 (Tim-3; ref. 11) are additional inhibitory receptors that can be upregulated in the setting of chronic infections or progressive cancers.

Tim-3 is an inhibitory receptor that plays a crucial role in mediating T-cell exhaustion in both viral infections and tumors (12–14). Indeed, Tim-3 blockade reverses the exhausted phenotype of CD4\(^+\) and CD8\(^+\) T cells in several chronic diseases, including melanoma (11, 15, 16). Unlike resting T cells, where it is minimally expressed and upregulated only after chronic stimulation, Tim-3 is constitutively expressed at considerably higher levels on resting natural killer (NK) cells (17). Recent publications, however, have reported conflicting data about Tim-3 function in NK cells (17, 18). Ndhllovu and colleagues showed that Tim-3 inhibits NK cell–mediated cytotoxicity (17), whereas another study suggested that Tim-3 may enhance IFN-\(\gamma\) production instead (18). Both studies only evaluated healthy donors and not patients with chronic diseases, such as cancer, where Tim-3 expression on NK cells may have a more significant immunomodulatory role.

NK cells, through IFN-\(\gamma\) production and direct cytotoxicity, are able to eliminate tumor cells. Accordingly, NK-cell infiltration in tumor tissue is associated with better prognosis (19–21), whereas low activity of peripheral blood NK cells is associated with increased risk of cancer (22). These findings have prompted interest in the development of cancer therapies based on NK cells, such as NK-cell adoptive transfer or targeting NK-cell inhibitory receptors (23). In this regard, a better understanding of Tim-3 function in the context of advanced tumors could potentially affect treatment.
In this study, we characterized Tim-3 expression and function in NK cells from patients with advanced melanoma. We found that NK cells from these subjects expressed high levels of Tim-3 and were functionally impaired/exhausted. Importantly, we showed that Tim-3 blockade reversed this exhausted phenotype. Furthermore, we found that Tim-3 expression in NK cells increases as the stage of melanoma advances and is higher in patients with melanoma with poor prognostic factors. These data open exciting avenues for new therapies targeting Tim-3 in tumor immunotherapy.

Materials and Methods

Reagents

For staining. Tim-3 antibody used for staining experiments was purchased from R&D Systems. Anti-CD279 (PD-1) was purchased from BD Biosciences and anti-CD152 (CTLA-4) was purchased from LifeSpan Biosciences. To check the purity of selected NK cells, they were stained for CD56, CD16, CD3, CD14, and CD19 purchased from BioLegend. Anti-CD25 was purchased from Miltenyi; anti-CD122, anti-CD132, and anti-interleukin-5 receptor (anti-IL-5R) were purchased from BioLegend. Anti-Cd107 (Lamp-1) and anti-IFN-γ-FITC antibodies were purchased from BioLegend. Anti-NKG2D, anti-NKp4H6, anti–DNAM-1, anti-KIR3DL1, and anti-KIR2DL3 antibodies (BD Biosciences) were used as indicated by the manufacturer. Anti-comesoderminal (Eomes) and anti–T-bet were purchased from eBioscience. Anti–galectin-9 (Gal-9) was purchased from BioLegend. CellTrace CFSE Cell Proliferation Kit was purchased from Life Technologies. The FITC Annexin V Apoptosis Detection Kit with 7-AAD (7-amino-actinomycin D) was purchased from BioLegend.

For blocking experiments. Ten or 20 μg/mL of Tim-3 blocking antibody (BioLegend, clone 2E2; R&D Systems; #AF2265), Gal-9 blocking antibody (BioLegend), IL-2 receptor (IL-2R) blocking antibody (α-β, γ-chains; R&D Systems), or CD16 blocking antibody (BioLegend) was added to the culture 1 hour before starting the functional assays.

For cross-linking experiments. The same anti-Tim-3 antibody (BioLegend, clone 2E2) was used. Anti-CD16 and anti-CD94 used for the cross-linking experiments (coated beads) and for the reverse antibody-dependent cell-mediated cytotoxicity (R-ADCC) assay were purchased from Biolegend. IgG1 isotype control used in the cross-linking and blocking experiments was purchased from BD Biosciences.

For stimulation. The cytokines recombinant human IL (rIL)-2, rIL-12, rIL-15, and rIL-18 were purchased from R&D Systems. Recombinant human Gal-9, purchased from R&D Systems, was used to treat NK cells 1 hour before the cytotoxicity assay.

Cell lines

K562 cells, Gmel, and FM29 cells were cultured in complete media (RPMI-1640; Life Technologies) supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. K562 is a chronic myelogenous leukemia cell line, which was used as target cells in experiments from Figs. 2B, 4A, 5A and B, and 6D and F; Supplementary Figs. S3A and S3D, S4B, S5A and S5C, S6A and S6B, S7A, S7D–S7G, and S8B. Gmel is a melanoma cell line that expresses Gal-9 intracellularly, and 5% of these cells express Gal-9 in the membrane. Gmel cells were used as target cells in the experiments shown in Supplementary Fig. S6C and S6D. FM29 cells were used as target cells in the experiment shown in Supplementary Fig. S7B. Melanoma cell lines WM1552, WM3248, and WM793b were purchased from the Wistar Institute (Philadelphia, PA). They were used as target cells in the experiment shown in Supplementary Fig. S7B. P815 cell line was purchased from the American Type Culture Collection (ATCC). This cell line is FeR+ and was used for the reverse-ADCC assay (Fig. 4C and Supplementary Figs. S5B and S5D). All cell lines were Mycoplasma-free; no other authentication assays were performed.

Human samples

Blood samples from healthy donors were purchased from the New York Blood Center. Blood samples were obtained under the Interdisciplinary Melanoma Cooperative Group Institutional Review Board (IRB)–approved protocols (#H10362) from 113 patients with untreated melanoma (melanoma donors; stages I, II, and III/IV).

NK-cell purification

Peripheral blood mononuclear cells were purified from healthy donor- or melanoma patient–derived buffy coats by Ficoll-Paque Plus centrifugation. NK-cell enrichment was performed by negative selection using the EasySep Human NK Cell Enrichment Kit (STEMCELL Technologies) according to the manufacturer’s recommendations, obtaining more than 95% CD3–CD56+ populations.

NK-cell stimulation

For short-term functional assays (cytotoxicity and IFN-γ production), purified NK cells were activated overnight in the presence of 1,000 U/mL of IL-2 complete media (RPMI-1640; Life Technologies) supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. For the long-term experiments and proliferation assays, NK cells were cultured in the same media and stimulated with 200 U/mL of IL-2.

Cell staining and flow cytometry analysis

Before staining, cells were washed twice with PBS supplemented with 0.5% bovine serum albumin and 2 mmol/L EDTA (staining buffer). For surface staining, cells were incubated with specific antibodies for 30 minutes at 4°C. For intracellular staining, cells were first fixed with 4% paraformaldehyde for 10 minutes at room temperature, permeabilized with staining buffer supplemented with 0.1% saponin, and stained with specific antibodies for 30 minutes at room temperature. Cells were then washed twice with staining buffer and analyzed by fluorescence-activated cell sorting (FACS). Data analysis was performed using FlowJo software.

Cell sorting

Gmel cells were stained for Gal-9, following the protocol described above. After Gal-9 staining, these cells were sorted into populations of either Gal-9–Gmel and Gal-9+Gmel by...
flow cytometry. We followed the same procedure to sort NK cells into Tim-3+ and Tim-3− NK cells.

**Cross-linking experiment**

**Coated beads.** Dynabeads Pan Mouse IgG (Invitrogen) were labeled with purified mouse IgG1, κ isotype control (BD Biosciences) or purified human anti–TIM-3 (BioLegend) according to the manufacturer’s instructions. Antibody-labeled beads were added to NK cells at a 1:1 ratio for 30 minutes at 4°C and then incubated at 37°C for 90 minutes. Functional assays were performed as described below.

**Reverse-ADCC.** NK cells were cocultured with FcR+ P815 cells and different antibodies were added to the reaction: either anti–Tim-3, anti-CD94 (negative control), or anti-CD16 (positive control) antibody.

**Endocytosis**

To assess whether Tim-3 was endocytosed after Tim-3 blockade, IL-2–stimulated NK cells from healthy donors were incubated with the Tim-3 blocking antibody or IgG1 isotype control, as previously described, for 15 minutes, 30 minutes, and 1 hour. Cells were then fixed, permeabilized or not, and stained with a phycoerythrin (PE) anti-mouse antibody to detect Tim-3 blocking antibody on the surface and/or in the cytoplasm of the cells.

**Cytotoxicity assay**

Purified NK cells were cocultured with target cells at a 5:1 effectortarget ratio in combination of anti–CD107 antibody (0.5 μg/mL) and monensin 1,000×. After 4 hours of incubation, CD107 expression on CD56+ cells was quantified by flow cytometry.

**IFN-γ production assay**

Purified NK cells were cultured for 4 hours in the presence of 1 μg/mL of IL-12 and brefeldin A (10 μg/mL). After 4 hours, cells were fixed (4% paraformaldehyde) and permeabilized (0.1% saponin). Permeabilized cells were stained for intracellular IFN-γ and analyzed by flow cytometry.

**Proliferation assay**

Purified NK cells were loaded with 2 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured in complete media supplemented with 200 U/mL of IL-2. After 6 days of culture, CFSE dilution was analyzed by flow cytometry as a measure of cell proliferation.

**Blocking experiment**

Ten or 20 μg/mL of Tim-3 blocking antibodies (BioLegend, clone 2E2 or R&D Systems; cat no. AF2365) was added to the culture 1 hour before starting the functional assays (before adding K562 cells in the cytotoxicity assay, IL-12 in the cytokine production assay, and IL-2 in the proliferation assay). We used IgG1 isotype control from BD Biosciences.

**Statistical analyses**

Separate analyses were performed for each experiment individually. Two-tailed t tests were used. Analyses take into account paired observations within donors when appropriate or unpaired observations when we compare parameters between healthy and melanoma NK cells. To analyze Tim-3 expression according to the demographic and clinical parameters, we used an unpaired t test to compare two groups, and ANOVA to compare more than two groups.

**Results**

**NK cells from melanoma donors display an exhausted phenotype**

Advanced tumors are characterized by an environment that promotes T-cell exhaustion (7). Exhausted T cells are characterized by (i) overexpression of inhibitory receptors such as CTLA-4 and PD-1; (ii) downregulation of cytokine receptors, rendering them refractory to cytokine stimulation; (iii) loss of function (cytotoxicity, cytokine production, and proliferation); and (iv) downregulation of transcription factors T-bet and Eomes (24). In metastatic melanoma, peripheral blood CD8+ T cells are functionally exhausted (11); however, NK-cell phenotype and function in the same context have not been evaluated.

To determine whether peripheral NK cells from patients with advanced melanoma display an exhausted phenotype, we examined whether they expressed features associated with T-cell exhaustion. NK cells were purified from the peripheral blood of patients with advanced melanoma and healthy donors (Supplementary Fig. S1A). Interestingly, melanoma donor NK cells downregulated the NK-cell markers CD16 and CD56 (Supplementary Fig. S1B) and, consequently, display a decrease in the percentage of both CD56+ high and CD56− dim NK-cell subsets (Supplementary Fig. S1C). We then measured the expression of activating and inhibitory receptors in NK cells from melanoma patients and healthy donors. Clearly, NK cells from melanoma patients expressed higher levels of inhibitory receptors (KIR3DL1 and KIR2DL3) and lower levels of activating receptors (NK2G2D, NKp46, and DNAM-1) when compared with NK cells from healthy donors (Fig. 1 and Supplementary Fig. S1D). We also evaluated PD-1 and CTLA-4 expression; however, there were no significant differences between melanoma patients and healthy donor NK cells (Supplementary Fig. S1E).

Second, we measured the levels of the three IL-2R chains [α- (CD25), β- (CD122), and γ-chain (CD132)]. The expression of each subunit was measured in steady-state conditions (day 0) and on days 2, 4, and 6 after IL-2 stimulation. Healthy or melanoma donor NK cells did not express significant levels of CD25 at rest. Interestingly, after IL-2 stimulation, healthy donor NK cells upregulated CD25 to very high levels, whereas melanoma donor NK cells failed to do so (Fig. 2A and Supplementary Fig. S2A). CD122 expression was similar in NK cells from healthy donors and melanoma patients and decreased after IL-2 addition (Supplementary Fig. S2A and S2B). CD132 expression was slightly higher in resting healthy donor NK cells compared with melanoma donor NK cells. After IL-2 stimulation, both sources of NK cells showed an increase of this receptor, followed by a plateau and then a decrease. However, the overall expression of CD132 on healthy donor NK cells was substantially higher than that of melanoma donor NK cells (Supplementary Fig. S2A and S2B). We next tested whether altered IL-2R expression translated into a differential response.
to IL-2 stimulation. Although cytotoxicity was induced in both sources of NK cells, the levels increased more significantly over time in healthy donor NK cells. IFN-γ production was induced by and increased in healthy donor NK cells in response to IL-2; however, it did not change from baseline in the case of melanoma donor NK cells (Fig. 2A). Moreover, healthy donor NK cells upregulated both NKG2D and KIR3DL1, whereas melanoma donor NK cells did not (Supplementary Fig. S2C). We also measured the levels of the IL-15 receptor (IL-15R), another significant NK-cell stimulatory molecule. As observed for IL-2R, IL-15R levels are significantly lower in melanoma donor NK cells when compared with healthy donor NK cells (Supplementary Fig. S2D).

Next, we determined whether NK cells from patients with melanoma were functionally impaired, by assessing cytotoxicity, IFN-γ production, and cell proliferation. We found that, in comparison with healthy donor NK cells, melanoma donor NK cells failed to efficiently kill target cells as assessed by LAMP-1 expression, after stimulation by either IL-2 ($P = 0.0158$) or IL-15 ($P = 0.04$; Fig. 2B; Supplementary Fig. S3A and S3D). In addition, they produced less IFN-γ in response to different stimuli: IL-12 ($P = 0.0312$; Fig. 2B; Supplementary Fig. S3A and S3D), the combination of IL-12 and IL-18 ($P = 0.001$), IL-15 ($P = 0.05$), or after coculture with K562 cells ($P = 0.0173$; Supplementary Fig. S3B). Finally, NK cells from patients with melanoma lost their ability to proliferate when cultured with IL-2 ($P = 0.0024$), IL-12

Figure 1. Melanoma donor (MD) NK cells upregulate inhibitory receptors and downregulate activating receptors. Graphs compare the expression of the inhibitory receptors KIR3DL1 [healthy donors (HD), $n = 18$; melanoma donors, $n = 8$] and KIR2DL3 (healthy donors, $n = 26$; melanoma donors, $n = 12$) and the activating receptors NKG2D (healthy donors, $n = 25$; melanoma donors, $n = 12$), Nkp46 (healthy donors, $n = 20$; melanoma donors, $n = 11$), and DNAM-1 (healthy donors, $n = 10$; melanoma donors, $n = 10$) in peripheral blood NK cells purified from healthy donors and from patients with melanoma. Right, plots depicting the expression of KIR3DL1, KIR2DL3, NKG2D, NKP46, and DNAM-1 according to CD56 expression in NK cells from a representative healthy donor and a representative melanoma patient. All experiments were performed in duplicate.
Figure 2. Melanoma donor (MD) NK cells are functionally impaired/exhausted. A, freshly purified NK cells [healthy donors (HD), n = 12; melanoma donors, n = 5] were stimulated with 200 U/mL of IL-2. Expression of IL-2R (α-chain), IFN-γ production, and cytotoxicity (Lamp-1 expression) were monitored every 2 days over 6 days (day 0, 2, 4, and 6) by flow cytometry. B, the percentage of Lamp-1⁺ NK cells from healthy (n = 30) and melanoma donors (n = 12) after a cytotoxic assay is shown using K562 cells as target cells (top left). The percentage of IFN-γ⁺ NK cells from healthy (n = 22) and melanoma donors (n = 9) is shown after 4 hours of stimulation with IL-12 (middle left). (Continued on the following page.)
(P = 0.04), IL-15 (P = 0.05), or IL-18 (P = 0.01; Fig. 2B; Supplementary Fig. S3C and S3D). The transcription factors T-bet and Eomes regulate the function of NK cells, and a recent study showed that downmodulation of these transcription factors is a characteristic of NK-cell exhaustion (25). As expected, Fig. 2C shows that the levels of Eomes and T-bet are lower in NK cells from melanoma donors when compared with NK cells from healthy donors.

These results clearly demonstrate that NK cells from patients with advanced melanoma are functionally exhausted as shown by overexpression of inhibitory receptors and down-regulation of activating receptors; an impaired response to IL-2 stimulation possibly due to IL-2R downmodulation; defects in cytokine production, proliferation and cytotoxicity; and a downmodulation of the transcription factors T-bet and Eomes.

**Tim-3 is upregulated in melanoma donor NK cells and is associated with clinical parameters that predict a poorer prognosis**

Tim-3 is another immune checkpoint expressed by CD8+ and CD4+ T cells in the context of advanced tumors, such as melanoma. However, the role of Tim-3 in NK cells under the same circumstances remains unknown. Therefore, we first measured Tim-3 expression in melanoma donor NK cells. Compared with healthy donor NK cells, they expressed significantly higher levels of surface Tim-3 (P = 0.0001; Fig. 3A). Moreover, we checked the expression of Tim-3 in steady-state conditions and after IL-2 stimulation in both CD56bright and CD56dim NK-cell subsets. In unstimulated conditions, both subsets of NK cells from patients with melanoma express higher levels of Tim-3 when compared with their counterparts from healthy donors [both percentage and mean fluorescent intensity (MFI)]. However, after stimulation, while Tim-3 expression increases significantly in both subsets in healthy donors (percentage and MFI), we did not observe any substantial increase in either subset from melanoma donors (Supplementary Fig. S4A).

We next measured the percentage of Lamp-1+ (Supplementary Fig. S4B) and IFN-γ+ cells (Supplementary Fig. S4C) in the fraction of Tim-3+ versus Tim-3− NK cells, in unstimulated and stimulated conditions. Interestingly, in unstimulated conditions, in both healthy donors and melanoma patients, the percentage of Lamp-1+ and IFN-γ+ cells was higher in the Tim-3− fraction; however, after IL-2 stimulation, the most active cells (Lamp-1+ or IFN-γ+ cells) were Tim-3− (Supplementary Fig. S4B and S4C). Indeed, as previously described (17, 18), Tim-3 is a marker of differentiation. However, when we compare the percentage of active cells in the Tim-3+ NK cells from healthy donors versus melanoma patients, clearly melanoma donor NK cells are less active, suggesting that these cells are exhausted.

We then evaluated whether Tim-3 expression was also increased at early stages of melanoma (stages I and II) and how its expression is related to clinical parameters. A cohort of 83 patients with melanoma, distributed as stage I (n = 47), II (n = 18), or III/IV (n = 18), was studied according to Tim-3 expression. As the stage of melanoma advanced, we observed a pattern of gradually increasing Tim-3− NK-cell numbers (percentage) and MFI (Fig. 3B). In the same cohort of patients, we were able to analyze Tim-3 expression (percentage and MFI) according to demographic and clinical parameters. Interestingly, the expression of Tim-3 is higher in patients with melanoma with poor prognostic factors, such as thickness >1 mm, mitotic rate >1/mm2, and ulceration. Moreover, the intensity of Tim-3 expression (MFI) was higher in patients with metastases. Significantly, Tim-3 expression did not differ according to age, gender, or lymph node status (Table 1).

In conclusion, our data suggest that higher Tim-3 expression is associated with clinical parameters such as thickness, mitotic rate, and ulceration, well-known poor prognostic factors.

**Tim-3 negatively regulates melanoma donor NK-cell function**

To determine the role of Tim-3 in NK-cell exhaustion, we developed a system to engage Tim-3 through cross-linking, by using anti–Tim-3-coated beads. As shown in Fig. 4A and B, Tim-3 activation in melanoma donor NK cells reduced cytotoxicity by approximately 20% (P = 0.0361) and IFN-γ production by 25% (P = 0.0176) as compared with controls. We performed a reverse-ADCC assay using FcγR-P815 cells that bind the Fc portion of antibodies, allowing them to cross-link their relevant ligands on other cells. We cocultured NK cells from patients with melanoma with P815 cells and added the following antibodies to the reaction: anti–Tim-3, anti–CD94 (negative control), or anti–CD16 (positive control). As expected, CD16 cross-linking activated NK cells, rendering them more cytotoxic, whereas CD94 cross-linking reduced NK-cell cytotoxicity. When Tim-3 was cross-linked, we observed a decrease in NK-cell cytotoxicity (P = 0.0002; Fig. 4C). This finding confirms that Tim-3 acts as an inhibitory receptor on NK cells and that its ligation downmodulates NK-cell function in the context of advanced melanoma. In healthy donors, Tim-3 ligation also resulted in a significant decrease of cytotoxicity as previously described (17). We used the same two systems to cross-link Tim-3 in healthy donor NK cells, anti–Tim-3-coated beads (Supplementary Fig. S5A), and reverse-ADCC (Supplementary Fig. S5B), as well as anti–Tim-3-coated K562 cells (Supplementary Fig. S5C), and showed an inhibition of Lamp-1 expression by NK cells (first two systems), and a decrease of K562 cell apoptosis (third system). It is important to point out that NK cells from patients with melanoma may have already undergone significant Tim-3–mediated inhibition in vivo; therefore, it is not surprising that Tim-3 ligation did not achieve greater inhibition of melanoma donor NK-cell function when compared with NK cells from healthy donors. Tim-3 also acts as an inhibitory receptor on T cells that, when chronically...
activated, induces T-cell apoptosis. However, in our system, chronic Tim-3 stimulation did not induce NK-cell apoptosis, as we show in Supplementary Fig. S5D.

Gal-9 is the most studied Tim-3 ligand identified thus far. Therefore, we used recombinant and surface-bound Gal-9 as Tim-3 agonists. NK cells from healthy donors were incubated with 25 and 50 nmol/mL of rh-Gal-9 1 hour before assessing cytotoxicity, and displayed a significant decrease in Lamp-1 expression, but without significant toxicity (Supplementary Fig. S6A). We were able to abrogate this effect by blocking Gal-9 with a specific blocking antibody or β-lactose (Supplementary Fig. S6B). Next, we used a more physiologic approach with the melanoma cell line Gmel as a source of Gal-9. Gmel cells were sorted according to their surface expression of Gal-9 into Gal-9⁺-Gmel and Gal-9⁻/C₀-Gmel (Supplementary Fig. S6C) and used as target cells. We found that NK cell–mediated cytotoxicity from both healthy donors and melanoma patients was lower in the presence of Gal-9⁺-Gmel cells compared with Gal-9⁻-Gmel cells.

Figure 3. Tim-3 is upregulated in melanoma donor (MD) NK cells. A, graph comparing Tim-3 expression in NK cells from healthy donors [healthy donors (HD), n = 45] and patients with melanoma [melanoma donors, n = 41]. Represented as the percentage of Tim-3⁺ cells (left) and the MFI of the Tim-3⁺ population (right). B, the graphs show the percentage (left) and MFI (right) of Tim-3⁺ NK cells from healthy donors (n = 30) and patients with melanoma stage I (n = 47), II (n = 18), and III/IV (n = 18). All experiments were performed in duplicate.

Figure 4. Tim-3 engagement inhibits NK-cell functions. A, the percentage of LAMP-1⁺ (n = 8) and B, the MFI of IFN-γ⁺ cells (n = 6) of NK cells from melanoma donors preincubated with IgG-coated beads or anti-Tim-3-coated beads for 2 hours before evaluating the cytotoxic function or IFN-γ production. C, reverse-ADCC assay using FcR⁺ P815 cells. NK cells from melanoma patients were cocultured with P815 cells and different antibodies were added to the reaction: anti-Tim-3, anti-CD94 (negative control), or anti-CD16 (positive control). Data were normalized to the values obtained for the condition: (A and B) with IgG-coated beads (100%); (C) with no antibody. All experiments were performed in duplicate.
We then sorted NK cells according to Tim-3 expression into Tim-3⁺ versus Tim-3⁻ NK cells, and repeated the same experiment. The suppressive effect of Gal-9 was only evident in the presence of Tim-3⁺ NK cells (Supplementary Fig. S6D). Therefore, our data indicate that following activation, Tim-3 functions as an inhibitory receptor in NK cells from patients with melanoma by reducing their cytotoxicity and cytokine secretion potential.

**Tim-3 Blockade Reverses Melanoma NK-Cell Exhaustion**

In T cells, Tim-3 has been described as a marker of T-cell exhaustion, which when blocked can reverse the function of these cells (11, 14, 15). To study whether Tim-3 blockade could also reverse the function of melanoma donor NK cells, we blocked the Tim-3 receptor by adding a soluble Tim-3 blocking antibody (10 and 20 µg/mL; clone 2E2) 1 hour before assessing NK-cell functions in various assays. Figure 5A shows that blocking Tim-3 significantly improved cytotoxicity by 20% to 25% (P = 0.002). We obtained the same results with a different Tim-3 blocking antibody (R&D Systems; #AF2365; Supplementary Fig. S7A). Both blocking antibodies were validated in a killing assay (Fig. 5B). IFN-γ production also increased in the presence of Tim-3 blocking antibody by 15% to 20% (Fig. 5C; P = 0.007). Finally, the percentage of proliferating cells dramatically increased by 30% and 60% with the addition of 10 and 20 µg/mL anti–Tim-3 antibody, respectively (Fig. 5D; P = 0.009). Our results were confirmed with another Tim-3 blocking antibody (R&D Systems; #AF2365; Supplementary Fig. S7A). Importantly, Tim-3 blockade improved NK-cell cytotoxicity against four different melanoma cell lines (Supplementary Fig. S7B). All four melanoma cell lines express Gal-9 (Supplementary Fig. S7C); however, the expression levels do not correlate with the increase of cytotoxicity that is observed with the Tim-3 blocking antibody. Isotype control antibody did not affect NK-cell cytotoxicity, IFN-γ production, or proliferation capacity (Supplementary Fig. S7D).

**Table 1.** Tim-3 expression (percentage and MFI) according to demographic and clinical parameters with well-known prognostic value

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<td>0.0056</td>
<td>29.28 (5.771)</td>
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<td>73.54 (10.21)</td>
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<td>6 (7)</td>
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<tr>
<td>Ulcerationᵇ</td>
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<td></td>
<td>33.61 (6.811)</td>
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<td>Positive</td>
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<td>72.64 (9.389)</td>
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<td>Metastasisᵇ</td>
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<tr>
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<td>Present</td>
<td>9 (11)</td>
<td>75.70 (8.996)</td>
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<td>36.31 (8.840)</td>
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Abbreviation: LN, lymph node.
ᵃOne-way ANOVA.
ᵇUnpaired t test.
As expected, Tim-3 blockade of NK cells from healthy donors also improved their cytotoxicity (Supplementary Fig. S7E and S7F). However, the addition of Gal-9 blocking antibody did not affect cytotoxicity, suggesting that other Tim-3 natural ligands may participate as K562 cells do not express Gal-9 (Supplementary Fig. S7G). Together, these results demonstrate that Tim-3 blockade enhances NK-cell function, including that of exhausted NK cells derived from patients with advanced melanoma.

Tim-3 blockade induces internalization of the receptor and upregulation of IL-2R

We investigated the mechanism underlying the reversal of NK-cell exhaustion by the blockade of Tim-3. NK cells treated with Tim-3 blocking antibody showed a clear decrease in surface membrane Tim-3 levels (Fig. 6A). We hypothesized that Tim-3 expression decreased due to internalization. To address this possibility, NK cells from healthy donors were first incubated with Tim-3 blocking antibody or isotype control, then fixed, permeabilized, and stained with a PE-conjugated anti-mouse IgG antibody to detect Tim-3 antibody both on the surface and in the cytoplasm of cells. As a control, we also assessed antibody levels on cells that were fixed but not permeabilized. These experiments demonstrated that soluble Tim-3 blocking antibody induced internalization of Tim-3 (Fig. 6B and C). To demonstrate that reduced Tim-3 levels in the membrane account for reversal of NK-cell exhaustion, NK cells were pretreated with soluble Tim-3 blocking antibody and residual Tim-3 was cross-linked using anti–Tim-3-coated...
beads. As expected, in this context, the beads had no effect on cytotoxicity, due to prior internalization of Tim-3 by the soluble blocking antibody (Fig. 6D).

The effect of Tim-3 blockade was not due to altered viability, as assessed after treatment with 5, 10, and 20 μg/mL of Tim-3 blocking antibody (Supplementary Fig. S8A). Moreover, we confirmed that this effect is really due to Tim-3 blockade and not consequent to the engagement of the Fc portion of our Tim-3 blocking antibody with CD16 receptor (FcγRIII) in NK cells. Indeed, before adding the Tim-3 blocking antibody, we incubated melanoma donor NK cells with a CD16 (FcγRIII) blocking antibody for 1 hour. This anti-CD16 antibody blocks the possibility that the Fc portion of other antibodies added to the reaction (in our case Tim-3 blocking antibody) binds to CD16 receptor (FcγRIII). Supplementary Fig. S8B shows an increase of cytotoxicity with Tim-3 blocking antibody, even after CD16 blockade.

Levels of activating and inhibitory receptors in the membrane of NK cells treated with Tim-3 blocking antibody were also measured. CD16 expression was increased by 10% after Tim-3 blockade (Supplementary Fig. S8C). Likewise, there was a significant increase in the expression of α- and γ-chains of the IL-2R (Fig. 6E). As shown in Fig. 2A and Supplementary Fig. S2C, healthy donor NK cells respond to IL-2 stimulation with increased expression of activating and inhibitory receptors, cytotoxicity, and IFN-γ production. To define the role of each chain of IL-2R in this NK-cell response, we cultured purified NK cells from healthy donors with 200 U/mL of IL-2, untreated or treated with blocking antibody for α-, β-, or γ-chain (n = 5). All experiments were performed in duplicate.
cytotoxicity were assessed. Our data demonstrate that all three chains affect the expression of the activating and inhibitory receptors, although the γ-chain seems to elicit the more pronounced effect (Supplementary Fig. SS): all three IL-2R chains also enhance NK cell–mediated cytotoxicity after IL-2 stimulation (Fig. 6F). Taken together, Tim-3 blockade increases the expression of IL-2R, making NK cells more responsive to IL-2 stimulation, and consequently improving NK cell–mediated cytotoxicity.

Collectively, our findings show that NK cells in patients with advanced melanoma display an exhausted phenotype. Furthermore, Tim-3 is overexpressed in melanoma donor NK cells, and its levels correlate with the stage of the disease and the poor prognostic factors. More importantly, through Tim-3 blockade it is possible to reverse exhaustion in NK cells from patients with advanced melanoma.

Discussion

T-cell exhaustion has been extensively studied in the context of chronic infectious diseases and different types of cancer; however, little is known about the exhaustion of NK cells. Recently Mamessier and colleagues have shown that NK cells from patients with breast cancer depict some dysfunctional phenotype (26); however, our results provide the first demonstration that NK cells from patients with advanced melanoma display the four main characteristics that define T-cell exhaustion and, more importantly, that Tim-3 is an exhaustion marker in NK cells. Therefore, similar to T cells, exhausted NK cells upregulate inhibitory receptors while downregulating IL-2 receptor and consequently are unable to respond appropriately to IL-2 stimulation. In addition, they are functionally impaired (reduced cytotoxicity, cytokine production, and proliferation) and express reduced levels of activating receptors and the transcription factors Eomes and T-bet. This exhaustion phenotype is associated with a higher expression of the inhibitory receptor Tim-3. Even though NK cells express Tim-3 in steady state, the NK cell–exhausted phenotype is characterized by an upregulation of this receptor. Therefore, the levels of Tim-3 in association with functional defects seem to be of key importance in defining the role of Tim-3. The exact mechanism of NK-cell exhaustion is still unclear; it is possible that NK cells become exhausted because of systemic production of cytokines, or within the tumor microenvironment in response to specific ligands. Indeed, chronic stimulation with IL-2 and IL-15 (27, 28), shed MICA (tumor-derived ligand for NKG2D; refs. 29, 30), or CD155 (a tumor-derived ligand for DNAM-1; ref. 31) has been described to induce NK-cell exhaustion. Exhausted T cells also upregulate CTLA-4 and PD-1; however, we found no significant expression of these receptors in the membrane of melanoma donor NK cells. Together, our results show that Tim-3 is constitutively expressed on NK cells from healthy donors. More importantly, we demonstrate for the first time that exhausted NK cells upregulate Tim-3, which functions as an inhibitory receptor/exhaustion marker, similar to its described role in T cells. We found that the expression of Tim-3 in NK cells is higher in patients with melanoma with bad prognostic factors, and increases as the disease stages progress.

Contrasting roles have been described for Tim-3 in NK cells from healthy donors. Although one study showed that Tim-3 inhibits normal donor NK cell–mediated cytotoxicity (17), another suggested that Tim-3 may instead enhance IFN-γ production (18). Although the two studies are difficult to align, the different model systems and contexts could explain these divergent results, as has been reported for other NK-cell receptors such as 2B4 and KIR2DL4 (32, 33). Our data are consistent with Tim-3 acting as an inhibitory receptor on NK cells from healthy and, most importantly, from melanoma donors. We also show that when triggered, Tim-3 does not promote NK-cell death. In our system, Tim-3 negatively regulates NK-cell function in a Gal-9–independent manner, suggesting a role for other Tim-3 ligands. Phosphatidylserine, exposed on the surface of apoptotic cells, has been reported as a Tim-3 ligand and may be a candidate for conferring NK-cell exhaustion in vivo after tumor cell death.

When we block Tim-3 receptor with a soluble antibody, we are able to recover, in part, NK cells’ function. This reversal is comparable with that in T cells after in vitro blockade of other immune checkpoints, such as PD-1 blockade (11, 34), which has been used in clinical trials with impressive clinical responses (35). The Tim-3 blocking antibody binds and internalizes the receptor, decreasing its expression in the membrane of NK cells and the possibility of binding to the natural ligands. Another possibility is that we are blocking the intrinsic inhibitory pathway of Tim-3, independently of any ligand. We also showed that Tim-3 blockade induces a 10% increase of CD16 expression (MFI) that could provide another explanation for the increase of NK-cell function. Thus, CD16, an activating receptor that is directly involved in the lysis of tumor cells, may function not only through ADCC but also independently of antibody binding. Finally, we demonstrated that Tim-3 blockade increases the expression of the IL-2R in the membrane of melanoma donor NK cells, augmenting their ability to respond to IL-2 stimulation. The enhanced responsiveness may contribute toward the partial reversal of melanoma donor NK-cell function after Tim-3 blockade.

Similar to CTLA-4 and PD-1, Tim-3 belongs to the group of immune checkpoint molecules and is a potential therapeutic target. Although there are no clinical data yet, Tim-3 has been reported to be coexpressed with PD-1 on human tumor-specific CD8 T cells, and dual blockade of both molecules significantly enhances the in vitro proliferation and cytokine production of human T cells (11). Furthermore, in vivo studies have shown that Tim-3 blockade alone, or in combination with PD-1 blockade, is able to control tumor growth in four different tumor models, including melanoma (14, 36). A recent study showed that Tim-3 blockade stimulates potent antitumor responses against established melanoma via NK cell–dependent mechanisms when associated with a vaccine (37). However, in those studies, it was not clear if Tim-3 had a direct effect on NK cells. Our findings provide the first evidence that Tim-3 blockade can directly reverse NK-cell exhaustion and improve the function of NK cells from patients with melanoma. Even though the recovery of melanoma NK-cell function is significant, it is not complete. It is possible that Tim-3 works with
other receptors to regulate NK-cell exhaustion, although we could not detect a role for either CTLA-4 or PD-1. Nevertheless, combinatorial strategies that also target other inhibitory NK-cell receptors may enable the recovery of NK-cell phenotype more completely. Our study has direct clinical relevance as it shows for the first time that blocking Tim-3 improves, *ex vivo*, the function of NK cells, which could be used for NK-cell adoptive transfer therapy. Moreover, our studies support the concept that systemic Tim-3 blockade could improve antitumor response in the context of melanoma, as is the case with systemic CTLA-4 and PD-1 blockade. Fewer adverse events should be expected with Tim-3 blockade as Tim-3-deficient mice are viable and do not develop autoimmune or lymphoproliferative diseases (12), as opposed to CTLA-4-deficient mice (38).

In conclusion, this study suggests that higher Tim-3 expression on NK cells is associated with advanced stages of melanoma and with poor prognostic clinical parameters. We show for the first time that Tim-3 is an exhaustion marker expressed in NK cells from patients with advanced melanoma and that its blockade reverses their exhausted phenotype. Tim-3, therefore, represents a promising therapeutic target that could enhance antitumor immunity with the potential to produce durable clinical responses that are dependent not only upon T cells but also on the innate immune system.

**Disclosure of Potential Conflicts of Interest**

A.C. Anderson is a consultant/ advisory board member for Celstim Pharmaceuticals, V.K. Kuchroo is the founder of Celstim Inc. and has ownership interest (including patents) in the same. No potential conflicts of interest were disclosed by the other authors.

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**Development of methodology:** S. Jimenez-Baranda, S. Khan

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** I.P. da Silva, A. Gallois, S. Jimenez-Baranda, I. Osman

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** I.P. da Silva, A. Gallois, S. Jimenez-Baranda, A.C. Anderson, V.K. Kuchroo

**Writing, review, and/or revision of the manuscript:** I.P. da Silva, A. Gallois, S. Jimenez-Baranda, A.C. Anderson, I. Osman, N. Bhardwaj

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** I. Osman

**Study supervision:** S. Jimenez-Baranda, N. Bhardwaj

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Reversal of NK-Cell Exhaustion in Advanced Melanoma by Tim-3 Blockade

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