

Adaptive NK Cells with Low TIGIT Expression Are Inherently Resistant to Myeloid-Derived Suppressor Cells

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Abstract

Human cytomegalovirus (CMV)-induced adaptive natural killer (NK) cells display distinct phenotypic and functional characteristics, including properties of immune memory. We hypothesized that these cells may be more resistant to suppression mediated by immunoregulatory cell subsets, making them attractive for use in cancer therapy. Here we report that relative to conventional NK cells, adaptive NK cells express lower levels of the inhibitory receptor T-cell Ig and ITIM domain (TIGIT), which results in resistance to immune suppression mediated by myeloid-derived suppressor cells (MDSC), as derived from cytokine induction in normal blood or patients with myelodysplastic syndrome. In contrast, conventional NK cells were potently suppressed by MDSCs, an effect abrogated completely by TIGIT

blockade. Mechanistically, TIGIT signaling in NK cells after MDSC coculture led to a decrease in the phosphorylation of ZAP70/Syk and ERK1/2. These effects were reversed by blocking TIGIT on NK cells or by inhibiting production of reactive oxygen species (ROS) by MDSCs, the latter of which upregulated the TIGIT ligand CD155 on MDSCs. Accordingly, the blunted cytotoxicity of NK cells cocultured with MDSCs against tumor cells could be reversed by blocking TIGIT or ROS production. Overall, our results show how adaptive NK cells arising in response to CMV infection can escape MDSC-mediated suppression, and defined TIGIT antagonists as a novel type of checkpoint inhibitor to enhance NK-cell-mediated responses against cancer and infection. *Cancer Res*; 76(19); 5696–706. ©2016 AACR.

Introduction

Natural killer (NK) cells are lymphocytes of the innate immune system (1, 2). Although they share similar mechanisms of killing with cytotoxic T cells (3, 4), NK cells recognize targets through families of activating and inhibitory receptors. The balance between these receptors determines the function of NK cells (5). A downregulation of MHC class I on damaged cells, or a mismatch between inhibitory subgroups of killer immunoglobulin-like receptors (KIR) and their respective human leukocyte antigen (HLA) ligands on cells will render targets susceptible to NK-cell killing (6, 7). Therefore, tuning down the expression of inhibitory receptors on NK cells would increase their response to tumor cells. Like T cells, NK-cell antitumor activity is limited by highly suppressive tumor microenvironment, which leads to dampened immunologic function and poor prognosis (8–11). Emerging studies indicate that inhibitory receptors such as cytotoxic T lymphocyte-associated 4 (CTLA-4), programmed cell death 1 (PD-1), and T-cell

Ig and ITIM domain (TIGIT) on T and NK cells can suppress antitumor responses (12–16). Although the physiologic role of inhibitory receptors is to maintain immune homeostasis, the goal in cancer immunotherapy is to unleash this control.

TIGIT is an inhibitory receptor that binds with high affinity to CD155 and with lower affinity to CD112. CD155 and CD112 are expressed in epithelial cells and antigen-activated T cells at steady state. However, these ligands are defined as "stress-induced" and their expression is increased upon viral infection and malignant transformation (17). Engagement of TIGIT with CD155 competes with the interaction between the activating receptor DNAM-1 and CD155, resulting in decreased NK-cell cytolytic activity and IFN γ production (16, 18). Recently, TIGIT was found to be highly expressed on tumor-infiltrating lymphocytes (TIL), and coblockade of TIGIT and PD-1 synergistically augmented CD8⁺ T-cell activity against autologous tumor cells (19). TIGIT is also expressed on polyclonal NK cells (20), but little is known with respect to how TIGIT regulates human NK-cell function in the tumor microenvironment.

We have recently identified heterogeneous subsets of highly specialized human NK cells that arise in response to cytomegalovirus (CMV) infection. We refer to these cells as "adaptive" and they are defined by epigenetic silencing of one or more of the proximal signaling molecules SYK, EAT-2, and Fc ϵ R γ along with silencing of the transcription factor PLZF. Interestingly, adaptive NK cells exhibit a whole-genome methylation signature that is remarkably similar to effector CD8⁺ T cells (21). Adaptive NK cells express the activating receptor NKG2C and maturation marker CD57 and these cells are virtually absent in CMV seronegative individuals. These cells produce markedly more inflammatory cytokines following CD16 ligation and are long-lived. Our group has recently shown that adaptive NK-cell

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expansion after CMV reactivation in hematopoietic cell transplant recipients is associated with lower relapse rates (22).

In this study, we examined the interaction between adaptive NK cells and myeloid-derived suppressor cells (MDSC). MDSCs are a heterogeneous population of myeloid progenitor cells and immature myeloid cells. In humans, MDSCs commonly express CD11b, CD33, low or no HLA-DR, and are either CD14⁺ [monocytic MDSCs (mMDSC)] or CD15⁺CD66b⁺ [granulocytic MDSCs (gMDSC); ref. 23]. These cells are induced by tumors and contribute to inhibition of both innate and adaptive antitumor immunity by producing TGF β , IL10, reactive oxygen species (ROS), and arginase (24). We found that, compared with conventional NK cells, adaptive NK cells expressed lower TIGIT and consequently resisted functional suppression by cancer patient-derived MDSCs. These data suggest that strategies to expand adaptive NK cells or approaches to block the CD155-TIGIT interaction should be considered for enhancement of antitumor NK-cell immune responses.

Materials and Methods

Patients and healthy donors

Peripheral blood mononuclear cells (PBMC) were obtained fresh from CMV-seropositive healthy subjects or cryopreserved from myelodysplastic syndrome patients ($n = 15$) obtained from the National Marrow Donor Program (NMDP)/Center for International Blood and Marrow Transplant Research Repository. All samples were de-identified and use was approved by the University of Minnesota and NMDP institutional review board in accordance with the Declaration of Helsinki.

Cell isolation

PBMC were seeded at 2×10^6 /mL in RPMI medium containing 10% heat-inactivated FBS, IL6 (10 ng/mL; Sigma-Aldrich) and GM-CSF (10 ng/mL; R&D systems) for a week and refreshed on day 3 of culture to generate MDSCs (25). Next, HLA-DR⁺ cells were isolated with anti-human HLA-DR microbeads (Miltenyi Biotec), and MDSCs were thereafter purified from the HLA-DR⁻ fraction using anti-CD33 microbeads (Miltenyi Biotec). mMDSCs were used in all shown experiments ($\geq 85\%$ CD14⁺HLA-DR⁻). NK and T cells were isolated from overnight rested PBMC by negative depletion (StemCell Technologies) or CD3 microbeads (Miltenyi Biotec). Control monocytes were isolated from overnight rested PBMCs using anti-CD33 microbeads.

Proliferation assays

Monocytes or MDSCs were seeded in 96-well U-bottom plates in duplicate at 1:1–1:16 ratios with CellTrace violet dye (5 μ mol/L; Invitrogen)-labeled autologous T or NK cells (1×10^5) in RPMI medium (Gibco) supplemented with 10% FBS (referred below as medium). T cells were stimulated with anti-CD3/CD28 activation beads (40 beads/well) and IL15 (1 ng/mL) or IL15 (10 ng/mL) alone for NK cells and cultured for 3 to 5 days. Cells were acquired by LSRII flow cytometer (BD Biosciences) and data analyzed by FlowJo (TreeStar).

Flow cytometry analysis

Purified NK cells were cultured with monocytes or MDSCs for 5 days, in contact or separated by transwell inserts (0.4 μ m pores; Corning), and in the presence of IL15 (10 ng/mL) prior to staining. In some experiments, NK cells were cultured overnight in medium alone or in the presence of IL15 (10 ng/mL), and with

the addition of IL18 (100 ng/mL) and IL12 (10 ng/mL), or agonistic anti-CD16 (3G8, 1 μ g/mL). Cryopreserved PBMCs were rested overnight in medium to recover from freezing and then cultured for 6 hours in the presence of IL15 (10 ng/mL) and anti-CD16 (1 μ g/mL) prior to staining. Cells were stained with fluorochrome-conjugated antibodies detailed in Supplementary Table S1. Detection of CD107a, Ki67, IFN γ , and TNF α production was performed following fixation and permeabilization (eBioscience) according to the manufacturer's instructions. In some experiments, MDSCs were cultured overnight and stained for CD155 (PVR) in the presence of reagents targeting MDSC-suppressive pathways (26) including 10 μ g/mL TGF β -neutralizing antibodies (R&D Systems), 200 IU/mL of the ROS scavenger catalase (Sigma-Aldrich) or superoxide dismutase (Sigma-Aldrich), 500 μ mol/L arginase inhibitor N(ω)-hydroxynor-L-arginine (nor-NOHA; Calbiochem), or iNOS inhibitor NG-monomethyl-L-arginine (L-NMMA; Sigma-Aldrich). All cells were acquired by LSRII and analyzed by FlowJo. Adaptive and conventional NK cells were gated and identified according to the gating strategy in Supplementary Fig. S1.

Confocal microscopy

MDSCs, monocytes, and NK cells were prelabeled with CellTracker Blue for 20 minutes (20 μ mol/L; Invitrogen) and cocultured overnight in the presence of IL15 (10 ng/mL). Cells were loaded onto poly-L-lysine pretreated chamber slides. After overnight culture, cells were stimulated with agonistic anti-CD16 for 6 hours, fixed in 4% paraformaldehyde for 30 minutes and then blocked with 3% BSA at room temperature. After blocking, cells were incubated with primary anti-TIGIT and anti-PVR (CD155) overnight at 4°C and then 1 hour with the fluorescence-labeled secondary antibodies before confocal microscopy (objective $\times 20$).

Phosflow

NK cells from healthy blood donors were cocultured with MDSCs or monocytes at a 2:1 ratio in the presence of IL15 (10 ng/mL) and in the presence or absence of catalase (200 IU/mL) or blocking antibodies against TIGIT (10 μ g/mL) for 5 days. Cells were then washed, rested for 4 hours, and stimulated with anti-CD16 agonist antibody for 10 and 30 minutes before analysis of Zap-70 and ERK1/2 phosphorylation, respectively. Cells were fixed and permeabilized with BD fixation buffer and permeabilization buffer III and stained for pZap-70 (pY319)/Syk (pY352) and pERK1/2 (pT202/pY204) according to the manufacturer's instructions (BD Biosciences).

Chromium release assays

Anti-TIGIT antibodies were functionally tested by cross-linking with chromium (⁵¹Cr)-labeled FcR11⁺ murine cell line p815 (authenticated from ATCC, used within 3 months of the first passage). NK-cell function was analyzed after 4 hours against p815 in the presence of, IgG, anti-TIGIT (10 μ g/mL), or an agonistic anti-CD158b (anti-KIR; 10 μ g/mL; Biolegend) control. After 5 days of coculture with monocytes or MDSCs in the presence or absence of blocking antibodies against TIGIT (10 μ g/mL) or ROS scavenger catalase (200 IU/mL), polyclonal-NK-cell cytotoxicity was analyzed by ⁵¹Cr-release assays (4 hours) against K562 (authenticated from ATCC, used within three months of the first passage) cells at a 5:1–2.5:1 effector:target ratios.

Statistical analysis

All data were first analyzed in the software mentioned above and summarized by Prism Version 6 software (Graph-Pad). All data were first tested for normal distribution. Thereafter, differences among groups were analyzed by a Student *t* test, one- or two-way ANOVA, or nonparametric Mann-Whitney *U* tests (as indicated in the figure legends). Representative histograms or images were chosen on the basis of the average values.

Results

MDSCs suppress T- and NK-cell proliferation and NK-cell function

To investigate the interaction between MDSCs and NK-cell subsets, MDSCs were generated from healthy donors (HD) cultured with IL6 and GM-CSF for 1 week (Supplementary Fig. S2A; ref. 25). Fresh monocytes were used as a myeloid cell

control for these experiments. Purified T and NK cells were cocultured with monocytes or MDSCs at different ratios and evaluated for proliferation following 3 to 4 days of culture. Although monocytes had a little effect on proliferation, MDSCs induced a 2.7 ± 1.6 -fold T-cell suppression ($P = 0.005$) of proliferation with a similar effect on NK cells (1.5 ± 0.27 -fold suppression, $P = 0.006$; Fig. 1A). Similarly, CD16 engagement stimulated NK-cell degranulation and IFN γ production that was significantly suppressed by MDSC (2.4 ± 1.4 -fold suppression, $P = 0.0001$ and 2.6 ± 1.5 -fold suppression, $P = 0.003$, respectively) relative to NK cells cultured with monocytes (Fig. 1B).

Adaptive NK cells resist MDSC suppression

We next examined whether adaptive NK cells could resist MDSC suppression compared with conventional NK cells. We defined adaptive NK cells by flow cytometry as NK cells from

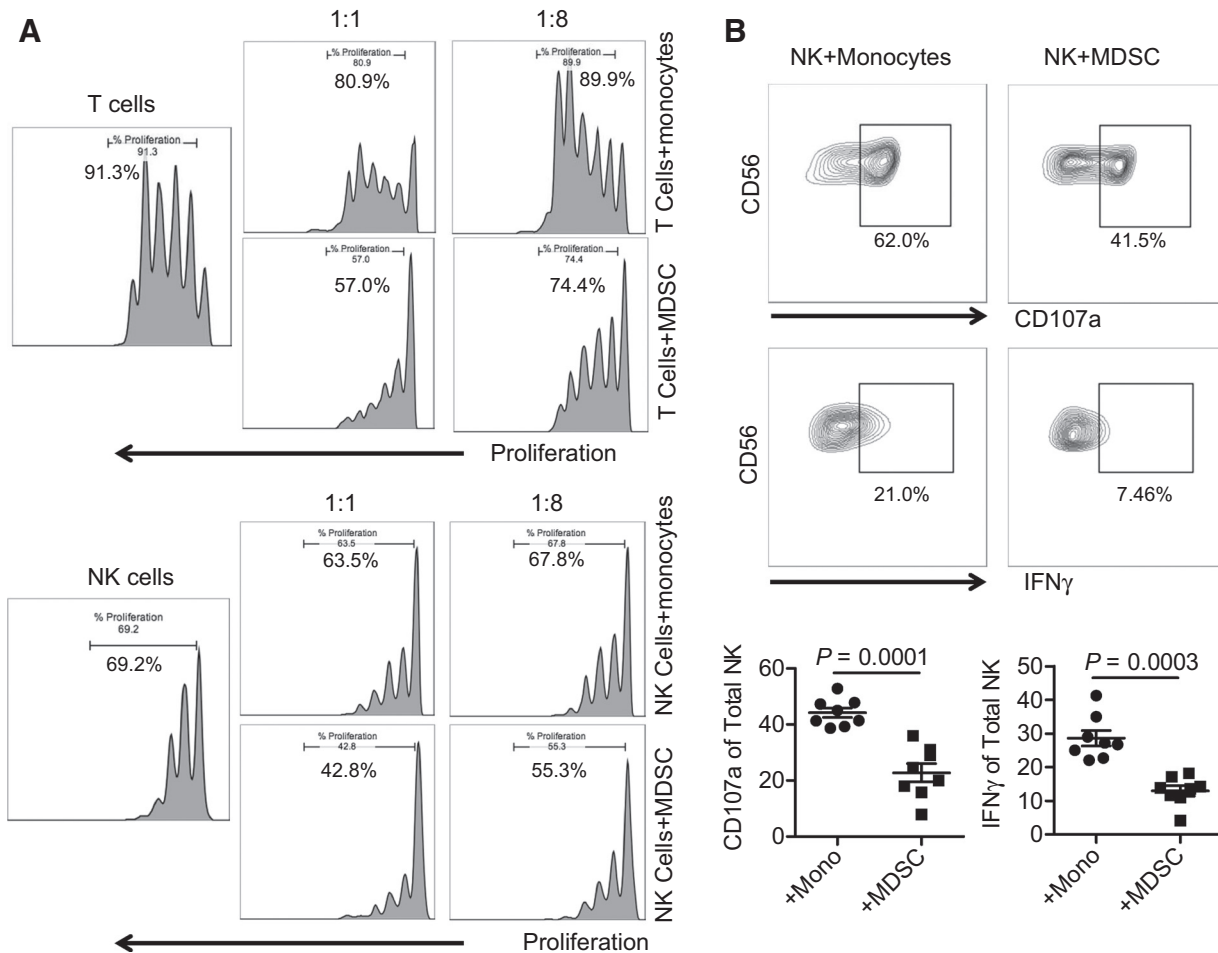


Figure 1.

MDSCs suppress T- and NK-cell proliferation and NK-cell functions. **A**, purified T and NK cells from healthy blood donors were labeled by CellTrace Violet and cocultured with MDSCs or monocytes at different ratios in the presence of CD3/CD28 beads (40 beads/ 1×10^5 cells) and IL15 (1 ng/mL) for T cells or IL15 (10 ng/mL) alone for NK cells. Proliferation was assessed on day 3 or 4, and representative data are shown of six independent experiments. **B**, purified NK cells were cocultured with monocytes or MDSCs at a 2:1 ratio in the presence of IL15 (10 ng/mL) for 5 days. Cells were stimulated with agonistic CD16 (1 μ g/mL) for 6 hours prior to staining and evaluated for degranulation (CD107a) and IFN γ production. Cumulative ($n = 8$) data are shown as mean \pm SEM. The Student *t* test was used for statistical analysis.

CMV-seropositive donors that were $CD57^+NKG2C^+Fc\epsilon R1\gamma^-$ (21) and conventional NK cells as $CD57^+NKG2C^-$. Because adaptive NK cells are less responsive to cytokines (21), they were instead stimulated with an anti-CD16 agonistic antibody. Purified polyclonal NK cells were cocultured with monocytes or MDSCs for 5 days and examined for degranulation, proliferation, and cytokine production following CD16 stimulation. Conventional and adaptive NK cells express equal amounts of CD16 (Supplementary Fig. S2B). Similar NK-cell function was observed when cultured alone or with the addition of monocytes in the presence of IL15 (Fig. 2A). Compared with monocyte controls, MDSCs mediated significant suppression of CD107a ($52.4 \pm 2.4\%$ vs. $33.5 \pm 3.1\%$, $P \leq 0.001$), IFN γ ($31.3 \pm 3.8\%$ vs. $13.8 \pm 3.0\%$, $P \leq 0.001$), TNF ($29.1 \pm 2.1\%$ vs. $13.6 \pm 3.3\%$, $P \leq 0.01$), and proliferation ($49.0 \pm 2.9\%$ vs. $23.0 \pm 3.5\%$, $P \leq 0.001$; measured by Ki67) within the population of conventional NK cells. However, adaptive NK cells were resistant to the same MDSC population (Fig. 2A). Moreover, conventional NK-cell degranulation and IFN γ production were completely restored when MDSC were separated

from NK cells by a transwell (Fig. 2B). Thus, CMV infection induces a population of adaptive NK cells that are resistant to contact-dependent MDSC suppression.

Adaptive NK-cell resistance to MDSC suppression correlates with lower TIGIT expression

NK cells were cultured overnight in the absence or the presence of IL15 (10 ng/mL) alone or with additional stimulation of IL12 (10 ng/mL) and IL18 (100 ng/mL) or anti-CD16 (1 μ g/mL) prior to staining. TIGIT expression was low without stimulation and was upregulated with IL15 stimulation alone. Additional stimulation by anti-CD16 further increased TIGIT expression. However, DNAM-1 could not be further increased due to high baseline expression level (Fig. 3A). Interestingly, the staining pattern for TIGIT on these polyclonal-activated NK cells showed a bimodal expression (Fig. 3A). To explore this phenomenon further, we examined the expression of TIGIT and the two counterparts DNAM-1 and CD96, and other receptors on adaptive and conventional NK cells when cultured with monocytes or MDSC. There were no expression differences between adaptive and

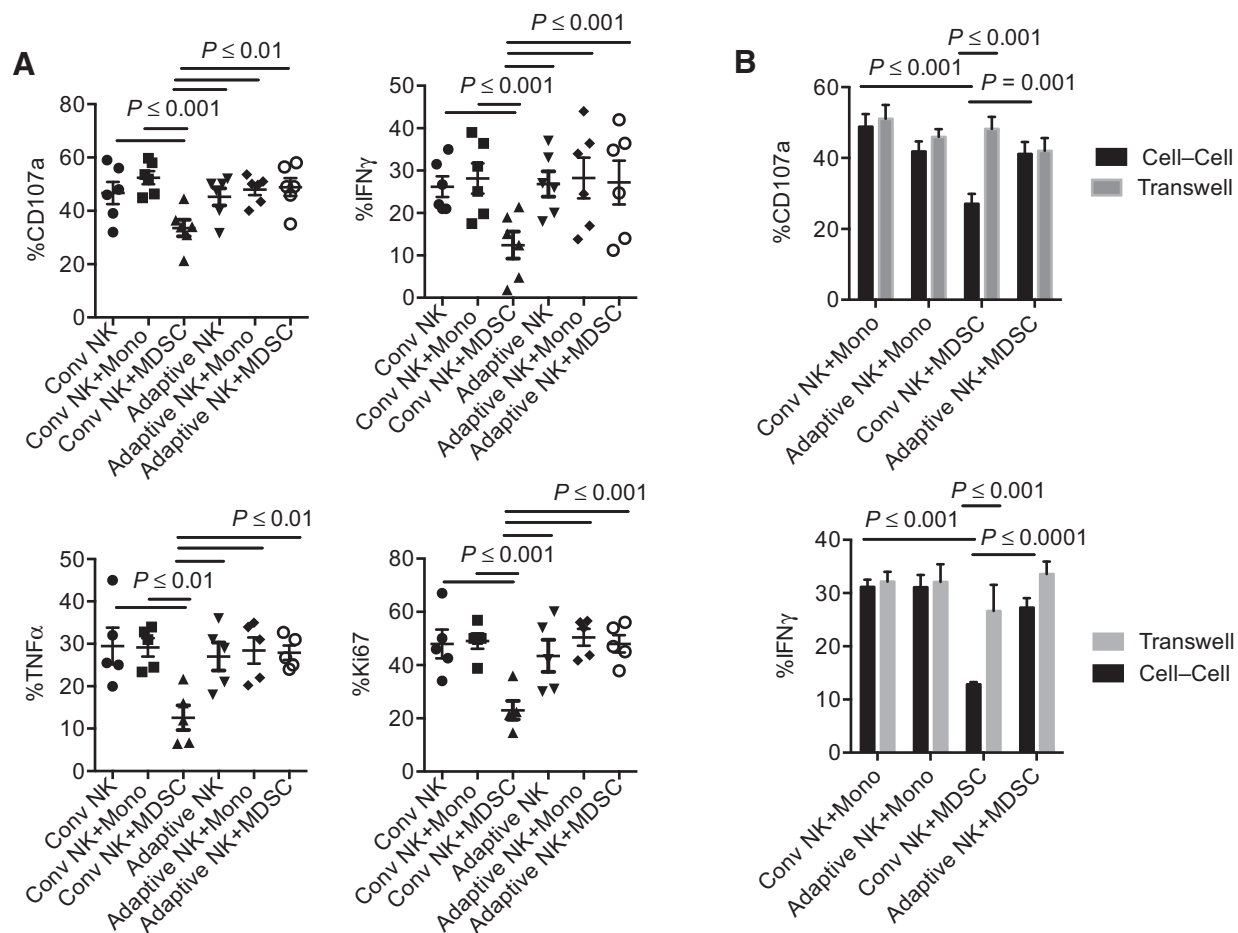


Figure 2.

Adaptive NK cells resist MDSC suppression. **A** and **B**, purified NK cells from healthy blood donors were cultured alone or in contact with MDSCs or monocytes at a 2:1 ratio in the presence of IL15 (10 ng/mL) for 5 days (**A**) or in transwells allowing exchange of soluble factors only (**B**). Cells were stimulated with anti-CD16 6 hours prior to staining, and degranulation, IFN γ and TNF production, as well as proliferation (Ki67) were assessed by flow cytometry. Pooled data of 5–7 independent experiments are shown as the mean \pm SEM and statistical analyses were done using the two-way ANOVA.

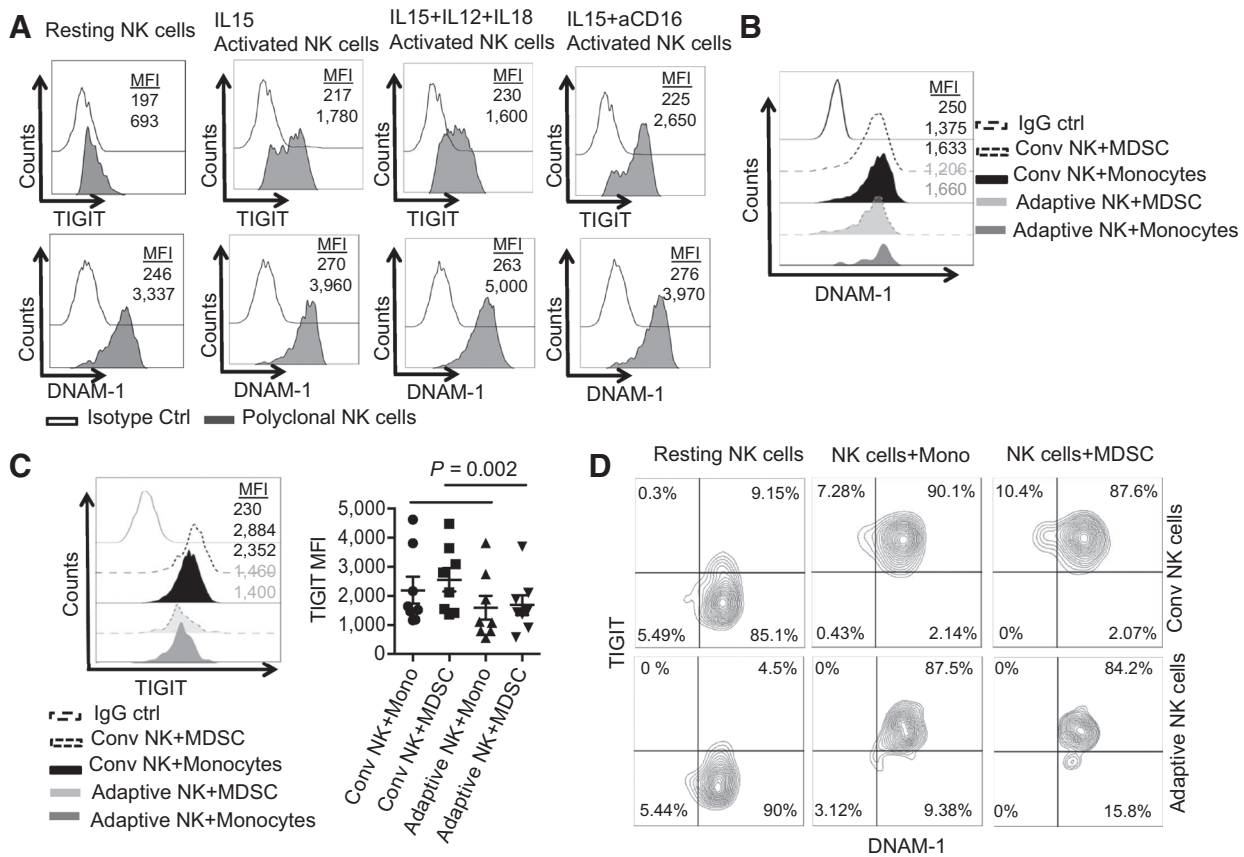


Figure 3. Conventional NK cells express higher TIGIT compared with adaptive NK cells. **A**, purified NK cells from healthy blood donors were cultured before staining in the absence or presence of IL15 (10 ng/mL) alone or with the additional stimulation of IL12 (10 ng/mL) and IL18 (100 ng/mL) for 18 hours or with stimulation with anti-CD16 (1 μ g/mL) for 6 hours. One of four independent experiments is shown. NK cells were cultured with MDSCs or monocytes at a 2:1 ratio in presence of IL15 (10 ng/mL) for 5 days. Cells were stimulated with anti-CD16 six hours prior to analysis. Representative histograms for DNAM-1 (**B**) and TIGIT (**C**) expression and aggregate data for TIGIT expression ($n = 8$) are shown as mean fluorescence intensity (MFI) \pm SEM. Two-way ANOVA was used for statistical analysis. **D**, NK cells before and after coculture with monocytes or MDSC were analyzed for coexpression of DNAM-1 and TIGIT. Representative data are shown of three independent experiments and seven replicates.

conventional NK cells for DNAM-1, CD96, NKp44, NKp46, PD-1, Tim3, or NKG2A (Fig. 3B and Supplementary Fig. S2C). In contrast, TIGIT expression was significantly less on adaptive versus conventional NK cells whether cocultured with monocytes ($P = 0.008$) or MDSCs ($P = 0.002$; Fig. 3C). Although, conventional and adaptive NK cells coexpressed TIGIT and DNAM-1 at similar levels before and after coculture with monocytes or MDSC (NK alone: 18% \pm 10% vs. 14% \pm 11.5%, NK+monocytes: 86% \pm 8% vs. 83% \pm 9%, NK+MDSC: 84% \pm 9% vs. 82% \pm 6%; Fig. 3D), adaptive NK-cell expression of TIGIT remained low.

TIGIT-dependent suppression of conventional NK cells by MDSCs

Monocytes, MDSCs, and NK cells were labeled with CellTracker Blue, cocultured in chamber slides overnight. Cells were stimulated with anti-CD16 prior stained with anti-CD155 (green) and anti-TIGIT (red) and distinguished by size. As expected, TIGIT on NK cells colocalized with CD155 on MDSCs as a result of high expression of CD155 on MDSCs compared with a minimal expression on monocytes (Fig. 4A). To assess whether TIGIT plays a role in MDSC-dependent regulation of NK cells, IFN γ produc-

tion was evaluated in conventional NK cells cocultured with MDSCs based on differential high versus low TIGIT expression after 6 hours of CD16 stimulation. Cells with low TIGIT expression produce significantly more IFN γ relative to NK cells with high TIGIT expression (36.2% vs. 19.9%, $P = 0.0005$; Supplementary Fig. S2D). Next, we examined whether engagement of TIGIT is responsible for driving the MDSC suppression of NK cells. We tested the function of the anti-TIGIT antibody as previously described in a P815 assay with normal NK cells (27). Although the presence of anti-CD158b control inhibited NK-cell cytotoxicity, NK-cell function was not affected in the presence of anti-TIGIT (Supplementary Fig. S3A), indicating the lack of agonistic function. NK cells were then cocultured with monocytes or MDSCs for 5 days in the presence or absence of blocking antibodies against TIGIT. MDSC-induced suppression of polyclonal NK-cell function was completely abrogated by blocking TIGIT (Fig. 4B). As TIGIT blockade had little effect on adaptive NK cells, this release of suppression was entirely based on the large conventional NK-cell population (Fig. 4C and D). Simultaneous blockade of TIGIT and DNAM-1 in conventional NK cells cocultured with MDSC reversed the effect of TIGIT-blockade and

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inhibited the degranulation and IFN γ of adaptive NK cells (Fig. 4E and Supplementary Fig. S3B), indicating a TIGIT-dependent inhibition of DNAM-1 signaling.

ROS induces CD155 expression on the surface of MDSCs

We further examined the expression of the TIGIT ligands CD155 and CD112 in monocytes and MDSCs. MDSCs expressed high levels of CD155 compared with almost no expression in monocytes (MFI: 675 ± 124 vs. 107 ± 23 ; $P = 0.015$). Moreover, CD112 expression was significantly higher in MDSCs compared with monocytes (MFI: 1714 ± 331 vs. 865 ± 196 ; $P = 0.015$; Fig. 5A). To further investigate the mechanisms of MDSC-induced conventional NK-cell suppression, we individually blocked pathways utilized by MDSCs including superoxide, arginase, ROS, TGF β , and iNOS overnight at the end of MDSC generation. Inhibition of ROS production with catalase resulted mainly in the significant decrease in the expression of CD155 on MDSCs down to the level seen in control monocytes ($55\% \pm 23$ decrease, $P = 0.03$; Fig. 5B).

Several studies have shown that increased ROS production in MDSCs correlates with suppression of T-cell function. We examined the ROS production levels in MDSCs compared with freshly isolated monocytes. Monocytes produced almost no ROS and were predominantly CD155 negative. In contrast, MDSCs produced high basal levels of ROS and were uniformly CD155 positive (Fig. 5C). Furthermore, inducing ROS production in monocytes by H $_2$ O $_2$ treatment induced the expression of CD155 in ROS $^+$ monocytes (Fig. 5D).

TIGIT engagement inhibits pZAP70/Syk and pERK1/2 and results in inhibition of NK-cell cytotoxicity

Given the strong suppressive effect of TIGIT engagement on conventional NK-cell function and proliferation, we next analyzed the CD16-induced signaling interaction with TIGIT in NK cells cocultured with MDSC. Compared to control monocytes, NK cells cocultured with MDSCs exhibited decreased phosphorylation of ERK1/2 ($P = 0.003$) and ZAP70/Syk ($P = 0.005$). Furthermore, blocking TIGIT or inhibiting ROS restored the

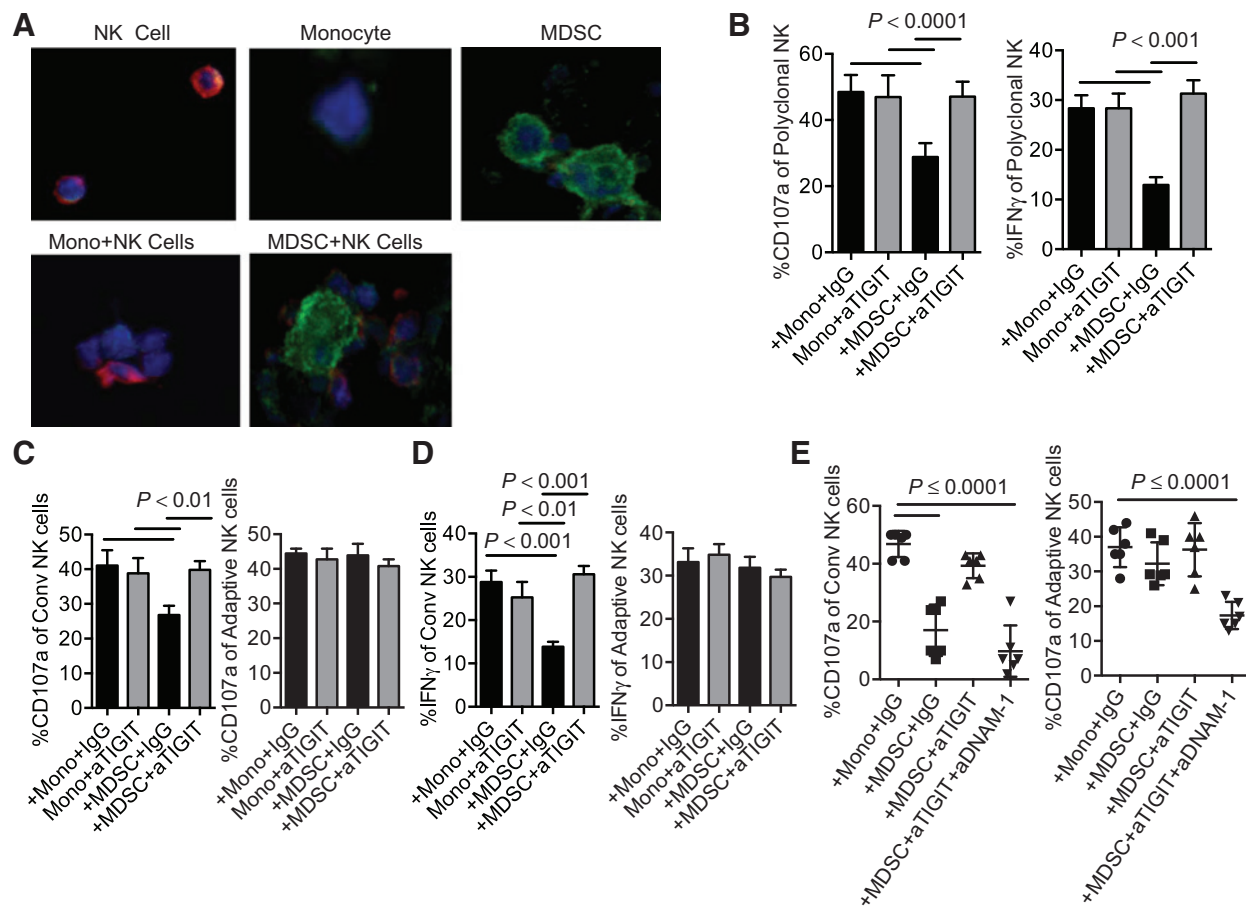


Figure 4.

TIGIT-dependent suppression of conventional NK cells by MDSC. **A**, monocytes, MDSCs, and NK cells were labeled with CellTracker Blue, cocultured on slides overnight, then stimulated with anti-CD16 prior stained with anti-CD155 (green) and anti-TIGIT (red), followed by confocal microscopy. Individual cell types are shown at the top or at the bottom when cocultured. Representative data of two independent experiments and six donors are shown. NK cells were cultured with monocytes or MDSCs in the presence of IL15 and IgG control (10 μ g/mL) or blocking antibodies against TIGIT (10 μ g/mL) for 5 days. Degranulation ($n = 9$) and IFN γ production ($n = 8$) were evaluated in polyclonal NK cells (**B**), conventional ($n = 8$; **C**), and adaptive NK cells ($n = 9$; **D**). **E**, alternatively, cells were coblocked by anti-TIGIT and anti-DNAM-1 (10 μ g/mL; $n = 6$). Pooled data are shown as mean \pm SEM of n number of replicates, and the two- and one-way ANOVA (**E**) were used for statistical analysis.

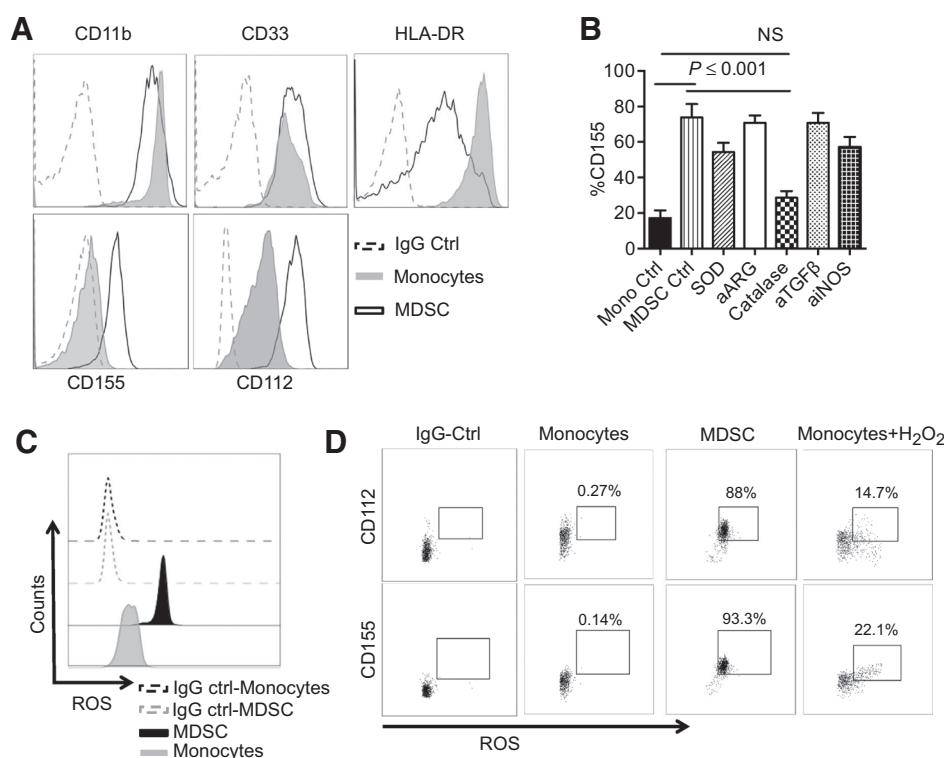


Figure 5.

ROS induce CD155 expression on MDSCs. **A**, MDSCs and monocytes were stained for the antigens shown. One representative example from 10 independent experiments is shown. **B**, induced MDSCs were stained for CD155 and analyzed by flow cytometry after overnight treatment with superoxide dismutase (SOD, 200 IU/mL), arginase inhibitor [α-ARG, arginase inhibitor N(ω)-hydroxy-nor-L-arginine, 500 μmol/L], ROS scavenger (catalase, 200 IU/mL), blocking antibodies against TGFβ (10 μg/mL), iNOS inhibitor (αiNOS, NG-monomethyl-L-arginine, 500 μmol/L), or left untreated compared with control monocytes. Pooled ($n = 4$) data from n independent experiments are shown as mean ± SEM, and statistical analysis was done using the two-way ANOVA. **C**, unstimulated monocytes and MDSCs were stained for total ROS and analyzed by flow cytometry. **D**, unstimulated or H₂O₂ (250 μmol/L) monocytes for 1 hour and unstimulated MDSCs were stained for total ROS, CD112, and CD155 and analyzed by flow cytometry. Cells double positive for ROS and CD112 or CD155 are shown. One representative donor of 6 is shown. One representative isotype control is shown for all groups for simplicity as individual controls were similar between conditions.

phosphorylation of ERK1/2 and ZAP70/Syk ($P \leq 0.02$; Fig. 6A and B). We next investigated whether blocking TIGIT or ROS could recover the cytotoxicity of NK cells cultured with MDSCs against tumor cells. Neither anti-TIGIT nor catalase had any effect on NK cells cultured alone (data not shown). NK cells cultured with either monocytes or MDSCs were pretreated with TIGIT blocking antibodies or catalase, washed, and then incubated with ⁵¹Cr-labeled K562 cells. NK-cell cytotoxicity was significantly decreased after coculture with MDSCs, whereas monocytes had no effect (Fig. 6C). Both TIGIT blockade and ROS inhibition completely reversed the suppressive effect mediated by MDSCs (Fig. 6C). Moreover, blocking TIGIT combined with catalase treatment in NK-cell and MDSC cocultures had no additive effect on either pZAP70/Syk and pERK1/2 or NK-cell cytotoxicity (data not shown).

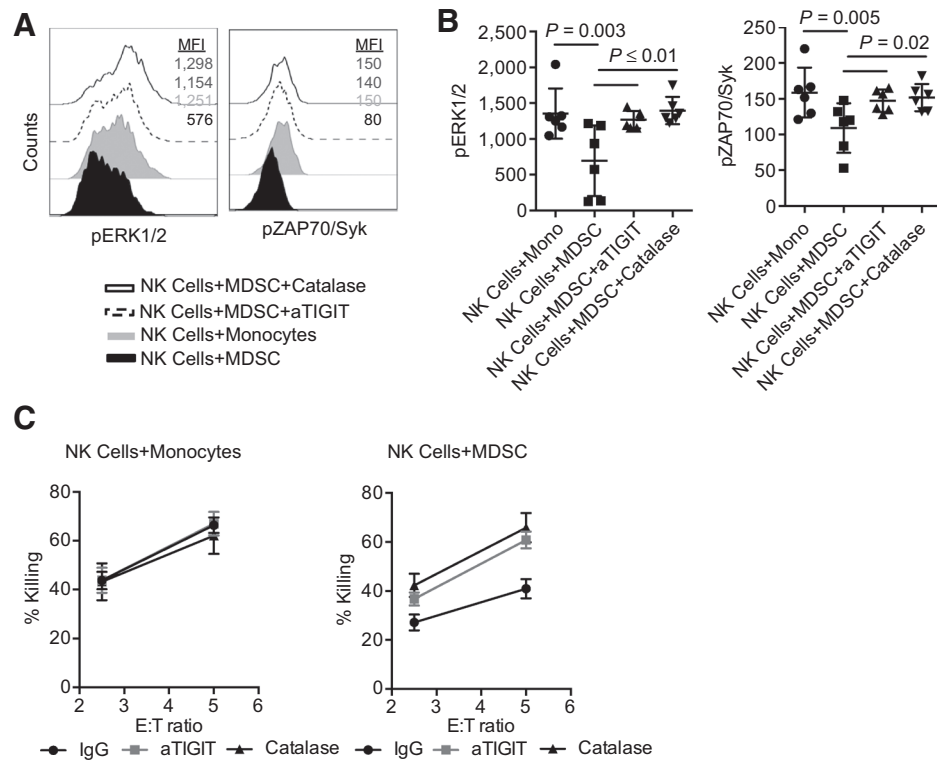
TIGIT-dependent suppression of conventional NK cells by myelodysplastic syndrome patient MDSCs

Having identified the contact-mediated suppressive mechanism of cytokine-generated MDSCs, we next investigated whether this mechanism was operant in a physiologic setting *in vivo*. PBMC from CMV-seropositive myelodysplastic syndrome patients and HDs were analyzed for the frequency of adap-

tive NK cells and MDSCs. Although there was a high frequency of adaptive NK cells in the blood of CMV⁺ myelodysplastic syndrome patients ($n = 10$, 17% ± 15% vs. 7% ± 5%), the total NK-cell frequency was significantly lower compared with HD ($n = 8$, 1.3% ± 1.2 vs. 8% ± 7%). mMDSCs were defined as CD45⁺Lin⁻CD11b⁺CD33⁺HLA-DR^{-low}CD14⁺ and gMDSCs as CD45⁺Lin⁻CD11b⁺CD33⁺CD15⁺. Compared with healthy blood donors, we found a significant increase in the frequency of both mMDSCs ($P = 0.02$) and gMDSCs ($P = 0.01$) in the blood of myelodysplastic syndrome patients (Fig. 7A). In addition, MDS-MDSCs have increased CD155 expression compared to MDS-monocytes (Fig. 7B). PBMC from myelodysplastic syndrome patients were then evaluated for the expression of TIGIT on conventional and adaptive NK cells. In patients with myelodysplastic syndrome, TIGIT expression was significantly lower on adaptive compared with conventional NK cells (MFI: 347 ± 189 vs. 660 ± 311; $P = 0.002$; Fig. 7C). Myelodysplastic syndrome patient CMV-induced adaptive NK cells exhibited significantly greater function after activation with IL15 and CD16 stimulation relative to myelodysplastic syndrome-conventional NK cells. Notably, adaptive NK cells displayed similar degranulation and IFNγ production as in HD NK cells (Fig. 7D). Blocking TIGIT signaling in conventional NK cells from

Figure 6.

TIGIT engagement inhibits pZAP70/Syk and pERK1/2 and results in inhibition of NK-cell cytotoxicity. **A**, purified NK cells were cocultured with MDSCs or monocytes at a 2:1 ratio in the presence of IL15 (10 ng/mL), IgG control (10 µg/mL), and in the presence or absence of blocking antibodies against TIGIT (10 µg/mL) or catalase (200 IU/mL) for 5 days. Cells were stimulated for 10 and 30 minutes with anti-CD16 and stained for pZAP/Syk or pERK1/2, respectively. Representative (**A**) or cumulative (**B**) data are shown from three independent experiments and six donors as mean ± SEM. Statistical analysis were done using the two-way ANOVA. **C**, NK cells from monocyte and MDSC cocultures in the presence or absence of anti-TIGIT or catalase were washed and incubated with ⁵¹Cr-labeled K562 for 4 hours to assess NK-cell cytotoxicity. Representative data from three independent experiments is shown as mean ± SEM.



myelodysplastic syndrome patients rescued their functional hyporesponsiveness, but there was little added effect on adaptive NK cells that have inherently low levels of TIGIT expression (Fig. 7D).

We next evaluated the suppressive capacity of MDSCs circulating in the blood of myelodysplastic syndrome patients on allogeneic NK cells from healthy volunteers. After 5 days of coculture in the presence of IL15 and CD16 stimulation, we observed a marked reduction in allogeneic conventional NK-cell function compared with that of adaptive NK cells in the same sample (Fig. 7E and Supplementary Fig. S3C). TIGIT blockade completely reversed the suppressive function of primary MDSCs from myelodysplastic syndrome patients on conventional NK cells, whereas no effect was seen on the CMV-induced adaptive NK cells (Fig. 7F). Thus, our data definitively demonstrate that the MDSC-suppressive mechanism observed with cytokine-generated MDSCs from normal donors is identical to those of primary MDSCs from myelodysplastic syndrome patients.

Discussion

There has been an explosion of new data in the immunotherapy literature describing the potential therapeutic benefits of NK- and T-cell immunotherapy in patients with active cancer (28–31). This excitement is fueled by unexpected strong clinical results with checkpoint blockade against T-cell PD-1/PD-L1 and/or CTLA-4 pathways (32, 33). Although it is well known that NK-cell responses to targets are determined by a counterbalance of signals via activating and inhibitory receptors, similar checkpoint blockade mechanisms are less understood. In this study, we identified TIGIT/CD155 as a key axis underlying

MDSC-induced suppression of conventional NK cells. Importantly, CMV-induced adaptive NK cells expressed low levels of TIGIT and were not susceptible to MDSC suppression. The function of MDSCs induced from normal blood by cytokine was equivalent to that of MDSCs naturally induced in myelodysplastic syndrome cancer patients, which highlights the physiologic relevance of these findings and their potential translational importance. There are at least two ways to overcome TIGIT-induced immunosuppression of NK cells. The first is TIGIT blockade, which restores CD16 signaling in conventional NK cells to normal levels. The second is the expansion of MDSC-resistant adaptive NK cells after CMV exposure.

Immunosuppressive cell types, including MDSCs, accumulate in the tumor microenvironment and exert suppression and diminished tumor clearance (34). In agreement with other studies (26, 35, 36), we show that cytokine-induced MDSCs suppress polyclonal NK-cell proliferation, degranulation, and IFN γ production. However, by segregating adaptive and conventional NK cells, we unexpectedly found that adaptive NK cells are resistant to MDSC suppression. We next investigated this mechanism. We found that TIGIT expression was significantly lower in adaptive NK cells compared with conventional NK cells. In a recent study, Wang and colleagues have shown that different expression levels of TIGIT in NK cells from healthy individuals was associated with functional heterogeneity, and high TIGIT expression inversely correlated with IFN γ production in response to IL12 stimulation (20). Our results are in agreement with this study. In addition, we found that TIGIT blockade interferes with DNAM-1-dependent signaling in conventional NK cells. Although CD96 detection decreased after coculture with MDSCs, CD96 blockade had no effect (data not shown), indicating a dominant role of TIGIT to suppress conventional NK cells by CD155-expressing MDSCs.

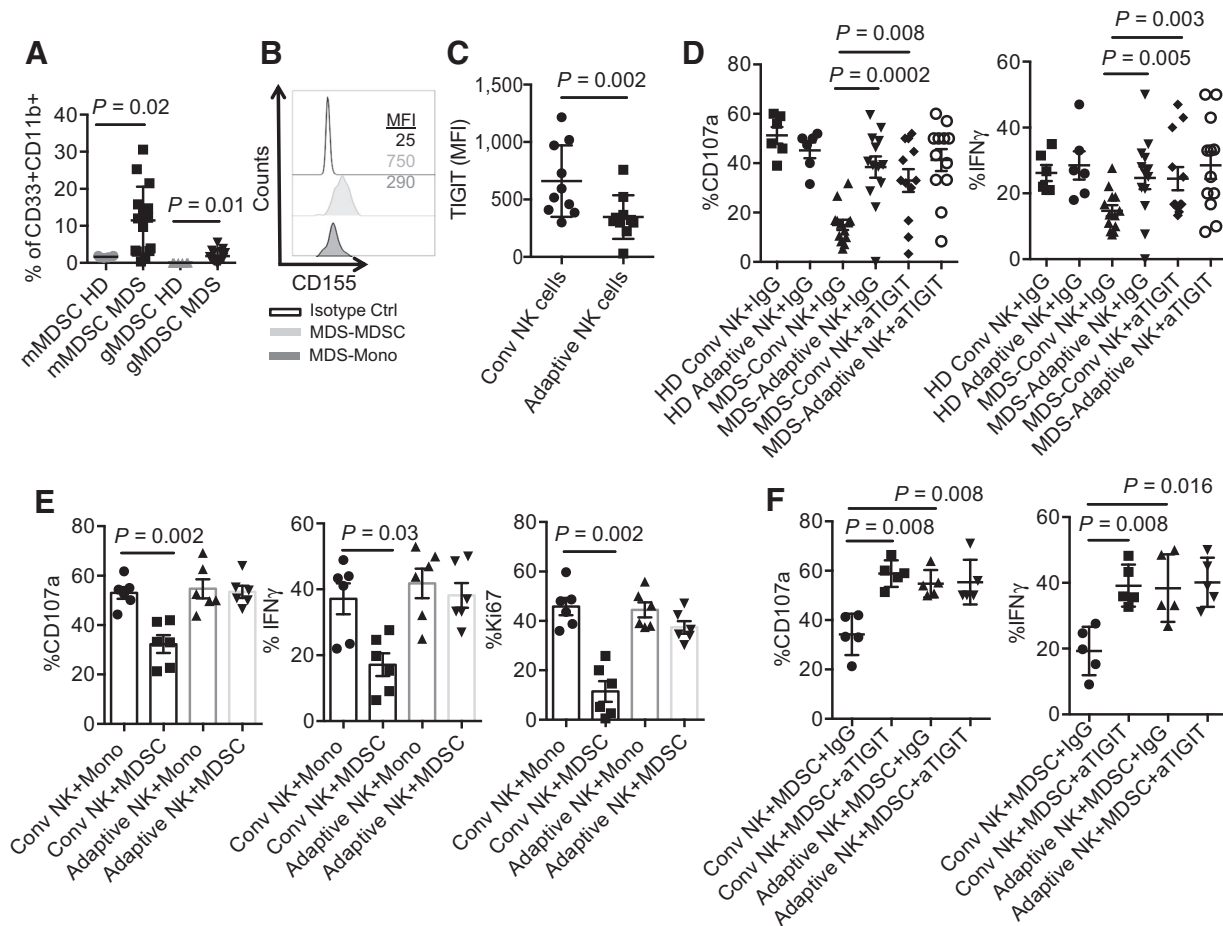


Figure 7.

TIGIT-dependent suppression of conventional NK cells by myelodysplastic syndrome (MDS) MDSCs. **A**, PBMC ($n = 15$) from myelodysplastic syndrome patients and HDs ($n = 6$) were rested overnight, stained, and the MDSC frequency was determined by flow cytometry. mMDSCs were defined as $CD45^{+}Lin^{-}CD11b^{+}CD33^{+}HLA-DR^{-/low}CD14^{+}$ and gMDSCs as $CD45^{+}Lin^{-}CD11b^{+}CD33^{+}CD15^{+}$. **B**, MDS-PBMC were stained for CD155 and gated for mMDSC and monocytes. Representative histograms are shown of 15. **C**, PBMCs ($n = 10$) from myelodysplastic syndrome patients were rested overnight and evaluated for TIGIT expression by flow cytometry. **D**, PBMCs from HDs ($n = 6$) or myelodysplastic syndrome patients ($n = 13$) were stimulated with IL15 (10 ng/mL) in the presence of IgG control or anti-TIGIT, and anti-CD16 (1 μ g/mL) for 6 hours and assessed for NK-cell degranulation and IFN γ production. **E**, purified NK cells ($n = 6$) from healthy blood donors were cocultured with autologous monocytes or allogeneic MDSCs enriched from the blood of myelodysplastic syndrome patients at a 2:1 ratio in the presence of IL15 (10 ng/mL) for 5 days. After 6-hour stimulation with anti-CD16, degranulation and IFN γ production was evaluated in conventional and adaptive NK cells by flow cytometry. **F**, purified NK cells ($n = 5$) from healthy blood donors were cocultured with allogeneic MDSCs enriched from the blood of myelodysplastic syndrome patients at a 2:1 ratio in the presence of IL15 (10 ng/mL), IgG control (10 μ g/mL), and in the presence or absence of anti-TIGIT (10 μ g/mL) for 5 days. Six hours prior staining, cells were stimulated with anti-CD16 and degranulation, and IFN γ production was evaluated in conventional and adaptive NK cells by flow cytometry. Representative data are shown as mean \pm SD, and statistical analyses were done on pooled data using the Student t test for **A**, **C**, **D**, and Mann-Whitney test for **E** and **F**.

CD112 and CD155 are regulated by cellular stress and bind TIGIT with low and high affinity, respectively. Both receptors are highly expressed on transformed cells (37–39). Here we show that conversion of monocytes into MDSCs is associated with increased CD112 and induction of CD155 expression. CD155 but not CD112 expression was dependent on ROS production, and MDSCs produced high levels of ROS. Corzo and colleagues have shown a substantial upregulation of ROS by MDSCs in different tumor models and in patients with head and neck cancer. These increases in ROS were explained by greater activity of NADPH oxidase (NOX2; ref. 40). In addition, an earlier study has revealed that the CD155 gene promoter contains a binding site for Nrf-1, a transcription factor regulated by oxidative stress (41). Hence, the

CD155 expression may indirectly be controlled by oxidative stress that is generated by an increase in ROS production.

TIGIT engagement on NK cells after coculture with MDSCs resulted in substantially less phosphorylation of ZAP70/Syk and ERK1/2 compared with NK cells cocultured with CD155 $^{-}$ monocytes. Importantly, blocking TIGIT or inhibiting ROS production reversed this defect in proximal signaling and restored K562 function. We also found that circulating MDSCs from patients with myelodysplastic syndrome were highly suppressive of conventional NK cells, an effect reversed by TIGIT blockade. This shows that the phenotypic MDSCs induced by myelodysplastic syndrome are functionally suppressive, a finding that may contribute to the clinical progression of this disease (42).

Our data suggest that it will be of value to consider blockade of TIGIT to enhance the antitumor role of NK cells in cancer immunotherapy. Alternatively, clonal expansion of TIGIT-low adaptive NK cells in cancer patients could improve antitumor immunotherapy with minimal suppression of the tumor microenvironment. Such expansion is seen after CMV reactivation in immunosuppressed patients (43, 44), and could be promoted by infusion of adaptive NK cells or by CMV vaccines. Treatment with CMV envelope glycoprotein B and genetic immunization with dendritic cell CMV vaccines have been shown to be safe and feasible (45–47). In summary, our novel data provide a new perspective on the suppression of different NK-cell subsets by MDSCs and highlight the importance of TIGIT in directing this interaction.

Disclosure of Potential Conflicts of Interest

J.S. Miller reports receiving a commercial research grant and is a consultant/advisory board member for Fate Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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