Published in final edited form as:

Cancer Immunol Immunother. 2020 October ; 69(10): 2147–2155. doi:10.1007/s00262-020-02615-7.

NKG2D-Fc fusion protein promotes antitumor immunity through the depletion of immunosuppressive cells

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Abstract

A major factor impeding the success of numerous therapeutic approaches in cancer is the immunosuppressive nature of the tumor microenvironment (TME). Hence, methods capable of reverting tumor immunosuppression through depletion or reprogramming of myeloid-derived suppressive cells (MDSCs) and regulatory T cells (Tregs) are of great clinical need. Here, we explore NKG2D-Fc as a modality to modulate antitumor immunity through the depletion of immunosuppressive MDSCs and Tregs in the TME. We have generated the NKG2D-Fc fusion protein and characterized its potential to mediate tumor control and overall survival in LL2 and

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Author Contributions:

PHF and BL equally contributed to the design of the study, the performance of the experiments, and the writing of the manuscript; SHT and YJK contributed to the performance of the experiments; EF and MC contributed to the writing and preparation of the manuscript; CH contributed to the design of the study.

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Conflict of interest: The authors do not declare any competing interests.

Ethics approval and consent to participate:

The housing and handling of mice follow guidelines established by Johns Hopkins Medical Institutions Animal Care and Use Committee and the National Institutes of Health. The Oncology Center Animal Facility has full-time veterinary support through the Department of Comparative Medicine. Animals are monitored daily for infection and other illnesses by trained animal technicians. The criteria include: infection, failure to thrive, perceived pain, respiratory distress, etc. Research support services include training classes and the capacity for full post-mortem analysis is available on request. Only trained laboratory personnel and animal technicians were allowed to handle laboratory animals. All individuals handling mice were registered to protocols at the Johns Hopkins Animal Care and Use Committee.

Consent for publication: Not applicable.

Availability of data and material:

All data recorded for this study was performed at the Johns Hopkins Medical Institutes.

MC38 murine models. Upon treatment of LL2 or MC38 tumor-bearing mice with NKG2D-Fc, we observe significant tumor control and enhanced survival compared to Fc control. When characterizing MDSCs and Tregs from tumor-bearing mice, we observe clear expression of NKG2D-ligand RAE1 γ and subsequent binding of NKG2D-Fc fusion protein to both MDSCs and Tregs. Examining the immune profile of mice treated with NKG2D-Fc reveals significant depletion of MDSCs and Tregs in the TME, as well as an increase in NK cells likely due to the reversed suppressive TME. In conclusion, NKG2D-Fc induces antitumor immunity and tumor control through the depletion of MDSCs and Tregs, subsequently providing a niche for the infiltration and expansion of proinflammatory cells, such as NK cells. Strategies capable of modulating the immunosuppressive state in cancer are in high clinical demand. NKG2D-Fc is a simple, single tool capable of depleting both MDSCs and Tregs and should be further investigated as a therapeutic agent for the treatment of cancer.

Précis:

NKG2D-Fc fusion protein mediates tumor control and overall survival, binds to MDSCs and Tregs, and modulates immune-suppressive and immune-stimulating cells. NKG2D-Fc induces antitumor immunity and tumor control through MDSC and Treg depletion.

Keywords

NKG2D; Fc fusion; immunosuppressive cells; Tregs; MDSCs; RAE1 γ

Introduction

The immune system exerts a strong selective pressure throughout tumor progression, and tumor cells consistently employ mechanisms to escape immune detection in a process known as immune editing (1). Immune editing explains how the immune system can drive the selection of immune-resistant cells, shaping and facilitating the development of tumors through three steps: elimination, equilibrium, and escape (1). Potent antitumor effect and effective tumor elimination require the engagement, activation, and expansion of cytotoxic immune cells in the tumor microenvironment. Tumors may down-regulate major histocompatibility complex (MHC) and costimulatory molecules, upregulate inhibitory checkpoint ligands such as program death-ligand 1 (PD-L1) (2), and generate a suppressive microenvironment. These cause advanced or distant metastatic disease and successful escape of immune-resistant tumor cells selected through immune editing (2).

An indispensable method by which tumor cells create a microenvironment permissive to escape from immune detection is through the recruitment, modulation, and expansion of anti-inflammatory cells (3). An example of this is the infiltration of macrophage precursors and subsequent polarization into “pro-tumor” M2 tumor-associated macrophages (TAMs) through the secretion of interleukin-10 (IL-10) and transforming growth factor beta (TGF- β) by tumor cells (3). Among suppressive cells recruited to the tumor, myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) play dominant roles in promoting tumor progression and metastasis as the tumor cytokine milieu promotes efficient infiltration and rapid expansion of these cells (4–7). Both MDSC and Tregs can rapidly outnumber

proinflammatory immune cells in the tumor, shifting the balance of immune editing to immune escape.

MDSCs consist of two groups of cells: granulocytic MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs). In mice, PMN-MDSCs are defined as CD11b⁺Ly6G⁺Ly6C^{lo} cells with high-side scatter, whereas M-MDSCs are defined as CD11b⁺Ly6G⁻Ly6C^{hi} cells with low-side scatter (6). In cancer patients, increased MDSCs correlated with poor clinical outcome and treatment response (8, 9). Many prominent factors are implicated in MDSC-directed suppressive activities, including arginase 1 (ARG1), nitric oxide (NO), upregulation of reactive oxidative species (ROS), and the production of prostaglandin E2 (PGE2) (10, 11). Intratumoral Tregs also exert a potent suppressive activity mediated by TGF- β and IL-10, while upregulating cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and inducible T-cell costimulatory (ICOS) (12).

Numerous strategies to promote immune elimination of tumors have been developed and function by blocking the individual mechanisms by which tumors promote immune escape. Recently, immune checkpoint blockade therapies, especially the PD-1/PD-L1 blockade, has been shown to overcome immune suppression in several types of cancers by blocking suppressive checkpoint signals (13). Targeting infiltrating anti-inflammatory cells in the tumor is an attractive solution as these cells can directly contribute to the suppressive tumor cytokine microenvironment while also expressing inhibitory checkpoint molecules such as PD-L1. In tumor-bearing mice, depleting MDSCs with anti-Gr-1 antibodies increases the amount of cytotoxic immune cells expressing interferon gamma (IFN γ) and perforin, leading to stronger antitumor responses (14). Additionally, the depletion of Tregs through numerous methods augments cytotoxic antitumor immunity, leading to superior tumor control (15).

NKG2D is a protein that can be expressed by tumor cells, as well as immune cell populations. Previously, it has been shown that a fusion protein of the single-chain variable fragment with the extracellular domain of NKG2D receptor (scFv-NKG2D) could engage tumor cells and T cells *in vitro*, subsequently stimulating IFN γ production by T cells. scFv-NKG2D also inhibited the growth of NKG2D ligand-negative B16F10 tumors through the increased infiltration of T cells and a reduction in suppressive cells (16). In our previous study, we found that NKG2D-Fc could accumulate in TC-1 tumors, which was shown to express RAE-1 γ , an NKG2D binding partner (17). However, the effects of NKG2D-Fc directly on immune cells expressing NKG2D ligands such as RAE-1 γ remain unclear. In this study, we used the NKG2D-Fc fusion protein to evaluate immune modulation and antitumor responses through the depletion of suppressive Tregs and MDSCs, promoting tumor immune elimination over immune escape.

Materials and Methods

Mice

Six-to-eight-week old female C57BL/6 mice were purchased from Taconic (Cambridge City, IN). All mice were maintained at the Johns Hopkins University School of Medicine Oncology Animal Facility in Cancer Research Building II (Baltimore, MD) under specific-

pathogen-free conditions. All animal procedures were performed according to approved protocols by the Johns Hopkins Institutional Animal Care and Use Committee and in accordance with recommendations for the proper use and care of laboratory animals.

Cells and antibodies

LL2 and MC38 cells were purchased from ATCC (Rockville, MD). Cells were cultured *in vitro* in RPMI-1640 media supplemented with 10% fetal bovine serum, 50 units/mL of penicillin/streptomycin, 2 mM of L-glutamine, 1 mM of sodium pyruvate, and 2 nM of non-essential amino acids, and grown at 37°C. For flow cytometric analyses, antibodies including CD11b (clone M1/70, Biolegend), Gr-1 (Clone RB6–8C5, Biolegend), Ly6C (clone HK1.4, Biolegend), Ly6G (Clone 1A8, Biolegend), RAE-1 γ (clone CX1, Biolegend), CD4 (clone GK1.5), CD3 ϵ (clone 145–2C11, BD Bioscience), NK1.1 (clone PK136, Biolegend), Foxp3 (clone PCH101, eBioscience), CD8 (53–6.7, Biolegend), IFN γ (XMG1.2, Biolegend) and CD45 (30-F11, Biolegend) were used. Live cell gating was performed using Zombie Aqua Fixable Viability Kit (Biolegend). To detect NKG2D-Fc, we used the PE goat anti-mouse antibodies (Multiple Adsorption, BD Bioscience Cat No: 550589).

NKG2D-Fc protein production and purification

The production of the NKG2D-Fc protein has previously been described (17). Briefly, to generate pFuse-NKG2D-Fc, the extracellular domain of murine NKG2D was amplified through PCR by primers AAAGAATTTCGAAAGAGACGTTTCAGCCAGT and TTTAGATCTCACCGCCCTT TTCATGCAGA with mouse NKG2D cDNA serving as the template DNA (Open Biosystems, Lafayette CO), and then cloned into EcoRI and Bgl II sites of pFuse-IgG2a-Fc (InvivoGen). For the production of the recombinant NKG2D-Fc or Fc control, 1×10^7 BHK-21 cells were transfected with 50 μ g of either the pFuse-NKG2D-Fc or pFuse-IgG2a-Fc plasmid in T-15 flasks using Lipofectamin 2000 (Invitrogen Corp. Carlsbad, CA, USA). Three days after transfection, the cultured media was collected, filtered with a 0.22- μ m syringe filter (Millipore, Billerica MA, USA) and concentrated with Amicon Ultra-15 50 kDa cut-off centrifugal filter units (Millipore, Billerica MA, USA). The concentrated recombinant proteins were loaded onto a HiTrap Protein G HP column (GE Healthcare) and immobilized via Fc-protein G binding. The column was washed with 20 mM of sodium phosphate buffer (pH 7.0) and the recombinant protein was eluted using 0.1 M glycine-HCl buffer (pH 2.8). Protein concentrations were determined with the Coomassie Plus protein assay (Pierce, Rockford, USA) and purity was estimated by SDS polyacrylamide gel electrophoresis.

In vivo tumor treatment experiments

For both MC38 and LL2 treatment protocol, female C57BL/6 mice (6-to-8 weeks old) were injected with 2×10^5 cancer cells subcutaneously over the right flank area. Experimental groups of MC38 or LL2 tumor-bearing mice were treated with 100 μ g of NKG2D-Fc on days 6, 9, and 13. They were compared with a treatment-matched control group treated with the Fc control protein alone. Tumors were measured ($1/2 \times$ long diameter $\times$ short diameter²) twice per week and were euthanized according to approved animal care and use protocol

guidelines to minimize distress. This corresponds to tumors measuring above 1.5 cm in any dimension or when hunching or body weight loss is observed in the mice.

Preparation of single-cell suspensions of intratumoral immune cells from MC38 and LL2 tumors and spleen

To collect intratumoral immune cells, MC38 and LL2 tumors were dissected into small pieces that can pass through a 1 mL pipette tip and immersed in a digestion buffer containing collagenase I and IV with a final concentration 100 U/mL and DNase I 100 µg/mL for 30 minutes at 37°C with periodic agitation. Single-cell suspensions were retrieved from tumor digests using C-tubes and a GentleMACS tissue dissociator (Miltenyi Biotech) as per manufacturer's instruction. Single cells were washed and resuspended with the FACS buffer; viable cells were determined using trypan blue dye exclusion. To collect single cell suspensions from the splenocytes of MC38 or LL2 tumor-bearing mice, spleens were macerated through the 70 µm cell strainer with the back of a syringe plunger. After washing with FACS buffer twice and RBC lysis, single cells were resuspended in FACS buffer for surface/intracellular staining.

Surface and intracellular staining for flow cytometry

After RBC lysis, PBMCs from peripheral blood of MC38 or LL2 tumor-bearing mice were resuspended in FACS buffer. Single-cell suspensions from tumor-infiltrating cells, splenocytes, and PBMCs were washed and adjusted to a cell concentration of 1×10^6 /mL, then stained with a Zombie Aqua™ Fixable viability dye (Biolegend) for 15 mins in 4°C. Cells were stained with surface antibodies for 30 mins at 4°C and prepared for flow cytometry analysis. For intracellular staining, cells were permeabilized and fixed with Foxp3/Transcription Factor staining buffer set (eBioscience) for 1 hr at 4°C, then stained with intracellular antibodies for 30 mins at 4°C. For IFNγ staining, cells were stimulated with PMA/Ionomycin + Brefeldin A prior to staining. After washing, the cells were analyzed by CytoFlex S and data were acquired using the CytExpert software (Beckman Coulter). For peripheral blood, only singlet and live cells were gated; for splenocyte and intratumor cell analysis, singlet, live, and CD45⁺ cells were gated for further analysis.

Statistical analysis

At least three samples per group were included in each of these experiments. Flow cytometry data and results of tumor treatment experiments were evaluated by analysis of variance (one-way ANOVA) and the Tukey-Kramer test. Individual data points were compared by Student's single-tailed *t*-test. Event-time distributions for mice were compared by the Kaplan-Meier method and the logrank test. All *p* values < 0.05 were considered statistically significant.

Results

NKG2D-Fc generates effective antitumor responses

To test the antitumor effects of NKG2D-Fc, C57BL/6 mice were injected with either 2×10^5 MC38 or LL2 cells subcutaneously and treated with either NKG2D-Fc or Fc protein (Fig. 1A). NKG2D-Fc was shown to be able to suppress MC38 tumor growth and improve

survival (Fig. 1B). The antitumor effects of NKG2D-Fc also confirmed in the LL2 tumor model (Fig. 1C). Additionally, to demonstrate the dose-dependency of NKG2D-Fc, MC38 tumor-bearing mice were treated with a lower dose of 30 ug of NKG2D-Fc to Fc control at the same time points. 30 ug of NKG2D-Fc was shown to be able to suppress MC38 tumor growth and improve survival, but to a lesser extent compared to the 100 ug dose (Sup Fig. 1). Overall, our results show that NKG2D-Fc improved the survival of tumor-bearing mice. Our previous study also highlighted NKG2D-Fc's ability to accumulate in tumors expressing NKG2D ligands, but NKG2D-Fc failed to recruit T cells into these tumors and had minimal antitumor effects. Both MC38 and LL2 cancer cells express NKG2D ligands, but antitumor effects are observed (18, 19); therefore antitumor effects of NKG2D-Fc may come from immune modulation of other immune cells.

NKG2D-Fc binds to NKG2D-ligand-expressing MDSCs

Previous studies showed that tumor-derived MDSCs and Treg cells expressed the NKG2D ligand RAE1 γ , and using a bi-specific NKG2D fusion protein could reduce the percentage of MDSCs and Tregs in peripheral blood and tumors (16). To prove that NKG2D-Fc could also bind to MDSCs (CD11b⁺Gr-1⁺) and Tregs (CD4⁺CD25⁺), MC38 tumor-bearing mice were euthanized and identified from PBMC, spleen, and tumor-infiltrating cells of tumor-bearing mice (Sup Fig. 2). Mice utilized for this staining had 0.5 cm diameter tumors. MDSCs and Treg cells of MC38 tumor-bearing mice expressed RAE1 γ (Fig. 2A, 2B) with the highest expression for MDSCs being from the tumor and highest expression for Tregs being from the spleen. RAE1 γ expression was also assessed from circulating MDSC and Tregs of naïve and tumor-bearing mice, where similar expression levels were observed (Sup Fig. 3). NKG2D-Fc could be detected by PE goat anti-mouse multiple adsorption antibodies on MDSCs and Treg cells (Fig. 2C, 2D). Tumor-derived MDSCs and Tregs bound the highest level of NKG2D-Fc. Our results indicate that NKG2D-Fc could interact with MDSCs and Tregs potentially through RAE1 γ , although other ligands may play a role.

NKG2D-Fc reduces peripheral blood and intratumor MDSCs and Tregs

As previously mentioned, MDSCs and Tregs could promote tumor progression and metastasis through the induction of a suppressive microenvironment. The depletion of MDSCs and Tregs was shown to improve tumor control in mouse models (14, 15). In our experiment, the NKG2D-Fc treatment group had a lower peripheral blood MDSC count (Fc CD11b⁺Gr-1⁺ vs. NKG2D-Fc CD11b⁺Gr-1⁺: 14.0 ± 1.9 vs. 6.7 ± 0.6 , $p < 0.05$, Fig. 3A) and a higher NK cell count (Fc CD3⁻ NK1.1⁺ vs. NKG2D-Fc CD3⁻ NK1.1⁺: 1.5 ± 0.1 vs. 2.5 ± 0.3 , $p < 0.05$; Fig. 3B), but no significant change in Treg count (Fc CD4⁺Foxp3⁺ vs. NKG2D-Fc CD4⁺Foxp3⁺: 1.2 ± 0.1 vs. 1.0 ± 0.04 , $p > 0.05$; Fig. 3C) and cytotoxic T cell count (Fc CD8⁺IFN- γ ⁺ vs. NKG2D-Fc CD8⁺IFN- γ ⁺: 21.6 ± 1.4 vs. 23.6 ± 1.7 ; Fig. 3D). We further analyzed MC38 tumor-infiltrating immune cells, and found that treatment with NKG2D-Fc also had a lower count of MDSCs (Fc CD11b⁺Ly6C⁺ vs. NKG2D-Fc CD11b⁺Ly6C⁺: 8.9 ± 0.6 vs. 6 ± 0.8 , $p < 0.05$; Fig. 4A) (Fc CD11b⁺Ly6G⁺ vs. NKG2D-Fc CD11b⁺Ly6G⁺: 1.1 ± 0.2 vs. 0.5 ± 0.1 , $p < 0.05$; Fig. 4A), lower Tregs (Fc vs NKG2D-Fc: 2.4 ± 0.3 vs 1.4 ± 0.1 , $p < 0.05$; Fig. 4B), and increased count of NK cells (Fc CD4⁺Foxp3⁺ vs. NKG2D-Fc CD4⁺Foxp3⁺: 2.1 ± 0.4 vs. 3.6 ± 0.4 ; $p < 0.05$; Fig. 4C). But NKG2D-Fc had no effect on T cells (Fc CD8⁺IFN- γ ⁺ vs. NKG2D-Fc CD8⁺IFN- γ ⁺: 0.9 ± 0.4 vs. 1.6 ± 0.8 , $p >$

0.05; Fig. 4D). All of these analyses were performed between 20–25 days after tumor injection, as this was the time point where the largest difference in tumor growth was observed between NKG2D-Fc or Fc treated mice. These results suggest that NKG2D-Fc is more effective in the TME than in peripheral blood. NKG2D-Fc could also reduce tumor-infiltrating MDSCs and Tregs while increasing NK cells, yet does not affect cytotoxic T cells, and post antitumor effects through these immune modulation effects.

Discussion

Traditional chemotherapy and radiotherapy treatments usually have significant cytotoxicity due to non-specific damage to both cancer and normal cells. Besides traditional therapies, recent immune checkpoint blockade (ICB) therapies that target PD-L1-expressing cancer cells have improved the overall survival of cancer patients with various types of cancer. Through the blockade of PD-1/PD-L1 pathway, the suppressive immune system in the TME was restored and subsequently activated tumor-specific T cells, resulting in better overall survival and tumor control (20). An alternative approach to promote antitumor immunity is through modulation of immunosuppressive cells in the TME, including MDSCs and Tregs.

There are several ways to alter the suppressive functions of MDSCs. One such way is to suppress STAT3, a key transcriptional factor vital to the suppressive functioning of MDSCs. AZD9150, a STAT3 oligonucleotide inhibitor, was applied in combination with ICB therapy in a phase I/II trial (21). Another approach is to block MDSC trafficking to the TME, mainly through blocking chemokine receptors such as CXCR2 and CCR5 (22, 23). By targeting the CCR5-CCR5L interaction, tumor growth and invasiveness could be suppressed in pancreatic, colorectal, prostate, and breast cancer (23, 24). Depleting MDSCs by normalization of myelopoiesis or inhibition of the conversion and differentiation of MDSCs into mature myeloid cells has also been shown to negate their suppressive function (25).

Another target to restore the TME is through modulation of Tregs. Tregs usually constitutively express the IL-2 receptor and other inhibitory molecules, including CTLA-4, GITR, PD-1, and LAG3. Neutralizing IL-2 antibodies can compromise Treg functioning and survival but also cause severe autoimmune disease in pre-clinical mouse models. Currently, the anti-CTLA-4 monoclonal antibody ipilimumab has been shown to deplete intratumoral Tregs through the indirect effect of antibody-dependent cell-mediated cytotoxicity (ADCC) (26). On the other hand, anti-PD-1 monoclonal antibodies were shown to downregulate FoxP3 expression and increase the ratio of effector T cells to Tregs (27).

NKG2D-Fc is a simple, easy to produce, single-agent capable of depleting both MDSCs and Tregs. Previous murine studies have shown that the expression of NKG2D ligand by target cells stimulates NK cell cytotoxicity and IFN- γ production (28), which potentially plays a role in our system as well. We believe that the fusion protein is unlikely to alter the function of MDSCs and Tregs, but rather, strengthens antitumor effects through the depletion of these immunosuppressive cells, likely through ADCC. Studies have shown how depleting MDSCs in several different cancer models activates antitumor immunity, such as through increasing granzyme B and IFN- γ production by effector T and NK cells (25). Similarly, Treg depletion also augments antitumor immunity by preventing the TGF- β -mediated suppression

of cytotoxic cells and down-regulation of natural cytotoxicity receptor NKp30 and NKG2D expression, allowing for the recognition of corresponding ligands on target cells (29). Our observations on MDSC and Treg depletion with NKG2D-Fc confirmed published results that the depletion of these immunosuppressive cell populations enhances tumor-immunotherapy approaches. However, there is also the possibility that NKG2D-Fc may alter the suppressive functions of MDSCs and Tregs, which could be the subject of investigation in future work. The dual functionality of NKG2D-Fc makes it an attractive molecule for clinical translation, as broad suppressive cell depletion can be easily achieved. The simple design also makes it an easy option for combination therapy. To achieve better antitumor effects in numerous types of cancers treated with traditional chemotherapy or newer immune checkpoint blockade therapies, depleting suppressive Tregs and MDSCs with NKG2D-Fc could easily be added to existing treatment regimens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

The authors would like to acknowledge Dr. T.-C. Wu for facilitating helpful science discussions and Mr. Christopher Carter Polston for providing administrative assistance. This study was supported by an NIH-supported research grant (R01CA233486).

List of Abbreviations

MHC	Major histocompatibility complex
PD-L1	Program death-ligand 1
TAMs	Tumor-associated macrophages
IL-10	Interleukin-10
TGF-β	Transforming growth factor beta
MDSCs	Myeloid-derived suppressor cells
Tregs	Regulatory T cells
PMN-MDSCs	Granulocytic MDSCs
M-MDSCs	Monocytic MDSCs
ARG1	Arginase 1
NO	Nitric oxide
ROS	Reactive oxidative species
PGE2	Prostaglandin E2
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4

ICOS	Inducible T-cell costimulatory
IFNγ	Interferon gamma
scFv-NKG2D	Single chain variable fragment with the extracellular domain of NKG2D receptor
ICB	Immune checkpoint blockade
ADCC	Antibody-dependent cell-mediated cytotoxicity

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Figure 1.

Anti-tumor effects of NKG2D-Fc. **(A)** Schematic diagram depicting the treatment regimen. On day 1, either 2×10^5 MC38 or LL2 cells were injected into C57BL/6 mice subcutaneously. The mice were treated with either NKG2D-Fc or Fc control protein on days 6, 9, and 13 following tumor injection. Tumor growth and Kaplan-Meier survival curves of C57BL/6 injected with either **(B)** MC38 or **(C)** LL2 treated with 100 µg of NKG2D-Fc or Fc control protein. Three independent experiments were performed. Data are represented as mean $\pm$ SEM, with $n=5$ in each group from one representative experiment.

Figure 2.

MDSCs and Tregs expressed the NKG2D ligand RAE1 γ and bound with the NKG2D-Fc fusion protein. (A) Histogram and (B) MFI represented as mean $\pm$ SEM of PE rat anti-mouse RAE1 γ gating on MDSCs and Tregs from PBMC, spleen, and MC38 tumor. The indicated sample is shown as a grey histogram, and FMO control is in red. (C) Histogram and (D) MFI represented as mean $\pm$ SEM of PE goat anti-mouse multiple adsorption Ig to NKG2D-Fc on MDSCs and Tregs from PBMC, spleen, and MC38 tumor. The indicated sample is shown as a grey histogram, and goat anti-mouse IgG secondary alone control is in red.

Figure 3.

NKG2D-Fc reduced circulating MDSCs and increased circulating NK cells of MC38 tumor-bearing mice. Shown are the summary of the level of (A) circulating MDSCs, (B) circulating NK cells, (C) circulating Treg cells, and (D) circulating cytotoxic T cells. Both representative expression profiles (*left*) and individual values (*right*) are shown. Data are represented as mean $\pm$ SEM, with $n = 5$ in each group.

Figure 4.

NKG2D-Fc reduced tumor-infiltrating MDSCs and Tregs, and increased NK cells of MC38 tumor-bearing mice. Shown are the summary of the level of (A) tumor-infiltrating MDSCs, (B) tumor-infiltrating Treg cells, (C) tumor-infiltrating NK cells, and (D) tumor-infiltrating cytotoxic T cells. Both representative expression profiles (*left*) and individual values (*right*) are shown. Upper panels are the Fc-treated control groups and lower panels are the NKG2D-Fc-treated experimental groups. Data are represented as mean $\pm$ SEM, with $n = 5$ in each group.