Published OnlineFirst July 19, 2017; DOI: 10.1158/2326-6066.CIR-16-0005

Research Article

Cancer Immunology Research

MICA-Expressing Monocytes Enhance Natural Killer Cell Fc Receptor-Mediated Antitumor Functions

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Abstract

Natural killer (NK) cells are large granular lymphocytes that promote the antitumor response via communication with other cell types in the tumor microenvironment. Previously, we have shown that NK cells secrete a profile of immune stimulatory factors (e.g., IFNγ, MIP-1α, and TNFα) in response to dual stimulation with the combination of antibody (Ab)-coated tumor cells and cytokines, such as IL12. We now demonstrate that this response is enhanced in the presence of autologous monocytes. Monocyte enhancement of NK cell activity was dependent on cell-to-cell contact as determined by a Transwell assay. It was hypothesized that NK cell effector functions against Ab-coated tumor cells were enhanced via binding of MICA on monocytes to NK cell NKG2D receptors. Strategies to block MICA–NKG2D interactions resulted in reductions in IFNγ production. Depletion of monocytes *in vivo* resulted in decreased IFNγ production by murine NK cells upon exposure to Ab-coated tumor cells. In mice receiving trastuzumab and IL12 therapy, monocyte depletion resulted in significantly greater tumor growth in comparison to mock-depleted controls ($P < 0.05$). These data suggest that NK cell–monocyte interactions enhance NK cell antitumor activity in the setting of monoclonal Ab therapy for cancer. *Cancer Immunol Res; 5(9); 778–89. ©2017 AACR.*

Introduction

Natural killer (NK) cells are innate immune cells that target and lyse virally infected cells as well as malignant cells. NK cells contain cytolytic granules and express an array of cellular adhesion molecules and cytokine receptors (1–4). They produce a variety of immunostimulatory cytokines, including IFNγ, TNFα, MIP-1α, RANTES, and GM-CSF (5, 6). Constitutive expression of multiple cytokine receptors facilitates their response to inflammatory events (7, 8). NK cell effector functions are regulated by a balance between activating and inhibitory receptors, as well as the expression of target cell MHC class I antigens (8). NK cells express an activating receptor for the Fc portion of immunoglobulin G (FcγRIIIa) that enables recognition of antibody (Ab)-coated targets. Support for the concept that antitumor activity of therapeutic monoclonal antibodies (mAb) is in part dependent on FcR-bearing cells stems from experiments conducted in FcR-deficient mice (9) and the observation that polymorphisms in the human FcR gene influence the clinical efficacy of therapeutic mAbs (10, 11). Studies from our group have shown that cytokine stimulation of NK cells enhances the immune response to Ab-coated tumor cells *in vitro*, in murine models, and in phase I clinical trials (12–15). Costimulation via the IL12 receptor (IL12R) and FcR has been shown to activate multiple cellular signaling pathways (e.g., JAK/STAT and PI3-kinase/Akt) and lead to maximal stimulation of NK cell effector functions (15).

NK cells express an activating, lectin-like receptor known as NKG2D (16). Human NKG2D binds to ligands MICA, MICB, and ULBPs that are expressed by a variety of tumor cell types (17, 18). Engagement of NKG2D on NK cells results in activation of the adapter protein DAP10, recruitment of the DAP10 binding partner Grb2 and signaling through Vav1, and also activation of PI3 kinase and the downstream serine threonine kinase Akt (19, 20). NK-cell interactions with NKG2D ligand-expressing tumor cells lead to NK cell–mediated cytotoxicity (21).

The role of reciprocal activation between monocytes and NK cells in immune responses is gaining increased recognition (22–25). NK cells function as one of the early sources of immunoregulatory cytokines (1). Upon activation, NK cells produce

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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doi: 10.1158/2326-6066.CIR-16-0005

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IFNγ, the prototypic macrophage activating factor, which optimizes monocyte/macrophage function (26, 27). Activated monocytes then produce cytokines like IL12 and IL15 that stimulate NK cells (28, 29). This positive feedback system supports the immune response against invading organisms and enables the rapid and effective clearance of infectious agents by monocytes/macrophages. We hypothesized that NK cell effector functions against Ab-coated tumor cells would be enhanced upon interactions with monocytes. We now report the ability of MICA-expressing monocytes to engage NKG2D on NK cells and promote NK-cell activity against Ab-coated tumor targets.

Materials and Methods

Cytokines and antibodies

Recombinant human IL12 (rhuIL12) was provided by Genetics Institute Inc. Recombinant human IL15 and IL18 were purchased from Peprotech, Inc. The anti-HER2 mAb trastuzumab was provided by Genentech Inc. Antibodies utilized for flow cytometry include APC anti-hNKG2D (BD Biosciences, 558071), PE anti-hIFNγ (BD Biosciences, 559326), PE anti-hMICA (R&D Systems, FAB1300P), and PE anti-hMICA/B (BioLegend, 320906). The anti-MICA human F(ab′)2 Ab and human IgG F(ab′)2 control utilized for neutralization studies were generated as described (30). sMICA used in monocyte coculture experiments was generated as described (31).

Cell lines

The SK-BR-3 and MDA-MB-468 human breast adenocarcinoma cell lines were obtained from American Type Culture Collection. The C1R and C1R-MICA cell lines were provided by Dr. Veronika Groh. The identity of these cell lines was not authenticated. Cells were maintained in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and C1R-MICA cultures were supplemented with G418 sulfate (Life Technologies Inc.). All cell lines used in this study were routinely tested for *mycoplasma* infection using the MycoAlert Mycoplasma Detection Kit (Lonza) and Plasmocin prophylactic (InvivoGen) was added to all growth media at a concentration of 2.5 μg/mL. Cell lines were maintained in culture for no more than 10 passages.

Isolation of human NK cells and monocytes

PBMC and NK cells were isolated from donor leukopacks (American Red Cross, Columbus, OH) as described (15). $CD14^+$ monocytes were isolated using anti-CD14 magnetic beads (Miltenyi Biotec, Auburn, CA). Immune cells were cultured in 10% HAB medium.

In vitro coculture with tumor cells

SK-BR-3 and MDA-MB-468 cells were cultured in 96-well plates. Cells were treated the following day with 100 μg/mL trastuzumab for 1 hour. Immune cells were added to wells (2×10^5 NK cells/well) in 10% HAB medium supplemented with IL12 (10 ng/mL). Cocultures were plated at a 2:1 NK cell to monocyte/PBMC ratio. Supernatants were harvested and analyzed for cytokines by ELISA (R&D Systems) as described (12).

Intracellular staining for IFNγ

NK-cell IFNγ production was analyzed using an IFNγ PE-conjugated mAb and a CD56 APC-conjugated mAb as described (13).

Real-time PCR

Cellular RNA was isolated using RNeasy Mini-kits (Qiagen). cDNA was generated with random hexamer primers and MMLV-RT according to manufacturer's recommendations (Life Technologies Inc.). Real-time PCR for huIFNγ transcript was performed using an ABI prism 7700 sequence detector (Applied Biosystems) with an 18S rRNA internal control (PE Applied Biosystems).

Transwell cocultures

SK-BR-3 cells were cultured in 24-well plates and treated the next day with 100 μg/mL trastuzumab for 1 hour. A Transwell insert with a 0.4-μm filter was placed in each well, and NK cells and/or monocytes were added in media ± IL12 (10 ng/mL). Supernatants were analyzed for IFNγ content by ELISA.

MICA siRNA and monocyte transfection

Monocytes were transfected using the Amaxa Nucleofector apparatus (Amaxa Biosystems). Cells were resuspended in Nucleofector Solution T and nucleofected with scrambled or human MICA siRNA from Ambion. Cells were transferred to RPMI media supplemented with 10% FBS and M-CSF (20 ng/mL). Cy3-labeled GAPDH siRNA was used to analyze transfection efficiency.

Murine studies

Mice received two intraperitoneal (i.p.) injections of F4/80 Ab (250 μg), isotype control Ab (250 μg), or PBS. CT-$26^{HER2/neu}$ cells were incubated in PBS plus 10% FBS with 4D5 (100 μg/mL) (6). A total of 4×10^6 cells in PBS containing 1 μg muIL12 were injected i.p. Serum was harvested at 24 hours and analyzed by ELISA. Splenocytes were collected to assess monocyte depletion by flow cytometry. For *ex vivo* studies, wild-type BALB/c splenocytes were cocultured with CT-$26^{HER2/neu}$ tumor cells. Culture supernatants were analyzed for muIFNγ by ELISA. NKG2D knockout mice were provided by Dr. David Raulet.

For the murine tumor study, mice received i.p. injections of control or clodronate-containing liposomes (1 mg/kg in 100 μL PBS) on days 0 and 4 with respect to tumor inoculation and every fourth day thereafter (32). On day 0, mice were inoculated with 8×10^5 EMT$6^{HER2/neu}$ cells in the mammary fat pad (33). On day 7 and every third day, mice received i.p. injections of trastuzumab and IL12 (10 mg/kg and 2.5 ng, respectively). Tumor volume = $0.5 \times [(\text{large diameter}) \times (\text{small diameter})^2]$. Upon completion of the study, mice were sacrificed and tumors were collected. Cells were labeled with F4/80 PE-conjugated Ab and CD11b APC-conjugated Ab to evaluate monocyte depletion.

Statistical analysis

Statistical analyses of cytokine levels were performed using Student *t* tests. Changes in tumor volume over time were assessed via a longitudinal model. Tumor data was log transformed, and a linear mixed effects model was applied to account for correlations of observations from the same mouse.

Results

NK cells secrete immune stimulatory cytokines in response to IL12 and tumor cells

To investigate the ability of monocytes to enhance NK-cell interactions with Ab-coated tumor cells, we first evaluated NK-cell cytokine production and lytic activity *in vitro* in response to a stimulatory strategy: a therapeutic mAb coupled with the cytokine originally referred to as NK-cell stimulatory factor, IL12. The

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HER2 overexpressing breast cancer cell line SK-BR-3 or the HER2 negative MDA-MB-468 cell line were cocultured with NK cells in the presence or absence of trastuzumab and IL12. NK-cell production of IFNγ in response to trastuzumab-coated SK-BR-3 cells was enhanced in the presence of IL12, as compared to control conditions; however, this was not observed with the HER2-negative cell line (Fig. 1A). The capacity of total peripheral blood mononuclear cells (PBMC) to respond to IL12 and Ab-coated tumor cells was also tested. PBMC (plated at the same cell density as pure NK cells) secreted more IFNγ compared to NK cells alone in response to dual stimulation via Ab-coated tumor cells and IL12. This relationship held true for other NK cell-derived cytokines including TNFα and MIP-1α (Fig. 1B). On average, PBMC IFNγ production was 200% higher than that of pure NK cells. The number of NK cells within the PBMC population added to each well was approximately 4-fold less than the number of pure NK cells plated (2×10^5 cells per well). Next, PBMC IFNγ production in response to various stimuli (e.g., IL1β, IL12, IL15, IL18, and/or trastuzumab-coated cells) was compared to that of NK cells. PBMC IFNγ production was greater than that of purified NK cells for all conditions tested (Fig. 1C). The most potent individual cytokine stimulus for NK-cell IFNγ production in response to Ab-coated tumor cells was IL12. Subsequent investigation sought to uncover which cellular compartment within total PBMC could be responsible for providing a stimulatory signal to increase NK cell antitumor activity in the presence of what was considered to be a strong stimulatory strategy, namely, FcR and IL12R coactivation.

Monocytes within a PBMC population enhance the NK-cell cytokine response

The impact of different immune cell populations on the NK-cell IFNγ response was investigated. Depletion of monocytes from

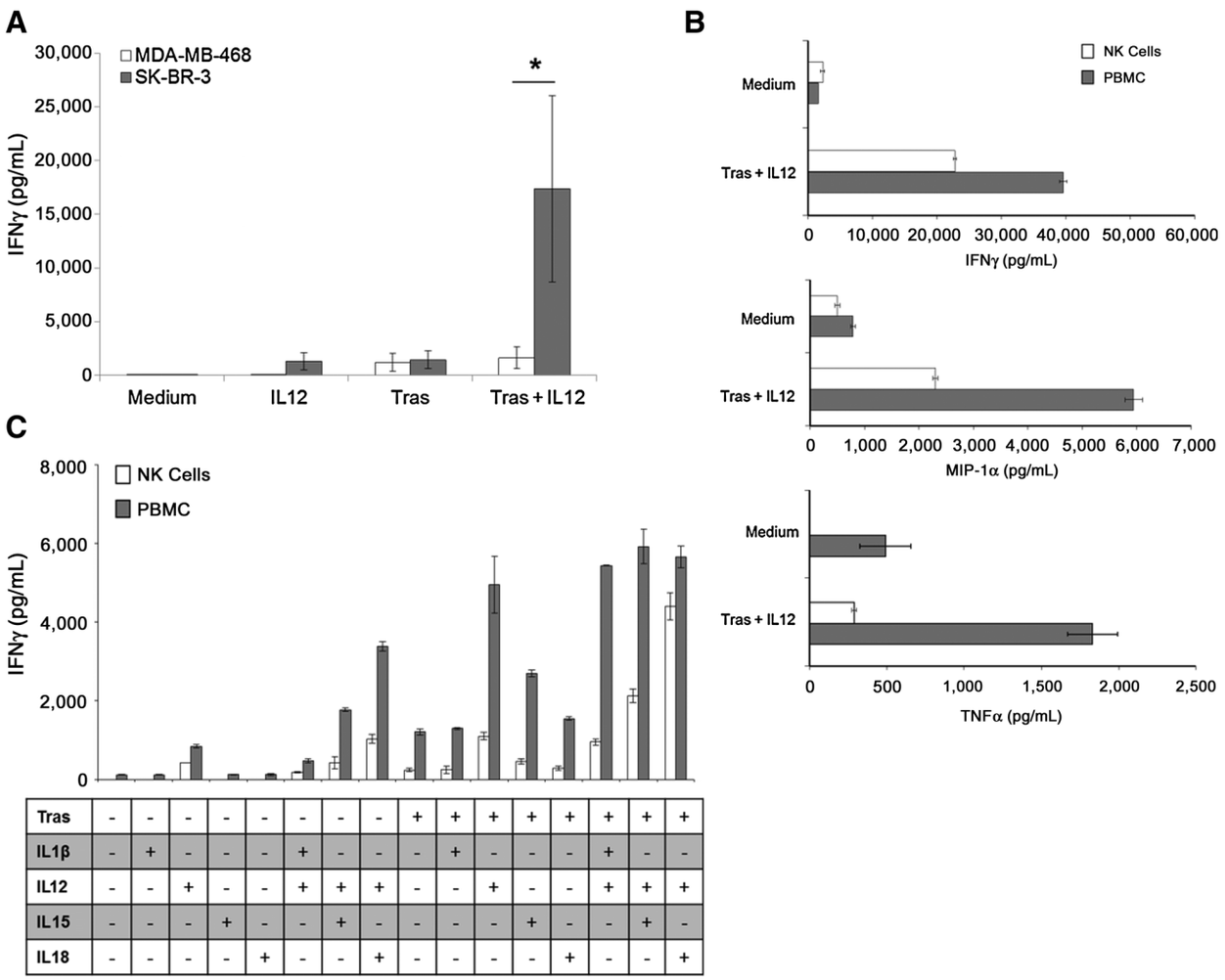

Figure 1.

Cytokine production is enhanced in the presence of PBMC. **A,** NK cells were cocultured with SK-BR-3 or MDA-MB-468 tumor cells in the presence of medium, rhuIL12 (IL12), trastuzumab (Tras), or the combination. **B,** NK cells or PBMC were cultured in medium alone or with trastuzumab-coated SK-BR-3 cells and IL12. **C,** NK cells or PBMCs were cultured in the presence of rhuIL1β, IL12, IL15, or IL18 and/or trastuzumab-coated SK-BR-3 cells. Bars represent the mean concentration ± SD of cytokine (IFNγ for **A**, **B**, **C**, MIP-1α for **B**, or TNFα for **B**) content from culture supernatants analyzed by ELISA. Data are representative of three independent experiments with similar results (**A**) or two experiments (**B** and **C**). *, $P < 0.05$.

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PBMCs, but not B cells or T cells, resulted in decreased IFNγ production following exposure to IL12 and trastuzumab-coated tumor cells (Fig. 2A, Supplementary Fig. S1). The ability of monocyte-depleted PBMC to induce IFNγ production in response to IL12 and Ab-coated tumor cells was restored in a dose-dependent fashion by the reintroduction of autologous monocytes (Fig. 2B). To confirm the effect of monocytes on NK-cell cytokine production, NK cells were cocultured with purified autologous monocytes. NK cells cocultured with monocytes in the presence of Ab-coated tumor cells and IL12 produced more IFNγ than NK cells alone (Fig. 2C). Monocytes produced no measureable amounts of IFNγ. Similar results were obtained with IL15 as the cytokine stimulus (Fig. 2D). Coculture with suppressive monocyte-derived cells such as tumor-associated

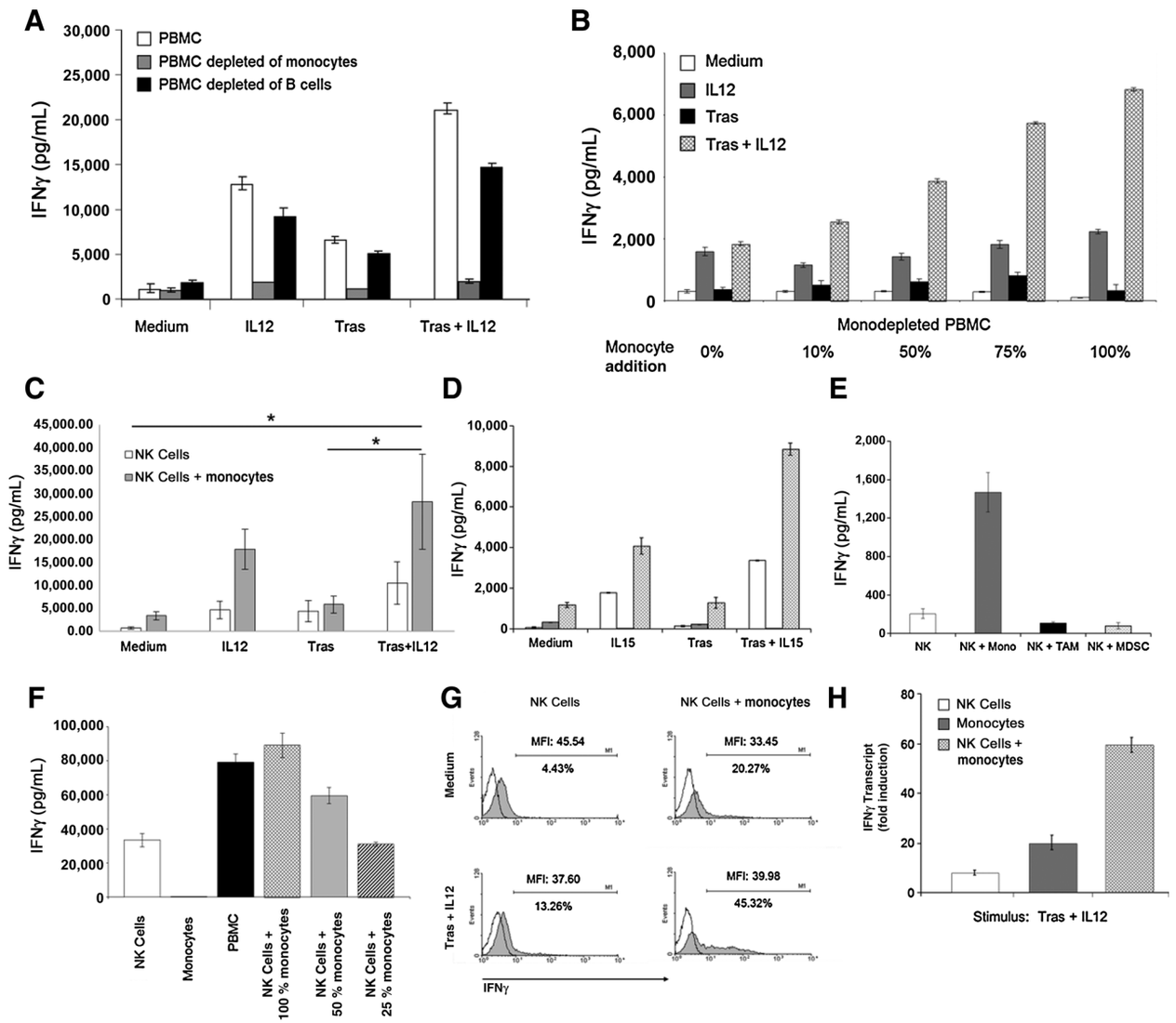

Figure 2.

Monocytes promote NK-cell IFNγ production. **A,** PBMC (~14% $CD14^+$ cells), PBMC depleted of monocytes (<0.03% $CD14^+$ cells), or PBMC depleted of B cells (>98% $CD19^-$ cells) were cocultured with SK-BR-3 cells in medium, IL12, trastuzumab, or the combination. **B,** Monocyte-depleted PBMCs were mixed with increasing numbers of autologous monocytes and cultured with SK-BR-3 cells in the presence of medium, IL12, trastuzumab, or the combination. **C,** NK cells, or NK cells plus monocytes were cultured with SK-BR-3 cells in the presence of medium, IL12, trastuzumab, or the combination. **D,** NK cells were mixed with monocytes and cocultured with SK-BR-3 cells in the presence of medium, IL15, trastuzumab, or the combination. **E,** NK cells were cultured in the presence of trastuzumab-coated SK-BR-3 cells and IL12, and monocytes and/or *in vitro*–generated autologous TAM or MDSC at a 2:1 ratio were added to the coculture (47, 48). **F,** NK cells were cultured with varying numbers of autologous monocytes in the presence of trastuzumab-coated SK-BR-3 cells and IL12. **G,** NK cells alone or in the presence of monocytes were cocultured with trastuzumab-coated SK-BR-3 cells and IL12. Cells were harvested and intracellular IFNγ was examined by flow cytometry. **H,** NK cells, monocytes, or both were cultured in the presence of trastuzumab-coated SK-BR-3 cells and IL12. Bars represent the mean concentration ± SD of IFNγ content from culture supernatants analyzed by ELISA (**A–F**) or IFNγ transcript as measured by PCR (**H**). Data shown are representative of two (**A, B**, **D**, **F, G**) or three (**E**) independent experiments with similar results, or the mean concentration ± SD of $n = 8$ donors (**c**). *, $P < 0.05$.

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macrophages (TAM) or myeloid-derived suppressor cells (MDSC) generated *in vitro* from autologous monocytes led to a reduction in NK-cell IFNγ production in this system (Fig. 2E). Thus, it appeared that monocytes, although not the direct source of IFNγ, were important for enhancement of NK-cell IFNγ production. The effect of monocyte addition was dose dependent, as the introduction of increasing numbers of monocytes led to NK-cell IFNγ production that was equivalent to the IFNγ output of unmanipulated PBMC (Fig. 2F). The effect of monocytes on NK-cell IFNγ production was confirmed by intracellular flow cytometry (Fig. 2G). Also, IFNγ transcript levels were evaluated in dual-stimulated NK cells in the presence and absence of monocytes. Addition of monocytes to coculture enhanced NK-cell IFNγ transcript levels (Fig. 2H). These results suggest that monocytes within the PBMC population are capable of enhancing NK-cell cytokine production in response to Ab-coated tumor cells and IL12.

Monocytes enhance the lytic activity of NK cells

The effect of monocytes on the ability of IL12-activated NK cells to lyse Ab-coated tumor cells was examined (Fig. 3). At the 50:1 effector to target ratio, IL12-activated NK cells mediated approximately 48% lysis of trastuzumab-coated SK-BR-3 target cells (Fig. 3, top). Monocytes alone exhibited negligible cytotoxic activity (Fig. 3, middle). It has been demonstrated that monocytes are capable of ADCC 8 to 14 hours after priming with IFNγ; however, this was not observed in our standard 4-hour chromium release assay conditions. Exposure of NK cells to monocytes (Fig. 3, bottom) prior to the addition of Ab-coated target cells and IL12 facilitated an increase in NK cell-mediated cytotoxicity to nearly 98% at the 50:1 effector to target ratio ($P < 0.05$).

NK cell activation is enhanced by direct contact between NK cells and monocytes

Increased NK-cell IFNγ production upon interaction with monocytes could be due to direct cell-to-cell contact or the diffusion of soluble mediators. To determine whether cell-to-cell contact was required for stimulation, a Transwell coculture experiment was performed. When NK cells and monocytes were separated by a cell-impermeable membrane, NK cells exposed to trastuzumab-coated tumor cells and IL12 produced normal amounts of IFNγ. However, coculture of NK cells and monocytes in the same compartment (permitting contact) led to significantly greater IFNγ production (Fig. 4A, $P = 0.03$). Figure 4B demonstrates the same experiment with a full set of controls, which was challenging to perform due to the requirement for a large number of NK cells from a single donor. The effect of monocytes on NK-cell cytokine production in Fig. 4B, although substantial and reproducible, was less than in previous experiments possibly due to the physical characteristics of larger Transwell cultures. In summary, monocyte enhancement of NK-cell IFNγ production in response to Ab-coated tumor cells and IL12 requires cell-to-cell contact, and this effect is lost when monocytes are separated from NK cells by a cell-impermeable membrane.

Ligand interactions essential for monocyte enhancement of NK-cell IFNγ production

Bauer and colleagues reported that interactions between NK cell NKG2D and its ligands MICA/B or ULBPs, expressed by tumor cells, enhances NK cell cytokine production (18). However, NKG2D ligands may also be expressed by cells of the

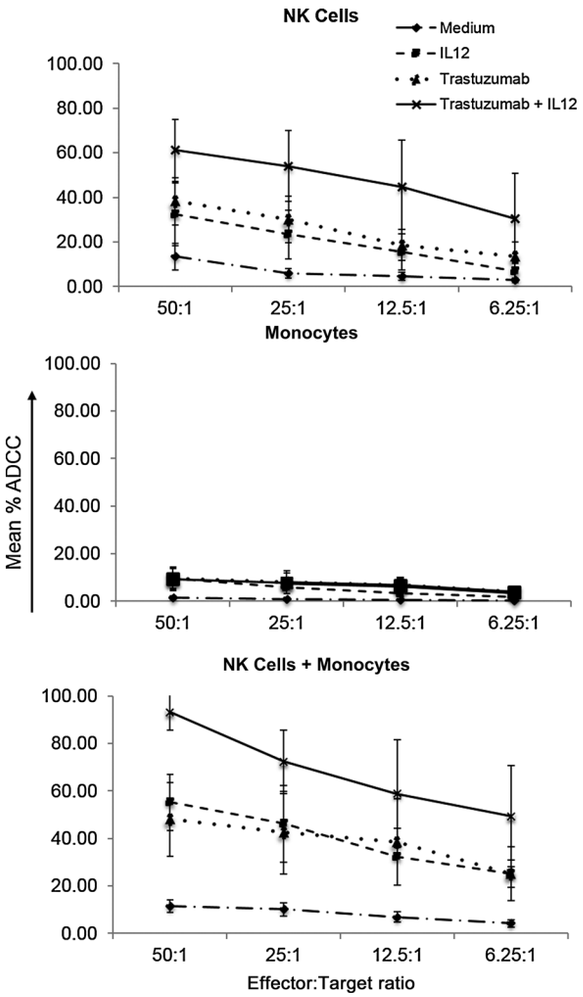

Figure 3.

Monocytes enhance NK cell ADCC. NK cells, monocytes, or both were incubated overnight in medium alone or medium containing IL12. NK cell ADCC was assessed in a standard 4-hour chromium release assay with trastuzumab-coated SK-BR-3 target cells. Symbols represent target cell mean percent lysis ± SD for three independent experiments.

innate immune system (34, 35). Thus, we hypothesized that monocytes could activate NK cells via expression of NKG2D ligands. Monocyte MICA surface expression was measured by flow cytometry. Though variability exists in NKG2D ligand expression among healthy donors, substantial MICA expression was detected in freshly isolated monocytes (Supplementary Fig. S2). Further, it was shown that MICA expression could be upregulated following stimulation of monocytes with IFNα or LPS (Fig. 5A). LPS treatment of monocytes enhanced their ability to augment NK-cell IFNγ production (Fig. 5B). To further explore the interaction between NKG2D and MICA, the MICA overexpressing cell line C1R-MICA was used and was confirmed to selectively overexpress MICA but not other NKG2D ligands (Supplementary Fig. S3). Coculture of NK cells with C1R-MICA cells in the presence of trastuzumab-coated SK-BR-3 cells and IL12 led to increased NK-cell IFNγ production that was comparable to that observed with the addition of

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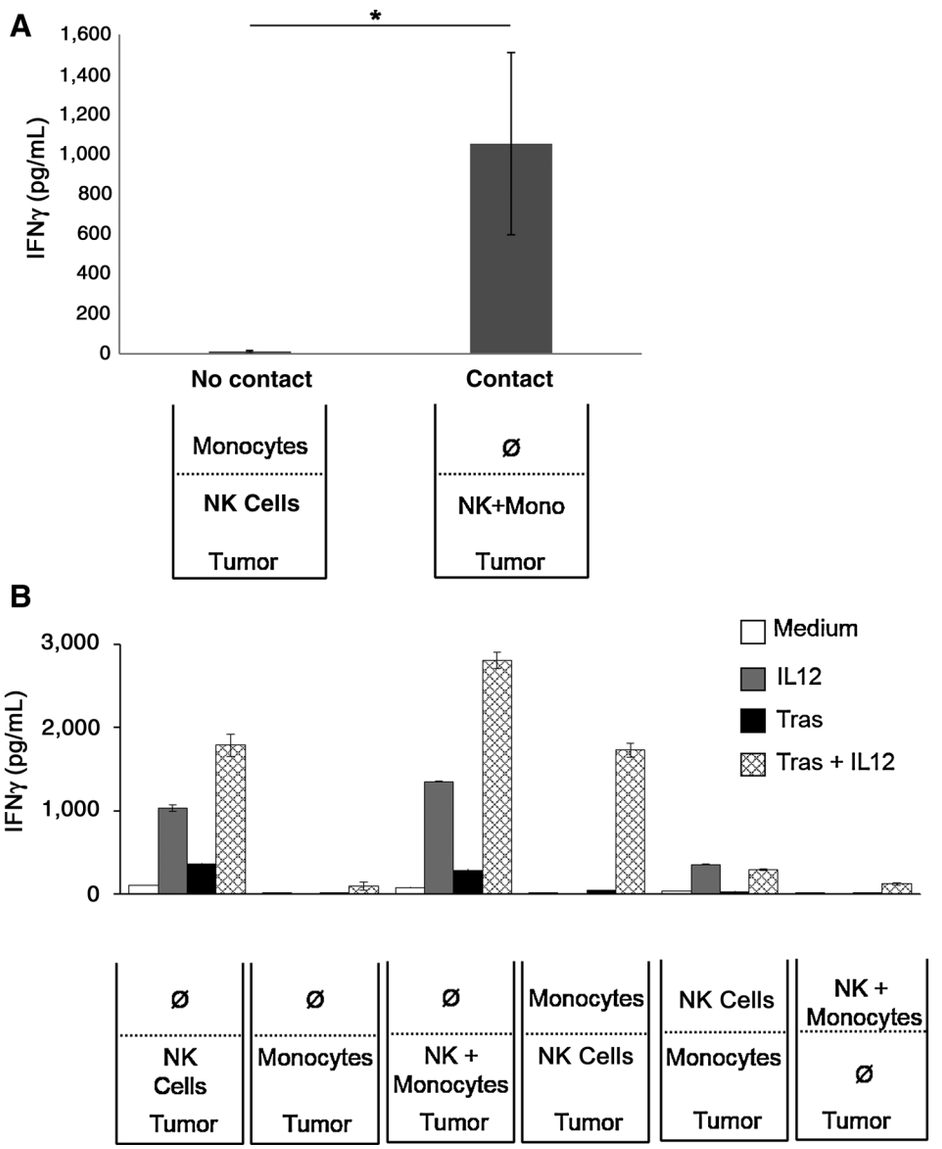

Figure 4.
Monocytes provide contact-dependent NK cell–activating signals. **A,** NK cells and monocytes from three separate leukopacks were cultured in Transwell plates. **B,** NK cells and monocytes from one representative donor were cultured in various combinations in a Transwell plate. Bars represent the mean concentration ± SD of supernatant IFNγ. *, $P < 0.05$.

monocytes. Coculture of NK cells with the parental cell line C1R (which lacks MICA expression) did not modify NK-cell IFNγ production (Fig. 5C). Introduction of an anti-MICA-neutralizing Ab abrogated the ability of C1R-MICA cells to enhance NK-cell IFNγ production (Fig. 5D).

Experiments involving an anti–MICA-neutralizing Ab were difficult to optimize with donor monocytes and NK cells due to FcR expression. An $F(ab')_2$ against MICA was designed and utilized in an attempt to block NKG2D–MICA interactions; however, unintended NK cell activation persisted. An anti-MICA siRNA was employed to study the interaction between NK cell NKG2D and monocyte MICA. MICA expression levels were downregulated in monocytes using this siRNA approach. Reduced MICA expression at the transcript level and on the surface of monocytes was confirmed by real-time PCR and flow cytometry (Fig. 5E and F). Down regulation of MICA impaired the ability of monocytes to augment NK-cell cytokine production and led to reduction in IFNγ levels in response to Ab-coated tumor cells and IL12 (Fig. 5G). The interaction between NK-cell NKG2D and monocyte MICA was also disrupted by pretreating NK cells with soluble MICA (sMICA) to block NKG2D receptor availability prior to coculture with monocytes (31). This strategy resulted in a dose-dependent decrease in IFNγ production, supporting the hypothesis that direct NKG2D–MICA interactions between NK cells and monocytes promote NK-cell activity (Fig. 5H). No correlation was found between MICA expression levels on donor monocytes and IFNγ production from NK cells, indicating that although MICA expression on monocytes is necessary for enhanced NK cell activation, the level to which MICA is expressed is not as important (Supplementary Fig. S4).

Modulation of NKG2D expression on NK cells

It was confirmed that NK cells express NKG2D, and expression may be upregulated upon NK-cell stimulation via IL12 or IL15 (Fig. 6A; ref. 36). Pretreatment of NK cells with IL15 (followed by

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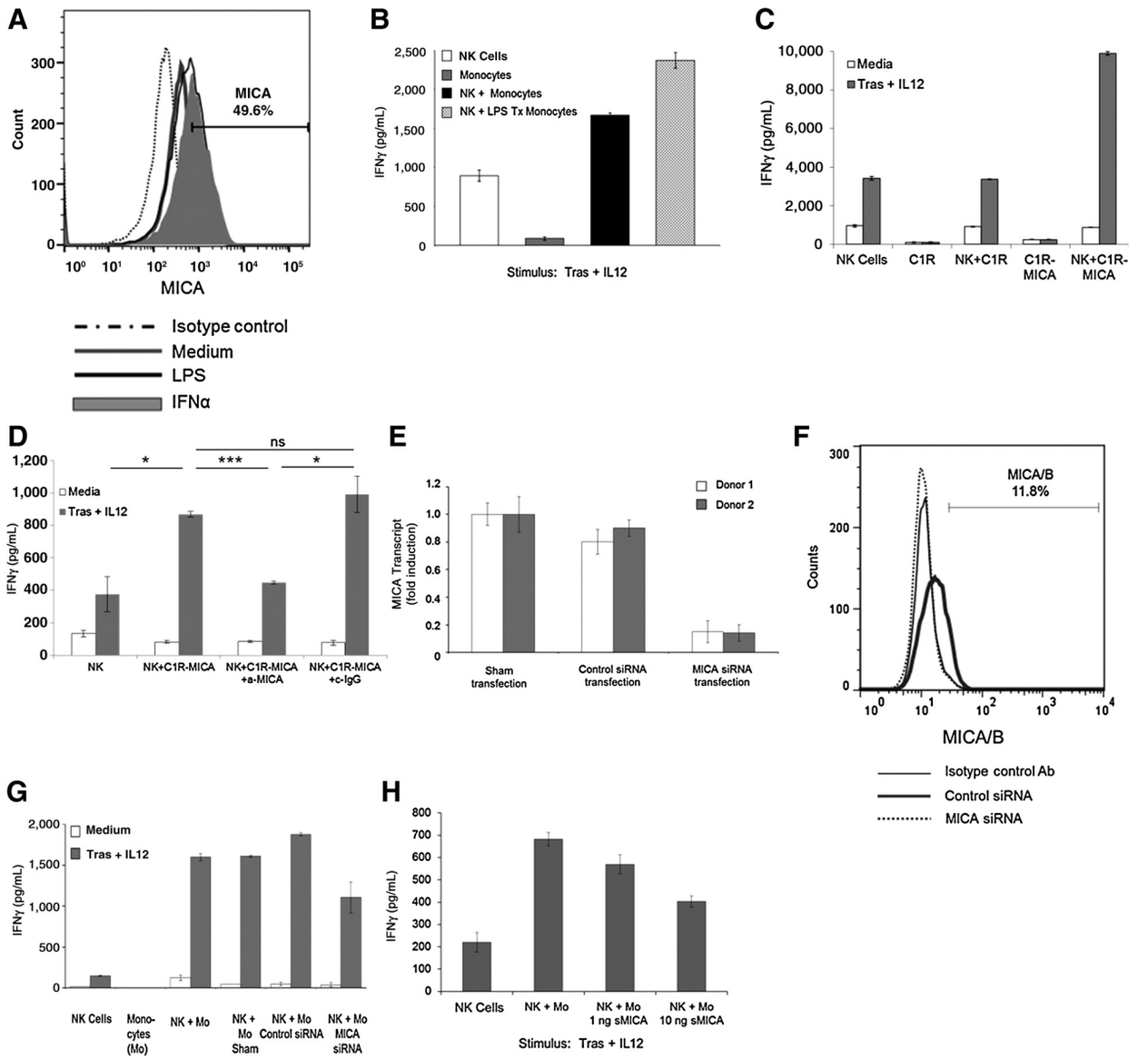

Figure 5.

Monocyte expression of the NKG2D ligand MICA promotes NK cell antitumor activity. **A,** Monocyte MICA expression at baseline (medium) and following treatment with LPS or IFNα was evaluated by flow cytometry. **B,** Untreated and LPS-treated monocytes were cocultured with NK cells, trastuzumab-coated SK-BR-3 cells, and IL12. **C,** NK cells were cocultured with C1R or C1R-MICA cells in the presence of trastuzumab-coated SK-BR-3 cells and IL12. **D,** C1R-MICA cells were incubated with control IgG or anti-MICA Ab. C1R-MICA cells then were cocultured with NK cells, trastuzumab-coated SK-BR-3 cells, and IL12. **E,** Monocytes were transfected with MICA siRNA or scrambled control siRNA. Transfected cells were analyzed for MICA transcript by real-time PCR or (**F**) MICA/B surface expression was examined by flow cytometry. **G,** Monocytes with downregulated levels of MICA were cocultured with NK cells in the presence of trastuzumab-coated SK-BR-3 cells and IL12. **H,** NK cells were pretreated with soluble MICA (sMICA) prior to coculture with monocytes in the presence of trastuzumab-coated SK-BR-3 cells and IL12. Bars represent the mean concentration ± SD of IFNγ content from supernatants analyzed by ELISA (**B, C, D, G, H**). Data shown for each panel are representative of two (**B, C, G**), three (**A**, **H**) independent experiments with similar results or the mean concentration ± SD of $n = 3$ donors (**D**). *, $P < 0.05$; ***, $P < 0.001$; ns, not significant.

an overnight rest) increased NKG2D expression and enhanced NK-cell IFNγ production in the presence of monocytes (Fig. 6B). Additionally, NK cell NKG2D expression was not decreased following interactions with MICA expressing cells *in vitro* (Supplementary Fig. S5). Further, an anti–NKG2D-neutralizing Ab was used to block activation of this receptor (37). NKG2D blockade reduced NK-cell IFNγ production in response to Ab-coated tumor cells and IL12 in the presence of C1R-MICA cells (Fig. 6C) as well as monocytes (Fig. 6D).

Murine NK-cell IFNγ production is enhanced in the presence of monocytes

Next, experiments were conducted using murine models. Though mice do not express MIC-derived molecules, they do

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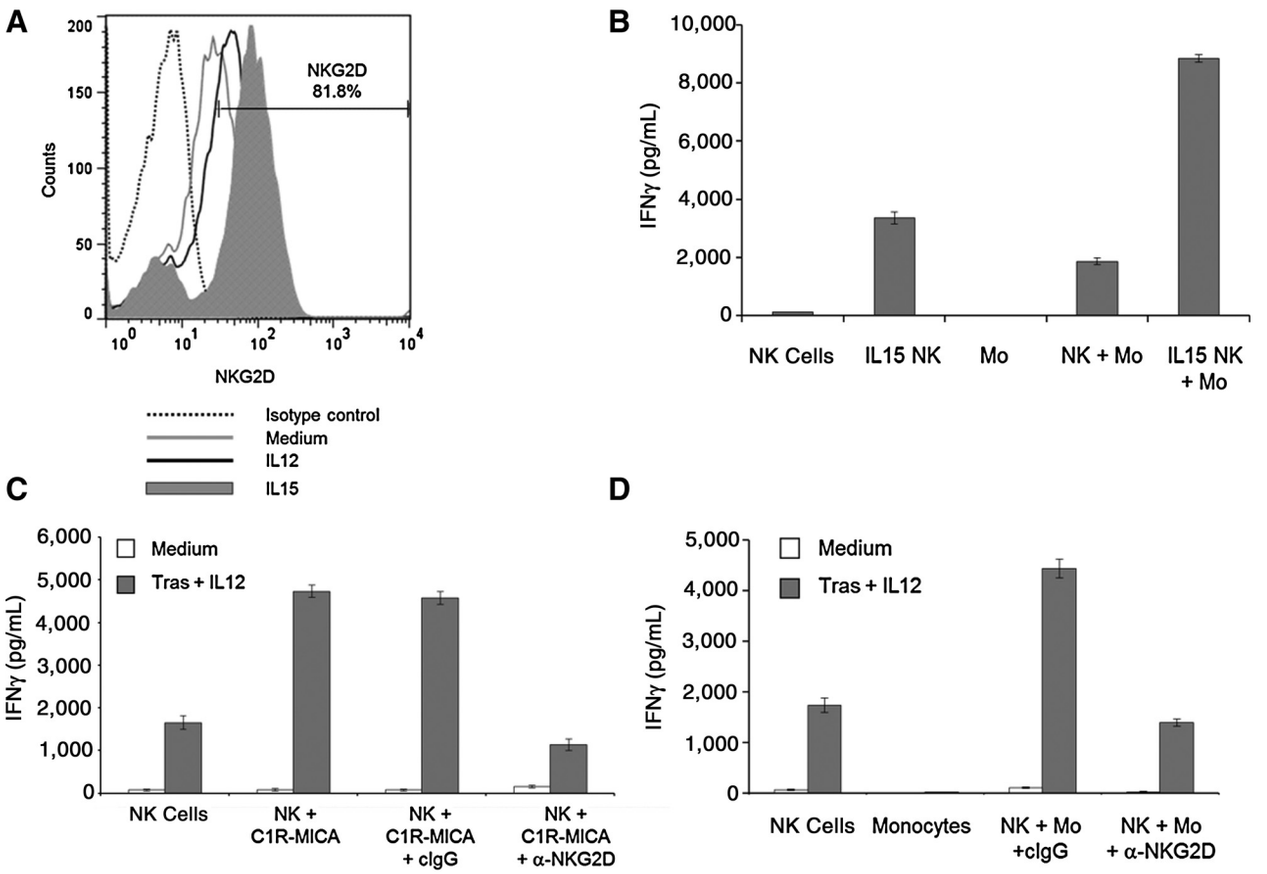

Figure 6.

NK cells interact with MICA on monocytes via the NKG2D receptor. **A,** NK cell NKG2D expression was evaluated at baseline (medium) and following stimulation via IL12 or IL15. **B,** NK cells were treated with IL15, rested overnight, then added to coculture with monocytes, trastuzumab-coated SK-BR-3 cells, and IL12. **C,** NK cells were incubated with a control IgG or anti-NKG2D Ab. NK cells then were cultured in the presence trastuzumab-coated SK-BR-3 cells, IL12, and C1R-MICA cells, or (**D**) monocytes. Bars represent the mean concentration ± SD of IFNγ content from supernatants analyzed by ELISA. Data shown for each panel are representative of two independent experiments with similar results.

express similar MHC class I-like molecules, including RAE-1, H60, and MULT-1, that engage murine NKG2D and promote NK-cell activation (38). Guerra and colleagues showed in murine models that tumor immunosurveillance is inhibited in NKG2D-deficient mice (39). Given that *in vitro* experiments in our laboratory demonstrated a relationship between NK cells and monocytes, we sought to evaluate whether interruption of this interaction would impact the NK-cell response to mAb therapy in murine models as well.

Splenocytes from wild-type mice were cultured in the presence of IL12 and 4D5 (a murine anti-HER2 mAb)-coated HER2-positive murine tumor cells. Splenocytes depleted of NK cells produced less IFNγ, identifying NK cells as the source of IFNγ, as described by our group previously (Fig. 7A, ref. 6). Clodronate-containing liposomes were used as a means of depleting mice of monocytes *in vivo*. Splenocytes isolated from mice treated with control liposomes retained normal monocyte numbers and produced IFNγ *ex vivo* in response to IL12- and 4D5-coated tumor cells. In contrast, treatment with clodronate-containing liposomes led to a 60% reduction in monocyte numbers and a reduction in NK-cell IFNγ production (Fig. 7B). An F4/80 antibody was used as an additional strategy to deplete monocytes. Control mice treated with an isotype Ab retained normal monocyte numbers, and the amount of IFNγ in the serum increased in response to i.p. administration of IL12 and 4D5-coated tumor cells. However, treatment with an F4/80 Ab led to a 61% reduction in monocytes in the spleen and resulted in a 2-fold reduction in serum IFNγ levels in response to dual stimulation (Fig. 7C). Next, mice rendered genetically deficient in NKG2D were used. Splenocytes were harvested from wild-type and NKG2D knockout mice and cultured in the presence of murine IL12- and 4D5-coated $CT26^{HER2/neu}$ tumor cells. IFNγ production was inhibited in mice lacking the NKG2D receptor (Fig. 7D). Lastly, a study was conducted in which mice bearing HER2-positive tumors were treated with trastuzumab and IL12 along with systemic administration of clodronate-containing liposomes or control liposomes. Treatment of mice with clodronate-containing liposomes led to significantly larger tumors as compared to mice receiving control liposomes ($P < 0.05$; Fig. 7E).

Published OnlineFirst July 19, 2017; DOI: 10.1158/2326-6066.CIR-16-0005

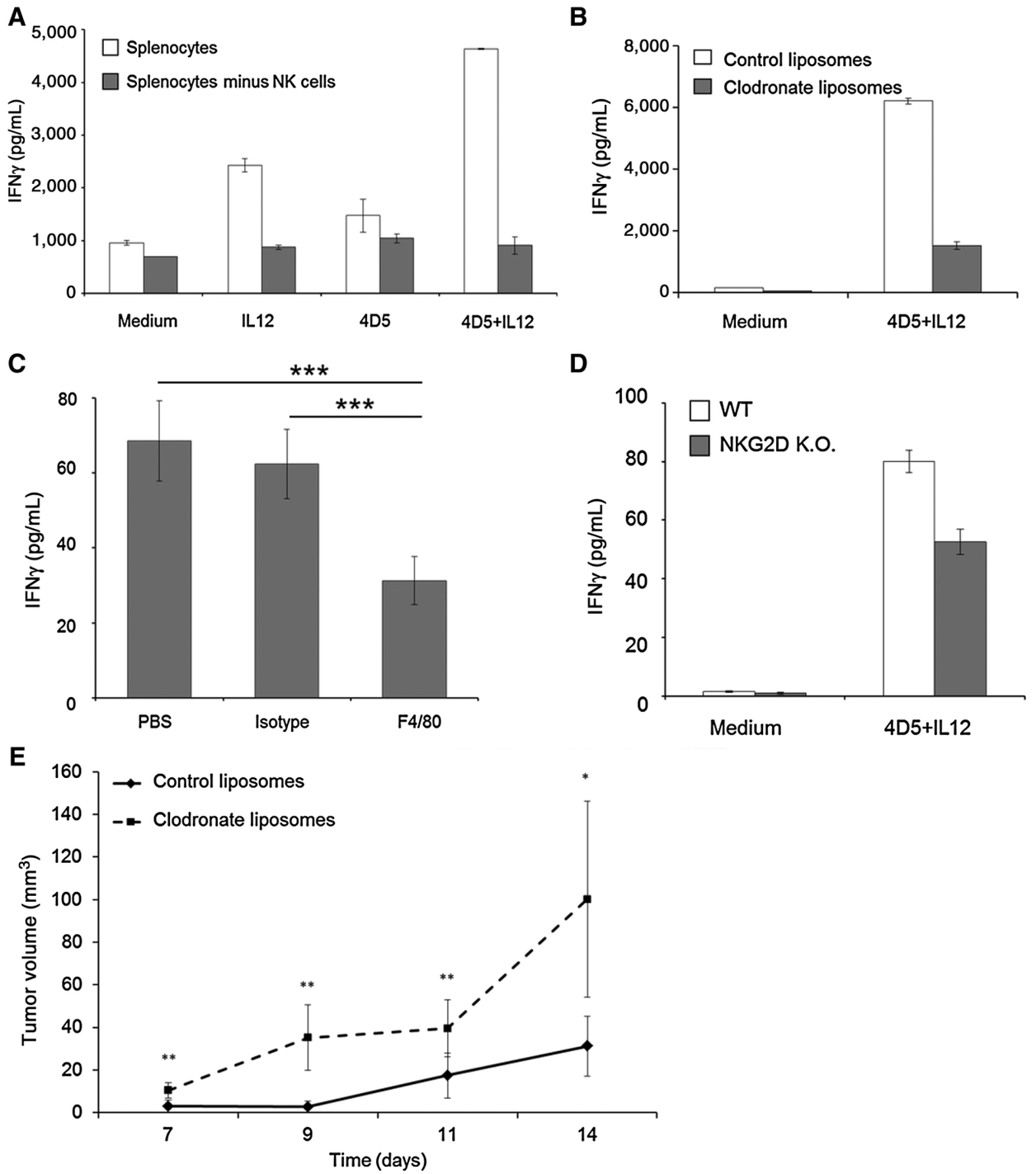

Figure 7.

Monocytes enhance NK cell function *in vivo*. **A,** Murine splenocytes were depleted of NK cells and cocultured with CT26$^{Her2/neu}$ tumor cells in the presence of medium, IL12, 4D5, or the combination. Bars represent the mean concentration ± SD of IFNγ content from supernatants analyzed by ELISA, $n = 2$ mice. **B,** Splenocytes from wild-type BALB/c mice treated with clodronate-containing or control liposomes were cocultured with 4D5-coated CT26$^{Her2/neu}$ tumor cells and IL12. Bars represent the mean concentration ± SD of IFNγ content, $n = 2$ mice. **C,** BALB/c mice were treated with PBS, an isotype control Ab, or an F4/80 Ab and were injected i.p. with 4D5-coated CT26$^{Her2/neu}$ tumor cells and IL12. Serum was collected 24 hours later and analyzed for IFNγ content by ELISA. The means ± SD are shown ($n = 5$ for each condition). **D,** Splenocytes were isolated from wild-type (WT) and NKG2D-deficient mice (NKG2D K.O.) and cocultured with 4D5-coated CT26$^{HER2/neu}$ tumor cells and IL12. Bars represent the mean concentration ± SD of IFNγ content, $n = 3$. **E,** Trastuzumab and IL12 were administered to EMT6$^{HER2/neu}$ tumor–bearing mice that were treated with clodronate-containing or control liposomes. Average tumor volume ± SEM is shown ($n = 5$ mice for each group). *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.001$.

Published OnlineFirst July 19, 2017; DOI: 10.1158/2326-6066.CIR-16-0005

Discussion

Here, we investigated the role of NK cell–monocyte interactions in the setting of mAb therapy for cancer. We have demonstrated previously that NK cell-derived IFNγ serves as a key mediator of the immune response to cancer. NK cells release IFNγ following costimulation via Ab-coated tumor cells and IL12, a therapeutic strategy proven to elicit NK cell activity *in vitro* and *in vivo*. Now, it has been shown that monocytes enhance NK-cell IFNγ production in response to a stimulus. This stimulatory interaction requires direct cell-to-cell contact between NK cells and monocytes and is mediated primarily by monocyte MICA engagement of the NKG2D receptor on NK cells. Lastly, it was demonstrated in murine models that the immune response to combination therapy with trastuzumab and IL12 is enhanced through NK cell–monocyte interactions. Taken together, these results suggest that NK cells and monocytes in the tumor microenvironment participate in crosstalk to optimize Ab-mediated antitumor activity, and mechanisms to promote these interactions may enhance the response to tumor-specific mAbs.

Interactions between NK cells and monocytes promote the immune response against invading pathogens and transformed cancer cells. However, the role of monocytes in modulating NK-cell activity in the setting of mAb therapy is not well understood. We noted that exposure of PBMC to various NK stimulatory conditions led to more IFNγ production than that of NK cells alone. Monocytes were the cells within the PBMC population responsible for this boost in NK-cell cytokine production. Others have demonstrated that reciprocal activation between NK cells and monocytes occurs via contact in the setting of infection as well as chronic arthritis (40, 41). However, the molecules responsible for this cross-talk were not determined. In the present study, a key interface between NK cells and monocytes, the NKG2D-MICA axis, was shown to be responsible for monocyte-mediated enhancement of NK cell antitumor activity. These results suggest that NK-cell NKG2D and monocyte MICA interactions play an important role in the immune response to mAb therapy.

Few studies have delved into the relationship between NK cells and monocytes in regard to cell-mediated cytotoxicity against tumor cells (42). We now show that interactions with monocytes promote NK cell-mediated cytotoxic activity. Bhatnagar and colleagues (43) described reciprocal cross-talk between NK cells and monocytes in the setting of viral infection, concluding that monocytes enhanced NK cell cytotoxicity against HIV-1–infected cells via direct contact in an FcγRII (CD32)-dependent manner. The role of NK cell recognition and lysis of NKG2D-ligand expressing tumor cells has been described previously (44). Pende and colleagues analyzed tumor expression levels of the NKG2D ligands MICA and ULBPs in melanoma, leukemia, neuroblastoma, and various carcinoma cell lines and correlated expression levels to increased tumor cell susceptibility to NK cell-mediated lysis. The impact of NKG2D ligand expression by circulating immune cells was not explored. Wang and colleagues evaluated IFNγ pretreated monocytes and determined that this strategy induced monocyte expression of MIC molecules and membrane-bound IL15 (45). These molecules promoted NK-cell IFNγ production and cytotoxicity against lymphoblastoid cells. This was observed only with IFNγ pretreated monocytes *in vitro*, and NK-cell activity was not enhanced in the presence of freshly isolated monocytes, as was the case in the present study. Also, Wang and colleagues utilized allogeneic NK cell-monocyte cocultures at a 5:1 NK cell to monocyte ratio, whereas the current study investigated autologous NK cells and monocytes cultured at a 2:1 ratio, modeling what might be found *in vivo*. Kloss and colleagues demonstrated that MICA-positive, LPS-activated monocytes enhanced NK cell cytokine production *in vitro*. The presence of monocytes led to increased NK cell proliferation, regardless of whether monocytes were untreated or primed with LPS (35). The current study demonstrates that in the setting of mAb therapy for cancer, interactions between MICA-positive monocytes and NKG2D-expressing NK cells facilitate NK-cell cytokine production and ADCC against Ab-coated tumor cells both *in vitro* and in murine models.

A recent study by Crane and colleagues described that myeloid cells, including monocytes isolated from glioblastoma multiforme (GBM) patients, expressed NKG2D ligands (46). It was shown that glioma cell line–derived lactate dehydrogenase-containing supernatants had the capacity to upregulate monocyte NKG2D ligand expression and promote NK-cell degranulation and cytokine production *in vitro*. The authors proposed that immune evasion in GBM patients may be due to NKG2D-dependent NK cell exhaustion. In contrast, this study demonstrated that interruption of NK cell-monocyte cross-talk *in vivo* inhibited the NK cell effector response against Ab-coated breast cancer cells. These contrasting results emphasize the need to further explore the possible effects of NKG2D ligand expression by myeloid cells.

This study has provided evidence that monocytes promote NK-cell cytokine production and tumor cell lysis in response to FcR and IL12R stimulation, an effective dual stimulation regimen with proven antitumor activity. The antitumor effects of mAb therapy may be enhanced by strategies aimed at maximizing monocyte expression of NK-cell activating molecules.

Disclosure of Potential Conflicts of Interest

D. Raulet is a consultant/advisory board member for Innate Pharma SAS. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.R. Campbell, M.C. Duggan, L.P. Suarez-Kelly, N. Bhave, K.S. Opheim, P. Trikha, R. Lee, S. Tridandapani, V. Groh, L. Yu, V. Yildiz, W.E. Carson III

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Acknowledgments

The authors thank The Ohio State University Comprehensive Cancer Center's Analytical Cytometry Shared Resource for providing equipment to facilitate flow cytometry analyses.

Grant Support

This work was supported by the Susan G. Komen Breast Cancer Foundation Dissertation Research Award (R. Parihar), the OSUCCC Pelotonia Graduate Fellowship (A.R. Campbell), and the NIH grants T32 GM068412 (M.C. Duggan) and P01 CA95426 (W.E. Carson).

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Received January 19, 2016; revised July 20, 2016; accepted July 5, 2017; published OnlineFirst July 19, 2017.

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Published OnlineFirst July 19, 2017; DOI: 10.1158/2326-6066.CIR-16-0005

Cancer Immunology Research

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Cancer Immunol Res 2017;5:778-789. Published OnlineFirst July 19, 2017.