The Journal of Immunology

Chimeric Antigen Receptor T Cells Shape Myeloid Cell Function within the Tumor Microenvironment through IFN-γ and GM-CSF

Paul Spear, Amorette Barber, Agnieszka Rynda-Apple and Charles L. Sentman

J Immunol 2012; 188:6389-6398; Prepublished online 14 May 2012;
doi: 10.4049/jimmunol.1103019
http://www.jimmunol.org/content/188/12/6389

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Chimeric Antigen Receptor T Cells Shape Myeloid Cell Function within the Tumor Microenvironment through IFN-γ and GM-CSF

Paul Spear, Amorette Barber, Agnieszka Rynda-Apple, and Charles L. Sentman

The infiltration of suppressive myeloid cells into the tumor microenvironment restrains anti-tumor immunity. However, cytokines may alter the function of myeloid lineage cells to support tumor rejection, regulating the balance between pro- and anti-tumor immunity. In this study, it is shown that effector cytokines secreted by adoptively transferred T cells expressing a chimeric Ag receptor (CAR) shape the function of myeloid cells to promote endogenous immunity and tumor destruction. Mice bearing the ovarian ID8 tumor were treated with T cells transduced with a chimeric NKG2D receptor. GM-CSF secreted by the adoptively transferred T cells recruited peripheral F4/80^{lo}Ly-6C^{+} myeloid cells to the tumor microenvironment in a CCR2-dependent fashion. T cell IFN-γ and GM-CSF activated local, tumor-associated macrophages, decreased expression of regulatory factors, increased IL-12p40 production, and augmented Ag processing and presentation by host macrophages to Ag-specific T cells. In addition, T cell-derived IFN-γ, but not GM-CSF, induced the production of NO by F4/80^{hi} macrophages and enhanced their lysis of tumor cells. The ability of CAR T cell therapy to eliminate tumor was moderately impaired when inducible NO synthase was inhibited and greatly impaired in the absence of peritoneal macrophages after depletion with clodronate encapsulated liposomes. This study demonstrates that the activation of host macrophages by CAR T cell-derived cytokines transformed the tumor microenvironment from immunosuppressive to immunostimulatory and contributed to inhibition of ovarian tumor growth. ***The Journal of Immunology*****, 2012, 188: 6389–6398.**

The tumor microenvironment is characterized by suppressive leukocytes that restrain anti-tumor immunity and promote tumor growth and survival (1). Tumor-associated macrophages (TAMs) comprise a large percentage of the cellular constituents within the tumor milieu. These cells promote tumor growth through secretion of proangiogenic cytokines and suppression of anti-tumor immunity (2). Despite their tumor-promoting properties, subpopulations of macrophages can support tumor rejection and promote anti-tumor immune responses elicited by cancer immunotherapies (3, 4). The dual function of macrophages is regulated by their immune environment (5). Lymphocytes regulate the immune environment and interact with macrophages to shape their activation, controlling the balance between pro- and anti-tumor immunity (6, 7). Transformation of the tumor milieu, including myeloid cells, supports tumor rejection and enhances immunotherapeutic strategies targeting cancer.

Chimeric Ag receptor (CAR) transduced T cells have been shown to be an effective means to reduce tumor burden and increase survival (8–10). CARs have been developed that recognize several different molecules, including CD19, Her2neu, mesothelin, and NKG2D ligands, and CARs use a variety of signaling motifs to enhance the efficacy of effector T cells (8, 11–14). Because such targeted T cells can mediate a variety of effector responses in addition to direct tumor lysis, CAR T cells have the potential to change the tumor microenvironment, induce host anti-tumor immunity, and lead to long-term tumor-free survival. Many studies have focused on their ability to kill tumor cells and how best to deliver them to tumor-bearing hosts (9). This study investigated the mechanisms of how adoptive T cell therapy altered local tumor myeloid cells to promote tumor destruction and anti-tumor immunity. CAR-bearing T cells engineered to express a chimeric NKG2D (chNKG2D) receptor, which consists of full-length NKG2D fused to CD3ζ, were used (13, 15, 16). The efficacy of these CAR-bearing T cells involves not only tumor lysis but cytokine-induced changes as well (14, 17, 18). This study demonstrates that one mechanism for CAR-bearing T cell efficacy is through cytokine-induced changes in the tumor microenvironment that cause recruitment and activation of tumor-associated myeloid cells to create an unfavorable milieu for tumor survival.

Materials and Methods

Mice

C57BL/6 and B6-Ly5.2Cr (CD45.1^{+}) were purchased from the National Cancer Institute (Frederick, MD). B6.129S7-Ifng^{tm1Agt}/J (IFN-γ$^{-/-}$) were purchased from The Jackson Laboratory. B6.129S4-Ccr2^{tm1Ifc}/J (CCR2$^{-/-}$) mice were provided by Dr. Brent Berwin (Dartmouth Medical School, Lebanon, NH), and GM-CSF–deficient mice on a C57BL/6 background were provided by Dr. Jeff Whitsett (University of Cincinnati, Cincinnati, OH). Mice used in experiments were between 7 and 12 wk of age. All animal work was performed in the Dartmouth Medical School Animal Facility (Lebanon, NH) in accordance with institutional guidelines.

Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Lebanon, NH 03756

Received for publication October 19, 2011. Accepted for publication March 30, 2012.

This work was supported by grants from the National Institutes of Health (CA130911, T32 AI007363).

The views in this article reflect the authors' opinions and do not necessarily reflect the opinions of the National Institutes of Health.

Address correspondence and reprint requests to Dr. Charles L. Sentman, Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, 6W Borwell Building, One Medical Center Drive, Lebanon, NH 03756. E-mail address: charles.sentman@dartmouth.edu

Abbreviations used in this article: CAR, chimeric Ag receptor; chNKG2D, chimeric NKG2D; iNOS, inducible NO synthase; KO, knockout; MHC II, MHC class II; TAM, tumor-associated macrophage; wtNKG2D, wild-type NKG2D.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1103019

Generation of CAR-expressing T cells

Mouse splenocytes were stimulated with Con A for 18 h (1 μg/ml), retrovirally transduced as previously described, and injected into mice 8 d postactivation (15, 17)

Injection of ID8-GFP cells and treatment of mice with genetically modified T cells

On day 0, ID8-GFP cells (2×10^6) were injected i.p. into mice. The ID8 ovarian carcinoma model recapitulates advanced stages of ovarian carcinoma. ID8 tumor cells express NKG2D ligands (13). Mice were treated with T cells on day +35, as described for each experiment. To inhibit NO production in vivo, mice were injected with the inducible NO synthase (iNOS) selective inhibitor L-nil (3 mg/kg) or PBS each day from day 35 to day 41. To deplete macrophage populations, 200 μl clodronate encapsulated liposomes or control PBS liposomes (gift of Roche Diagnostics, Mannheim, Germany) were injected i.p. on day 33 into ID8 tumor-bearing mice, which was 2 d prior to T cell inoculation. Clodronate liposomes were prepared as previously described (19). The number of solid tumors on the peritoneal wall and the number of GFP^+ tumor cells in the peritoneal wash was determined 8 wk (day 56) after ID8 tumor inoculation.

Flow cytometry and F4/80 cell isolation

Cells isolated by peritoneal wash were incubated with anti-CD16/CD32 mAbs and mouse γ-globulin (Jackson ImmunoResearch) and stained with anti-CD45.1 (clone A20), anti-CD45.2 (clone 104), anti-F4/80 (clone BM8), anti–Ly-6C (clone HK1.4), anti-MHC class II (clone M5/114.15.2), anti-CD86 (clone GL-1), anti–IFN-γR (clone MOB-47), anti-CD3e (clone 145-2C11), anti-CD4 (clone GK1.5), anti-CD8b (clone YTS156.7.7), anti-CXCR3 (clone CXCR3-173), anti-CD11c (clone N418), or anti–IL-12p40 (clone C15.6). $F4/80^+$ cell production of reactive oxygen species was measured by the oxidation-sensitive dye CM-H_2DCFDA (Invitrogen). Peritoneal cells were labeled with 10 μM DCFDA for 30 min, washed twice, and stained with anti-F4/80 mAbs before running flow cytometry. For intracellular staining, 10^6 peritoneal cells were cultured in complete medium for 24 h in a 48-well plate. Brefeldin A (10 μg/ml; Sigma-Aldrich, St. Louis, MO) and LPS (10 μg/ml) were added to the cultures for the last 5 h of incubation. Peritoneal cells assayed for IL-6 expression were not stimulated with LPS. Cells were stained with PE or allophycocyanin-conjugated anti-F4/80 mAbs, fixed with 1.0% paraformaldehyde, permeabilized with 0.1% saponin, and stained with allophycocyanin-conjugated anti–IL-12p40, anti–IL-10–PE, anti–IL-6–PE, or anti-NOS2–Alexa Fluor 647 mAbs. Peritoneal $F4/80^+$ cells were isolated from tumor-bearing mice by magnetic bead selection and anti-F4/80 biotin Ab (Miltenyi). The purity of cells after isolation was >90%.

Adoptive transfer experiments

$CD45.1^+$ mice bearing tumors for 5 wk were injected i.p. with wild-type NKG2D (wtNKG2D) or chNKG2D T cells. At 5 wk after tumor inoculation in this murine tumor model, there are both free tumor cells and solid tumors present. One hour prior to T cell treatment, a mixture of bone marrow (5×10^6) and spleen cells (5×10^6) isolated from naive $CD45.2^+$ C57BL/6 or $CCR2^{-/-}$ mice was injected i.v. into $CD45.1^+$ mice. Three days after T cell treatment, the number of $CD45.2^+$, $F4/80^+$ cells in the peritoneum was assessed to determine myeloid cell recruitment. To assess chNKG2D T cell activation of TAMs, $CD45.2^+$ mice bearing 5-wk ID8 tumors were injected i.p. with peritoneal cells isolated from $CD45.1^+$ mice bearing 5-wk ID8 tumors. One hour after transfer of tumor-associated $CD45.1^+$ cells, wtNKG2D or chNKG2D T cells were injected i.p. into $CD45.2^+$ mice. The phenotype of $CD45.1^+$ tumor-associated cells was assessed by flow cytometry 3 d after T cell treatment.

Real-time PCR

$F4/80^+$ peritoneal cells were isolated, and total RNA was extracted using RNeasy Mini Kit (Qiagen). cDNA was synthesized using random hexamer primers (Fermentas) in accordance with the manufacturer's protocol. cDNA (5 ng) was used as a template for RT-PCR amplification using SYBR-Green Master Mix (Applied Biosystems) in 25 μl of volume according to the manufacturer's protocol. Statistics were performed using the ΔCT values.

Cytokine secretion

$F4/80^+$ cells (2×10^5) from tumor-bearing mice were cultured for 24 h in a 96-well plate in complete medium. Cell-free conditioned media was assayed for IL-6 and IL-10 using multiplex analysis (Millipore) by the DartLab of the Norris Cotton Cancer Center (Lebanon, NH). NO production was measured using Griess reagent for nitrite according to the manufacturer's protocol (Sigma-Aldrich). Peritoneal cells (10^6) were cultured in a 48-well plate for 24 h, and cell-free supernatants were assayed for IFN-γ by ELISA using mouse DuoSet ELISA kits (R&D Systems).

Ag processing and presentation

Ag processing assays were performed using peritoneal cells (5×10^5) stained for F4/80 prior to pulsing cells with 50 μg/ml DQ-OVA at 37°C for 15 min. Cells were washed three times in PBS at 4°C and incubated for 15 min at 37°C before Ag processing by $F4/80^+$ cells was assessed by flow cytometry. For Ag presentation assays, $F4/80^+$ cells (2×10^5) were isolated from peritoneal wash cells and cultured with CFSE-labeled OT-I cells (10^5) positively selected by magnetic beads for $CD8b^+$ cells. Prior to culture, $F4/80^+$ cells were pulsed with 10^{-10} M $OVA_{257–264}$ peptide. T cell proliferation was measured after 4 d based on CFSE dilution.

Cytotoxicity assay

Purified $F4/80^+$ cells were cultured with ID8 tumor cells at an E:T ratio of 25:1 with or without 20 μM of the NOS2 selective inhibitor L-nil (Cayman Chemical) for 24 h. Macrophage cytotoxicity was measured by lactate dehydrogenase release using the manufacturer's protocol (Promega).

Statistical analysis

Differences between groups were analyzed using a Student *t* test or ANOVA. A Wilcoxon two-group test was used to compare ΔCT values in real-time PCR experiments. The *p* values <0.05 were considered significant.

Results

CAR T cell GM-CSF recruits myeloid cells to the tumor site in a CCR2-dependent manner

To determine mechanisms whereby CAR T cells regulated myeloid cell populations in the tumor microenvironment, tumor-bearing mice were injected with CAR T cells, and the role of specific cytokines was determined. Adoptive transfer of CAR chNKG2D T cells increased the number of peritoneal cells expressing low levels of F4/80 in the tumor microenvironment 3 d posttreatment (Fig. 1A). Approximately 40% of the $F4/80^{hi}$ and 50% of the $F4/80^{lo}$ macrophages expressed Ly-6C, a marker of recent monocyte recruitment (Fig. 1B). Note that less than 5% of the $F4/80^+$ cells in the tumor microenvironment expressed the granulocytic neutrophil marker Ly-6G (data not shown). The low expression of F4/80 can distinguish newly recruited monocytes from $F4/80^{hi}$ resident macrophages (20–22). The increase in $F4/80^{lo}$ myeloid cell numbers may be attributed to increased proliferation of local myeloid cells or the recruitment of peripheral monocytes that differentiate into $F4/80^+$ tissue macrophages. To confirm that the increase in $F4/80^{lo}Ly\text{-}6C^+$ cells after adoptive T cell treatment was due to recruitment of myeloid cells, $CD45.1^+$ bone marrow and spleen cells (two sources of peripheral myeloid cells) were injected i.v. into $CD45.2^+$ tumor-bearing mice. These mice were also injected at the tumor site i.p. with chNKG2D T cells or control wtNKG2D T cells. Treatment with CAR T cells preferentially recruited $CD45.1^+F4/80^{lo}Ly\text{-}6C^+$ myeloid cells to the peritoneum within 3 d after T cell treatment (Fig. 1C). GM-CSF–deficient T cells failed to increase the number of $F4/80^{lo}Ly\text{-}6C^+$ cells compared with GM-CSF–sufficient chNKG2D T cells indicating that the secretion of GM-CSF by the transferred T cells, but not IFN-γ, was necessary for the recruitment of $F4/80^{lo}Ly\text{-}6C^+$ cells (Fig. 1D). These data indicate that CAR T cell treatment recruits $F4/80^{lo}Ly\text{-}6C^+$ myeloid cells to the tumor microenvironment through secretion of GM-CSF.

Inflammatory monocytes have been shown preferentially to express the chemokine receptor CCR2, which they use to migrate

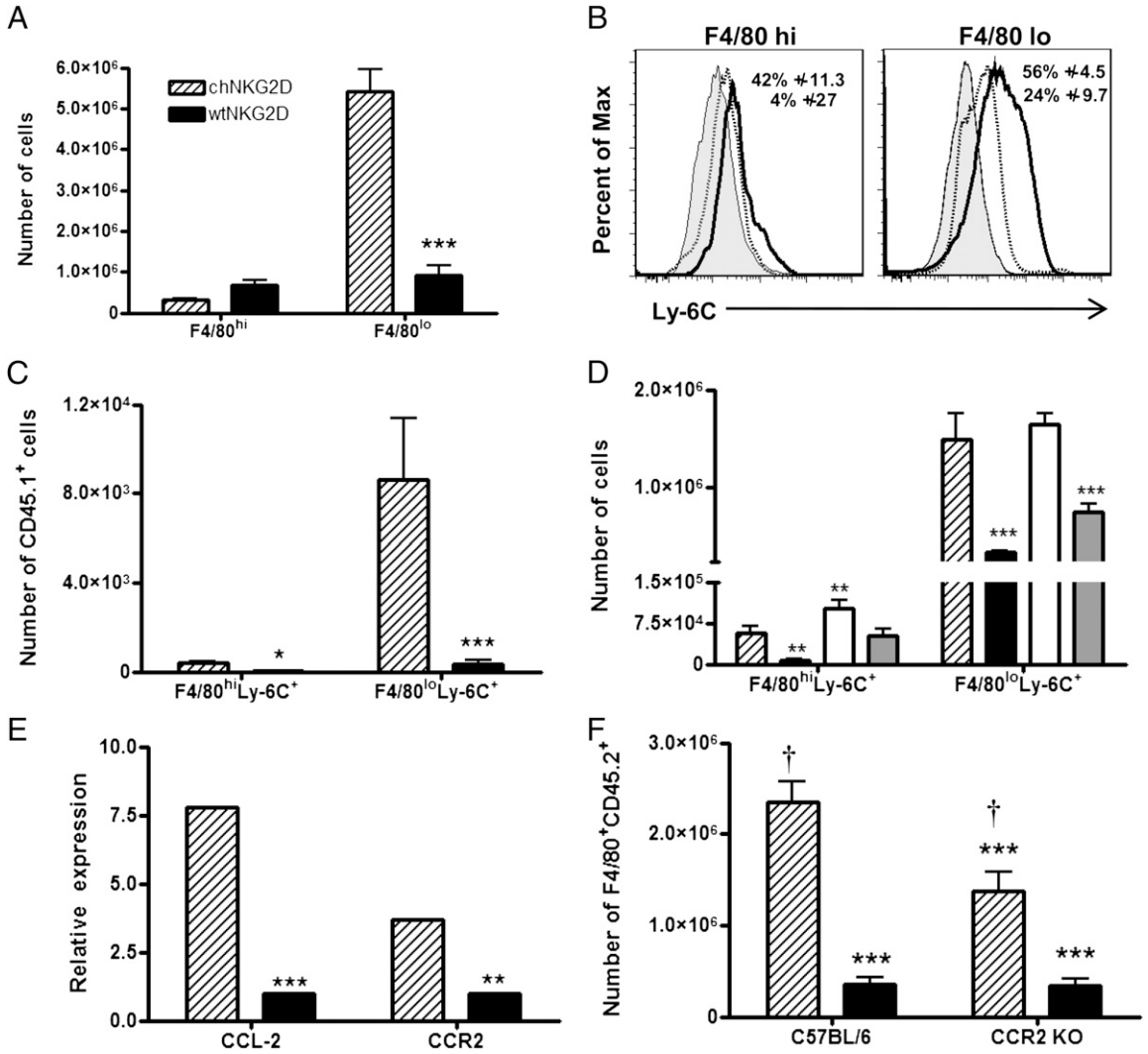

FIGURE 1. T cell-derived GM-CSF recruits Ly-6C$^+$ myeloid cells to the tumor microenvironment in a CCR2-dependent manner. ID8 tumor-bearing mice were treated with chNKG2D (hatched), wtNKG2D (filled), chNKG2D IFN-γ KO (open), or chNKG2D GM-CSF KO (gray) T cells on week 5. (**A**) The number of F4/80^{hi} and F4/80^{lo} cells in the peritoneum was determined 3 d after T cell treatment ($n = 4$). ***$p < 0.001$ (versus F4/80^{lo} chNKG2D). (**B**) Representative flow cytometry plots show the expression of Ly-6C on the F4/80^{hi} and F4/80^{lo} populations after treatment with chNKG2D (solid) or wtNKG2D (dashed) T cells. CD45.2$^+$ tumor-bearing mice were injected i.v. with a 1:1 mixture of CD45.1$^+$ bone marrow and spleen cells 1 h before i.p. injection with chNKG2D or wtNKG2D T cells. (**C**) The number of CD45.1$^+$ F4/80^{hi} and F4/80^{lo} cells expressing Ly-6C in the peritoneum was assessed 3 d after T cell treatment ($n = 4$). ***$p < 0.001$ (versus F4/80^{lo}Ly-6C$^+$ chNKG2D), *$p < 0.05$ (versus F4/80^{hi}Ly-6C$^+$ chNKG2D). (**D**) The number of F4/80^{hi} and F4/80^{lo} cells expressing Ly-6C was determined after T cell treatment. ***$p < 0.001$ (versus F4/80^{lo}Ly-6C$^+$ chNKG2D), **$p < 0.01$ (versus F4/80^{hi}Ly-6C$^+$ chNKG2D). (**E**) F4/80$^+$ cells were isolated from tumor-bearing mice, and the expression of CCL2 and CCR2 was determined by RT-PCR. ***$p < 0.001$, **$p < 0.01$ (versus chNKG2D). (**F**) Tumor-bearing CD45.1$^+$ mice were injected i.v. with CD45.2$^+$ bone marrow and spleen cells from naive C57BL/6 or CCR2$^{-/-}$ donors 1 h before i.p. injection with chNKG2D or wtNKG2D T cells. The number of F4/80$^+$CD45.2$^+$ cells recruited to the peritoneum was assessed 3 d after T cell treatment ($n = 4$). ***$p < 0.001$ (versus C57BL/6 chNKG2D), $^{\dagger}p < 0.05$ (versus C57BL/6 wtNKG2D). (A), (B), (C), and (F) are representative data of at least two independent experiments. (D) and (E) are cumulative results of two independent experiments. SD is shown ($n = 8$).

into inflamed tissue and the ovarian tumor microenvironment (20, 22, 23). Macrophages isolated from the peritoneum of chNKG2D T cell-treated mice expressed higher amounts of the chemokine receptor CCR2 and produced more of the CCR2 ligand, CCL2 (Fig. 1E). Tumor-bearing mice treated with chNKG2D CAR T cells increased total CCL2 production by cells within the tumor microenvironment, and this was dependent upon adoptive T cell production of GM-CSF but not IFN-γ (18). To determine if the recruitment of myeloid cells after CAR T cell therapy was CCR2-dependent, CD45.2$^+$ bone marrow and spleen cells from C57BL/6 or CCR2 knockout (KO) mice were injected i.v. into tumor-bearing CD45.1$^+$ mice 1 h before i.p. treatment with chNKG2D or wtNKG2D T cells. CCR2-deficient cells had a reduced ability to traffic to the tumor site after chNKG2D T cell treatment (Fig. 1F). However, chNKG2D T cell treatment still recruited significantly more CCR2$^{-/-}$ myeloid cells relative to wtNKG2D T cell-treated mice, indicating the recruitment of myeloid cells from the periphery was not solely CCR2-dependent.

CAR T cell IFN-γ is required to upregulate expression of MHC and costimulatory molecules on endogenous macrophages

Cytokines secreted by T cells shape macrophage activation upon tissue entry. To determine if cytokines secreted by adoptively transferred T cells regulated macrophage activation, tumor-bearing

mice were treated with chNKG2D T cells deficient in IFN-γ or GM-CSF, and the expression of cell surface proteins was determined. IFN-γ from the adoptively transferred chNKG2D T cells, but not GM-CSF, was required to upregulate MHC class II (MHC II), CD86, and IFN-γR expression on F4/80^{hi} macrophages after chNKG2D T cell treatment (Fig. 2A). There was a trend that F4/80^{hi} cells isolated from GM-CSF–deficient chNKG2D T cells expressed lower amounts of activation markers compared with F4/80^{hi} cells from mice treated with cytokine-sufficient chNKG2D T cells. Transfer of cytokine-deficient T cells resulted in reduced expression of these activation markers on F4/80^{lo} cells relative to cytokine-sufficient T cells (Fig. 2B). F4/80^{lo} cells represent a population of newly recruited macrophages, and they were similar between chNKG2D and control wtNKG2D T cell-treated mice. The mature F4/80^{hi} macrophage population consists of both tumor-associated and newly recruited macrophages that upregulate F4/80 upon maturation. The upregulation of activation markers on F4/80^{hi} macrophages indicated that IFN-γ from CAR T cells activated host macrophages.

CAR T cells alter the activation of TAMs present at the tumor site at the time of treatment

After treatment with CAR-expressing T cells, two populations of endogenous macrophages within the tumor microenvironment are present. Newly recruited macrophages constitute the majority of F4/80^{+} cells 3 d after adoptive therapy. A second population of tumor-associated myeloid cells that differentiate in the tumor milieu prior to T cell treatment have been shown to suppress tumor-specific T cell responses (2). However, their re-education through activating signals has been shown to stimulate adaptive immunity and promote regression of advanced tumors (4, 24). It is controversial as to what extent TAMs are malleable and can be induced to become anti-tumor effector cells. To determine if T cell effector cytokines could activate suppressive TAMs already present within a tumor, 5-wk tumor-bearing CD45.2^{+} mice were injected locally with peritoneal cells isolated from 5-wk tumor-bearing CD45.1^{+} mice prior to CAR T cell transfer. The majority of the transferred peritoneal cells from the tumor-bearing CD45.1^{+} mice expressed F4/80 (data not shown). ChNKG2D T cell treatment activated CD45.1^{+} TAMs as evidenced by an increase in the expression of MHC II, CD86, and IFN-γR on F4/80^{hi} macrophages (Fig. 3A). Both IFN-γ and GM-CSF secreted by CAR T cells were necessary to activate TAMs. Treatment with chNKG2D T cells had no significant effect on tumor-associated F4/80^{lo} cells (Fig. 3B). However, ~75% of the transferred TAMs were F4/80^{hi} (data not shown). These data indicate that both newly recruited and tumor-associated suppressive macrophages within a tumor can be activated through a combination of IFN-γ and GM-CSF produced by the CAR-bearing T cells.

CAR T cell treatment inhibits TAM production of regulatory proteins

Ovarian cancer cells have been shown to polarize macrophages toward a tumor-associated phenotype (25). To determine the effect of adoptive CAR T cell treatment to modify the suppressive capabilities of tumor-associated regulatory macrophages, macrophage production of suppressive factors was assessed 3 d after T cell treatment. F4/80^{hi} and F4/80^{lo} macrophages produced lower amounts of IL-10 and IL-6 after treatment with chNKG2D T cells (Fig. 4A, 4B). CAR T cell treatment decreased the production of reactive oxygen species by F4/80^{hi} and F4/80^{lo} cells (Fig. 4C), which can suppress T cell function through downregulation of CD3ζ (1). In addition to direct suppression of anti-tumor immune responses, TAMs can recruit CCR8^{+} and CCR4^{+} regulatory T cells to potentiate suppression (26, 27). ChNKG2D T cell treatment reduced macrophage expression of the CCR8 and CCR4 ligands, CCL1 and CCL17 (Fig. 4D). Macrophages also expressed less YM-1, HIF-1α, and VEGF, indicating a less alternatively activated phenotype (Fig. 4D) (28). The decrease in expression of suppressive and angiogenic factors by macrophages at the tumor site indicates that chNKG2D T cell treatment activated host macrophages resulting in a reduced ability to suppress immune responses.

CAR T cells increase Ag presentation capacity of TAMs in a cytokine-dependent manner

The ability of TAMs to present Ag and induce a T cell response is often impaired (29, 30). T cell effector cytokines can regulate Ag processing and presentation in the tumor microenvironment and promote endogenous immunity (31, 32). A higher percentage

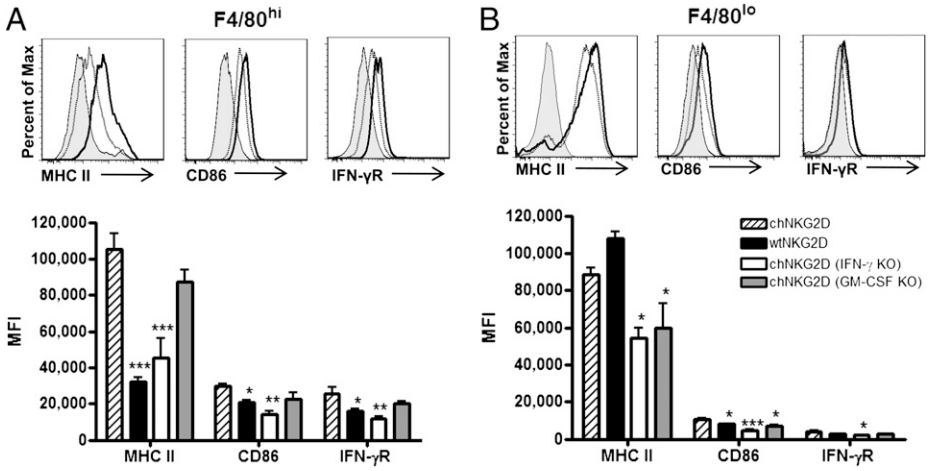

FIGURE 2. T cell-derived IFN-γ is required to activate host macrophages. ID8 tumor-bearing mice were treated with chNKG2D (hatched), wtNKG2D (filled), chNKG2D IFN-γ KO (open), or chNKG2D GM-CSF KO (gray) T cells at week 5, and peritoneal washes were performed 3 d after T cell treatment. (**A** and **B**) *Top*, Representative flow cytometry plots showing the expression of MHC II, CD86, and IFN-γR on F4/80^{hi} and F4/80^{lo} cells from mice treated with chNKG2D (solid) or wtNKG2D (dashed) T cells, or isotype control (gray histogram). *Bottom*, The mean fluorescence intensity of F4/80^{hi} and F4/80^{lo} cells expressing MHC II, CD86, and IFN-γR was determined after adoptive T cell treatment ($n = 4$). Flow plots and data are representative of two to four independent experiments. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ (versus chNKG2D).

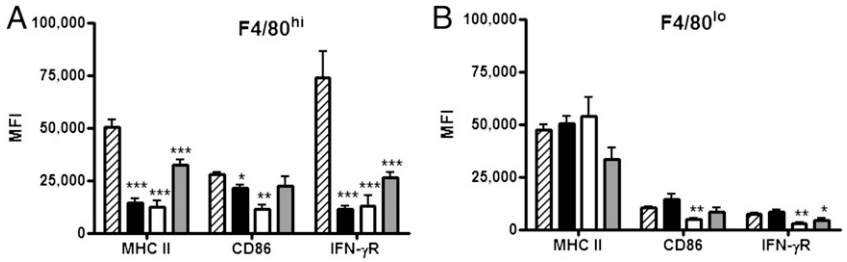

FIGURE 3. Both GM-CSF and IFN-γ secreted by chNKG2D T cells are required to activate TAMs. Peritoneal cells isolated from CD45.1$^+$ tumor-bearing mice were injected i.p. into CD45.2$^+$ tumor-bearing mice 1 h before injection of chNKG2D (hatched), wtNKG2D (filled), chNKG2D IFN-γ KO (open), or chNKG2D GM-CSF KO (gray) T cells. Peritoneal washes were performed 3 d after T cell treatment, and the mean fluorescence intensity of MHC II, CD86, and IFN-γR was determined on CD45.1$^+$F4/80^{hi} (**A**) and CD45.2$^+$F4/80^{lo} (**B**) macrophages. Representative results and SD of two independent experiments are shown ($n = 4$). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ (versus chNKG2D).

of both F4/80^{hi} and F4/80^{lo} macrophages from chNKG2D T cell-treated mice effectively processed Ag as measured by processing of DQ-OVA, a protein that fluoresces upon proteolytic degradation and is widely used as a measure of endosomal protein processing. Both IFN-γ and GM-CSF secretion by adoptively transferred T cells were required to enhance Ag processing by macrophages after CAR T cell treatment (Fig. 5A).

To determine the effects of chNKG2D T cell cytokines on the ability of TAMs to stimulate Ag-specific T cells, F4/80$^+$ cells were isolated from the tumor site after T cell injection, pulsed with SIINFEKL peptides, and the proliferation of naive OT-I T cells was determined. F4/80$^+$ cells isolated from mice treated with cytokine-deficient CAR T cells induced less OT-I T cell proliferation than F4/80$^+$ cells isolated from mice treated with cytokine-sufficient CAR T cells (Fig. 5B). Macrophages from mice treated with IFN-γ– or GM-CSF–deficient chNKG2D T cells had an impaired ability to stimulate OT-I proliferation compared with cytokine-intact chNKG2D T cells. F4/80$^+$ cells from wtNKG2D and IFN-γ–deficient T cell-treated mice induced less OT-I proliferation than F4/80$^+$ cells from naive mice, indicative of the suppressive nature of these TAMs. These data, together with previous findings showing enhanced Ag presentation in the draining lymph node and tumor site, indicate that CAR-bearing T cells stimulated Ag processing and presentation in a cytokine-dependent manner (17, 18).

T cell-derived IFN-γ and GM-CSF induce macrophage production of IL-12 to potentiate IFN-γ secretion and Th1 differentiation (33). However, tumor-associated macrophages demonstrate a reduced ability to produce IL-12 upon stimulation with IFN-γ or LPS (34, 35). Intracellular flow cytometry using an anti–IL-12 Ab showed that both mature F4/80^{hi} and newly recruited F4/80^{lo} macrophages produced more IL-12 after treatment with CAR T cells. The increased IL-12 production by newly recruited F4/80^{lo} cells was dependent on chNKG2D T cell-derived GM-CSF, but not IFN-γ (Fig. 5C). In contrast, both chNKG2D-derived GM-CSF and IFN-γ were required to increase IL-12p40 production by F4/80^{hi} TAMs. Thus, CAR-bearing T cells can promote local macrophages to produce IL-12 and support activation of Th1

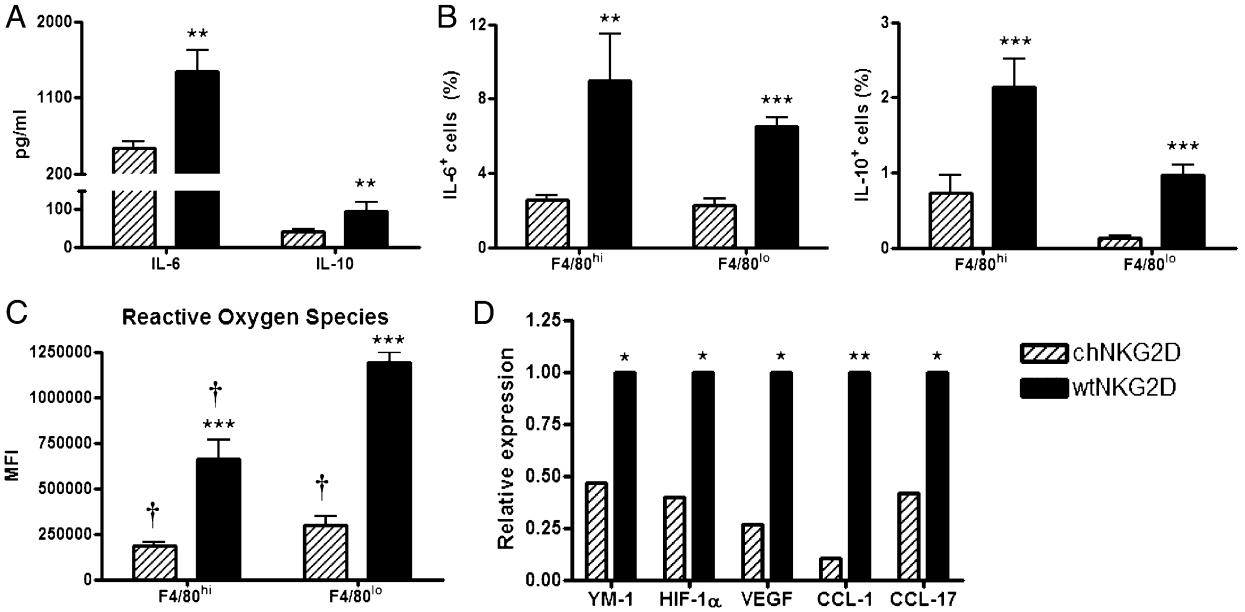

FIGURE 4. ChNKG2D T cell treatment reduces macrophage expression of regulatory factors. F4/80$^+$ cells were isolated from the peritoneum of tumor-bearing mice 3 d after T cell treatment and cultured in medium for 24 h. (**A**) Cell-free supernatant was assayed for IL-10 and IL-6. (**B**) F4/80^{hi} and F4/80^{lo} peritoneal cells were assessed for IL-6 and IL-10 expression by intracellular flow cytometry. (**C**) F4/80^{hi} and F4/80^{lo} peritoneal cells were assessed for reactive oxygen species production using a DCFDA dye. (**D**) The expression of YM-1, HIF-1α, VEGF, CCL1, and CCL17 on purified F4/80$^+$ cells was determined by real-time PCR. Cumulative data and SD of at least two independent experiments are shown. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ (versus chNKG2D), $^{\dagger}p < 0.05$ (versus F4/80^{lo} wtNKG2D).

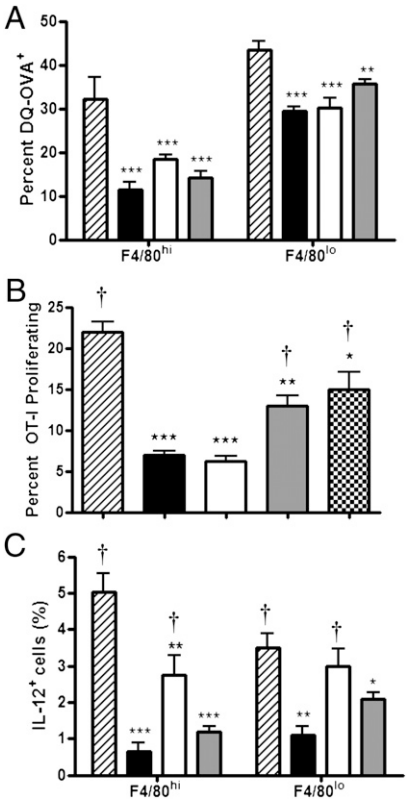

FIGURE 5. ChNKG2D T cell treatment induces macrophage production of proinflammatory cytokines and enhances Ag processing and presentation. Tumor-bearing mice were treated with chNKG2D (hatched), wtNKG2D (filled), chNKG2D IFN-γ KO (open), or chNKG2D GM-CSF KO (gray) T cells. (**A**) F4/80$^+$ cells isolated from the peritoneum 3 d after T cell treatment were pulsed with DQ-OVA, and the percent of F4/80^{hi} and F4/80^{lo} cells processing Ag was assessed after 15 min by flow cytometry. (**B**) Purified peritoneal F4/80$^+$ cells isolated from naive (checkered) or tumor-bearing mice treated with chNKG2D (hatched), wtNKG2D (filled), chNKG2D IFN-γ KO (open), or chNKG2D GM-CSF KO (gray) T cells were pulsed with 10^{-10} M $OVA_{257–264}$ peptide and cultured with CFSE-labeled OT-I T cells. OT-I cell proliferation was measured by CFSE dilution and flow cytometry after 4 d of culture. (**C**) Three days after T cell treatment, peritoneal cells were isolated and cultured with brefeldin A for 24 h. During the last 5 h of culture, cells were stimulated with LPS, and the percent of F4/80^{hi} and F4/80^{lo} cells producing IL-12p40 was determined by intracellular flow cytometry. Representative data and SD of at least two independent experiments are shown ($n = 4$). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ (versus chNKG2D), $^{\dagger}p < 0.01$ (versus wtNKG2D).

T cells and NK cells through their production of IFN-γ and GM-CSF.

CAR T cells activate macrophages within the tumor microenvironment to kill tumor cells through NO production

T cell effector cytokines can regulate macrophage cytotoxicity of tumor cells (36, 37). It was previously shown that IFN-γ secreted by chNKG2D T cells, but not GM-CSF, was required to increase host NO production after T cell transfer (18). After chNKG2D T cell treatment, macrophages were the major source of NO in the tumor microenvironment and produced significantly more NO than wtNKG2D T cell-treated mice (Fig. 6A). F4/80^{hi} cells expressed higher amounts of iNOS compared with F4/80^{lo} cells after chNKG2D T cell therapy (Fig. 6B). CAR T cell treatment enhanced macrophage lysis of ID8 tumor cells compared with control CAR T cell-treated mice (Fig. 6C). The increased direct tumoricidal activity of macrophages was dependent on secretion of NO because inhibition of iNOS production through the selective inhibitor L-nil impaired their ability to kill ID8 tumor cells in vitro (Fig. 6C). To determine the role of NO on tumor elimination in vivo, tumor-bearing mice were treated with L-nil 1 h before CAR T cells, and for the following 6 d. The ability of CAR T cell therapy to eliminate free tumor cells was significantly, but moderately, impaired when iNOS was inhibited by L-nil at the tumor site (Fig. 6D). However, inhibition of iNOS had no effect on the ability of chNKG2D T cells to inhibit the growth of solid tumors. Thus, treatment with CAR T cells resulted in an increased ability of local macrophages to kill tumor cells, which enhanced the anti-tumor effects of CAR T cell therapy at the tumor site.

CAR T cell treatment activates macrophages to inhibit ovarian tumor growth and promote endogenous immunity

To determine the role of macrophages in tumor elimination, tumor-bearing mice were injected with clodronate liposomes to deplete host macrophages. Mice treated with clodronate liposomes and CAR T cells had a higher number of solid tumors on the peritoneal wall and more free tumor cells compared with mice treated with CAR T cells and nondepleting PBS liposomes (Fig. 7A). However, mice treated with chNKG2D T cells and clodronate liposome had lower tumor burden than mice that received control CAR T cells. Macrophage depletion by itself had no effect on tumor growth, as mice treated with control CAR T cells and clodronate liposomes had similar tumor burdens as those of mice injected with PBS liposomes. These data indicate that host macrophages are required for complete tumor elimination by CAR T cell therapy.

In addition to direct tumor cell killing, macrophages can activate endogenous leukocytes to promote tumor elimination. Host macrophages present Ag and secrete IL-12 to activate lymphocytes. Because host NK and T cells secrete higher amount of IFN-γ at the tumor site after chNKG2D T cell therapy, the effect of macrophage depletion on IFN-γ production was assessed (18). Peritoneal cells isolated from tumor-bearing mice treated with CAR T cells and clodronate liposomes produced lower amounts of IFN-γ compared with mice treated with CAR T cells and PBS liposomes (Fig. 7B). Peritoneal cells produced higher amounts of IFN-γ after CAR T cell treatment and clodronate liposome-mediated macrophage depletion compared with mice treated with control CAR T cells, which demonstrated that CAR T cells led to host lymphocyte activation through other mechanisms as well. These results demonstrate that in addition to direct tumor cell killing, macrophages promote endogenous immunity to eliminate established tumors.

Discussion

The therapeutic success of adoptive T cell therapy is often attributed to direct tumor destruction. However, T cell effector cytokines may have an equally important function in tumor elimination through indirect effects on tumor cells mediated by endogenous leukocytes. T cell effector cytokines alter the tumor microenvironment by shaping leukocyte populations and eliciting endogenous immunity to support CAR T cell-mediated tumor rejection. Treatment with chNKG2D T cells transformed tumor macrophages from immunosuppressive to immunostimulatory through the secretion of specific cytokines. Macrophages after chNKG2D T cell treatment not only promoted anti-tumor immunity but also directly assisted in tumor destruction via NO. These cytokine-induced changes in myeloid cells were necessary for complete inhibition of ovarian tumor growth.

T cell-derived GM-CSF and IFN-γ have been shown tightly to correlate with anti-tumor efficacy after adoptive T cell therapy (38). It is hypothesized that these cytokines induce potent host

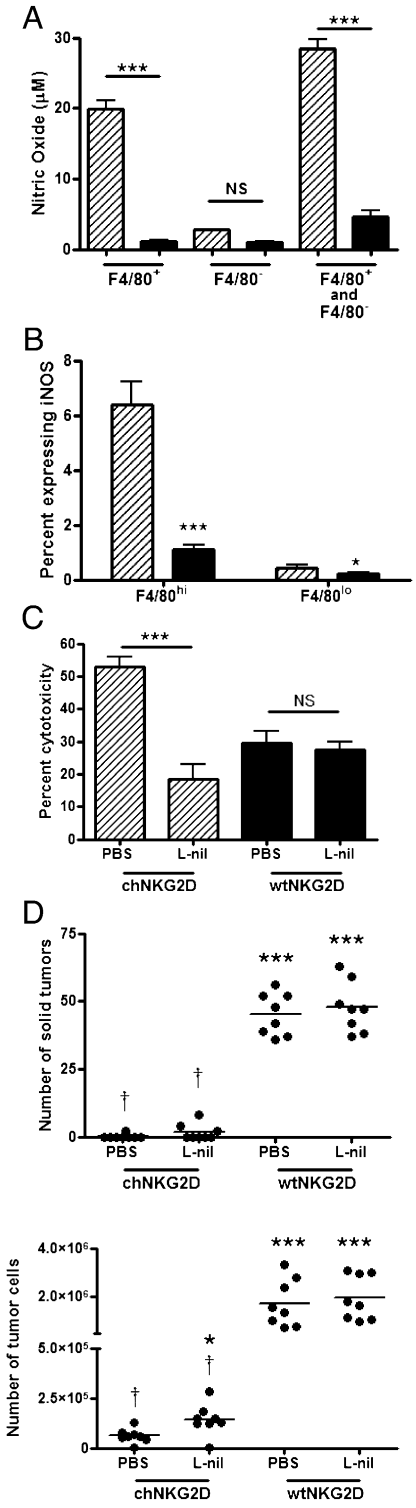

FIGURE 6. ChNKG2D T cell treatment enhances macrophage cytotoxicity. Tumor-bearing mice were treated with CAR T cells, and F4/80$^+$ cells were purified from the peritoneum 3 d after T cell treatment. (**A**) F4/80$^+$ cells (positive fraction), F4/80$^-$ cells (negative fraction), or both F4/80$^+$ and F4/80$^-$ cells were cultured for 24 h, and cell-free supernatant was assayed for NO production by Griess reagent ($n = 8$). (**B**) F4/80^{hi} and F4/80^{lo} peritoneal cells were assessed for iNOS expression by intracellular flow cytometry. $^{***}p < 0.001$ (versus F4/80^{hi} chNKG2D), $^{*}p < 0.05$ (versus F4/80^{lo} chNKG2D). (**C**) F4/80$^+$ cells isolated from T cell-treated mice were cultured with ID8 tumor cells in the presence or absence of the iNOS inhibitor L-nil to determine the effect of NO blockade on macrophage cytotoxicity of tumor cells ($n = 8$). (**D**) Tumor-bearing mice were treated with chNKG2D or wtNKG2D T cells on day 35. Mice were injected i.p. with L-nil for 1 wk beginning on the day of T cell treatment. The number of solid tumors on the peritoneal wall and the number of tumor cells in ascites was determined 3 wk after T cell transfer. Cumulative results and SD of at least two independent experiments are shown. $^{***}p < 0.001$, $^{*}p < 0.05$ (versus chNKG2D + PBS), $^{\dagger}p < 0.01$ (versus wtNKG2D + PBS).

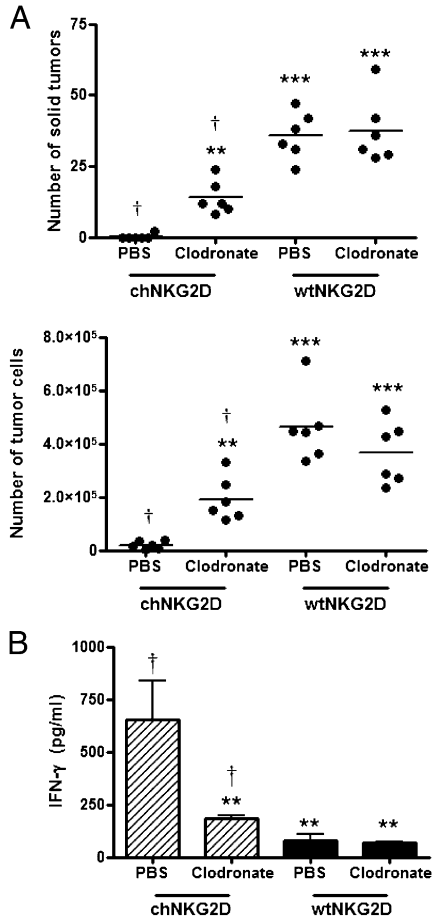

FIGURE 7. ChNKG2D T cell treatment requires host macrophages for tumor elimination. Tumor-bearing mice were treated with clodronate liposomes or PBS liposomes 2 d before CAR T cell transfer. Tumor burden was assessed 3 wk after T cell therapy. (**A**) The number of solid tumors on the peritoneal wall and the number of free tumor cells in the ascites was determined. (**B**) Peritoneal cells were cultured for 24 h, and cell-free supernatants were assayed for IFN-γ production. Representative data and SD of two independent experiments are shown. $^{***}p < 0.001$, $^{**}p < 0.01$ (versus chNKG2D + PBS), $^{\dagger}p < 0.05$ (versus wtNKG2D + PBS).

anti-tumor immunity to destroy the tumor (39). We show that IFN-γ and GM-CSF secreted by CAR-expressing T cells exert unique and synergistic effects on host macrophages, which are likely responsible for the increased therapeutic benefit of these cytokines after adoptive T cell therapy. Together, IFN-γ and GM-CSF function in concert to regulate endogenous macrophage recruitment, tumoricidal activity, and activation of an endogenous anti-tumor immune response. Newly recruited F4/80$^+$ cells constitute a large percentage of cellular infiltrate that enters the tumor after chNKG2D T cell-induced inflammation (18). The profile of cell surface markers and production of inflammatory cytokines by macrophages indicates that newly recruited F4/80^{lo}Ly-6C$^+$

monocytes differentiate into inflammatory macrophages (M1) with anti-tumor functions (22, 40). Few of these cells expressed Ly6G, a molecule associated with granulocytic cells, but they expressed Ly6C, which is associated with monocytic myeloid cells. Ly-6C^{hi} inflammatory monocytes have been shown to have a dependency on the chemokine receptors CCR2 and CCR5 for recruitment to sites of inflammation (20). CCR2 deficiency reduced, but did not completely ablate, the increase in myeloid cell trafficking to the tumor microenvironment after chNKG2D T cell treatment. The partial ablation may be due to recruitment of myeloid cells through another chemokine receptor, such as CCR5, at the same time. It was previously shown that chNKG2D T cell treatment increased the amount of both CCL2 and CCL5 in the tumor microenvironment (18). Treatment with GM-CSF– and IFN-γ–deficient T cells demonstrated that GM-CSF was more important than IFN-γ to increase the total chemokine amounts in the peritoneum. Thus, GM-CSF from adoptively transferred CAR T cells induces chemokine production that increases myeloid cell recruitment.

Although IFN-γ was not important for the recruitment of F4/80^{hi}Ly-6C^{+} myeloid cells to the tumor microenvironment, its secretion by adoptively transferred CAR T cells was important in the activation of both newly recruited myeloid cells and local TAMs. These data support a report showing that IFN-γ reverses the protumoral properties of TAMs and that their re-education promotes regression of advanced tumors (4). The activation of macrophages through T cell secretion of IFN-γ and GM-CSF may trigger a feedback loop in which these macrophages promote endogenous T cell IFN-γ and GM-CSF secretion to maintain macrophage proinflammatory activity. The sustained endogenous IFN-γ response for up to 8 wk after chNKG2D T cell treatment may be initiated and maintained by these macrophages (17).

The requirement of IFN-γ, but not GM-CSF, to activate F4/80^{lo}Ly-6C^{+} macrophages may be explained by differences in cytokine activation of signaling pathways and transcription factors that regulate monocyte to macrophage maturation and activation (41–43). STAT1 has been shown to function as a central regulator of inflammatory macrophage activation. Because IFN-γ potently induces STAT1 activation during maturation, it may be critical in the activation of F4/80^{lo}Ly-6C^{+} macrophages recruited to the peritoneum. GM-CSF secreted by CAR-expressing T cells may not be an important signal in F4/80^{lo}Ly-6C^{+} macrophage activation because it only weakly induces STAT1 activation during macrophage maturation (44).

The increase in IL-12p40 production after treatment with chNKG2D T cells may contribute to the IFN-γ response by stimulating NK cell production of IFN-γ and inducing a Th1 response to promote endogenous anti-tumor immunity (33). The increased expression of IL-12 after chNKG2D T cell treatment was dependent on their production of both IFN-γ and GM-CSF. However, GM-CSF deficiency had a greater impact on IL-12p40 production compared with T cell-derived IFN-γ. The greater dependence on GM-CSF may be attributed to the cytokine's preference for priming IL-12p40 production, whereas IFN-γ favors IL-12p35 production over p40 (45–47). Despite its preference for IL-12p35, IFN-γ has been shown to prime the IL-12p40 gene promoter, which may be the reason for the suboptimal IL-12p40 response elicited by IFN-γ–deficient CAR T cells (48). Both chNKG2D T cell-derived IFN-γ and GM-CSF were required to activate endogenous macrophages and enhance stimulation of Ag-specific T cell proliferation. The enhanced Ag processing and presenting abilities of macrophages after chNKG2D T cell therapy in concert with increased IL-12p40 may be responsible for the increase in endogenous T cell activation and trafficking of Ag-specific CD8^{+} T cells to the tumor site (17, 18).

In addition to upregulating IL-12, chNKG2D T cell therapy increased macrophage production of NO. NO may prevent tumor metastasis and synergize with IL-12 to enhance NK cell IFN-γ secretion and cytotoxicity (49, 50). The enhanced NO-mediated macrophage cytotoxicity of tumor cells after CAR T cell therapy is consistent with previous data showing that macrophages lyse ID8 tumor cells through secretion of NO (4, 51). It was previously shown that adoptive T cell-derived IFN-γ, but not GM-CSF, was responsible for the increase in NO production (18). Thus, macrophage NO cytotoxicity against ID8 tumor cells is IFN-γ dependent.

The impaired ability of CAR T cell therapy to eliminate tumors in the absence of macrophages (clodronate liposomes) demonstrates the importance of host myeloid cells in tumor destruction. Although macrophages were necessary for complete tumor elimination, chNKG2D T cell treatment significantly inhibited tumor growth in the absence of macrophages. The lower tumor burden seen in mice treated with clodronate liposomes and CAR T cells may be attributed to chNKG2D T cell killing of tumor cells through perforin and granzymes (17). CAR T cell therapy may activate macrophages to enhance host immunity against tumor Ags that are freed upon tumor cell lysis. The inability of CAR T cell therapy to initiate host immunity in the absence of macrophages likely contributes to the impaired ability of CAR T cell therapy to eliminate tumor. Because host NK cells and T cells secrete higher amounts of IFN-γ after chNKG2D T cell treatment, the lower amount of IFN-γ produced at the tumor site in the absence of host macrophages corroborates the role of these local macrophages in initiating host immunity (18). In addition, the greater effect of macrophage depletion on tumor elimination compared with iNOS inhibition indicates additional roles for macrophages in tumor elimination independent of NO-mediated killing.

The reduction in macrophage secretion of IL-10 after treatment with chNKG2D T cells may be due to negative regulation of IL-10 production by IFN-γ (52). The decrease in the total amount of IL-10 in the tumor microenvironment is likely a combination of reduced macrophage secretion of IL-10 and lysis of NKG2D ligand-expressing Foxp3^{+} regulatory T cells capable of producing IL-10 (18). Limiting of IL-10 production likely enhances tumor-specific CTL responses and inhibits tumor growth (53, 54). In addition to reduced IL-10 secretion, macrophages produced less IL-6 after treatment with chNKG2D T cells (Fig. 5A). In ovarian cancer, Th17 cytokines have been shown to contribute to tumor pathogenesis (55, 56). The reduction in global and macrophage-derived IL-6 may prevent the induction of a Th17 response and enhance the differentiation and maturation of APCs (18, 57).

The data from this study demonstrate that effector cytokines secreted by adoptively transferred CAR T cells induced changes in myeloid cell recruitment and function to transform the tumor microenvironment and inhibit ovarian tumor growth. The results demonstrate both the unique and redundant effects of T cell-derived IFN-γ and GM-CSF on tumor-associated myeloid cells and how these cytokines shape subsequent endogenous immunity in a tumor-bearing host. The mechanism by which adoptively transferred T cell-derived effector cytokines shape endogenous immunity via effects on local myeloid cells should be considered more broadly in optimizing adoptive T cell therapies for the treatment of cancer.

Acknowledgments

We thank DartLab for helpful assistance in Luminex analysis (Norris Cotton Cancer Center, Lebanon, NH), Dr. Mitsuhiro Iyori for technical assistance and advice, Dr. Brent Berwin for thoughtful suggestions on the manuscript,

and the National Institutes of Health Biological Resource Branch for providing recombinant human IL-2.

Disclosures

The chimeric NKG2D technology used in this study is licensed to Celdara Medical LLC. C.L.S. and Celdara are developing the technology for clinical use. If they are successful, C.L.S. will receive compensation. The arrangement is under compliance with Dartmouth College. The other authors have no financial conflicts of interest.

References

1. Gabrilovich, D. I., and S. Nagaraj. 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* 9: 162–174.
2. Mantovani, A., S. Sozzani, M. Locati, P. Allavena, and A. Sica. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 23: 549–555.
3. Ostrand-Rosenberg, S. 2008. Immune surveillance: a balance between protumor and antitumor immunity. *Curr. Opin. Genet. Dev.* 18: 11–18.
4. Hagemann, T., T. Lawrence, I. McNeish, K. A. Charles, H. Kulbe, R. G. Thompson, S. C. Robinson, and F. R. Balkwill. 2008. "Re-educating" tumor-associated macrophages by targeting NF-kappaB. *J. Exp. Med.* 205: 1261–1268.
5. Mosser, D. M., and J. P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8: 958–969.
6. DeNardo, D. G., P. Andreu, and L. M. Coussens. 2010. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev.* 29: 309–316.
7. Biswas, S. K., and A. Mantovani. 2010. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol.* 11: 889–896.
8. Sadelain, M., R. Brentjens, and I. Rivière. 2009. The promise and potential pitfalls of chimeric antigen receptors. *Curr. Opin. Immunol.* 21: 215–223.
9. Sadelain, M., I. Rivière, and R. Brentjens. 2003. Targeting tumours with genetically enhanced T lymphocytes. *Nat. Rev. Cancer* 3: 35–45.
10. Barber, A., T. Zhang, and C. L. Sentman. 2008. Immunotherapy with chimeric NKG2D receptors leads to long-term tumor-free survival and development of host antitumor immunity in murine ovarian cancer. *J. Immunol.* 180: 72–78.
11. Brentjens, R. J., J. B. Latouche, E. Santos, F. Marti, M. C. Gong, C. Lyddane, P. D. King, S. Larson, M. Weiss, I. Rivière, and M. Sadelain. 2003. Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. *Nat. Med.* 9: 279–286.
12. Ahmed, N., M. Ratnayake, B. Savoldo, L. Perlaky, G. Dotti, W. S. Wels, M. B. Bhattacharjee, R. J. Gilbertson, H. D. Shine, H. L. Weiss, et al. 2007. Regression of experimental medulloblastoma following transfer of HER2-specific T cells. *Cancer Res.* 67: 5957–5964.
13. Barber, A., T. Zhang, L. R. DeMars, J. Conejo-Garcia, K. F. Roby, and C. L. Sentman. 2007. Chimeric NKG2D receptor-bearing T cells as immunotherapy for ovarian cancer. *Cancer Res.* 67: 5003–5008.
14. Zhang, T., A. Barber, and C. L. Sentman. 2007. Chimeric NKG2D modified T cells inhibit systemic T-cell lymphoma growth in a manner involving multiple cytokines and cytotoxic pathways. *Cancer Res.* 67: 11029–11036.
15. Zhang, T., B. A. Lemoi, and C. L. Sentman. 2005. Chimeric NK-receptor-bearing T cells mediate antitumor immunotherapy. *Blood* 106: 1544–1551.
16. Zhang, T., A. Barber, and C. L. Sentman. 2006. Generation of antitumor responses by genetic modification of primary human T cells with a chimeric NKG2D receptor. *Cancer Res.* 66: 5927–5933.
17. Barber, A., and C. L. Sentman. 2009. Chimeric NKG2D T cells require both T cell- and host-derived cytokine secretion and perforin expression to increase tumor antigen presentation and systemic immunity. *J. Immunol.* 183: 2365–2372.
18. Barber, A., A. Rynda, and C. L. Sentman. 2009. Chimeric NKG2D expressing T cells eliminate immunosuppression and activate immunity within the ovarian tumor microenvironment. *J. Immunol.* 183: 6939–6947.
19. Van Rooijen, N., and A. Sanders. 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J. Immunol. Methods* 174: 83–93.
20. Tacke, F., D. Alvarez, T. J. Kaplan, C. Jakubzick, R. Spanbroek, J. Llodra, A. Garin, J. Liu, M. Mack, N. van Rooijen, et al. 2007. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J. Clin. Invest.* 117: 185–194.
21. Nibbering, P. H., P. C. Leijh, and R. van Furth. 1987. Quantitative immunocytochemical characterization of mononuclear phagocytes. II. Monocytes and tissue macrophages. *Immunology* 62: 171–176.
22. Geissmann, F., M. G. Manz, S. Jung, M. H. Sieweke, M. Merad, and K. Ley. 2010. Development of monocytes, macrophages, and dendritic cells. *Science* 327: 656–661.
23. Hart, K. M., S. P. Bak, A. Alonso, and B. Berwin. 2009. Phenotypic and functional delineation of murine CX(3)CR1 monocyte-derived cells in ovarian cancer. *Neoplasia* 11: 564–573.
24. Scarlett, U. K., J. R. Cubillos-Ruiz, Y. C. Nesbeth, D. G. Martinez, X. Engle, A. T. Gewirtz, C. L. Ahonen, and J. R. Conejo-Garcia. 2009. In situ stimulation of CD40 and Toll-like receptor 3 transforms ovarian cancer-infiltrating dendritic cells from immunosuppressive to immunostimulatory cells. *Cancer Res.* 69: 7329–7337.
25. Hagemann, T., J. Wilson, F. Burke, H. Kulbe, N. F. Li, A. Plüddemann, K. Charles, S. Gordon, and F. R. Balkwill. 2006. Ovarian cancer cells polarize macrophages toward a tumor-associated phenotype. *J. Immunol.* 176: 5023–5032.
26. Iellem, A., M. Mariani, R. Lang, H. Recalde, P. Panina-Bordignon, F. Sinigaglia, and D. D'Ambrosio. 2001. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. *J. Exp. Med.* 194: 847–853.
27. Curiel, T. J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, et al. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* 10: 942–949.
28. Carmeliet, P., Y. Dor, J. M. Herbert, D. Fukumura, K. Brusselmans, M. Dewerchin, M. Neeman, F. Bono, R. Abramovitch, P. Maxwell, et al. 1998. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 394: 485–490.
29. Gabrilovich, D. 2004. Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nat. Rev. Immunol.* 4: 941–952.
30. Watson, G. A., and D. M. Lopez. 1995. Aberrant antigen presentation by macrophages from tumor-bearing mice is involved in the down-regulation of their T cell responses. *J. Immunol.* 155: 3124–3134.
31. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* 179: 1109–1118.
32. Hamilton, J. A. 2008. Colony-stimulating factors in inflammation and autoimmunity. *Nat. Rev. Immunol.* 8: 533–544.
33. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3: 133–146.
34. Sica, A., A. Saccani, B. Bottazzi, N. Polentarutti, A. Vecchi, J. van Damme, and A. Mantovani. 2000. Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. *J. Immunol.* 164: 762–767.
35. Sica, A., P. Larghi, A. Mancino, L. Rubino, C. Porta, M. G. Totaro, M. Rimoldi, S. K. Biswas, P. Allavena, and A. Mantovani. 2008. Macrophage polarization in tumour progression. *Semin. Cancer Biol.* 18: 349–355.
36. Grabstein, K. H., D. L. Urdal, R. J. Tushinski, D. Y. Mochizuki, V. L. Price, M. A. Cantrell, S. Gillis, and P. J. Conlon. 1986. Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. *Science* 232: 506–508.
37. Spitalny, G. L., and E. A. Havell. 1984. Monoclonal antibody to murine gamma interferon inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. *J. Exp. Med.* 159: 1560–1565.
38. Aruga, A., S. Shu, and A. E. Chang. 1995. Tumor-specific granulocyte/macrophage colony-stimulating factor and interferon gamma secretion is associated with in vivo therapeutic efficacy of activated tumor-draining lymph node cells. *Cancer Immunol. Immunother.* 41: 317–324.
39. Nagoshi, M., P. S. Goedegebuure, U. L. Burger, N. Sadanaga, M. P. Chang, and T. J. Eberlein. 1998. Successful adoptive cellular immunotherapy is dependent on induction of a host immune response triggered by cytokine (IFN-gamma and granulocyte/macrophage colony-stimulating factor) producing donor tumor-infiltrating lymphocytes. *J. Immunol.* 160: 334–344.
40. Martinez, F. O., L. Helming, and S. Gordon. 2009. Alternative activation of macrophages: an immunologic functional perspective. *Annu. Rev. Immunol.* 27: 451–483.
41. Hagemann, T., S. K. Biswas, T. Lawrence, A. Sica, and C. E. Lewis. 2009. Regulation of macrophage function in tumors: the multifaceted role of NF-kappaB. *Blood* 113: 3139–3146.
42. Ohmori, Y., and T. A. Hamilton. 2001. Requirement for STAT1 in LPS-induced gene expression in macrophages. *J. Leukoc. Biol.* 69: 598–604.
43. Vakkila, J., R. A. Demarco, and M. T. Lotze. 2008. Coordinate NF-kappaB and STAT1 activation promotes development of myeloid type 1 dendritic cells. *Scand. J. Immunol.* 67: 260–269.
44. Coccia, E. M., N. Del Russo, E. Stellacci, U. Testa, G. Marziali, and A. Battistini. 1999. STAT1 activation during monocyte to macrophage maturation: role of adhesion molecules. *Int. Immunol.* 11: 1075–1083.
45. Trinchieri, G. 1998. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv. Immunol.* 70: 83–243.
46. Hayes, M. P., J. Wang, and M. A. Norcross. 1995. Regulation of interleukin-12 expression in human monocytes: selective priming by interferon-gamma of lipopolysaccharide-inducible p35 and p40 genes. *Blood* 86: 646–650.
47. Sweet, M. J., C. C. Campbell, D. P. Sester, D. Xu, R. C. McDonald, K. J. Stacey, D. A. Hume, and F. Y. Liew. 2002. Colony-stimulating factor-1 suppresses responses to CpG DNA and expression of toll-like receptor 9 but enhances responses to lipopolysaccharide in murine macrophages. *J. Immunol.* 168: 392–399.
48. Ma, X., J. M. Chow, G. Gri, G. Carra, F. Gerosa, S. F. Wolf, R. Dzialo, and G. Trinchieri. 1996. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *J. Exp. Med.* 183: 147–157.
49. Diefenbach, A., H. Schindler, M. Röllinghoff, W. M. Yokoyama, and C. Bogdan. 1999. Requirement for type 2 NO synthase for IL-12 signaling in innate immunity. *Science* 284: 951–955.
50. Weiss, J. M., L. A. Ridnour, T. Back, S. P. Hussain, P. He, A. E. Maciag, L. K. Keefer, W. J. Murphy, C. C. Harris, D. A. Wink, and R. H. Wiltrout. 2010. Macrophage-dependent nitric oxide expression regulates tumor cell detachment and metastasis after IL-2/anti-CD40 immunotherapy. *J. Exp. Med.* 207: 2455–2467.

51. Vicetti Miguel, R. D., T. L. Cherpes, L. J. Watson, and K. C. McKenna. 2010. CTL induction of tumoricidal nitric oxide production by intratumoral macrophages is critical for tumor elimination. *J. Immunol.* 185: 6706–6718.
52. Hu, X., P. K. Paik, J. Chen, A. Yarilina, L. Kockeritz, T. T. Lu, J. R. Woodgett, and L. B. Ivashkiv. 2006. IFN-gamma suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins. *Immunity* 24: 563–574.
53. Curiel, T. J. 2007. Tregs and rethinking cancer immunotherapy. *J. Clin. Invest.* 117: 1167–1174.
54. Saraiva, M., and A. O'Garra. 2010. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* 10: 170–181.
55. Langowski, J. L., X. Zhang, L. Wu, J. D. Mattson, T. Chen, K. Smith, B. Basham, T. McClanahan, R. A. Kastelein, and M. Oft. 2006. IL-23 promotes tumour incidence and growth. *Nature* 442: 461–465.
56. Kryczek, I., S. Wei, L. Zou, S. Altuwaijri, W. Szeliga, J. Kolls, A. Chang, and W. Zou. 2007. Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. *J. Immunol.* 178: 6730–6733.
57. Menetrier-Caux, C., G. Montmain, M. C. Dieu, C. Bain, M. C. Favrot, C. Caux, and J. Y. Blay. 1998. Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. *Blood* 92: 4778–4791.

Corrections

Barber, A., T. Zhang, and C. L. Sentman. 2008. Immunotherapy with chimeric NKG2D receptors leads to long-term tumor-free survival and development of host antitumor immunity in murine ovarian cancer. *J. Immunol.* 180: 72–78.

Barber, A. and C. L. Sentman. 2009. Chimeric NKG2D T cells require both T cell- and host-derived cytokine secretion and perforin expression to increase tumor Ag presentation and systemic immunity. *J. Immunol.* 183: 2365–2372.

Barber, A., A. Rynda, and C. L. Sentman. 2009. Chimeric NKG2D expressing T cells eliminate immunosuppression and activate immunity within the ovarian tumor microenvironment. *J. Immunol.* 183: 6939–6947.

Spear, P., A. Barber, A. Rynda-Apple, and C. L. Sentman. 2012. Chimeric antigen receptor T cells shape myeloid cell function within the tumor microenvironment through IFN-γ and GM-CSF. *J. Immunol.* 188: 6389–6398.

We recently discovered through single-nucleotide polymorphism analysis that the GM-CSF–deficient mice used in these studies were not backcrossed onto the C57BL/6 (B6) background and appear to be a B6/129/FVB genetic mix. These mice have the MHC region of B6 origin. Because it is theoretically possible that the other genes in this mixed strain could account for some of the phenotypes we observed using these mice, we have done several experiments to test this hypothesis. We have performed in vivo experiments on acute chimeric Ag receptor (CAR) T cell effects and anti-tumor efficacy studies using 129/Sv, (B6 × 129/Sv)F1, or C3H as sources of CAR T cells, and these F1 and allogeneic CAR T cells gave similar readouts as B6 CAR T cells in the same experiments with B6 recipients. GM-CSF–deficient mice as hosts in the ID8 ovarian tumor model or the RMA-RG lymphoma model resulted in a similar outcome as when B6 CAR T cells are used in B6 hosts. These data suggest that different background genes do not affect the outcomes in these types of experiments. However, we cannot rule out that a unique combination of genes in this strain may have some effect, so we want the scientific community to be aware that the GM-CSF–deficient mouse strain used in these was of a mixed genetic background.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1490025