Combination of CD40 Agonism and CSF-1R Blockade Reconditions Tumor-Associated Macrophages and Drives Potent Antitumor Immunity

Karla R. Wiehagen1, Natasha M. Girgis1,2, Douglas H. Yamada1, Andressa A. Smith1, Szeman Ruby Chan1, Iqbal S. Grewal1, Michael Quigley1,3, and Raluca I. Verona1

Abstract

Efficacious antitumor immune responses must overcome multiple suppressive mechanisms in the tumor microenvironment to control cancer progression. In this study, we demonstrate that dual targeting of suppressive myeloid populations by inhibiting CSF-1/CSF-1R signaling and activation of antigen-presenting cells with agonist anti-CD40 treatment confers superior antitumor efficacy and increased survival compared with monotherapy treatment in preclinical tumor models. Concurrent CSF-1R blockade and CD40 agonism lead to profound changes in the composition of immune infiltrates, causing an overall decrease in immunosuppressive cells and a shift toward a more inflammatory milieu. Anti-CD40/anti-CSF-1R–treated tumors contain decreased tumor-associated macrophages and Foxp3+ regulatory T cells. This combination approach increases maturation and differentiation of proinflammatory macrophages and dendritic cells and also drives potent priming of effector T cells in draining lymph nodes. As a result, tumor-infiltrating effector T cells exhibit improved responses to tumor antigen rechallenge. These studies show that combining therapeutic approaches may simultaneously remove inhibitory immune populations and sustain endogenous antitumor immune responses to successfully impair cancer progression. Cancer Immunol Res; 5(12): 1109–21. ©2017 AACR.

Introduction

To escape detection by the immune system, tumors foster an immunosuppressive environment and produce signals that subdue antitumor immune responses (1). Tumor-associated macrophages (TAMs) are a heterogeneous population of myeloid cells that differentiate in the tumor microenvironment and become sensitized to tumor-derived suppressive signals (2). TAMs, in turn, act to prevent local immune responses by inefficiently presenting antigen and generating suppressive signals such as IL10, indoleamine-pyrrole 2,3-dioxygenase (IDO), and TGFβ that inhibit lymphocyte function and metabolism (3). Therefore, for adaptive immune responses to persist, tumor-infiltrating lymphocytes (TILs) must overcome a suppressive cytokine milieu and mechanisms of tolerance propagated by TAMs to successfully attack cancerous cells.

CSF-1 (M-CSF) is a cytokine that supports differentiation, proliferation, and function of monocyte and macrophage populations (4). In tumors, CSF-1 promotes immune suppression by expanding and promoting differentiation of myeloid-derived suppressor cells (MDSC) and alternatively activated MHC IIlo TAMs that express its cognate receptor, CSF-1R (5, 6). High CSF-1R expression on infiltrating immune cells in the TME correlates with immune dysfunction and increased immunosuppression (7, 8), as well as poor prognosis in breast, ovarian, pancreatic cancers, and lymphoma (9–11). Although inhibiting the CSF-1/CSF-1R axis alone constrains tumor growth in preclinical models by partially decreasing TAMs and encouraging T-cell infiltration (12–16), it has often proven insufficient to clear tumors. Pairing CSF-1R blockade with chemotherapeutic agents or checkpoint inhibitors has enhanced efficacy of the monotherapy activity of CSF-1R (17).

CD40 is a highly conserved costimulatory receptor expressed on antigen-presenting cells (APCs) including dendritic cells (DC), macrophages, monocytes, B cells, as well as a number of non-hematopoietic cell types and some tumor cells (18, 19). The ligand for CD40 (CD40L) is expressed on activated T cells and B cells and, under inflammatory conditions, is upregulated on innate immune cells as well as endothelial and epithelial cells (20–24). CD40 ligation drives “licensing” (maturation and activation) of APCs, enabling effective antigen presentation and stimulation of CD4+ Th1 and CD8+ T cells. This is a key step in mounting antitumor responses, as supported by emerging preclinical and clinical data with agonist anti-CD40 in multiple tumor types (18, 20, 25). However, a number of studies have shown that potency of CD40 agonists can be enhanced by combinations with other immune modulatory therapies such as TLR agonists, cytokines including type I interferons and IL2, adoptive cell transfer, and chemotherapy (18, 26–30). 

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

Corresponding Author: Raluca I. Verona, Janssen R&D, 1400 McKean Road, Spring House, PA 94777. Phone: 215 628-5211; E-mail: rverona@its.jnj.com

doi: 10.1158/2326-6066.CIR-17-0258

©2017 American Association for Cancer Research.
also demonstrated that CD40 agonism following CSF-1R blockade leads to enhanced antitumor efficacy, suggesting these two therapies may cooperate when coadministered (30).

In this study, we sought to investigate whether dual targeting of suppressive myeloid populations via CSF-1/CSF-1R inhibition and APC activation with agonist anti-CD40 treatment confers enhanced antitumor efficacy compared with monotherapy treatments. Tumor profiling after anti-CD40/anti-CSF-1R treatment revealed significant changes in the composition of CD11b+ myeloid populations, decreased frequencies of regulatory Foxp3+ T cells (Treg), and increased infiltration of effector TILs. Analysis of the cytokine milieu in combination-treated tumors demonstrated an inflammatory TME, which corresponded with improved control of tumor burden. The combination of starving TAMs while activating APCs resulted in a cascade of proinflammatory responses and significant gains in effector T-cell responses and tumor clearance. Thus, the combination of anti-CD40 and anti-CSF-1R simultaneously removes inhibitory immune populations and drives endogenous antitumor immune responses to improve tumor clearance and significantly lengthen overall survival.

**Materials and Methods**

**Tumor cell lines**

CT26 and MC-38 cell lines were obtained from ATCC. Cell lines were authenticated and certified by ATCC (February 2015) and regularly tested by Analytical Biological Services, Inc. MAP/RAP PCR results (February 2016) were negative, and <1 EU/mL by limulus amebocyte lysate (LAL; ATCC, ABS). CT26 and MC-38 cell lines were cultured in vitro under conditions specified by the ATCC and passaged up to 3 times before implant.

**Syngeneic tumor studies**

In all in vivo studies, age-matched female mice from Charles River Laboratories were implanted subcutaneously with $5 \times 10^5$ cultured cells of the indicated tumor line suspended in PBS. Animal welfare (grooming, activity) was monitored daily, and animal weights and caliper measurements to calculate tumor volume ($L \times W / 2$) were collected at least twice per week until end of the study or until tumor burden limit approached 2,000 mm$^3$. All studies were conducted in accordance with Institutional Animal Care and Use Committees of Janssen R&D and in compliance with federal guidelines. Female Balb/c mice, 6 to 9 weeks old, bearing subcutaneous CT26 tumors 50 to 100 mm$^3$ in volume were randomized into groups of 10 to 12 mice and treated with 200 μg anti-IgG2a (clone 2A3; BioXcell), 100 μg anti-CD40 (clone FGK4.5; BioXcell), or 200 μg anti-CSF1R (clone AF598; BioXcell) per dose. The combination group received 100 μg FGK4.5 and 200 μg AF598 given at the same time but in separate injections on opposite sides of the abdomen (treatment was not premixed before injection). Doses were spaced four days apart for a total of three doses. CD8$^+$ T-cell depletion studies were carried out with randomized groups of mice bearing CT26 tumors, which were treated with 200 μg anti-CD8 (clone 2.43; BioXcell) or PBS intraperitoneally 24 hours before immunotherapy was initiated and concurrently with immunotherapy. Dosing began day 11 and continued every four days as described above. For MC-38 tumor studies, female C57BL/6 mice, 6 to 9 weeks old, harboring MC-38 tumors 75 to 125 mm$^3$ in volume were randomized and treated identically to the CT26 studies.

**Tumor and tissue processing**

Tumors were excised and dissociated into single-cell suspensions using the Miltenyi Tumor Dissociation Kit for murine tissues (catalog no. 130-096-730) per the manufacturer’s instructions. Samples were filtered and mechanically processed through 70-μm nylon filters. The following draining lymph nodes (dLN) were pooled per mouse: ipsilateral brachial, axillary, and inguinal lymph nodes. Spleens or pooled dLNs were processed through 70-μm nylon filters with syringe plungers. Samples were stained for flow cytometry the same day, and remaining suspensions were stored on ice at 4°C overnight for subsequent analyses.

**Flow cytometry and antibodies**

For flow cytometry profiling, 2 × 10$^6$ tumor samples or 1 × 10$^6$ lymphoid tissue samples were plated in tissue culture nontreated polystyrene round bottom 96-well plates (Corning). Viability was assessed by Live/Dead Fixable Aqua Dead Cell Stain (ThermoFisher). Antibody cocktails were assembled with the following antibodies:

- **Myeloid stains.** FITC-CD206 (clone C66852), PE-IL4Rx (clone L105F8), PE-CD115 (clone AF598), PE-NOS2 (clone SC1B52), PE-CCR7 (clone 4B12), PE-IL10 (clone 1B1.3a), PE-IIlO (clone JES3-12G8), PE-IL6 (clone MP5-20F3), PE-CD103 (clone 2E7), PerCP-Cy5.5-F4/80 (clone BM8), PerCP-Cy5.5-CD80 (clone 16-10A1), PerCPCy5.5-TNFR (clone MP6-XT22), PE-Cy7-CD103 (clone 2E7), APC-IDO (clone 2E2/IDO1), APC-TGF/LAP (clone TW7-16B4), APC-CD86 (clone GL-1), APC-F4/80 (clone BM8), AF700-Ly6C (clone HK1.4), APCCy7-F-AI-E (MHc II; clone M5/114.15.2), BV421-Ly6G (clone 1A8), BV421-TGF/LAP (clone TW7-16B4), BV605-CD24 (clone 30-F1), BV650- or BV711-CD11b (clone M1/70), BV786-CD11c (clone N418), BV395-CD45 (clone 30-F11), and dump stain BV711 for CD90.2 (clone 53-2.1) or TCRβ (clone H57-597), Siglec-F (clone E50-2440), and CD62L (clone V65).

- **Lymphoid stains.** FITC-Granzyme B (clone GB11), FITC-CD8 (clone 53-6.7), PE-IFNy (clone XMG1.2), PE-Tbet (clone 4B10), PerCP-Cy5.5-CD4 (clone GK1.5), PECy7-Foxp3 (clone FJK-16s), PECy7-Eomes (clone Dan11mag), APC-IFNy (clone XMG1.2), APC-TIM3 (clone RMT3-23 or B8.2C12), AF700-CD90.2 (clone 53-2.1), AF700-CD4 (clone GK1.5), AF700-CD44 (clone 1M7), APCCy7-CD90.2 (clone 53-2.1), APCCy7-CD4 (clone GK1.5), Pacific Blue-Foxp3 (clone FJK-16s), BV605-PD-1 (clone 2F.1A12), BV605-CD4, BV711-dump, BV786-CD8, BV786-CD62L, BV395-CD45 (clone 30-F11), and BV711 for CD11b, CD11c, CD49b (clone DX5), 68CR, and Siglec-F. AH1-tetramer: PE-conjugated H-2Ld MuLV-gp70 tetramer (SP5Y-VHYQE; MBL International Corporation).

All antibodies were sourced from BD Biosciences, Biolegend, R&D systems, or ebioscience. Intracellular staining was performed after cellular fixation and permeabilization using either the BD Cell Fix (catalog no. 554714) or ebioscience Transcription Factor/ Foxp3 staining buffer set (catalog no. 00-5523-00) according to the manufacturer’s instructions. All flow cytometry data were collected on BD Fortessa instruments and analyzed by FlowJo software v9.9.4 (TreeStar).

**Ex vivo functional assays**

**T-cell stimulation.** Tumor and lymphoid tissues were processed into single-cell suspensions, and $2 \times 10^6$ tumor samples or...
1 × 10^6 lymphoid tissue samples were plated in tissue culture nontreated polystyrene round bottom 96-well plates (Corning). Stimulation cocktail containing BD GolgiPlug (catalog no. 555029) and BD GolgiStop (catalog no. 554724) was prepared per manufacturer’s instruction (final concentration 1 μL/mL for each), with or without AH11 peptide (final concentration of 5 μg/mL). Cell stimulation cocktail (500×; catalog no. 00-4970-93; eBiosciences) served as the positive control. All conditions were incubated at 37°C for four hours.

**TAM intracellular staining.** Tumor suspensions were plated 2 × 10^6 per well with GolgiStop and GolgiPlug as stated above for four hours at 37°C. Cells were surface-stained, fixed, and permeabilized. Samples were then stained intracellularly for IL6, TNFα, and nitric oxide synthase 2 (NOS2, iNOS). TAMs (viable, Dump CD11b^+ Ly6G^- Ly6C^lo F4/80^+ ) were assessed for IL6, TNFα, and NOS2.

**Statistics and graphing with GraphPad Prism v6**

On all graphs, mean and standard error of the mean (SEM) are shown unless otherwise indicated. Differences between treatment groups measured at endpoint were determined using analysis of variance (ANOVA) followed by post hoc all-pairwise comparison tests with a Tukey adjustment for multiple comparisons. Survival analysis was performed using log-rank test. Statistical analyses were run using GraphPad Prism version 6 (GraphPad Software). Statistical significance was accepted at P < 0.05. All comparisons stated in results are versus isotype, unless otherwise specified.

**Results**

**Combined anti-CD40 and CSF-1R blockade improves tumor control and survival**

Tumor-infiltrating myeloid cells, including TAMs, suppress antitumor immunity, and these populations rely on CSF-1/CSF-1R signaling for survival, development, and differentiation. To determine which myeloid subsets depend on the CSF-1/CSF-1R axis, CSF-1R expression was assessed in distinct populations of myeloid cells in established, untreated CT26 tumors. We used a gating strategy previously described by Movahedi and colleagues, which uses Ly6C and MHC class II (MHC II) expression to discriminate populations in the Ly6C^hi and Ly6C^lo gates (31, 32). Specifically, in this analysis, MHC II expression distinguishes Ly6C^lo monocytes (Ly6C^lo MHC II^lo) from Ly6C^int TAMs (Ly6C^int MHC II^hi; Supplementary Fig. S1A). Ly6C^hi F4/80^- TAMs can be separated into proinflammatory MHC II^hi TAMs (Ly6C^hi F4/80^+ MHC II^hi) and alternatively activated, suppressive MHC II^lo macrophages (Ly6C^hi F4/80^- MHC II^lo; Supplementary Fig. S1A). Compared with isotype control staining, DCs, Ly6C^hi MHC II^hi TAMs, Ly6C^lo MHC II^hi, and MHC II^lo TAMs demonstrated increased surface expression of CD11b, whereas Ly6C^lo neutrophils maintained lower expression (Supplementary Fig. S1B). The highest frequencies of CSF-1R^hi cells were detected in Ly6C^lo MHC II^hi and Ly6C^int TAMs, with no significant differences in CSF-1R expression between MHC II^lo or MHC II^hi TAMs (Supplementary Fig. S1B). Together, these expression profiles correlate with data in the literature and suggest mature TAMs would be sensitive to CSF-1R blockade.

We hypothesized that blocking the CSF-1/CSF-1R pathway in CT26 tumors would result in significant changes in TAMs, and that coupling CSF-1R inhibition with CD40 agonism would result in enhanced antitumor activity. Anti–CSF-1R monotherapy failed to significantly control CT26 tumor burden compared with isotype control, whereas anti–CD40 agonist treatment delayed tumor growth (Fig. 1). Treatment with the combination resulted in the most significant control of tumor growth and extended survival (Fig. 1A–C and E). Similar results were seen in MC-38 tumors. Combination anti-CD40/anti–CSF-1R treatment elicited superior control of tumor growth compared with isotype or monotherapy-treated groups. As in CT26 studies, anti–CSF-1R monotherapy treatment failed to limit MC-38 tumor burden, whereas anti–CD40 monotherapy significantly controlled progression but not to the extent of combination treatment (Fig. 1F and G). Although these therapies targeted myeloid cells that expressed CD40 and CSF-1R, depletion of CD8^+ T cells in the CT26 model abrogated the antitumor efficacy of anti-CD40 monotherapy and combination treatment, as well as eliminated survival benefits observed with combination treatment (Fig. 1C–E), suggesting adaptive immune responses are critical for these responses.

**Combination immunotherapy results in reduced TAMs and alters myeloid populations**

To understand mechanisms underlying the improved efficacy and survival mediated by anti-CD40 and anti–CSF-1R therapy, we profiled the immune composition of tumors harvested at two time points during treatment: 24 hours after the first dose and 24 hours after the final dose. At both time points, groups that received anti-CD40 or combination anti-CD40/anti–CSF-1R exhibited similar frequencies of CD45^- hematopoietic cells compared with isotype controls. However, tumors treated with anti–CSF-1R monotherapy contained approximately 10% fewer CD45^- cells (Supplementary Fig. S2A). No changes were observed in the frequencies of CD11b^+ cells, targeted by anti-CD40 and anti–CSF-1R, at either time point across treatment groups (Supplementary Fig. S2B).

We used Ly6C and Ly6G expression to subdivide CD11b^+ populations into monocytes, macrophages, and neutrophils and identified broad changes in the immune infiltrates across groups. Combination-treated tumors exhibited the greatest reduction in Ly6G^- cells, which includes neutrophils and polymorphonuclear MDSGs, and Ly6C^lo F4/80^- TAMs (Fig. 2A and B). In contrast, Ly6C^hi cells increased significantly over isotype in all treatment groups. The greatest increase was seen in combination-treated tumors, which was also significantly greater than both monotherapies (Fig. 2B). According to this model of subset analysis, the Ly6C^hi fraction consists of both monocytes and Ly6C^int tumor–associated macrophages, but the accumulation of Ly6C^hi cells in combination-treated tumors was not due to proportionate increases of both cell types (Fig. 2C–E). Analysis of MHC II expression revealed a nonsignificant increase in frequencies of Ly6C^hi MHC II^lo
monocytes in monotherapy- and combination-treated tumors (Fig. 2D) and that increased Ly6C\textsuperscript{int} MHC II\textsuperscript{lo} TAMs were primarily responsible for the overall increase in Ly6C\textsuperscript{hi} TAMs detected after combination treatment (Fig. 2E). The most significant changes were found in combination-treated tumors, where 4-fold more Ly6C\textsuperscript{hi} macrophages accumulated over the course of treatment compared with isotype controls and were significantly higher than both monotherapies (Fig. 2E).

Approximately 70\% of CD11b\textsuperscript{+} Ly6G\textsuperscript{-} myeloid lineage cells in isotype-treated tumors consisted of Ly6C\textsuperscript{lo} TAMs, which were detected in both MHC II\textsuperscript{lo} and MHC II\textsuperscript{hi} populations (Fig. 2C, F and G). All treatment approaches decreased frequencies of MHC II\textsuperscript{lo} TAMs compared with the isotype group, with the fewest MHC II\textsuperscript{lo} TAMs detected after combination treatment (Fig. 2C and F). Relative to isotype controls, we observed a 33\%, 50\%, and 70\% reduction in MHC II\textsuperscript{lo} suppressive TAMs in anti-CD40, anti-CSF-1R, and combination-treated groups, respectively (Fig. 2F).

Frequencies of MHC II\textsuperscript{hi} TAMs in monotherapy groups were not significantly different from isotype-treated groups, whereas nearly half of MHC II\textsuperscript{hi} TAMs were reduced with combination treatment (Fig. 2G). Together, these data indicate that anti-CSF-1R and CD40 agonism differentially affect TAM and monocyte populations and that anti-CD40/anti-CSF-1R combination treatment significantly skews the composition of tumor-infiltrating CD11b\textsuperscript{+} myeloid cells.

**CD40 agonism or combination immunotherapy increases proinflammatory TAMs**

Low frequencies of Ly6C\textsuperscript{hi} TAM subsets led us to investigate the polarization status of the total Ly6C\textsuperscript{hi} F4/80\textsuperscript{+} TAM population. We first assessed expression of cell-surface markers associated with inflammatory MHC II\textsuperscript{lo} TAMs and alternatively activated MHC II\textsuperscript{lo} macrophages on total Ly6C\textsuperscript{hi} F4/80\textsuperscript{+} TAMs after treatment concluded. Typically, alternatively activated TAMs have high expression of IL4R\textalpha, MMR (CD206), MerTK, and IL10R, consistent with MHC II\textsuperscript{lo} TAM sensitivity to suppressive signals. TAMs from tumors treated with anti-CD40 or combination anti-CD40/anti-CSF-1R exhibited decreased expression of IL4R\textalpha, IL10R, and CD206 compared with anti-CSF-1R and isotype groups (Fig. 3A–D). Expression of inhibitory ligands, PD-L1 and PD-L2, did not change significantly across treatment groups in the majority of the experiments performed (Fig. 3E and F). In contrast, TAMs in anti-CD40 and combination-treated tumors expressed markers consistent with activation (CD83) and maturation (CD80 and CD86) of proinflammatory MHC II\textsuperscript{hi} macrophages (Fig. 3G–I), with highest expression observed in anti-CD40 and combination-treated groups, consistent with CD40 ligation-induced activation (ref. 33; Fig. 3G–I). Anti-CSF-1R monotherapy elicited increased expression of CD80 and CD83 markers compared with isotype controls, but did not affect activation markers in alternatively activated MHC II\textsuperscript{lo} TAMs. These data suggest that combination treatment aids polarization of remaining TAMs.
populations from the MHC IIlo suppressive TAM phenotype toward MHC IIhi proinflammatory TAMs.

Based on expression of markers affiliated with proinflammatory TAMs, we hypothesized that macrophages from anti-CD40 and combination anti–CD40/anti–CSF-1R would be primed to generate proinflammatory signals. Anti-CD40 and anti-CD40/anti–CSF-1R treatment induced increased frequencies of TNFα+ and NOS2+ TAMs compared with isotype- or anti–CSF-1R-treated groups (Fig. 3J–L). Latency-associated protein (LAP) was evaluated as a surrogate for the immunosuppressive cytokine TGFβ. Anti-CD40 and combination treatment decreased the fraction of TAMs poised to produce TGFβ in tumors, whereas anti–CSF-1R monotherapy was not appreciably different from isotype treatment (Fig. 3M).

IL10/IL10R signaling is associated with suppression of antitumor immune responses (34, 35). Reduced frequencies of IL10-producing TAMs were observed in all treated groups compared with isotype controls (Fig. 3N), with only the combination-
treated group reaching significance. Therefore, combination treatment reduced levels of LAP/TGFβ, IL10, and IL10R in suppressive TAM populations, increased frequencies of activated MHC IIhi macrophages, and skewed the TME toward a proinflammatory phenotype.

Combination therapy induces accumulation of activated CD103+ DCs in dLNs

Efficient antigen presentation by DCs is critical to priming effective antitumor T-cell responses, and CD40 agonism is known to drive DC activation and maturation (36, 37). In anti-CD40 or anti-CD40/anti-CSF-1R-treated tumors harvested after the end of treatment, CD83, CD80, and CD86 expression was increased on intratumoral DCs compared with isotype-treated DCs, whereas anti-CSF-1R monotherapy did not significantly change the activation status of these APCs (Fig. 4E). Lower frequencies of DCs (CD24hi MHC IIhi CD11c+) were observed in tumors from anti-CD40 and combination-treated groups compared with isotype controls 24 hours after start of treatment (Fig. 4A). We hypothesized that DCs could be trafficking to lymphoid tissues after activation via CD40 agonism and, therefore, examined dLNs 24 hours after the initial dose. Consistent with our hypothesis,
In dLN, CD103⁺ DCs express increased markers of activation and accumulate in dLN after one dose. Twenty-four hours after initial dosing, mice were sacrificed and tumors and dLNs were harvested. A and B, Frequency of DCs (CD24⁺ MHC II⁺ CD11c⁺) of total CD45⁺ infiltrates in (A) tumors or (B) dLNs. C and D, Frequency of CD103⁺ DCs of total DCs in (C) tumor or (D) dLNs. E, Expression of activation markers on intratumoral DCs at the conclusion of treatment. F-H, Expression of activation markers on DCs in the dLNs from animals treated with isotype (gray filled histogram), anti-CSF-1R (blue line), anti-CD40 (green line), or combination (red line). F, CCR7 expression and compiled frequencies of CCR7⁺ DC in dLNs. G and H, Expression and compiled gMFI of (G) CD80 or (H) CD86 on DC in dLNs. (A-H) *, P < 0.05; **, P < 0.01; ***, P < 0.0005; ****, P < 0.0001. (A-H) isotype (white), anti–CSF-1R (blue), anti-CD40 (green), combination (red). Significance between treatment groups measured at endpoint was determined using ANOVA followed by post hoc all-pairwise comparison tests with a Tukey adjustment for multiple comparisons. Data are representative of four experiments.
significant DC accumulation was observed in the dLNs of animals treated with anti-CD40 or combination therapy but not isotype or anti-CSF-1R monotherapy (Fig. 4B).

CD103+ DCs were reduced in tumors one day after anti-CD40 or combination treatment began but made up a significant fraction of total DCs in matched dLNs at the same time point (Fig. 4C and D). A higher proportion of these DCs in the dLNs expressed markers of migration and maturation: CCR7, CD83, CD80, and CD86 (Fig. 4F–H). Together, these findings demonstrate anti-CD40 agonism with CSF-1R blockade drives accelerated maturation and sustained activation of DCs and macrophages in tumors and increased frequencies of mature DCs in draining lymph nodes.

Enhanced CD8+ T-cell effector function in the dLNs of combination-treated mice

Although the individual treatments in this combination approach targeted myeloid cells, control of tumor growth by anti-CD40/anti-CSF-1R therapy was mitigated when CD8+ T cells were depleted in vivo. Therefore, adaptive immune responses are critical for tumor rejection mediated by anti-CD40/anti-CSF-1R therapy. Given the increased accumulation and activation of DCs in the dLNs of anti-CD40 and anti-CD40/anti-CSF-1R-treated mice, we next investigated whether T cells in dLNs and tumors mounted improved antitumor effector responses. After treatment concluded, increased frequencies of CD8+ T cells were detected in the dLNs of anti-CD40 and anti-CD40/anti-CSF-1R-treated animals but not after anti-CSF-1R treatment alone (Fig. 5A).

Because CD103+ DCs are capable of trafficking tumor antigen to dLNs (38) and increased CD103+ DCs were found in the dLNs of combination-treated animals, we sought to evaluate tumor antigen–specific CD8+ T-cell responses in dLNs. CT26 tumor cells express AH1 peptide, which is found in an envelope glycoprotein (gp70) of murine leukemia virus. Tetramer staining revealed an increased frequency of AH1-specific CD8+ T cells in the dLNs of anti-CD40/anti-CSF-1R-treated mice after treatment concluded (Fig. 5B), indicating that combination treatment expanded the tumor AH1-specific T cells.

Responses against AH1 serve as a surrogate measurement of tumor antigen–specific CD8+ T-cell activity. To assess T-cell function, CD8+ T cells from dLNs were rechallenged with AH1 peptide ex vivo. Only CD44hi effector CD8+ T cells from combination-treated mice, but not from either monotherapy, exhibited improved T-cell function on rechallenge (Fig. 5C–F). Significantly higher frequencies of IFNγ CD44hi or TNFα CD44hi CD8+ T cells were found in the dLNs of combination-treated animals compared with monotherapy or isotype groups (Fig. 5C–F).

Along with increased effector function, CD8+ T cells also demonstrated increased expression of activation markers and upregulation of checkpoint molecules PD-1, TIM-3, LAG-3, and TIGIT (Supplementary Fig. S3A and S3B). Patterns of increased PD-1 and TIM-3 coexpression on CD8+ T cells were similar in anti-CD40 and anti-CD40/anti-CSF-1R-treated groups, whereas <1% of CD8+ T cells expressed either PD-1 or TIM-3 in the dLNs of anti-CSF-1R- or isotype-treated groups. CD8+ T cells from anti-CD40 or combination-treated animals expressed multiple checkpoint markers, whereas <3% of CD8+ T cells expressed more than one of these receptors after anti-CSF-1R or isotype treatment (Supplementary Fig. S3B). More CD8+ T cells from the dLNs of combination-treated animals exhibited an activated cell-surface phenotype and increased expression of the T-box transcription factor (T-bet) and Eomesodermin (Eomes), two transcription factors critical for effector function (Supplementary Fig. S3C–S3F). Anti-CD40 treatment was sufficient to drive increased expression of these transcription factors relative to isotype and anti-CSF-1R-treated groups but not to the extent observed in combination-treated groups. Taken together, anti-CD40/anti-CSF-1R treatment triggers enhanced APC priming resulting in expanded antigen-specific effector CD8+ T cells in dLNs.

Combined immunotherapy increased CD8+ TILs and enhanced CD8+ effector responses

We next investigated how improved antigen responses in dLNs of treated animals correlated to effector lymphocyte responses in tumors. First, we assessed frequencies of total T cells of CD45+ infiltrating immune cells in tumors. After treatment concluded, anti–CD40/anti-CSF-1R tumors contained more T cells on average than isotype-treated tumors, whereas anti-CSF-1R and anti-CD40 monotherapies drove smaller but reproducible increases in T-cell frequencies (Fig. 5G). Within the Thy1+ fraction, CD8+ TIL frequencies were significantly increased across all treatment groups compared with isotype-treated tumors, with the most significant increase detected in the anti-CD40/anti-CSF-1R group that exhibited almost 40% more CD8+ TILs compared with isotype controls (Fig. 5H). Increased Thy1+ and CD8+ T cells resulted in increased CD8+ T cells within the total CD45+ immune infiltrate in tumors treated with anti-CD40/anti-CSF-1R therapy (Supplementary Fig. S2C). Moreover, the absolute number of CD8+ T cells was increased per gram of tumor after combination or anti-CD40 treatment (Supplementary Fig. S2F). These data demonstrate that more CD8+ T cells infiltrate tumors after CD40 agonism or CSF-1R blockade, but the combination is more effective than either monotherapy. However, unlike the findings in dLNs, the proportion of AH1 tetramer+ CD8+ cells was not significantly altered in tumors across treatment groups (Fig. 5I).

To determine whether intratumoral T-cell effector responses improved with combination immunotherapy, we assessed the phenotype and function of CD8+ TILs in CT26 tumors by measuring expression of activation markers in total CD8+ TILs and AH1 tetramer+ CD8+ T cells. Similar to CD8+ T cells in dLNs, activation markers and checkpoint molecules, PD-1, TIM-3, TIGIT, and LAG-3, were increased on CD8+ TILs after anti-CD40 or combination immunotherapy, relative to isotype-treated tumors (Supplementary Fig. S3G and S3H). Combination treatment led to increased frequencies of CD8+ TILs coexpressing PD-1 and TIM-3, and nearly half of CD8+ TILs coexpressed multiple checkpoint molecules, indicating enhanced effector activation (Supplementary Fig. S3H).

Restimulation with AH1 peptide ex vivo revealed that anti-CD40/anti-CSF-1R combination immunotherapy elicited significantly higher frequencies of antigen-specific CD8+ TILs primed to produce IFNγ, TNFα, or granzyme B in response to tumor antigen (Fig. 5I). A higher proportion of CD8+ TILs from combination-treated tumors were also able to mount polyfunctional responses in response to AH1 peptide (Fig. 5J). Together, these data suggest that anti-CD40/anti-CSF-1R immunotherapy enhances both the quality and quantity of CD8+ TILs.
Tumor-infiltrating regulatory T cells are decreased in tumors with combination treatment

Anti-CD40/anti-CSF-1R treatment resulted in increased frequencies and improved function of CD8+ T cells in dLNs. A, Frequency of CD8+ T cells in dLNs compiled from three independent experiments. n = 12 for each group. B, Frequency of AH1 tetramer+ CD8+ T cells in dLNs. C, Intracellular staining for IFNγ and TNFα after rechallenge ex vivo with AH1 peptide. Numbers within gates represent relative frequencies of total CD44hi CD8+ T cells that produced (D) IFNγ, (E) TNFα, or (F) IFNγ and TNFα in response to AH1 peptide rechallenge. A–F: *, p < 0.05; **, p < 0.01; ***, p < 0.005; ****, p < 0.0005. Significance was determined using ANOVA followed by post hoc all-pairwise comparison tests with a Tukey adjustment for multiple comparisons. Representative of four independent experiments. G, Compiled frequency of helper CD4+ T cells of total CD45+ immune infiltrates in tumors. n = 13 for each group. H, Compiled frequencies of CD8+ T cells in tumors of Thy1+ events. n = 13 for each group. I, Compiled frequency of AH1-tetramer antigen-specific CD8+ T cells of total Thy1+ events in tumors. n = 22 per group. J, Compiled frequencies of IFNγ+ or IFNγ+ TNFα+ or IFNγ+ TNFα+ or IFNγ+ TNFα+ or IFNγ+ TNFα+ or TNFα+ or Granzyme B+ CD8+ TILs after rechallenge with AH1 peptide. G–J: *, p < 0.05; **, p < 0.01; ***, p < 0.005; ****, p < 0.0005. A–J, Isotype (white), anti-CSF-1R (blue), anti-CD40 (green), combination (red). Significance was determined using ANOVA followed by post hoc all-pairwise comparison tests with a Tukey adjustment for multiple comparisons. G–J, Data are compiled from three studies. J, Data are representative of four experiments.

Tumor-infiltrating regulatory T cells are decreased in tumors with combination treatment

Anti-CD40/anti-CSF-1R treatment resulted in increased frequencies and improved function of CD8+ T cells, as well as decreased frequencies of Foxp3+ conventional CD4+ TILs. Although frequencies of CD4+ T cells were decreased in the immune infiltrate of anti-CD40 or combination-treated tumors, the number of helper T cells per gram of tumor was increased, reflecting overall increased immune infiltration induced by anti-CD40 or combination treatment (Supplementary Fig. S2F). Additionally, the remaining helper CD4+ Foxp3- T cells in both dLNs and tumors treated with anti-CD40/anti-CSF-1R exhibited significantly increased activation and effector function. After treatment concluded, more CD4+ T cells in combination-treated animals produced IFNγ, TNFα, and IL2 in unstimulated ex vivo cultures (Fig. 5A and B). Anti-CSF-1R monotherapy had a role in driving improved CD4+ helper responses compared with isotype controls (Fig. 6B).

Previous reports described decreased immunosuppressive Foxp3+ Tregs in tumor models after either anti-CSF-1R or anti-CD40 treatment (40, 41). We assessed whether similar changes were observed in this subset of CD4+ T cells after...
Consistent with published data, tumors treated with either anti–CSF-1R or anti-CD40 monotherapy consistently exhibited decreased frequencies of CD4+ Foxp3+ Tregs of total lymphocytes or total CD45+ immune cells after treatment concluded (Fig. 6C and E; Supplementary Fig. S2E), and the most dramatic decrease was seen after anti-CD40/anti-CSF-1R combination treatment, which had a higher ratio of helper CD4+ T cells to T regs in combination-treated animals. A 4-fold higher ratio of CD8+ TILs to Tregs was seen in combination-treated tumors compared with isotype controls (Fig. 6F). Although frequencies of AH1 tetramer+ CD8+ TILs did not significantly change, combination treatment resulted in a
significantly higher ratio of AH1-specific CD8+ TILs to Tregs (Fig. 6G). Together, these data suggest that helper CD4+ TILs and effector CD8+ TILs may be able to sustain improved antitumor functions due to the decreased Treg frequency in the TME.

The cytokine milieu is more inflammatory with combination treatment

Changes we observed in both myeloid and lymphoid populations demonstrate that anti-CD40/anti-CSF-1R immunotherapy resulted in significant alterations to the immune infiltrate in CT26 tumors. To determine the sum effect of the observed changes on the TME cytokine milieu, we measured cytokine analytes in homogenized tumors and normalized analyte concentration per gram of tumor harvested. Proinflammatory cytokines TNFα, IL6, IL23, and IL12 were increased in tumors that received anti-CD40 or combination immunotherapy (Supplementary Fig. S4A). In anti-CD40 or combination-treated tumors, increased GM-CSF was also observed, which supports MHC II+ TAM polarization. In contrast, levels of immunosuppressive cytokine IL10 were decreased in all treatment groups compared with isotype-treated tumors, including anti-CSF-1R treatment alone (Supplementary Fig. S4B). Mean levels of IL4 were also reduced ~30% in anti-CD40 and combination-treated tumors compared with isotype controls, but the amount of IL4 was relatively minimal in all groups (Supplementary Fig. S4B). These data suggest that the total effect of combination treatment skewed the TME toward increased inflammatory cytokine production and reduced suppressive signals. Together, the phenotype and functional analysis of TAMs, monocytes, DCs, and tumor-infiltrating T cells demonstrate that anti-CD40/anti-CSF-1R immunotherapy drives a cumulative polarization of the TME toward an antitumor, proinflammatory environment, resulting in improved immune responses, overall enhanced antitumor efficacy, and extended survival.

Discussion

In this report, we investigated the efficacy of combining CD40 agonism and CSF-1R blockade to modulate endogenous antitumor immunity and control tumor growth. We demonstrated enhanced inhibition of tumor growth and extended survival with combination treatment compared with monotherapies. The improved antitumor efficacy with anti-CD40/anti-CSF-1R therapy was characterized by significant changes to the immune infiltrate that disrupted suppressive immune mechanisms and promoted inflammatory innate and adaptive antitumor responses.

The most dramatic changes in the immune infiltrate after anti-CD40/anti-CSF-1R treatment were observed in macrophage and monocyte populations. Consistent with the highest CSF-1R expression on Ly6Ch TAMs, combining anti-CSF-1R inhibition and CD40 agonism resulted in significantly reduced frequencies of MHC IIhi and MHC IIlo TAMs in tumors. We observed a concomitant increase in MHC IIlo Ly6Glo macrophages, which suggested that combination therapy reduced the suppressive tumor-educated TAMs, while leaving newly differentiated, proinflammatory macrophages to repopulate the TME. The remaining macrophages in tumors had higher expression of costimulatory molecules CD80 and CD86, inflammatory cytokines, and lower levels of MHC IIlo markers, including IL10R. IL10 signaling, through its receptor IL10R, not only drives IL10 production by Foxp3+ Tregs, but also antagonizes IL1- and TNF-α-induced pathways and suppresses transcription of proinflammatory genes in a wide range of immune cells (34). Since increased expression of IL-10R is associated with poor control of tumor progression, the decreased IL-10R levels observed across cell types suggest that combination therapy supports resistance to IL-10-dependent suppression. Macrophages and myeloid subsets infiltrating the TME also demonstrated upregulated costimulatory molecules that potentially propelled effector T-cell responses within the tumor. Together, these data demonstrated broad changes in the balance of suppressive and inflammatory myeloid subsets functioning in tumors and illustrated how targeting CD40 pathways, together with CSF-1R inhibition, generated potent downstream effects beyond TAM depletion.

Although anti-CD40/anti-CSF-1R therapy does not directly target T cells, it has a profound effect on boosting T-cell responses. To investigate how combination treatment affected antigen-presenting cells responsible for initiating T-cell responses, we explored changes in DCs across treatment groups. We found CD40 agonism alone or in combination with anti-CSF-1R reduced frequencies of DCs in tumors but increased frequencies in dLNs. DCs exhibited an activated, mature APC phenotype with increased levels of MHC II, CD80, and CD86 in both tumor and dLNs. Studies from other groups have described CD103+ DCs immune surveillance and migration through nonlymphoid tissues. Upon receiving activating signals, migratory DCs traffic from surrounding tissues to dLNs to present antigens that prime lymphocytes. Accumulation of CD103+ DCs was most increased with combination therapy, which may indicate CD40 agonism improves activation of CD103+ DCs residing in tissues around tumor sites, enabling them to pick up and process tumor antigens for T-cell priming. Consistent with this hypothesis, AH1-specific CD8+ T-cell responses were increased in magnitude. Our observation that T cells in combination-treated mice exhibited greater levels of activation and function than those in the anti-CD40 monotherapy group indicates that skewing of the macrophage population by CSF-1R contributes to the overall antitumor response. A prominent effect of combination treatment included lower quantities of IL10. IL10 signaling in DCs potently inhibits maturation and antagonizes IL12 production, resulting in poor activation of T cells and increased promotion of Treg differentiation (34, 42). Alternatively, we hypothesized that blocking CSF-1R signals may alter the composition of DCs by reducing frequencies of suppressive tolerogenic DCs that rely on this cytokine for development and survival. Together, these direct and indirect effects of anti-CD40/anti-CSF-1R treatment may increase frequencies of mature immunogenic DCs and improve T-cell priming. Therefore, the quantity and quality of T-cell responses found in combination-treated animals could result from improved APCs that couple more efficient antigen presentation with increased expression of costimulatory molecules. Our data highlight how generating antitumor T-cell responses not only within the tumor, but also in local lymphoid tissues, may support long-lived adaptive immunity.

We found combination therapy boosted the function not only of CD8+ cytotoxic T cells but also of conventional CD4+ T cells. These activated helper CD4+ T cells exhibited an inflammatory Th1 differentiation profile, based on production of prototypical Th1 cytokines IFNγ and TNFα. The shift in inflammatory cytokines seen in combination-treated tumors is likely driven not only by myeloid and CD8+ effector activity but also by increased Th1 inflammatory innate and adaptive myeloid populations. Consistent with the highest CSF-1R expression on Ly6Ch TAMs, combining anti-CSF-1R inhibition and CD40 agonism resulted in significantly reduced frequencies of MHC IIhi and MHC IIlo TAMs in tumors. We observed a concomitant increase in MHC IIlo Ly6Glo macrophages, which suggested that combination therapy reduced the suppressive tumor-educated TAMs, while leaving newly differentiated, proinflammatory macrophages to repopulate the TME. The remaining macrophages in tumors had higher expression of costimulatory molecules CD80 and CD86, inflammatory cytokines, and lower levels of MHC IIlo markers, including IL10R. IL10 signaling, through its receptor IL10R, not only drives IL10 production by
effect function. CD40 ligand signaling has a critical role in preserving CD4+ T-cell help [37, 43]. Increased CD4+ helper T-cell function could be induced by enhanced antigen presentation and MHC–peptide complex stability via CD83 upregulation, costimulation with increased expression of CD80 and CD86, and IL12 production [20, 21, 37]. Decreased frequencies of Tregs could also contribute to the increased magnitude of CD4+ T-cell effector function in anti-CD40/anti–CSF-1R-treated tumors. Future investigation will focus on whether combination therapy directly reduces Treg numbers by stimulating Th1 differentiation or changing availability of cytokines critical for maintaining Tregs’ Foxp3 expression and transcriptional programs [44]. As suppressive TAMs were eradicated and more inflammatory cells infiltrated combination-treated tumors, we found lower levels of IL10 and IL4 and higher levels of IFNγ, TNFα, and TGFβ in tumors, which may lend to the instability of Foxp3+ Treg numbers and support differentiation of Th1 CD4+ T cells.

Combination therapy induced significant activation of lymphocytes, concomitant with upregulation of checkpoint receptors PD-1, TIM-3, TIGIT, and LAG-3. Although coinhibitory receptors normally contribute to contraction of immune responses following pathogen clearance, checkpoint molecules in the context of cancer and chronic infection are associated with T-cell exhaustion and immune dysfunction. A critical question emerging from these mechanistic data is how to reconcile improved antitumor efficacy with TILs that phenotypically resemble exhausted lymphocytes vulnerable to intratumoral suppressive mechanisms. T-cell exhaustion is not purely a product of expression of inhibitory receptors, but a complex, progressive syndrome that develops over time in environments with multiple obstacles, including reduced CD4+ T cell help, chronic exposure to antigen, and inhibitory signaling via checkpoint ligands and suppressive cytokines [45, 46]. Although coexpression of checkpoint receptors was elevated on both CD4+ and CD8+ T cells following combination therapy, we found that expression of checkpoint ligands, PD-L1 or PD-L2, was not significantly different in the anti-CD40/anti-CSF-1R group from isotype controls. It has also been shown that increased CD4+ T cell help preserves CD8+ T-cell effector functions during chronic infection or in cancer [47]. Therefore, exhaustion may be avoided despite upregulation of inhibitory receptors associated with immune dysfunction in cancer. Nonetheless, given the expression of numerous checkpoint receptors on T cells, antitumor efficacy of the combination of CD40 agonism and CSF-1R blockade may be further improved by including checkpoint blockade. Checkpoint blockade has complemented TAM-directed therapies in various tumor models [48–50], and future experiments will investigate whether including anti-PD-1 checkpoint blockade in combination with anti-CD40/anti–CSF-1R combination therapy will provide additional benefits.

The present studies demonstrated the advantage of combining CD40 agonism with CSF-1R blockade to produce the most effective tumor control and extend survival. Using a rigorous flow cytometry approach that enables in-depth characterization of distinct immune subsets, we have extensively profiled the phenotypic and functional changes in tumor-associated myeloid and lymphocyte populations in response to treatment. Previous reports have described the effects of CD40 agonist or anti–CSF1R monotherapy alone in murine tumor models by using the general phenotypic markers, CD11b and Gr-1, to characterize changes in the myeloid infiltrate. Our data improved the resolution on the heterogeneous myeloid populations that infiltrate tumors and detailed the manner in which anti-CD40/anti–CSF-1R immunotherapy altered endogenous immunity. Tumors treated with both anti-CD40 and anti–CSF-1R maintained features of both monotherapies, but the combination regimen increased the magnitude of changes in myeloid and lymphoid responses. Our data highlight how successful antitumor efficacy might require both the removal of suppressive populations and the delivery of activating signals for immune cells in the TME. The cumulative effect of CD40 agonism and CSF-1R inhibition conditions immune infiltrates to counteract immunosuppression in the tumor environment and successfully attack tumor cells.

Disclosure of Potential Conflicts of Interest
M. Quigley has ownership interest in Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: K.R. Wiehagen, N.M. Girgis, D.H. Yamada, I.S. Grewal, M. Quigley, R.I. Verona Development of methodology: K.R. Wiehagen, N.M. Girgis, D.H. Yamada Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.R. Wiehagen, N.M. Girgis Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.R. Wiehagen, N.M. Girgis, D.H. Yamada, A.A. Smith, S.R. Chan, I.S. Grewal, M. Quigley, R.I. Verona Writing, review, and/or revision of the manuscript: K.R. Wiehagen, N.M. Girgis, D.H. Yamada, A.A. Smith, S.R. Chan, I.S. Grewal, M. Quigley, R.I. Verona Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.R. Wiehagen, A.A. Smith Study supervision: K.R. Wiehagen, I.S. Grewal, M. Quigley, R.I. Verona

Acknowledgments
The authors acknowledge the contributions of Cassandra L. Lowenstein, Nikki A. DeAngelis, and Catherine A. Ferrante in support of ex vivo experiments. Amy Wong, Leopoldo L. Luistro, Darlene J. Pizutti, and Diana Chin supported in vivo studies. We thank Enrique Zudaire and Jonathan Ceniza for expert advice and helpful discussions, and Enrique Zudaire for critical reading of this article.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 22, 2017; revised September 1, 2017; accepted October 24, 2017, published OnlineFirst November 2, 2017.

References
Combination of CD40 Agonism and CSF-1R Blockade Reconditions Tumor-Associated Macrophages and Drives Potent Antitumor Immunity


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-17-0258

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2017/11/01/2326-6066.CIR-17-0258.DC1

Cited articles
This article cites 49 articles, 16 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/5/12/1109.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerimmunolres.aacrjournals.org/content/5/12/1109. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.