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Cancer Research

Combined Anti-CD40 and Anti–IL-23 Monoclonal Antibody Therapy Effectively Suppresses Tumor Growth and Metastases

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Abstract

Tumor-induced immunosuppression remains one of the major obstacles to many potentially effective cancer therapies and vaccines. Host interleukin (IL)-23 suppresses the immune response during tumor initiation, growth, and metastases, and neutralization of IL-23 causes IL-12–dependent antitumor effects. Here, we report that combining agonistic anti-CD40 monoclonal antibodies (mAb) to drive IL-12 production and anti–IL-23 mAbs to counter the tumor promoting effects of IL-23 has greater antitumor activity than either agent alone. This increased antitumor efficacy was observed in several experimental and spontaneous lung metastases models as well as in models of *de novo* carcinogenesis. The combination effects were dependent on host IL-12, perforin, IFN-γ, natural killer, and/or T cells and independent of host B cells and IFN-αβ sensitivity. Interestingly, in the experimental lung metastases tumor models, we observed that intracellular IL-23 production was specifically restricted to MHC-II^{hi}CD11c^{+}CD11b^{+} cells. Furthermore, an increase in proportion of these IL-23–producing cells was detected only in tumor models where IL-23 neutralization was therapeutic. Overall, these data suggest the clinical potential of using anti-CD40 (push) and anti–IL-23 mAbs (pull) to tip the IL-12/23 balance in established tumors. *Cancer Res; 1–10. ©2014 AACR.*

Introduction

Interleukin (IL)-23 is a member of a small family of proinflammatory heterodimeric cytokines that also includes IL-12 (1). Both cytokines share a common p40 subunit that is covalently linked to a p35 subunit to form IL-12 or to a p19 subunit to form IL-23. IL-23 is required for the terminal differentiation of Th17 cells, a subset of CD4^{+} T cells distinct from classical Th1 and Th2 cells, generated following exposure to IL-6 and TGF-β1 (2). Th17 cells produce IL-17, a proinflammatory cytokine that stimulates the production of proinflammatory molecules such as IL-1, IL-6, TNF, NOS-2, and chemokines resulting in inflammation. We and others have previously demonstrated that IL-23 is a critical cytokine in tumor initiation, growth, and metastases, and that IL-23 can inhibit natural killer (NK) cell effector functions (3, 4). Recently, we reported that a neutralizing monoclonal antibody (mAb) to mouse IL-23p19 alone suppressed early experimental B16F10 melanoma lung metastases and modestly inhibited subcutaneous tumor growth. More importantly, the use of anti–IL-23p19 mAb in combination with IL-2 or anti-erbB2 mAb treatment significantly inhibited subcutaneous growth of established mammary carcinoma and suppressed established experimental and spontaneous lung metastases (5). Overall, these data suggest that IL-23 is an important immunosuppressive cytokine in developing tumors and metastases and that targeting IL-23 in combination with other promising immune targets is a rational and effective means to treat cancer.

A more recent study of tumors in a state of cancer:immune equilibrium (6, 7) or immune-mediated tumor dormancy, where minimal residual disease exists, has demonstrated that IL-12 and IL-23 critically determine the balance between tumor elimination and tumor escape, respectively (8). The ability of anti-CD40 to prevent tumors emerging from equilibrium and the known ability of anti-CD40 to provoke IL-12 production by antigen-presenting cells (APC; ref. 9), suggest that there may be some merit in preventing tumor outgrowth by using a combination of anti-CD40 and anti–IL-23p19 mAbs. Therefore, here we have evaluated the efficacy of an anti-CD40/anti–IL-23p19 combination in mouse models of minimal residual cancer, including metastases and tumor initiation. Using these models, we now show that the anti-CD40 and anti–IL-23p19

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mAb combination is superior to either therapy alone and the effect is NK/T- and IL-12p35 dependent.

Materials and Methods

Mice

Inbred C57BL/6 wild-type (WT), C57BL/6 IL-12p35–deficient (IL-12p35$^{-/-}$), C57BL/6 RAG-1–deficient (RAG-1$^{-/-}$), C57BL/6 RAG-2-common γ-chain–deficient (RAG-2$^{-/-}$x γc$^{-/-}$), C57BL/6 μMT (μMT$^{-/-}$), C57BL/6 perforin-deficient (pfp$^{-/}$) and C57BL/6 Type I IFN-deficient (IFNAR1$^{-/-}$), C57BL/6 IL-17A deficient (IL-17A$^{-/-}$), and BALB/c WT mice were bred and maintained at the Peter MacCallum Cancer Centre (Peter Mac; Victoria, Australia) or QIMR Berghofer Medical Research Institute (QIMR; Queensland, Australia) as described previously (4, 10). Mice (6- to 14-weeks-old) were used in all experiments. BALB/c female mice were used for all experiments with the 4T1.2 tumor and all experiments were performed in accordance with guidelines set out by the Peter Mac and QIMR Animal Experimental Ethics Committee guidelines.

Tumor models

Experimental metastases tumor models. To examine metastatic tumor growth, WT or gene-targeted mice were inoculated intravenously (i.v.) with the indicated dose of B16F10 melanoma, MC38 colon adenocarcinoma, or RM-1 prostate carcinoma cells. Mice were monitored and lungs were harvested at day 14 and metastases quantified as previously described (11). Some mice were treated with control Ig (cIg; 2A3 or MAC4) or anti–IFN-γ (H22, kindly provided by Dr. Schreiber; Washington University, St Louis, MO), anti-CD8 (53.6.7), anti-asialoGM1 (anti-ASGM1; Wako Chemicals; as indicated in the legends) to neutralize IFN-γ or deplete cell subsets as previously described (4). Anti-AGP3 (cIg, 4D2), anti–IL-23p19 (16E5), and anti–IL-12/23p40 (C17.8) were all grafted on the same murine immunoglobulin G-1 background and kindly provided by AMGEN as previously described (4). Agonistic anti-mouse CD40 mAb (FGK45) was purchased from Bioxcell.

Spontaneous tumor metastases model. 4T1.2 mammary carcinoma cells (2×10^5) were injected into the mammary fat pad of BALB/c WT mice. Some mice were treated with anti-AGP3, cIg, anti–IL-23p19, anti-CD40, or anti–IL-12/23p40 as indicated in the legends.

Carcinogenesis model. Groups of 15 male C57BL/6 WT mice were inoculated subcutaneously (s.c.) in the hind flank with 400 μg of 3-methylcholanthrene (MCA; Sigma Fine Chemicals) in 0.1 mL of corn oil as previously described (12). Mice were injected with anti-AGP3, cIg, anti–IL-23p19, or anti-CD40 as indicated in the legends. Development of fibrosarcomas was monitored weekly over the course of 250 to 300 days. Tumors > 3 mm in diameter and demonstrating progressive growth were recorded as positive. Measurements were made with a caliper square as the product of two perpendicular diameters (cm^2) and individual mice are represented.

Cell preparations and flow cytometry

After euthanizing mice, naïve or tumor-bearing lungs were perfused with ice cold PBS via the right ventricle to remove peripheral blood. The perfused lungs were removed, minced finely, and incubated in PBS containing 1.3 mmol/L EDTA for 30 minutes at 37°C. Cell suspension were pelleted and resuspended in RPMI digestion media containing 1 mg/mL collagenase type IV (Worthington), 1 mmol/L $MgCl_2/CaCl_2$ for 60 minutes at 37°C. Digested lung tissue was passed through a 70 μmol/L cell strainer and washed with PBS twice before being resuspended in RPMI-1640 [Gibco supplemented with 2.5 mmol/L HEPES, 5.5×10^{-5} mol/L 2-mercaptoetanol, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 mmol/L glutamine, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate (all from Gibco Invitrogen) and 10% FBS]. Single-cell suspensions (5×10^6 cells/mL) were incubated alone or with lipopolysaccharide (LPS; 50 μg/mL; Sigma-Aldrich) in the presence of GolgiPlug (1 μl/mL; BD Pharmingen), in nontissue cultured treated plates (Corning). After overnight incubation, cells were harvested for surface and intracellular cytokine staining. For surface staining, cells were incubated with the following antibodies (all from eBioscience or BD Biosciences): FITC-anti-MHC II (2G9), APC-eF780-anti-CD11b (MI/70), and eF450-anti-CD11c (N418) in the presence of 2.4G2 (anti-CD16/32, to block Fc-receptors) on ice. Intracellular staining (ICS) was performed using reagents from BD Cytofix/Cytoperm Kit (BD Biosciences) and the following antibodies: PE-anti–IL-12p40/p70 (C15.6), PE-isotype control (MOPC-173), and eF660-isotype control (P3.6.2.1). Mouse anti-mouse IL-23 (clone 16E5) antibody was labeled with Alexa 647 (Invitrogen) per the manufacturer's instructions and used at 1:50 dilution. CD40 expression was not detected by flow cytometry on B16F10, RM-1, and 4T1.2 tumor cells, nor do they produce IL-23 as assayed by ELISA (data not shown). Cells were acquired on the BD FACSCanto II (BD Biosciences). Analysis was performed using the software program FCS Express.

Statistical analysis

Significant differences in metastases, survival, or proportion of IL-23$^+$ cells were determined by the Mann–Whitney *U* test or log-rank sum test. Values of *, $P < 0.05$ and **, $P < 0.01$ were considered significant.

Results

Growth inhibition of MCA-induced fibrosarcomas following anti–IL-23p19/anti-CD40 combination therapy

Anti-CD40 mAb therapy prevents MCA-induced fibrosarcomas emerging from equilibrium (8) and induces IL-12 production by APCs (9). Given that IL-23 plays a critical role in opposing the antitumor effects of IL-12 (8), we wished to determine whether IL-23 neutralization could further potentiate the antitumor effects of anti-CD40–derived IL-12 in mouse models of minimal residual tumor. Thus, we first assessed the antitumor efficacy of anti-CD40/anti–IL-23p19 combination therapy using the MCA-induced fibrosarcoma model, where we have demonstrated that host immunity is a critical factor in suppressing tumor initiation (13) and progression (12). We tested whether prophylactic treatment of the combination therapy was efficacious in groups of MCA-inoculated C57BL/6 WT mice (Fig. 1). Mice treated with anti–

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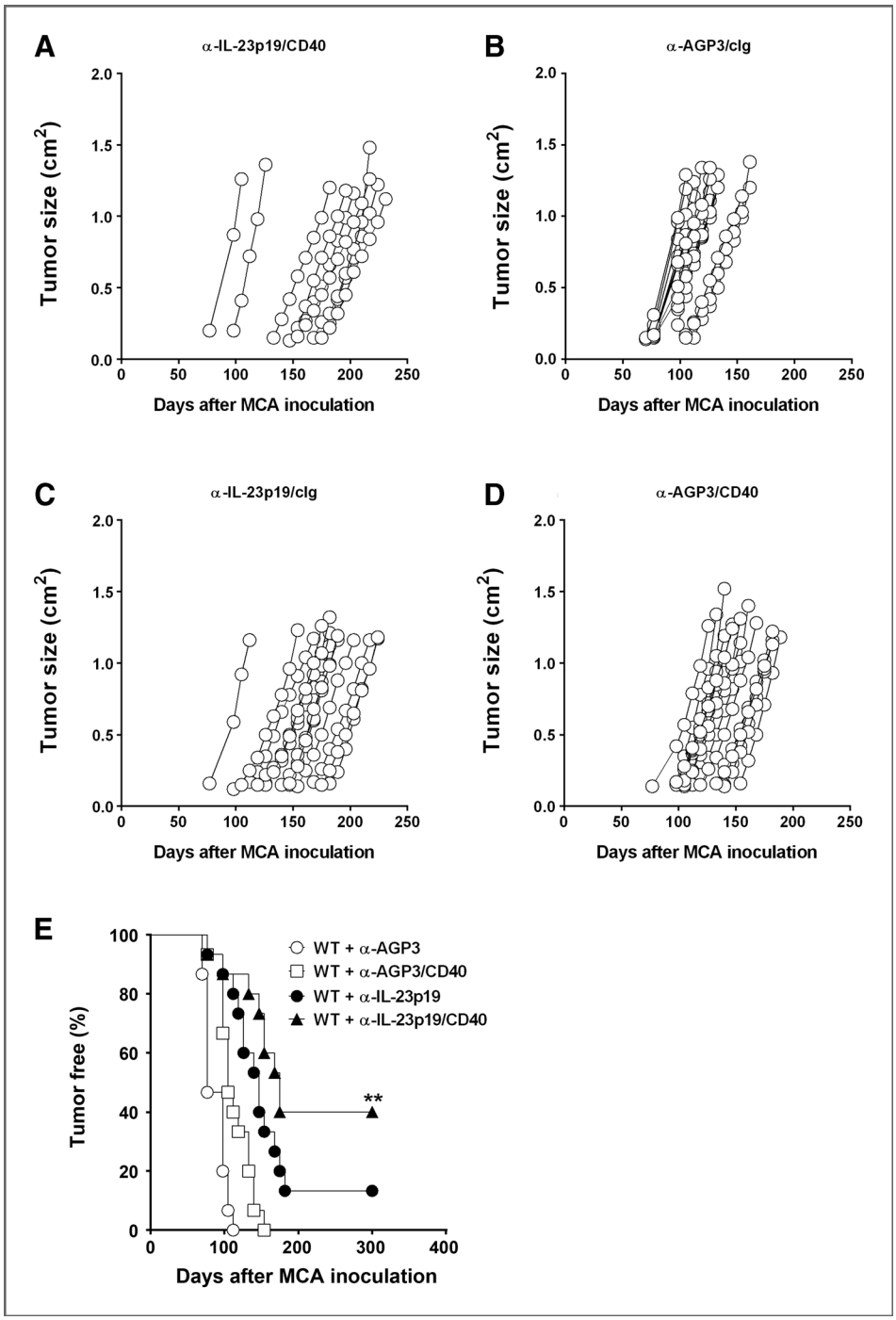

Figure 1. Prophylactic treatment with anti-CD40/anti–IL-23p19 combination mAb therapy increases survival of mice with MCA-induced fibrosarcomas. Groups of 15 male C57BL/6 mice were injected subcutaneously with 400 μg MCA on day 0. On day 1, groups of mice were injected intraperitoneally with either cIg/anti-CD40 (100 μg) and/or anti-AGP3/anti–IL-23p19 (500 μg). On day 0 and twice weekly after until day 30, groups of mice were injected intraperitoneally with either cIg/anti-CD40 (100 μg) and/or anti-AGP3/anti–IL-23p19 (250 μg). Mice were subsequently monitored for tumor development over 300 days and individual tumor growth curves (A–D) and their survival (E) is plotted. An asterisk indicates the significant difference between anti–AGP3-treated mice and anti-CD40/anti–IL-23p19-treated mice as determined by the log-rank sum test, **, $P < 0.0001$.

IL-23p19/anti-CD40 mAb combination therapy had significantly reduced tumor incidence and delayed tumor onset compared with cIg-treated groups (Fig. 1A, B, and E). MCA-injected mice that received anti–IL-23p19 or anti-CD40 mAb therapy alone also displayed delayed tumor onset compared with cIg-treated groups (Fig. 1B, C, and D) but neither single therapy was as impressive as the combination therapy at reducing tumor incidence (Fig. 1E). We also assessed the antitumor efficacy of anti–IL-23p19/anti-CD40 combination mAb therapy in mice with established MCA-induced fibrosarcomas (~0.25–0.45 cm^2; Fig. 2). Impressively, the combination therapy was able to strongly inhibit tumor growth compared with cIg-treated groups and interestingly, some mice displayed long periods of tumor suppression following treatment, with one mouse actually being cured (Fig. 2A and B). Treatment of MCA tumor-bearing mice with either therapy alone also resulted in good tumor growth suppression but was clearly not as potent as the combination therapy (Fig. 2C and D).

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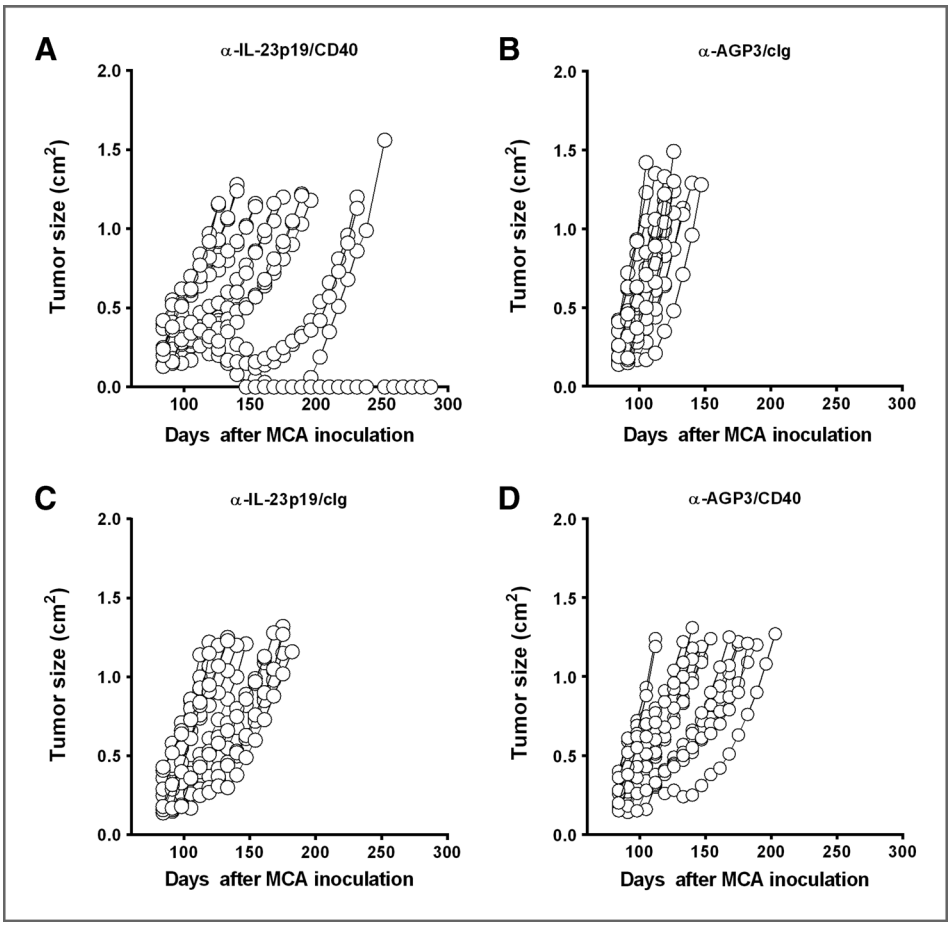

Figure 2. Combined anti-CD40/anti–IL-23p19 mAb therapy suppresses growth of established MCA-induced fibrosarcomas. Groups of 15 male C57BL/6 mice were injected subcutaneously with 400 μg MCA on day 0. Individual mice were treated with the indicated therapy clg/anti-CD40 (100 μg) and/or anti-AGP3/anti–IL-23p19 (500 μg) after their second palpable tumor measurement (~0.25–0.45 cm^2). Mice were subsequently monitored for tumor development over 300 days and their individual tumor growth curves (A–D) are plotted.

Combined anti–CD40/IL-23p19 mAb therapy suppresses established experimental and spontaneous lung metastases

Experimental B16F10 lung metastasis is a tractable short-term model of minimal tumor burden, where host NK cells protect the host. We have previously reported that neutralization of IL-23 using a mouse anti-mouse IL-23p19 mAb in WT mice injected intravenously with B16F10 melanoma cells reduced lung metastases in a NK cell-dependent manner compared with similar groups of mice treated with isotype control mAb. In addition, we demonstrated proof-of-principle in this study that anti–IL-23p19 mAb synergized with immunotherapies (e.g., IL-2) that induced NK cell activation (5). Considering the ability of IL-12 to activate NK cells, we therefore combined neutralizing anti–IL-23p19 mAb with agonistic anti-CD40 mAb (Fig. 3). Treatment of WT mice with established B16F10 experimental lung metastases with anti–IL-23p19 plus CD40 mAbs significantly decreased tumor burden compared with control-treated groups (Fig. 3A). Treatment of similar groups of tumor-bearing mice with anti–IL-23p19 mAb or anti-CD40 mAb alone also reduced tumor burden, although their effects were more modest (Fig. 3A). The increased therapeutic effect of combining anti–IL-23p19 mAb with anti-CD40 mAb was also observed in WT mice bearing established RM-1 prostate carcinoma experimental lung metastases (Fig. 3B). As we previously reported, anti–IL-23p19 mAbs had no impact against WT mice bearing established MC38 colon adenocarcinoma experimental lung metastases (Fig. 3C; ref. 4). Furthermore, anti–IL-23p19 did not further enhance the suppression of metastases mediated by anti-CD40 alone (Fig. 3C).

Having shown that the anti–IL-23p19 plus anti-CD40 combination mAb therapy suppressed established experimental lung metastases, we next wanted to determine the therapeutic efficacy of this combination on 4T1.2 spontaneous mammary carcinoma metastases following primary tumor resection. This model mimics a setting of surgery and adjuvant treatment of residual metastatic disease in humans. The 4T1.2 mammary carcinoma cell line was injected into the mammary fat pad of groups of BALB/c mice, and after 28 days, the primary tumors were resected. The next day, these mice commenced a 3-day treatment schedule with anti–IL-23p19 plus anti-CD40 mAbs, which resulted in significant survival over similar groups of mice treated with clg or mice treated with either therapy alone. In some experiments, groups of mice with resected tumors treated with the combined anti–IL-23p19/anti-CD40 mAbs were additionally treated with anti–IL-12/23p40 mAbs that neutralized both IL-12 and IL-23 (Fig. 3D). Here, loss of IL-23 could not compensate for the loss of IL-12 because the length of

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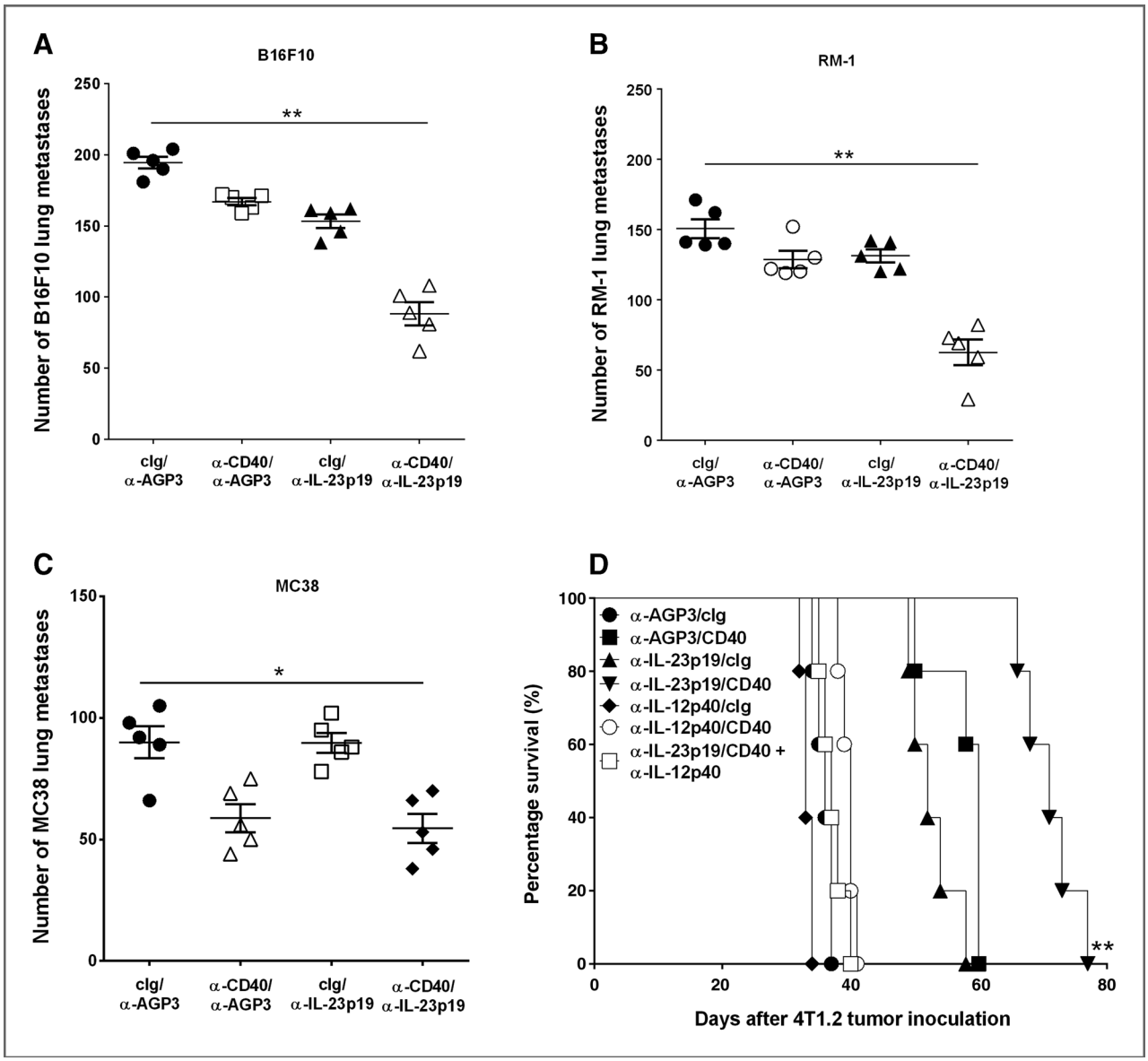

Figure 3. Combined anti–CD40/IL-23p19 mAb therapy suppresses established experimental and spontaneous lung metastases. Groups of C57BL/6 mice ($n = 5$) were injected intravenously with either the B16F10 melanoma cell line (7.5×10^5; A), the RM-1 prostate carcinoma cell line (2×10^4; B), or the MC38 colon cell line (1×10^5; C). On days 5, 6, 7, the indicated groups of mice were treated intraperitoneally with either cIg/anti-CD40 (100 μg) and/or anti-AGP3/anti–IL-23p19 (500 μg). Fourteen days after tumor inoculation, the lungs of these mice were harvested and fixed, and the colonies counted and recorded for individual mice as shown. In D, groups of 5 female BALB/c mice were injected subcutaneously in the mammary fat pad with the mammary carcinoma cell line 4T1.2 (2×10^4). On day 28, the primary tumor was resected and mice were treated intraperitoneally with either cIg/anti-CD40 (100 μg) and/or anti-AGP3/anti–IL-23p19 (500 μg) on day 29, 30, 31. In one group, mice were additionally treated with anti–IL-12/23p40 (500 μg). Survival of the mice was monitored for 80 days. Asterisks indicate the groups that are significantly different between anti-AGP3/cIg-treated mice and anti-CD40/anti–IL-23p19-treated mice as determined by the Mann–Whitney *U* test or log- rank sum test, *, $P < 0.05$; **, $P < 0.01$.

survival of these treated mice was similar to control-treated groups.

Efficacy with anti–IL-23p19/anti-CD40 mAb therapy is dependent upon NK cells, IL-12, and the effector molecules IFN-γ and perforin

Across different mouse models of cancers, we have clearly demonstrated that anti–IL-23p19 mAb synergizes with anti-CD40 mAb. We next wanted to determine the mechanism of action of this combination therapy using the B16F10 experimental lung metastases tumor model (Fig. 4). Using IL-12p35$^{-/-}$ mice, we confirmed that IL-12 was required for the antitumor efficacy of the combination therapy. We observed no decrease in lung metastases in the IL-12p35–deficient mice following combination therapy compared with groups of similarly treated WT mice (Fig. 4A). Indeed, the level of metastases

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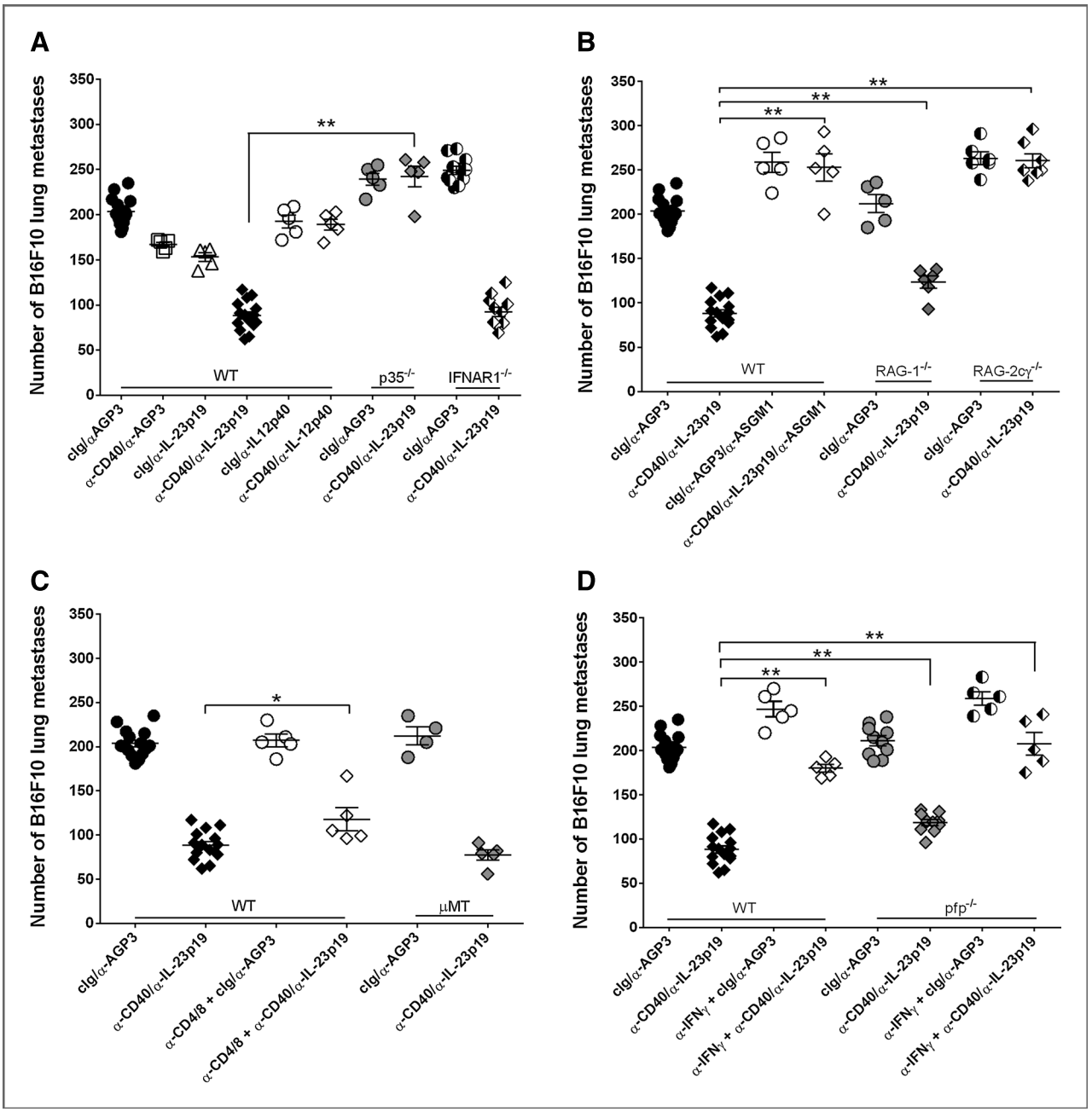

Figure 4. Anti-CD40/anti–IL-23p19 combination mAb therapy is dependent on NK cells, IL-12p70 and effector molecules IFN-γ and perforin. Groups of 5 C57BL/6 or gene targeted mice were injected intravenously with either the B16F10 melanoma cell line (7.5×10^5; A–D). On days 5, 6, 7, the indicated groups of mice were treated intraperitoneally with either cIg/anti-CD40 (100 μg) and/or anti-AGP3/anti–IL-23p19 (500 μg). In some groups, mice were additionally treated intraperitoneally with anti–IL-12/23p40 (days 4, 5, 6, and 7, 500 μg), anti-ASGM1 (days 4 and 11, 100 μg), anti–IFN-γ (days 4, 5, and 12, 250 μg), or anti-CD4 plus anti-CD8 (days 4 and 11, 100 μg each). Fourteen days after tumor inoculation, the lungs of these mice were harvested and fixed, and the colonies counted and recorded for individual mice as shown. Results are pooled from a number of experiments. Asterisks indicate the groups that are significantly different between anti-CD40/anti–IL-23p19 mAb-treated mice and indicated groups as determined by the Mann–Whitney *U* test, **, $P < 0.01$.

was significantly higher in IL-12p35$^{-/-}$ mice compared with WT mice, further illustrating the importance of IL-12 in natural host control of lung metastases. In contrast, type I IFNs (IFN-αβ) were not required for the antitumor efficacy of the combination therapy. Although type I IFNs can initiate antitumor immunity independent of IL-12 (14), we did not observe any loss of ability to control lung metastases in tumor-bearing IFNAR1$^{-/-}$ mice (which are unable to respond to type I IFNs) following combination therapy (Fig. 4A).

NK cells seemed most important for controlling lung metastases in the B16F10 experimental metastases model (Fig. 4B). We observed loss of antimetastatic activity with the

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combination therapy in RAG-2$^{-/-}$c$\gamma^{-/-}$ mice (which lack T cells, B cells, and NK cells) compared with retained activity in RAG-1$^{-/-}$ mice (lacking T and B cells only). This was further substantiated in WT tumor-bearing mice that were additionally treated with anti-ASGM1 to deplete NK cells. In contrast, neutralizing antibodies to CD4 and CD8 demonstrated that CD4$^+$ and CD8$^+$ T cells played a very minor role and through the use of μMT$^{-/-}$ mice, which lack B cells, we showed that B cells were not critical for the antimetastatic effect of the combination therapy (Fig. 4C). Similarly, the cytokine IL-17A was not required, as the combination therapy was equally effective in tumor-bearing IL-17A$^{-/-}$ or WT mice (Fig. 4C). Given the importance of NK cells, we next examined whether key NK cell effector functions contributed to the antimetastatic effect. Combination therapy was only mildly affected in mice lacking perforin, whereas those neutralized for IFN-γ or lacking both effector molecules did not respond to the therapy (Fig. 4D). We observed similar requirements for NK cells and IL-12, but not T cells or type 1 IFN or IL-17A, in mice bearing established RM-1 lung metastases treated with combination therapy (Supplementary Fig. S1). In contrast, both NK and CD8$^+$ cells were required for the full efficacy of the combination therapy in mice bearing spontaneous 4T1.2 lung metastases (Supplementary Fig. S2; ref. 15). Interestingly, in mice bearing established subcutaneous B16F10 tumors, the efficacy of the combination therapy was dependent primarily on T cells, with a minor requirement for NK cells (Supplementary Fig. S3). Thus, we demonstrate that the improved efficacy of the anti–IL-23p19/CD40 combination therapy requires NK cells and/or T cells depending upon the model examined. This is consistent with the ability of IL-12 to activate either of these effector lymphocyte populations.

Expression of IL-23 in tumor-bearing lungs is specifically restricted to MHC-II^{hi} CD11c$^+$CD11b$^+$ cells

To investigate which cells are producing IL-23, we first performed a comprehensive analysis of *in vitro* bone marrow-derived APCs and *ex vivo* purified immune cell populations stimulated with a range of agonists and measured their capacity to produce IL-23 by ELISA and intracellular cytokine staining (Supplementary Figs. S4–S8). IL-23 was detected within 2 hours after stimulation with LPS or curdlan (Supplementary Fig. S4A) and plateaued between 8 to 24 hours. In contrast, IL-12p70 was not detected until 8 hours and was maximal at 24 hours after LPS stimulation (Supplementary Fig. S4B). We next tested the ability of a range of cell types to secrete IL-23 following overnight culture with a range of stimuli. Surprisingly, we found that amongst the *in vitro* derived populations examined, only activated granulocyte macrophage colony-stimulating factor (GM-CSF)–derived bone marrow dendritic cells (BMDC) were capable of producing IL-23 (Supplementary Fig. S5). Next, we conjugated the anti–IL-23p19 mAb to the Alexa647 fluorochrome to allow for ICS of IL-23. The specificity of the IL-23 ICS was validated using BMDCs from WT and IL-23p19$^{-/-}$ mice (Supplementary Fig. S6). Similar to our ELISA results (Supplementary Fig. S5), we detected intracellular IL-23 in stimulated BMDCs (Supplementary Fig. S7). CpG proportionately generated far more single IL-12p40$^+$ cells than LPS, suggesting that different stimuli may generate quite a different profile of IL-12 versus IL-23 production. To determine whether cells other than BMDCs could produce IL-23, we next assessed for its presence in *ex vivo* derived immune cells. Intracellular IL-23 was detected in LPS-stimulated CD11b$^+$F4/80$^+$ peritoneal exudate cells (PEC) and liver mononuclear cells (MNC), but to lower levels than in stimulated BMDCs (Supplementary Figs. S7 and S8). In addition, we confirmed that no IL-23 was produced by naïve or stimulated NK cells, B cells, T cells, and NKT cells or by a range of tumor types (EG7, B16F10, RM-1; data not shown). Overall, these data suggest that mouse IL-23 production is quite restricted to specific immune cell types and is very context/environment dependent.

Although dendritic cells (DC) and macrophages are thought to be the source of IL-23 (16), it is currently not clear which mononuclear phagocyte populations produce IL-23 in the tumor setting. Given our ability to now detect intracellular IL-23, we utilized this approach to characterize IL-23–producing cells using the experimental lung metastases tumor models (Fig. 5). Single-cell lung suspensions from tumor-bearing or naive lungs were generated and cultured overnight. In some groups, LPS was used as a stimulus to maximize our ability to detect intracellular IL-23 given our findings from above. We observed that the proportion of LPS-stimulated IL-23–expressing cells was statistically elevated in B16F10 and RM-1 tumor-bearing lungs compared with naive lungs (Fig. 5A and B). Interestingly, in both tumor models, we detected IL-23 in MHC-II$^+$CD11c$^+$CD11b$^+$ but not MHC-II$^+$CD11c$^+$CD11b$^-$ cells, although both populations clearly were capable of IL-12p40 production, which may be secreted as homodimers or other heterodimers (Supplementary Fig. S9). In contrast, we saw no increase in the proportion of IL-23$^+$–producing cells in MC-38 tumor-bearing lungs (Fig. 5C). This increase in expression correlated directly with the previously described relative importance of IL-23 in promoting B16F10 and RM-1, but not MC38, tumor growth/metastases (Fig. 3C; ref. 4).

Discussion

In this study, we demonstrated in four different tumor models of experimental and spontaneous metastasis or *de novo* carcinogenesis, that a combination of anti-CD40 and anti–IL-23 mAbs had greater antitumor activity than either agent alone. The combination effects were dependent on host IL-12, IFN-γ, NK, and/or T cells, and independent of host B cells and IFN-αβ sensitivity. IL-23 production was regulated very specifically and restricted to MHC-II^{hi}CD11c$^+$CD11b$^+$ cells in the tumor. The combination therapy further demonstrates the value of increasing the IL-12:IL-23 ratio in tumors and the potential of using anti–IL-23 mAbs to improve the immunostimulatory effects of anti-CD40 mAbs in the current management of some advanced human cancers (17).

It is generally thought that dendritic cell and phagocytic myeloid cell populations that produce IL-12p70 are also capable of producing IL-23, as measured by their increase in p19 subunit gene expression by real-time PCR or Western blot analyses (18). However, increased expression of p19 mRNA

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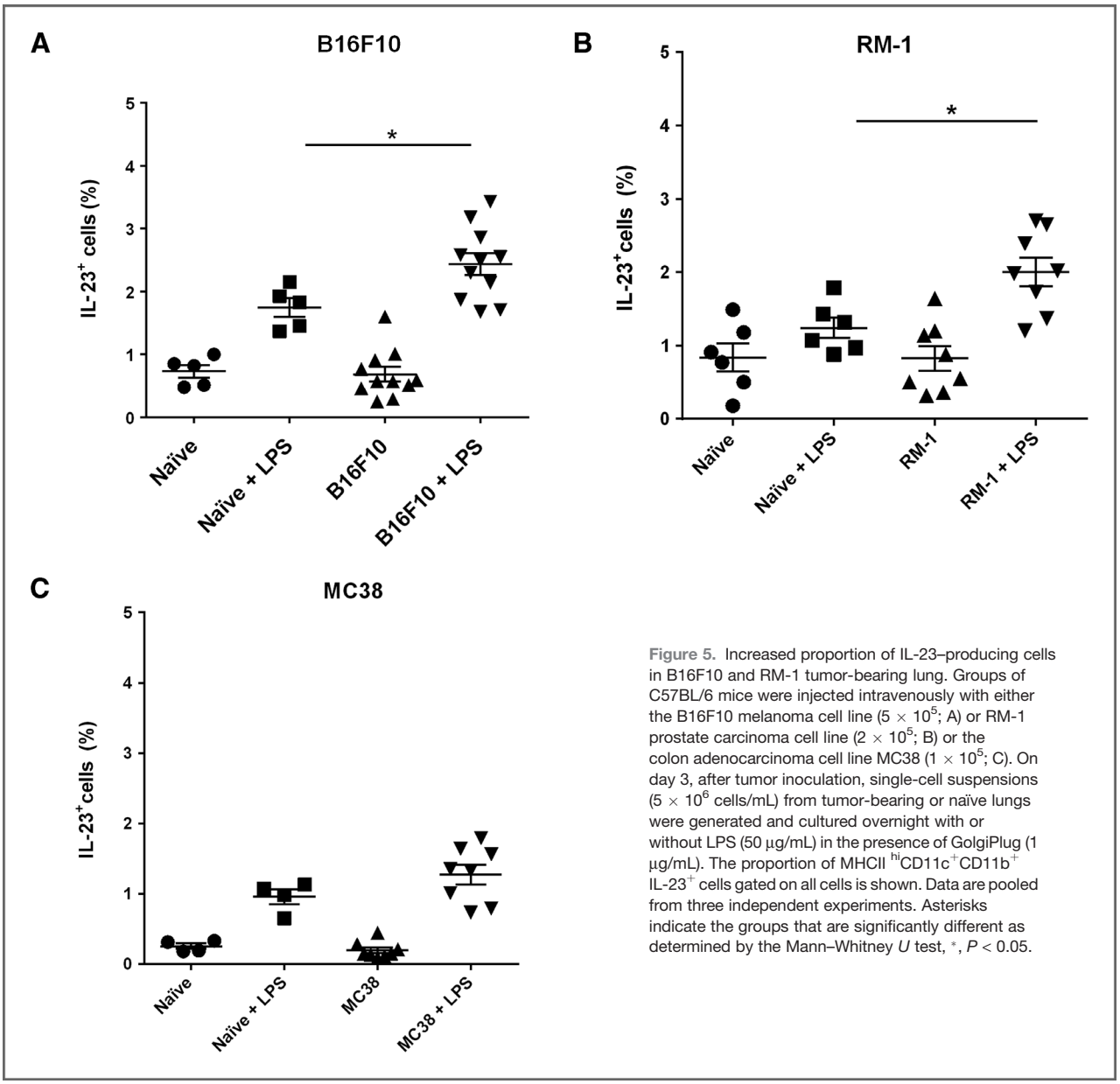

Figure 5. Increased proportion of IL-23–producing cells in B16F10 and RM-1 tumor-bearing lung. Groups of C57BL/6 mice were injected intravenously with either the B16F10 melanoma cell line (5×10^5; A) or RM-1 prostate carcinoma cell line (2×10^5; B) or the colon adenocarcinoma cell line MC38 (1×10^5; C). On day 3, after tumor inoculation, single-cell suspensions (5×10^6 cells/mL) from tumor-bearing or naïve lungs were generated and cultured overnight with or without LPS (50 μg/mL) in the presence of GolgiPlug (1 μg/mL). The proportion of MHCII ^{hi}CD11c$^+$CD11b$^+$ IL-23$^+$ cells gated on all cells is shown. Data are pooled from three independent experiments. Asterisks indicate the groups that are significantly different as determined by the Mann–Whitney *U* test, *, $P < 0.05$.

may not correlate to the translation of p19 protein and/or formation of bioactive IL-23 (19). In this study, we performed a comprehensive analysis of IL-23 expression on a range of *in vitro* derived APCs and *ex vivo* purified immune cell populations stimulated with a range of agonists to determine their capacity to produce IL-23. Intriguingly, GM-BMDCs, but not Flt3L-cultured BMDCs or M-CSF–cultured bone marrow macrophages were capable of producing IL-23 following stimulation. Our studies confirm a previous study by Waibler and colleagues, who had reported that GM-BMDCs stimulated with LPS produced IL-23, but not Flt3L-derived plasmacytoid dendritic cells (pDC; ref. 19) as measured by Western blot analyses and ELISA. Interestingly, this study also reported that p19 mRNA was expressed constitutively in GM-BMDCs and pDCs irrespective of whether the cells were activated or not, suggesting the obvious caveat of using an increase in mRNA gene expression as a readout for functional protein production.

Given that GM-BMDC are considered representative of dendritic cells that emerge after inflammation *in vivo* (20), we also examined the capacity of CD11c$^+$ and CD11b$^+$ cells isolated *ex vivo* from lymphoid organs, liver, or the peritoneal cavity to produce IL-23. Intracellular IL-23 was detected in LPS-stimulated PECs or liver MNCs isolated from WT mice, but not from stimulated ex vivo purified CD11c$^+$ or CD11b$^+$ cells isolated from lymphoid organs. Although we saw no IL-23 from stimulated splenic CD11c$^+$ cells in our experiments either by ELISA or ICS, one study reported detection of IL-23 from these cells stimulated with CpG, anti-CD40 mAb, and GM-CSF, but not LPS. Notably however, the levels of IL-23 measured were very low (10–60 pg/mL; ref. 21). Recently

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Qian and colleagues reported increased IL-23 production in splenic-derived $CD11c^+$ cells after stimulation with tumor-derived conditioned media and LPS (22). The authors further showed that tumor-derived prostaglandin E2 was the factor responsible for upregulating IL-23p19 mRNA expression. In this study, we demonstrated for the first time, detection of intracellular IL-23 in tumor-bearing lungs. Importantly, we observed significant increase in the proportions of $IL\text{-}23^+$ cells only from B16F10 and RM-1 tumor-bearing lungs, but not lungs harboring MC38 tumors. Although a basal level of intracellular IL-23 was detected across all three experimental lung tumor models, the increase in $IL\text{-}23^+$ cells in B16F10 and RM-1 suggests that these tumors induce a tumor microenvironment that expands $IL\text{-}23^+$ cells. It may be interesting to assess if any differences exist in cell infiltrates, growth factors, and cytokines between B16F10/RM-1 and MC38 tumor-bearing lungs. Strikingly, IL-23 was detected in MHC-$II^{hi}CD11c^+CD11b^+$ cells, but not in other populations, such as MHC-$II^{hi}CD11c^+CD11b^-$ cells. In nonlymphoid tissues such as the lung, it has been suggested that cells costaining for the above markers may be quite different in both origin and function to cells from lymphoid tissues (23). Indeed, Schlitzer and colleagues recently reported that MHC-$II^{hi}CD11c^+CD11b^+$ cells in lungs consisted of equal proportion of $CD24^+CD64^-$ dendritic cells and $CD24^-CD64^+$ macrophages. Furthermore, they showed that lung $CD11b^+$ dendritic cells, but not lung $CD11b^+$ macrophages, were required for the induction of a Th17 response to fungal infection. Studies are currently under way to determine whether the $IL\text{-}23^+$ cells in our study are lung $CD11b^+$ dendritic cells or macrophages.

Preclinically, anti-CD40 mAb has been combined with many other approaches, including surgery, chemotherapy, cytokines (IL-2 or IL-15), Toll-like receptor stimulation, anti–CTLA-4, anti-CD137, and adoptive T-cell therapy (reviewed in ref. 24). Of these combination approaches with agonistic anti-CD40 mAb, clinical trials have focused on various chemotherapies (e.g., gemcitabine, cisplatin and pemetrexed, paclitaxel, and carboplatin). Early clinical trials of agonistic CD40 mAb have shown highly promising results in the absence of disabling toxicity, both in single-agent studies and in combination with chemotherapy (25). These findings suggest that CD40 agonists can mediate both T-cell–dependent and T-cell–independent immune mechanisms of tumor regression in mice and patients. Although dendritic cells have been identified as key player in anti–CD40-driven tumor-specific T-cell responses in mice, the role of dendritic cell in human response to agonistic anti-CD40 is not clear. Although the primary mechanistic rationale invoked for agonistic anti-CD40 mAb is to activate the host APC, particularly dendritic cells, to induce antitumor T-cell responses in patients, macrophage-dependent triggering of tumor regression may also be important (17, 26). Recent insights into the mechanism of action also implicate Fc:FcγR interactions as key (25).

Our study with the anti-CD40/anti–IL-23p19 combination suggests a mechanism of action that is IL-12 dependent and capable of promoting both NK cell and T-cell antitumor activities likely as a result of anti-CD40 agonizing IL-12 production from APC. Given the specialized conditions of IL-23 production in tumors, it is likely that the combination of anti-CD40 and anti–IL-23 serves to drive IL-12p70 production and neutralize IL-23, thus promoting effector lymphocyte activity in the tumors. The responding lymphocyte (NK cell or T cell) depends upon the model examined. Despite the critical importance of host IFN-γ, we detected equivalent NK cell IFN-γ in the lungs of mice bearing established B16F10 lung metastases, after treatment with anti-CD40 or the combination therapy (data not shown). This suggested that the mechanism by which IL-23 neutralization enhances anti-CD40 mAb is not by increasing NK cell IFN-γ production. Clearly, when anti–IL-23p19 was not effective alone (e.g., MC38 metastases), there was no benefit in combination with anti-CD40 mAb. How IL-23 neutralization promotes even greater effector cell function remains elusive.

A study by Uhlig and colleagues (15) suggested that targeting IL-23 may be effective for the treatment of tissue-restricted inflammatory conditions such as inflammatory bowel disease, while maintaining the ability to mount protective immune responses. Macrophage effector responses may be potentially limited by blockade of IL-23 because IL-23 has been previously shown to synergize with anti-CD40 to induce TNF, IL-1-β, and IFN-γ production by macrophages (27), but here in our models, NK cells and T cells seem to be the major effectors of antitumor immunity. This consideration may have to be made in translation of the anti-CD40/anti–IL-23 combination into humans where macrophages seem critical effectors of anti-CD40 mAb (16). Although we have not assessed autoimmunity and other immune pathology directly in tumor-burdened mice receiving anti-CD40 and anti–IL-23, we believe this will be a very important and interesting future study to undertake.

As an alternative strategy to anti-CD40 mAb, the possibility of promoting the NKT cell:DC axis using agonist NKT cell ligands (28) may also combine well with IL-23 neutralization. Future experiments will consider the comparative effectiveness of local administration of NKT cell ligands and anti–IL-23 mAbs, and the scheduling of these treatments to achieve optimal combination activity. Anti–IL-23 mAbs are being tested in clinical trials in the context of autoimmune and inflammatory conditions such as psoriasis and inflammatory bowel disease. An established safety profile there will allow exploration of this therapy in the context of cancer.

Disclosure of Potential Conflicts of Interest

J.E. Towne has received commercial research grant from Amgen Inc. M.J. Smyth has received commercial research grant from AMGEN Inc. No potential conflicts of interest were disclosed by the other authors.

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