In vivo CD40 ligation can induce T cell-independent antitumor effects that involve macrophages

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Abstract: We have previously demonstrated T cell-independent antitumor and antimetastatic effects of CD40 ligation that involved natural killer (NK) cells. As CD40 molecules are expressed on the surface of macrophages (Mφ), we hypothesized that Mφ may also serve as antitumor effector cells when activated by CD40 ligation. Progression of subcutaneous NXS2 murine neuroblastomas was delayed significantly by agonistic CD40 monoclonal antibody (anti-CD40 mAb) therapy in immunocompetent A/J mice, as well as in T and B cell-deficient severe combined immunodeficiency (SCID) mice. Although NK cells can be activated by anti-CD40 mAb, anti-CD40 mAb treatment also induced a significant antitumor effect in SCID/beige mice in the absence of T and NK effector cells, even when noncytolytic NK cells and polymorphonuclear cells (PMN) were depleted. Furthermore, in vivo treatment with anti-CD40 mAb resulted in enhanced expression of cytokines and cell surface activation markers, as well as Mφ-mediated tumor inhibition in A/J mice, C57BL/6 mice, and SCID/beige mice, as measured in vitro. A role for Mφ was shown by reduction in the antitumor effect of anti-CD40 mAb when Mφ functions were inhibited in vivo by silica. In addition, activation of peritoneal Mφ by anti-CD40 mAb resulted in survival benefits in mice bearing intraperitoneal tumors. Taken together, our results show that anti-CD40 mAb immunotherapy of mice can inhibit tumor growth in the absence of T cells, NK cells, and PMN through the involvement of activated Mφ. J. Leukoc. Biol. 79: 1181–1192; 2006.

Key Words: antitumor · anti-CD40 mAb · tumor immunotherapy

INTRODUCTION

Weakly immunogenic tumors are poorly recognized by T cells. Methods to effectively enhance the antitumor response of the innate immune system may provide alternative approaches for tumor immunotherapy. With increasing evidence supporting the importance of the CD40-CD40 ligand (CD40L) interaction in immune regulation, including maturation of antigen-presenting cells (APC) [1] and T cell priming and activation [2–4], CD40 ligation has been investigated for its role in the induction of antitumor immunity. In T cell-dependent antitumor mechanisms, ligation of CD40 expressed on APC by the CD40L on CD4+ T cells has been shown to result in maturation and production of interleukin (IL)-12 by APC [2–5], which enables activated APC to cross-prime CD8+ cytotoxic T lymphocytes (CTL) [4]. The cross-linking of CD40 on APC by agonistic anti-CD40 monoclonal antibody (mAb) eliminated the requirement for T helper stimulation in the production of a CTL-dependent antitumor response [2, 6]. Alternatively, we have reported a role for treatment with an agonistic anti-CD40 mAb in T cell-independent activation of natural killer (NK) cells for antitumor effects against established murine NXS2 neuroblastoma (NB) [7]. This activation was indirect, suggesting that the observed antitumor mechanism involved anti-CD40 mAb activation of macrophages (Mφ) and/or dendritic cells to release IL-12, which stimulated antitumor NK cells.

Tumor-associated Mφ are able to exert pro- and antitumor effects in the tumor microenvironment depending on their activation state [8]. Direct ligation of CD40 on Mφ has been shown to play a role in activating Mφ for in vitro antitumor activity [9, 10]. CD40 ligation on Mφ can lead to the production of nitric oxide (NO) [11] and several cytokines, including IL-1, IL-6, tumor necrosis factor α (TNF-α), and IL-12 [12]. Of these, NO and TNF-α have been shown to be involved in the cytokotoxic effector mechanisms of Mφ [13]. We have recently shown that CD40 ligation on Mφ causes Mφ activation through an interferon-γ (IFN-γ)-dependent mechanism, which results in Mφ-mediated tumor cell cytotoxicity in vitro [10]. Based on these findings, we hypothesized that anti-CD40 mAb therapy may activate Mφ in vivo for antitumor effects in a T cell-independent manner. In this study, we investigated the immune effector cells involved in agonistic anti-CD40 mAb treatment of immunocompetent mice, as well as severe combined immunodeficiency (SCID) and SCID/beige mice, bearing murine NXS2 NB or B16 melanoma. In addition, we characterized the antitumor Mφ following CD40 ligation.
MATERIALS AND METHODS

Mice and cell lines

Female A/J, C57BL/6, C.17-SCID, and C.17 SCID/beige mice were obtained from Harlan Sprague Dawley (Madison, WI). All animals were housed in university-approved facilities and were handled according to National Institutes of Health (NIH; Bethesda, MD) and University of Wisconsin-Madison Research Animal Resource Center guidelines. NXS2 is a poorly immunogenic, highly metastatic, murine NB cell line, which is histocompatible to and grows progressively in A/J mice. This cell line is sensitive to NK cell-mediated therapies [14] and was a gift from Dr. Ralph Reisfeld ( Scripps Research Institute, La Jolla, CA). The murine NXS2 cell line was grown in Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA). The murine lymphoma yeast artificial chromosome (YAC)-1 and B16 melanoma cell lines were cultured in RPMI-1640 medium (Mediatech). All media were supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM; all from Life Technologies, Inc., Grand Island, NY), and 10% heat-inactivated fetal calf serum (FCS; Sigma Chemical Co., St. Louis, MO). Cells were maintained at 37°C in a humidified 5% CO2 atmosphere.

Agnostic anti-CD40 mAb and antitumor immunotherapy

The FGK 45.5 hybridoma cells producing an agonistic anti-mouse CD40 mAb [15] were a gift from Dr. Fritz Melchers (Basel Institute for Immunology, Switzerland). Anti-CD40 mAb was purified and confirmed to be specific as described previously [7]. For in vivo subcutaneous (s.c.) tumor models, A/J, C.17-SCID, or C.17-SCID/beige mice, 7–10 weeks old, were injected s.c. with 2 × 106 NXS2 NB cells in 100 µl phosphate-buffered saline (PBS) in the middle of the abdomen. Five and 12 days after tumor implantation, mice were injected intraperitoneally (i.p.) with 0.25–0.5 mg anti-CD40 mAb or control immunoglobulin G (IgG; Sigma Chemical Co.). Tumor size was measured every third day with a digital caliper, and volume was calculated by applying the formula [volume (mm³) = length × width × height/2]. For i.p. antigen challenge studies, A/J mice or SCID/beige mice, 7–10 weeks old, were injected i.p. with 2–3 × 106 NXS2 tumor cells in 0.5 ml PBS, or C57BL/6 mice, 7–8 weeks old, were injected i.p. with 1 × 106 B16 cells in 0.5 ml PBS. Mice were injected with anti-CD40 mAb or control rat IgG as described in the text. The end-point of these studies was death of the animal or excessive tumor burden as determined by tumor size and the condition and behavior of the animal. The decision to kill an animal was made by an independent observer without regard for treatment group.

NK cytotoxicity assay

SCID mice were injected i.p. with 0.5 mg anti-CD40 mAb or rat IgG 3 days before sacrifice. Spleens were harvested and prepared as a single-cell suspension, and red blood cells (RBC) were lysed. Effector cell preparation and NK cytolytic assays using 51Cr-labeled YAC-1 target cells were performed as described previously [16].

Peritoneal Mφ preparation and cytostatic assay

Antitumor cytostatic activity of Mφ was determined by the inhibition of 3H-thymidine ([3H]-TdR) incorporation in target tumor cells, as described previously [7]. In brief, peritoneal exudate cells (PEC) were collected by peritoneal lavage with 5 ml cold RPMI-1640 complete medium from groups of three to four mice treated 5 days earlier with 0.5 mg anti-CD40 mAb or control rat IgG (i.p.). PEC were plated at 2–2.5 × 105 cells in 0.1 ml per well in a 96-well flat-bottom plate (Corning Inc., Corning, NY). After 2 h, the monolayer was washed three times with warm RPMI to remove nonadherent cells. Flow cytometry revealed that 95% of adherent cells were Mφ, based on F4/80 expression. NXS2 tumor cells or B16 tumor cells (1 × 105/well) were added in triplicate for 48 h to wells with or without adherent cells in the presence of varying concentrations of lipopolysaccharide (LPS; Sigma Chemical Co.). As a control, tumor cells were cultured with or without LPS in the absence of effector cells. To estimate DNA synthesis, cells were pulsed with [3H]-TdR (1 µCi/well, PerkinElmer, Boston, MA) during the last 6 h of incubation. [3H]-TdR incorporation was determined by β-scintillation of total cells harvested from the wells onto glass fiber filters (Packard, Meriden, CT) using the Packard Matrix 9600 Direct β-counter (Packard). Results are expressed as mean counts per 5 min of triplicate wells ± SE. In these assays, adherent Mφ cultured in the absence of tumor cells showed negligible [3H]-TdR incorporation (data not shown).

Detection of intracellular cytokines by flow cytometry

PEC, from A/J mice and C57BL/6 mice injected i.p. with 0.5 mg anti-CD40 mAb or rat IgG, were obtained 3 or 5 days after treatment. PEC were seeded into six-well cell-culture clusters (Costar, Corning Inc.) at a concentration of 1 × 106 cells/ml, 5 ml/well, and enriched for Mφ by adherence to plastic for 1.5 h prior to staining. To enable accumulation of cytoplasmic cytokines in the endoplasmic reticulum, cells were incubated in medium containing monensin (1 µl/ml) for 4 h. Cells were harvested by gentle scraping with a rubber policeman and assayed for intracellular IFN-γ, IL-12, TNF-α, IL-4, and IL-10 as described elsewhere [17, 18] and according to the e Bioscience 2004 Catalog and Reference Manual (e Bioscience, San Diego, CA). Briefly, Mφ were resuspended in PBS with 2% FCS (flow buffer) at a concentration of 1 × 106/ml and stained with anti-F4/80-APC mAb (B220), 2 µg/1 × 106 cells at 4°C for 40 min. Rat IgG2a-APC was used as an isotype control. Cells were centrifuged, the cell pellet was resuspended in 0.1 ml flow buffer, and 0.1 ml fixation buffer was added for 20 min. After fixation, cell membranes were permeabilized with permeabilization buffer for 5 min and washed in the same buffer two additional times. After the final cell wash, the pellet was resuspended in 0.1 ml permeabilization buffer, and cells were stained with anti-IFN-γ phycoerythrin (PE) mAb (XM1G.1), isotype control rat IgG1-FITC, anti-IL-12 p40/70-PE mAb (C17.5), isotype control rat IgG2a-PE, anti-TNF-α fluorescein isothiocyanate (FITC) mAb (MP6-XT22), isotype control rat IgG1-PE, anti-IL-4-PE mAb (11B11), isotype control rat IgG1-PE, anti-IL-10 mAb (JES5-16E3), or isotype control rat IgG2b-PE, 2 µg/1 × 106 cells, at 4°C for 40 min. All mAbs and reagents for intracellular staining were purchased from e Bioscience. Finally, cells were resuspended in 0.3 ml flow buffer and analyzed using a FACSscan cytometer (Becton Dickinson, San Jose, CA). Analyses of data collected for 10,000 events/sample were performed using the Cell Quest software (Becton Dickinson).

Flow cytometric analysis

Peritoneal cells (0.5–1 × 106) collected from A/J mice 5 days after i.p. injection of 0.5 mg anti-CD40 mAb or control rat IgG were stained for 40 min at 4°C with the following mAbs (all from e Bioscience, unless otherwise stated) to detect cell surface expression levels: rat anti-F4/80-FITC mAb (B220), isotype-control rat IgG2a-FITC, anti-CD80-FITC mAb (16-10A4), isotype control Armenian hamster IgG-FITC, anti-CD86-PE mAb (GL1), isotype control rat IgG2a-PE, anti-CD40-PE mAb (IC10), isotype control rat IgG2a-PE (PharMingen), anti-Toll-like receptor 4 (TLR)-PE mAb (M1S510), or isotype control rat IgG2a-PE. Propidium iodide (PI) was added to stain dead cells, which were excluded subsequently from the analysis. Stained cells were analyzed as described above. Some findings are presented as mean fluorescent intensity (MFI) ratios (MFI of staining with a specific antibody/MFI of staining with the corresponding isotype control antibody).

In vivo cell depletion

Mice were implanted s.c. on Day 0 with 2 × 106 NXS2 NB cells and treated with 0.25–0.5 mg anti-CD40 mAb or control rat IgG as described above. NK cells were depleted by i.p. injection with 50 µl (in a total volume of 0.5 ml PBS) rabbit polyclonal anti-asialo ganglioside I (GM1) antibody (ASGM1; Wako Chemicals, Richmond, VA) on Days 3, 7, 11, and 15 post-tumor injection, as described previously [16]. Control animals received 0.5 mg rabbit IgG (Sigma Chemical Co.). Mice were depleted of polymorphonuclear cells (PMN) with 0.5 mg rat anti-Gr-1 (RB6-8C5) mAb, injected i.p. on Days 3, 7, 11, and 15 as described [19]. Control animals were similarly administered 0.5 mg rat IgG. Mφ were inactivated by i.p. injection of 25 mg silica (Sigma Chemical Co.) in 0.5 ml PBS, beginning on Day −1 and continuing every 4 days until Day 15 [20]. Control animals received 0.5 ml PBS by this schedule. The quality of effector cell depletions was tested by flow cytometry, where PEC or splenocytes were collected 3 days after specific antibody or silica treatment and returned to the animal. Results are expressed as mean counts per 5 min of triplicate wells ± SE. In these assays, adherent Mφ cultured in the absence of tumor cells showed negligible [3H]-TdR incorporation (data not shown).
and stained for NK cells [anti-CD49b-FITC mAb (DX5)], PMN [anti-Gr-1-PE mAb (RB6-8C5)], or Mφ [anti-F4/80-APC mAb (BM8)]. Expression of asialo GM1 on Mφ was determined by binding rabbit ASGM1 to PEC, washing cells with flow buffer, and staining with goat anti-rabbit IgG-PE (eBioscience) and anti-F4/80-APC mAb. Analyses were performed as outlined above.

Nitrite production
Peritoneal Mφ were prepared and cocultured with NXS2 cells or B16 cells for 48 h, as described above in the Mφ cytotoxic assay. Supernatants were collected, and nitrite accumulation was determined using Griess reagent (Sigma Chemical Co.). Equal volumes of supernatants and Griess reagent were mixed for 10 min, and the absorbance at 540 nm was measured by a microplate reader and compared with a standard nitrite curve ranging from 0 to 120 μM.

Statistical analysis
A two-tailed Student’s t-test was used to determine significance of differences between experimental and relevant control values. Kaplan-Meier survival curves were generated and analyzed by the log-rank test using the MedCalc statistics program (MedCalc Software, Mariakerke, Belgium). Data are presented as mean ± s.e. and considered significant for P values less than 0.05.

RESULTS
Anti-CD40 mAb therapy results in antitumor effects that do not require T cells or NK cells
We have recently shown that anti-CD40 mAb treatment induced antimetastatic effects in mice with experimentally induced metastatic NXS2 NB disease via a NK cell-depen-
dent mechanism [7]. To extend our studies to include localized solid tumors and specifically characterize the immune effector cells involved in the antitumor effects of anti-CD40 mAb therapy, we first determined whether anti-CD40 mAb is able to induce an antitumor effect against s.c. NXS2 tumors. Conventional A/J mice were implanted with 2 × 10⁶ NXS2 cells s.c. on Day 0, and all mice developed solid tumors, which were palpable on Day 5 and grew aggressively over a period of 3 weeks. Mice were treated by i.p injection with 0.5 mg anti-CD40 mAb or control rat IgG on Days 5 and 12 of tumor growth. Anti-CD40 mAb treatment of s.c NXS2 tumors effectively slowed tumor growth (Fig. 1A) with significant differences in mean tumor volume observable by Day 16 (P < 0.001). Comparison of the survival times of NXS2 tumor-bearing mice, based on when mice required euthanasia as a result of excessive tumor size, revealed a statistical difference between the median survival times of control and anti-CD40 mAb-treated animals, which survived 4 additional days (P = 0.0008, data not shown). Complete and nonrecurring tumor regression of a slowly growing and smaller-than-average NXS2 tumor was observed in one of the anti-CD40 mAb-treated mice. In addition, anti-CD40 mAb treatment of C57BL/6 mice bearing s.c. B16 melanoma tumors resulted in suppression of tumor growth in several experiments (data not shown), supporting our data in the NXS2 tumor model as well as our previous work [7].

To determine whether the antitumor efficacy of anti-CD40 mAb required T cells, T and B cell-deficient C.B-17 SCID

\[ \text{Fig. 1. Antitumor effects of anti-CD40 mAb in the treatment of s.c. tumors are T and NK cell-independent. (A) Conventional A/J mice (n=8) and (B) C.B-17 SCID mice (n=8) were implanted with 2 × 10⁶ NXS2 tumor cells (Day 0). Anti-CD40 mAb (open symbols) or rat IgG (solid symbols) was administed i.p. on Days 5 and 12 (indicated by arrows). Tumor volume was recorded as described in Materials and Methods and is expressed in mm}^3\). Data shown are representative of four experiments in A/J mice and two experiments in SCID mice. *, P < 0.001; **, P < 0.005. (C) SCID mice (n=6) were implanted with 2 × 10⁶ NXS2 cells s.c. on Day 0, and two groups were depleted of NK cells, as described in Materials and Methods, and also received 0.5 mg anti-CD40 mAb or rat IgG on Days 5 and 12. To control for the effect of antibody-mediated depletion on anti-CD40 mAb treatment, one group of animals received anti-CD40 mAb and control rabbit IgG. +, P < 0.01, beginning on Day 16. \]
mice were implanted with s.c. NXS2 tumors and similarly injected with anti-CD40 mAb. Figure 1B shows that anti-CD40 mAb treatment induced an antitumor response against murine NXS2 NB tumors, independent of T cells and B cells (P<0.005). Here, the difference in median survival times between the two groups was 5 days (P=0.001, data not shown). Anti-CD40 mAb treatments retarded tumor growth in one animal to enable it to survive as long as Day 28. Growth of s.c. B16 tumors in SCID animals was also inhibited significantly by anti-CD40 mAb treatment (data not shown). Furthermore, as the NXS2 tumor cell line expresses few or no CD40 molecules [7], the antitumor effect demonstrated by anti-CD40 mAb cannot be a result of direct, complement-mediated lysis with bound anti-CD40 mAb or related to direct CD40 ligation on the tumor cell surface. Altogether, these data suggest that the antitumor effect is mediated by non-T and non-B cell immune effector cells via an indirect mechanism.

Having documented a T cell-independent antitumor effect of anti-CD40 mAb, we next examined whether NK cells are

![Diagram](image-url)

**Fig. 2.** Efficacy of NK cell depletion following ASGM1 treatment. Groups of SCID mice (n=2) were treated i.p. with PBS or ASGM1 (50 μl/mouse) in 0.5 ml PBS on Days 0 and 4. Anti-CD40 mAb or rat IgG (0.5 mg) was administered i.p. on Day 5. Spleens were harvested on Day 8. Single-cell suspensions were prepared, and RBC were lysed osmotically. Splenocytes were incubated with anti-F4/80-APC mAb and anti-CD49b-FITC mAb and analyzed by flow cytometry as described in Materials and Methods. (A) Plot shows the gating (shown in the lower right) of viable cells, which was used in subsequent analyses, FSC, Forward-scatter. (B) Plot shows control isotype antibody staining on splenocytes from A. Ellipses labeled R2 and R3 depict gating used to distinguish F4/80<sup>+</sup> M<sub>φ</sub> and CD49b<sup>+</sup> NK cells, respectively, in all analyses shown in C. (C) Plots show specific gating for each treatment group, as indicated in figure. Numbers reflect the percentage of positive cells (of the cells gated and analyzed from A) located in each region.
required as effector cells for tumor rejection in vivo. This is an essential consideration, as NK cells can be activated by anti-CD40 mAb [7], and C.b-17 SCID mice may have a substantial, allogeneic, NK cell-mediated response to the NXS2 tumor cells. To address this, T and B cell-deficient SCID mice were implanted with s.c. NXS2 tumors and depleted of NK cells with ASGM1 (Fig. 1C). The efficacy of NK cell depletion has been confirmed by flow cytometric analyses and is shown in Figure 2. Here, SCID mice were treated with ASGM1 and then rat IgG or anti-CD40 mAb. Analysis of the NK cell population, based on CD49b expression, showed >93% reduction in the percentage (Fig. 2C) and absolute number (Table 1) of NK cells following ASGM1 treatment in control and anti-CD40 mAb-treated animals. Furthermore, NK cytolytic activity was also found to be reduced following ASGM1 administration (Table 1). NK activity in PEC of SCID animals treated with ASGM1 was also reduced (data not shown). The depletion of NK cells in anti-CD40 mAb-treated NXS2 tumor-bearing SCID mice did not cause a reduction in antitumor activity compared with nondepleted, anti-CD40 mAb-treated mice, arguing that NK cells are not required for in vivo antitumor effects in this model.

Anti-CD40 mAb induces antitumor effects in the absence of T cells, NK cells, and PMN

To confirm our results that T and NK cells are not required for the antitumor effect of anti-CD40 mAb against s.c. NXS2 NB tumors, C.b-17 SCID/beige mice, which are genetically deficient of T cells and cytolytic NK cells, were implanted with NXS2 tumor cells and treated with anti-CD40 mAb or control rat IgG on Days 5 and 12. As shown in Figure 3A, CD40 ligation induced a significant inhibition of tumor growth in SCID/beige mice compared with control rat IgG (P<0.015). Although NK cells in SCID/beige mice are able to secrete cytokines, this finding confirms that the cytolytic function of NK cells is not required for the antitumor effects. This experiment also included the treatment of tumor-bearing SCID/beige mice with LPS (25 μg, i.p.) as a positive control for Mφ activation. In vivo administration of this dose of LPS resulted in an antitumor effect similar to that of anti-CD40 mAb in SCID/beige animals. Together, these results show that anti-CD40 mAb therapy is effective in the absence of T cells and cytolytic NK cells, suggesting that other immune cells such as Mφ may be playing a role in the antitumor effect of anti-CD40 mAb.

Although SCID/beige mice are genetically deficient of T cells and cytotoxic NK cells, these animals retain a population of NK cells that are capable of producing inflammatory cytokines [21]. Experiments performed in our laboratory have confirmed that NK cytolytic activity in splenocytes from SCID/beige mice following anti-CD40 mAb treatment was significantly lower than that found in splenocytes from SCID mice (data not shown). However, it has been shown that CD40 ligation can activate APC to produce IL-12, which can stimulate NK cells to produce IFN-γ [22], a known activator of Mφ. Therefore, we tested whether anti-CD40 mAb therapy of s.c. NXS2 tumors in SCID/beige mice requires NK cells for the indirect activation of antimur Mφ. Following implantation of s.c. NXS2 tumors, C.b-17 SCID/beige mice received rabbit ASGM1 to eliminate NK cells or control rabbit IgG followed by anti-CD40 mAb therapy. Here, the efficacy of NK cell depletion was confirmed by flow cytometric analyses of depleted and nondepleted SCID/beige animals, where the percentage of CD49b+ NK cells was reduced from 2.33% to 0.53% among splenocytes 3 days after ASGM1 treatment (data not shown). Figure 3B, the results of the in vivo antitumor model show that the depletion of NK cells in SCID/beige animals did not change the antitumor effect of anti-CD40 mAb therapy, which was significant beginning on Day 13 of tumor growth (P<0.0075, ▲ vs. △). Although the effect of ASGM1 treatment on NK cells and Mφ in SCID mice is shown in Table 1, we further analyzed the effect of ASGM1 on PEC from SCID/beige mice. Here, 37.4% of total viable PEC expressed F4/80, and 25.9% of total viable PEC were F4/80+/asialo-GM1+ (data not shown). After ASGM1 treatment, the F4/80− Mφ population was reduced to 11.9% of total PEC, where 5.4% of total PEC were F4/80−/asialo-GM1+ cells, documenting the effect of ASGM1 depletion on asialo GM1+ cells. To determine whether the remaining Mφ population was still able to exert an antitumor effect, in a separate control in vitro experiment, we have shown that even a 75% reduction in the number of activated Mφ exerted equivalent tumoristatic effects (data not shown), suggesting that even following ASGM1 treatment, Mφ may still account

**Table 1. Analysis of NK Cells and Mφ Following Treatment with ASGM1 or Silica and Anti-CD40 mAb**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% NK cells (%)</th>
<th>% Mφ (%)</th>
<th>NK activity (%)</th>
<th>% NK cells (%)</th>
<th>% Mφ (%)</th>
<th>NK activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>50.3 (2.9)</td>
<td>20.2 (1.2)</td>
<td>29.5</td>
<td>41.6 (2.5)</td>
<td>12.0 (0.71)</td>
<td>52.0</td>
</tr>
<tr>
<td>ASGM1</td>
<td>3.31 (0.19)</td>
<td>36.3 (2.3)</td>
<td>2.8</td>
<td>1.41 (0.11)</td>
<td>26.7 (2.14)</td>
<td>6.6</td>
</tr>
<tr>
<td>Silica</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>23.0 (6.4)</td>
<td>7.56 (2.1)</td>
<td>30.3</td>
</tr>
</tbody>
</table>

Groups of C.b-17 SCID mice (n = 2) were treated with PBS, ASGM1 (50 μl/mouse in 0.5 ml PBS), or silica (25 mg/mouse in 0.5 ml PBS), all i.p., on Days 0, and 4. Anti-CD40 mAb or rat IgG (0.5 mg) was administered i.p. on Day 5. Spleens were harvested on Day 8. Splenocytes were stained with anti-F4/80-APC mAb and anti-CD49b-FITC mAb and analyzed by flow cytometry as described in Materials and Methods. Numbers of total viable cells per SCID spleen (excluding RBC and counted by eosin exclusion) were as follows (in millions of cells): PBS + rat IgG—5.7; PBS + anti-CD40 mAb—5.9; ASGM1 + rat IgG—5.3; ASGM1 + anti-CD40 mAb—8.0; silica + anti-CD40 mAb—27.7. N.D. = Not determined. 1 Percentage of CD49b+ cells of gated viable cells. 2 Total number of NK cells or Mφ per spleen (×106) is shown in parentheses. 3 Percentage of F4/80+ cells of gated viable cells. 4 NK activity in splenocytes as measured by a 4-h 51Cr assay against YAC-1 targets as described in Materials and Methods. Data shown reflect a 50:1 effector:target cell ratio.

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for the antitumor effect seen in anti-CD40 mAb-treated SCID/beige mice. Furthermore, as only peritoneal MΦ were analyzed following i.p. ASGM1 treatment, the extent of asialo GM1+ MΦ in the tumor microenvironment in these mice with s.c. tumors is not known.

As PMN are also able to exert an antitumor response in some murine tumor models [23], NXS2 tumor-bearing SCID/beige mice were depleted of Gr-1+ PMN and treated with anti-CD40 mAb therapy (Fig. 3C). The efficacy of depletion was confirmed by flow cytometric analysis, where SCID/beige mice treated with anti-Gr-1 mAb showed a reduction in Gr-1+ cells from 12% to 1.4% of total PEC (data not shown). The results of the in vivo experiment showed that PMN were not required in the observed antitumor effect; a significant antitumor effect of anti-CD40 mAb treatment persisted even when PMN were depleted (P<0.019, * vs. □). Anti-CD40 mAb treatment of SCID and SCID/beige mice, as presented in Figures 1C and 2, A–C, resulted in significant tumor growth retardation that prevented s.c. NXS2 tumors in these mice from reaching an excessive size an average of 4–5 days beyond that of control-treated groups for all experiments. Thus, in total, these data show that the antitumor effect as a result of anti-CD40 mAb therapy in SCID/beige animals is mediated by cells other than T, B, and NK cells and PMN. As MΦ (and other monocyte-derived cells) are the only CD40+ effector cells in these SCID/beige mice, these results suggest that MΦ are involved in the anti-CD40 mAb-induced in vivo inhibition of murine NXS2 NB.

In vivo anti-CD40 mAb activates peritoneal MΦ

The next series of experiments focused on the effect of in vivo anti-CD40 mAb treatment on peritoneal MΦ. In considering reports demonstrating that MΦ and monocytes could be activated in vitro by CD40 ligation to develop tumoricidal activity [9, 24], we investigated the tumorstatic effect of in vivo anti-CD40 mAb-activated peritoneal MΦ against NXS2 and B16 tumor cells in vitro. In these experiments, groups of A/J mice or C57BL/6 mice were administered anti-CD40 mAb or control IgG 5 days before PEC were collected and tested for tumoricidal activity in a [3H]-TdR incorporation assay as outlined in Materials and Methods. Figure 4A demonstrates that anti-CD40 mAb-activated MΦ from A/J mice could inhibit the proliferation of NXS2 target cells compared with control rat IgG-treated PEC in a LPS dose-dependent manner (P<0.001 at 10 ng/ml LPS). It is interesting that anti-CD40 mAb-activated MΦ from C57BL/6 mice exhibited strong antitumor effects against B16 melanoma cells even in the absence of LPS (P<0.001, rat IgG vs. anti-CD40 for all conditions). In addition, these CD40-ligated MΦ showed enhanced activation with...
10 or 0.1 ng/ml concentrations of LPS as a second signal (Fig. 4A).

Cytokine production was evaluated to assess the in vivo effect of anti-CD40 mAb on peritoneal Mφ. We have recently shown that in vitro stimulation with anti-CD40 mAb for 24 h causes a significant increase in the percentage of Mφ, which express cytoplasmic IFN-γ; this IFN-γ expression is IL-12-dependent [14]. Here, A/J mice or C57BL/6 mice were injected in vivo with anti-CD40 mAb or control rat IgG, and Mφ were stained for intracellular IFN-γ, IL-12 p70, TNF-α, IL-4, and IL-10. In Figure 4B, anti-CD40 mAb-activated Mφ from A/J mice showed enhanced expression of intracellular IFN-γ, IL-12 p70, and TNF-α compared with Mφ from rat IgG-treated mice (Fig. 4B, upper row vs. lower row). However, there was no detectable increase in IL-4 or IL-10 expression. The percentage of F4/80+ cells expressing each cytokine after in vivo treatment with rat IgG or with anti-CD40 mAb is presented for three experiments in Table 2. Significant differences were seen for IL-12 and TNF-α, and the P value for IFN-γ was 0.0685. In summary, in vivo anti-CD40 mAb treatment resulted in peritoneal Mφ, which produced members of the Th1 cytokine profile and resembled classically activated effector or M1 phenotype, based on cytokine expression patterns. In addition, we evaluated the expression of several Mφ cell surface markers, including MHC class II, CD80, CD86, CD40, and TLR4. Figure 4C shows that treatment with anti-CD40 mAb increased the expression of MHC class II, CD80, CD86, and TLR4 on the surface of F4/80+ Mφ, as seen by increases in MFI ratios. This documented the activated state of Mφ, as well as suggested that these cells may enhance their responsiveness to anti-CD40 mAb and LPS (the ligand for TLR4). Specifically, further increase in CD40 expression after stimulation with anti-CD40 mAb provided a rationale for the immunotherapy protocol used in this study.

Fig. 4. Characterization of in vivo anti-CD40 mAb-activated Mφ. (A) PEC were harvested from A/J mice (left panel) and C57BL/6 mice (right panel) injected with anti-CD40 mAb or control rat IgG 5 days earlier (four per group). A Mφ cytostatic assay on NXS2 cells (left panel) and B16 cells (right panel) was performed as described in Materials and Methods. These results are representative of three similar experiments.* P < 0.001. (B) PEC were collected from A/J mice (four per group) 5 days after rat IgG (upper row) or anti-CD40 mAb injection (lower row). Histograms show F4/80+ Mφ stained for intracellular cytokines (filled gray peaks): IFN-γ, IL-12, TNF-α, IL-10, or IL-4 compared with isotype controls (solid black lines). (C) Total PEC were harvested from A/J mice (four per group) 5 days after rat IgG (upper row) or anti-CD40 mAb (lower row) treatment and stained for cell surface expression of F4/80+ and MHC class II, CD80, CD86, CD40, and TLR4, as described in Materials and Methods. Histograms show F4/80+ Mφ analyzed for surface staining with isotype control mAb (solid black lines) or specific cell surface markers (filled gray peaks). Numbers represent MFI ratios for each cell surface marker.
whereby multiple injections of anti-CD40 mAb were administered for the purpose of increasing the antitumor effect.

**Anti-CD40 mAb-activated Mφ are involved in antitumor effects**

Having observed the persistent antitumor effects induced by anti-CD40 mAb in the absence of T and NK cells and PMN, we next addressed whether anti-CD40 mAb activates Mφ in T cell-deficient and NK cell-dysfunctional SCID/beige mice. Groups of C.b-17 SCID/beige mice were injected i.p. with anti-CD40 mAb or control rat IgG, and peritoneal Mφ were tested for tumorstatic activity in vitro by $[^{3}H]$-TdR incorporation assay. The results in Figure 5A show that PEC collected from anti-CD40 mAb-treated SCID/beige mice demonstrated increased antitumor activity against NXS2 cells and B16 cells in vitro compared with control PEC, which was evident in the presence of LPS. In these control-treated SCID/beige animals, high doses of LPS alone resulted in substantial Mφ activation. However, the degree of tumorstatic activity following in vivo anti-CD40 mAb treatment was increased significantly with low and high concentrations of LPS compared with Mφ from control animals ($P<0.025$).

In agreement with studies that have shown enhanced NO production following activation of Mφ by CD40 ligation [9, 11], we also observed increased nitrite levels following in vivo anti-CD40 mAb treatment, which correlated with Mφ tumorstatic activity. Figure 5B shows significantly elevated nitrite levels in supernatants collected from anti-CD40 mAb-activated SCID/beige Mφ cocultured with NXS2 or B16 target cells, compared with control-treated PEC. Similar results have also been observed following CD40 ligation of PEC from conventional A/J and C57BL/6 mice (data not shown). The results presented in Figure 5, A and B, demonstrate that Mφ can be activated by CD40 ligation in SCID/beige mice, including demonstration of enhanced responsiveness to LPS, suggesting that these activated Mφ may be involved as effector cells against s.c. tumors following anti-CD40 mAb therapy.

To determine whether Mφ are involved in the antitumor effect of anti-CD40 mAb therapy, silica was used to inhibit the function of Mφ in NXS2 tumor-bearing A/J mice treated with anti-CD40 mAb. First, however, we verified the in vivo effects of silica on Mφ. The effect of silica on the percentage of Mφ was somewhat variable. The percentage of F4/80$^+$ Mφ in PEC from silica-treated A/J mice was not reduced when assessed by flow cytometric analyses (data not shown). There was a small reduction in the percentage of Mφ but an increase in the total number of Mφ seen in splenocytes from SCID mice as a result of activation with anti-CD40 mAb (Table 1). However, we demonstrated the efficacy of Mφ inactivation by silica by assessing a global response to LPS-induced weight loss, as also described by Pulaski et al. [25]. In four separate experiments, we observed a reduced susceptibility of silica-treated mice to LPS-induced cachexia. In a typical experiment, groups of A/J mice (n=4) were treated with 25 mg silica 3 days before being challenged with 100 μg LPS i.p. Body weight of each mouse was measured before the LPS challenge and on the next 3 days. Animals that received silica before LPS challenge showed a greater percent of body mass maintained over 3 days (90.3%±1.2 on Day 2; 94.3%±0.9 on Day 3) compared with PBS-treated, control mice (82.9%±1.2 on Day 2; 87.9%±0.9 on Day 3; data not shown). The decreased weight loss in silica-treated animals was significant ($P<0.009$ on Day 2 and $P<0.0027$ on Day 3), demonstrating that silica inhibited Mφ-mediated, LPS-induced cachexia.

Therefore, having shown that silica can inhibit Mφ function in vivo, we used silica to assess the role of Mφ in the antitumor effects of anti-CD40 mAb. In this model, silica treatment resulted in significant reduction in the antitumor effect of anti-CD40 mAb on Days 16 and 19 (Fig. 5C; $P=0.038$ and $P=0.041$, respectively, anti-CD40 mAb vs. anti-CD40 mAb+silica). Silica alone did not significantly affect the growth of NXS2 tumors at any time-point (i.e., rat IgG vs. rat IgG+silica). Animals treated with anti-CD40 and silica did show reduced tumor growth compared with animals that received rat IgG and silica ($P<0.0063$, Day 16; $P<0.011$, Day 19); however, the percentage of mean tumor growth inhibition as a result of anti-CD40 therapy was less in mice that also received silica (51.1%) compared with mice without silica treatment (68.5%) on Day 16. Thus, the reduction in the antitumor effect induced by anti-CD40 mAb by silica suggests that Mφ play a role in the efficacy of this treatment.

**TABLE 2. Intracellular Cytokine Expression of Anti-CD40 mAb-Activated Mφ**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>A/J mice (Day 3)</th>
<th>C57BL/6 mice (Day 3)</th>
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<tbody>
<tr>
<td>Rat IgG</td>
<td>Anti-CD40</td>
<td>Rat IgG</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>IL-12</td>
<td>TNF-α</td>
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<tr>
<td>64</td>
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<td>0</td>
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<tr>
<td>68</td>
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<tr>
<td>6</td>
<td>3</td>
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<tr>
<td>73</td>
<td>90</td>
<td>68</td>
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PEC were harvested from A/J mice or C57BL/6 mice (three per group), 3 or 5 days after i.p. treatment with 0.5 mg anti-CD40 mAb or rat IgG. All PEC were stained for intracellular cytokine expression as described in Materials and Methods, except C57BL/6 PEC were cultured in medium for 24 h before staining. Numbers shown represent the percentage of total F4/80$^+$ cells that expressed the cytokine of interest compared with isotype antibody staining. As a result of the need to obtain sufficient cell numbers to perform these assays, PEC were pooled from three animals per group, and thus, intraexperimenter statistical comparisons were not possible. Therefore, $P$ values of each cytokine expression are compared between mice receiving rat IgG versus mice treated with anti-CD40 mAb for three combined experiments.
Anti-CD40 mAb-activated Mϕ respond to associated tumor cells

To address whether anti-CD40 mAb-activated Mϕ could exert effector activity as intratumoral Mϕ, we developed an in vivo model system, where Mϕ in the tumor environment were activated by anti-CD40 mAb. Specifically, mice were challenged with tumor cells in the peritoneal cavity, and 5 days later, peritoneal Mϕ were activated by i.p. injection of anti-CD40 mAb. A/J mice implanted i.p. with 3 × 10⁵ NXS2 cells (Day 0) were treated with anti-CD40 mAb or rat IgG on Days 5 and 12. Median survival time for the anti-CD40 mAb-treated animals was 4 days longer than the control-treated animals (Fig. 6, P=0.0038). We also addressed the antitumor efficacy of Mϕ activated by anti-CD40 mAb on tumor initiation and progression. Here, peritoneal Mϕ of C57BL/6 mice were activated by anti-CD40 mAb 3 days before i.p. inoculation with B16 cells. The difference in median survival time of 8 days was significant (P=0.0058, data not shown). In addition to these results observed in immunocompetent mice, we have also studied the effects of intratumoral anti-CD40 mAb-activated Mϕ in SCID/beige animals. Here, groups of eight SCID/beige mice were challenged i.p. with 2.5 × 10⁵ NXS2 cells and treated with anti-CD40 mAb or control rat IgG on Days 3, 5, 9. Although all of the animals died or needed to be killed by Day 22 as a result of toxicities related to large i.p. tumor burden, the median survival of the anti-CD40 mAb treatment group was slightly longer (2 days) than the control group; this difference was statistically significant (P=0.0492, data not shown). Thus, in total, we have seen in three similar in vivo models of i.p. tumor growth, a role for anti-CD40 mAb-activated intratumoral Mϕ in providing a modest but significant antitumor effect.

Having determined that anti-CD40 mAb-activated Mϕ can be effective in the presence of tumor cells in vivo, we next addressed whether the tumor itself might be providing additional stimuli to the CD40-ligated Mϕ, accounting for in vivo antitumor effects that occurred in the absence of exogenous...
LPS. As an extension of the results shown in Figure 4A, which showed in vitro tumoristatic activity of anti-CD40 mAb-activated Mφ when cultured with LPS and tumor cells, we sought to determine the role of the tumor on the in vitro activation of anti-CD40 mAb-activated Mφ. To test this hypothesis, we measured the NO production of anti-CD40 mAb-activated PEC from C57BL/6 mice cultured in the presence or absence of B16 tumor cells for 42 h. The results shown in Figure 7 show that the presence of B16 cells activated CD40-ligated PEC to release significant levels of NO, even in the absence of LPS; however, PEC in the absence of B16 cells did not produce detectable levels of NO unless LPS was also present (P = 0.0011). As a negative control, B16 cells cultured alone did not produce measurable levels of NO. Thus, these data suggest that CD40-activated Mφ can be activated further by associated tumor cells.

**DISCUSSION**

CD40 ligation induces the maturation of APC, which enables these cells to function in various essential immune responses, including antitumor effects. In the present study, we investigated the antitumor effects of agonistic anti-CD40 mAb to further understand the effector cells involved in anti-CD40 mAb-induced primary antitumor immune defenses. We observed significant antitumor efficacy of anti-CD40 mAb therapy against s.c. NXS2 tumors in the absence of T, B, NK cells, or PMN, suggesting that Mφ might play a role in this antitumor effect. In addition, we found that in vivo CD40 ligation resulted in increased Mφ-mediated tumoristatic activity against NXS2 and B16 tumor cells in vitro, as well as an activated Mφ phenotype characterized by production of proinflammatory cytokines and NO production, all supporting the role of Mφ as antitumor effector cells in anti-CD40 mAb immunotherapy. Anti-CD40 mAb-activated intratumoral Mφ exerted an antitumor effect, and the CD40-activated Mφ were able to be activated further by associated tumor cells.

Inhibition of Mφ function in A/J mice by silica substantially inhibited the antitumor effect of anti-CD40 mAb, showing an in vivo role for Mφ. These results have been confirmed and extended in separate studies examining the role of Mφ in the antitumor effect of anti-CD40 mAb and CpG-oligodeoxynucleotides (CpG-ODN). The in vivo antitumor efficacy of the combined anti-CD40 mAb and CpG-ODN therapy was also reduced significantly by silica inhibition of Mφ [26]. This finding supports our conclusion that anti-CD40 mAb activates Mφ, which can serve as antitumor effector cells. In addition, in separate studies, we have shown that depletion of Mφ in NXS2 tumor-bearing A/J mice by clodronate liposomes [27] reduced the antitumor effect of anti-CD40 mAb such that anti-CD40 mAb + clodronate liposome-treated animals had the largest and fastest growing tumors (data not shown). We also investigated the role of silica on NK activity. Our data in Table 1 show that silica treatment somewhat reduced NK cell activity. We believe that this decrease in NK activity may be a result of the indirect effects of Mφ inactivation by silica. In separate experiments (not shown), activation of NK cells by in vivo IL-2 administration was not inhibited by concurrent silica administration, suggesting that silica does not have a direct effect on NK cells. Further investigation of the effects of silica on NK cells from anti-CD40 mAb-treated animals is needed.

Recent reports have shown that CD40 ligation of monocytes and Mφ in vitro results in the development of tumoricidal effects [9, 24]. Furthermore, we have recently found that in vivo, anti-CD40 mAb activates peritoneal Mφ in an IFN-γ-dependent manner to kill tumor cells in vitro via the induction of apoptosis [10]. In this study, we have documented differences in sensitivity to anti-CD40 mAb activation of Mφ from different mouse strains (Fig. 4A). In particular, CD40 ligation in C57BL/6 mice, in contrast to A/J mice, resulted in significant tumoristatic activity in the absence of LPS. The degree of effectiveness of anti-CD40 mAb likely may vary depending on mouse host strain, tumor origin, and tumor location. Antitumor Mφ may secrete various factors that inhibit tumor growth, such as cytokines, lysozymes, proteases, and complement components [8]. Activated Mφ have been shown to demonstrate tumor cytotoxicity in vitro by cell contact-dependent and independent mechanisms [20]. However, the mechanisms by which anti-CD40 mAb-activated Mφ kill tumor cells and limit tumor growth in vivo remain unclear and warrant further investigation. NO and TNF-α are known soluble, cytotoxic effector molecules and are examples of nonspecific, contact-independent, Mφ-mediated tumour cytotoxicity mechanisms [29, 30]. Although our results show that in vivo activation of Mφ with anti-CD40 mAb induced tumoristatic activity and NO production, these effects could be inhibited in vitro by the NO inhibitor L-arginine-methyl ester or anti-TNF-α-neutralizing mAb (unpublished results) but not by the pan-caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (unpublished results). This suggests a role for NO and TNF-α in the caspase-independent tumor cytotoxicity induced by anti-CD40 mAb-activated Mφ. Further studies to evaluate the roles of these soluble, cytotoxic effector molecules in the anti-CD40 mAb-mediated, antitumor

**Fig. 7.** Anti-CD40 mAb-treated Mφ are activated by tumor cells. PEC were harvested from C57BL/6 mice (three per group) 5 days after injection of 0.25 mg anti-CD40 mAb or control rat IgG. Adherent PEC were cultured in the presence or absence of 1 × 10⁶ B16 cells and 10 ng/ml LPS for 42 h. Supernatants were collected and assayed for NO concentration by Griess reagent. #, NO concentration was below detectable levels. Results shown are representative of two experiments. *, P = 0.0011.
effect are needed to understand the molecular mechanisms of the antitumor effects mediated by anti-CD40 mAb-activated Mφ.

We have shown that anti-CD40 mAb activation of peritoneal Mφ resulted in production of Th1 cytokines (IFN-γ, IL-12, and TNF-α) and increased expression of Mφ cell surface molecules, suggesting that CD40 ligation results in the development of proinflammatory, M1-like, classically activated effector cells. The relevance of this finding is underscored by the knowledge that the presence of tumor often causes immunosuppression, possibly mediated by tumor-associated M2 Mφ. Future studies will address the effect of anti-CD40 mAb on Mφ from tumor-bearing mice and whether CD40 ligation can change the phenotype of resident versus activated Mφ. In addition, i.p. treatment with anti-CD40 mAb activated resident peritoneal Mφ to serve as intratumoral effector cells, resulting in antitumor effiacy against peritoneal tumors. Thus, we have observed local and systemic effects of anti-CD40 mAb therapy. The mechanisms of this effect, whether by stimulating antitumorm Mφ infiltration into the tumor or Mφ production of cytokines and other effector molecules, remain to be determined.

In vitro evaluation of in vivo anti-CD40 mAb-activated Mφ showed enhanced Mφ tumoricidal activity in the presence of LPS (Figs. 4A and 5A). This suggests that CD40 ligation on Mφ primes the cells to be more sensitive to a strong second triggering signal such as LPS or CpG-ODN (data not shown). However, LPS is not required for activation of effector functions by CD40-ligated Mφ; we have recently demonstrated that endotoxin-resistant C3H/HeJ mice showed effective tumoricidal activity against B16 cells by anti-CD40 mAb-activated Mφ, which was similar to LPS-sensitive control C3H/HeQueJ mice [26]. Furthermore, the presence of significant inhibition of tumor growth observed in vivo as a result of anti-CD40 mAb therapy without addition of LPS suggested that other factors may provide further activation to stimulate in vivo CD40-ligated Mφ. Our finding that the presence of tumor cells cocultured with anti-CD40 mAb-activated Mφ induced NO production (Fig. 7) suggests that the tumor itself may present factors or ligands that can activate anti-CD40 mAb-treated Mφ. An alternative possibility is that endogenous TNF-α, IFN-γ, and IFN-α, as well as other factors produced by anti-CD40 mAb-activated Mφ (Fig. 4B) may serve as autocrine or paracrine second stimuli in vivo. Indeed, we repeatedly observe decreases in NO production by anti-CD40-activated Mφ stimulated in vitro with LPS in the presence of tumor cells, when blocking antibodies to TNF-α or IFN-γ are also present in the tissue-culture medium (data not shown).

It is important to note that although this study has found that in vivo activation of Mφ by anti-CD40 mAb therapy resulted in significant antitumor activity detectable in the absence of T and NK cells, it does not rule out a role for NK cells in the antitumor effects of anti-CD40 mAb [7]. Immune control of primary versus metastatic disease may involve distinct mechanisms, and studies have identified NK cells as among the critical components of innate immunity against metastatic tumors [31, 32]. Thus, although NK cells are activated by anti-CD40 mAb and involved in limiting the number of liver metastases in a model of NXS2 NB disease following intravenous injection of tumor cells [7], the role of NK cells in a s.c. tumor model following anti-CD40 mAb may not be readily detectable, as suggested by the findings in this study. In addition, NK cells may be more involved in antitumor effects in other s.c. tumor models as we showed previously [7].

In conclusion, we have shown that anti-CD40 mAb therapy induced antitumor effects against s.c. and i.p. NXS2 NB, which did not require T, B, and NK cells or PMN and involved Mφ. In addition, in vivo anti-CD40 mAb-activated Mφ from conventional and T and NK cell-deficient mice demonstrated increased tumorstatic activity and NO production in vitro. CD40 ligation primed Mφ to further stimulation by LPS or the tumor, which resulted in strong antitumor effects. Taken together, our results suggest that anti-CD40 mAb is able to limit tumor growth by a T cell-independent immune mechanism that involves activated Mφ.

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REFERENCES


