Published in final edited form as:

J Immunother. 2013 January ; 36(1): 29–40. doi:10.1097/CJI.0b013e3182780f61.

Intratumoral delivery of low doses of anti-CD40 mAb combined with monophosphoryl lipid A induces local and systemic antitumor effects in immunocompetent and T cell-deficient mice

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Abstract

In this study, an agonistic anti-CD40 monoclonal antibody was combined with monophosphoryl lipid A (MPL), a nontoxic derivative of LPS and agonist of toll-like receptor 4, to assess the immunomodulatory and antitumor synergy between the two agents in mice. Anti-CD40 was capable of priming macrophages to subsequent *ex vivo* activation by MPL in immunocompetent and T cell-depleted mice. Intraperitoneal injections of anti-CD40+MPL induced additive to synergistic suppression of poorly immunogenic B16-F10 melanoma growing subcutaneously in syngeneic mice. When anti-CD40+MPL were injected directly into the subcutaneous tumor, the combination treatment was more effective, even with a 25-fold reduction in dose. Low-dose intratumoral treatment also slowed the growth of a secondary tumor growing simultaneously at a distant, untreated site. Antitumor effects were also induced in immunodeficient SCID mice and in T cell-depleted C57BL/6 mice. Taken together, our results show that the antitumor effects of anti-CD40 are enhanced by subsequent treatment with MPL, even in T cell-deficient hosts. These preclinical data suggest that an anti-CD40+MPL combined regimen is appropriate for clinical testing in human patients, including cancer patients that may be immunosuppressed from prior chemotherapy.

Keywords

immunotherapy; anti-CD40; monophosphoryl lipid A; macrophages; intratumoral

Introduction

The CD40 receptor protein is expressed on antigen-presenting cells and is an important co-stimulatory molecule for T cell, B cell, dendritic cell, and macrophage (MΦ) activation. Many experimental cancer immunotherapies are designed to activate the CD40 pathway in order to enhance the potency of tumor-specific cytotoxic T lymphocytes.¹ Unfortunately, effective preclinical T cell-mediated therapies have been difficult to translate into clinical

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Conflicts of Interest: The authors declare no existing conflicts of interest.

settings²⁻⁷ because of immunosuppressive factors in the tumor microenvironment^{6,8} and immunosuppression caused by other factors, including conventional cancer treatments.^{9,10} However, innate immune cells, including MΦ, have been shown to be more durable and recover from chemotherapy more quickly than B or T lymphocytes,³ suggesting that T cell-independent antitumor strategies based on the innate immune system may be of value as alternatives to T cell-dependent immunotherapies. Indeed, T cells are not the only type of cells that can be activated by CD40 ligation; our lab¹¹⁻¹⁴ and others¹⁵ have shown that T cells may not be necessary for the antitumor effects observed when this pathway is activated. The importance of an innate immune system-based approach is highlighted by a recent paper by Beatty et al. showing regression of pancreatic carcinoma in patients treated with anti-CD40 and gemcitabine and establishing a role for MΦ in the antitumor effects.¹⁵

Previous *in vitro* studies in our lab showed that anti-CD40 stimulated MΦ to mediate antitumor effects in an IFNγ-dependent manner.¹² Anti-CD40 was also found to initiate T cell-independent antitumor effects against intraperitoneal (i.p.)¹³ and subcutaneous (s.c.)¹⁴ B16 tumors in mice. When combined with a toll-like receptor (TLR) 9 agonist, CpG, the antitumor effects of anti-CD40 were synergistically enhanced, retarding tumor growth and prolonging survival in C57BL/6 and SCID/beige mice bearing either B16 melanoma or NXS2 neuroblastoma tumors, respectively. The antitumor effects persisted in the absence of T cells, cytolytic NK cells, and neutrophils.¹⁴ CpG has been used as a T cell adjuvant preclinically¹⁶ and clinically;¹⁷ however, while the ability of CpG to activate murine MΦ has been documented by our group^{14,18} and others,¹⁹ it seems less effective in activating human MΦ²⁰, necessitating the search for other MΦ-activating TLR agonists which would synergize with anti-CD40 for clinical cancer immunotherapy development. As an activator of the TLR4 pathway, lipopolysaccharide (LPS) activates MΦ^{21,22} and also synergizes with anti-CD40 to activate MΦ *in vitro*,¹² but the therapeutic potential of LPS *in vivo* is limited because of its severe toxicity in mammals. However, the component of LPS that is primarily responsible for its immunologic effects, Lipid A, can be chemically modified to produce monophosphoryl lipid A (MPL), a potent immunostimulant which is significantly less toxic than LPS.^{23,24}

TLR agonists have potential as adjuvants for future cancer therapies, especially when combined with other agents.¹⁹ MPL has been effective as a vaccine adjuvant,^{5,25-30} but its role in promoting the immune response against cancer has not been fully explored. The first goal of this study was to determine if MPL, in a manner similar to CpG or LPS, could be combined synergistically with anti-CD40 to prompt immune cells, specifically MΦ, to inhibit tumor cell proliferation *in vitro*. Using the immunotherapy protocol that we established for anti-CD40+CpG, we then also assessed the *in vivo* antitumor effects of anti-CD40 combined with MPL. Two treatment approaches were explored: a high-dose, systemic treatment injected i.p.; and a local, low-dose treatment injected directly into a growing tumor. In addition, we tested whether T cells were required for MΦ activation and the resulting antitumor effects after treatment with anti-CD40+MPL. The results show that the antitumor effects of anti-CD40 are enhanced by subsequent treatment with MPL, even in T cell-deficient hosts. These data suggest that anti-CD40+MPL could be a clinically-promising immunotherapy for immunosuppressed cancer patients.

Materials and Methods

Mice and cell lines

Female C57BL/6 and CB-17 SCID mice (6 to 8 weeks old), were obtained from Taconic Farms (Germantown, NY) or from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in the University of Wisconsin-Madison animal facilities at the Wisconsin Institutes for Medical Research. All experimentation was performed in accordance to protocols

approved by the National Institutes of Health and by the Animal Care and Use Committees of UW-Madison. The B16-F10 melanoma tumor cell line was used as a tumor model because it is weakly immunogenic and syngeneic to the C57BL/6 strain of mice. B16-F10 cells were grown in RPMI 1640 complete medium supplemented with 10% FCS (Sigma Chemicals, St. Louis, MO), 2 mM L-glutamine and 100 U/ml of penicillin/streptomycin (all from Life Technologies, Inc., Grand Island, NY) at 37°C in a humidified 5% CO₂ atmosphere.

Antibodies and reagents

FGK 45.5 hybridoma cells capable of producing the agonistic anti-CD40 Ab were a gift from Dr. F. Melchers (Basel Institute for Immunology, Basel, Switzerland). The mAb was obtained from ascites of nude mice injected previously with the hybridoma cells, and the ascites was then enriched for IgG by ammonium sulfate precipitation.¹² MPL from *Salmonella enterica* serotype minnesota (#L6895) was purchased from Sigma-Aldrich (St. Louis, MO) and reconstituted as previously described.³¹ Rat IgG and LPS (from *Salmonella enteritidis*) were purchased from Sigma-Aldrich (St. Louis, MO). Unmethylated CpG-1826 oligodeoxynucleotide (TCCATGACGTTCTGACGTT) was purchased from TriLink Biotechnologies (San Diego CA).

Activation of MΦ with anti-CD40 and MPL

Mice were injected i.p. with 0.5 mg of either anti-CD40 or Rat IgG in 0.5 ml calcium- and magnesium-free phosphate buffered saline (PBS). After 3 days, peritoneal exudate cells (PECs) were obtained by peritoneal cavity lavage with 5 ml of cold RPMI 1640 complete medium. Collected PECs were placed into 96 well flat-bottom cell culture plates (Corning Inc, Corning, NY) at a concentration of 2×10^5 cells per well in 0.1 ml medium. The peritoneal MΦ population was enriched by allowing PECs to adhere to the plastic wells for 1½ to 2 h, followed by careful aspiration and removal of non-adherent cells. In our previous studies, flow cytometry indicated that approximately 40% of the PECs adhere and 95% of these adherent cells were MΦ, based on their expression of the F4/80 MΦ marker.¹² For our studies, we choose not to take MΦ off the plastic after adherence to get accurate cells counts, as this process can reduce their viability and can induce the “danger” signal, which could activate MΦ and interfere with our studies on MΦ activation with anti-CD40 and MPL. Adherent MΦ were then co-cultured with B16 melanoma cells (10^4 cells per well) for 48 h in complete RPMI 1640 medium, alone or in the presence of MPL (5 µg/ml), CpG (5 µg/ml), or LPS (10 ng/ml). Assay plates were incubated at 37°C in a humidified 5% CO₂ atmosphere. To neutralize cytotoxic factor produced by anti-CD40+MPL-activated MΦ, 10 µg/ml of functional grade blocking antibodies to TNF-α (clone MP6-XT3), TRAIL (clone N2B2) or FasL (clone MFL3), (eBioscience, San Diego, CA.), and 5 mM of the inducible nitric oxide synthase (iNOS) inhibitor L-nitro-arginine-methyl esterase (L-NAME, Sigma), alone or in combination, were added to PECs from anti-CD40-treated mice 30 min before adding of MPL and B16 tumor cells.

MΦ-induced tumor cytostasis assay

The antitumor effect of mouse MΦ activated by anti-CD40 and MPL was determined by co-culturing MΦ and B16 cells for 48 h and pulsing the wells with tritiated thymidine ([³H]-TdR, PerkinElmer, Boston, MA, 1 µCi/well) for the last 6 h of incubation. [³H]-TdR incorporation was determined by liquid β-scintillation counting of total cells after harvesting onto glass fiber filters (Packard, Meriden, CT), using the Packard Matrix 9600 Direct β-counter (Packard, Meriden, CT). MΦ incorporate negligible amounts of [³H]-TdR compared to rapidly-dividing B16 cells.^{12,32}

Nitric oxide (NO) production assay

Peritoneal MΦ were prepared and co-cultured with B16 cells as described in the MΦ cytostatic assay above. Supernatants were collected after 42 h, and nitrite accumulation was measured using Griess Reagent (Sigma, St. Louis, MO). Equal volumes of supernatants and Griess Reagent (50 μL each) were mixed for 10 min, and the absorbance at 570 nm was measured by a microplate reader and compared to a standard nitrite curve ranging in concentration from 0–125 μM.

Flow cytometric analysis of TLR expression

PECs from C57BL/6 mice injected on day -1, -3, or -5 with anti-CD40 or Rat IgG were collected on day 0. As TLR4 can only bind LPS or MPL when it is associated with MD-2 (lymphocyte antigen 96),³³ PECs were stained with anti-TLR4-MD-2-PE (clone MTS510, eBioscience, San Diego, CA), and also with anti-F4/80-APC (clone BM8), for 40 min at 4°C. After surface staining, cells were washed, fixed, and permeabilized as described previously.¹² Flow cytometry was performed on a FACSCalibur flow cytometer (BD, Franklin Lakes, NJ) and analyzed for TLR4 expression within the F4/80⁺ cell population using FlowJo Software (Ashland, OR).

Fluorescence-activated cell sorting

PECs from anti-CD40-treated C57BL/6 mice were collected and stained for 40 min at 4°C with anti-CD11b-APC (clone M1/70, BioLegend, San Diego, CA), anti-Gr-1-PE (clone RB6-8C5), anti-F4/80-FITC (clone BM8), and anti-CD19-PECy7 (clone 1D3) fluorochromes (eBioscience, San Diego, CA). Cells were then sorted into purified populations according to their immunophenotype or forward scatter and side scatter characteristics. FACS sorting was performed with a FACS Aria cell sorter (BD, Franklin Lakes, NJ). The purity of the sorted populations was confirmed by standard flow cytometry. For functional assays involving sorted cells, 5×10⁴ sorted PECs and 1×10⁴ B16 tumor cells were plated in triplicate in 96-well flat-bottomed plates. The [³H]-TdR incorporation and NO assays were performed as described in the sections above.

Immunohistochemistry

The cytospin preparations of sorted PECs were stained with Wright-Giemsa Stain (Sigma) as described.³⁴ Briefly, PECs were centrifuged at 800 rpm for 3 min, fixed in 100% methanol for 2 min, and stained horizontally with Wright-Giemsa Stain (Sigma) for 45 s. Cells were further stained with an equal volume mixture of Wright-Giemsa Stain and glass filtered water for 10 min, washed, and destained for 5 min. Pictures of cells were taken at 40x magnification using the Magnafire 2.1 computer software (Optronics, Goleta, California).

In vivo tumor model and therapy

C57BL/6 mice or CB-17 SCID mice were injected subcutaneously (s.c.) in the lower-right quadrant of the shaved abdomen with 1×10⁵ viable B16 melanoma cells in 0.1 ml PBS on day 0. In some experiments, a primary tumor was implanted s.c. in the right flank of the animal on day 0, followed by a secondary s.c. tumor injection in the left flank on day 3. Mice were randomized into treatment groups after tumors were injected. For mice receiving i.p. treatment, 0.5 mg anti-CD40 or 0.5 mg rat IgG was injected i.p. in 0.5 ml PBS on days 4 and 11, and 50 μg MPL in 0.5 ml or 0.5 ml PBS was injected i.p. on days 7 and 14. For mice receiving intratumoral (i.t.) therapy, 20 μg anti-CD40 or 20 μg rat IgG was injected i.t. into the primary tumor on days 5 and 12, and either 2 μg MPL or PBS was given i.t. on days 8 and 15. Intratumoral injections were administered in 0.1 ml PBS. The perpendicular diameters of s.c. tumors were measured every 3 to 4 days using digital calipers. Tumor

volumes were estimated according to the formula: $(1/2) \times \text{tumor length} \times \text{tumor width}$.² Mice that had no visible or palpable tumors by day 8 post-tumor cell inoculation were excluded from experimental analysis.

In vivo T cell depletion

For certain experiments, C57BL/6 mice were depleted of T cells with a mixture of 250 μg anti-CD4 mAb (clone GK1.5) and 250 μg anti-CD8 mAb (clone 2.43) injected i.p. 2 days before the start of treatment. Injections of depleting Abs were repeated every 5 days for the remainder of the experiment. Non-depleted mice received 500 μg rat IgG as a control. The efficacy of T cell depletion was confirmed by flow cytometry showing an 83.4% reduction of CD4⁺ lymphocytes and an 89.3% reduction of CD8⁺ lymphocytes compared to CD4⁺ and CD8⁺ lymphocytes from IgG control mice.

Statistical analysis

A two-tailed Student's t-test was used to determine significant differences between experimental and relevant control means for *in vitro* experiments. Survival data were analyzed with the log-rank test. Differences in the mean tumor growth rate of treatment groups were determined by fitting the tumor growth curve of each mouse to an exponential curve using the equation for exponential growth ($V_t = V_0 e^{rt}$), where V_0 is tumor volume, t is time, and the rate constant r is the parameter taken to describe the overall tumor growth rate for each mouse. The parameter V_0 was calculated to be the same for all mice within an experiment. The group mean r was compared between groups using a two-tailed Student's t-test. For all tests, $p < 0.05$ was considered statistically significant. In all figures, p -values are represented with asterisks (*) as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Graphs were generated and significance tests were performed using the GraphPad Prism 5.04 software.

Results

Anti-CD40 and MPL synergize to activate M Φ

Our first set of experiments tested whether an i.p. injection of 0.5 mg anti-CD40 into naïve C57BL/6 mice could prime M Φ to further stimulation by MPL *ex vivo*. Our previous studies showed that anti-CD40 upregulated TLR9 expression in M Φ in a time-dependent manner, peaking 3 days after anti-CD40 and correlating with anti-CD40/CpG-induced antitumor effects.¹⁴ Since the receptor for MPL is TLR4, we looked for a similar pattern of anti-CD40-mediated upregulation of TLR4. The level of MPL-responsive TLR4 was indeed enhanced in F4/80⁺ M Φ when analyzed by flow cytometry on days 1, 3, and 5 post-treatment with anti-CD40 (Fig 1A). A similar effect was seen for TLR9 expression (data not shown), in agreement with our previous data.¹⁴ Two mice were tested for each time point and gave consistent results. The baseline TLR4 expression from one representative naïve mouse had a mean fluorescence intensity (MFI) of 9.6, compared to MFIs of 25 on day 1, 33.2 on day 3, and 24.4 on day 5 following anti-CD40 (Fig. 1A).

Since TLR4 expression seemed to reach peak levels 3 days after *in vivo* treatment with anti-CD40, we hypothesized that M Φ from anti-CD40-treated mice would show higher levels of antitumor activity upon exposure to MPL on day 3, compared to M Φ from rat IgG-treated mice. Indeed, M Φ from anti-CD40-treated mice were further activated by MPL *in vitro*, almost completely inhibiting B16 proliferation (Fig 1B) and secreting a significant amount of NO (Fig 1C). A comparable synergistic effect with anti-CD40 was also seen when the cells were stimulated by LPS or CpG, which were used as positive controls. In addition to their ability to suppress proliferation of B16 murine melanoma, M Φ that were primed by anti-CD40 and activated by MPL also suppressed the proliferation of human tumor cell

lines, including M21 melanoma, ECC-1 endometrial carcinoma, SKOV-3 and OVCAR-3 ovarian carcinomas, K562 myelogenous leukemia, and RPMI-8226 multiple myeloma (data not shown).

To address the mechanism of tumor cell inhibition by anti-CD40+MPL-activated M Φ , we inhibited potential cytotoxic molecules secreted by M Φ : NO, TNF- α , FasL and TRAIL, and measured the effects in the tumor cytostasis assay (Fig. 1D) and in the production of NO (Fig. 1E). As shown in Fig 1D, blocking of NO with iNOS inhibitor L-NAME (Fig. 1E) caused partial reduction of B16 cell cytostasis mediated by anti-CD40+MPL-activated M Φ . Inhibition of NO by L-NAME reduced the antitumor activity of anti-CD40-activated M Φ with MPL vs. media by 72.7%, 24.5%, and 11.3% in 3 separate experiments. Neutralization of TNF- α , FasL, or TRAIL caused no detectable reversal of M Φ -mediated tumoristasis. The combination of L-NAME and anti-TNF- α was no more effective in reducing the antitumor effect as L-NAME alone (Fig 1D). These data indicate that NO participates in the antitumor effect mediated by anti-CD40+MPL-activated M Φ .

CD11b⁺ M Φ and monocytes are activated following anti-CD40 and MPL treatment

In order to identify the specific subset of effector cells that can be activated by MPL on day 3 after anti-CD40 priming, PECs were collected from mice that had been injected with anti-CD40 3 days earlier, stained, gated on CD11b^{high} cells, sorted into 4 sub-populations based on their expression of immunophenotypic surface markers (Fig. 2A), and were further characterized by histological staining (Fig. 2B) and functional analysis (Fig. 2C and 2D). Subpopulation 1 consisted of monocytes and naïve M Φ that did not suppress B16 proliferation (Fig. 2C) or secrete NO (Fig. 2D) following anti-CD40 treatment (black bars), but could be induced to do so by MPL (grey bars). Subpopulation 2 expressed slightly less F4/80 and slightly more Gr-1, and was shown by histological staining (Fig 2B) to be a distinct population from subpopulation 1. Subpopulation 2 consisted of a relatively pure population of activated M Φ that strongly inhibited B16 proliferation (Fig. 2C) and secreted large quantities of NO (Fig. 2D).

In a separate prior experiment (not shown), CD11b⁺ Gr-1⁺ cells were sorted as one population that consisted of a mixture of monocytes and polymorphonuclear cells, according to histology. These cells were capable of inhibiting B16 proliferation and secreting modest amounts of NO. In order to identify more precisely which cells were activated in the experiment shown in Fig. 2, this population of CD11b⁺ Gr-1⁺ cells was further purified by forward scatter and side scatter qualities into two distinct populations, one predominantly consisting of granulocytes (Fig. 2B, 3) and the other, monocytes (Fig. 2B, 4). The granulocytes showed no antitumor activity (Fig 2C and 2D). The monocytes were able to suppress B16 proliferation after stimulation by anti-CD40, but these cells did not synergize with MPL to cause further suppression of B16 proliferation (Fig. 2C). CD11b⁺ cells in anti-CD40-injected not tumor-bearing mice were found to be mostly CD19⁺ B cells that neither mediated antitumor effects nor produced NO following stimulation by MPL (data not shown).

Similar results were obtained when PEC were collected from anti-CD40-treated tumor-bearing mice on day 7 (Fig. 2E–H) or day 14 (data not shown) post tumor cell implantation, in that activated M Φ (Fig. 2E, F; subpopulation 1) in the presence of MPL exhibited the strongest inhibition of B16 proliferation (Fig. 2G), and NO secretion (Fig. 2H). Gr-1^{high} monocytes (Fig. 2E, F; subpopulation 2) were less effective than activated M Φ , and CD19⁺ B cells (Fig. 2E, F; subpopulations 3, 4) were ineffective. MPL stimulated anti-CD40-activated M Φ better than CpG, whereas MPL and CpG had comparable effects on monocytes (Fig. 2G, H).

Combining anti-CD40 and MPL in mice with intact immune systems slows s.c. B16 tumor growth and prolongs survival more effectively than treating with either agent individually

The above experiments established synergy between anti-CD40 and MPL *in vitro*, indicating that the two immunostimulants could be therapeutically efficacious when used as a dual-agent cancer treatment. Since the route of administration for anti-cancer therapy may play a critical role in determining a treatment's outcome,^{5,16,35,36} we looked at two methods of administering anti-CD40 and MPL, treating mice either intraperitoneally (i.p.) or intratumorally (i.t.). C57BL/6 mice were first engrafted with s.c. tumors on day 0 and then treated i.p. with anti-CD40 and MPL, either alone or in combination, as described in *Materials and Methods*. The mice that received both agents had a significantly reduced tumor burden compared to any other group, based on the rate at which the tumors grew after beginning treatment (Fig. 3A). Anti-CD40 alone induced a statistically significant anti-tumor effect ($p=0.037$), but i.p. treatment with MPL alone did not have an antitumor effect ($p=0.49$). The mice treated with the combination therapy survived longer than the PBS+IgG and MPL+IgG groups, but not compared to the anti-CD40+PBS group ($p=0.27$) (Fig. 3B).

Next, we targeted immune cells in the tumor microenvironment by injecting anti-CD40 and MPL directly into the tumor. A dose titration pilot study was performed to determine suboptimal doses for anti-CD40 and MPL in order to reduce their ability to overpower the tumor individually and mask a possible synergistic effect of the combination. Suboptimal doses of 20 μ g anti-CD40 and 2 μ g MPL were chosen (data not shown), each of which was 25 times less than the doses we used for i.p. therapy. When injected locally into the growing tumor, the combination of anti-CD40 and MPL resulted in a significant reduction in tumor volume (Fig. 3C). Moreover, 2 of 8 combination-treated mice rejected the tumor and remained tumor-free, whereas none of the mice from other groups survived (Fig. 3D). When injected i.t., MPL alone slowed tumor growth ($p=0.017$), whereas the antitumor effect of anti-CD40 was not statistically significant ($p=0.42$) (Fig. 3C). Thus, anti-CD40 and MPL were synergistic *in vivo* in immunocompetent mice, and the antitumor effects of i.t. therapy with anti-CD40 and MPL were more pronounced than those of i.p. therapy.

MPL with and without anti-CD40 can activate M Φ in the absence of T cells

In the next series of experiments we tested whether T cells played a role in activating M Φ or mediating the antitumor effects induced by anti-CD40 and MPL. Peritoneal M Φ from immunocompetent C57BL/6 mice were primed with anti-CD40 for further activation by MPL, resulting in greatly augmented tumoristasis (Fig. 1B) and NO production (Fig. 1C). Because CD40 ligation may require additional T cell help to fully sensitize M Φ to MPL, we depleted T cells in C57BL/6 mice with anti-CD8 and anti-CD4 depleting mAbs (250 μ g each, injected i.p. in 0.5 ml) 2 days before giving a single dose of anti-CD40 or control rat IgG (0.5 mg, i.p.) on day 0. On day 3, PECs from these mice were collected and tested *in vitro* for their ability to suppress B16 proliferation (Fig. 4A) and produce NO (Fig. 4B). M Φ from T cell-depleted C57BL/6 mice were activated by anti-CD40 and MPL, resulting in an almost complete inhibition of B16 proliferation (** $p=0.009$) (Fig. 4A). However, this anti-proliferative effect was equally strong in M Φ obtained from T cell-depleted mice that received MPL and non-specific rat IgG instead of anti-CD40 (* $p=0.035$). Furthermore, in these CD4/CD8 depleted animals, the rat IgG treatment alone also induced some tumor inhibition, similar to that in anti-CD40-treated mice (Fig. 4A). As pretreatment of immunocompetent mice with the control rat IgG alone did not cause tumoristasis, NO production, or priming to MPL (Fig. 1B and Fig. 1C), we hypothesized that some systemic effect was induced by the CD4/CD8 depletion resulting in M Φ priming similar to that induced by anti-CD40. In terms of NO levels, the combination of anti-CD40 and MPL was more effective than rat IgG and MPL in inducing NO production (* $p=0.019$) (Fig. 4B). Even

without anti-CD40, MΦ from IgG-treated mice secreted more NO when cultured in the presence of MPL than when cultured with medium alone (* $p=0.018$).

In order to eliminate any potential stimulating effects of CD4⁺ and CD8⁺ T cell-depleting antibodies, we repeated these experiments using T and B cell-deficient CB-17 SCID mice. SCID mice were injected i.p. with either anti-CD40 or control rat IgG (0.5 mg in 0.5 ml PBS) and the PECs harvested 3 days later. Similar to the results observed with T cell-depleted C57BL/6 mice (Fig. 4A), anti-CD40-MΦ and rat IgG-MΦ from SCID mice showed an equally strong response to MPL (* $p<0.05$) (Fig. 4C). Anti-CD40, however, unlike its effects on NO production by MΦ from T cell-depleted mice (Fig. 4B), was unable to induce SCID MΦ to produce NO, with or without MPL, (Fig. 4D) for reasons that remain to be investigated.

Intratumoral MPL with or without anti-CD40 slows tumor growth in SCID mice

When combined into a single treatment regimen, anti-CD40 and MPL mediated potent antitumor effects in immunocompetent mice. Our previous studies showed that anti-CD40 could exert antitumor effects in the absence of T cells when given i.p.^{11,14,32} In order to explore the requirement for T cells in i.t. treatment, anti-CD40 and MPL were given i.t. to CB-17 SCID mice bearing subcutaneous B16 tumors. The tumors were treated i.t. with anti-CD40 (20 μg/mouse) on day 5 and day 12 and with MPL (2 μg/mouse) on day 8 and day 15. Untreated mice received rat IgG in place of anti-CD40 and PBS in place of MPL. When CB-17 SCID mice were treated i.t. with the combination of anti-CD40 and MPL, there was a significant reduction in the growth of their tumor (Fig. 5A), but this antitumor benefit was no better than what was seen in mice given MPL without anti-CD40. Intratumoral MPL by itself seemed capable of slowing B16 tumor growth, consistent with our *in vitro* observations (Fig. 4C). In this setting, treatment with anti-CD40+MPL, or MPL alone did not cause a significant prolongation of survival (Fig. 5B).

Intratumoral anti-CD40 and MPL can delay the growth of distant, untreated B16 tumors in immunodeficient hosts

Even though locally-administered MPL mediates T cell-independent antitumor effects in SCID mice, T cells may still be involved when immunocompetent mice receive this treatment. In order to determine whether T cells affect the growth of distant tumors, we injected primary s.c. tumors into C57BL/6 mice (Fig. 6A-C), anti-CD4/CD8-treated C57BL/6 mice (Fig. 6D-F), and, in a separate experiment, CB-17 SCID mice (Fig. 6G-I). Secondary tumors were injected s.c. into the opposite flanks of all mice on day 3. Primary tumors were treated i.t. with anti-CD40 on day 5 and with MPL on day 8, followed by a second round of anti-CD40 on day 12 and MPL on day 15. In all 3 groups of mice (immunocompetent, T cell-depleted, and SCID), i.t. treatment resulted in a substantial retardation of the growth of the primary (Figs. 6A, D, G) and secondary tumors (Figs. 6B, E, H), and significantly prolonged survival (Figs. 6C, F, I). In contrast with the results of single-tumor experiments, in which the low i.t. doses of anti-CD40+MPL occasionally resulted in complete tumor eradication and long-term survival (Fig. 3D), complete tumor rejection was not seen in these two-tumor experiments, with the exception of one treated SCID mouse. Collectively, these results suggest that the antitumor effects at the treated and distant secondary tumors appear, at least in this tumor model at the doses tested, to be mediated by cells other than T cells.

Discussion

Anti-CD40^{15,37} and MPL^{19,38} have been used in clinical settings as individual therapies, but have not, to our knowledge, been combined into a single immunotherapy regimen for experimental or clinical cancer treatment. In this study, we show that anti-CD40 mAb and

MPL can activate monocytes and MΦ in both immunocompetent and T cell-deficient hosts, and induce suppression of tumor growth *in vitro* and *in vivo*. Moreover, the synergy between anti-CD40 and MPL was observed in immunocompetent mice.

This study is a clinically-relevant follow up to our lab's earlier work on the potential cellular effector mechanisms of CD40 ligation.^{11–14,32,39,40} Most current therapies based on CD40 ligation are aimed toward the activation of T cells as the main effector cells,⁴¹ but we established that MΦ and NK cells can also be activated by CD40 ligation to mediate antitumor effects that persist even when T cells are absent.^{13,39} T cells were still unnecessary when we combined anti-CD40 *in vivo* with CpG,¹⁴ a TLR9 agonist, yielding results consistent with those presented here for TLR4 agonist MPL.¹⁴ Even though CpG is a powerful vaccine adjuvant in preclinical studies^{16,35} and activates mouse MΦ,¹⁸ it seems to be less effective in activating human MΦ.²⁰ Alternatively, MPL is relatively non-toxic^{24,42}, has a history of use in clinical trials as a single agent,⁴³ and activates monocytes/MΦ in human patients.⁴³ Because of these characteristics and our results, we believe that a combination of anti-CD40 and MPL may prove to be more clinically beneficial than a combination of anti-CD40 and CpG. In addition to our previously published data, these results show that the local i.t. injection of both anti-CD40 and MPL can induce a more powerful antitumor effect than substantially higher doses of these agents administered systemically. This suggests that i.t. injection of anti-CD40 and MPL in patients with accessible tumors may be effective and would circumvent the toxicity of systemic anti-CD40 observed in clinical trials.³⁷

In this study we present evidence that anti-CD40+MPL treatment results in antitumor effects in which T cells are not the main effector cells, since MΦ activation and antitumor effects against primary and distant tumors were observed in mice in the absence of T cells. In addition, our preliminary data indicate that subcutaneous injections of anti-CD40+MPL into healthy skin is similarly effective against a distant, untreated tumor as compared to i.t. injections of anti-CD40+MPL, arguing against a necessary role of local T cell activation in the systemic antitumor effect of this combination against B16-F10 tumors. However, while not required for tumor suppression in this tumor model, T cells still may be activated. For instance, MPL has been reported to induce IFNγ release from T cells.⁴⁴ MPL can also inhibit suppressor T cells, possibly leading to a strengthened immune response against the tumor.³¹ This activation of T cells by anti-CD40 and MPL may contribute to rejection of tumors that are more immunogenic than B16, as we have shown previously for anti-CD40.³² In addition, our data showing a greater tumor reduction by anti-CD40+MPL in immunocompetent mice (Fig. 3B) than in SCID mice (Fig. 5A) suggest that T cells play a small but discernible role when anti-CD40 and MPL are injected into the tumor. The nature of this T cell contribution in this model needs further investigation. It also remains possible that in addition to MΦ, NK cells play a role against primary and/or distant tumors in this model.

The T cell-independent, MΦ-dependent mechanism of antitumor effects induced by anti-CD40 and MPL is in agreement with the recent clinical and preclinical results on anti-CD40 therapy of pancreatic cancer.¹⁵ A Phase I trial of the first fully-humanized anti-CD40 agonistic mAb (CP-870,893)³⁷ combined with gemcitabine chemotherapy resulted in tumor regression in 4 out of 21 pancreatic ductal adenocarcinoma (PDA) patients.¹⁵ Biopsies showed that regressing tumors had been infiltrated by MΦ, but lacked signs of lymphocytes. In immunocompetent KPC mice that spontaneously developed PDA, anti-CD40 treatment resulted in MΦ (but not lymphocyte) infiltration into tumors, failed to produce protective T cell immunity, and required MΦ for anti-CD40-induced tumor regression,¹⁵ suggesting that the targets for the anti-CD40 mAb are MΦ and monocytes. Similar to our previous studies on a synergistic combination of anti-CD40 and LPS, the antitumor effect of anti-CD40 and

MPL, a non-toxic derivative of LPS, was mediated by activated MΦ partially via secretion of NO^{13,40}, whereas TNF-α, TRAIL, and FasL did not seem to play a role.

During the course of our experiments, we observed that MΦ from SCID and T cell-depleted C57BL/6 mice responded strongly to MPL *in vitro*, with or without initial priming by anti-CD40 (Fig. 4). F4/80⁺ MΦ in SCID mice were shown to express TLR4, but little or low CD40⁴⁵, which is consistent with our data. SCID MΦ might be primed as a compensatory defense mechanism in the absence of T and B cells. If SCID MΦ are primed, then further priming by anti-CD40 may have no discernible effect. The results obtained from T cell-depleted C57BL/6 mice showing MΦ activation (Fig. 4C) are potentially explained by the mechanism of T cell-depletion. The anti-CD8 and anti-CD4 depleting antibodies opsonize CD8⁺ and CD4⁺ T cells, leading to MΦ-mediated antibody-dependent cellular cytotoxicity. Because MΦ would be responsible for the depletion of T cells, they might still be in an activated or primed state upon removal from these T-cell depleted mice. This would explain why MPL by itself was a sufficient stimulus for MΦ from T cell-depleted mice (Fig. 4C), but not from immunologically intact mice (Fig. 1B).

Compared to untreated controls, it appears that i.p. treatment with anti-CD40 alone was effective against tumors in immunocompetent mice, but i.p. treatment with MPL alone did not have an effect (Fig. 3A). Conversely, when we injected these agents i.t. in lower doses, MPL slowed tumor growth, but anti-CD40 did not (Fig. 3B). These observations suggest a potential benefit of injecting anti-CD40 systemically in order to prime large numbers of monocytes or MΦ that would then migrate to and infiltrate the tumor. If systemic anti-CD40 is followed by an i.t. injection of MPL, it might introduce a high local concentration of the activating signal in the tumor site for the newly-arrived primed MΦ. By combining alternate routes of administering anti-CD40 and MPL, it might be possible to further augment the therapeutic potential of this combination. However, the results of this present study demonstrate that injecting both agents intratumorally, even at very small doses, can still elicit a strong antitumor response. This suggests that local delivery is a potential strategy for reducing or eliminating the toxicity of anti-CD40 in the clinical setting. Comparable effectiveness of MPL injected i.t., alone or with anti-CD40, in slowing growth of B16 tumors in immunocompetent (Fig. 3B) and SCID mice (Fig. 5A) suggest that T cells do not play an essential role in MPL and MPL+anti-CD40-induced antitumor effects.

Another promising use for anti-CD40 is combining it with other agents in addition to MPL.⁴⁶ For example, the efficacy of anti-CD40 and CpG was enhanced in combination with cyclophosphamide¹¹ and other chemotherapeutic agents⁴⁷ which are normally immunosuppressive in high doses. Stone et al. showed synergistic antitumor effects of a triple combination of CD40L, CpG and Poly (I:C) injected into the B16 tumor. The lack of synergy between CD40L and MPL in that study, in contrast to our results, could be explained by a different experimental design which favored CD8⁺ T cell involvement.⁴⁸ Since in our study anti-CD40 and MPL were effective at slowing tumor growth in immunocompromised CB-17 SCID mice and in C57BL/6 mice depleted of CD4⁺ and CD8⁺ T cells, this combination immunotherapy may be appropriate for future testing in cancer patients that have become functionally immune suppressed by their chemotherapy regimens.

Acknowledgments

Source of Funding: This study was supported by NIH-NCI grants CA87025 and CA32685, a grant from the Midwest Athletes Against Childhood Cancer (MAACC) Fund, support from The Crawdaddy Foundation, and a UW-Madison Hilldale Undergraduate Research Fellowship (to TJV).

The authors would like to thank the UW Flow Cytometry staff for their assistance with sorting, Dr. Kory Alderson for helpful discussions and experimental advice, and Xiaoyi Qu and Nicholas Kalogiropoulos for technical help.

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FIGURE 1.

Synergistic activation of MΦ with anti-CD40 and MPL. **A**, Expression of TLR4 on MΦ from mice treated with anti-CD40. C57BL/6 mice were injected i.p. with anti-CD40 (0.5 mg/mouse in 0.5 ml PBS), and their PECs were collected 1, 3, or 5 days later and tested by flow cytometry for TLR4 expression. Results are presented as histograms of viable MΦ (PI⁻ F4/80⁺) from 1 representative mouse out of the 2 mice injected on each day. On histogram: black peak, baseline TLR4 expression from naïve mouse, MFI 9.6; open peaks, overlapping TLR4 expression 1 day or 5 days after anti-CD40, MFI 25 (1 d) and 24.4 (5 d); grey peak, TLR4 on MΦ collected 3 days after anti-CD40, MFI: 33.2. **B**, Tumorstatic synergy between anti-CD40 and MPL *in vitro*. C57BL/6 mice were injected with anti-CD40 (500 μg i.p.) or rat IgG (500 μg i.p.) on day 0. On day 3, PECs were collected and enriched for MΦ by allowing cells (2×10⁵ per well) to adhere to plastic wells for 2 hours, followed by gentle pipetting to remove non-adherent cells. Adherent cells were then co-cultured in triplicate wells for 48h with 10⁴ B16 cells and stimulated with either MPL (5 μg/ml), CpG (5 μg/ml), or LPS (10 ng/ml). Wells were pulsed with [³H] thymidine (1 μCi/well) for the last 6 h of incubation in order to measure proliferation of B16 cells. Results are presented as the mean total number of β counts over 5 min ± SE from 10 mice combined from 3 identical experiments. Cells from each mouse were tested in all 4 *in vitro* conditions. **C**, Anti-CD40+MPL-MΦ secrete NO in a synergistic manner. After 42 h of stimulation with MPL (5 μg/ml), CpG (5 μg/ml), or LPS (10 ng/ml), supernatants were tested for nitrite concentration

using the Griess test. Results from 2 identical experiments are presented. Each bar represents the mean nitrite concentration $\pm$ SE from 6 IgG-treated mice and 6 anti-CD40-treated mice. Cells from each mouse were tested in all 4 *in vitro* conditions. **D** and **E**, Adherent PEC from anti-CD40-treated mice were pre-incubated with L-NAME, anti-TNF α , anti-TRAIL, or anti-FasL neutralizing antibodies, followed by 48 h of co-culture with B16 cells in the presence of MPL. [3 H]-thymidine incorporation (D) and NO production (E) were tested as described for (B) and (C). The results of a representative experiment (of three experiments) is shown. A two-tailed Student's t test was used to compare the populations indicated by the horizontal lines (* p <0.05; ** p <0.01; *** p <0.001).

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FIGURE 2.

CD11b⁺ MΦ and monocytes are activated following anti-CD40 and MPL treatment. C57BL/6 mice were injected i.p. with 500 μg anti-CD40 on day 0. **A**, On day 3, mice were euthanized and their PECs were collected, stained, gated on CD11b^{high} cells and sorted into 4 sub-populations by a FACSARIA cell sorter. The cells were divided as follows: (1) CD11b⁺ F4/80⁺ Gr-1^{low} “monocytes”, (2) CD11b⁺ F4/80⁺ Gr-1^{dim} “activated MΦ”. A separate group of cells showed a CD11b⁺ F4/80⁻ Gr-1^{high} phenotype; these were further divided by forward and side scatter into populations (3) “granulocytes”, and (4) “monocytes”. **B**, Each cell population was stained with a Wright-Giemsa stain to identify cell types based on morphology. The relative sizes of each sorted cell population have been maintained, and these 4 micrographs all show 40x magnification. Each sorted population was tested in medium or MPL (5 μg/ml) for the cells’ ability to inhibit B16 proliferation ([³H] thymidine

incorporation) (**C**) and produce NO (**D**). This experiment was performed 3 times; data from 1 representative experiment is shown. FACS sorting was also performed on PEC from tumor-bearing mice (TBM) (**E** – **H**). C57BL/6 mice were inoculated with 10^5 B16 cells (i.p.) on day 0, treated i.p. with 500 μ g anti-CD40 on day 4, and euthanized on day 7. B16 tumor cells ($CD45^-$) were gated out, and $CD45^+$ host cells were subsequently sorted (**E**) into the following 4 sub-populations: (1) $CD11b^+$ Gr-1^{low} “macrophages”, (2) $CD11b^+$ Gr-1^{high} “monocytes”, (3) $CD19^+$ $CD11b^{int.}$ Gr-1⁻ “activated B cells,” and (4) $CD19^+$ $CD11b^{low}$ Gr-1⁻ “naïve B cells”. Wright-Giemsa stains of these 4 populations are shown (**F**). Assays for [3 H]-thymidine incorporation (**G**) and NO production (**H**) were performed for these 4 populations. The assays for tumor cytostasis and NO production shows activation with MPL as well as with CpG. A two-tailed Student’s T test was used to compare [3 H]-TdR counts or nitrite concentration. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

FIGURE 3.

Anti-CD40 and MPL synergistically induce antitumor effects *in vivo*. **A**, C57BL/6 mice ($n=8-10$, combined from two identical experiments) were injected s.c. with 10^5 B16 melanoma cells on day 0 and treated i.p. with 0.5 mg anti-CD40 (or 0.5 mg rat IgG as control) on days 4 and 11. MPL (or PBS as control) was given on days 7 and 14. Graph shows the mean tumor volume $\pm$ SE. The tumor growth curve of each mouse was fit to an exponential curve, and the mean specific growth constant from the combination-treated mice was compared to each of the 3 other groups individually using a two-tailed Student's T test. Asterisks indicate significant differences in growth rates ($***p<0.001$, Anti-CD40+MPL vs. IgG+PBS; $*p<0.019$, Anti-CD40+MPL vs. anti-CD40+PBS; $***p<0.001$, Anti-CD40+MPL vs. MPL+IgG). **B**, Overall survival of the mice shown in (A) ($**p<0.0075$, Anti-CD40+MPL vs. IgG+PBS; $p=0.27$, Anti-CD40+MPL vs. anti-CD40+PBS; $**p=0.0048$, Anti-CD40+MPL vs. IgG+MPL). **C**, C57BL/6 mice ($n=8-10$, combined from two identical experiments) were implanted with a B16 tumor and treated i.t. with 20 μ g anti-CD40 on days 5 and 12, and with 2 μ g MPL on days 8 and 15. Control mice received 20 μ g rat IgG or PBS, respectively, injected i.t. ($***p<0.001$, Anti-CD40+MPL vs. IgG+PBS; $***p<0.001$, Anti-CD40+MPL vs. anti-CD40+PBS; $**p=0.0099$, Anti-CD40+MPL vs. MPL+IgG). **D**, Overall survival of the mice shown in (C) ($***p<0.001$, Anti-CD40+MPL vs. IgG+PBS; $***p<0.001$, Anti-CD40+MPL vs. anti-CD40+PBS; $**p=0.0032$, Anti-CD40+MPL vs. IgG+MPL).

FIGURE 4.

PECs from T cell deficient mice treated with anti-CD40 *in vivo* cause tumoristatic effects and produce NO after *in vitro* culture with MPL. **A**, C57BL/6 mice were injected i.p. with anti-CD4 (250 μg) and anti-CD8 (250 μg) mAbs on day -1. On day 0, mice were divided into two groups ($n=4$) and injected i.p. with either anti-CD40 or rat IgG (0.5 mg/mouse in 0.5 ml). Mice were euthanized on day 3. PECs were collected and enriched for adherent cells (M Φ) by plating 2×10^5 cells per mouse into each well of a 96-well plate. Non-adherent cells were removed after 1.5 – 2 h and adherent cells were co-cultured with 10^4 B16 cells per well and stimulated with MPL at a concentration of (5 $\mu\text{g/ml}$) for 48 hrs. Wells were

pulsed with [^3H]-thymidine (1 $\mu\text{Ci}/\text{well}$) for the last 6h of incubation in order to measure proliferation of B16 cells. Results are presented as the mean total number of β counts over 5 min $\pm$ SE from 4 mice per group. **B**, T cells are not required for anti-CD40-induced NO production. After 42 h of stimulation, supernatants were taken from the wells described in (A) and tested for nitrite concentration using the Griess test. The experiments described in (A) and (B) were also repeated in SCID mice (C, D). **C**, [^3H]-Thymidine incorporation into B16 cells in the presence of rat IgG- or anti-CD40-treated adherent SCID PEC. **D**, Nitrite production of the same PEC as in (C). Mean $\pm$ SE of 9 mice from 3 separate experiments are included in (C) and (D). In all panels, groups were compared using a two-tailed Student's T test (* $p<0.05$; ** $p<0.01$).

FIGURE 5.

MPL with and without anti-CD40 slows tumor growth in SCID mice when injected i.t. **A**, CB-17 SCID mice (*n*=5) were implanted with a B16 tumor and treated i.t. with 20 µg anti-CD40 on days 5 and 12, and with 2 µg MPL on days 8 and 13. Control mice received (20 µg rat IgG or PBS, respectively, injected i.t.) The graph shows the mean tumor volume ± SE. The tumor growth curve of each mouse was fit to an exponential curve, and the mean specific growth constants from the combination-treated group and the rat IgG+MPL group were compared to the rat IgG+PBS control group using a two-tailed Student's T test. Asterisks indicate significant differences in growth rates (**p* < 0.05). **B**, Survival of the mice shown in (A). Although the combination-treated mice and the rat IgG+MPL had higher

median survival times than the other two groups, the survival benefit was not statistically significant by the log-rank test.

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FIGURE 6.

I.t. anti-CD40 and MPL treatment is effective against local and distant tumors in immunocompetent C57BL/6 mice, T cell-depleted C57BL/6 mice, and CB-17 SCID mice. Mice were implanted with 10^5 B16 melanoma cells on day 0 and with a second inoculum of 10^5 B16 into the opposite flank on day 3. Groups of mice received i.t. treatment with either the anti-CD40+MPL combination (triangles) or control rat IgG+PBS (circles) into the primary tumor for two rounds corresponding to days 5 and 12 for anti-CD40 and days 8 and 15 for MPL. **A**, Primary tumor growth of immunologically intact C57BL/6 mice (injected i.p. with 500 μ g rat IgG on days 3, 9, and 14 as control for T cell depletion) (** $p=0.008$). **B**, Secondary tumor growth of the same mice as in (A) (* $p=0.03$). **C**, Survival of the same mice

as in (A) and (B) (** $p=0.0054$). **D**, Primary tumor growth of C57BL/6 mice (injected i.p. with 250 μg of anti-CD4 and 250 μg anti-CD8 depleting mAbs on days 4, 9, and 14) (** $p<0.001$). **E**, Secondary tumor growth of the same mice as in (D) (** $p=0.004$). **F**, Survival of the same mice as in (D) and (E) (** $p=0.0022$). The experiments with C57BL/6 mice that are shown in (A) through (F) were performed in parallel using 5 – 7 mice per treatment group. **G**, In a separate experiment, CB-17 SCID mice ($n=4$ for rat IgG+PBS; $n=5$ for anti-CD40+MPL) were injected s.c. with 10^5 B16 cells on day 0 and in the opposite flank on day 3, and treated i.t. with two rounds of treatment: anti-CD40 (20 μg) on day 5 and 12 and MPL (2 μg) on day 8 and 15 (* $p<0.031$). **H**, Secondary tumor growth of the same mice as in (G) (* $p=0.012$). **I**, Survival of the same mice as in (G) and (H) (** $p=0.0054$).