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Synergistic Activation of Macrophages via CD40 and TLR9 Results in T Cell Independent Antitumor Effects

Ilia N. Buhtoiarov, Hillary D. Lum, Gideon Berke, Paul M. Sondel, and Alexander L. Rakhmilevich

We have previously shown that macrophages (Mφ) can be activated by CD40 ligation to become cytotoxic against tumor cells in vitro. Here we show that treatment of mice with agonistic anti-CD40 mAb (anti-CD40) induced up-regulation of intracellular TLR9 in Mφ and primed them to respond to CpG-containing oligodeoxynucleotides (CpG), resulting in synergistic activation. The synergy between anti-CD40 and CpG was evidenced by increased production of IFN-γ, IL-12, TNF-α, and NO by Mφ, as well as by augmented apoptogenic effects of Mφ against tumor cells in vitro. The activation of cytotoxic Mφ after anti-CD40 plus CpG treatment was dependent on IFN-γ but not TNF-α or NO, and did not require T cells and NK cells. Anti-CD40 and CpG also synergized in vivo in retardation of tumor growth in both immunocompetent and immunodeficient mice. Inactivation of Mφ in SCID/beige mice by silica treatment abrogated the antitumor effect. Taken together, our results show that Mφ can be activated via CD40/TLR9 ligation to kill tumor cells in vitro and inhibit tumor growth in vivo even in immunocompromised tumor-bearing hosts, indicating that this Mφ-based immunotherapeutic strategy may be appropriate for clinical testing.


Macrophages (Mφ) can participate in antitumor responses as APCs to activate T cell immunity (1). Mφ can also be involved in antitumor immune reactions as nonspecific effectors stimulated by IFN-γ produced by activated T cells or by NK cells (2). In addition, Mφ can be directly activated by immunotherapy to serve as antitumor effectors (3). Thus, we have recently shown that Mφ can be effectively activated via CD40 ligation, even in the absence of other immune cells, to produce IFN-γ and kill tumor cells in vitro (4).

Classical activation of cytotoxic Mφ requires two signals. First, Mφ have to be “primed” by IFN-γ. IFN-γ activates signal transducing molecules (e.g., MyD88 and NF-κB) (5, 6, 7), induces expression of certain TLRs (TLR2, TLR4, and TLR9) (5, 6, 8), and activates selected enzymes (9) facilitating the responsiveness to a second signal. The second signal, typically provided by bacterial derivatives or TNF-α (5, 10, 11), then activates the spectrum of biological responses attributable to effector Mφ. These include secretion of NO, IL-12, IL-1β, TNF-α, Mφ tumor cytotoxicity-170 kDa, chemokines (e.g., CCL2), as well as expression of costimulatory molecules (CD40, CD80, and CD86) and cell death-inducing ligands (Fas ligand, TRAIL, and membrane-bound TNF-α) (5, 12–17).

Activated Mφ can kill tumor cells and orchestrate antitumor reactions (18, 19). Effector Mφ can mediate Ab-dependent cellular cytotoxicity and ligate death receptors (including CD95, TNF-R1, and TRAIL-R1/R2) on target cells upon direct tumor cell-Mφ contact (16, 20–22). In addition, soluble factors such as IFN-γ, TNF-α, and NO, produced by Mφ can damage tumor cells or the tumor vasculature (23–25). Some of these factors can also participate in antitumor reactions indirectly by stimulating immune cells in the intratumoral microenvironment (19, 26).

We have shown that antitumor effects of anti-CD40 (4) may not require T cells (27), although in some tumor models T cells may be involved in CD40 ligation-induced tumor regression (28). In addition, we have recently shown that ligation of CD40 on Mφ by anti-CD40-induced production of IFN-γ that primed Mφ to subsequent stimulation with LPS, rendering them capable of inducing apoptosis in tumor cells in vitro (4). These data suggested that Mφ activated in vivo with anti-CD40 and LPS could be effective for cancer therapy. However, because of the limited clinical utility of LPS due to its toxicity in vivo, we hypothesized that a similar TLR-dependent stimulator of Mφ, that has already shown clinical tolerability, might substitute for LPS and provide synergistic antitumor effects when combined with anti-CD40.

CpG, a synthetic analog of bacterial DNA, is capable of activating innate immunity, including Mφ (29, 30). The recognition of CpG requires expression of intracellular (IC) TLR9 that is constitutively expressed by Mφ (29). Upon internalization, CpG binds to TLR9 in early endosomes and triggers a signaling cascade mediated via MyD88 and NF-κB (31, 32). Consequently, CpG-stimulated Mφ secretes NO, TNF-α, IL-12, IFN-γ, IL-1β, as well as other factors involved in immune reactions (33). Thus, the effect of CpG on Mφ can be similar to that elicited by LPS, although the mechanisms of Mφ activation by CpG and LPS appear to be different (34).
Antitumor effects of CpG, similar to those of anti-CD40, have been mainly attributable to activated T and NK cells (35, 36). In the present study, we demonstrate that anti-CD40 and CpG synergize in their activation of cytotoxic Mφ. CD40 ligation primed Mφ to CpG via an IFN-γ-dependent mechanism by inducing expression of TLR9. Anti-CD40 + CpG-stimulated Mφ (anti-CD40 + CpG-Mφ) induced apoptosis in tumor cells in vitro. Combining anti-CD40 and CpG treatment in vivo similarly resulted in synergistic activation of Mφ and potent antitumor effects mediated by Mφ, even in the absence of T cells, NK cells, or polymorphonuclear cells (PMN).

Materials and Methods

Mice and cell lines

C57BL/6, A/J, C3H/HeJ, C3H/HeQu1 mice, and IFN-γ–/–, TNF-α–/–, iNOS–/–, CD40–/–, CD40–/–, and Fcrγ chain–/– mice of C57BL/6 background (Harlan Sprague Dawley or The Jackson Laboratory), and CB17 and CB17 SCID/beige mice (Taconic Farms or Charles River Laboratories) were housed, cared for, and used in accordance with the Guide for Care and Use of Laboratory Animals. The murine B16 melanoma, L5178Y lymphoma, and Renca carcinoma cells, and human M21 melanoma cells were grown in RPMI 1640 complete medium; NXS2 murine neuroblastoma and NIH-OVCAR-3 human ovarian carcinoma cells were grown in DMEM complete medium at 37°C in a humidified 5% CO2 atmosphere as described (4).

Abs and reagents

The FGK 45.5 hybridoma producing anti-CD40 was a gift from Dr. F. Melchers (Basil Institute for Immunology, Basel, Switzerland). Anti-CD40 was produced as previously described (4). Endotoxin-free CpG1826 (TC CATGAGGTTTCTTGACGGTT; CpG motifs that are absent in control non-CpG1982, bold and underlined) was purchased from Coley Pharmaceuticals Group and Sigma-Genosys; non-CpG1982 (TCCAGGACTTTTACCAGTT). Anti-CD40 was a gift from Dr. F. Melchers (Basel Institute for Immunology, Basel, Switzerland). Anti-CD40 mAb AND CpG-ODN ACTIVATE CYTOTOXIC Mφ

CpG1826. For activation of Mφ, 0.5 ml of PBS was injected i.p. In preliminary experiments, no difference was observed throughout the manuscript) in all subsequent experiments. For activation of Mφ and depletion of NK cells was determined in the 4-h51Cr cytotoxicity assay against YAC-1 cells. Mφ were inactivated by i.p. injection of 25 mg of silica (silicon dioxide; Sigma-Aldrich) in 0.5 ml of PBS on days 1, 3, 7, 11, and 15 relative to tumor cell implantation. Control treatment consisted of 0.5 ml of PBS.

αHTr incorporation assay

Adhesion-purified Mφ (1.5–2 × 10^6 per well) from mice treated with anti-CD40, CpG, IgG, or PBS were incubated with tumor cells (1 × 10^4 per well) for 24–48 h in medium with or without CpG (0–50 μg/ml), LPS (0–10 ng/ml), or anti-CD40 (0–100 μg/ml). For the last 6 h, tumor cells were pulsed with 1 μCi/well of [3H]Tdr, and counted by β-scintillation of total cells as previously described (4). Results are presented as counts per minute for triplicate wells ± SE. Mφ alone incorporated negligible amounts of [3H]Tdr.

NO detection

IgG- or anti-CD40-Mφ were cocultured for 48 h in vitro with B16 cells in medium with or without CpG. Nitrite accumulation in cell culture supernatants was determined using Griess reagent (Sigma-Aldrich) as described (4).

Immunophenotypic analysis of Mφ

Mφ activated in vivo and/or in vitro with anti-CD40 and CpG, were stained with eFluor 80-APC (eBioscience) and eFluor 111-PE (eBioscience) or irrelevant control rabbit IgG (Sigma-Aldrich), developed with goat anti-rabbit-FITC mAb (BD Pharmingen), F4/80+ Mφ were analyzed on the FACScan flow cytometer with CellQuest software (BD Biosciences) (4).

FC analysis for IC TLR9

Purified IgG- or anti-CD40-Mφ from C57BL/6 or IFN-γ–/– mice were stained with eFluor 80-PE (eBioscience) on ice for 40 min. After repeated washing, Mφ were fixed and permeabilized according to the eBioscience 2004 Catalog & Reference Manual and as previously described (4), followed by staining for IC TLR9 and rabbit anti-mouse TLR9 Ab (BMG-431; Imgenex) or irrelevant control rabbit IgG (Sigma-Aldrich), developed with goat anti-rabbit-FITC mAb (BD Pharmingen). F4/80+ Mφ were analyzed on the FACScan flow cytometer with CellQuest software.

FC analysis for IC IFN-γ, TNF-α, and IL-12

PC were obtained from mice 3 days after injection of anti-CD40 or IgG. Mφ were cultured for 24 h in medium with or without 5 μg/ml CpG and with 1 μl/ml monensin (eBioscience) for the last 4 h to enable accumulation of IFN-γ, TNF-α, and IL-12 in the endoplasmic reticulum. IC cytokines in F4/80+ Mφ were detected and data were analyzed as described above for TLR9, by using the following mAbs (all from eBioscience): αIFN-γ-PE (XM11.2), αTNF-α-FITC (MP6-XT22), and αIL-12p70-PE (C17.8).

Assay for tumor cell apoptosis

L5178Y cells were coincubated for 24 h with IgG- or anti-CD40-Mφ in medium with or without 5 μg/ml CpG. FC analysis of apoptotic changes of F4/80+ L5178Y cells was performed as previously described (4).

Statistical analysis

A two-tailed Student’s t test was used to determine significance of differences between experimental and relevant control values.

Results

Anti-CD40 and CpG synergize in Mφ activation in vitro

We first asked whether CD40 ligation of Mφ would up-regulate the expression of TLR9. As shown in Fig. 1A, CD40 ligation up-regulated expression of TLR9 in Mφ in a time-dependent manner, with a maximal effect noted on day 3. These anti-CD40-activated Mφ inhibited the proliferation of cocultured B16 cells in vitro (Fig. 1B). Notably, additional stimulation of anti-CD40-Mφ with CpG led to complete inhibition of B16 cell proliferation (shown on Fig. 1B by asterisks). This effect was identical to that of LPS used as a positive control for synergistic activation of anti-CD40-Mφ (4). Maximal activity of anti-CD40 + CpG-Mφ was observed 3–8 days after CD40 ligation. The enhanced tumorstatic activity of Mφ induced by CpG was still observed 11 days after anti-CD40 injection, albeit to a lesser degree, and disappeared by day 21 (data not shown). This enhanced in vitro tumorstatic

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FIGURE 1. Synergistic activation of M\(\phi\) with anti-CD40 and CpG. A, Kinetics of TLR9 expression in anti-CD40-M\(\phi\). PC from C57BL/6 mice treated i.p. with IgG or anti-CD40 collected on day 1 (IgG and anti-CD40) and day 3, 5, 8, and 11 (anti-CD40) after the treatment, were tested for IC TLR9. Results are presented as mean \(\pm\) SE of triplicate wells of \([^{3}H]TdR\) incorporation into tumor cells. A combined graph of two separate experiments is shown. Anti-CD40-M\(\phi\) and CpG synergized in IFN-\(\gamma\), Anti-CD40 and CpG in TNF-\(\alpha\), and IL-12p70 production by M\(\phi\) (Fig. 1A). Similar results were obtained in three repeat experiments. Therefore, it appears that the synergy between anti-CD40 and CpG in M\(\phi\) activation requires that anti-CD40 treatment precedes stimulation it appears that the synergy between anti-CD40 and CpG in M\(\phi\) activation requires that anti-CD40 treatment precedes stimulation. In contrast, anti-CD40 had no effect on M\(\phi\) by the combination of anti-CD40 and CpG depended upon the sequence of their delivery. The in vitro tumoristatic activity of M\(\phi\) from mice injected with anti-CD40 (Fig. 2, A and B) or CpG (Fig. 2, C and D), and with control IgG (Fig. 2, A and B) or PBS (Fig. 2, C and D), was tested against B16 cells in the presence of LPS (Fig. 2, A and C), CpG (Fig. 2B), or anti-CD40 (Fig. 2D). Both CpG and LPS effectively augmented the effects mediated by anti-CD40-M\(\phi\). In contrast, anti-CD40 had no effect on M\(\phi\) from mice injected with CpG. Similar results were obtained in three repeat experiments. Therefore, it appears that the synergy between anti-CD40 and CpG in M\(\phi\) activation requires that anti-CD40 treatment precedes stimulation.

Table I. Sensitivity of various murine and human tumor cell lines to anti-CD40 + CpG-M\(\phi\)\(^a\)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>No M(\phi)</th>
<th>Anti-CD40-M(\phi)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>CPG</td>
</tr>
<tr>
<td>B16</td>
<td>189,000 ± 3,214</td>
<td>169,666 ± 887</td>
</tr>
<tr>
<td>L5178Y</td>
<td>260,000 ± 9,999</td>
<td>207,666 ± 7,622</td>
</tr>
<tr>
<td>RENCA</td>
<td>138,000 ± 2,081</td>
<td>137,000 ± 3,464</td>
</tr>
<tr>
<td>M21</td>
<td>131,666 ± 1,855</td>
<td>121,000 ± 2,516</td>
</tr>
<tr>
<td>OVCAR</td>
<td>17,343 ± 930</td>
<td>9,399 ± 157</td>
</tr>
</tbody>
</table>

*Pertitoneal M\(\phi\) were activated with anti-CD40 and purified in vitro as described in Materials and Methods. Adherent anti-CD40-M\(\phi\) were cocultured with the murine (B16 melanoma, L5178Y lymphoma, Renca carcinoma) or human (M21 melanoma, OVCAR carcinoma) tumor cells for 48 h in medium alone or with 5 \(\mu\)g/ml CpG. Control wells contained tumor cells without macrophages (No M\(\phi\)). \([^{3}H]TdR\) was added to the wells 6 h before harvesting. The results are presented as mean \(\pm\) SE of triplicate wells of \([^{3}H]TdR\) incorporation into tumor cells. A combined graph of two separate experiments is shown. Anti-CD40-M\(\phi\) stimulated with CpG mediated stronger antitumor effects than anti-CD40-M\(\phi\) without additional stimulation.

\(^{*}, p < 0.001.

\(^{**}, p < 0.05\).
with CpG. Although the levels of Mϕ activation by anti-CD40 alone and CpG alone varied considerably between experiments (for reasons that remain to be determined), stimulation of anti-CD40-Mϕ with CpG invariably resulted in their enhanced activation and augmented antitumor function in vitro.

Stimulation of Mϕ with anti-CD40 and CpG might promote differentiation of Mϕ into dendritic cells. We phenotyped the population of adherent anti-CD40 + CpG-Mϕ for expression of CD11b and CD11c, specific for Mϕ and DC, respectively (8). We found that 97% of the adherent Mϕ were F4/80CD11bCD11c+, indicating that the tumoristasis in vitro was mediated by activated Mϕ rather than DC.

**Molecular and cellular requirements for synergistic activation of Mϕ with anti-CD40 and CpG**

To exclude the possibility of nonspecific activation of Mϕ with anti-CD40, we next determined whether the CD40 molecule was required for Mϕ activation with anti-CD40 and with and without CpG. C57BL/6 control mice or CD40−/− mice were treated with anti-CD40 or CpG. Treatment with CpG was used as a control for the ability of CD40−/−Mϕ to be activated in vivo. Mϕ from control mice readily responded to treatment with anti-CD40 or CpG in vivo, as detected by the tumoristatic activity (Fig. 3A). The antitumor effect of anti-CD40-Mϕ could be further augmented with CpG in vitro. In contrast, CD40−/−Mϕ did not respond to in vivo treatment with anti-CD40. Their tumoristatic effect was only slightly enhanced by CpG added in vitro. However, the CD40−/−Mϕ were functionally responsive to stimuli other than anti-CD40 because they responded to in vivo treatment with CpG, indicating that they constitutively express TLR9. Thus activation of Mϕ with anti-CD40 required ligation of CD40, but CD40 was not required for Mϕ activation with CpG. In addition, we found similar high levels of in vitro tumoristasis induced by anti-CD40 + CpG-Mϕ from FcRγ-chain−/− mice (data not shown), confirming that the activation of Mϕ with anti-CD40 resulted from the ligation of CD40 by the Ag-binding component of the anti-CD40, and was not from nonspecific interaction of the Fc end of anti-CD40 with FcR.

To rule out that anti-CD40-mediated Mϕ activation was due to contaminating endotoxin (38), we tested Mϕ activation in endotoxin-resistant C3H/HeJ mice. As control, C57BL/6 mice and C3H/HeQuJ mice were used in two independent experiments with similar results. Anti-CD40-Mϕ from control and from C3H/HeJ mice induced almost identical tumoristasis in vitro (Fig. 3B). As expected, the effect of anti-CD40-Mϕ from control mice was further augmented with CpG or LPS in vitro. In contrast, the effect of anti-CD40-Mϕ from C3H/HeJ mice could be enhanced only with CpG but not with LPS. These results prove that the observed activation of Mϕ from C3H/HeJ mice with anti-CD40 plus CpG cannot be attributed to contamination with endotoxin.

T and NK cells may play a role in Mϕ activation (3, 39). To determine whether T and NK cells were essential for activation of Mϕ with anti-CD40 plus CpG, we tested Mϕ activation from C17 SCID/beige mice. As control, C57BL/6 mice and C3H/HeQuJ mice were used in independent experiments with similar results. Anti-CD40-Mϕ from control and from C3H/HeJ mice induced almost identical tumoristasis in vitro (Fig. 3B). As expected, the effect of anti-CD40-Mϕ from control mice was further augmented with CpG or LPS in vitro. In contrast, the effect of anti-CD40-Mϕ from C3H/HeJ mice could be enhanced only with CpG but not with LPS. These results prove that the observed activation of Mϕ from C3H/HeJ mice with anti-CD40 plus CpG cannot be attributed to contamination with endotoxin.
augmented the antitumor effect by these Mϕ similar to that of anti-CD40 + CpG-Mϕ from CB17 mice. In addition, similar levels of NO were produced by anti-CD40 + CpG-Mϕ from both CB17 and CB17 SCID/beige mice (data not shown), indicating that T and NK cells are not essential for Mϕ activation by anti-CD40 and CpG.

**Role of IFN-γ, TNF-α, and NO in the synergy between anti-CD40 and CpG and Mϕ-mediated tumoristatic effects**

In previous studies, we showed that endogenous IFN-γ was required for Mϕ activation by anti-CD40 and LPS (4). We hypothesized that the synergy between anti-CD40 and CpG might similarly depend upon endogenous IFN-γ. Exposure of Mϕ to exogenous IFN-γ up-regulates TLR9 expression (6). As shown in Fig. 4A, similar to Fig. 1A, Mϕ from control IFN-γ+/+ mice readily responded to anti-CD40 by up-regulating TLR9 expression. In contrast, the level of TLR9 expression in anti-CD40-Mϕ from IFN-γ−/− mice was similar to that in IgG-Mϕ from IFN-γ−/− and IFN-γ−/− mice. Thus, up-regulation of TLR9 in anti-CD40-Mϕ requires endogenous IFN-γ. Fig. 4B shows that IFN-γ is also essential for the synergistic activation of Mϕ by anti-CD40 and CpG; whereas anti-CD40-Mϕ from IFN-γ−/− mice effectively suppressed proliferation of B16 cells in medium alone and to a much greater extent in the presence of CpG, anti-CD40-Mϕ from IFN-γ−/− mice were not capable of suppressing tumor cell proliferation even in the presence of CpG. In addition, IFN-γ−/− anti-CD40-Mϕ, alone or in the presence of CpG, produced no measurable levels of NO (data not shown).

Anti-CD40 + CpG-Mϕ are able to induce production of TNF-α and NO (Fig. 1, C and D). Therefore, we asked whether these molecules are required for the tumoristatic effects of anti-CD40 + CpG-Mϕ. To answer this question, IgG- and anti-CD40-Mϕ from TNF-α+/− or iNOS−/− (deficient of inducible NO synthase) mice were tested for tumoristatic activity in vitro in medium with or without CpG. The results show that anti-CD40 + CpG-Mϕ from TNF-α−/− mice (Fig. 4C) and iNOS−/− mice (Fig. 4D) inhibited proliferation of B16 cells at levels similar to those induced by anti-CD40 + CpG-Mϕ from control TNF-α+/+ or iNOS+/+ mice. Furthermore, anti-CD40-Mϕ from TNF-α−/− and iNOS−/− mice demonstrated a significant antitumor effect that was slightly reduced from that of C57BL/6 mice (Fig. 4, C and D). Hence, these results indicate that TNF-α and NO may be involved but are not essential for activation of Mϕ via CD40 and TLR9 or for the antitumor effects mediated by anti-CD40 + CpG-Mϕ.

**Anti-CD40 + CpG-ODN-Mϕ induce tumor cell apoptosis in vitro**

We next tested whether tumoristasis (detected in [3H]Tdr incorporation assays) mediated by anti-CD40 + CpG-Mϕ was associated with apoptosis of tumor cells. L5178Y lymphoma cells were chosen as targets as described previously (4). In brief, these cells are highly sensitive to anti-CD40-Mϕ (Table I), grow in suspension in contrast to B16 cells and thus can be harvested for accurate assessment of apoptotic changes without trypsinization. L5178Y cells were cultured with anti-CD40-Mϕ in medium with or without CpG. Twenty four or 48 h later, they were assessed for expression of phosphatidylserine (PS) by staining with Annexin VFITC as a measure of early apoptotic changes, and tested for changes in tumor cell membrane integrity by staining with DNA-binding dye 7-aminoactinomycin D (7-AAD) as a measure of late apoptotic changes (41). During the first 24 h of coculture, anti-CD40-Mϕ (Fig. 5A, left column) induced more apoptosis (48% annexin V+ cells in the two right quadrants) than did IgG-Mϕ (23% annexin V+ cells in the same two right quadrants).

Apoptotic changes induced in the tumor cells were enhanced when IgG- or anti-CD40-Mϕ were additionally stimulated with CpG (Fig. 5A, right column). After 24 h, 63% of tumor cells were annexin V+ (33% annexin V−7-AAD− and 30% annexin V−7-AAD+ cells) when cultured with anti-CD40 + CpG-Mϕ vs 24% (10% annexin V−7-AAD− and 14% annexin V−7-AAD+ cells) for tumor cells cultured with IgG + CpG-Mϕ. By 48 h, 87% of the L5178Y cells cultured with the anti-CD40 + CpG-Mϕ underwent apoptotic death (annexin V−7-AAD+) vs only 28% in the control culture (data not shown).

In a parallel [3H]Tdr incorporation assay performed with the same cultures, L5178Y cells showed retarded proliferation after coculture with anti-CD40-Mϕ, and this antitumor effect was further enhanced by CpG (Fig. 5B). Even though 37% of L5178Y...
of affected tumor cells was observed after 48, 72, and 96 h of coincubation of tumor cells with anti-CD40 + CpG-Mφ (data not shown). In addition to L5178Y cells, B16 cells were found to be killed by anti-CD40 + CpG-Mφ via apoptosis, as determined in a different apoptosis assay that was based on flow-cytometric evaluation of tumor cell membrane integrity by staining CFSE-labeled tumor cells with 7-AAD (data not shown). Altogether, these results demonstrate that anti-CD40 and CpG synergistically activated Mφ to mediate tumoricidal effects that were associated with the tumor cell apoptosis.

**Anti-CD40 and CpG synergize in vivo in inducing Mφ-mediated antitumor effects**

In the above experiments, synergy was demonstrated when anti-CD40 was administered in vivo followed by stimulation of Mφ by CpG in vitro. To determine whether combining anti-CD40 and CpG in vivo would result in similar activation of Mφ, mice were treated with anti-CD40 on day 0 followed by CpG on day 3 (D0/D3 schedule). This schedule was based on in vivo data (Fig. 1, A and B). One day after the CpG-treatment, Mφ were tested in vitro for tumoricidal activity against B16 cells. As shown in Fig. 6A, the combined treatment with anti-CD40 and CpG induced greater Mφ activation than anti-CD40 and CpG given separately. A separate experiment (Fig. 6B) showed less Mφ activation when CpG was given 3 h (D0/D0), 1 day (D0/D1), or 2 days (D0/D2) after the anti-CD40. Comparing Fig. 6, A and B, it can be seen that synergy between anti-CD40 and CpG in vivo occurred in a time-dependent manner with a maximal effect observed when using a D0/D3 schedule. Although the level of the tumoricidal effects varied between experiments, the combined in vivo treatment was more effective than CD40 and CpG given separately in four different experiments.

As cancer immunotherapy frequently relies on multiple immunostimulations, we tested whether repeated administration of anti-CD40 + CpG augments the effector function of Mφ. As shown in Fig. 6C, two courses of anti-CD40 + CpG led to activation of tumoricidal Mφ that were much more potent than that induced by a single course of combined treatment. This in vivo treatment did not induce differentiation of Mφ into DC as all adherent cells were found to be F4/80+CD11b+CD11c− (data not shown).

We next determined whether the combination of anti-CD40 and CpG would be effective for tumor therapy in vivo. Treatment of C57BL/6 mice bearing s.c. B16 tumors with anti-CD40 or CpG alone resulted in similar retardation of tumor growth (Fig. 7A). However, when anti-CD40 was combined with CpG, the antitumor effect was statistically greater than that of each single treatment (p < 0.025). This combined immunotherapy was well-tolerated. The synergistic anti-tumor effect of anti-CD40 and CpG was also observed in A/J mice bearing s.c. NXS2 tumors (Fig. 7B). Three of eight A/J mice treated with combination anti-CD40 + CpG rejected their tumors, whereas no complete tumor regression was achieved in any mouse receiving treatment with anti-CD40 alone or CpG alone.

Similarly, SCID/beige mice bearing B16 tumors were treated with two courses of anti-CD40 and CpG given either alone or in combination. As in immunocompetent mice, anti-CD40 + CpG induced significant synergistic antitumor effects in SCID/beige mice that resulted in retardation of tumor growth (Fig. 7C) and prolonged survival (Fig. 7D). The effects induced by anti-CD40 + CpG occurred independently of T and NK cells as determined in an experiment with B16 tumor-bearing C57BL/6 mice depleted of T and NK cells in vivo. Combined anti-CD40 + CpG treatment caused 93.5% inhibition of tumor growth in immunocompetent

**FIGURE 5.** Anti-CD40 and CpG synergistically activate Mφ to kill tumor cells via apoptosis. A, Mφ were obtained from C57BL/6 mice 3 days after treatment with rat IgG or anti-CD40. L5178Y cells cultured for 24 h alone (No Mφ) or with IgG- or anti-CD40-Mφ in medium with or without 5 μg/mL CpG, were stained for PS by Annexin VFITC and for altered membrane permeability by 7-AAD. Numbers indicate the percentage of L5178Y cells that are viable (left lower quadrant, annexin V−, 7-AAD−), “early” apoptotic (right lower quadrant, annexin V−, 7-AAD+), or dead (right upper quadrant, annexin V+, 7-AAD+). B, The same IgG or anti-CD40-Mφ were tested for antitumor activity in vitro against L5178Y cells in medium with or without CpG in [3H]ThD incorporation assay.

cells cultured with anti-CD40 + CpG-Mφ remained nonapoptotic after 24 h (Fig. 5A, lower left quadrant of the bottom right dot plot), these cells did not proliferate (Fig. 5B, last bar). No recovery
of anti-CD40 + CpG, we selectively inactivated Mφ in vivo by silica administration (43). The effectiveness of silica in inhibiting Mφ activity was confirmed by the reduction of LPS-induced weight loss in silica-treated mice (data not shown). As shown in Fig. 7F, treatment of tumor-bearing SCID/beige mice with silica abrogated the antitumor effect of anti-CD40 + CpG treatment \( (p < 0.001) \), indicating that this effect was mediated by Mφ. The results of control experiments indicated that the abrogation of the anti-CD40 and CpG-mediated antitumor effect in silica-treated mice was not due to the limited distribution of anti-CD40 and CpG from the i.p. site of administration. Thus, mAbs against T cells, NK cells, or PMN injected i.p. effectively depleted these cells in the spleen even when given to mice that had also received silica i.p. (data not shown).

Discussion

In the present study, we show that anti-CD40 can synergize with class B CpG in activating tumoricidal Mφ, which, in turn, mediated antitumor effects in vitro and in vivo. Class B CpGs are potent activators of both murine and human mononuclear phagocytes, and Mφ in particular (30, 33, 34, 44). Although CpG and LPS share many immunostimulatory properties, CpG seems to be less toxic in vivo due to stimulation of mononuclear phagocytes in a much more restricted manner (33, 34). Our data suggest that CpG and LPS might synergize with anti-CD40 in activating Mφ via different mechanisms, because CpG, but not LPS, synergized with anti-CD40 in C3H/HeJ mice. Our results also show that anti-CD40 synergized with CpG in activation of cytotoxic Mφ in a time- and order-dependent manner. The observed time-dependent up-regulation of TLR9 in response to anti-CD40 could account, in part, for the synergy between anti-CD40 and CpG. Thus, CD40-ligation of B cells resulted in augmented expression of TLR9 (45), and CD40/TLR9-stimulated B cells secreted increased amounts of cytokines and Ig (46). DC cells also responded to CD40 and TLR9 ligations by increased production of cytokines and expression of costimulatory molecules (47). Consistent with the known role of TLR9 as the primary receptor for CpG, the data show that the maximal level of TLR9 expression in anti-CD40-Mφ on day 3 corresponds with the maximal sensitivity to CpG in vitro, as demonstrated by the abrogation of tumor proliferation (Fig. 1). However, TLR9 up-regulation may only partially explain the synergy between anti-CD40 and CpG. Thus, Fig. 1A shows a similar level of TLR9 expression on day 8 after anti-CD40 treatment as on day 1, which was about half of the level seen on day 3; however, the level of CpG-augmented tumoricidal effect of anti-CD40-Mφ was similar for days 3 and 8, and substantially stronger than that seen on day 1. It is possible that this time-dependent synergy takes place at a pretranslational level, for example, involving phosphorylation of STAT-1 and NF-κB, or merely depends upon posttranslational events (7, 48).

While testing the requirements for Mφ activation induced by anti-CD40 and CpG, we found that these ligands synergize in an order-dependent fashion where CD40-ligation must precede stimulation with CpG (Fig. 2). This stimulation results in activation of Mφ capable of secreting increased amounts of IFN-γ, NO, TNF-α, and IL-12, and able to suppress tumor cell proliferation in vitro. This activation was not seen when the two stimuli were given in the reverse order. CpG-Mφ did not respond to challenge with anti-CD40 in vitro (Fig. 2), even though CpG-stimulation induces increased CD40 expression on murine Mφ, as demonstrated by others (49, 50) and us (data not shown). This paradox, yet unexplained, might be due to insufficient phosphorylation of NF-κB and STAT1 (51) in response to CD40-ligation of CpG-Mφ.

C57BL/6 mice, and 85% inhibition of the tumor growth in C57BL/6 mice depleted of T and NK cells (data not shown).

As PMNs express TLR9 (42) and might, therefore, be engaged in antitumor effects by the treatment with CpG, SCID/beige mice were additionally depleted of PMN and noncytotoxic NK cells that might secrete cytokines in response to anti-CD40 or CpG. Under these circumstances, Mφ would be the only possible effector cells capable of mediating the antitumor effects. Treatment of B16 tumor-bearing SCID/beige mice with a combination of anti-CD40 and CpG resulted in marked inhibition of tumor growth (Fig. 7E) and prolonged survival (data not shown) even when these mice were depleted of PMN and NK cells. To directly test whether activated Mφ are required in antitumor effects induced by the combination of anti-CD40 + CpG, we selectively inactivated Mφ in vivo by silica administration (43). The effectiveness of silica in inhibiting Mφ activity was confirmed by the reduction of LPS-induced weight loss in silica-treated mice (data not shown). As shown in Fig. 7F, treatment of tumor-bearing SCID/beige mice with silica abrogated the antitumor effect of anti-CD40 + CpG treatment \( (p < 0.001) \), indicating that this effect was mediated by Mφ. The results of control experiments indicated that the abrogation of the anti-CD40 and CpG-mediated antitumor effect in silica-treated mice was not due to the limited distribution of anti-CD40 and CpG from the i.p. site of administration. Thus, mAbs against T cells, NK cells, or PMN injected i.p. effectively depleted these cells in the spleen even when given to mice that had also received silica i.p. (data not shown).

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The order-dependent activation of cytotoxic Mφ described in this study may have important biological implications. It is known that TLRs are essential for initial recognition of pathogens and subsequent host defense (52). It is also established that CD40-ligation of APC occurs in the course of immune response via CD40L expressed on CD4+ T cells (53). Our data suggest the interesting possibility that optimal induction of an immune response requires that T cell activation precedes activation via TLR4 and TLR9. This hypothesis is consistent with strong rapid recall responses involving memory T cells and warrants further investigation.

Our experiments also show the nonessential role of NK and T cells in activating Mφ with anti-CD40 and CpG. Exposure of Mφ to exogenous IFN-γ in vitro induces TLR9 up-regulation (6). Consistent with that, we demonstrate here that CD40 ligation of Mφ in vivo leads to TLR9 up-regulation, which is not seen in Mφ from IFN-γ−/− mice. Similarly, anti-CD40 + CpG-Mφ from IFN-γ−/− mice did not mediate antitumor activity in vitro, indicating that IFN-γ plays an essential role for Mφ activation with anti-CD40 and CpG. Importantly, IFN-γ appears to be provided by the anti-CD40-Mφ themselves (Fig. 1D), as they were the only source of IFN-γ in our in vitro system.

Whereas inhibition of [3H]TdR incorporation into tumor cells is a reliable assessment of tumoristasis, it does not provide information about the mechanisms of the antitumor effects. The results show that the tumorstatic activity of anti-CD40 + CpG-Mφ was associated with apoptosis of the tumor cells. Thus, after 24 h, 63% of tumor cells incubated with anti-CD40 + CpG-Mφ demonstrated depolarized cell membranes as revealed by positive staining for PS; 30% of those tumor cells were dead, based on their membrane permeability for the 7-AAD (Fig. 5A). However, the remaining 37% of cells that retained intact membranes (annexin V−7-AAD−) did not incorporate [3H]TdR (Fig. 5B). These findings are in agreement with reports that cell-cycle arrest precedes apoptotic death (54).

The exact mechanism of tumor cell inhibition and killing by anti-CD40 + CpG-Mφ is not clear. Upon activation, Mφ can express a number of factors, such as TNF-α, NO, IFN-γ, IFN-α, IL-1-α, IL-1β, TRAIL, and Fas ligand, that may be involved in Mφ-mediated tumor toxicity. The spectrum and magnitude of expression of these factors depend upon the mechanism of Mφ activation. In this regard, anti-CD40 + CpG-Mφ may differ from the anti-CD40 + LPS-Mφ we have recently described (4) or “classically activated” Mφ induced by IFN-γ + LPS (5). Thus, when compared with Mφ from immunocompetent mice, anti-CD40-Mφ from TNF-α−/− and iNOS−/− mice demonstrated a slightly reduced antitumor effect, whereas the antitumor effect of anti-CD40 + CpG-Mφ from the same mice appeared similar (Fig. 4, C and D). Although these results demonstrate the nonessential role of TNF-α and NO in anti-CD40 + CpG-Mφ effects, they do not preclude the involvement of these molecules in antitumor effects. It is also known that various tumor cell lines have different sensitivity to different cytotoxic molecules (22–24). In agreement with this, different tumor cell lines showed different levels of response to anti-CD40 + CpG-Mφ (Table I). In contrast, the cytotoxic mechanism of anti-CD40 + CpG-Mφ might not depend on soluble cytotoxic molecules but could be similar to that of IL-12-induced effector Mφ, which were effective in iNOS−/− mice and killed various tumor cells via a novel contact-dependent, paraformaldehyde fixation-resistant, apoptosis-inducing mechanism (3). These
and other possible mechanisms of tumor cell recognition and killing by anti-CD40 + CpG-Mφ are currently under investigation. Anti-CD40 and CpG have been used separately in experimental tumor immunotherapy. Most of these approaches focused on inducing T cell-mediated antitumor effects by giving either anti-CD40 (28) or CpG (55). In this respect, ligation of CD40 and TLR9 was shown to synergistically augment Ag presentation by APC, possibly via induction of IL-12 (47, 56). Less attention has been paid to non-T cell-mediated antitumor effects of these two therapeutic modalities. We have shown that anti-CD40 could induce antemetastatic effects in vivo mediated by NK cells (27). Similarly, the antitumor efficacy of B class CpG has been shown to involve NK cells (57), Mφ (58), and PMN (59). In this study, we demonstrate that combining anti-CD40 and CpG in vivo resulted in B16 tumor growth retardation that was significantly greater than the effects induced by each treatment alone. The synergistic antitumor effect of anti-CD40 and CpG against B16 melanoma was observed in immunocompetent C57BL/6 mice and in immunocompromised SCID/beige mice, indicating that T cells were not required. The induction of durable tumor resolution after anti-CD40 and CpG treatment in three of eight A/J mice bearing Nx2 tumors suggests that the induced antitumor effect may vary for different tumor types and for different strains of tumor-bearing mice. In SCID/beige mice depleted of PMN and cytokine-producing NK cells and treated with anti-CD40 + CpG, potent retardation of B16 tumor growth was still observed (Fig. 7E). In contrast, in vivo inactivation of Mφ with silica nearly abrogated that antitumor effect (Fig. 7F). Although in vivo Mφ depletion with silica has limitations (as do other in vivo methods of Mφ depletion or inactivation), these results confirm that the antitumor effect of the combination of anti-CD40 and CpG in SCID/beige mice was mediated primarily by activated Mφ. These results are in agreement with our previous findings suggesting the potential involvement of Mφ in the in vivo antitumor effects of anti-CD40 given alone (27). Our findings also support reports suggesting a role for Mφ in tumor regression induced by or facilitated by CpG (58). In the present study we demonstrate (to our knowledge, for the first time) that CD40 ligation and CpG can synergize in inducing T and NK cell-independent, Mφ-mediated antitumor effects in vivo. In addition, the findings show that Mφ can be effectively activated to mediate antitumor effects in the absence of other immune cells, suggesting that this immunotherapeutic strategy may be appropriate for clinical trials in immunocompromised cancer patients. For example, as Mφ may be more resistant than other immune cells to cytotoxic drugs (60), a combination of anti-CD40 and CpG may be considered for clinical testing as adjuvant treatment after cytotoxic chemotherapy.

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Disclosures
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References


