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Anti-CD40 Antibody Induces Antitumor and Antimetastatic Effects: The Role of NK Cells

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We assessed the effect of the stimulatory anti-CD40 Ab on NK cell activation in vivo and the therapeutic potential of activated NK cells in tumor-bearing mice. Single-dose i.p. injection of the anti-CD40 Ab resulted in production of IL-12 and IFN-γ in vivo, followed by a dramatic increase in NK cell cytolytic activity in PBLs. NK cell activation by anti-CD40 Ab was also observed in CD40 ligand knockout mice. Because NK cells express CD40 ligand but not CD40, our results suggest that NK activation is mediated by increased cytokine production upon CD40 ligation of APCs. Treatment of tumor-bearing mice with anti-CD40 Ab resulted in substantial antitumor and antimetastatic effects in three tumor models. Depletion of NK cells with anti-asialo GM1 Ab reduced or abrogated the observed antitumor effects in all the tested models. These results indicate that a stimulatory CD40 Ab indirectly activates NK cells, which can produce significant antitumor and antimetastatic effects. The Journal of Immunology, 2001, 166: 89–94.

The activation of antitumor immune responses in cancer patients has been well documented using in vitro analysis. However, in the absence of exogenous stimulation this immune response is rarely effective at causing an antitumor effect in vivo. Therefore, many approaches have been used to activate the immune cells of cancer patients in efforts to achieve a therapeutic effect. Recently, the involvement of a CD40-CD40 ligand (CD40L) interaction in the induction of a cellular immune response has been reported (1–3). It has been shown that interaction of CD40L expressed on CD4+ T cells with the CD40 receptor expressed on APCs results in activation of APC. Activated APC produce IL-12 and costimulatory molecules B7-1 and B7-2, which are necessary for activating effector CD8+ T cells (1–3). CD40-CD40L interaction has also been demonstrated to be required for the protective immunity induced by tumor vaccines (4, 5).

These recently identified mechanisms have prompted attempts to activate antitumor immune T cells by means of CD40 ligation. Indeed, it has recently been shown that direct activation of APC using either CD40L (6, 7) or anti-CD40 Ab (8–10) resulted in CD8+ T cell-mediated antitumor effects in murine tumor models. However, it is not yet clear whether CD40 triggering can induce the activation of other immune cells that have the potential for exerting antitumor effects. NK cells have been shown to mediate strong antitumor activities (11–13). Although early activation of NK cells in mice implanted with tumor cells transduced with the CD40L gene has been observed (7), the role of NK cells in inducing therapeutic antitumor effects as a result of CD40 ligation has not been systematically investigated.

In this study we investigated how CD40 ligation via an anti-CD40 Ab might activate NK cells and determined the potential role of the activated NK cells in mediating antitumor and antimetastatic effects. Our results demonstrate that a stimulatory anti-CD40 Ab is able to indirectly activate NK cells, apparently via induction of Th1 cytokines, and that the activated NK cells can mediate antitumor and antimetastatic activities.

Materials and Methods

Mice and cell lines

Female C57BL/6 mice (National Cancer Institute, Bethesda, MD, and Taconic Farms, Germantown, NY), BALB/c and SCID/DB1BL/c mice (Harlan-Sprague-Dawley, Madison, WI), and A/J mice (The Jackson Laboratory, Bar Harbor, ME), 5–8 wk old, were used for the experiments. CD40L-deficient mice (14) were provided by Dr. Richard Flavell (Howard Hughes Medical Institute, Yale University, New Haven, CT). Mice were housed in American Association for Accreditation of Laboratory Animal Care-accredited, specific pathogen- and viral Ab-free facilities located at the University of South Florida. H. Lee Moffitt Cancer Center and Research Institute (Tampa, FL) and at the University of Wisconsin Medical School (Madison, WI). CT26-Ep21.6 is a low MHC class I-expressing clone of CT26 adenocarcinoma stably transfected with EpCAM cDNA (15). NXS2 is a poorly immunogenic, highly metastatic, syngeneic neuroblastoma in A/J mice that is sensitive to NK cell-mediated therapies (12) and is a gift from Dr. R. Reisfeld (Scripps Research Institute, La Jolla, CA). The murine B16 cell line and the murine lymphoma cell line YAC-1 were grown in RPMI 1640 complete medium with 10% FBS and 100 U/ml of penicillin/streptomycin at 37°C in a humidified 5% CO2 atmosphere.

Anti-CD40 stimulatory Ab

The FGK 45.5 hybridoma cells producing a stimulatory anti-CD40 Ab (16) were a gift from Dr. Fritz Melchers (Basel Institute for Immunology, Basel, Switzerland). For in vivo studies, the Ab was obtained from ascites of nude mice injected with the hybridoma cells and enriched for IgG by ammonium sulfate precipitation. To confirm the specificity of the FGK 45.5 anti-CD40 Ab, mouse spleen cells were dual stained with both the FGK45.5 CD40 Ab and a FITC-conjugated hamster anti-mouse CD40 mAb, clone HM40-3 (PharMingen, San Diego, CA). The two Abs bind to different epitopes of the CD40 receptor. Development of the FGK45.5 Ab was performed using biotinylated goat anti-rat IgG with detection via streptavidin-PE.
NK cytolytic assay

BALB/c mice were injected with 0.25 mg of anti-CD40 Ab i.p. at 1, 2, 3, 4, 5, 6, and 7 days before sacrifice. BALB/c SCID mice were also injected with 0.25 mg of anti-CD40 Ab. Effector cell preparation and NK cytolytic assays using 51Cr-labeled YAC-1 target cells were performed as described previously (17).

Flow cytometry

PBMC from mice receiving 0.5 mg of either anti-CD40 Ab or rat IgG i.p. was obtained 5 days after the treatment. PBMCs (2.5 × 10^5) were stained with FITC-conjugated rat anti-mouse Ab against pan-NK cells (DX5), FITC-conjugated rat anti-mouse Mac-1 Ab (M1/70), FITC-conjugated rat anti-mouse Ly-6A/E Ab (E13-161.7), and FITC-conjugated rat IgG2a Ab (all Abs from PharMingen) for 40 min at 4°C. Propidium iodide (2 μg/ml) was added to stain dead cells, which were subsequently excluded from the analysis. Stained cells were analyzed using a FACScan cytofluorometer (Becton Dickinson, San Jose, CA), and data were collected for 10,000 events/sample.

Depletion of CD4^+ and CD8^+ T cells in vivo

A mixture of anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 2.43) was administered i.p. at a dose of 250 μg/mouse for each mAb, on days −2, 3, 8, and 13 relative to B16 tumor cell implantation. Control mice received 500 μg of rat IgG (Sigma, St. Louis, MO). Blood samples were obtained from the experimental mice on day 7 post-tumor cell implantation.

Cytokine production and measurement

C57BL/6 mice were injected i.p. with 0.5 mg of CD40 Ab at 24, 48, and 72 h before sacrifice. Serum prepared from these mice was then assessed by murine IL-12 (p70) and IFN-γ ELISAs (Genzyme, Cambridge, MA).

Peritoneal macrophage preparation and cytostatic test

Peritoneal cells were prepared from mice treated with either anti-CD40 Ab or rat IgG as described above. The peritoneal macrophage population was enriched by adhesion on plastic plates, followed by washing and aspiration of nonadherent cells. The percentage of macrophages among adherent cells was estimated by morphological criteria using Giemsa staining (>95%).

FIGURE 1. Injection of CD40 Ab results in NK cytolytic activities. A, PBMCs were obtained from BALB/c mice 1–7 days after a single i.p. injection of anti-CD40 Ab or from control mice (day 0). PBMCs pooled from three mice per group were included in NK cytolytic assays. This experiment was repeated once with similar results. B, NK cytotoxicity in SCID mice. SCID/BALB/c mice and regular BALB/c mice were injected i.p. with 0.25 mg of anti-CD40 mAb or rat IgG (three mice per group). Five days later the mice were sacrificed, and PBMC cytotoxicity was tested against YAC-1 cells.

FIGURE 2. Cell activation by CD40 Ab. Flow cytometric analysis of PBMC from BALB/c mice treated with either rat IgG (A, C, and E) or anti-CD40 Ab (B, D, and F). Data represent PBMC pooled from three mice per group. For A and B, FITC-conjugated rat anti-mouse Ab against pan-NK cells was used. For C and D, PBMC was stained with anti-Ly-6A/E Ab. Anti-macrophage Ab (Mac-1) was used to detect macrophages (E and F). These results were reproduced in a separate experiment. The numbers in the figure indicate the percentages of cells expressing the marker after subtracting background (appropriate isotype controls).
Antitumor cytostatic activity of macrophages was determined by the inhibition of DNA synthesis by target tumor cells. Briefly, B16 tumor cells (2 × 10^6/well) were cocultured for 48 h with and without macrophages prepared from individual mice. To estimate DNA synthesis, the cells were pulsed with [3H]Tdr (0.25 μCi/well) during the last 6 h of incubation. [3H]Tdr incorporation was determined using a liquid scintillation beta counter (Pharmacia Wallac, Finland). Results are expressed as the percent inhibition of [3H]Tdr incorporation by B16 cells incubated with macrophages compared with [3H]Tdr incorporation by B16 cells incubated in medium alone.

NO production

Peritoneal macrophages (2 × 10^5/ml) from mice subjected to various treatments were incubated for 48 h. Nitrite accumulation in macrophage supernatants was determined using Griess reagent.

Anti-CD40 Ab tumor therapy

C57BL/6 female mice, 5–8 wk old, were challenged with 5 × 10^4 or 1 × 10^5 B16 melanoma cells injected intradermally on the abdomen. CT26-Ep21.6 adenocarcinoma cells (4 × 10^6) were injected i.v. into BALB/c mice to establish lung metastases. NXS2 neuroblastoma cells (1 × 10^3) were injected i.v. into A/J mice to induce liver metastasis. Five days after tumor implantation, all mice were injected i.p. with 0.25–0.5 mg of either anti-CD40 Ab (FGK45) or rat IgG control Ab (Sigma). To determine whether anti-CD40 Ab-mediated antitumor effects were due to NK cell activation, mice treated with anti-CD40 Ab received injections of either rat IgG or anti-asialo GM1 Ab (1.5 mg; Wako, Richmond, VA). To neutralize IFN-γ in vivo, mice were injected i.p. with 0.5 mg of anti-IFN-γ Ab R4-6A2 (NXS2 tumor model only) on days 4 and 9 post-tumor challenge. B16 melanoma tumor volume was determined as previously described (18). Lung metastases in mice receiving CT26-Ep21.6 tumor cells were enumerated 19 days post-tumor challenge as previously described (19). NXS2 neuroblastoma liver metastases were evaluated on day 28 post-tumor cell injection as previously described (12).

Results

Anti-CD40 Ab administration results in NK cell activation

Mice receiving CD40 Ab were evaluated for NK activity. The results presented in Fig. 1A show that PBMC acquired an increased ability to lyse NK cell-sensitive YAC-1 target cells 2 days after anti-CD40 Ab treatment, and maximal NK activity was observed on days 2 and 3 after anti-CD40 Ab treatment. As a negative control, mice were also injected with anti-IgG Abs. No enhanced PBMC NK activity was detected on days 3 and 5 in the control mice. NK activity of the spleen cells from mice treated with anti-CD40 Ab was also increased, with a pattern similar to that of PBMC, although to a lesser degree (data not shown). Anti-CD40 Ab-stimulated NK activity was also observed in both T cell-deficient SCID mice (Fig. 1B) and B16 tumor-bearing mice (data not shown). Flow cytometric analysis of PBMC from mice treated with anti-CD40 Ab 5 days earlier showed an elevated percentage of NK cells (Fig. 2, A and B). As DX5 mAb can sometimes stain a population of T cells, we performed the dual staining of PBMC with FITC-conjugated DX5 mAb and PE-conjugated anti-TCRβ-chain mAb, clone H57-795 (PharMingen). Results from the dual staining confirmed that NK-positive cells did not express TCR (data not shown). In addition, staining of PBMC with the Ab against the Ly-6A/E Ag, a marker for IFN-induced cell activation (20), revealed a marked activation of PBMC from anti-CD40 Ab-treated mice (Fig. 2, C and D). Staining PBMC with Mac-1 Ab revealed an increased number of macrophages in anti-CD40 Ab-treated mice (Fig. 2, E and F). NK cells from both control mice and anti-CD40 Ab-treated mice did not express CD40 (data not shown).

Activation of APC precedes the activation of NK cells following anti-CD40 Ab treatment

It has been reported that CD40 ligation results in activation of APC, as evidenced by increased production of IL-12 and increased expression of B7-1/B7-2 costimulatory molecules (1, 7). To determine whether anti-CD40 Ab administration resulted in activation of APC, we assessed these two parameters. A substantial increase in IL-12 and IFN-γ levels in the serum was detected 24 h after anti-CD40 Ab injection (Fig. 3A). Peritoneal macrophages obtained from mice treated with anti-CD40 Ab exhibited elevated levels of nitrite, indicating NO production (Fig. 3B) and a potent cytostatic effect against tumor target cells (Fig. 3B). Activation of APC was also confirmed by increased expression of B7-1 on the surface of PBMC and spleen cells derived from anti-CD40 Ab-treated mice (data not shown). Although virtually no NK cytolytic activity was observed until day 2 (Fig. 1), macrophage activation was readily detectable 24 h after anti-CD40 Ab treatment (Fig. 3), indicating that activation of APC, including macrophages, preceded maximal activation of NK cells.

**FIGURE 3.** Anti-CD40 Ab-induced APC activation and cytokine production precede NK cytolysis activity. A, IL-12 and IFN-γ levels in plasma pooled from three mice per group as determined by ELISA. Plasma samples were taken at different time points after a single i.p. injection of anti-CD40 Ab. These assays were repeated once with similar results. B, Cytostatic activity (CSA) and NO production by peritoneal macrophages from control and anti-CD40 Ab-treated mice (n = 3 for each group). CSA was determined by inhibition of [3H]Tdr incorporation in B16 tumor cells. Nitrite concentration and percentage of CSA in mice on day 0 (before anti-CD40 Ab treatment) are considered the negative control. Similar results were obtained in an independent experiment.
NK cells are involved in antitumor and antimetastatic effects induced by anti-CD40 Ab administration

Although T cells and NK cells are two main subsets of immune cells able to mediate clinically relevant tumor regression (21–23), antitumor effects as a result of anti-CD40 Ab administration have been largely attributed to T cells (8–10, 24). Given the observed dramatic activation of NK cells following anti-CD40 Ab treatment, we sought to determine whether NK cells were involved in anti-CD40 Ab-induced antitumor effects. To do this we chose three distinct tumor models, namely B16 melanoma, CT26-Ep21.6 colon adenocarcinoma, and NXS2 neuroblastoma. NK-mediated antitumor effects have been reported in each of these models in vivo (12, 15, 25). Our results show that treatment of mice bearing established tumors with anti-CD40 Ab induced significant antitumor (Fig. 4A) and antimetastatic (Fig. 4, B and C) effects. Depletion of NK cells with anti-asialo GM1 Ab during the course of anti-CD40 therapy either reduced (Fig. 4, A and B) or abrogated (Fig. 4C) the observed antitumor/antimetastatic effects. In the NXS2 tumor model, neutralization of IFN-γ in vivo also resulted in loss of the antimetastatic effect induced by anti-CD40 therapy, suggesting that NK cell-mediated tumor rejection was IFN-γ dependent in this treatment model (Fig. 4C). Furthermore, injection of anti-CD40 Ab into mice depleted of CD4+ and CD8+ T cells elicited an antitumor effect (Fig. 5). B16, CT26-Ep21.6, and NXS2 tumor cell lines, like NK cells, express no or little CD40 molecules on their membranes, as evidenced by flow cytometric analysis (data not shown). The same three tumor cell lines were sensitive to NK cell lytic activity in vitro by PBMC obtained from mice 5 days after anti-CD40 Ab injection (data not shown). These experiments suggest that tumor killing mediated by CD40-activated NK cells in

**FIGURE 4.** Anti-CD40 Ab-mediated antitumor and antimetastatic effects involve NK activity. A, Tumor growth inhibition in the B16 tumor model. Data are the mean of five mice per group ± SEM. These results were confirmed in another experiment. B, Lung metastasis in CT26-Ep21.6 adenocarcinoma model. Mice were given rat IgG (upper row) or anti-CD40 Ab (middle and bottom rows) on days 5 and 12. Depletion of NK cells was performed in mice shown in the bottom row. Mice were sacrificed on day 19, and lungs were stained with India ink and bleached with acetic acid. C, Liver metastases in the NXS2 neuroblastoma model. On days 5 and 12 (post-NXS2 tumor cell injection) mice were injected with anti-CD40 Ab or rat IgG. To deplete NK cells or neutralize IFN-γ, anti-asialo GM1 Ab or anti-IFN-γ R4–6A2 mAb, respectively, were injected into mice that had received anti-CD40 Ab. Mice were sacrificed, and liver metastases were evaluated on day 28. The results are presented as the mean ± SEM of five mice per group, except in the rat IgG group (four mice).
these tumor models does not involve CD40-CD40L interaction between tumor cells and NK cells.

**CD40 ligation activates NK cells via an indirect mechanism**

It has been shown that NK cells can express CD40 (26, 27). Recognition of CD40 on other cells can provide an activation pathway for such NK cells (26, 27). Because anti-CD40 Ab treatment in vivo resulted in increased expression of CD40 on B cells and macrophages (data not shown), the observed activation of NK cells might result from direct interaction of the CD40 molecule on APC (or other cells) with CD40L on NK cells. To test this hypothesis, CD40 Ab-induced NK cytolytic activities were evaluated in both normal C57BL mice and C57BL mice deficient in CD40L. Fig. 6 shows that NK cell-mediated cytolytic activities of PBMC in both CD40L knockout mice and normal C57BL/6 mice 5 days after anti-CD40 Ab administration were nearly the same. These data demonstrate that the anti-CD40 Ab can activate NK cells via an indirect mechanism that does not require the CD40L molecule on NK cells.

**Discussion**

CD40-CD40L interactions can be crucial in generating antitumor immune responses (1–3). Modification of tumor cells to express CD40L or administration of a stimulatory anti-CD40 Ab have been demonstrated to inhibit tumor growth (6–10, 24, 28). In all these studies the antitumor effect has been shown to involve CD8 T cells. Although a previous study showed that rejection of a murine P815 tumor transfected with the CD40L gene was mediated by NK cells (7), the therapeutic potential of NK cells activated by CD40-CD40L ligation was not assessed.

In this study we show that systemic NK cell activation can be one of the main mechanisms of immunotherapeutic action of anti-CD40 Ab in vivo. Thus, triggering CD40 with a stimulatory anti-CD40 Ab can result in a dramatic in vivo systemic activation of NK cells. Moreover, anti-CD40 Ab treatment of tumor-bearing mice that had established tumors in the skin (B16 melanoma), the lungs (CT26-Ep21.6 colon adenocarcinoma), or the liver (NXS2 neuroblastoma) resulted in NK cell-dependent antitumor or antimetastatic effects in all three tumor models tested. Among these tumor models, activated NK cells were the most effective against NXS2 neuroblastoma (Fig. 4C). This is in agreement with a previous report that the NXS2 tumor model was highly sensitive to the NK cell-mediated antitumor effects of targeted IL-2 therapy (12).

Whereas the antimetastatic effect was clearly dependent on NK cells in the NXS2 tumor model, anti-CD40 Ab-mediated antitumor effects were only partially reduced by anti-asialo GM1 treatment in the B16 and CT26 models, suggesting that in addition to NK cells, other NK-independent immune cells participate in inhibition of tumor growth. Our results indicate that anti-CD40 Ab can also stimulate antitumor activity in mice depleted of both CD4+ and CD8+ T cells. However, the role of cells other than NK and T cells in mediating anti-CD40 Ab-induced antitumor effects has not been determined.

Previous studies (7) and our own results (data not shown) indicated that activated NK cells did not express CD40. Therefore, direct activation of NK cells by anti-CD40 Ab is highly unlikely. It has been shown that NK cells express CD40L (26) and can be activated to kill CD40-expressing tumors in vitro (26). Therefore, it is possible that increased production of IL-12 by activated APC can up-regulate expression of CD40L on NK cells in a similar way as was reported for CD4+ T cells (29). Consequently, those activated NK cells may kill tumor cells via CD40-CD40L interaction.
However, because administration of anti-CD40 Ab resulted in similarly high NK activities in both normal mice and CD40L knockout mice, direct interaction of CD40-CD40L is not required for NK cell activation or NK-mediated cytolytic activity. These data are in agreement with a previous study (27) showing that NK cells from conventional or CD40L knockout mice were similarly activated by the CD40 molecule expressed on tumor cells. Secondly, all three tumors used in our study were CD40 negative, and while IFN-γ treatment in vitro induced a low level of CD40 expression in B16 cells, both CT26 and NXS2 cells remained CD40 negative despite IFN-γ treatment (data not shown). Because CD40 expression was absent in these tumor cells, the antitumor effect of the anti-CD40 Ab in our models did not involve the presence of CD40 on the tumor cells. Therefore, our data document an activation of antitumor NK cells different from what was shown previously with CD40+ tumor models, in which CD40-CD40L interaction can result in direct growth inhibition and apoptosis of CD40+ tumor cells in vitro and in vivo (30, 31).

The results presented here demonstrate that in vivo treatment with anti-CD40 Ab activates NK cells, which results in potent therapeutic antitumor and antimetastatic effects. Our results also indicate that anti-CD40 Ab stimulates NK-mediated antitumor activities in the absence of CD40L and CD40 on NK cells as well as on tumor cells. Because NK activation was preceded by the production of IL-12 and IFN-γ, a role for these cytokines in activating NK cells is implicated.

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