ABSTRACT

Interleukin (IL)-12 activates a T-cell-dependent antitumor immune response that is able to eradicate established large tumors in a number of immunogenic tumor models. The effector mechanisms in these dramatic antitumor responses have not yet been identified. In this report, we show that the effector mechanism of IL-12-induced rejection of established MCA207 tumors is unique in that it is not dependent on perforin, Fas/Fas ligand, and nitric oxide. Study of cyclophosphamide plus IL-12 (Cy + IL-12)-induced rejection of ascites Sa1 tumor demonstrates that macrophages are the predominant immune cell infiltration in the ascites. These macrophages possess nonspecific tumoricidal activity in vivo as immune distinct MCA207 tumor cells inoculated i.p., but not s.c., in mice bearing regressing Sa1 ascites tumors after Cy + IL-12 therapy are rejected. Furthermore, Cy + IL-12-treated Sa1 ascites cells or macrophages, but not spleen macrophages from the same mouse or inflammatory macrophages induced by thioglycollate, are able to suppress the development of immune-irrelevant s.c. tumors in a Wynn assay. These macrophages kill various tumor cells in a contact-dependent manner in vitro, and the cytotoxicity is preserved after fixation with paraformaldehyde. These results demonstrate that activated macrophages function as effector cells in an IL-12-induced, T-cell-dependent eradication of established tumors through a novel contact-dependent, paraformaldehyde fixation-resistant, apoptosis-inducing mechanism.

INTRODUCTION

The broad antitumor activity of IL-12 is likely attributable to its ability to target multiple cell populations including NK and NKT cells (1, 2), antigen-specific T cells (3), and antigen-presenting cells (4). IL-12 can directly activate NK and NKT cells to reject nonestablished tumors (1, 5). On the other hand, IL-12 also activates a T-cell-dependent antitumor response that is able to eradicate established palpable s.c. tumors (6–10). We have shown recently that IL-12 treatment alone is able to induce complete rejection of small (4–8 mm in diameter) s.c. immunogenic MCA207 tumors established for 10 days (9) but is ineffective once the tumor is >10 mm in diameter. However, when Cy is combined with IL-12 (Cy + IL-12), long-term (>3 weeks) established large (15–20 mm) MCA207 tumors are completely eradicated (10). The dramatic antitumor effects of Cy + IL-12 are not limited to the s.c. tumor model, because late-stage MCA207 tumor burden established by the i.p. and i.v. routes are also completely eradicated (11). In addition to MCA207, the curative effects of IL-12 and Cy + IL-12 are also found in several other immunogenic, but not nonimmunogenic, tumor models (11).

In the T cell-independent response induced by IL-12, NK and NKT cells were often found as effectors through a perforin-dependent cytotoxic pathway (12). However, the effector mechanisms in T-cell-dependent responses induced by IL-12 and Cy + IL-12 is not known. Analysis of regressing tumors after IL-12 and Cy + IL-12 treatments reveals significant infiltration of tumors by T cells and iNOS-expressing activated macrophages (9, 10) at levels that are rarely seen in other antitumor responses. Although T cells are required for IL-12-induced tumor rejection (11), their role as effector cells in this tumor rejection model has not been established. Because complete rejection of both small and large tumors by IL-12 alone and Cy + IL-12, respectively, can be mediated by the single CD4 or CD8 subset of T cells under both conditions of antibody depletion and specific gene knockout hosts (6, 11), critical questions regarding the potential effector mechanism as opposed to the classic CD8-mediated CTL response have been raised. The most well-studied T-cell killing pathways are the perforin/granzyme and Fas/FasL pathways (13–14). These pathways have been shown to be involved in antitumor responses in vivo in other tumor models (15–17). On the other hand, based on the presence of activated macrophage and requirement of IFN-γ, we have proposed involvement of macrophages as effector cells in the IL-12-induced tumor rejection, probably through macrophage-mediated release of nitric oxide (9). The current study was carried out to determine the key effector cell population and the tumor killing mechanisms for the elimination of large tumor burdens after IL-12 and Cy + IL-12 treatments. We found that the effector mechanism(s) in IL-12-induced rejection of established tumors is a unique one, not dependent on previously identified antitumor effector pathways. Furthermore, our experiments show that macrophages are involved in tumor cell killing through a novel cell contact-dependent and fixation-resistant apoptosis-inducing pathway.

MATERIALS AND METHODS

Murine Tumors. MCA207 sarcoma is a methylcholanthrene-induced transplantable tumor in C57BL/6 mice (18). It was obtained from the Surgery Branch, National Cancer Institute (a generous gift of Dr. J. Yang). Sa1 is a chemically induced sarcoma in A/J mice. Lewis lung carcinoma is a spontaneously occurring lung cancer in C57BL/6 mice. These tumors were obtained from the Tumor Depository at the Biological Testing Branch, Division of Cancer Treatment, National Cancer Institute, NIH (Frederick, MD).

Tumor cells were maintained in cell culture from in vivo harvested tumor implants. RPMI 1640 tissue culture medium was supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 5 × 10−5 M 2-mercaptoethanol. All wild-type C57BL/6 and A/J × C57BL/6 (AB6F1) mice were obtained from Biological Testing Branch, National Cancer Institute, NIH (Frederick, MD). All mutant and gene knockout mice are of the C57BL/6 background and were obtained from Jackson Laboratory (Bar Harbor, ME). Experiments were performed in female mice, 8–12 weeks of age.

In Vivo Treatment Models. In s.c. tumor models, 5 × 105 tumor cells in 0.2 ml of saline were injected s.c. on the flank of syngeneic or semisyngeneic F1 mice. Tumor size was assessed in two directions with calipers. Recombinant murine IL-12 (Peprotech, Rocky Hill, NJ) was administered i.p. at a dose of 300 μg in 0.5 ml of 1% mouse serum in saline given once every other day for three doses. Cy + IL-12 treatment was composed of a single i.p. injection of 3 mg (120 mg/kg) of Cy (Sigma Chemical Co., St. Louis, MO) in 0.5 ml of...
saline, followed 3 days later by a course of IL-12 as described above. In some cases, an additional single dose of IL-12 (300 ng i.p.) was given weekly during tumor regression.

In the ascites tumor model, 5 × 10^5 Sa1 tumor cells in 0.2 ml of saline were injected i.p. in A66F1 mice. Visible signs of ascites development could be seen 8 days after tumor inoculation. Cy treatment was given by i.p. injection of 3 mg in 0.5 ml saline. Two days later, IL-12 treatment was given s.c. at a dose of 500 ng every other day for three doses. Cy + IL-12 treatment was composed of a single i.p. Cy treatment was followed 3 days later by s.c. IL-12. For in vivo depletion of IFN-γ, tumor-bearing mice were treated i.p. with 1 mg of antibody to IFN-γ (clone R4-6A2) 1 day before Cy and 1 day before IL-12 treatments. Rat IgG was used as control.

Collection and Preparation of Ascites Macrophage Cells. Ascites cells were collected by peritoneal lavage of euthanized tumor-bearing mice with heparin-containing (10 units/ml) saline. The RBCs were lysed by osmotic shock, and the remaining cells were washed with culture medium two times before use in other in vitro assays. For isolation of macrophages, ascites cells were stained with antimouse CD11b antibody conjugated with microbeads in buffer as recommended by the manufacturer (Miltenyi Biotec, Auburn, CA). The CD11b/Mac-1-positive cells were collected on a magnetic column according to the protocol of the manufacturer. The purity of the isolated cells was analyzed by flow cytometry. The cells were washed once with medium and used for other in vitro assays.

Flow Cytometry Analysis. Ascites cells were collected as described above. For each analysis, 5 × 10^5 cells were stained with FITC- or PE-conjugated antibody specific for T cells (CD3), macrophages (CD11b/Mac-1), and NK cells (DX5). Isotype-matched irrelevant antibodies were used as control. The specific antibodies used were: hamster antimouse CD3 (clone 145–2C11), rat antimouse CD11b (clone M1/70), rat antimouse NK marker (clone DX5), and rat antimouse CD80 (clone 16-10A). All antibodies were from PharMingen (San Diego, CA). Antibody-stained cells were analyzed by flow cytometry (FACStar; Becton Dickinson).

Winn Assay. Effector and target cells were washed twice separately in saline before final suspension in saline. The two cells were mixed at designated ratios and centrifuged. They were suspended in final concentration (2 × 10^6 effector cells and 1–2 × 10^5 target cells in 0.2 ml of saline) for inoculation. The mixture was injected s.c. in naive mice in the flank. As a control, target cells alone were also inoculated in naive mice.

In Vitro Cytotoxicity Assay. Ascites cells and macrophages were obtained as described above. Thy1.2-labeled-induced peritoneal macrophages were harvested from naive mice 3 days after mice had been given 1 ml of 10% Difco thioglycollate medium i.p. Ascites cells or purified macrophages were washed once in saline and fixed with 1% paraformaldehyde in saline at a final concentration of 1 × 10^6 cells/ml as described in previous studies (19, 20). The cells were incubated at room temperature for 20 min, washed one time with culture medium, and cultured in medium at 2 × 10^4/ml in 100-mm culture dishes for 3–16 h. This fixation was found to be sufficient to kill all cells, because no viable adherent cells were seen after overnight culture in medium. For cytotoxicity assay, the fixed effector cells were harvested and washed twice with medium. After suspension in medium at a concentration of 1 × 10^6 cells/ml (E:T, 10:1), the cells were diluted to give concentrations of 5 × 10^5, 2.5 × 10^5, and 1.25 × 10^5 cells/ml in medium. Effector cells (0.1 ml) were added to triplicating wells in 96-well, flat-bottomed plates containing 1 × 10^4 MCA207 target cells in 0.1 ml added 10–30 min before the effector cells. Control wells contained only effector or target cells. Plates were incubated at 37°C for 1 day, at which time a graded number of target cells ranging from 1 × 10^3 to 4 × 10^4 were added to triplicating empty wells to generate a standard curve for counting cell numbers. The plate was returned to incubation for another day, at which time the nonadherent effector and dead tumor cells were removed through gentle washing of the wells with medium for three to four times. The number of adherent cells was determined by CellTiter 96 Aqueous One Cell proliferation kit (Promega Corp., Madison, WI). The absolute number of viable cells in each well was determined against the standard curve with known numbers of cells. The percentage of viable cells in each well was expressed as mean number of viable cells/mean number of target cells alone.

RESULTS

Perforin-, FasL-, and iNOS-mediated Cytotoxicity Pathways Are Not Essential in Tumor Rejection by IL-12 and Cy + IL-12.

We have shown in our previous studies that established MCA207 tumors are rejected by therapy with IL-12 alone or Cy + IL-12 (9, 10). To investigate the possible roles of some of the previously established T-cell and macrophage effector mechanisms in these tumor rejection models, s.c. MCA207 tumors were established in normal C57BL6 mice and B6-derived mice lacking perforin (Pp−/−), FasL, (gld/gld), and iNOS (NOS2−/−)., respectively. Because FasL mutant mice develop an autoimmune disorder, which may affect tumor rejection indirectly, the Fas mutant mice (lpr/lpr), which develop the same autoimmune disease as gld/gld mice because of the loss of the function of a FasL receptor, were used as controls. Tumor-bearing mice were treated with IL-12 alone at days 9–10 when tumors were 3–6 mm in diameter or with Cy + IL-12 after 4 weeks when tumor sizes were 15–20 mm in diameter. As Table 1 shows, despite the lack of function of these previously known effector molecules, rejection of small tumors by IL-12 alone and large tumors by Cy + IL-12 was preserved in nearly all of the mutant mice. The incomplete tumor rejection found in some of the FasL mutant (gld/gld) mice with large tumors during Cy + IL-12 treatment is likely attributable to the development of autoimmune disease, because both gld/gld and lpr/lpr mice had similar reduction in complete tumor rejection. Furthermore, both of these types of mice developed enlarged (>10 mm) lymph nodes during the later period of tumor regression. Subsequent experiments were also carried out with mice double deficient in two of the three effector molecules. Lack of iNOS/Pp and iNOS/FasL did not affect tumor rejection (not shown). On the other hand, mice with double deficiency in perforin and FasL died early in life because of severe autoimmune diseases as reported recently (21) and were not tested for tumor rejection.

Predominant Presence of Macrophages during Rejection of Ascites Tumors by Cy + IL-12.

The lack of essential involvement of these previously established antitumor effector mechanisms in the solid tumor regression model suggests that there may be a novel effector mechanism(s). Identification of the effector cell population through direct analysis of the T cells and macrophages at the site of regressing solid tumors is problematic. We therefore turned to an ascites tumor model in which the tumor-infiltrating immune cells can be readily isolated from the peritoneal cavity at various time points during tumor rejection and directly analyzed in vitro. For this, we used the Sa1 tumor that forms either solid or ascites tumors, depending on the route of implantation. s.c. Sa1 tumors have been shown to respond

<table>
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<tr>
<th>Table 1 Lack of perforin, Fas ligand, and iNOS does not abrogate tumor rejection by IL-12 and Cy + IL-12 treatments</th>
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MCA207 tumor cells (5 × 10^5) were inoculated s.c. into normal C57BL6 or perforin gene knockout (Pp−/−) mice. Lact of Fas ligand mutant (gld/gld), Fas mutant (lpr/lpr), and iNOS gene knockout (iNOS−/−) mice.

Nine to 10 days after tumor inoculation when tumors reached 3-6 mm in diameter (small) or >4 weeks after tumor inoculation when tumors reached 15-20 mm in diameter (large) IL-12 or Cy + IL-12 treatment was initiated. Cure is defined as complete tumor regression after treatment, while animals remained tumor-free for the entire observation period (>60 days).
to IL-12/Cy + IL-12 therapy in our previous study (11). In the Sa1 ascites model, the tumor burden at day 7 after i.p. inoculation of 5 × 10^5 tumor cells is completely eradicated by IL-12 treatment alone in ~40% of mice. However, eradication of the heavy tumor burden at days 9–11 of tumor establishment requires the combined treatment with Cy and IL-12. Untreated mice died of Sa1 ascites tumor rapidly between days 12 and 15 after i.p. inoculation of 5 × 10^5 tumor cells (Fig. 1A). In contrast, 9 of the 10 mice treated with Cy + IL-12 starting on day 10 of tumor inoculation rejected late-stage Sa1 ascites tumors and were cured (Fig. 1A). Treatment of day 10 tumor-bearing mice with Cy alone significantly prolonged survival without cure. Treatment with IL-12 alone was ineffective. Although day 10-established Sa1 ascites tumors are efficiently eradicated by Cy + IL-12 therapy, the same treatment given early at day 3 of tumor establishment resulted in tumor eradication in only 20% of mice (Fig. 1A), despite much lighter tumor burden at the earlier time point. Similar to the MCA207 tumor rejection model (10), rejection of ascites Sa1 tumor by Cy + IL-12 is dependent on endogenous IFN-γ, because in vivo depletion of IFN-γ with antibody abolished the curative effects of Cy + IL-12 on day 10-established Sa1 ascites tumors (Fig. 1B).

To analyze the immune cells at the site of ascites tumor rejection, we established Sa1 ascites tumors with 5 × 10^5 tumor cells. Ten days after tumor establishment, ascites cells were collected from the peritoneal cavities of tumor-bearing mice immediately before Cy + IL-12 treatment. Ascites cells were also collected 8 and 12 days after the initiation of Cy + IL-12 treatment. The cells were stained with antibodies to T cells (CD3, CD4, and CD8), macrophages (Mac-1), and NK cells (DX5) and analyzed by flow cytometry. T cells occupied <1% of total number of cells in untreated Sa1 ascites, and T-cell presence increased to ~7% after Cy + IL-12 treatment (Fig. 2, T cell). In comparison, macrophages occupied from 9% of total cells in untreated Sa1 ascites (Fig. 2, macrophage, before Cy + IL-12), and both the number and percentage of macrophages increased after Cy + IL-12 treatment. Thus, at 8 days after the start of IL-12 treatment, macrophages occupied about two-thirds of total cells (Fig. 2, macrophage, 8 days after Cy + IL-12). By 12 days after Cy + IL-12 treatment, macrophages increased to >75% of total number of ascites cells with diminished (<1%) tumor cell presence. By 12 days after Cy + IL-12, the Mac-1-positive cells also express high levels of MHC class II molecules and costimulatory molecule B7.1. They adhere rapidly and continuously to plastic culture dishes in vitro (not shown). These characteristics and the morphology of the cells are consistent with the notion that the Mac-1-positive cells are macrophages and not other antigen-presenting cells, such as myeloid dendritic cells. However, the expression of a dendritic cell marker such as CD11c by the Mac-1-positive cells in our experiments was not examined. No DX5-positive NK cells were detected in the Sa1 ascites before or after Cy + IL-12 treatment. No sign of increased T-cell and macrophage infiltration was found in mice bearing 10-day-established Sa1 ascites treated with IL-12 alone.

Macrophages from Cy + IL-12-treated Sa1 Ascites Suppress Tumor Growth in Vivo. The large numbers of macrophages, but not T cells, in the ascites fluid during tumor regression support the possibility that macrophages may carry out the effector function. Two experiments were therefore carried out to test whether macrophages from Cy + IL-12-treated Sa1 ascites are tumoricidal in vivo. In the first experiment, we tested the killing of immune irrelevant tumor cells by Cy + IL-12-treated Sa1 ascites cells. To avoid antigen-specific killing by T cells, we inoculated the H-2b MCA207 tumor cells to AB6F1 mice bearing regressing Sa1 (H-2a) ascites immediately after Cy + IL-12 treatment at both i.p. and s.c. locations. Control mice included naïve F1 mice and F1 mice treated with thiglycollate medium for induction of peritoneal exudate macrophages. The development of s.c. MCA207 tumors in mice bearing regressing Sa1 ascites tumors was delayed as compared with naïve mice, but all mice eventually developed progressive tumors (Fig. 3A). In contrast, MCA207 tumor cells inoculated i.p. into mice bearing regressing Sa1 ascites tumors were completely rejected in 80% of mice, resulting in long-term survival (Fig. 3B). Naïve mice developed i.p. sarcomatosis and died of tumor between 3 and 5 weeks. Tumor development and death were accelerated in mice bearing thiglycollate-induced macrophages.

In the second in vivo experiment, we isolated total ascites cells and Mac-1-positive ascites cells from Cy + IL-12-treated mice and tested...
coculture experiments in which 2 tumor cells. In the initial experiments, cytotoxicity was tested in

Table 2 Antigen nonspecific suppression of tumor development by Cy + IL-12-
activated macrophages in Winn assay

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<tr>
<th>Effector(^a)</th>
<th>Target(^b)</th>
<th>Tumor take(^c) (no. total)</th>
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<tr>
<td>None</td>
<td>Sa1</td>
<td>5/5</td>
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<td>None</td>
<td>MCA207</td>
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<td>None</td>
<td>LLC</td>
<td>5/5</td>
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<tr>
<td>Sa1 ascites/Cy + IL-12(^d)</td>
<td>Sa1</td>
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<tr>
<td>Sa1 ascites/Cy + IL-12(^d)</td>
<td>MCA207</td>
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<td>Sa1 ascites/Cy + IL-12(^d)</td>
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<td>Macrophage from(^e)</td>
<td>Sa1</td>
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<tr>
<td>Sa1 ascites/Cy + IL-12(^d)</td>
<td>MCA207</td>
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<tr>
<td>Thiglycollate induction</td>
<td>MCA207</td>
<td>5/5</td>
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<tr>
<td>Spleen of regressing Sa1(^f)</td>
<td>MCA207</td>
<td>5/5</td>
</tr>
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</table>

\(^a\) Cells (2 × 10\(^5\)) or purified Mac-1\(^+\) cells were mixed with tumor cells and inoculated s.c. into naive AB6F1 mice.

\(^b\) MCA207 (2 × 10\(^5\)) and LLC (1 × 10\(^5\)) tumor cells were mixed with various effector cells in the experiments.

\(^c\) Number of mice with palpable (>1 mm) tumors/number of mice tested. Pooled results from two similarly executed experiments are shown.

\(^d\) Eight days after Cy + IL-12 treatment started.

\(^e\) Mac-1\(^+\) (>80\%) cells isolated by antibody-conjugated magnetic beads.

\(^f\) Three days after i.p. injection of 1 ml of 10\(^%\) thiglycollate medium.

\(\) Adherent spleen cells of mice bearing regressing Sa1 ascites tumors 1 day after Cy + IL-12 treatment.

the ability of these cells to suppress tumor development by mixing them with tumor cells and inoculating the mixture s.c. in a Winn assay. Again, to avoid any involvement of antigen-specific T cells in tumor killing, we used two H-2\(^b\) tumor lines, MCA207 and LLC in addition to the autologous Sa1 tumor. The ascites cell/tumor mixture was inoculated in AB6F1 mice that are semisyncytic to both H-2\(^a\) and H-2\(^b\) tumors. The MCA207 and Sa1 tumors are responders to Cy + IL-12 treatment, whereas LLC is not (11). Despite the differences among these tumors, development of all three tumors in the Winn assay was similarly suppressed by total ascites cells from Cy + IL-12-treated Sa1 ascites at ratios of 10–20:1 (ascites:tumor; Table 2). Furthermore, purified macrophages from regressing Sa1 ascites, but not the spleen of the same mouse, showed similar suppression of tumor development as the total ascites cells. In contrast, macrophages from thiglycollate-induced, non-tumor-bearing mice accelerated tumor development in all animals. Tumor rejection in the Winn assay did not involve T-cell-mediated immunity and had left no immunological memory, in that all mice that rejected the MCA207 and LLC tumors in the Winn assay were subsequently challenged with 5 × 10\(^5\) cells of the same tumor on the opposite flank and developed tumors in the same way as naïve control mice (not shown).

Paraformaldehyde-fixed Cy + IL-12-activated Sa1 Ascites Macrophages Kill Tumor Cells in Vitro by a Contact-dependent Mechanism. We carried out a series of in vitro experiments to measure the cytotoxicity of Sa1 ascites cells or purified macrophages from these cells against Sa1 as well as a number of immune-irrelevant tumor cells. In the initial experiments, cytotoxicity was tested in coculture experiments in which 2 × 10\(^6\) effector cells were cocultured with 1–10 × 10\(^4\) tumor target cells in 24-well plates. Under such conditions, macrophages in the coculture did not become adherent, and the efficiency of tumor cell killing was indicated by the number of adherent tumor cells 2 days after the start of the coculture. By this measurement, all tumor lines tested were efficiently (>80%) killed by total ascites cells or purified macrophages from Cy + IL-12-treated regressing Sa1 ascites but not by the nonadherent T-cell fraction of the ascites cells. However, if the target cells were cultured in the same well but separated from the effector cells by a membrane permeable to soluble factors, no tumor killing was seen, indicating a contact-dependent tumor cell killing mechanism.

Subsequent experiments were carried out using primarily the MCA207 tumor cells as targets. To assess the requirement of energy and protein synthesis by macrophages during in vitro cytotoxicity, we have tested killing of MCA207 cells by macrophages from Cy + IL-12-treated Sa1 ascites that had been treated with protease and metabolism inhibitors that are known to inhibit CTL and NK cell-mediated cytotoxicity (22–24). The results again indicate efficient tumor cell killing in the presence of these inhibitors. These observations led to the fixation of effector cells with paraformaldehyde, which preserves membrane protein function but excludes any secretion of cytokytic factors and other energy-dependent processes in the subsequent cytotoxicity assay. As the experiment in Fig. 4 indicates, even under such harsh conditions, we still observed efficient killing (>75%) of MCA207 cells by macrophages isolated from Cy + IL-12-treated Sa1 ascites at E:T ratios as low as 2:5:1. Significant tumor cell killing was also observed in macrophages from untreated and Cy alone-treated Sa1 ascites under higher (10:1) E:T ratios. In contrast, inflammatory macrophages induced by thiglycollate did not show cytotoxicity in these assays. Importantly, the strong macrophage cytotoxicity observed in vitro is transient and is associated with the presence of residual Sa1 ascites tumor cells in vivo. Macrophages isolated 8 days after the start of Cy + IL-12 therapy from Sa1 ascites, which still contained significant portion of Sa1 tumor cells, or ascites, in which almost all tumor cells have regressed, demonstrated significantly different cytotoxic activities in vitro. Whereas macrophages from the former source showed high levels of cytotoxicity, macrophages from the latter source lost cytotoxicity (Fig. 5). This association between in vitro cytotoxicity and in vivo presence of Sa1 tumor cells is also observed in macrophages isolated from ascites 12 days after treatment.
mediated effector mechanism, especially CTL-mediated effector
IL-12/Cy
of preexisting tumor-sensitized T cells for IL-12-induced tumor re-
ment (7–10 days) than when given early (3 days) after tumor inocu-
is more effective when given after a brief period of tumor establish-
observation in the Sa1 ascites tumor model that IL-12-based therapy
process, as our recent study has indicated (11). The paradoxical
antitumor effect of Cy plus endotoxin is T cell dependent, because it
this reduction in tumor cell number by Cy treatment alone is transient,
this dramatic antitumor efficacy is not limited to the MCA207 tumor
model, because large (>15 mm in diameter) s.c. Sa1 tumors and other
immunogenic tumors are also eradicated by Cy + IL-12 treatment
(11). In the current study, we demonstrate that late-stage Sa1 ascites
tumors are also effectively eradicated by Cy + IL-12 treatment (Fig.
1). This observation of dramatic antitumor effect in the Sa1 ascites
tumor model is consistent with an earlier study (25) showing that day
7-established Sa1 ascites tumors were eradicated by treatment with Cy
followed with endotoxin but not Cy or endotoxin treatment alone.
The direct cytotoxicity of Cy to tumor cells may play a role in reducing
the number of tumor cells as shown by a previous study (25). However,
this reduction in tumor cell number by Cy treatment alone is transient,
and tumor cells rebound quickly (25). We have observed similar
kinetics in the current study, in that Cy treatment alone is only able to
reduce tumor number by about two-thirds, when the pretreatment
tumor burden is >2 × 10^6 cells. Our previous study with s.c. tumor
models has indicated that the major effect of Cy in the dramatic
antitumor response of Cy + IL-12 is not its direct cytotoxicity to
tumor cells but its ability to potentiate a T-cell response (10). The
antitumor effect of Cy plus endotoxin is T cell dependent, because it
is lost in T-cell-deficient mice. Because endotoxin is able to induce
IL-12 production from macrophages and dendritic cells (26), the
antitumor effect of endotoxin may be mediated partially or entirely by
IL-12. Although not directly tested in this study, the rejection of
established ascites tumors is also likely to be a T-cell-dependent
process, as our recent study has indicated (11). The paradoxical
observation in the Sa1 ascites tumor model that IL-12-based therapy
is more effective when given after a brief period of tumor establish-
ment (7–10 days) than when given early (3 days) after tumor inocu-
lation (Fig. 1A) is consistent with the similar observation seen in the
MCA207 tumor model (9) and may be explained by the requirement of
preexisting tumor-sensitized T cells for IL-12-induced tumor re-
jection (11).
Although T cells are required for rejection of established tumors by
IL-12/Cy + IL-12 (11), the involvement of an established T-cell-
mediated effector mechanism, especially CTL-mediated effector
mechanisms, in tumor rejection is not supported by evidence from this
and other previous studies: (a) MCA207 tumors are rejected by IL-12
or Cy + IL-12 treatment in mice in the absence of either CD4 or CD8,
but not both, T cells (6, 11); (b) CTL activity is not consistently
observed in IL-12-treated mice bearing regressing tumors (9, 10); (c)
immunohistochemical analysis of IL-12-treated regressing tumors
shows preferential peripheral distribution of T cells that are separated
from tumor tissue by a layer of macrophages, an observation reported
in two different tumor models (9, 27); and (d) the current study shows
that s.c. MCA207 tumors are rejected completely in mice deficient in
either perforin or FasL, the two effector pathways identified in T-cell-
mediated, contact-dependent killing of tumor cells. Thus, either T
cells are involved mainly as regulatory cells or they participate in
tumor cell killing through a yet unknown mechanism. One possible
role for T cells in tumor rejection is that they control an IFN-γ-
dependent and antigen-specific activation of macrophages that in turn
serve as effector cells. This hypothesis is supported by the require-
ment of IFN-γ in tumor rejection (Fig. 1B; Ref. 10). An adoptive
transfer experiment in our recent study (28) shows that tumor-specific
T cells, but not other host cells, and their production of IFN-γ are both
necessary and sufficient for IL-12-induced rejection of established
MCA207 tumors. Furthermore, our recent experiments using IFN-γ
receptor knockout mice indicate that host cell response to IFN-γ is
required for IL-12-induced tumor rejection. However, once macro-
phages are activated, tumor cells are eradicated in an antigen-nonspe-
cific manner, as shown in the current study. This nonspecific killing
by macrophages, however, is limited to site of tumor rejection (Table
2), is short-lived, and terminates when the activation antigen is de-
pleted (Fig. 5). Thus, the entire tumor rejection process is T cell
dependent and appears to be antigen specific.
Evidence from the current study suggests that macrophages are
involved as effector cells in the rejection of Sa1 ascites tumor. The
predominance of macrophages in Sa1 ascites after Cy + IL-12 treat-
ment is the result of both a direct reduction of tumor cells by Cy
treatment and an increase in macrophage migration into the peritoneal
cavity. Cy treatment does not suppress the number and function of
macrophage (29). Thus, the numbers of macrophages increase from
1–2 × 10^7 before Cy + IL-12 treatment to >5 × 10^7 after the
approach to test tumor rejection after in vivo elimina-
tion of macrophages by some methods reported previously (30, 31)
had been attempted. However, none of these methods could eliminate
the intense infiltration of macrophages in regressing MCA207 tumors
after IL-12 treatment, despite a significant reduction of macrophages
in the spleen. The difference between the current and previous
studies by others may be at the level of the antitumor responses. It
seems that the IL-12-induced antitumor response is highly efficient at
recruiting T cells and macrophages to the site of tumor. Alternatively,
the macrophages involved in tumor rejection by IL-12 may be less
phagocytic than normal resting macrophages, which would make
them less susceptible to depletion by methods based on macrophage
phagocytosis. Although involvement of macrophages in tumor rejec-
tion in vivo has been implicated in many previous studies (32–40) and
macrophage-mediated tumor cell lysis has been extensively studied in
many in vitro experiments, the mechanism by which macrophages kill
tumor cells in vivo remains unclear. Macrophages kill tumor cells in vitro
by both contact-dependent and contact-independent mechanisms
(41–42). The soluble factors responsible for tumor cell killing by
activated macrophages are TNF-α and nitric oxide (42). In the
MCA207 tumor model, depletion of TNF-α in vivo did not affect
tumor rejection by IL-12 (6). On the other hand, inhibition of nitric

Fig. 5. Association of in vitro macrophage cytotoxicity with in vivo presence of tumor
antigen. Mac-1-positive macrophages were isolated from Sa1 ascites 8 or 12 days after the
start of Cy + IL-12 treatment. Macrophages were fixed with paraformaldehyde. 1 × 10^5
(E/T; 10:1) fixed effector cells were cocultured with 1 × 10^6 MCA207 target cells in
quadroplets for 2 days. Columns, fractions (means) of viable cells relative to the number
of target cells alone (100%); bars, SD. Cy + IL-12/d8/TB, macrophages from ascites
containing Sa1 tumor cells 8 days after start of Cy + IL-12 treatment; Cy + IL-12/d12/NT,
macrophages from ascites in which minimal (<5%) Sa1 tumor cells were present 8 days
after start of Cy + IL-12 treatment; Cy + IL-12/d12/NT, macrophages from ascites in
which <1% Sa1 tumor cells were present 12 days after start of Cy + IL-12 treatment.

with Cy + IL-12 in which Sa1 tumor has totally regressed (Fig. 2, 12
days after Cy + IL-12; Fig. 5).

DISCUSSION
IL-12 and Cy + IL-12-induced tumor rejection has demonstrated the
most significant antitumor efficacy in tumor models. For example, in
a recent study we have shown that late-stage MCA207 tumor burdens
established by s.c., i.p., and i.v. routes of inoculation treated with
Cy + IL-12 are completely eradicated and cured (11). Furthermore,
this dramatic antitumor efficacy is not limited to the MCA207 tumor
model, because large (>15 mm in diameter) s.c. Sa1 tumors and other
immunogenic tumors are also eradicated by Cy + IL-12 treatment
(11). In the current study, we demonstrate that late-stage Sa1 ascites
tumors are also effectively eradicated by Cy + IL-12 treatment (Fig.
1). This observation of dramatic antitumor effect in the Sa1 ascites
tumor model is consistent with an earlier study (25) showing that day
7-established Sa1 ascites tumors were eradicated by treatment with Cy
followed with endotoxin but not Cy or endotoxin treatment alone.
The direct cytotoxicity of Cy to tumor cells may play a role in reducing
the number of tumor cells as shown by a previous study (25). However,
this reduction in tumor cell number by Cy treatment alone is transient,
and tumor cells rebound quickly (25). We have observed similar
kinetics in the current study, in that Cy treatment alone is only able to
reduce tumor number by about two-thirds, when the pretreatment
tumor burden is >2 × 10^6 cells. Our previous study with s.c. tumor
models has indicated that the major effect of Cy in the dramatic
antitumor response of Cy + IL-12 is not its direct cytotoxicity to
tumor cells but its ability to potentiate a T-cell response (10). The
antitumor effect of Cy plus endotoxin is T cell dependent, because it
is lost in T-cell-deficient mice. Because endotoxin is able to induce
IL-12 production from macrophages and dendritic cells (26), the
antitumor effect of endotoxin may be mediated partially or entirely by
IL-12. Although not directly tested in this study, the rejection of
established ascites tumors is also likely to be a T-cell-dependent
process, as our recent study has indicated (11). The paradoxical
observation in the Sa1 ascites tumor model that IL-12-based therapy
is more effective when given after a brief period of tumor establish-
ment (7–10 days) than when given early (3 days) after tumor inocu-
lation (Fig. 1A) is consistent with the similar observation seen in the
MCA207 tumor model (9) and may be explained by the requirement of
preexisting tumor-sensitized T cells for IL-12-induced tumor re-
jection (11).
Although T cells are required for rejection of established tumors by
IL-12/Cy + IL-12 (11), the involvement of an established T-cell-
mediated effector mechanism, especially CTL-mediated effector

4 Unpublished results.
oxide synthase with 1-NMMA was shown to inhibit tumor rejection by IL-12 in our previous study (9). However, the results of using iNOS knockout mice in the current study contradict this finding (Table 1). The use of high dose of 1-NMMA in vivo is toxic to mice, and the partial loss of tumor rejection by IL-12 in the presence of 1-NMMA may have been attributable to a side effect of the chemical. In our in vitro cytotoxicity assay, the inclusion of iNOS inhibitor 1-NMMA did not affect killing of tumor targets by various macrophages.

In any case, complete tumor rejection in the absence of the nitric oxide pathway demonstrates the presence of another efficient tumor killing pathway(s) by activated macrophages. Indeed, our in vitro experiments have revealed a macrophage killing mechanism that is contact dependent and does not require the macrophage viability. Because significant contact-dependent cytotoxicity is preserved after paraformaldehyde fixation, it is likely that the activated macrophages from Cy + IL-12-treated Sa1 ascites kill various tumor cells through a simple membrane receptor/ligand interaction. This is consistent with the macrophage effector mechanism in IL-12-induced, T cell-dependent antitumor response by hosts bearing immunogenic tumors such as Sa1 ascites. Future studies are needed to:

1. Determine the contribution of nNOS to the antitumor response by hosts bearing immunogenic tumors such as Sa1 ascites.
2. Compare the contribution of iNOS and nNOS to the antitumor response by IL-12-activated antitumor CD8 cells.
3. Determine the contribution of iNOS and nNOS to the antitumor response by other cytokines.
4. Determine the contribution of iNOS and nNOS to the antitumor response by other cytokines.

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