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Immune Response Against Large Tumors Eradicated by Treatment with Cyclophosphamide and IL-12

Kangla Tsung, Jennifer B. Meko, Ying L. Tsung, Gary R. Peplinski, and Jeffrey A. Norton

Previous studies have demonstrated eradication of small (4–8 mm) established murine MCA207 sarcomas by treatment with systemic IL-12. Analysis of the mechanism has revealed a cellular and molecular immune response at the tumor typical of a Th1 cell-mediated, macrophage-effected, delayed-type hypersensitivity (DTH) response. In the current study we investigate the immune response against long term established, large MCA207 tumors induced by combined treatment with IL-12 and cyclophosphamide (Cy), an agent known to potentiate the DTH response. Our results demonstrate that s.c. large MCA207 tumors (15–20 mm) that are refractory to treatment by either IL-12 or Cy alone can be completely eradicated by the combination of Cy and IL-12. IL-12 is apparently the only cytokine capable of mediating tumor eradication, and the effect is dependent on IFN-γ. The contribution of Cy is probably due to immunopotentiation of DTH rather than to direct cytotoxicity to the tumor. The regression of these large tumors takes >4 wk and, in many cases, is self-sustained, in that little or no additional IL-12 is needed beyond the initial week of administration. Analysis of the cellular and molecular events at the tumor site suggests that the mechanism is a Th1-mediated antitumor immune response. The Journal of Immunology, 1998, 160: 1369–1377.

Among all previously studied cytokines and biologic response modifiers, IL-12 has demonstrated the most potent antitumor effects in multiple experimental tumor models (1–5). Nevertheless, the current antitumor efficacy of IL-12 is far from satisfactory, in that complete tumor eradication is mainly limited to animals bearing small established tumors. It is important to understand the mechanism(s) by which IL-12 exerts antitumor effects, so that the knowledge obtained can guide further studies in which more effective immunotherapy can be developed. The mechanism of the antitumor effect of IL-12 is difficult to pinpoint because of the multiple biologic effects assigned to this cytokine. For example, besides the essential role of IL-12 in the development of Th1 cells (6), IL-12 is known to activate IFN-γ production by NK (7) and T cells (8), and CTL development in vitro (9). It is also found to possess antiangiogenesis activity, apparently through the induction of IFN-γ-inducible protein 10 (IP-10) (10). Until recently, IL-12 was thought to contribute to antimicrobial responses by activating the production of IFN-γ by B or NK cells (11). However, by using IFN-γ-deficient hosts, it was found that IL-12 contributed directly to early antimicrobial responses independent of IFN-γ (12, 13). Each of these properties of IL-12 may contribute to the antitumor activity. Recently, using a complete tumor regression model, we have analyzed the mechanism by which IL-12 induces the regression of established murine MCA207 s.c. tumors <10 mm in size (5). Our results indicate that IL-12 induces a Th1 cell-directed, macrophage-effected, delayed-type hypersensitivity (DTH) response at the site of the regressing tumors. The IL-12-mediated response is greatest when initiated after the establishment of a host immune response to the incipient tumor. Classic CTL are unlikely to be involved in tumor regression, as tumor-immune animals fail to demonstrate detectable CTL activity in vitro. Such a mechanism for IL-12-induced tumor regression is consistent with the well-recognized role of IL-12 as an inducer of Th1 responses in antimicrobial studies (6), but is contrary to the widely held view that CTL-mediated direct lysis of tumor cells in vivo is essential for tumor eradication.

Realizing that a DTH response is effective against established MCA207 tumors, we went further in this study to enhance the IL-12-mediated antitumor response with the use of a well-known DTH-potentiating agent, cyclophosphamide (Cy) (14). We show that long term (>3 wk) established, large (15–20 mm), s.c. MCA207 tumors that are refractory to treatment with either IL-12 or Cy alone regress completely when a brief treatment with IL-12 is given within 5 days after a single dose of Cy. The regression of the large MCA207 tumors appears to be a self-sustained process that takes >4 wk to complete. This tumor regression model has provided a rare opportunity to study a complete antitumor immune response against a syngeneic, weakly immunogenic tumor. It also further demonstrates the great potential of cancer immunotherapy based on the biologic activities of IL-12.

Materials and Methods

Tumor model

Murine MCA207 sarcoma, a methylcholanthrene-induced transplantable tumor in C57BL/6 mice, was obtained from the Surgery Branch, National Cancer Institute (Dr. J. Yang, Frederick, MD). The tumor was maintained free of Mycoplasma contamination. Tumor cells were cultured from in vivo-harvested tumor implants in RPMI 1640 medium supplemented with 10% heat-inactivated, 2 mM glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 5 × 10^3 M 2-ME. For tumor implantation, 5 × 10^3 tumor cells were injected in a 0.2-ml volume in saline s.c. on the side of 10- to 16-wk-old female C57BL/6 mice or female C57BL/6 IFN-γ gene knock-out mice (The Jackson Laboratory, Bar Harbor, ME). Tumor development and growth were followed by palpation and measurement of perpendicular tumor diameters. Cure is defined as complete tumor regression following treatment and the absence of recurrent tumor for the entire follow-up period (4–6 mo).

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2 Abbreviations used in this paper: IP-10, IFN-γ-inducible protein 10; DTH, delayed-type hypersensitivity; Cy, cyclophosphamide; 5-FU, 5-fluorouracil; m, murine; h, human; iNOS, inducible nitric oxide synthase.

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In vivo treatment of tumors

MCA207 tumors were grown for 10 days (small tumor) to reach tumor sizes of 4 to 8 mm or for 3 to 4 wk to reach tumor sizes of 15 to 20 mm (large tumor) before the start of various treatments, as defined in the text and figure legends. Cy (Sigma Chemical Co., St. Louis, MO) and 5-fluorouracil (5-FU; U.S. Biochemical Corp., Cleveland, OH) were given at a single dose of 100 to 125 mg/kg (2.5 mg/mouse) in 0.5 ml of saline via i.p. injection. Cytokines were given by i.p. injection in 0.5 ml of saline according to the following doses and schedule: mIL-12 (Genetics Institute, Cambridge, MA), 500 ng, once every other day for three to five total injections; hIL-2 (Cetus), 50,000 IU/day for 10 days; mIL-4 (Biologic Response Modifier Program, National Cancer Institute), 1 μg/day for 10 days; and mIL-10 (Biologic Response Modifier Program), 500 ng/day for 10 days.

In vitro cytotoxicity assay

After lysis of RBC, spleen cells (5 × 10^6/ml) were cultured in vitro in RPMI medium with irradiated (20,000 rad) MCA207 tumor cells (1 × 10^6/ml) in 24-well plates for a total of 7 days. IL-2 was added to the culture at a final concentration of 40 IU/ml 2 days after the start of restimulation. At the end of restimulation, cells were collected and washed extensively to remove dead cells.3Cr-labeled MCA207 tumor cells were incubated with the restimulated spleen cells at the E:T ratios indicated in a 5-h chromium release assay. The percent specific lysis was calculated from triplicate samples as follows: ((experimental counts per minute−spontaneous counts per minute)/ maximal counts per minute− spontaneous counts per minute) × 100.

Immunohistochemistry

Tumors were resected and snap-frozen immediately. Cryostat sections, 6 μm thick, were fixed in cold acetone, dried at −20°C, and stored at −70°C before use. Before staining with Ab, the frozen sections were dried at room temperature. After rehydration in PBS, the sections were blocked with 1% normal goat serum and 1% BSA in PBS for 20 min. The sections were stained with primary Abs diluted in blocking solution for 30 min at room temperature or overnight at 4°C. The primary Abs and the working dilutions used in this study are as follows: partially diluted rabbit anti-mouse macrophage iNOS polyclonal serum (1/2000; Calbiochem, San Diego, CA); partially purified rabbit anti-mouse IP-10 (1/4000; Dr. Farber, National Institute of Allergy and Infectious Diseases, Bethesda, MD); biotinylated rat anti-mouse CD11b/Mac-1 (5 μg/ml; clone M1/70, PharMingen, San Diego, CA); and purified monoclonal rat anti-mouse CD4 (15 μg/ml; clone H129.19), CD8 (15 μg/ml; clone 53-6.7), IFN-γ (2.5–5 μg/ml; clone R4-6A2), IL-4 (10 μg/ml; clone 11B11), and isotype-matched control rat IgG (10–40 μg/ml; PharMingen). Biotinylated goat anti-rabbit IgG (1/1000; Vector Laboratories, Burlingame, CA) and goat-anti-rat IgG (5 μg/ml; PharMingen) were used as secondary Abs for detection of unlabeled primary Abs followed by the alkaline phosphatase-conjugated ABC system (Vector Laboratories). Color was developed using the Vector Red substrate (Vector Laboratories). The sections were then counterstained with hematoxylin, cleared with xylene, and mounted permanently. Isotype-matched control Abs/sera were also used to ensure that the positive staining obtained with each Ab was Ag specific.

Results

Cy enhances IL-12-induced tumor regression

Previous studies by us (5) and others (2) have indicated that IL-12 treatment can induce complete regression of small (<10 mm) MCA207 tumors that are established for a short period of time (7–10 days). When tested on larger tumors (>10 mm) grown for >12 days, treatment with IL-12 alone causes inhibition of tumor growth, but is unable to induce complete tumor regression. Since we have previously identified a Th1 cell-directed DTH immune response associated with the regression of small MCA207 tumors treated with IL-12 alone, we asked whether Cy, a well-known DTH-potentiating agent (14), can enhance the antitumor efficacy of IL-12 in a large MCA207 tumor model. In several experiments, summarized in Table I, we tested the antitumor effects of Cy and IL-12 in combination on MCA207 tumors that had been established for 24 to 34 days and were 12 to 28 mm in diameter. The responses of tumors to various treatments listed in Table I are categorized into four types as defined in Table I. As Table I shows, all untreated tumors grew progressively. Treating large tumors with Cy alone caused a transient tumor regression that lasted for 2 wk, and all tumors resumed growth subsequently. On the other hand, treatment of large tumors with IL-12 alone only minimally inhibited tumor growth for a short period (3–7 days). The few tumors that regressed with IL-12 treatment were smaller ones with sizes near 12 mm at the beginning of the treatment. Among mice treated with the combination of IL-12 and Cy, when IL-12 was administered 1, 4, or 5 days, but not 7 or 10 days, after a single dose of Cy, the majority of tumors were completely eradicated. The few tumors that did not regress completely were the largest ones with sizes >22 mm in diameter at the initiation of treatment. In contrast, the curative effect of Cy and IL-12 in combination was lost if IL-12 was given before, instead of after, the single dose of Cy (Table I, IL-12, 10 days, Cy). Following the initial three to five doses of IL-12 given over 5 to 7 days, a single dose of IL-12 was again administered to some tumor-bearing animals 7 to 14 days after the previous IL-12 injection. This “maintenance” injection was especially necessary for complete eradication of larger (>18 mm) tumors. Regression of these tumors slowed down and stopped if the maintenance IL-12 injection was withheld (not shown). In most instances, complete tumor regression took approximately 1 mo or longer to complete. The patterns of tumor responses to some of the treatments summarized in Table I are illustrated in Figure 1. Untreated tumors grew rapidly to reach sizes of >500 mm^3. Treatment of similar sized tumors (>15 mm in diameter) with IL-12 alone barely caused an inhibition of tumor progression during the first week of IL-12 administration, and progressive tumor growth followed. Cy alone, as summarized in Table I, induced transient tumor regression that lasted for 2 wk, and then all tumors resumed growth. Finally, the combination treatment of a single dose of Cy followed by IL-12 induced continued tumor regression until complete eradication occurred 1 mo later. Mice that completely eradicated tumor were resistant to subsequent rechallenge with MCA207 tumor cells, indicating the presence of an immunologic memory.

The role of direct cytotoxicity of Cy in the combination treatment was addressed by replacing Cy with another cytotoxic drug, 5-FU, with in vivo direct tumor cytotoxicity similar to Cy (15). As

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Prog</th>
<th>Inhib</th>
<th>Trans. reg.</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
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<td>2/15</td>
<td>13/15</td>
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<td>3/15</td>
<td>8/15</td>
<td>2/15</td>
<td>2/15</td>
</tr>
<tr>
<td>Cy-1 day-IL-12</td>
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<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Cy-4 day-IL-12</td>
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<td>0/17</td>
<td>2/17</td>
<td>15/17</td>
</tr>
<tr>
<td>Cy-5 day-IL-12</td>
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<td>0/5</td>
<td>0/5</td>
<td>5/5</td>
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<tr>
<td>Cy-7 day-IL-12</td>
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<td>0/5</td>
<td>0/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Cy-10 day-IL-12</td>
<td>0/5</td>
<td>1/5</td>
<td>4/5</td>
<td>0/5</td>
</tr>
<tr>
<td>IL-12-10 day-Cy</td>
<td>0/5</td>
<td>5/3</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td>5FU-4 day-IL-12</td>
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<td>0/5</td>
<td>0/5</td>
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<tr>
<td>Cy + IL-2</td>
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<td>0/5</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Cy + IL-4</td>
<td>0/5</td>
<td>1/5</td>
<td>4/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Cy + IL-10</td>
<td>0/5</td>
<td>0/5</td>
<td>5/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

*5C7BL/6 mice were inoculated with 5 × 10^3 MCA207 cells s.c. Treatments were started at 24 to 34 days after tumor inoculation when tumors reached average diameters of >15 mm. Results are combined from four experiments.

*Cy and 5-FU were given i.p. at a single dose of 125 to 150 mg/kg. Recombinant mIL-12 was given i.p. at a dose of 500 ng/mouse three to five times once every other day. Recombinant hIL-2, mIL-4, and mIL-10 were given i.p. daily at a dose of 50,000 IU. 5FU, 500 mg, and 500 ng, respectively, for a total of 10 days.

*Responses were categorized in four groups: prog., progressive growth of tumor, no response to treatment; inhib., tumor growth was inhibited without obvious regression; trans. reg., transient regression of tumor followed by resumed growth; and cure, complete regression of tumor, and animals remained tumor free (>60 days).
The antitumor activity of Cy alone in the MCA207 tumor model

Since Cy alone was able to induce transient regression of large MCA207 tumors (Table I and Fig. 1), and it has been reported to induce complete tumor regression in other studies (16, 17), we tested the antitumor activity of Cy in the 3- and 10-day established small MCA207 tumor models. Similar to a previous report by others in a different tumor model (16), treatment of 3-day established nonpalpable MCA207 tumors resulted in only a delay in tumor appearance, while treating palpable tumors (4–8 mm) 10 days after tumor implantation resulted in initial regression of all tumors and complete eradication of tumor in 12 of 17 mice (Table II). Since mice with greater (10-day established) tumor burdens responded better to Cy treatment than those with lesser (3-day nonpalpable) burdens, it suggests that direct cytotoxicity of Cy was not the main effector mechanism for tumor regression as was also suggested in previous studies (16, 17). Finally, similar to observations in another tumor model treated with Cy (16), MCA207 tumors initially treated with Cy 3 days after tumor inoculation did not respond to a subsequent second treatment when the previously treated tumors became palpable (Table II).

IFN-γ dependence of Cy- and IL-12-mediated tumor regression

Previous studies have shown that regression of MCA207 tumors induced by IL-12 requires the presence of IFN-γ (2). We confirmed this finding with the use of IFN-γ gene knockout mice in the 10-day MCA207 tumor regression model. In this experiment three of five normal mice bearing 6- to 10-mm tumors treated with IL-12 alone were rendered tumor free, while none of nine tumors (6–12 mm) in the IFN-γ gene knockout mice had a complete response (Table III). Although previous studies (18) and our preliminary results (data not shown) have demonstrated that daily injection of high doses of IL-12 (500–1000 ng/day) caused significant treatment mortality within 1 wk in IFN-γ gene knockout mice, the lack of response to IL-12 treatment in IFN-γ gene knockout mice in this experiment was not due to treatment-associated toxicity, because no toxicity was observed when 500 ng/injection of IL-12 was administered i.p. every other day for three doses. In contrast, Cy treatment of IFN-γ knockout mice bearing 10-day established MCA207 tumors resulted in the same pattern of regression as that seen in normal mice and tumor eradication, albeit at a lower rate (20%) than that in normal mice (70%; Table III), was achieved in some animals. Further, IFN-γ gene knockout mice that were rendered tumor free by Cy treatment were resistant to subsequent challenge with $1 \times 10^6$ MCA207 tumor cells 1 mo later, indicating the presence of T cell-mediated immune memory independent of IFN-γ. We next studied the combination of Cy and IL-12 in the

table II. Antitumor effect of Cy given at different times after tumor inoculation

<table>
<thead>
<tr>
<th>Treatment Schedulea</th>
<th>Prog.</th>
<th>Trans. reg.</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Late</td>
<td>0/17</td>
<td>5/17</td>
<td>12/17</td>
</tr>
<tr>
<td>Early-latea</td>
<td>4/5</td>
<td>1/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

a C57BL/6 mice were inoculated with $5 \times 10^6$ MCA207 tumor cells. Cy was given i.p. at a single dose of 125 mg/kg. Results are combined from two experiments.

b Cy was given either at 3 days (early) after tumor inoculation or at 10 days (late).

c Prog., progressive growth; no antitumor effect seen; trans. reg., tumor regressed in response to treatment but regrew later; cure, complete regression and animals remained tumor free (>60 days).

d Palpation of tumors was delayed at least 1 wk.

e Complete regression and animals remained tumor free (>60 days).

FIGURE 1. Responses of large MCA207 tumors to treatments with Cy, IL-12, and Cy plus IL-12. Subcutaneous MCA207 tumors established in vivo for 28 days were treated with saline (open square), Cy alone (open circle), IL-12 alone (open triangle), or Cy followed 5 days later by IL-12 (solid circle). Individual growth curves of three tumors from each of the treatment groups that share similar tumor sizes at the start of the treatments are shown. The tumor growth curves depicted here are derived from one of the experiments summarized in Table I.

FIGURE 2. The expression of Mac-1, iNOS, and IP-10 in regressing small MCA207 tumors treated with Cy. Ten-day established MCA207 tumors were treated with a single dose of Cy (on day 0), and 2, 4, 6, and 9 days after the treatment, tumors were harvested and analyzed by immunohistochemical staining with Abs to Mac-1, iNOS, and IP-10. Positive cells are red. Magnifications: day 2, Mac-1, ×100; all others, ×40.
Table III. **Comparison of IL-12 and Cy-mediated antitumor effects in normal and IFN-γ knockout (KO) mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>In normal mice</th>
<th>In IFN-γ KO mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prog.</td>
<td>Inhblt.</td>
</tr>
<tr>
<td>None</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Cy alone</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td>IL-12 alone</td>
<td>0/5</td>
<td>1/5</td>
</tr>
</tbody>
</table>
| Cy + IL-12
            | 0/5   | 0/5     | 0/5         | 5/5  | 0/5   | 2/5     | 3/5         | 0/5  |

*a C57BL/6 normal and IFN-γ KO mice were inoculated with $5 \times 10^5$ MCA207 cells on day 0. Treatments were started between day 8 and day 11 when the tumors were 4 to 8 mm in diameter. Results are combined from two experiments.

*b Cy was given i.p. at a single dose of 125 mg/kg. Recombinant mIL-12 was given i.p. at a dose of 500 ng/mouse for a total of three doses once every other day. When combined with Cy, IL-12 was started at the 4th day after Cy administration.

*c Categories of responses are same as defined in Table I.

*d Tumors receiving this treatment were 11 to 17 mm in diameter.

**IL-12-MEDIATED TUMOR REGRESSION**

IFN-γ gene knockout mice with large MCA207 tumors. As indicated in Table III, the initial transient regression of MCA207 tumors did take place after Cy treatment, but the tumor resumed growth after 12 days despite the combined treatment with IL-12, suggesting that tumor eradication by the Cy and IL-12 combination treatment is dependent on IFN-γ.

The effector mechanisms of Cy-, IL-12-, and Cy- plus IL-12-induced tumor regression

We (5) and others (19) have reported extensive iNOS expression by activated macrophages at the site of tumors treated with IL-12 alone. Other recent studies have suggested the presence of another putative effector molecule, IP-10, associated with tumor regression induced by IL-12 (20). In this study we examined the expression of both iNOS and IP-10 by macrophages in MCA207 tumors treated with Cy alone, IL-12 alone, and Cy plus IL-12. Compared with the peripheral presence of macrophages in the untreated tumors (Fig. 2, day 0, Mac-1), in the small regressing MCA207 tumors treated with Cy alone, massive macrophage infiltration, known to be the hallmark of Cy treatment of tumors (21, 22), appeared as early as 2 days after Cy treatment (Fig. 2, day 2, Mac-1) and remained in the regressing tumor throughout the entire period (9 days) of tumor regression. Despite this massive macrophage infiltrate, no expression of iNOS was detected throughout the entire period of tumor regression (Fig. 2, iNOS, days 2–9), and mild, but clear, expression of IP-10 by infiltrating macrophages was seen at all stages of tumor regression (Fig. 2, IP-10, days 2–9). To determine whether IP-10 is also involved in IL-12-mediated small tumor regression, IP-10 staining was repeated on samples collected from the previous study in which extensive iNOS expression by macrophages in the small regressing MCA207 tumors treated with IL-12 was observed (5). Despite the presence of massive numbers of activated macrophages and clear expression of iNOS (5), only sporadic expression of IP-10 by macrophages was seen (not shown), suggesting that IP-10 is unlikely to be essential for the regression of small MCA207 tumors treated with IL-12 alone. Thus, evidence collected to date indicates that nitric oxide is unlikely to be an effector molecule in Cy-mediated tumor regression, and IP-10 may be involved in the regression of tumors treated with Cy but not IL-12.

The expression of both iNOS and IP-10 was also examined in the large regressing tumors collected before and at various time points after the start of treatment with Cy plus IL-12. Except in a very few cells, iNOS expression was not detectable in the untreated large MCA207 tumors (Fig. 3A) and in the large tumors treated with Cy for 4 days before initiation of IL-12 (Fig. 3B) or in tumors treated with Cy alone for 8 days (Fig. 3C). The
iNOS expression was not obvious 2 days after the start of IL-12 treatment (Fig. 3D), but became consistently detectable in the tumors at all stages of tumor regression 4 days after the start of IL-12 treatment (Fig. 3, E-G, I, and K). Note that the tumors shown in Figure 3, C and E, were both treated with Cy for 8 days, except that the tumor shown in Figure 3E had received additional IL-12 treatment 4 days after Cy, indicating that IL-12 administration was responsible for the observed iNOS expression. Similar to the previous observation, iNOS was mainly expressed by macrophages (Mac-1; compare Fig. 3, J and J). Interestingly, in the large MCA207 tumor model, the expression of iNOS seemed to correlate with the use of IL-12 rather than with the regression of the tumors, as a nonregressing tumor treated with IL-12 alone 15 days after the start of treatment also showed intense local expression of iNOS (Fig. 3H). Even 25 days after the start of IL-12 treatment, some expression of iNOS, albeit limited in locality, was still visible in progressively growing tumors treated with IL-12 alone (Fig. 3L). In contrast to the lack of iNOS expression in tumors treated with Cy alone (Fig. 3, B and C), extensive IP-10 expression by infiltrating lymphocytes was seen associated with the heavily necrotic area of large tumors treated with Cy and 8 days previously (Fig. 4, B and C). The IP-10-expressing lymphocytes found in the heavily necrotic area of the Cy-treated tumors were probably macrophages, as they stained positive for the Mac-1 marker (match Fig. 4, K and L). Interestingly, this tumor necrosis with disrupted tumor cell nuclei and a heavy cellular infiltration of macrophages expressing IP-10, but not iNOS, was only seen in Cy-treated large tumors. In the same size tumors treated with Cy plus IL-12, there was an increase in iNOS (Fig. 3, E vs C) and a down-regulation of IP-10 (Fig. 4, E vs C) expression. This down-regulation of IP-10 expression was obvious as early as 2 days after the start of IL-12 treatment at which time no iNOS expression could be detected (Figs. 3D and 4D). In contrast to the association of iNOS expression with IL-12 treatment of tumors (Fig. 3H), no clear IP-10 expression was seen in tumors treated with IL-12 alone (Fig. 4H). This is consistent with findings from small regressing tumors treated with Cy or IL-12 (Fig. 2).

In the small MCA207 tumor regression model, animals cured of tumors developed T cell memory, yet the involvement of a CTL response could not be demonstrated in vitro (5). In the current study we asked whether CTL response or memory was detectable in animals treated with Cy or Cy plus IL-12. As observed before, spleen cells from animals in which small MCA207 tumors were eradicated by treatment with IL-12 alone failed to demonstrate significant lysis (<8%) toward MCA207 tumor cells in vitro (Fig. 5). Surprisingly, splenocytes from Cy-cured IFN-γ-deficient animals that had resisted the subsequent challenge with MCA207 tumors also failed to demonstrate CTL activity (Fig. 5). The lack of demonstrable CTL activity in these two cases was not due to a failure of experimental procedures, as splenocytes from animals rendered tumor free by Cy plus IL-12 treatment assayed in the same experiment did show appreciable levels of CTL activity at a E:T ratio of 40:1 (27%; Fig. 5). Thus, it appears that a measurable CTL response was activated during the regression of large MCA207 tumors treated by Cy plus IL-12, but not in small regressing MCA207 tumors treated with either Cy or IL-12 alone. Further, despite the fact that the IFN-γ gene knockout mice were able to reject a subsequent tumor rechallenge following Cy-induced complete eradication of small tumors, we did not detect any CTL activity in these mice (Fig. 5). Therefore, it remains to be determined how an immunologic memory against MCA207 tumor independent of both CTL and IFN-γ is established and functional in the IFN-γ gene knockout mice treated with Cy alone.
IFN-\( \gamma \) cells within the tumor expressed either cytokine (Fig. 6, day 0, Cy-treated tumors that expressed IFN-\( \gamma \), days 6/2, CD4 and CD8). As in the tumors treated only with a single CD8 T cell appeared within the tumor (Fig. 6, Cy plus IL-12, days 6/2, CD4 and CD8). The expression of both IFN-\( \gamma \) and IL-4 by non-T cells decreased significantly 4 days later; thus, by 12 days after Cy treatment or 8 days after the start of IL-12 treatment, T cell-mediated cytokine expression with an imbalance toward more production of IFN-\( \gamma \) became obvious in the tumors (not shown). A clear dominance of IFN-\( \gamma \) and lack of IL-4 production by T cells, the hallmark of a Th1 response, took place during the third week following the start of treatment (Fig. 6, Cy plus IL-12, days 19/15, IFN-\( \gamma \) and IL-4). Also at this time, peak numbers of CD4 and CD8 T cells were seen in the regressing tumors (Fig. 6, Cy plus IL-12, days 19/15, CD4 and CD8; note that a low magnification of \( \times 40 \) was used). This time period coincided with the regrowth of tumors treated with Cy alone following a transient regression and a continued rapid regression of tumors treated with Cy plus IL-12 (Fig. 1). On the other hand, tumors treated with IL-12 alone without Cy failed to show any regression over the pretreatment sizes (Fig. 1), and these tumors contained T cell infiltrates limited in location and number (Fig. 6, IL-12, day 15, CD4 and CD8; note that a higher magnification of \( \times 100 \) was used). These tumors also lacked intratumoral cytokine production (Fig. 6, IL-12, day 15, IFN-\( \gamma \) and IL-4). For the Cy- plus IL-12-treated tumors, the last week of tumor regression was associated with a gradual diminution of T cell infiltrates (Fig. 6, Cy plus IL-12, days 29/25, CD4 and CD8). However, not proportional to the reduction of T cells was the almost complete cessation of IFN-\( \gamma \) production and the appearance of IL-4 production by a few T cells (Fig. 6, Cy plus IL-12, day 29/25, IFN-\( \gamma \) and IL-4).

Thus, the entire process of tumor regression appears to have undergone three distinct phases of intratumoral cytokine production. In the early phase, both IFN-\( \gamma \) and IL-4 were produced, mainly by non-T cells. During the midphase and at the peak of tumor regression, a Th1 cytokine response dominated. In the final phase toward the completion of tumor eradication, IFN-\( \gamma \) production was turned off, and IL-4 production by some T cells occurred. Expression of other cytokines in the regressing tumors were also analyzed. Similar to the observation in the small MCA207 tumor model (5), we did not see any significant expression or increase in levels of expression of IL-2 and IL-10 at any time point analyzed (not shown).

**Discussion**

Complete eradication of several established tumors has been previously reported with IL-12 (2, 4, 5, 19, 23), but in many other instances, antitumor activity was minimal (1). The methylcholanthrene-induced MCA207 sarcoma used in this study is immunogenic by the classic concomitant immunity test (24), but the immunogenicity of the tumor is weak in that s.c. immunization of a naïve host with irradiated MCA207 tumor cells alone fails to protect against subsequent challenge with viable tumor cells. In addition, tumor-reactive T cells have been isolated from s.c. growing MCA207 tumors (24) and lymph nodes draining the tumor site (25), indicating that a host T cell response to the incipient tumor is primed. We have previously demonstrated that small (4-8 mm) MCA207 tumors (established in vivo for 7-10 days) are completely eradicated by the administration of either IL-12 protein or a recombinant vaccinia virus that expresses IL-12 (5). However,
IL-12 is ineffective against large MCA207 tumors (>10 mm). Of interest is the striking similarities between tumor regression models described in the past for some of the biologic response modifiers, such as endotoxin, and the MCA207 tumors treated by IL-12. For example, it has been shown that endotoxin-induced tumor regression is most effective when administered after the establishment of tumor during the period in which concomitant immunity is detectable (26). However, if tumors are allowed to continue to grow, the curative effect by endotoxin disappears, and the animals enter a state of apparent immunosuppression. In light of the recent finding that several bacterial products, including endotoxin, are potent inducers of IL-12 production by macrophage (27), these past studies add support to the concept that IL-12-mediated tumor regression requires a pre-existing host T cell response to the incipient tumors. They also suggest that the curative effect of IL-12 is limited to a narrow window beyond which large tumors become refractory to IL-12 treatment. Since most human cancers more closely resemble the long term established, large tumor animal models, the complete eradication of large tumors by treatment with the combination of Cy and IL-12, but not with either agent alone, as described in the current report represents a novel observation and an improvement in immunotherapy based on the biologic activity of IL-12. It also provides a rare opportunity to study an effective immune response against a large tumor. Because IL-12 has had significant antitumor effects in many experimental models, a better understanding of its mode of action is likely to contribute to better immunotherapy against a broad range of tumors.

The essential role of IL-12 in this large tumor regression model is supported by the fact that other cytokines (IL-2, IL-4, and IL-10) combined with Cy did not have similar antitumor effects (Table I). We chose IL-4 and IL-10 because these cytokines are instrumental in Th2- and CTL-mediated immune responses and IL-2 because Cy had been shown previously to augment the IL-2-mediated antitumor effect. With the same dose of IL-4 used in our experiment, others have shown a significant antitumor effect by systemic administration (28). Although enhanced antitumor effects have been previously reported with the combination of Cy and IL-2 (29, 30), the tumors were small (<8 mm). In the current study the combination of Cy and IL-2 was
in the necrotic area of the IL-12-treated tumor, it seems that the cytokines were not simultaneously produced by a single macrophage. As a result, the preferential production of IFN-γ, but not IL-4, in the necrotic area of the tumors may in some way favor the induction of a Th1, but not a Th2, response. In this respect it would be interesting in future experiments to see whether induction of a Th1 response by Cy plus IL-12 is interrupted in mice lacking the IFN-γ gene.

As in our previous study (5), the presence of a pre-existing host T cell response to the incipient tumors or, in other words, the presence of a tumor-specific memory seems to play an important role in an effective T cell response activated by Cy plus IL-12. Since it has been shown that Cy treatment can eliminate primary, but not secondary, T cell responses (31, 32), and since direct cytotoxicity of Cy to T cells (33, 34) was confirmed by the total lack of T cells in Cy-treated tumors for at least 8 days (Fig. 6, Cy, day 8), it is likely that the tumor-infiltrating T cells found after treatment with Cy and IL-12 were derived from memory T cells that had survived the cytotoxicity of Cy (32). Such a pre-existing host response, albeit not able to reject the incipient tumor, is essential for endotoxin-induced tumor regression, and it has also been implicated as the major factor distinguishing a responder from a nonresponder (26). In this study the known cytotoxicity of Cy toward the majority of T cells makes priming of tumor-specific T cells from naive precursors following Cy treatment unlikely. Instead, the rapid recovery of T cells in tumors treated with both Cy and IL-12 (Fig. 6, Cy plus IL-12, days 6/2–8/4), but not in tumors treated with Cy alone (Fig. 6, Cy, day 8), further argues for the involvement of memory, but not naive, T cells as targets of IL-12 action in the MCA207 tumor regression model. Consistent with this view, one recent study indicates that IL-12 promotes the proliferation of Ag-stimulated CTL, but not unstimulated T cells (35).

If, indeed, the pre-existing T cell response is a major factor influencing the likelihood of a tumor’s response to IL-12-based treatment, the question then is how to detect this pre-existing immunity before treatment. In this regard, the appearance of cytokine-producing T cells associated with the tumor before the start of treatment may be an indicator of such a pre-existing host T cell response. Future experiments will focus on the comparison of pretreatment cellular and cytokine profiles of IL-12-responding and nonresponding tumors to determine whether a marker for the likelihood of a treatment response can be identified.

In conclusion, we have described a large tumor treatment model in which MCA207 tumors grown >3 wk to sizes as large as 20 mm in diameter can be completely eradicated by treatment with Cy and IL-12. This model offers an opportunity to study the immune response capable of eradicating large, weakly immunogenic tumors. In addition, the model extends previous findings concerning the antitumor activity of IL-12 by demonstrating that IL-12 activity can be dramatically enhanced by DTH-potentiating agents such as cyclophosphamide. Through better understanding of the IL-12-mediated antitumor immune response as seen in this model, it may be possible to identify factors that are either missing or inadequate in other less responsive or nonresponsive tumors. With this knowledge, it is hoped that improved antitumor immune responses may be extended to other animal tumors and, eventually, to patients with cancer.

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References