Pre-Existing Tumor-Sensitized T Cells Are Essential for Eradication of Established Tumors by IL-12 and Cyclophosphamide Plus IL-12

Hop N. Le, Natalie C. Lee, Kangla Tsung and Jeffrey A. Norton


http://www.jimmunol.org/content/167/12/6765

**References**

This article *cites 39 articles*, 31 of which you can access for free at: http://www.jimmunol.org/content/167/12/6765.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Pre-Existing Tumor-Sensitized T Cells Are Essential for Eradication of Established Tumors by IL-12 and Cyclophosphamide Plus IL-12

Hop N. Le, Natalie C. Lee, Kangla Tsung,2 and Jeffrey A. Norton

The antitumor immune response activated by IL-12, especially by a combination of cyclophosphamide and IL-12 (Cy+IL-12), is clinically significant in certain experimental tumor models, in that a number of well-established (10–20 mm in diameter) s.c. tumors are completely eradicated. Furthermore, Cy+IL-12 treatment is also able to eradicate well-established grossly detectable experimental lung metastases and advanced ascites tumors. Despite the dramatic antitumor effects seen in some tumor models, Cy+IL-12 fails to induce regression of other established tumors. Characterization of tumor immunogenicity shows that all tumors responding to IL-12 and Cy+IL-12 treatments are immunogenic tumors, in that an antitumor immune response is detectable in tumor-bearing hosts upon tumor establishment. In contrast, none of the nonimmunogenic tumor responds to IL-12 and Cy+IL-12 treatments. Analysis of cellular requirements for successful tumor rejection through an adoptive cell transfer approach reveals that the presence of tumor-sensitized, but not naive, T cells is essential for tumor rejection by IL-12 and Cy+IL-12. Transfer of these tumor-sensitized T cells must be conducted before, but not after, IL-12 treatment in order for tumor rejection to occur. The requirement of sensitized T cells is also tumor specific. In mice bearing immunogenic tumors, the presence of pre-existing tumor-sensitized T cells is demonstrated by adoptive cell transfer experiments using purified spleen T cells from these mice. Results from our study show that Cy+IL-12-based immunotherapy of cancer may be highly effective and that pre-existing tumor-sensitized T cells are essential for the success of the therapy. The Journal of Immunology, 2001, 167: 6765–6772.

Immunotherapy of cancer may eradicate local as well as disseminated tumors. Despite reported observations of spectacular tumor regression in sporadic cancer patients following nonspecific immune activation during responses against infection (1), the conditions under which consistent eradication of established tumors in animal models and cancer patients have yet to be identified. Previously, most studies of antitumor immune responses have been performed in minimal or nonestablished experimental tumor models in which the tumor burden and information obtained may not reflect the clinical situation in which tumors are well established and metastatic. In fact, it is not currently clear whether or not the host immune system can eradicate established large tumors. Thus, it is important to identify and study established tumor models in which the immunologic mechanism of rejection of long-term established large tumors can be elucidated. In this paper, we further characterize our previously reported dramatic antitumor immune response activated by treatment with IL-12, especially a combination of the immunopotentiating agent cyclophosphamide (Cy)3 and IL-12 (Cy+IL-12), against clinically significant large tumor burdens (2). Furthermore, we identify the conditions that are essential for tumor eradication.

Due to the ability of IL-12 to target, among other cells of the immune system, NK, NKT, and T cells, the antitumor activities of IL-12 have been demonstrated in both established and nonestablished tumor models. IL-12-induced antitumor activity in nonestablished tumor models is mediated mainly by NK and NKT cells, and conventional T cells are not required (3–5). Because both immunogenic (FBL-3, leukemia) and nonimmunogenic (Lewis lung carcinoma (LLC) and B16 melanoma) tumors were found to respond to IL-12 treatment equally in these nonestablished tumor models, tumor immunogenicity does not seem to play a significant role in the first type of IL-12-induced antitumor response. In contrast, unlike NK and NKT cells, in which the antitumor effect of IL-12 affects the development of newly inoculated tumor cells, the second type of IL-12-mediated antitumor effect is found in established s.c. tumor models (2, 6–10). In this second type of IL-12-induced tumor rejection, T cells (6, 11) and IFN-γ (2, 6, 12) are essential for tumor rejection. Clearly different from the NKT-mediated antitumor response, rejection of established large nonimmunogenic tumors has not been reported.

In our previous report, we have shown that a single dose (100–125 mg/kg) of Cy followed by three injections of IL-12 results in complete eradication of long-term (3–4 wk) established, large (15–20 mm in diameter) s.c. tumors that are resistant to treatment with either Cy or IL-12 alone (2). Although the antitumor effect of Cy+IL-12 is much stronger than that of IL-12 alone, it is still based on a mechanism similar to that of IL-12, and the role of Cy is likely to be a potentiating agent through an as yet unknown mechanism (2). Because most previous antitumor studies using the MCA207 tumor model use experimental lung metastases to measure the antitumor efficacy (13–16), it remains a possibility that the CY+IL-12-mediated tumor regression is limited to certain anatomic sites but is not effective against pulmonary metastases. In
addition, even if superior antitumor efficacy by Cy+IL-12 can be demonstrated in various anatomic sites of the MCA207 tumor model, it is critical to know whether similar effects of Cy+IL-12 can be obtained in other tumor models. In the first part of this study, we tested the antitumor activity of Cy+IL-12 in a number of tumor models and under different tumor locations. The results indicate that Cy+IL-12 is highly effective in eradicating established tumors in all anatomic sites in immunogenic tumor models. In contrast, Cy+IL-12 fails to induce regression in established palpable nonimmunogenic tumors. In the second part of the paper, we describe experiments leading to the identification of tumor regression conditions that explain this differential response to IL-12-based therapy by immunogenic and nonimmunogenic tumors. Findings from this study are critical to our understanding of the dramatic antitumor effects by Cy+IL-12 and bear clinical relevance in identifying potential responders to IL-12-based immunotherapy.

Materials and Methods

Murine tumors and animals

Murine tumors and animals

In vivo treatment models

In s.c. tumor models, 5 × 10^3 tumor cells in 0.2 ml of saline were injected s.c. on the flank of syngeneic or semisynthetic F1 mice. Tumor size was assessed with calipers. Recombinant murine IL-12 (Genetics Institute, Cambridge, MA) was administered i.p. at a dose of 200 ng 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-Aldrich, St. Louis, MO) in 0.5 ml of saline followed 3–4 days later by a course of IL-12 as described above. In some cases, additional single-dose IL-12 injection was given weekly following the initial IL-12 treatment during tumor regression.

In the experimental lung metastases model, 5 × 10^3 MCA207 tumor cells in 0.5 ml of saline were injected i.v. via tail vein. Cy+IL-12 treatment, same as described for the s.c. model above, was initiated on the days described in the text. Survival of animals was followed by daily inspection. Visualization of pretreatment tumor burden in the lung was conducted by injection of india ink into the lungs of euthanized animals followed by bleaching as described before (13).

In the peritoneal sarcomatosis model, 5 × 10^3 MCA207 tumor cells in 0.2 ml of saline were injected i.p. Cy+IL-12 treatment, as described for the s.c. model above, was initiated on the day described in the text. The survival of the animals was followed by daily inspection. Pretreatment tumor burden was determined by inspection and photography of the peritoneal cavity of euthanized animals.

Concomitant and prophylactic immunity test

Concomitant immunity tests were conducted as described previously (18, 19). Briefly, s.c. tumors were first established in naive mice in one flank with 5 × 10^3 tumor cells. At the indicated time points after first tumor inoculation, another inoculation of 5 × 10^3 tumor cells was given to naive or tumor-bearing mice in the contralateral flank. The development of tumors from the second inoculation in both naive and tumor-bearing mice was assessed. Protective immunity was tested by immunizing naive mice with 1 × 10^6 irradiated (5000 rad) tumor cells s.c. once a week for two times followed by challenge with 5 × 10^3 live tumor cells on the opposite flank 1 wk after the second immunization. Naive mice were used as control at the time of live tumor challenge.

Adoptive cell transfer model

TCRβ gene knockout mice were used as recipients of adoptively transferred spleen and T cells from various donor sources. TCRβ knockout mice were first inoculated with 5 × 10^3 MCA207 tumor cells s.c. Fourteen days after tumor establishment when most tumors were 7–12 mm in diameter, tumor-bearing TCRβ mice received indicated numbers of spleen or purified T cells from indicated donor mice via i.v. tail vein injection. Two to 7 days after adoptive cell transfer, recipients were treated with IL-12 (200 ng i.p. three times). The donor T cells were isolated from single cell suspension of spleen cells by anti-Thy1.2-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA). Five to ten million (5–10 × 10^6) purified T cells (>93% CD3 positive) were used for each adoptive transfer. Spleen cells were prepared by removing RBCs from single cell suspension by osmolysis followed with extensive washing with saline. Tumor-immune donor mice were generated by immunization of naive mice twice with 0.5–1 × 10^6 irradiated (5000 rad) tumor cells s.c. The immunized mice were challenged with 5 × 10^3 live tumor cells 1 wk after the second immunization.

Results

The effect of Cy+IL-12 treatment under various tumor establishment conditions

We have previously demonstrated that large (established 3–4 wk) s.c. MCA207 tumors can be completely eradicated by a single low dose of Cy (125 mg/kg) followed by three injections of recombinant murine IL-12 (Cy+IL-12) (2). In subsequent experiments reported in this work we found that Cy+IL-12 treatment was also highly effective against MCA207 tumors established by the i.p. and i.v. routes. The antitumor effects in these treatment models are truly clinically significant and surpass other previously developed immunotherapies in this tumor model, in that premortem tumor-bearing mice can be cured by Cy+IL-12 treatment. Fig. 1 shows the pretreatment tumor burden in mice following s.c., i.p., and i.v. tumor inoculation. In the case of experimental lung metastases induced by i.v. tumor inoculation, there has been no previously demonstrated therapy that is curative in this tumor model when administered 14 days after tumor inoculation when lung metastases are grossly visible, as shown in Fig. 1C. It is under these advanced tumor-bearing conditions that we are able to demonstrate the curative effects of Cy+IL-12 treatment (Table I).

First, as we have reported in our previous study (2), a single dose of Cy plus a short course of IL-12 can cure long-term established large (17–25 mm) MCA207 s.c. tumors such as the ones established for 32 days shown in Fig. 1A. Treatment with either Cy or IL-12 alone inhibited tumor growth only temporarily and no mice were cured (data not shown). Second, mice bearing i.p. sarcomas died of tumor burden between 20 and 27 days, but treatment with Cy+IL-12 started on day 18 at a time of massive i.p. tumor burden (Fig. 1B) cured all treated mice. Even very late treatment started on day 20 cured three of five mice. Finally, in the experimental lung metastases model, untreated mice died of tumor burden between 21 and 31 days, corresponding to the varying pulmonary tumor burden on day 14, as seen in Fig. 1C. Nevertheless, all mice treated with Cy+IL-12 on days 7 and 14 were cured.

The effect of Cy+IL-12 treatment in other tumor models

Cy+IL-12 treatment was tested in a panel of other murine tumors, each of which, except the Sa1 tumor, is derived in the C57BL/6
background like the MCA207 tumor. Although Cy+IL-12 was effective in eradicating established s.c. tumors of Sa1, MCA105, MCA203, and MCA205, it was ineffective against s.c. tumors of B16 melanoma, LLC, and Pan02 pancreatic carcinoma (Table II). For most of these other tumors that respond to Cy+IL-12, the size of the tumor that completely responds to therapy is smaller than that seen in the MCA207 tumor model. These results show that the curative response to Cy+IL-12 treatment is not limited to MCA207 tumor model alone, but varies among different tumor models.

Several factors may account for the failure of the nonresponders to be eradicated by IL-12/Cy+IL-12 treatment. Because we have previously seen strong immune infiltration at the site of regressing MCA207 tumors responding to IL-12 (10) and Cy+IL-12 (2), we compared the immune cell infiltration in a responder tumor, MCA207, and a nonresponder tumor, LLC, before and after Cy+IL-12 treatment. As Fig. 2 shows, there were already a few CD4 and CD8 T cells present in day-21 MCA207 tumor of 13–17 mm before treatment with Cy+IL-12. The infiltration by T cells and Mac-1-positive macrophages increased dramatically following Cy+IL-12 treatment. In contrast, s.c. day-14 LLC tumors of 10–14 mm had almost no sign of T cells and macrophages before treatment with Cy+IL-12. However, following Cy+IL-12 treatment there were a few CD4-positive cells and no CD8 T cells and macrophages in the tumor. This vivid contrast of immune infiltration between a responding and a nonresponding tumor has been observed in several other responding and nonresponder tumor models.

**Responders to IL-12/Cy+IL-12 are immunogenic tumors**

Why do some tumors respond to Cy+IL-12 so well and others not at all? One suggestion from the list of responders and nonresponders to Cy+IL-12 (Table II) is that all responders are tumors

---

**Table I. Antitumor effects of Cy+IL-12 in MCA207 models established by s.c., i.p., and i.v. routes**

<table>
<thead>
<tr>
<th>Route of Implant</th>
<th>Treatment*</th>
<th>Cure Rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Start day)</td>
<td>(Survival time in days)</td>
</tr>
<tr>
<td>s.c.</td>
<td>None</td>
<td>0/5</td>
</tr>
<tr>
<td>s.c.</td>
<td>Cy+IL-12 (28–35)</td>
<td>10/10</td>
</tr>
<tr>
<td>i.p.</td>
<td>None</td>
<td>0/5 (20–27)</td>
</tr>
<tr>
<td>i.p.</td>
<td>Cy+IL-12 (14)</td>
<td>5/5</td>
</tr>
<tr>
<td>i.p.</td>
<td>Cy+IL-12 (18)</td>
<td>5/5</td>
</tr>
<tr>
<td>i.p.</td>
<td>Cy+IL-12 (20)</td>
<td>3/5 (23,24)</td>
</tr>
<tr>
<td>i.v.</td>
<td>None</td>
<td>0/8 (21–31)</td>
</tr>
<tr>
<td>i.v.</td>
<td>Cy+IL-12 (7)</td>
<td>5/5</td>
</tr>
<tr>
<td>i.v.</td>
<td>Cy+IL-12 (14)</td>
<td>8/8</td>
</tr>
</tbody>
</table>

*a MCA207 tumor cells (5 × 10⁶) were inoculated s.c., i.p., or i.v. into naive C57BL/6 mice.
*b Cy+IL-12 treatment was initiated on the day indicated in parentheses following tumor inoculation.
*c Cure rate is defined as the number of mice that are tumor-free with survival of >100 days over the number of mice in each group. The survival time in days of each animal that died is given in parentheses.

---

**Table II. Response of other established marine transplantable tumors to Cy+IL-12 treatment**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tumor Size (mm)*</th>
<th>Cure Rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responders</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sa1</td>
<td>17–22 (21)</td>
<td>9/10</td>
</tr>
<tr>
<td>MCA105</td>
<td>13–17 (14)</td>
<td>5/5</td>
</tr>
<tr>
<td>MCA203</td>
<td>10–14 (14)</td>
<td>7/10</td>
</tr>
<tr>
<td>MCA205</td>
<td>9–15 (14)</td>
<td>8/10</td>
</tr>
<tr>
<td>Non-responders</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B16</td>
<td>4–13 (7–14)</td>
<td>0/10</td>
</tr>
<tr>
<td>Lewis lung</td>
<td>5–14 (7–14)</td>
<td>0/10</td>
</tr>
<tr>
<td>Pan02</td>
<td>6–10 (14)</td>
<td>0/10</td>
</tr>
</tbody>
</table>

*a Cultured tumor cells (5 × 10⁶) of the indicated tumor line were inoculated s.c. in naive mice.
*b Tumor size is given as the diameter of the tumor. The number of days between tumor establishment and treatment is given in parentheses.
*c Cure is defined as the complete regression of the s.c. tumor, and the animal remains tumor-free for at least 90 days thereafter. Cure rate is given as the number of cored mice over the total number of mice tested.

---

**FIGURE 1.** Examples of pretreatment tumor burdens in mice following s.c., i.p., and i.v. inoculation of MCA207 tumor cells. A, s.c. MCA207 tumors at day 32 after tumor inoculation. Tumor diameter as shown is 22 mm in diameter. B, Presence of massive i.p. sarcomatosis 18 days after i.p. tumor inoculation. Clusters of tumor nodules are white in color. C, Presence of grossly visible tumor nodules (white in color) in lungs of mice 14 days after i.v. tumor inoculation.

**FIGURE 2.** Intratumoral cellular immune infiltration before and after Cy+IL-12 treatment in a responder (MCA207) and a nonresponder (LLC). Frozen tumor sections from untreated day-21 MCA207, day-14 LLC and Cy+IL-12-treated MCA207 and LLC (14 days after the start of treatment) were stained with Ab to CD4, CD8, and Mac-1 by immunohistochemistry. Photomicrograph magnification: MCA207 before Cy+IL-12, 100×; MCA207 after Cy+IL-12, 40×; LLC before Cy+IL-12, ×100; LLC after Cy+IL-12, ×100. Positive cells are red in color.
that have been characterized as immunogenic, whereas the nonresponders are tumors that are usually described as poorly immunogenic or nonimmunogenic. An immunogenic tumor is able to induce an antitumor immune response upon establishment in normal, but not T cell-deficient, hosts. This T cell-mediated antitumor immunity is usually not strong enough to eliminate the primary tumor but is detectable by the rejection of a secondary challenge with the homologous tumor cells in a classic concomitant immunity test (18, 20). Using this criterion, we could see that all of the responders to Cy+IL-12 treatment are indeed immunogenic tumors. As Table III shows, at 7–8 days following tumor establishment, mice bearing tumors that respond to Cy+IL-12 treatment (MCA203, MCA205, MCA207, and Sa1) resisted a rechallenge with the same tumor cells, whereas the same tumor inoculation resulted in 100% tumor take in control naive mice. The resistance of rechallenge in three of the four tumor models (all except for the Sa1 model) was found to be dependent on T cells, as it was not observed in αβ T cell-deficient mice. T cell-dependent concomitant immunity in the Sa1 tumor model has already been described in previous studies (19). In contrast, none of the three tested nonresponders (LLC, B16, and Pan02) showed resistance to rechallenge in a T cell-dependent manner following primary tumor establishment. There was an inhibition of second tumor development by a primary Lewis lung tumor. However, this inhibition was also seen in T cell-deficient mice, indicating that the inhibition is not due to a T cell-mediated mechanism, but may be caused by angiogenesis factor secreted by the tumor itself (21). To test the immunogenicity of various tumors without the interference by a primary tumor, we conducted another test of passive immunization and rechallenge. In this prophylactic immunity test, Lewis lung, together with B16 and Pan02, behaved like nonimmunogenic tumors, in that repeated immunization of naive mice with irradiated tumor cells failed to protect the immunized mice from subsequent challenge with live tumor cells. In contrast, immunization of naive mice with the three immunogenic tumors MCA207, MCA205, and Sa1, showed 100, 80, and 100% protection, respectively, against a subsequent live tumor challenge.

**Tumor-sensitized, but not naive, T cells are essential for tumor rejection induced by IL-12/Cy+IL-12**

Why are only immunogenic tumors able to respond to Cy+IL-12? The major difference between an immunogenic and a nonimmunogenic tumor is that the former induces a T cell-mediated antitumor response upon tumor establishment whereas the latter does not. T cells from mice bearing immunogenic tumors are thus tumor-sensitized. To study the role of T cells, especially tumor-sensitized T cells, in tumor rejection induced by IL-12 and Cy+IL-12, we tested tumor rejection by Cy+IL-12 in the MCA207 tumor model in various T cell-deficient mice. Using athymic nude mice that are defective in thymus-derived T cells, but not extra-thymus-derived NKT cells (22), we found that tumor growth was only transiently inhibited by Cy+IL-12 treatment, and the curative effect of Cy+IL-12 against large s.c. MCA207 tumors was abolished (Table IV). This is clearly in contrast to the NKT-mediated antitumor response that is equally functional in both normal and nude mice (3), and suggests that thymus-derived T cells are essential for tumor rejection in this model. Further analysis using specific TCR gene knockout mice showed that the curative effects of Cy+IL-12 found in normal mice was completely abolished in TCRβ knockout mice lacking the classic αβ T and NKT cells, but not in TCRβ knockout mice lacking γδ T cells (Table IV). As expected, rejection of established MCA207 tumors was also abolished in TCRβ double knockout mice lacking all T cells. Among subsets of T cells, either CD4 or CD8 T cells alone were found to be able to mediate complete tumor rejection (Table IV). This observation is consistent with previous studies by others who also reported that depletion of either CD4 or CD8 T cells using Abs did not abolish the antitumor response by IL-12–12 in the MCA207 small tumor model, but depletion of both T cell subsets eliminated the antitumor effects (6). Finally, complete tumor rejection was obtained in mice lacking β2-microglobulin (Table IV), which affects the development of MHC I-dependent CD8 T cells and CD1-dependent NKT cells (23–25). These results indicate that rejection of established large tumors by Cy+IL-12 requires conventional T cells of either the CD4 or CD8 subset.

However, this requirement alone does not explain why only immunogenic tumors respond to Cy+IL-12, because conventional T cells are present in mice bearing nonimmunogenic tumors. So we wanted to know whether tumor rejection induced by Cy+IL-12 requires tumor-sensitized T cells. For this, we developed an adoptive cell transfer model in which we could selectively transfer naive or tumor-sensitized T cells to MCA207 tumor-bearing TCRβ knockout mice. For creating a tumor-bearing host containing naive, but not tumor-sensitized, T cells, we transferred naive spleen cells or Thy1.2+ T cells purified from naive spleen into TCRβ knockout mice bearing established s.c. MCA207 tumors. In parallel, we transferred spleen cells or purified spleen Thy1.2+ T cells from tumor-immune donors to tumor-bearing TCRβ knockout mice to create the situation of a tumor-bearing host containing tumorsensitized T cells. Because splenocytes show very low NKT-mediated antitumor effects due to the lack of this cell population (4) and the Thy1.2+ T cells we have purified are NK1.1−

**Table III. Concomitant immunity in mice bearing Cy+IL-12-responsive and nonresponsive tumors**

<table>
<thead>
<tr>
<th>Tumora</th>
<th>Second Tumor Takeb</th>
<th>Normal mouse</th>
<th>TCRβ KO mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCA203</td>
<td>0/5</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>MCA205</td>
<td>1/5</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>MCA207</td>
<td>0/5</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>Sa1</td>
<td>0/5</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Nonresponder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lewis lung</td>
<td>5/5</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>B16</td>
<td>5/5</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Pan02</td>
<td>5/5</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

a Tumor cells (5 × 104) were inoculated s.c. in the indicated hosts. Seven to 8 days after the tumors became palpable, a second inoculation of the same tumor cells was given on the opposite flank to both tumor-bearing and naive mice.

b Tumor take in tumor-bearing, but not naive, normal and TCRβ knockout (KO) mice is shown. All naive control mice that received the second inoculation alone developed tumors. ND, Not done.

**Table IV. Tumor rejection by Cy+IL-12 is dependent on αβ T cells**

<table>
<thead>
<tr>
<th>Hosta</th>
<th>Cure Rateb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10/10</td>
</tr>
<tr>
<td>Nude</td>
<td>0/3</td>
</tr>
<tr>
<td>TCRβ KO</td>
<td>0/20</td>
</tr>
<tr>
<td>TCRβ KO</td>
<td>4/4</td>
</tr>
<tr>
<td>TCRδ6 KO</td>
<td>0/10</td>
</tr>
<tr>
<td>CD4 KO</td>
<td>9/10</td>
</tr>
<tr>
<td>CD8 KO</td>
<td>7/10</td>
</tr>
<tr>
<td>β2m KO</td>
<td>5/5</td>
</tr>
</tbody>
</table>

a MCA207 tumor cells (5 × 104) were inoculated into normal C57BL/6 and various indicated gene knockout (KO) mice of the B6 background. Tumors were treated with Cy+IL-12 when they reach 15–20 mm in diameter in 3–4 wk. β2m, β2 microglobulin.

b Results are from four experiments.
by staining (data not shown), the reconstituted recipients with purified T cells or spleen cells remained NKT deficient. Following adoptive cell transfer, the recipients were treated with either saline or IL-12 or Cy+IL-12, and the sizes of tumors were assessed. As Table V shows, T cell-deficient MCA207 tumor-bearing mice reconstituted with naive spleen cells or naive T cells did not respond to either IL-12 or Cy+IL-12 treatment, in that none of the tumors in these recipients were eradicated by IL-12 and Cy+IL-12 treatments. In contrast, reconstitution of tumor-bearing T cell-deficient mice with spleen cells or T cells from tumor-immune donors restored the response of tumor-bearing mice to both IL-12 and Cy+IL-12 treatments. It should be noted that complete tumor rejection was achieved in T cell-deficient recipients of tumor-sensitized T cells treated with IL-12 alone (Table V). Because Cy is not necessary for tumor rejection in the adoptive transfer model, we conducted future adoptive transfer experiments using IL-12 treatment alone instead of Cy+IL-12.

Not only are tumor-sensitized T cells required, but they are also required before, but not after, IL-12 treatment, as the experiment in Fig. 3 shows. Thus all MCA207 tumors progressed in T cell-deficient mice treated with IL-12 before receiving T cells from tumor-immune donors. In contrast, in recipient mice that received tumor-sensitized T cells before IL-12 treatment, all tumors were completely eradicated. Furthermore, tumor rejection by IL-12 not only requires tumor-sensitized T cell from tumor-immune donors, but the T cells must be tumor-specific. In an experiment we transferred T cells from MCA205 and MCA207 tumor-immune donors to either MCA205- or MCA207-bearing recipients. All four of four MCA207 tumors in mice receiving T cells from MCA207-immune donors and zero of four tumors in mice receiving T cells from MCA205-immune donors were eradicated by IL-12 following adoptive cell transfer. In the same experiment, three of five MCA205 tumors in mice receiving T cells from MCA205-immune donors and zero of five tumors in mice receiving T cells from MCA207-immune donors were eradicated by IL-12 following adoptive cell transfer. The combined results from these adoptive transfer experiments show that pre-existing tumor-sensitized Ag-specific T cells are essential for rejection of established large tumors by IL-12/Cy+IL-12 treatment.

Finally, if this requirement for tumor-sensitized T cells is true, then tumor-sensitized T cells from spleens of mice bearing tumors that respond to Cy+IL-12 should be demonstrable in an adoptive transfer experiment. This was confirmed in the experiment shown in Table VI, in which spleen T cells from normal mice bearing early (day-12) or late (day-28) MCA207 tumors were isolated and transferred into T cell-deficient mice bearing established MCA207 tumors. It is shown in Table VI that T cells from mice bearing early (day-12) MCA207 are able to prime T cell-deficient recipients for tumor rejection following IL-12 treatment. Furthermore, when spleen cells from mice bearing long-term established large tumors were tested as donor cells in the adoptive transfer experiment, complete tumor eradication in the recipients required Cy+IL-12 instead of IL-12 alone. This is similar to the situation in normal mice bearing long-term established large tumors in which treatment with Cy+IL-12 is better than IL-12 alone.

**Discussion**

IL-12 possesses the most significant antitumor activity among all cytokines and biological response modifiers tested. In general, there are two types of tumor models in which IL-12 induces different cellular responses. The NK/NKT-mediated antitumor response is mainly seen in nonestablished tumor models in which IL-12 treatment is started 1–3 days after tumor inoculation and the antitumor effects are measured as the degree of inhibition of tumor development but not tumor regression and cure (3). The response is not dependent on classic T cells, as it is fully preserved in nude mice (3). Correlated to this lack of T cell involvement, tumor immunogenicity does not seem to play a role in the model, as most studies were conducted in nonimmunogenic tumor models. The effector mechanism seems to be mediated by NK-like, nonspecific cytotoxicity dependent on perforin (26). Finally, although IFN-γ is produced by IL-12-activated NKT cells (5), it is not clear that it is

Table V. Tumor rejection by IL-12 and Cy+IL-12 requires tumor-sensitized T cells

<table>
<thead>
<tr>
<th>Donor Cell</th>
<th>Treatment</th>
<th>Cure Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>0/10</td>
</tr>
<tr>
<td>None</td>
<td>IL-12</td>
<td>0/10</td>
</tr>
<tr>
<td>None</td>
<td>Cy+IL-12</td>
<td>0/10</td>
</tr>
<tr>
<td>Naive T cell</td>
<td>None</td>
<td>0/5</td>
</tr>
<tr>
<td>Naive T cell</td>
<td>IL-12</td>
<td>0/5</td>
</tr>
<tr>
<td>Naive T cell</td>
<td>Cy+IL-12</td>
<td>0/5</td>
</tr>
<tr>
<td>Naive spleen</td>
<td>None</td>
<td>0/5</td>
</tr>
<tr>
<td>Naive spleen</td>
<td>IL-12</td>
<td>0/5</td>
</tr>
<tr>
<td>Naive spleen</td>
<td>Cy+IL-12</td>
<td>0/5</td>
</tr>
<tr>
<td>Immune T cell</td>
<td>None</td>
<td>1/10</td>
</tr>
<tr>
<td>Immune T cell</td>
<td>IL-12</td>
<td>9/10</td>
</tr>
<tr>
<td>Immune T cell</td>
<td>Cy+IL-12</td>
<td>10/10</td>
</tr>
<tr>
<td>Immune spleen</td>
<td>None</td>
<td>1/10</td>
</tr>
<tr>
<td>Immune spleen</td>
<td>IL-12</td>
<td>9/10</td>
</tr>
<tr>
<td>Immune spleen</td>
<td>Cy+IL-12</td>
<td>10/10</td>
</tr>
</tbody>
</table>

a MCA207 tumor cells (5 x 10^5) were inoculated in TCRβ knockout mice on day 0. On day 14 when most tumors were 10-13 mm in diameter, donor cells (1/4 equivalent of spleen cells or 5-10 x 10^6 purified spleen T cells) were transferred.

b IL-12 treatment was initiated 2 days after T cell transfer and 7 days after spleen cell transfer. Cy+IL-12 treatment was initiated 7 days after T and spleen cell transfer.

c Results are from five experiments.

Table VI. Priming for tumor rejection by IL-12 and Cy+IL-12 with T cells and spleen cells from tumor-bearing mice

<table>
<thead>
<tr>
<th>Donor Cell</th>
<th>Treatment</th>
<th>Cure Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day-12 MCA207</td>
<td>None</td>
<td>0/5</td>
</tr>
<tr>
<td>Day-12 MCA207</td>
<td>IL-12</td>
<td>5/5</td>
</tr>
<tr>
<td>Day-28 MCA207</td>
<td>None</td>
<td>0/4</td>
</tr>
<tr>
<td>Day-28 MCA207</td>
<td>IL-12</td>
<td>0/4</td>
</tr>
<tr>
<td>Day-28 MCA207</td>
<td>Cy+IL-12</td>
<td>4/4</td>
</tr>
</tbody>
</table>

a Purified spleen T cells (10 x 10^6) from day-12 MCA207 tumor-bearing mice were transferred to TCRβ knockout mice bearing day-12 MCA207 tumors. In the case of day-28 tumor-bearing donors, one spleen equivalent of cells were transferred into TCRβ knockout mice bearing day-14 MCA207 tumors.

b IL-12 treatment in recipients of day-12 tumor-bearing spleen T cells was started 2 days after cell transfer. IL-12 and Cy+IL-12 treatments in recipients of day-28 tumor-bearing spleen cells were started on day 7 after spleen cell transfer.

FIGURE 3. Tumor-sensitized T cells are required before, but not after, IL-12 treatment. MCA207 tumor cells (5 x 10^5) were inoculated in TCRβ knockout mice. A. Tumor-bearing animals were first treated with IL-12 on days 10, 12, and 14 before receiving T cells from tumor-immune donors on day 15. B. Tumor-bearing animals received adoptive T cell transfer on day 14 followed by IL-12 treatment on days 16, 18, and 20. Individual tumor growth by tumor area (length x width) is shown.
essential for the antitumor effects. In contrast, the second type of IL-12-induced antitumor immune response, including the one described in the current study, is demonstrated in established palpable tumor models in which s.c. tumors are treated with IL-12 or Cy+IL-12 at 7–35 days after tumor inoculation when tumor sizes are 4–25 mm in diameter (2, 6–10). Unlike in the nonestablished tumor models, complete tumor rejection (cure) is often achieved in responders only when IL-12 treatment is initiated at least 7 days, but not 1–4 days, after tumor establishment (8, 10, 27, 28). The antitumor response in the established tumor model is dependent on T cells (Refs. 6 and 11 and Table IV) and IFN-γ (2, 6, 9). The effector mechanism in these models is not yet elucidated, but is unlikely to be dependent on perforin, because rapid and complete tumor rejection was found to take place in both normal and perforin gene knockout mice (our unpublished results).

As the current study shows, the antitumor activity of Cy+IL-12, when observed under the right conditions, is dramatic and clinically relevant (Fig. 1 and Table I). In terms of efficacy, there is no other previously described immunotherapy that is equal to the antitumor effects of Cy+IL-12 in any of the responder tumor models. For example, previous studies involving the use of MCA207 tumor model have been mainly limited to adoptive T cell transfer (13–15) and active immunization (16) with dendritic cells in experimental lung metastasis model. In these experiments, antitumor activity was detected only when the tumor burden was minimal at 3, but not 7 or 10, days after tumor inoculation. Successful immunotherapy treatments against established s.c. tumors are rare and are limited to small (days 3–5) tumors (16). Similarly, a number of previous studies using the Sa1 tumor model have demonstrated limited therapeutic effects against established small s.c. tumors (29). In contrast, treatment with Cy+IL-12 in both these tumor models leads to complete eradication of advanced tumor burdens established at various anatomic sites. The degree of antitumor efficacy shown in the current study even surpasses those observed in most previous reported studies with IL-12 alone (6, 27). This is due to the inclusion of Cy before IL-12 treatment as an immunopotentiating agent, as we have described in a recent study (2). Some of the tumors used in the current study, such as MCA203 and MCA205, responded only to Cy+IL-12, but not to IL-12 or Cy alone even when treated at a stage of small (3–6 mm) tumor (our unpublished observation). Although the mechanism by which Cy enhances IL-12-induced antitumor response is still under investigation, our previous study has shown that tumor rejection induced by both IL-12 alone and Cy+IL-12 is similar and is mediated by a Th1 response (2). The difference between the responses activated by IL-12 alone and Cy+IL-12 is likely at the level of response, in that Cy+IL-12 activates a stronger Th1 response than IL-12 alone does.

Despite the ability of Cy+IL-12 to activate a strong antitumor response in responding tumors, the same treatment barely affects other tumors, including the two nonimmunogenic tumors, LLC and B16 melanoma, that are used in most studies of IL-12-induced, NKT-mediated antitumor response in nonestablished tumor models (3, 4). What are the conditions that render some established tumors responsive to Cy+IL-12 and others not at all? One important finding from the current study is the demonstration that the presence of tumor-sensitized T cells at the time of IL-12 treatment is essential for tumor rejection in these models. This conclusion is supported by our adoptive transfer experiments in which we created two situations differing in the presence or absence of tumor-sensitized, but not total, T cells. If IL-12 is able to activate an antitumor response from naive T cells that is able to reject the established tumors, we would expect to see this in T cell-deficient mice receiving naive T cells. Our data show that this is not the case, in that tumors in recipients of naive T cells did not regress in response to subsequent IL-12 treatment (Table V). Furthermore, our study demonstrates that the T cells must be not only tumor-sensitized, but also tumor-specific. It should be noted that although eradication of 2-wk established MCA207 tumors of >10 mm in diameter in normal mice requires combined treatment of Cy+IL-12 (2), IL-12 alone is sufficient to induce complete eradication of 14-day established MCA207 tumors in the adoptive cell transfer model. Some of the tumors in the adoptive transfer experiments had progressed to >15 mm before exhibiting complete regression following IL-12 treatment. This is consistent with our previous hypothesis that the direct cytotoxicity of Cy to tumor cells is not critical for the dramatic antitumor effect of Cy+IL-12, because large tumors in the adoptive transfer experiments can be eradicated by IL-12 treatment alone. A possible explanation is that Cy is required in normal mice to counteract a down-regulation of antitumor immunity that cannot be overcome by IL-12 treatment alone. In the adoptive transfer model, tumor-sensitized T cells were from mice immunized with irradiated tumor cells. It is possible that the down-regulation of antitumor immunity found in normal long-term tumor-bearing mice does not develop in these mice. Furthermore, it does not seem to develop in the T cell-deficient recipients. Therefore, there is no need for Cy treatment following adoptive T cell transfer in the T cell-deficient recipients. Indeed, when spleen cells from long-term tumor-bearing mice were transferred into T cell-deficient recipients, tumor rejection required the addition of Cy, because IL-12 alone was found ineffective (Table VI). The presence of a down-regulation of antitumor immunity in long-term tumor-bearing hosts is indicated by the loss of concomitant immunity after 3 wk of tumor establishment. It seems that a suppression of antitumor immunity is cotransferred with spleen cells.

One important implication coming from the requirement of tumor-sensitized T cells for tumor rejection is that, without further immune intervention, only immunogenic tumors will likely meet this requirement. This is because only these tumors are able to induce a host T cell response to the incipient tumor upon tumor establishment (Table III) (19). It should be noted that although sensitive in vitro assays have been used to detect host response to tumors (30), we chose the in vivo assays of prophylactic and concomitant immunity for two reasons. First, these assays have been used to assign the immunogenicity of a tumor historically. Thus B16 melanoma has been referred to as poorly immunogenic or nonimmunogenic by all investigators not because it does not induce any kind of detectable host response to the tumor, but because it does not induce the kind of response that is able to reject a subsequent tumor challenge. Secondly, these assays are rather stringent in terms of measuring antitumor response, and they seem to provide more meaningful correlation between tumor immunogenicity and response to immunotherapy in our study. The antitumor immunity induced by immunogenic tumors rarely affects the progression of the incipient tumor but is detectable by the ability of the immunity to reject a second tumor challenge in a concomitant immunity test. The onset of this tumor-induced immunity takes >3 days after tumor inoculation, because second tumor challenge given 3 days after first tumor establishment is not rejected in the concomitant immunity test in both MCA207 and Sa1 tumor models (our unpublished results). Without further manipulation, this spontaneously generated antitumor immunity dissipates after a period of 3 wk. Thus second tumor challenge given to mice bearing 3-wk MCA207 tumors is no longer rejected (our unpublished result). North et al. (31) have attributed the dissipation of this spontaneous antitumor primary response to negative regulation by suppressor cells. In immunogenic tumor models, the effectiveness
of treatment with IL-12 seems to correlate to this window of primary response. For example, it has been repeatedly seen by several investigators that IL-12 treatment initiated too early (day 1–3) in tumor establishment before the establishment of the primary antitumor response is less effective than that initiated later (7–10 days), at the peak of the primary response (8, 10, 27, 28). This phenomenon may be explained by the requirement of the pre-existing tumor-sensitized T cells for tumor rejection as identified in the current study. Although treatment initiated early after tumor establishment faces a lighter tumor burden, the absence of an adequate number of tumor-sensitized T cells is such a limiting factor at this early time that it does not allow the activation of a tumor-specific T cell response by IL-12. In contrast, IL-12 treatment initiated after the onset of tumor-sensitization of T cells faces a larger tumor burden, but the presence of tumor-sensitized T cells make it possible for a strong T cell response, resulting in eradication of established tumors. When IL-12 treatment is further delayed until the dissipation of the primary response after 3 wk of tumor growth, the response to IL-12 alone by immunogenic tumors such as MCA207 is again lost (2). Different from the early tumor establishment situation, the presence of tumor-sensitized T cells in late tumor-bearing hosts is preserved, most likely in the form of resting memory T cells. Treatment of late tumor-bearing mice with Cy+IL-12, but not IL-12 alone, seems to reactivate these memory cells and result in a strong antitumor response.

Will the requirement of tumor-sensitized T cells explain why nonimmunogenic tumors do not respond to IL-12/Cy+IL-12? On the one hand, unlike immunogenic tumors such as MCA207, non-immunogenic tumors are unable to induce a strong antitumor host immune response upon establishment of the tumor. This is not necessarily the result of a lack of antigenicity, because introduction of well-defined viral surface Ag in the nonimmunogenic tumors of LLC (32) and B16 melanoma (33) did not make these tumors more immunogenic. It seems that mice bearing nonimmunogenic tumors tend to lack the levels and type of tumor-sensitized T cells required for a response to IL-12/Cy+IL-12. This can be seen from immunohistochemical analysis of nonresponder tumors (Fig. 2), which indicates that there is no sign of a strong T cell response at the site of tumors both before and after Cy+IL-12 treatment. On the other hand, it is not clear that this lack of pre-existing immunity is the sole reason for the lack of response to IL-12/Cy+IL-12 therapy by nonimmunogenic tumors. Gao et al. (34) recently showed that some immunogenic tumors with demonstrated presence of tumorsensitized T cells still do not respond to IL-12. They have attributed the failure to the lack of certain forms of tumor stroma (35). Alternatively, we have seen evidence in our preliminary studies to support the hypothesis that enhancing host recognition of nonimmunogenic would directly contribute to a better response of these tumors to Cy+IL-12. For example, by immunizing mice with tumor vaccine made with tumor-derived heat shock proteins (36), we were able to eradicate large (>10 mm) s.c. LLC tumors in the immunized, but not naive, mice (our unpublished results). In another study we found that the spontaneously derived BALB/c breast carcinoma 4T1 is nonimmunogenic and refractory to Cy+IL-12 therapy in normal BALB/c mice. However, in Stat6-deficient mice in which host recognition of 4T1 is enhanced (37), 4T1 tumor behaves like a typical immunogenic tumor and 14-day established 4T1 tumors were completely eradicated by Cy+IL-12 therapy (our unpublished results). Thus, the answer to the above question may not be a simple one, and may differ among different tumor models.

Why are tumor-sensitized T cells necessary for IL-12-induced tumor rejection? One likely explanation is that T cells are primary targets of IL-12 during tumor rejection. Because IL-12R is selectively expressed in activated, but not naive, T cells (38, 39), targeting of T cells by IL-12 during tumor rejection requires that these T cells be in activated state. Sensitization by tumor Ag before the start of IL-12 treatment would satisfy this requirement. Consistent with this view, we have observed in the current study that tumor-sensitized T cells must be present before, but not after, IL-12 treatment in order for tumor rejection to occur (Fig. 3). This view is further supported by a recent study by Iwasaki et al. (11) in other IL-12-responding tumor models in which they showed a correlation between spleen T cell response to IL-12 in vitro and tumor response to IL-12 in vivo. Finally, if indeed tumor-sensitized T cells are targets of IL-12 during tumor rejection, we would expect to see a loss of IL-12-induced tumor rejection by T cell-deficient mice receiving tumor-sensitized T cells from IL-12R knockout donor in an adoptive transfer experiment. Results from our preliminary study support this prediction (our unpublished results). However, the proof of this hypothesis calls for an adoptive transfer experiment in which tumor-sensitized T cells from normal mice transferred into T cell-deficient and IL-12R knockout mice are able to mediate tumor rejection following IL-12 treatment.

Despite the fact that IL-12 has been shown to possess significant antitumor activities in a large number of animal tumor models, clinical application of IL-12 in cancer patients has not met with great success. Results from our previous and current studies suggest that at least two factors are responsible. First, to eradicate clinically significant large tumor burden, T cell-mediated antitumor response is more effective than those mediated by NK and NKT cells. But the activation of such T cell-mediated antitumor responses by IL-12 requires a pre-existing immunity that is only present in a limited number of tumor-bearing hosts (patients). Second, clinical trials thus far were conducted with IL-12 alone. Even if IL-12 treatment is occasionally effective against established tumors in animal tumor models, its efficacy is still limited to small (<10 mm in diameter) tumors in most cases. Thus, even in potential responders, IL-12 alone may not be effective due to the late tumor-bearing states of these hosts (patients). Our repeated demonstration of better antitumor effects by Cy+IL-12 than by IL-12 alone provides one solution to the low efficacy of IL-12 alone in potential responders. However, the current study also shows that this potential curative treatment is limited only to hosts bearing immunogenic tumors. Thus better clinical response to IL-12/Cy+IL-12-based treatment will likely depend on accurate selection of potential responders and conversion of nonresponders. The latter may be achieved through various forms of immunization with tumor vaccines. Previous immunotherapy trials have indicated that either cytokine treatment or active immunization with tumor vaccine alone is not sufficient to induce complete rejection of long-term established large tumor burdens consistently. It would be interesting to see whether the enhancement of a pre-existing antitumor immunity through immunization followed by treatment with Cy+IL-12 will bring more success in the laboratory as well as in the clinic.

Acknowledgments

We thank Genetics Institute for providing the murine rIL-12 used in this study.

References

activated by IL-12 as a major effector in inhibition of experimental tumor me-
metastasis by adoptive transfer of IL-12-activated Vo14 NKT cells. Int. J. Can-
cer 91:523.
ri12 induces complete tumor regression and protective immunity: response is cor-
related with a striking reversal of suppressed IFN-γ production by anti-tumor
T cells. Int. Immunol. 7:1135.
tiveness of T cells but not of NK cells from tumor-bearing mice in IL-12-re-
derlying IFN-γ-mediated tumor growth inhibition induced during tumor immu-
otherapy with rIL-12. Int. Immunol. 8:855.
phage colony-stimulating factor and interferon γ secretion is associated with in 
vivo therapeutic efficacy of activated tumor-draining lymph node cells. Cancer
161:2187.
15. Berendt, M. J., R. J. North, and D. P. Kirstein. 1978. The immunological basis of 
edoxygen-induced tumor regression: requirement for a pre-existing state of con-