Adoptive Transfer of Anti-CD3–Activated CD4+ T Cells Plus Cyclophosphamide and Liposome-Encapsulated Interleukin-2 Cure Murine MC-38 and 3LL Tumors and Establish Tumor-Specific Immunity

By Mark L. Saxton, Dan L. Longo, Holly E. Wetzel, Henry Tribble, W. Gregory Alvord, Larry W. Kwak, Arnold S. Leonard, Claudio Danský Ullmann, Brendan D. Curti, and Augusto C. Ochoa

The infusion of anti-CD3–activated murine T cells plus interleukin-2 (IL-2) exerts antitumor effects against several tumors in murine immunotherapy models. This study compares the therapeutic efficacy of anti-CD3–activated CD4+ or CD8+ T-cell subsets, when given with cyclophosphamide (Cy) and liposome-encapsulated IL-2 (L-IL2) in a murine model. C57BL/6 mice bearing subcutaneous (SC) MC-38 colon adenocarcinoma, 3LL Lewis lung carcinoma, or 38C13 lymphoma for 7 to 14 days were pretreated with low-dose intraperitoneal (IP) Cy before intravenous (IV) injection of anti-CD3–activated T cells or T-cell subsets. Cell administration was followed by IP administration of L-IL2 for 5 days. Mice receiving activated CD4+ T cells showed significantly reduced tumor growth or complete remissions with prolonged disease-free survival in MC-38, 3LL, and 38C13. The timing of Cy doses in relation to adoptive transfer was critical in obtaining the optimal antitumor effect by CD4+ cells. Injecting Cy 4 days before the infusion of CD4+ T cells greatly enhanced the antitumor effect of the CD4+ cells and improved survival of the mice compared with other Cy regimens. C57BL/6 mice cured of MC-38 after treatment with CD4+ T cells developed tumor-type immunologic memory as demonstrated by their ability to reject rechallenges with MC-38, but not 3LL. Similarly, mice cured of 3LL tumors rejected rechallenges of 3LL, but not MC-38. The immunologic memory could be transferred with an IV injection of splenocytes from mice cured of MC-38 or 3LL. No cytotoxic T-lymphocyte activity was detected in T cells or T-cell subsets from mice cured of MC-38 or 3LL. Increased IL-2 and interferon-γ (IFN-γ) production was observed from CD4+ subsets in cured animals when stimulated in vitro with the original tumor, but not with an unrelated syngeneic tumor. These results suggest that tumor-specific immunity can be achieved in vivo with anti-CD3–stimulated CD4+ T cells in this cellular therapy model.

This is a US government work. There are no restrictions on its use.

MATERIALS AND METHODS

Mice. Inbred female C57BL/6 and C3H/HeNCr mice (6 to 10 weeks of age) were obtained from the Animal Production Facility, NCI-Frederick Cancer Research and Development Center, Frederick, MD.

Tumor preparation and tumor cell lines. MC-38 and 3LL were maintained by subcutaneous (SC) passage in C57BL/6 mice. 38C13 was maintained in C3H/HeNCr mice. MC-38 is a weakly immunogenic murine colon adenocarcinoma induced by the SC injection of dimethylhydrazine in C57BL/6 mice. The 3LL cell line (3LL-M2) used in this study was a generous gift of Dr Ronald Hornung (NCI-FCRDC, Frederick, MD) and has been described previously. The carcinogen-induced 38C13 B-cell lymphoma of C3H origin has been previously described. Tumor volumes were calculated with the following formula 1/2: Total Volume = (0.5) (larger diameter) × (smaller diameter).2

Activated lymphocytes. C57BL/6 murine splenocytes were harvested and activated with antinmurine CD3 monoclonal antibody (MoAb) (145-2C11, a generous gift from Dr Jeffrey Bluestone, University of Chicago, Chicago, IL) and rIL-2 (Hoffmann-LaRoche, Inc, Nutley, NJ) at a dose of 100 U/mL overnight before adoptive transfer, as previously described. Briefly, mouse spleens were crushed in culture dishes to obtain a single-cell suspension. The splenocytes were resuspended in cold RPMI, and red blood cells (RBCs) were lysed using ACK lysing buffer (Quality Biological, Inc, Gaithersburg, MD). The resulting suspension was filtered through sterile nytex mesh to remove cellular debris, washed twice with phosphate-buffered saline (PBS) (GIBCO-BRL, Grand Island, NY), and counted. T cells were enriched by passing splenocyte suspensions over nylon wool columns using RPMI 1640 plus fetal calf serum (FCS) 10% vol/vol, or T-cell negative selection chromatography columns (Collect Mouse T Cell Enrichment Immunocol...
All mice that received anti-CD3–activated cells were also treated with L-IL-2 (50,000 U IP) for 5 days after cells. Control groups for each experiment are shown in the accompanying figures or are described in Results. * Refers to number of days before cell dose. † Splenocytes were obtained from mice bearing 3LL or MC-38 tumors for 7 days.

Table 1. Experimental Conditions

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Tumor Line</th>
<th>Tumor Size Pretreatment Median (range) μL</th>
<th>Tumor Age Pretreatment (d)</th>
<th>Cy Dose (mg/kg) Day Given*</th>
<th>No. Anti-CD3–Activated Cells Given</th>
<th>No. of Treatment Courses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MC38</td>
<td>18 (6-32)</td>
<td>7</td>
<td>100/1</td>
<td>3.2 × 10^7</td>
<td>2 × 10^7 / 6.5 × 10^7</td>
</tr>
<tr>
<td>2</td>
<td>3LL</td>
<td>16 (13-32)</td>
<td>7</td>
<td>150/4</td>
<td>5 × 10^7</td>
<td>5 × 10^7 / —</td>
</tr>
<tr>
<td>3</td>
<td>3LL</td>
<td>23 (13-32)</td>
<td>16</td>
<td>150/4</td>
<td>5 × 10^7†</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>MC38</td>
<td>4 (4-14)</td>
<td>8</td>
<td>100/4</td>
<td>3.1 × 10^7†</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>MC38</td>
<td>48 (44-75)</td>
<td>9-11</td>
<td>100/2 or 4</td>
<td>5.5 × 10^7</td>
<td>—</td>
</tr>
</tbody>
</table>

All mice that received anti-CD3–activated cells were also treated with L-IL-2 (50,000 U IP) for 5 days after cells. Control groups for each experiment are shown in the accompanying figures or are described in Results. * Refers to number of days before cell dose. † Splenocytes were obtained from mice bearing 3LL or MC-38 tumors for 7 days.

...and gram-negative bacterial cell wall lipopolysaccharide...
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Fig 1. Normal C57BL/6 mice are inoculated with MC-38 SC in the right hindflank (Table 1, experiment 1). After 7 days, mice received T cells (6.5 \times 10^7 cells/injection) or T-cell subsets (3.2 \times 10^7 cells/injection) as indicated. Tumor volumes were measured over 31 days. Median tumor volumes are shown for each treatment group. There were 10 mice per group.

RESULTS

Therapeutic effect of T-cell subsets. Mice bearing subcutaneous MC-38 (Fig 1, Table 1, experiment 1) or 3LL (Fig 2, Table 1, experiment 2) tumors and treated with CD4\(^+\) T cells showed a marked antitumor effect. Repeated experiments in MC-38 confirmed that the infusion of anti-CD3 activated CD4\(^+\) T cells + L-IL-2 had a significantly higher therapeutic efficacy compared with CD8\(^+\) T cells (P = .0036), unselected T cells (P = .0077) or Cy + L-IL-2 (P = .055). In 3LL, CD4\(^+\) cellular therapy caused a significantly greater antitumor effect than CD8\(^+\) therapy (P = .0034), or controls using Cy (P = .0079), L-IL-2 (P = .0168) or Cy + L-IL-2 (P = .0112). When two courses of CD4 therapy were given, a cure rate of 60% to 90% was achieved in several experiments with MC-38 or 3LL tumors that had been established for as long as 16 days (Fig 3 and Table 1, experiment 3). No cures were seen with single courses of unseparated T cells or CD8\(^+\) T cells, or Cy + L-IL-2. However, multiple courses of treatment with Cy + L-IL-2 with or without unselected T cells cured between 30% to 60% of animals, com-
pared with 80% to 90% with CD4-based therapy. CD4+ T cells obtained from 7-day tumor-bearing donor mice also displayed this antitumor effect. Similar results were obtained in the 38C13 lymphoma model (data not shown).

Development of tumor-type specific immunologic memory. Cures were defined as those animals having established 7- to 14-day MC-38 or 3LL SC tumor deposits that were measurable before treatment, and were tumor-free after treatment for at least 80 days. Cured animals were rechallenged to determine the presence of immunologic memory. As shown in Table 2 all animals cured from MC-38 rejected subsequent challenges with MC38, but not an unrelated tumor (3LL or B-16 melanoma). Similarly, all animals cured of 3LL rejected subsequent challenges with 3LL, but not MC-38. All control mice were previously untreated and died of progressive tumor, indicating that the injected MC-38, 3LL, and B-16 cell lines were viable.

This immunologic memory could be transferred by the IV injection of a single cell suspension of splenocytes from cured mice into naïve (ie, nontumor-bearing and untreated) normal adult C57BL/6 mice. Three hours after the splenocyte transfer, tumor lines were injected SC as indicated in Table 3. None of the recipient mice developed tumor over a 30-day period when rechallenged with the same tumor from which the donor mice were cured, but not when challenged with a different tumor. All of the naïve control animals died of progressive tumor.

Treatment response and the timing of Cy doses. Pretreatment with low-dose Cy or radiation is routinely used in most adoptive immunotherapy models. Experiments to optimize the timing of Cy administration were done (Table 1, experiment 4). Figure 4A and B shows that the infusion of Cy 4 days before the adoptive transfer of CD4+ cells + L-IL-2 had a significantly better antitumor effect (P = .0006) and improved survival (P = .0003) compared with animals given Cy 2 days before CD4+ cell infusion. Also the administration of Cy 4 days before cellular therapy resulted in 80% tumor cures, not simply delays in tumor growth. The effect did not appear to be related only to debulking of the tumor, as a similar debulking effect was seen with Cy given 2 or 4 days before cellular therapy. Cy alone at 100 mg/kg usually does not cure any mice and rarely induces durable tumor remissions.

Functional characteristics of T cells from cured animals. T-cell subsets were obtained from naïve or cured animals, stimulated with irradiated MC-38 tumor cells, and cultured for 2 to 5 days as described above. Supernatants from these cultures were obtained after 2 and 5 days. CD4+ T cells from cured mice (n = 2) showed significantly higher peak IL-2, and IFN-γ production compared with CD8+ cells from cured animals, or CD4+ and CD8+ T cells from naïve animals (Fig 5). Similar cytokine production profiles were seen in three experiments.

The incorporation of [3H]-thymidine after stimulation with irradiated MC-38 was also greater in CD4+ subsets from

Table 2. Rechallenge and Cross-Challenge Experiments

<table>
<thead>
<tr>
<th>Test Group</th>
<th>No.</th>
<th>Rechallenge (no. of cells)</th>
<th>Tumor Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC-38 cured</td>
<td>8</td>
<td>MC-38 (1 x 10^6)</td>
<td>0/8</td>
</tr>
<tr>
<td>Naive</td>
<td>5</td>
<td>MC-38 (1 x 10^6)</td>
<td>5/5</td>
</tr>
<tr>
<td>3LL cured</td>
<td>4</td>
<td>3LL (2 x 10^6)</td>
<td>0/4</td>
</tr>
<tr>
<td>Naive</td>
<td>5</td>
<td>3LL (2 x 10^6)</td>
<td>5/5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test Group</th>
<th>No.</th>
<th>Cross-Challenge (no. of cells)</th>
<th>Tumor Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC-38 cured</td>
<td>8</td>
<td>3LL (2 x 10^6)</td>
<td>8/8</td>
</tr>
<tr>
<td>MC-38 cured</td>
<td>8</td>
<td>B-16 (1 x 10^5)</td>
<td>4/4</td>
</tr>
<tr>
<td>3LL cured</td>
<td>4</td>
<td>MC-38 (1 x 10^5)</td>
<td>4/4</td>
</tr>
</tbody>
</table>
cured animals (620 ± 58 cpm) compared with CD4+ subsets from untreated animals (194 ± and 482 ± 124 cpm, respectively). Although CD4+ cells from cured mice showed the ability to proliferate and produce cytokines after exposure to tumor, very low cytotoxicity was detected in total splenocytes or enriched CD4+ or CD8+ T-cell populations (Table 4). Even repeated experiments in a 4- or 18-hour lytic assay with IFN-γ (for class I upregulation) failed to demonstrate any significant cytotoxicity (data not shown). Supernatants were collected from CD4+ T cells isolated from cured animals after activation with anti-CD3 and IL-2 as described in Materials and Methods. MC-38 tumor cells were cultured with the CD4 supernatant to determine if cytokines secreted by the CD4 cells would inhibit tumor growth. The proliferation of MC-38 was not inhibited using pure CD4 supernatant or supernatant diluted 1:2 or 1:4 with RPMI (data not shown).

**DISCUSSION**

Previous studies using whole T-cell preparations were done in mice with MC-38 liver metastases. This anatomic location favored the trafficking of the infused cells and required killing mice to count metastases. For the present study, all tumors were implanted SC; therefore, effector cells would have to traffic to a site outside the liver, spleen, or lymph nodes to be effective. Also, this approach allowed easy assessment of antitumor effects and survival after treatment.

The present report demonstrates that anti-CD3–activated CD4+ T cells induce a strong antitumor effect in vivo when injected with L-IL-2. This marked therapeutic effect, confirmed in three different tumor models, not only produces a reduction in tumor size, but results in a significant number of cured animals. The mice that achieved a total regression of the tumor also developed a tumor-specific immunologic response. Although we did not select antigen-specific CD4+ T cells, the results are similar to those previously reported showing that antigen-specific CD4+ T cells can cure tumors in mice and facilitate the response of CD8+ T cells. In humans, there is also increasing evidence of the presence of antigen-specific CD4+ tumor-infiltrating lymphocytes (TIL) in a variety of tumors including melanomas and breast cancer.

Possible mechanisms for the antitumor effect by CD4+ T cells include the production of high concentrations of cytokines at the site of the tumor and the induction of tumor-specific effector cells. Preliminary data from immunohistochemical studies show a significant infiltration by CD4+ T cells in mice with regressing tumor (Danksy Ullmann et al, manuscript in preparation). However, the precise effector mechanisms are as yet unknown, as no significant in vitro cytotoxic activity against the tumors was detected even in cured mice who had successfully rejected a tumor rechallenge. The development of long-term immunologic memory, as well as the ability to transfer immunity by the infusion of splenocytes, suggests the presence of antigen-specific T cells. It is unlikely that the adoptively transferred polyclonally activated CD4+ T cells inducing the antitumor activity are the same ones that develop the long-term memory. It is possible that primed host cells cannot develop effective antitumor activity because of the weakly immunogenic nature of the tumors, or from T-cell alterations induced by the malignant cells. Therefore, the presence of high concentrations of cytokines produced by the activated CD4+ T cells could stimulate these partially activated host cells to become fully responsive and develop immunological memory.

Another interesting observation from this study is the development of tumor-specific memory in the cured animals. The immunity was readily transferred via splenocytes from cured animals. The transfer of the tumor-specific immunity did not require any other manipulation of the recipient animals and specifically did not require Cy pretreatment. Hosts receiving adoptively transferred cells such as lymphokine-activated killer (LAK) or TIL rarely develop specific memory, except when the T cells are cloned antigen-specific cells. The cells that mediate the immunologic memory or transferred immunity in this model are unlikely to be the same anti-CD3–activated CD4+ cells that originated from naive donor mice. However, CD4+ T cells in the cured animals are clearly responsive to stimulation with the tumor cells in vitro. It is possible that the cytokines produced by the transferred CD4+ cells expand host CD4+ or CD8+ T cells that have been primed to tumor antigens. Although we were unable to identify cells with cytotoxic activity in vitro, this does not rule out the participation of cytokotic T lymphocytes or other cytotoxic immune cells in the in vivo antitumor effect. Another possible explanation is that cytokines secreted by transferred CD4+ T cells induce the infiltration of macrophages or other antigen presenting cells, which prime cytotoxic T cells to tumor antigens. Ongoing in vivo depletion experiments and immunohistochemical studies should elucidate the mechanisms involved in the antitumor response.

Activated CD4+ cells did not induce a significant antitumor response unless Cy pretreatment was given. The use of Cy as an adjuvant in adoptive cellular immunotherapy models has been described for over a decade.

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**Table 3. Splenocyte Transfer Experiments**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Test Group</th>
<th>Source of Splenocytes</th>
<th>Splenocyte Dose</th>
<th>Rechallenge Tumor Type</th>
<th>Dose of Tumor Inoculum</th>
<th>Outcome/Length of Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naive</td>
<td>3</td>
<td>MC-38</td>
<td>MC-38</td>
<td>1 x 10^6</td>
<td>3/3 tumor free at 30 d</td>
</tr>
<tr>
<td></td>
<td>Naive</td>
<td>3</td>
<td>None</td>
<td>None</td>
<td>1 x 10^6</td>
<td>3/3 dead of tumor at 30 d</td>
</tr>
<tr>
<td>2</td>
<td>Naive</td>
<td>3</td>
<td>3LL</td>
<td>None</td>
<td>5 x 10^6</td>
<td>3/3 tumor free at 30 d</td>
</tr>
<tr>
<td></td>
<td>Naive</td>
<td>3</td>
<td>None</td>
<td>None</td>
<td>3LL</td>
<td>3/3 dead of tumor at 30 d</td>
</tr>
</tbody>
</table>

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Fig 4. Normal C57BL/6 mice were inoculated with MC-38 SC in the right hind flank (Table 1, experiment 4). After 7 days, mice received Cy. Cellular therapy + L-IL-2 was started either 2 or 4 days after Cy. (A) Median tumor volumes for each treatment group. (B) Graph of disease-free survival for each treatment group.

showed that immunized Thy-1⁺ splenocytes induce permanent regression of some murine tumors when tumor bearers were pretreated with Cy. The proposed mechanism was that Cy eliminated suppressor lymphocytes. Studies from our laboratory have not shown suppressor cells in mice with MC-38 tumors. Instead, the T cells from mice bearing MC-38 and other tumors had alterations in signal transduction characterized by decreases in intracellular p56LK and the zeta-chain subunit of the T-cell receptor. Abnormalities in nuclear transcription factors were also seen, which correlated with a marked decrease in in vivo antitumor activity. These changes appear to be more significant in T cells infiltrating tumor sites. The dose of Cy used here has a modest antitumor effect, but also produces a marked leukocyte nadir.
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Fig 5. IL-2, and IFN-γ production indexed per million cells in culture. Cells were obtained from the spleens of normal and cured animals. CD4⁺ or CD8⁺ T-cell subsets were cultured with irradiated MC-38 for 2 or 5 days. Supernatants from these cultures were used to measure cytokine levels. Similar cytokine production profiles were seen in the CD4⁺ cells of 3 of 3 cured animals studied.

on day 4 after infusion, a finding similar to that reported by Katsanis et al. It is possible that Cy eliminates T cells with signaling defects, facilitating the adoptive transfer of more reactive cytokine-secreting lymphocytes. Preliminary experiments support this mechanism. Partial tumor debulking by Cy could be important by exposing immune cells to antigens from dying tumor cells. The debulking effect seems to be only a part of the Cy’s action, because Cy given 2 or 4 days before cellular therapy debulks by the same amount, yet the therapeutic outcome is markedly different.

Table 4. Cytotoxicity of T Cells or T-Cell Subsets Using ⁵¹Chromium Against MC-38 Targets

<table>
<thead>
<tr>
<th>Experiment No. 1</th>
<th>Experiment No. 2</th>
<th>Experiment No. 3</th>
<th>Experiment No. 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>E:T ratio</td>
<td>% Specific Lysis</td>
<td>E:T Ratio</td>
<td>% Specific Lysis</td>
</tr>
<tr>
<td>25:1</td>
<td>6.6</td>
<td>42:1</td>
<td>11.2</td>
</tr>
<tr>
<td>12.5:1</td>
<td>5.8</td>
<td>21:1</td>
<td>10.9</td>
</tr>
<tr>
<td>6.25:1</td>
<td>5.3</td>
<td>10:1</td>
<td>11.4</td>
</tr>
<tr>
<td>3:1</td>
<td>5.8</td>
<td>5:1</td>
<td>13.6</td>
</tr>
</tbody>
</table>

Effectors in experiment 1: CD4⁺ T cells from naive mice; effectors in experiment 2: splenocytes from cured mice; effectors in experiment 3: CD4⁺ T cells from cured mice; effectors in experiment 4: CD8⁺ T cells from cured mice.
The degree of immunogenicity plays an important role in the antitumor response in most murine models. MC-38 and 3LL are only weakly-to-moderately immunogenic tumors, thus it is significant that CD4+ cells were able to induce cures in models where CD8- subsets or unselected T cells could not. Activated CD4+ cells, Cy, and L-IL-2 were also tested in C3H mice (H-2k) bearing 38C13 lymphomas, another weakly immunogenic model. Cure rates of 50% were obtained, similar to the antitumor effects in the MC-38 and 3LL models.

Studying the mechanisms for CD4-mediated antigen-specific immunity and tumor responses may yield significant insight into regulatory functions of the immune system. In addition, this approach might be important in immunotherapy, as it could circumvent the difficulties in the detection, isolation, and purification of tumor-specific antigens and antigen-specific T cells. A clinical trial is under way to evaluate the immunologic and antitumor effects of activated CD4+ cells with Cy and IL-2 in humans.

REFERENCES


Adoptive Transfer of Anti-CD3–Activated CD4+ T Cells Plus Cyclophosphamide and Liposome-Encapsulated Interleukin-2 Cure Murine MC-38 and 3LL Tumors and Establish Tumor-Specific Immunity


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