Synergism of Interleukin-2 and Cyclosporine A in Induction of a Graft-Versus-Tumor Effect Without Graft-Versus-Host Disease After Syngeneic Bone Marrow Transplantation

By Bishan S. Charak, Ravin Agah, and Amitabha Mazumder

Interleukin-2 (IL-2) therapy generates killer cells with major histocompatibility complex (MHC)-unrestricted cytotoxicity against most tumors but not normal tissues. Cyclosporine A (CsA) has been reported to break tolerance to self and to induce killer cells with specificity against class II MHC (Ia) antigens both on the host and the tumor cells, resulting in a mild graft-versus-host disease (GVHD) in an autologous bone marrow transplantation (BMT) setting in the rat. We used these two agents in a syngeneic BMT model in a strain of histocompatibility complex (MHC)-unrestricted cytotoxicity against most tumors but not normal tissues. Cyclosporine A (CsA) has been reported to break tolerance to self and to Interleukin-2 mild graft-versus-host disease (GVHD) in an autologous bone marrow transplantation (BMT) setting in the rat. We used these two agents in a syngeneic BMT model in a strain of mice that does not develop GVHD with CsA. Therapy with either agent alone was ineffective, whereas a combination of CsA plus IL-2 after BMT induced a potent graft-versus-tumor (GVT) effect against a melanoma and an acute myeloid leukemia. The antitumor effect could be adoptively transferred by infusing spleen cells harvested from mice treated with CsA plus IL-2 into secondary recipients that received chemoradiotherapy. The cytotoxicity of these cells was not influenced by treatment of tumor cells with interferon or the antibody. The cytotoxic effect was mediated by Thy 1+ and asialo GM1+ cells. There was no GVHD either in the primary recipients of CsA and IL-2 or in those receiving the adoptively transferred spleen cells. Our findings show that combination therapy with CsA and IL-2 after syngeneic BMT induces a potent GVT effect in a non-MHC-restricted manner, and point to the existence of differences between the mechanisms of GVT and GVHD.

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MATERIALS AND METHODS

Design of the study. Each experiment was replicated three times. For evaluation of antitumor effect or for survival there were at least four mice under each treatment arm. In experiments for adoptive transfer studies, there was one donor (an age-matched normal mouse, an untreated mouse, or previously treated mouse) and four recipients (tumor-bearing mice) under each treatment arm. A control group that received no treatment was included in every experiment.

Animals. The animals used in this study were female C57 BL/6 (H-2b) mice between the age of 8 and 12 weeks (Jackson Laboratory, Bar Harbor, ME). Mice were housed in open rooms and food and water were allowed ad libitum.

Tumor cells. B16 (murine melanoma), C1498 (murine acute myeloid leukemia), and P815 (murine mastocytoma) cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). B16 and C1498 cell lines are syngeneic to H-2b mice, whereas P815 is an H-2k bearing tumor. The cells were propagated in complete medium (CM) consisting of RPMI 1640, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 0.03% L-glutamine (Irvine Scientific, Santa Ana, CA), 5 × 10^-5 2-mercaptoethanol (Sigma Chemical Co, St Louis, MO), penicillin (100 U/mL), streptomycin (100 pg/mL), and 10% heat-inactivated fetal calf serum (FCS; JR Scientific, Woodland, CA), and were cryopreserved in 10% dimethylsulfoxide (Sigma) and 90% FCS at -80°C. They were thawed immediately before in vitro experi.

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ments. For in vivo experiments, the cells were allowed to grow in CM for 24 hours at 37°C with 100% humidity and 5% CO₂ in air. The cells were then harvested, washed three times in RPMI, and suspended appropriately in RPMI to infuse 2 × 10⁸ cells/mouse intravenously (IV) in a volume of 0.5 mL via the lateral tail vein.

**BM and spleen cells.** BM and spleen cells were collected as described before. Briefly, BM was collected by flushing femurs and tibias with RPMI. Spleen was crushed in RPMI with the piston of a syringe. The respective cell suspensions were passed through a single layer of nylon mesh. Erythrocytes were lysed by distilled water.

**BMT.** BMT was performed 3 days after the induction of tumor. The myeloablative regimen consisted of cyclophosphamide (C; Bristol-Myers, Evansville, IN) 100 mg/kg of body weight intraperitoneally (IP) followed 4 hours later by 850 rad of total body irradiation delivered at a rate of 150 rad/min from a Gammacel Cesium irradiator (Atomic Energy of Canada, Ottawa, Ontario, Canada). BM (5 × 10⁶ cells in 0.5 mL of RPMI) was infused via the lateral tail vein 8 to 10 hours after C.

**IL-2.** Recombinant IL-2 was a generous gift of Cetus Corporation (Emeryville, CA). It had a specific activity of 3 × 10⁸ U/mg (1.2 mg/vial), a purity of greater than 98%, and an endotoxin level of less than 0.1 ng/vial. IL-2 therapy was started immediately after BMT and continued for 14 days. It consisted of 10⁶ Units IP twice a day.

**CsA.** CsA was obtained from Sandoz (East Hanover, NJ). CsA therapy was started immediately after BMT. It was administered in a dose of 20 mg/kg body weight IP once a day for 14 days. This dose has been reported to induce GVHD in a rat model.

**Chromium release assay.** The in vitro cytotoxic potential of spleen cells harvested from mice undergoing various treatments was evaluated in a 4-hour chromium release assay. The target cells were labeled with 500 μCi of Na⁵¹Cr O₄ (ICN Radiochemical, Irvine, CA) in 1 mL of CM for 1 hour. The cells were washed three times with CM and placed in 96-well round-bottomed microtiter plates (Corning Glassware, Corning, NY) at a concentration of 5 × 10⁶ cells/well. Various numbers of effector cells were added in a final volume of 200 μL/well. Maximum isotope release was measured by addition of 0.1 N HCl to the target cells; spontaneous release was measured by the addition of CM alone. The plates were incubated for 4 hours at 37°C in a humid atmosphere with 5% CO₂ in air. The culture supernatant was harvested from the Skatron Titertek System (Skatron AS, Lier, Norway) and was counted in a gamma counter (Packard Auto Gamma-500; United Technologies Packard, Laguna Hills, CA). The percentage specific lysis was calculated by the formula:

\[
\text{Percentage specific lysis} = \left( \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximum cpm} - \text{Spontaneous cpm}} \right) \times 100
\]

All determinations were made in triplicate at various effector to target ratios. The data were calculated as mean ± SEM. Lytic units (LU) were calculated by a computer program (Curvetit) as described by Mitchel et al and expressed as LU/10⁶ effector cells causing lysis of 30% of the target cells. The data have been shown to be representative in vitro experiments.

**Adoptive transfer experiments.** Tumor-bearing mice were subjected to BMT 3 days after tumor induction followed by infusion of splenocytes (1 × 10⁷/mouse IV) harvested from tumor-bearing untreated mice or mice previously treated by BMT and CsA and/or IL-2 therapy as above or from normal mice. The splenocytes for adoptive transfer were harvested immediately after the completion of therapy.

**Ia antigen enhancing/blocking experiments.** Murine γ-interferon (IFN) was obtained from Genentech, Inc (San Francisco, CA). Ia antibody (M5-114) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). For enhancing the Ia antigen, the tumor cells were incubated with IFN (1,000 U/10⁶ cells) in microtiter plates at 37°C in a humid atmosphere for 48 hours. This procedure has been reported to increase the Ia expression significantly. IFN-treated cells were harvested, washed twice with RPMI, and incubated with Ia antibody for 1 hour at 4°C. Tumor cells not treated with IFN were also incubated with Ia-antibody similarly. The cells were washed with RPMI and finally suspended in CM. Tumor cells without any treatment or after treatment with IFN and/or Ia antibody were labeled with ⁵¹Cr and used as targets in a 4-hour ⁵¹Cr release assay as described above. Spleen cells harvested from mice after completion of therapy with IL-2 plus CsA after BMT were used as effectors.

**Phenotype analysis.** Immunophenotype analysis of effector cells in vivo was performed by endogenous depletion of respective cell populations. Hybridoma secreting antibodies against T cells (Thy-1 antibody) was obtained from ATCC. Rabbit antiserum against asialo GM1 was obtained from Wako Chemicals (Dallas, TX). The stock solutions were diluted 1:10 with RPMI and 0.2 mL of this dilution per mouse was injected IV immediately after BMT and, thereafter, on alternate days for two more doses. This protocol has previously been reported to cause the depletion of specific cell populations in vivo.

**Monitoring the GVHD and antitumor effect.** Mice were examined daily for signs of GVHD in the form of erythoderma, alopecia, or obvious weight loss. Some mice from each treatment group were subjected to histologic examination of skin and gut for signs of GVHD. They were also followed-up for survival. Mice living for ≥ 100 days were considered cured. For evaluation of pulmonary metastases in B16-bearing mice, the mice were killed on day 21 postinduction of tumor and their lungs were harvested and fixed in 70% ethyl alcohol.

**Statistical analysis.** P values for comparison of results in various groups were determined by a two-sided log rank test. Survival data are based on Cox regression analysis.

**RESULTS**

The data from three sets of in vivo experiments have been pooled. There was no interexperiment variability as determined by the two-sided log rank test (P = .58).

**Antitumor effect of IL-2 and CsA.** In the B16 melanoma model, BMT alone did not reduce the pulmonary metastases or prolong the survival over the control group. After BMT IL-2 therapy alone or CsA therapy alone also did not significantly enhance the antitumor effect. A combination of IL-2 and CsA after BMT led to a significant reduction in the pulmonary metastases and survival prolongation of the tumor-bearing mice (Table 1). In the C1498 leukemia model, BMT improved the survival over the control group. However, the addition of IL-2 or CsA therapy alone after BMT did not improve the survival further. A combination of IL-2 and CsA after BMT improved the survival as well as the cure rate as compared with BMT alone (Table 2).

**Antitumor efficacy of spleen cells from mice treated with IL-2 and CsA.** In vitro cytotoxic activity of spleen cells
IL-2 AND CsA IN ABMT

Mice were infused with $2 \times 10^5$ B16 cells/mouse IV. BMT was performed on day 3 after induction of tumor. Therapy with IL-2 and/or CsA was administered for 14 days as described in Materials and Methods. Mice were either killed on day 21 and their lungs harvested for counting the pulmonary metastases, or they were followed-up for survival. Control group received no treatment. Values are mean ± SE. P values comparing various treatment groups: 1 v 2, v 3 v 4, P = .84; 1 v 5, P < .001.

Table 1. Antitumor Efficacy of IL-2 and/or CsA Against Melanoma in Mice Undergoing BMT

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Pulmonary Metastases</th>
<th>Survival (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>65 ± 13</td>
<td>29 ± 8</td>
</tr>
<tr>
<td>2. BMT alone</td>
<td>62 ± 11</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>3. BMT + IL-2</td>
<td>63 ± 17</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>4. BMT + CsA</td>
<td>59 ± 12</td>
<td>27 ± 6</td>
</tr>
<tr>
<td>5. BMT + IL-2 + CsA</td>
<td>15 ± 7</td>
<td>46 ± 7</td>
</tr>
</tbody>
</table>

Mice were infected with $2 \times 10^5$ C1498 cells/mouse IV. BMT was performed on day 3 after induction of leukemia. IL-2 and/or CsA therapy was started immediately after BMT and continued for 14 days. Control groups received no treatment. Values are mean ± SE. P values comparing various treatment groups in respective columns: 1 v 2, P = .94; 1 v 3, P = .01; 1 v 4, P = .01; 1 v 5, P < .001.

Table 2. Antitumor Efficacy of IL-2 and/or CsA Against Acute Myeloid Leukemia in Mice Undergoing BMT

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Median Survival (d) (range of days to death)</th>
<th>Cure Rate + SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>22 (19-24)</td>
<td>0</td>
</tr>
<tr>
<td>2. BMT alone</td>
<td>28 (25-34)</td>
<td>22.2 ± 9.4</td>
</tr>
<tr>
<td>3. BMT + IL-2</td>
<td>29 (26-36)</td>
<td>22.2 ± 9.4</td>
</tr>
<tr>
<td>4. BMT + CsA</td>
<td>31 (26-35)</td>
<td>11.1 ± 8.8</td>
</tr>
<tr>
<td>5. BMT + IL-2 + CsA</td>
<td>47 (37-66)</td>
<td>38.8 ± 5.5</td>
</tr>
</tbody>
</table>

Mice were infected with $2 \times 10^5$ C1498 cells/mouse IV. BMT was performed on day 3 after induction of leukemia. IL-2 and/or CsA therapy was started immediately after BMT and continued for 14 days. Control groups received no treatment. Mice were followed-up for survival. P values comparing various treatment groups: 1 v 2, P < .001; 2 v 3 v 4, P = .54; 2 v 5, P < .001.

Table 3. In Vitro Cytotoxic Potential (LU) of Splenocytes Harvested From Mice Previously Treated With Various Forms of Therapy

<table>
<thead>
<tr>
<th>Previous Treatment</th>
<th>Cytotoxicity Against B16</th>
<th>Cytotoxicity Against C1498</th>
<th>Cytotoxicity Against P815</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>2. BMT alone</td>
<td>0.1 ± 0.3</td>
<td>0.3 ± 0.3</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>3. BMT + IL-2</td>
<td>6.1 ± 2.2</td>
<td>4.2 ± 2.7</td>
<td>3.2 ± 2.9</td>
</tr>
<tr>
<td>4. BMT + CsA</td>
<td>3.4 ± 2.2</td>
<td>2.2 ± 2.9</td>
<td>3.7 ± 4.1</td>
</tr>
<tr>
<td>5. BMT + IL-2 + CsA</td>
<td>26.4 ± 23.7</td>
<td>23.7 ± 25.3</td>
<td>26.8 ± 22.9</td>
</tr>
</tbody>
</table>

Mice with established B16 melanoma (A) or C1498 leukemia (B) were subjected to BMT on day 3 or no treatment (none). IL-2 and/or CsA therapy was started immediately after BMT and continued for 14 days. Spleen cells were harvested from these mice immediately after cessation of therapy and tested for their cytotoxic potential in vitro in a 4-hour 51Cr release assay. P values comparing various treatment groups in Table 2: 1 v 2, P = .04; 1 v 3, P = .001; 1 v 4, P = .04; 1 v 5, P < .001.

Adoptive transfer of antitumor effect. Infusion of spleen cells harvested from untreated B16-bearing mice did not show any antitumor effect in vivo (Table 4). Spleen cells from mice having undergone BMT + IL-2 or BMT + CsA therapy also did not show any antitumor effect in the secondary recipients. However, spleen cells from mice previously treated with IL-2 plus CsA after BMT showed significant antitumor effect in the secondary recipients with established B16 melanoma. A similar pattern was seen in the C1498 leukemia model (Table 5); as a control, spleen cells from normal mice were used in these experiments. Spleens from untreated, leukemic mice showed greater than 50% myeloblasts at this point of time and, thus, were not considered suitable for adoptive transfer studies. Only the spleen cells harvested from leukemic mice undergoing BMT followed by IL-2 plus CsA therapy showed antileukemic effect in the secondary recipients. Interestingly, these cells continued to maintain their antitumor effect even in the absence of further exogenous IL-2 and/or CsA administration. Also, the adoptively transferred cells showed antitumor efficacy only in a BMT setting. They did not show any antitumor effect in tumor-bearing mice that were not subjected to chemoradiotherapy.

Cytotoxicity after treatment of targets with IFN and/or Ia antibody. Preincubation of any of the tumor cells with IFN did not increase, and preincubation with Ia antibody did not decrease the cytotoxic effect of spleen cells harvested from B16-bearing mice undergoing BMT and therapy with IL-2 plus CsA (Table 6). Similar results were seen with spleen cells from leukemic mice undergoing BMT followed by IL-2 plus CsA therapy (data not shown).

Phenotype of effector cells after IL-2 plus CsA therapy after BMT. Depletion of Thy-1+ cells led to a reduction in the antitumor effect by approximately 65%, whereas depletion of Lyt-2+ cells did not have a significant effect on the antitumor effect.

Table 4. Adoptive Transfer of Antitumor Effect of Spleen Cells Harvested From Mice Treated With IL-2 and CsA After BMT for Melanoma

<table>
<thead>
<tr>
<th>Previous Treatment of Donor Mice*</th>
<th>Pulmonary Metastases in Secondary Recipient†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMT Group</td>
<td>Non-BMT Group</td>
</tr>
<tr>
<td>1. None</td>
<td>61 ± 12</td>
</tr>
<tr>
<td>2. BMT alone</td>
<td>59 ± 8</td>
</tr>
<tr>
<td>3. BMT + IL-2</td>
<td>53 ± 7</td>
</tr>
<tr>
<td>4. BMT + CsA</td>
<td>57 ± 8</td>
</tr>
<tr>
<td>5. BMT + IL-2 + CsA</td>
<td>25 ± 5†</td>
</tr>
</tbody>
</table>

*Mice with established B16 melanoma were subjected to BMT on day 3 or no treatment. IL-2 and/or CsA therapy was instituted immediately after BMT and continued for 14 days. Spleen cells were harvested from these mice immediately after cessation of therapy and infused (1 x 10^7 cells per mouse IV) into another group of tumor-bearing mice. 

†P value comparing previous treatment group 1 v 5, P < .001. P value comparing all other groups in both columns, P = .73.
of asialo GM1+ cells reduced the antitumor effect by approximately 75% against B16 melanoma in vivo (Table 7).

GVHD. GVHD was not seen in any mice whether or not they received CsA and/or IL-2. It was also not seen in any mice receiving adoptive transfer of spleen cells from mice previously treated with CsA and IL-2.

### DISCUSSION

CsA is a potent immunosuppressive agent widely used for suppressing GVHD after allogeneic BMT. Paradoxically, when used after syngeneic BMT in a Lewis rat model, it induces a syndrome that is clinically and histologically similar to GVHD, and induces cells with antitumor effect in vitro.4-6 The conditions necessary for the development of this syngeneic GVHD include the use of CsA, irradiation of the host, and inclusion of the thymus in the irradiation field.4,5,19 However, this syndrome develops in only some strains of rats and mice.7,20 This was the rationale for selecting C57BL/6 mice for our experiments, as CsA does not induce GVHD in this strain.21,22 In our previous studies, we have reported that IL-2 therapy did not induce GVHD in a syngeneic BMT setting.10,11 Thus, the present study provided an experimental model to evaluate the role of CsA in inducing GVT effect without GVHD and its possible synergism with IL-2 in a BMT setting.

In the CsA-induced GVHD, the autoreactive potential of the effector cells has been explained on the basis of recognition of Ia antigen on the cell surface.1 The tumoricidal potential of these cells is also related to the expression of Ia antigen as it did not increase the lysis of targets mediated by the effector cells in our study was not related to the recognition of Ia antigen as it did not increase even by attempts to enhance the Ia expression on the tumor cells. Furthermore, the antitumor effect of these cells was nonspecific and was not restricted to the tumor of the host only, unlike that seen with CsA alone.6

The exact cellular mechanisms involved in the GVT effect in our model are not known at present. CsA has been reported to cause involution of the thymic medulla, reduced expression of Ia antigen in the thymus, and impairment of the intrathymic differentiation of T cells.23-25 This results in an increase in double-negative (CD3+, CD4−, CD8−) cells both in the thymus as well as in the peripheral blood.23 These cells have been reported to have a high degree of autoreactivity.25 Clonal deletion of the cells that have a high affinity for self-MHC antigens takes place in the thymus by the process of apoptosis. It has been proposed that CsA inhibits apoptosis by inhibiting thymocyte activation via the T-cell receptor.26 Excessive numbers of double-negative cells eventually could lead to a syndrome of GVHD. However, recent data suggest that the effector cells mediating syngeneic GVHD are predominantly CD8+ and their...
The antitumor effect of CsA-generated cells has also been attributed to the CD8+ cells. Thus, it is possible that some of the double-negative cells mature ringherally into single-positive (CD4+ or CD8+) cells.

IL-2 has been reported to generate CD3+ NK1.1+ cells, both from the spleen as well as from BM. The double-negative cells also appear to be precursors in the IL-2 activation system. Thus, the CsA and IL-2 may be synergistic in generating a cell population with antitumor effect. Our findings show that the antitumor effector cells consisted of Thy-1+, asialo GM-1+ cells. Preliminary data showed that there was an increase in the percentage of double-negative cells in the spleen of the animals treated with the combination of CsA and IL-2 as compared with the animals treated with either agent alone. These cells might have been responsible for the higher antitumor effect of spleen cells harvested from mice receiving a combination therapy after BMT. Alternatively, it is possible that CsA-induced maturation arrest of T cells in our model was enough to break tolerance (including tolerance to the syngeneic tumors), but was inadequate to cause overt GVHD. At the same time, IL-2-activated cells might have become more cytotoxic to the tumor on the background of a break in tolerance because of CsA and chemoradiotherapy. In this regard, it is interesting to note that the adoptive transfer of spleen cells from mice treated with IL-2 plus CsA was effective only in recipients that had been subjected to chemoradiotherapy and not in intact mice. In our previous studies, we were unable to adoptively transfer the GVT effect from mice undergoing BMT with IL-2-activated BM plus IL-2 therapy. IL-2 by itself did not increase the GVT effect of BMT with FB in either this study or in our previous studies. These observations suggest that a break in tolerance may be a factor contributing to the higher antitumor effect of IL-2 plus CsA after BMT. The present study points to the possibility of manipulation of host mechanisms to optimize the IL-2-induced GVT effect and to maintain it without requiring exogenous IL-2.

GVHD and GVT effect have generally been considered to be inseparable from each other. Our observations suggest the possibility of differences in the cellular mechanisms involved in the two processes. Future studies should be performed to define the effector mechanisms responsible for the GVT effect induced by CsA and IL-2 after BMT and why the adoptive transfer worked only in mice subjected to chemoradiotherapy.

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