Antitumor Protection from the Murine T-Cell Leukemia/Lymphoma EL4 by the Continuous Subcutaneous Coadministration of Recombinant Macrophage-Colony Stimulating Factor and Interleukin-2

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ABSTRACT

Combined continuous subcutaneous coadministration of macrophage-colony stimulating factor (M-CSF) plus interleukin-2 (IL-2) by osmotic pump provided an effect that was likely attributed to murine effector cells. To determine how M-CSF + IL-2 provided this effect, we performed immunohistochemical and functional analyses in a nude mouse model of antitumor cells. Cytokine phenotyping experiments revealed that the highest levels of cytokines were present in mice given the cytokine combination rather than either M-CSF or IL-2 alone. In vivo depletion of natural killer cells ablated the antitumor protective effect of M-CSF and IL-2. T-cells were also important for M-CSF + IL-2 efficacy, since adult thymectomy/T-cell depletion significantly inhibited the ability of cytokine coadministration to protect against EL4. Coadministration of the cytokines significantly elevated in vivo levels of CD3+CD4+, CD3+CD8+, CD3+NK1.1+ T-cells, and CD3+CD25+ (activated) T-cells, and elevated anti-EL4 cytotoxic T-cell activity measured in vivo. Although WBC counts and fluorescence-activated cell sorter studies showed that M-CSF + IL-2 treatment significantly elevated neutrophils, the ability of granulocyte-colony stimulating factor at doses sufficient to induce neutrophilia was unable to confer anti-EL4 protection. These studies indicate that macrophages, T-cells, and natural killer cells are all important in the M-CSF + IL-2 anti-EL4 response. The superior antitumor effect of this cytokine combination along with the ability of M-CSF to diminish the toxicity of IL-2 in this model suggests that further investigations into the clinical potential of this combination treatment are warranted.

INTRODUCTION

Macrophage-colony stimulating factor is a glycoprotein that acts on progenitor and mature cells of monocyte/macrophage lineage via specific cell surface receptors (1). M-CSF receptor (c-fms) is distributed most on monocytes/macrophages, myeloid precursors, and placental trophoblasts. Thus, M-CSF stimulates murine macrophages to secrete a variety of products important in the immune response (2-4). M-CSF induces enhanced expression of Fc receptors (5), cytotoxic activity of macrophages and monocytes (6), enhanced chemotaxis (7), and respiratory burst activity (8), and can be used for viral immunotherapy (9). In murine models, M-CSF has resulted in tumoricidal activity when used alone (10) or in combination with other therapies (11). Monocytes/macrophages are important in antitumor responses. In the effector phase of response, macrophages process antigens and present tumor-specific peptides in conjunction with major histocompatibility complex antigens to the T-lymphocyte. Macrophages can also regulate lymphocytes by releasing stimulatory and inhibitory cytokines. In the effector phase, macrophages can serve as important effector cells in antitumor responses.

IL-2 receptors are distributed on T-cells, B-cells, monocytes, myeloid precursors, and NK cells. Cells that are targets for IL-2 have also been known to play a very important role in antitumor immune responses. LAK cells with IL-2 treatment have been used for cancer therapy (12-15). Circulating NK cells have been proposed as the circulating in vivo cytotoxic effectors in studies in which IL-2 has been administered for cancer therapy (16-20).

In these studies, we tested the ability of coadministration of M-CSF + IL-2 to heighten antitumor responses. Following i.v. injection of the T-cell leukemia/lymphoma EL4, we observed that there is an enhancement of antitumor protection with M-CSF + IL-2 that is significantly greater than that observed with either cytokine alone. Then, using immunohistochemistry, functional assays, and other immunological techniques, we also evaluated the effector cells responsible for the antitumor effect.

MATERIALS AND METHODS

Mice and Tumors. Female 5–8-week-old C57Bl/6 mice (Thy1.2+), or B6.PL-thy-1/Cy mice (Thy1.1+) were obtained from The Jackson Laboratory (Bar Harbor, ME) and used for all experiments. The chemically induced C57Bl/6 EL4 lymphoma (21) originally obtained from the ATCC (Rockville, MD) was propagated in suspension culture in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mm glutamine, and 100 U/ml penicillin/streptomycin at 37°C in a 5% CO2/95% air atmosphere. We have designated the subline used in these studies as EL4/University of Minnesota.

Antibodies for IF Studies. Antibodies for IF studies included: anti-Thy-1.2 monoclonal antibody (clone 30-H12, rat anti-mouse IgG2b from ATCC) (22), anti-Ly-1 (clone 53–7.313, rat IgG2a from ATCC) (22), anti-Ly-2.2 (clone 2.43, rat IgG2b from Dr. Frank Fitch, University of Chicago, Chicago, IL) (23), anti-CD4 (clone OK1.5 also from Dr. Fitch) (24), anti-CD2 (clone RM2.2, rat IgG2b from Dr. Frank Fitch, University of Chicago, Chicago, IL) (23), anti-CD3-ε (clone 1452C11, hamster IgG from Dr. Jeffery Bluestein, University of Chicago, Chicago, IL) (26), anti-CD3-ε (clone RA3-58B2, rat IgG2a from ATCC) (27), anti-neutrophil (clone RB6–8C5, rat IgG2b from Pharmingen) (28), anti-Ly8.2 (clone CY-34–1.1, mouse IgG1, from Dr. Kevin Holmes, NIH) (29), anti-LF-A1 (clone FD4218, rat IgG2b from Dr. Frank Fitch) (30), anti-macrophage/monocyte (clone F4/80, rat IgG2b provided by ATCC) (31), anti-IL-2 receptor (clone 7D4, rat IgG from Pharmingen) (32), and NK1.1 (clone PK136 mouse IgG2a from Dr. Gloria Koo, Rahway, NJ) (33). The monoclonal antibodies were linked to FITC or phycoerythrin for 2-color IF as reported (34). Direct IF studies were performed as previously described, by incubating EL4 cells in 1 h at 4°C with an optimal concentration of labeled antibody. Cells were washed 3 times and then suspended in 0.5 ml of 1% paraformaldehyde. Binding was quantitated on a FACScan (Becton Dickinson, Mountain View, CA). A minimum of 20,000 cells (determined by forward light scatter) was analyzed. Anti-human CD7 (3A1e, mouse IgG2b provided by Dr. Barton Haynes, Duke University, Durham, NC) was conjugated to FITC or phyco-
curves generated over 100 days (post-tumor injection) showed that a

tumor inoculation); HP EIA cells were given. Two hundred j.L. volumes M-

cells (by NK cells), and of H-2d P815 cells (by LAK cells) was measured at

RESULTS

Flow Cytometric Analysis of EL4 Reveals Absence of IL-2 and
M-CSF Receptor. EL4 cells were reacted with a panel of labeled
monoclonal antibodies directed against lymphoid cell surface anti-
gens. EL4 cells expressed the following markers: 95% Thy1.2, 96%
Ly1, 85% CD2, 93% CD3, and 96% CD11a (LFA-1) surface markers,
which are all associated with T-cells. The cells did not express B-cell
antigens or the low affinity IL-2 receptor (CD25). As a control, CD25
was found to be present on 88% of day 3 phytohemagglutinin-acti-
vated splenocytes, but only 7% of nonactivated splenocytes. IL-2
was found to be present on 88% of day 3 phytohemagglutinin-acti-
antigens or the low affinity IL-2 receptor (CD25). As a control, CD25
was detected in bone marrow, lymph node, peripheral blood, spleen,
or thymus, suggesting that the levels of propagation were not suffi-
cient for detectability. On day 14, tumor appeared in bone marrow,
lymph node, and/or peripheral blood, but not the spleen or thymus
in 3 of 4 mice. On day 21, tumor was in the spleen and thymus of 2 of
4 mice. No reactivity was observed when anti-Thy1.2-FITC was re-
acted with bone marrow, lymph node, peripheral blood, spleen, or
C57Bl/6 mice receiving i.v. injections of EL4 in other experiments has
frequently demonstrated the presence, at autopsy, of EL4 tumor in the
peritoneum, mesentary, uterus, kidney, liver, and muscle.

Low Dose IL-2 Administration Elicits Antitumor Effect. To test the
ability of s.c. administered IL-2 to protect mice against EL4
lethality, IL-2 was administered to mice given 10^5 EL4 cells (Fig. 1).
Pumps were inserted on day -3 so that circulating IL-2 levels would
presumably reach an equilibrium state at the time of EL4 injection on
day 0. Mice given either 0.625 /j.Lg/day or 2.5 /j.Lg/day in 14-day pumps
showed significant (P < 0.02), albeit partial, EL4 protection com-
pared to PBS-treated controls. In both groups, 40-50% of the mice
succeeded to the EL4 tumor by day 60 postinjection. In a second experi-
ment (n = 15/group) shown in Fig. 2A, higher doses of IL-2
(5 /j.Lg/day) in 14-day pumps were studied. The higher dosage resulted
in significant toxicity, with the majority of the animals dead by day 10
postinjection. The mean survival time of these mice was significantly
(P < 0.009) shorter (14 days) than control PBS-treated mice.

Coadministration of M-CSF and IL-2 Protects against Tumor
and Reduces IL-2-mediated Toxicity. To test our hypothesis that
monocytes, NK cells, and T-cells together augment antitumor effec-
tors, we coadministered M-CSF and this higher dose of IL-2. Survival
was significantly (P < 0.0005) improved in comparison to PBS-
treated controls (Fig. 2A). Seventy % of the M-CSF + IL-2-treated
mice survived to day 100 (MST > 100 days). Some antitumor pro-
tection was conferred in this experiment by 20 /j.Lg/day M-CSF treat-

Determination of a Tumor Dose. To determine the susceptibility
of the C57Bl/6 mice to EL4 tumor, groups of mice were given injec-
tions of varying doses of EL4 tumor cells (n = 10/group). Survival
survival curves generated over 100 days (post-tumor injection) showed that a
dosage of 10^6 cells/mouse was mostly lethal. After 100 days, 10^4
cells/mouse was an 83% lethal dose (a dose at which 83% of the mice
died), 10^3 cells/mouse was a 63% lethal dose, and 10^2 cells/mouse
was a 15% lethal dose. There was little, if any, difference in the dose
response curves of mice given injections by either the i.v. or i.p. route.

Tumor Localization Studies. To determine the localization of
Thy1.2+ EL4 tumor cells, tumor was injected into Thy1.1+ congenic
mice. On day 7 after i.v. injection of 2 x 10^6 tumor cells, no tumor
was detected in bone marrow, lymph node, peripheral blood, spleen,
or thymus, suggesting that the levels of propagation were not suffi-
cient for detectability. On day 14, tumor appeared in bone marrow,
lymph node, and/or peripheral blood, but not the spleen or thymus
in 3 of 4 mice. On day 21, tumor was in the spleen and thymus of 2 of
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peritoneum, mesentary, uterus, kidney, liver, and muscle.

In Vivo Administration of Recombinant M-CSF and Recombinant
IL-2. For continuous s.c. delivery of cytokines, 14-day miniosmotic pumps
(ALZA Corp., Palo Alto, CA) were implanted under general anesthesia as described
(36) in control and experimental mice, on day -3 with day 0 the day of
tumor inoculation); 10^6 EL4 cells were given. Two hundred j.L units
M-CSF and recombinant IL-2 were injected into the pumps. Control pumps
were injected with PBS. The pumps were implanted in the dorsal lumbar area.

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tumor inoculation); 10^6 EL4 cells were given. Two hundred j.L units
M-CSF and recombinant IL-2 were injected into the pumps. Control pumps
were injected with PBS. The pumps were implanted in the dorsal lumbar area.

Hematological Evaluation of Recipients. Fifty j.L of peripheral blood
were obtained by retro-orbital venipuncture on days 7, 14, and 28. Leukocyte
number and morphology were determined by examination of Wright-Giemsa
stained slides (37).

Lysis of Targets by CTLs, NK Cells, and LAK Cells. Mice were given
implants of pumps containing M-CSF, IL-2, M-CSF + IL-2, or control PBS,
as described. Groups of mice were given injections of EL4 tumor cells and
sacrificed on day 7 postinjection, at which time spleenocytes were isolated and
tested for their ability to lyse 51Cr-labeled target cells. Spleenocytes were pooled
from 3 mice. Cytolysis of H-2a EL4 cells (by specific CTL), of H-2b YAC-1
cells (by NK cells), and of H-2b P815 cells (by LAK cells) was measured at
an effector:target ratios of 50:1, 25:1, 12:5:1, and 6.25:1. Percent cytolysis was
determined as described previously (38). For EL4, spontaneous and maximum
release was 470 and 4957 cpm, respectively. For YAC-1 targets, spontaneous
and maximum release was 551 and 5443 cpm, respectively. For P815 targets,
spontaneous and maximum release was 327 and 3576 cpm, respectively.

Statistical Analyses. Groupwise comparisons of continuous data were
made by Student's t-test. For plotting actuarial survival, the computer program
for compiling life table and statistical data analyzed by a log-rank test was
written by Dr. Bruce Bostrom, Department of Pediatrics, University of Min-
nesota.

RESULTS

Flow Cytometric Analysis of EL4 Reveals Absence of IL-2 and
M-CSF Receptor. EL4 cells were reacted with a panel of labeled
monoclonal antibodies directed against lymphoid cell surface anti-
gens. EL4 cells expressed the following markers: 95% Thy1.2, 96%
Ly1, 85% CD2, 93% CD3, and 96% CD11a (LFA-1) surface markers,
which are all associated with T-cells. The cells did not express B-cell
antigens or the low affinity IL-2 receptor (CD25). As a control, CD25
was found to be present on 88% of day 3 phytohemagglutinin-acti-
vated splenocytes, but only 7% of nonactivated splenocytes. IL-2
(1000 units/ml) or M-CSF (2000 units/ml) was unable to stimulate
EL4 cells. No proliferation as measured by incorporation of tritiated
thymidine was observed on day 1 or 2.

Determination of a Tumor Dose. To determine the susceptibility
of the C57Bl/6 mice to EL4 tumor, groups of mice were given injec-
tions of varying doses of EL4 tumor cells (n = 10/group). Survival
survival curves generated over 100 days (post-tumor injection) showed that a
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Fig. 2. Continuous s.c. administration of M-CSF + IL-2 by pump protects against EL4 tumorigenicity. Groups of C57Bl/6 mice were given pumps containing M-CSF + IL-2, M-CSF, IL-2, or PBS on day -3 and injections of $10^5$ EL4 cells i.v. on day 0. Independent experiments ($n = 15$ group) are shown in A and B and the pooled data ($n = 30$ group) are shown in C. Actuarial survival was plotted.

A. Experiment 1

B. Experiment 2

C. Pooled Data

Fig. 2. Continuous s.c. administration of M-CSF + IL-2 by pump protects against EL4 tumorigenicity. Groups of C57Bl/6 mice were given pumps containing M-CSF + IL-2, M-CSF, IL-2, or PBS on day -3 and injections of $10^5$ EL4 cells i.v. on day 0. Independent experiments ($n = 15$ group) are shown in A and B and the pooled data ($n = 30$ group) are shown in C. Actuarial survival was plotted.

- In a different experiment (Fig. 2B), groups ($n = 15$) of mice treated identically to those in Fig. 2A showed similar results. Early deaths due to toxicity were observed with IL-2 treatment as compared to PBS controls. The mean survival time of the IL-2-treated mice was 32 days shorter than that of the PBS-treated mice. M-CSF + IL-2 treatment gave the best antitumor protection with nearly 90% of the mice alive more than 90 days after tumor injection. The level of protection was significantly greater than that obtained with M-CSF alone ($P = 0.0026$), IL-2 alone ($P = 0.000005$), or PBS ($P = 0.0027$) treatment. In contrast to the experiment in Fig. 2A, M-CSF treatment alone did not protect in this experiment. Here, not all of the animals succumbed to EL4 in the PBS control-treated group.

- Since the experiments in Fig. 2, A and B, were performed identically, the data were pooled in Fig. 2C ($n = 30$ group). M-CSF + IL-2-treated mice showed 78% survival 100 days (MST > 100 days) as compared to PBS.
Table 1  Coadministration s.c. of M-CSF + IL-2 enhances neutrophil in the peripheral blood of EL4-injected mice

<table>
<thead>
<tr>
<th>Cells</th>
<th>PBS</th>
<th>M-CSF</th>
<th>IL-2</th>
<th>M-CSF + IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 2B experiment</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WBC</td>
<td>13.6±5.1</td>
<td>17.6±3.7^a</td>
<td>10.1</td>
<td>37.4±35.3^ab</td>
</tr>
<tr>
<td>ANC</td>
<td>3.4±1.7</td>
<td>5.3±3.1^a</td>
<td>5.2</td>
<td>21.0±25.1^ab</td>
</tr>
<tr>
<td>ALC</td>
<td>11.2±2.8</td>
<td>12.3±3.4</td>
<td>5.0</td>
<td>16.5±11.6</td>
</tr>
<tr>
<td>n</td>
<td>15</td>
<td>15</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Fig. 2A experiment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC</td>
<td>8.9±2.1</td>
<td>10.4±3.3</td>
<td>14.2±4.3^a</td>
<td>15.5±11.2^a</td>
</tr>
<tr>
<td>ANC</td>
<td>0.8±0.3</td>
<td>1.9±0.9^a</td>
<td>5.1±2.7^a</td>
<td>6.4±7.6^ab</td>
</tr>
<tr>
<td>ALC</td>
<td>8.0±1.8</td>
<td>8.4±3.0</td>
<td>8.0±1.9</td>
<td>8.1±5.2</td>
</tr>
<tr>
<td>n</td>
<td>15</td>
<td>15</td>
<td>5</td>
<td>14</td>
</tr>
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</table>

^a P < 0.04 as compared to PBS controls.
^b P < 0.05 as compared to M-CSF group.
^c ANC, absolute neutrophil counts; ALC, absolute lymphocyte counts.

Table 2 Coadministration of M-CSF and IL-2 Stimulates Peripheral Blood Neutrophilia. To determine the effects of M-CSF + IL-2 treatment on peripheral blood cell populations, the mice in Fig. 2, A and B, were evaluated at the end of infusion (day 11) for WBC counts, absolute neutrophil counts, and absolute lymphocyte counts (Table 1) (day 11 is the last day of infusion, since 14-day pumps were inserted on day -3.) There were enough mice available from the PBS-, M-CSF-, and M-CSF + IL-2-treated groups for hematological analysis on day 11. However, there was only one survivor from the IL-2-treated group in Fig. 2B, and 5 mice from the IL-2-treated mice in Fig. 2A. In both experiments, M-CSF + IL-2 treatment significantly (P < 0.04) elevated WBC levels in comparison to PBS-treated controls, and the M-CSF- and IL-2-treated groups showed higher WBC values than the IL-2 or M-CSF treatment alone. In fact, in the mice from Fig. 2B, mean WBCs were elevated about 3-fold in the M-CSF + IL-2-treated group as compared to the PBS control. In both experiments, the WBC elevation in the M-CSF + IL-2-treated group was the result of a significant (P < 0.04) increase in peripheral blood neutrophil, but not lymphocyte, levels.

Elevated neutrophil levels induced by M-CSF + IL-2 were not responsible for the protective anti-EL4 effect in vivo, since G-CSF, a powerful enhancer of neutrophil number and function, was not able to protect mice given G-CSF by pump. In the experiment shown in Fig. 2A, an additional group of mice given G-CSF died at a similar rate to mice given PBS (Fig. 3). The P value (P = 0.34) derived from the comparison of the 2 groups was not significant. The neutrophil levels measured on day 11 (also shown in Fig. 3) in the G-CSF mice were significantly higher than in the PBS mice. In fact, higher levels of neutrophil stimulation were achieved in the G-CSF mice than in the mice given M-CSF + IL-2 which were subsequently protected from the EL4 tumor.

M-CSF and IL-2 Coadministration Stimulates Splenic Localized NK Cell and Macrophage Compartments in Normal and Tumor-injected Mice. To further explore the cell populations affected by M-CSF + IL-2 treatment, normal animals that had received pumps were sacrificed on day 7 and the spleen cells were isolated and examined by FACS (Table 2). These studies revealed about a 2.0- to 2.5-fold increase in T-cell populations (CD3+, CD4+, CD8+) in mice given M-CSF + IL-2 as compared to the PBS control. A similar elevation was noted with IL-2 administration alone. In comparison to IL-2 treatment alone, M-CSF + IL-2 treatment elevated the cell numbers in the macrophage compartment (181 × 10^3/μl versus 116 × 10^3/μl) and in the NK compartment (106 × 10^3/μl versus 79 × 10^3/μl). Similar observations were made on day 7 with EL4-injected
ANTITUMOR EFFECTS WITH M-CSF + IL-2 THERAPY

Table 2. Coinadministration s.c. of M-CSF and IL-2 enhances various leukocyte subpopulations in the spleen of normal mice as measured by FACS

<table>
<thead>
<tr>
<th>Day 7</th>
<th>Granulocyte cell</th>
<th>Macrophage cell</th>
<th>NK cell</th>
<th>B-cell</th>
<th>T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-CSF + IL-2</td>
<td>35</td>
<td>181</td>
<td>106</td>
<td>124</td>
<td>115</td>
</tr>
<tr>
<td>IL-2</td>
<td>45</td>
<td>116</td>
<td>79</td>
<td>116</td>
<td>120</td>
</tr>
<tr>
<td>M-CSF</td>
<td>12</td>
<td>61</td>
<td>13</td>
<td>50</td>
<td>31</td>
</tr>
<tr>
<td>PBS</td>
<td>13</td>
<td>58</td>
<td>9</td>
<td>61</td>
<td>46</td>
</tr>
<tr>
<td>B6 No pump</td>
<td>5</td>
<td>37</td>
<td>4</td>
<td>4</td>
<td>33</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 14</th>
<th>Granulocyte cell</th>
<th>Macrophage cell</th>
<th>NK cell</th>
<th>B-cell</th>
<th>T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-CSF + IL-2</td>
<td>11</td>
<td>95</td>
<td>52</td>
<td>39</td>
<td>37</td>
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<tr>
<td>IL-2</td>
<td>13</td>
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<td>32</td>
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<tr>
<td>M-CSF</td>
<td>32</td>
<td>97</td>
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<td>PBS</td>
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<td>50</td>
<td>11</td>
<td>39</td>
<td>38</td>
</tr>
<tr>
<td>B6 No pump</td>
<td>4</td>
<td>42</td>
<td>5</td>
<td>47</td>
<td>38</td>
</tr>
</tbody>
</table>

animals (Table 3). On day 14, responses had normalized since this was 3 days past the delivery lifespan of the pumps. The FACS studies demonstrate that the addition of M-CSF to IL-2 resulted in the stimulation of multiple lineages of cells with antitumor capabilities including macrophages, T-cells, and NK cells.

NK Cells Are Required for M-CSF + IL-2-mediated Protection in EL4-injected Mice. To determine whether NK cells were responsible for the M-CSF + IL-2 effect, groups of mice (n = 10/group) were NK cell-depleted by administrating a known depletion dose of 400 µg anti-NK 1.1 monoclonal antibody 4 days before tumor injection and then weekly thereafter for 2 additional injections (Fig. 4). A group of mice was depleted of T-cells by adult thymectomy followed by a depleting dose of anti-CD4 plus anti-CD8 monoclonal antibody (400 µg each). When mice depleted in this fashion were given 10³ EL4 cells i.v., the NK cell-depleted animals died earlier (MST = 24 days) than control mice given PBS (MST = 31 days) (P < 0.006). These earlier deaths were unaffected by the administration of M-CSF + IL-2. Since the nondepleted control mice given M-CSF + IL-2 were protected against EL4-related mortality, these data argue that NK cells were at least one of the antitumor effector cells affected by M-CSF and IL-2 treatment. The removal of T-cells also significantly (P < 0.03) reduced M-CSF + IL-2 efficacy, indicating that T-cells are also involved in M-CSF + IL-2-mediated protection.

M-CSF + IL-2 Coinadministration Augments NK and CTL Splenic Lytic Activity in EL4-injected Mice. On day 7 after tumor injection, splenocytes were removed and tested for their activity against NK ⁵¹Cr-labeled target cells (YAC-1) or against CTL ⁵¹Cr-labeled targets (EL4 cells). The cytolytic response to YAC-1 target cells was elevated in both M-CSF + IL-2-treated mice and IL-2-treated mice, as compared to PBS controls. At an E:T ratio of 25:1, EL4-injected mice treated with M-CSF + IL-2, IL-2, M-CSF, or PBS showed cytolytic values of 25, 17, 5, or 5%, respectively. At an E:T ratio of 12:1, EL4-injected mice treated with M-CSF + IL-2 or IL-2 alone showed cytolytic values at least 6.5-fold and 4.7-fold greater than PBS controls, respectively. Again, M-CSF treatment alone did not elevate NK response. Similar responses were observed in treated mice not given EL4.

CTL response (measured with the labeled EL4 cells) was observed only by M-CSF and IL-2 treatment. At an E:T ratio of 50:1, mice given injections of 10⁵ EL4 cells and treated with M-CSF + IL-2, IL-2, M-CSF, or PBS showed cytolytic values of 27, 6, 1, or 3%, respectively. Mice given injections of 10⁶ EL4 cells and treated with M-CSF + IL-2, IL-2, M-CSF, or PBS showed cytolytic values of 19, 5, 2, or 1%, respectively. Mice treated with M-CSF + IL-2, IL-2, M-CSF, or PBS, and were not given EL4 showed no anti-EL4 cytolytic activity (2, 3, 1, or 0%, respectively). These functional studies support our FACS findings (which only measure cell number). They also support the hypothesis that M-CSF + IL-2 together induce T-cells and NK cells that are capable of recognizing and killing EL4 cells in vivo.

DISCUSSION

We chose EL4 for our studies since EL4 cells injected in vivo provide a well-established model for exploring antitumor responses of a T-cell cancer. Kinetic studies have shown that EL4 elicits immunological responsiveness through a variety of effector cells, including...
cytolytic T-cells, NK cells, LAK cells, and suppressor cells (39). EL4 was also selected because we were interested in studying the immunobiology of M-CSF + IL-2 coadministration without the complication of a tumor that expresses M-CSF receptor (c-fms) and IL-2 receptor. Others have reported that EL4 does not express IL-2 receptor (40), and in our own studies we were unable to detect IL-2 receptor using FACS and could not activate EL4 tumor cells in vitro with recombinant M-CSF and IL-2. Although this argues that IL-2 is not acting directly on EL4 cells, we cannot rule out the possibility that IL-2 receptor β- or γ-chains may some how allow these cells to respond to IL-2.

We observed that M-CSF + IL-2 coadministration stimulated several cell populations with potential antitumor effector cell properties. Our data were consistent with an effect on monocytes and macrophages. Phenotyping studies using FACS showed that macrophage numbers (F4/80+ cells) were increased by coadministration as compared to macrophage numbers obtained when the cytokines were administered individually. This observation was not surprising, since investigators reported that c-fms and IL-2 receptor are both present on monocytes (41). Their data demonstrated that IL-2 enhanced c-fms mRNA and c-fms glycoprotein expression, suggesting that IL-2, by augmenting expression of c-fms, can lead to prolongation of monocyte-mediated tumoricidal activity. Investigators have also shown that peripheral blood cells from cancer patients given IL-2 treatment have enhanced expression of mRNA for M-CSF (42). It is therefore possible that IL-2 treatment increases M-CSF production, which in turn increases macrophage numbers and/or macrophage activity in our system. Human monocytes in culture with IL-2 have shown high expression of mRNA for M-CSF (42). It is therefore possible that IL-2 treatment increases M-CSF production, which in turn increases macrophage numbers and/or macrophage activity in our system. Human monocytic cells with IL-2 have shown high activities against various tumors (43). Macrophage effector cells against EL4 have previously been reported in mice (39) and in these studies, function was diminished in late tumor-bearing mice, suggesting that suppression occurs as the tumor expands. Together, these findings suggest that macrophage/monocytes could play a primary role in the anti-EL4 response, while the role of other cell types could be more indirect, i.e., secondary provision of cytokines such as interferon-γ or tumor necrosis factor that could further activate monocytes. However, in our model, macrophage stimulation with M-CSF alone was only partially protective against EL4. This suggests that other cells are operative in the antitumor effect of M-CSF + IL-2.

NK cells were also important in the coadministration of M-CSF + IL-2. Phenotyping studies showed that in either normal or EL4-infected hosts, the highest level of NK1.1+CD3+ cells was obtained with M-CSF + IL-2 treatment rather than IL-2 or M-CSF treatment alone. A more direct link of NK cells to the anti-EL4 leukemia effect was established in the in vivo depletion experiments using anti-NK1.1 injections to eliminate NK cells. This established method of depletion (44) significantly augmented the rates of EL4-related deaths and also totally prevented a successful M-CSF + IL-2 response. M-CSF was synergistic with IL-2 in inducing NK cell numbers and/or function. Thus, while IL-2 probably had a direct effect stimulating IL-2 receptor-positive NK cells and their precursors, the M-CSF effect was likely indirect through interferon. M-CSF is known to stimulate the production of interferon (45), and investigators have established a role of monocytes in the IL-2 activation of human NK cells (46). It has been shown that interferon is a potent stimulator of NK activity in tumor models (47). Interferon has also been shown to act through NK cells in other EL4 studies (48). Administration of interferon did enhance in vivo anti-EL4 effect in normal C57B1/6 mice and in T-cell-deficient nude mice, but not in NK-deficient beige mice. It may be important that other investigators (49) have shown that macrophage precursors enriched from murine bone marrow show strong proliferation in response to treatment with M-CSF and IL-2 in vitro, and that IL-2 exposure induced NK-like activity in these cells. When IL-2 was withdrawn from the culture media, cells reverted to properties of typical macrophages.

Interestingly, our phenotyping studies showed that the CD3−NK1.1+ cells (50) that express both NK marker (NK1.1) and the T-cell receptor, behaved more like T-cells than NK cells: they were equally expanded by M-CSF + IL-2 treatment or IL-2 treatment alone. This might not be so surprising in light of the fact that these cells have been shown to differentiate into a CD3+CD8−NK1.1− phenotype. Thus, these cells were apparently generated in response to IL-2 and not augmented by the M-CSF + IL-2 combination. Taken
together, our data indicate that NK cells are key effector cells in the M-CSF + IL-2 response. 

T-cells were also affected in our system. The relationship between T-cells and anti-EL4 protection was established in vivo in experiments with thymectomized adult mice that were further T-cell-depleted with in vivo injections of anti-CD4 and anti-CD8 monoclonal antibody. (A group of 60 of these mice had a mean of only 1.4% CD4 expressing T-cells and 1.5% CD8 expressing T-cells and no T-cell function.) In these mice given EL4 cells, M-CSF + IL-2 treatment resulted in a 40% day 96 survival rate, which was significantly lower (P < 0.003) than the survival rate in nonthymectomized/non-T-cell-depleted mice given M-CSF + IL-2. Thus, the removal of T-cells diminished, but did not eliminate M-CSF + IL-2 antitumor efficacy. Previously, a role for specific T-cell responses in the EL4 model has been reported (48, 51). 

In our in vitro studies, the highest levels of anti-EL4 CTL function were measured from mice given injections of both M-CSF + IL-2 and not the individual cytokines. In our phenotyping studies of EL4-injected mice, we examined the number of several independent T-cell populations in the spleen, including CD3⁺ (T-cell receptor positive) cells, CD4⁺ helper T-cells, CD8⁺ CTL, and CD3⁺ IL-2 receptor-expressing (activated) T-cells. All were significantly elevated. However, we achieved the same increase in cell numbers with IL-2 alone as we did with the M-CSF + IL-2 combination. Identical findings were obtained in separate experiments in normal mice treated with M-CSF + IL-2. Together, these data indicate that although IL-2 alone might be chiefly responsible for the increase in T-cell number, the M-CSF + IL-2 combination was the cause of the enhanced anti-EL4 CTL function and that T-cells are clearly important in the M-CSF + IL-2 response. 

Neutrophil levels were also high in M-CSF- and IL-2-treated mice. This was shown in stained blood smears and supported by our phenotyping experiments. However, levels were similarly elevated in IL-2-treated mice. Since our previous studies indicated that G-CSF (37, 52) infusion induced neutrophilia in mice, we administered G-CSF to mice given EL4 in the same experiment shown in experiment 2A. No protection against EL4 was afforded, despite the fact that high levels of neutrophils were measured in the G-CSF-treated mice on day 11. In fact, higher levels of neutrophils were seen in the G-CSF-treated mice than in the mice given M-CSF + IL-2. Thus, although neutrophil levels are elevated by M-CSF and IL-2 treatment, these cells do not appear to play an important role in protecting our mice from EL4. On the other hand, we cannot rule out an anti-EL4 effect mediated by neutrophils, since M-CSF or the M-CSF + IL-2 combination may be affecting neutrophil function in a qualitatively different fashion than that induced by G-CSF. We do not exactly know why neutrophil levels were elevated, but it is possible that accessory cytokines such as IL-1, G-CSF, granulocyte macrophage-colony-stimulating factor, and IL-6 were elevated by M-CSF + IL-2 treatment. Investigators have shown that in cancer patients given IL-2 pronounced increases in G-CSF and IL-6 were observed (53). 

We found that lower doses of IL-2 cured a percentage of mice given injections of EL4. However, at higher IL-2 doses, early toxic deaths were observed. This finding was not surprising in light of clinical studies demonstrating vascular leak syndrome and other toxic effects related to clinical IL-2 treatment (54). We know that IL-2 can induce the release of other cytokines such as tumor necrosis factor (55), IL-1 (56), and interferon-γ (57), which can be toxic. We do not know why M-CSF was able to protect mice from the early IL-2-related toxic deaths. One could speculate that M-CSF stimulates the monocyte pool and increases their numbers. Since IL-2 stimulates IL-2 receptor expression on monocytes (58), IL-2 might preferentially bind to this expanded monocyte pool. Less IL-2 might be available to bind to other targets that stimulate cytokine release and toxicity. Perhaps the addition of M-CSF to IL-2 changes the cytokine profile. Future studies will be performed to learn more about the role of other cytokines in mice given M-CSF + IL-2. We do not favor the explanation that the M-CSF protein directly slowed the release of IL-2 from the pump, because phenotypic studies show that M-CSF + IL-2 promote different populations of cells than either M-CSF or IL-2 treatment alone. For example, F4/80⁺ macrophages and NK1.1⁺ CD3⁻ NK cells were highest with M-CSF + IL-2 treatment. Also, protection against the tumor was obtained when M-CSF and IL-2 were delivered in separate pumps placed in the same animal (data not shown). Our in vivo M-CSF dose of 20 μg/day was derived from our previous mouse studies (59). A dose of 30 μg/day was toxic. 

In conclusion, our data support an important role for macrophages, T-cells, and NK cells in the anti-EL4 response induced by the M-CSF + IL-2 combination. Macrophages are important since macrophage numbers were highest when the combination was used. Also it is known that IL-2 enhances c-fms expression, and receptors for both cytokines are found on macrophages. Our data also show that T-cell function is important since T-cell anti-EL4 CTL function was highest in the M-CSF + IL-2 combination (although the removal of T-cells did not entirely ablate the efficacy of the M-CSF + IL-2 combination against EL4). Neutrophils appeared to play a minor role in the anti-tumor response. Although neutrophil levels were elevated, G-CSF was unable to confer any protection against EL4. Thus, their role is debatable. The studies in this manuscript support the argument that combined M-CSF + IL-2 therapy represents a strong immunostimulatory regimen, which, based on findings in an aggressive systemic tumor model, shows some degree of promise. The beneficial effect of this cytokine combination is not restricted to chemically induced tumors, since reports show that combined M-CSF + IL-2 treatment can be used to protect 7-day-old C57Bl/6 mice from HSV-1 infection (60). Perhaps of greater importance is the observation that the addition of M-CSF reproducibly diminished toxic deaths attributed to IL-2. This may provide a means to enhance IL-2 dosages in future trials. An M-CSF + IL-2 regimen may be interesting in light of clinical trials with IL-2 (13) and M-CSF (61) alone. 

REFERENCES


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