Granulocyte-Macrophage Colony-Stimulating Factor Augmentation of T-Cell Receptor–Dependent and T-Cell Receptor–Independent Thymocyte Proliferation

By Ann M. Stewart-Akers, J. Scott Cairns, David J. Tweardy, and Susan A. McCarthy

The effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) are not confined to cells of the myeloid lineage. GM-CSF has been shown to have effects on mature T cells and both mature and immature T-cell lines. We therefore examined the GM-CSF responsiveness of murine thymocytes to investigate whether GM-CSF also affected normal immature T lymphocytes. The studies presented here indicate that GM-CSF augments accessory cell (AC)-dependent T-cell receptor (TCR)-mediated proliferation of unseparated thymocyte populations. To identify the GM-CSF responsive cell type, thymic AC and T cells were examined for GM-CSF responsiveness. We found that GM-CSF augmentation of TCR-induced thymocyte proliferation appears to be mediated via augmentation of AC function, and not via direct effects on mature single-positive (SP) thymocytes. Enriched double-negative (DN) thymocytes were also tested for GM-CSF responsiveness. GM-CSF induced the proliferation of adult and fetal DN thymocytes in an AC-independent and TCR-independent single-cell assay. Thus, in contrast to the SP thymocytes, a DN thymocyte population was directly responsive to GM-CSF. GM-CSF therefore may play a direct role in the expansion of DN thymocytes and an indirect role in the expansion of SP thymocytes.

© 1994 by The American Society of Hematology.

MATERIALS AND METHODS

Animals. Six to 12-week-old C57BL/6J (B6) mice (Jackson Laboratories, Bar Harbor, ME) of either sex were used. B6 mice were bred in our animal facility to obtain fetal mice. Pregnancies were timed, and day 0 was defined as the day that a vaginal plug was observed. All animals were housed in a specific pathogen-free animal facility, and provided with Purina rodent chow and tap water ad libitum. Animals were killed by cervical dislocation or CO₂ inhalation.

Medium. RPMI 1640 (GIBCO, Grand Island, NY) was supplemented with 50 to 75 μmol/L L-2-mercaptoethanol (GIBCO), 2 to 4 mmol/L glutamine (GIBCO), 8 to 10 U/mL penicillin, and 8 to 10 μg/mL streptomycin (GIBCO), and 10% heat inactivated fetal

From the Departments of Molecular Genetics and Biochemistry, Surgery, Pathology, and Medicine, and the Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA.

Submitted April 15, 1993; accepted September 29, 1993.

Supported by Grants No. CH-503 (D.J.T.) and JFRA-355 (S.A.M.) from the American Cancer Society.

Address reprint requests to Susan A. McCarthy, PhD, W1554 BioScience Tower, DeSoto and Terrace Streets, University of Pittsburgh, Pittsburgh, PA 15213.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1994 by The American Society of Hematology.
0006-4971/94/8303-0024/3 $00/0

Blood, Vol 83, No 3 (February 1, 1994): pp 713-723
bovine serum (FBS; GIBCO), hereafter referred to as complete medium (CM).

**Cytokines and antibodies.** Human recombinant (r)IL-2 (specific activity, 3 × 10⁶ U/mg) was donated by Cetus Oncology (Emeryville, CA). COS-7 supernatant containing murine rGM-CSF was the generous gift of DNAX Research Institute (Palo Alto, CA) or was produced by us as described previously. Murine rGM-CSF (specific activity, 5 × 10⁷ colony-forming-units·committing cell [CFU/C]/mg) was purchased from Genzyme (Boston, MA) and used both in experiments and as a standard for titrating the other murine rGM-CSF–containing material on the GM-CSF–dependent cell line DA3.21,24 Anti-CD4 monoclonal antibody (MoAb) (clone RL/172),25 anti–IL-2 receptor α chain MoAb (clone 7D4),26 anti–IL-4 MoAb (clone 11B11),27 anti-la MoAb (clone BP107.2.2),28 and J11d.2 MoAb29 were used as hybridoma culture supernatants. Anti-CD8 MoAb was used as ascites fluid (clone 3.15)30 or culture supernatant (clone 3.168).30 Anti-CD3 MoAb (clone 145-2C11)31 was used as hybridoma culture supernatant and as protein A–purified material. Anti-Fc receptor MoAb (clone 2.4G2)32 was used as protein A–purified material. Goat anti-mouse GM-CSF polyclonal antiserum33 was a gift from DNAx Research Institute. Hamster anti-murine IFN-γ MoAb was purchased from Genzyme. Rat anti-mouse TNF-α MoAb was purchased from PharMingen (San Diego, CA). The following fluorescence-tagged antibodies were used as indicated: goat anti-rat (GAR) immunoglobulin–fluorescein isothiocyanate (FITC) (Caltag, South San Francisco, CA); anti-CD8–FITC (Becton Dickinson, Mountain View, CA); anti-CD4–PE (Becton Dickinson); anti–Thy-1–FITC (Becton Dickinson); Leu-4–FITC (antihuman CD3) (Becton Dickinson); and Leu-4 phycoerythrin (Becton Dickinson).

**Comitogenic assay.** Single-cell suspensions of thymocytes were obtained by gentle abrasion of the thymus on a sterile stainless steel screen and were washed twice in phosphate-buffered saline (PBS) plus 5% fetal bovine serum (FBS) before use. Thymocytes were cultured at 3 × 10⁵ cells per well in CM in flat-bottom microtiter plates (GIBCO) in the presence or absence of anti-CD3 MoAb hybridoma culture supernatant or Concanavalin (Con) A (Sigma, St Louis, MO), as indicated. Cytokines and additional antibodies were added as indicated. The plates were incubated at 37°C in 5% CO₂ for 4 days unless indicated otherwise. The plates were labeled with 1 μCi of [³H]-thymidine (NEN, Dupont, Boston, MA) for 24 hours, the cultured AC-enriched cells were harvested, washed, counted, and added to freshly isolated thymocytes enriched for responder cells (see following) (3 × 10⁵ wells) in the presence of 1.25 μg/mL Con A in 96-well plates. The cultures were incubated for an additional 3 days and labeled with 1 μCi [³H]-thymidine for the last 18 hours of the culture period.

The thymocyte responder population was depleted of most ACs by passage on nylon wool (NW) essentially as described previously.34 The NW-nonadherent cells were further treated with a 1:2 dilution of anti-la MoAb (anti-MHC class II) hybridoma supernatant for 30 minutes on ice followed by a 45-minute incubation with a 1:20 dilution of rabbit complement at 37°C. The cells were washed extensively and viable cells were recovered by separation on Lympholyte M and 50% Percoll. After 24 hours, the cultured AC-enriched cells were harvested, washed, counted, and added to freshly isolated thymocytes enriched for responder cells (see following) as indicated (3 × 10⁵ wells) for 3 days and labeled with 1 μCi [³H]-thymidine for the last 18 hours of the culture period.

Kinetic experiments. For addition experiments, thymocytes were plated into microtiter plates at 3 × 10⁵/well in the presence of 1.25 μg/mL Con A. GM-CSF 100 U/mL or medium was added at 0, 1, 2, or 3 days. The cultures were labeled for the last 18 hours of culture and harvested on day 4 as described above.

For subtraction experiments, 1 × 10⁶ thymocytes were cultured in 1 mL of CM in the presence of 1.25 μg/mL Con A or the combination of (1.25 μg/mL Con A + 100 U/mL GM-CSF) in 24-well plates (GIBCO) for 0, 1, 2, or 3 days. The cells were harvested, washed to remove excess factors, and recultured at 3 × 10⁵ (assuming no cell loss) in microtiter plates in CM in the presence or absence of 1.25 μg/mL Con A for the remainder of the 4-day culture period. Anti-GM-CSF antiserum at a concentration of 0.1% was added to cells recultured in Con A to neutralize residual GM-CSF. The cultures were labeled for the last 18 hours of culture and harvested on day 4 as described above.

**Flow-cytometric analysis of thymocyte cultures.** Thymocytes, 5 × 10⁵/mL, were cultured in the presence of 1.25 μg/mL Con A or (0.01% anti-CD3 MoAb hybridoma supernatant + 20 U/mL IL-2) in CM in 25-cm² tissue culture flasks (GIBCO). GM-CSF 100 U/mL was added as indicated. Cells were harvested at the times indicated, and viable cells were recovered by separation on Lympholyte M (Cedarlane, Accurate, Westbury, NY). After extensive washing, the cells were stained with anti–CD3-PE, anti–CD8-FITC, or anti–IL-2 receptor α chain (IL-2R) MoAb for 30 minutes at 4°C. Anti-IL-2R MoAb was followed by a 30-minute incubation at 4°C with GAR-FITC before analysis. A total of 5 × 10⁵ events were collected on a FACStar (Becton Dickinson) or FACStar Plus (Becton Dickinson). Viable cells based on forward and 90° angle light scatter were analyzed with logarithmic signal amplification.

**Accessory cell enrichment protocol.** A thymocyte accessory cell (AC)-enriched population was obtained by treating thymocytes at 5 × 10⁵ cells/mL with a 1:20 dilution of anti-CD4 MoAb hybridoma supernatant and a 1:20 dilution of anti-CD8 MoAb hybridoma supernatant for 30 minutes on ice. The cells were then spun down, washed once, and resuspended in a 1:20 dilution of rabbit complement (Cedarlane). The cells were incubated for an additional 45 minutes at 37°C. The cells were washed extensively, and viable cells were recovered by separation on Lympholyte M and cultured in 100 U/mL GM-CSF on medium in 24-well plates for 24 hours. After 24 hours, the cultured AC-enriched cells were harvested, washed, counted, irradiated (3,000 rad), and added to freshly isolated thymocytes enriched for responder cells (see following) (3 × 10⁵/well) in the presence of 1.25 μg/mL Con A in 96-well plates. The cultures were incubated for an additional 3 days and labeled with 1 μCi [³H]-thymidine for the last 18 hours of the culture period.

For fetal thymocytes, single-cell suspensions of 14-day fetal thymic lobes were prepared in PBS plus 5% FBS with a microtissue grinder, followed by one or two passages through a 23-gauge needle.
The cells were washed twice and counted before use. In some experiments, the cells were stained with anti-CD4-PE, anti-CD8-FITC, and anti-Thy-1-FITC.

Microculture assay. Single-cell suspensions of the cell populations described above were prepared at 160 cells/mL with additives at the indicated concentrations in CM further supplemented with 10 mmol/L of nonessential amino acids (GIBCO) and 1 mmol/L of sodium pyruvate (GIBCO). Ten microliters of the cell suspensions were plated in each well of a Terasaki plate (60-well HL-A plate; Nunc, Naperville, IL). For some experiments, anti-CD3 MoAb was linked to the plates by preincubating 10 μL of purified anti-CD3 MoAb, at the concentrations indicated, in 50 mmol/L of Tris, pH 8.8, for at least 1 hour at RT. The plates were washed twice with PBS and once with PBS plus 2% FBS before use.

After the cells were distributed, the plates were spun at 750g for 5 minutes to pellet the cells, after which each well was examined microscopically (200× magnification on an Olympus BH-2 microscope). Only wells containing one cell were included in subsequent analyses. The plates were incubated at 37°C in 10% CO2 in humidity chambers, constructed from square Petri dishes (Falcon), with PBS-saturated sterile gauze strips in the bottom. At the times indicated, wells were reexamined microscopically for proliferation. A well was scored positive if it contained more than one cell. This assay has been shown to be sensitive enough to detect the effects of cytokines on single cells.35 Statistical analysis of these experiments was performed using a chi-square test. Results were considered statistically significant at P ≤ .05.

RESULTS

Characterization of GM-CSF augmentation of TCR-mediated thymocyte proliferation. A number of stimuli, including antibodies to the TCR/CD3 complex and the mitogen Con A, can be used to mimic the antigen-MHC engagement of TCR on T cells or thymocytes and induce proliferation. Murine thymocytes proliferated in a concentration-dependent manner to soluble anti-CD3 MoAb (Fig 1A). In the presence of a suboptimal concentration of anti-CD3 MoAb (0.15%), IL-2 augmented thymocyte proliferation in a concentration-dependent manner (Fig 1B). GM-CSF augmented thymocyte proliferation when added to cultures containing suboptimal concentrations of both anti-CD3 (0.15%) and IL-2 (0.5 U/mL) (Fig 1C). GM-CSF also augmented anti-CD3-dependent proliferation in the presence of optimal IL-2 concentrations (data not shown), suggesting that GM-CSF does not act by inducing IL-2 production in these cultures. In the absence of anti-CD3 MoAb, neither IL-2 nor GM-CSF, alone or together, induced thymocyte proliferation (Fig 1C). These observations are consistent with reports that adult thymocyte responses to a number of cytokines, including IL-1, IL-2, IL-4, and IL-6, require a costimulus.

GM-CSF also augmented the Con A–induced proliferation of murine thymocytes (Fig 2A). This augmentation was seen only at mitogenic concentrations of Con A; when Con A was used at submitogenic concentrations, no augmentation by GM-CSF was seen (Fig 2A). Furthermore, when GM-CSF was added in the absence of Con A, no thymocyte proliferation was measured (Fig 2A). At a suboptimal, but mitogenic concentration of Con A (1.25 μg/mL), augmentation of the thymocyte response by GM-CSF was dose-dependent, with augmentation consistently measured at 100...
The kinetics of thymocyte proliferation were compared in cultures stimulated with either Con A or Con A plus GM-CSF (Fig 3). No differences were found in either the time at which proliferation was first detectable, day 3 (Fig 3A), or the time at which peak levels of proliferation occurred, days 4 and 5 (Fig 3B). Thus, GM-CSF does not appear to augment Con A–induced thymocyte proliferation by altering the kinetics of the proliferative response.

To define the period in the Con A comitogenic assays when GM-CSF was required, GM-CSF was added to, or removed from, cultures at various times. To induce an enhanced response in a 4-day culture, GM-CSF was required at the initiation of culture. Addition of GM-CSF on day 1, 2, or 3 failed to induce greatly augmented proliferation (Fig 4A). In reciprocal experiments, thymocytes were stimulated with Con A or Con A plus GM-CSF on day 0; on day 0 (immediately after set up), and on day 1, 2, and 3 of culture, samples were harvested, washed to remove additives, and recultured in Con A or medium for the remainder of the 4-day culture period. Anti–GM-CSF antiserum was added to the samples recultured in Con A to ensure that any residual

U/mL GM-CSF (Fig 2B). An anti–GM-CSF antiserum at a concentration of 0.1% inhibited GM-CSF augmentation of Con A–induced thymocyte proliferation, but not basal Con A–induced proliferation (Fig 2C). Normal goat serum did not inhibit thymocyte proliferation induced by Con A or by Con A plus GM-CSF (data not shown).
GM-CSF AUGMENTS THYMOCYTE PROLIFERATION

GM-CSF would be neutralized. GM-CSF augmentation was observed even if the GM-CSF had been removed from cultures after only 1 day (Fig 4B). In contrast, if Con A was removed from the cells at any time, almost no proliferation was measured. These results indicate that the GM-CSF-responsive cells require early delivery of the GM-CSF signal, and that delivery of this signal occurs within the first day of culture.

Two of the early consequences of the activation of T cells by mitogens, such as Con A or MoAb to the TCR/CD3 complex, are the upregulation of the expression of the IL-2R on the surface of the cells and induction of the production and secretion of IL-2. To test if IL-2 was important in the GM-CSF-induced augmentation of thymocyte proliferation, thymocytes were stimulated with Con A or Con A plus GM-CSF in the presence and absence of anti-IL-2R MoAb hybridoma supernatant. Both the Con A and the Con A plus GM-CSF responses of the thymocytes were greatly inhibited in the presence of anti-IL-2R α chain MoAb (Fig 5A), indicating that IL-2 is required for the maximal proliferation in the GM-CSF augmented thymocyte cultures. Although thymocyte proliferation in this system is largely IL-2-dependent, GM-CSF does not appear to act solely by inducing the production of IL-2 in the thymocyte cultures, since GM-CSF did not augment anti-CD3 MoAb-induced proliferation in the absence of IL-2 (Fig 1C), but did augment proliferation in the presence of optimal IL-2 concentrations (data not shown). We also examined whether GM-CSF induced the upregulation of IL-2R α chain expression on the thymocytes. Although Con A did upregulate IL-2R α chain expression (Table 1, experiment 1), no differences were seen in the IL-2R α chain expression on thymocytes cultured with either Con A or anti-CD3 plus IL-2 in the presence or absence of GM-CSF. These results suggest the IL-2R α chain is upregulated in this system by the costimulus and that GM-CSF does not have an effect or this upregulation.

IL-4, IFN-γ, and TNF-α have all been shown to augment thymocyte proliferation in the presence of comitogens. MoAbs to IL-4, IFN-γ, and TNF-α were therefore added to thymocyte cultures supplemented with Con A and Con A plus GM-CSF to test whether any of these growth factors was required for GM-CSF augmentation of Con A-induced proliferation. In contrast to anti-IL-2R MoAb (Fig 5A) and anti-GM-CSF antiserum (Fig 2C), none of these antibodies inhibited Con A plus GM-CSF-induced thymocyte proliferation (Fig 5B, C, and D). These results suggest that IL-4, IFN-γ, and TNF-α are not involved in the GM-CSF augmentation of thymocyte proliferation.

To examine whether GM-CSF acted by recruiting additional thymocyte subsets into the response, the phenotype

---

**Table 1. GM-CSF Does Not Induce IL-2R α Receptor Expression on Thymocytes**

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Costimulus*</th>
<th>Length of Culture (h)</th>
<th>Percentage of IL-2R α+ Chain Positive Cells</th>
<th>MFI of IL-2R α Chain Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>---</td>
<td>20</td>
<td>5</td>
<td>44</td>
</tr>
<tr>
<td>Con A</td>
<td>---</td>
<td>20</td>
<td>23</td>
<td>88</td>
</tr>
<tr>
<td>Con A +</td>
<td>20</td>
<td>18</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Con A -</td>
<td>48</td>
<td>20</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Con A +</td>
<td>48</td>
<td>19</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>Con A</td>
<td>---</td>
<td>96</td>
<td>18</td>
<td>607</td>
</tr>
<tr>
<td>Con A +</td>
<td>96</td>
<td>24</td>
<td>562</td>
<td></td>
</tr>
<tr>
<td>αCD3 + IL-2</td>
<td>96</td>
<td>52</td>
<td>782</td>
<td></td>
</tr>
<tr>
<td>αCD3 + IL-2 +</td>
<td>96</td>
<td>57</td>
<td>839</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: MFI, mean fluorescence intensity.

The following concentrations were used: Con A, 1.25 μg/mL, anti-CD3 MoAb, 0.01%, IL-2, 20 U/mL, and GM-CSF, 100 U/mL. This is a different batch of anti-CD3 MoAb hybridoma supernatant than the one used in Figs 1A through C. This batch had a higher titer of anti-CD3 MoAb.

*Thymocytes were stained with anti-IL-2R α chain MoAb, followed by goat anti-rat Ig-FITC (GAR-FITC).
of thymocyte responders was determined after a 4-day culture in the presence of Con A or Con A plus GM-CSF. CD4 and CD8 expression on thymocytes stimulated with Con A and Con A plus GM-CSF was examined by flow cytometry. There was no consistent or dramatic effect of GM-CSF on the phenotype of cells after culture (data not shown). Thus, GM-CSF does not recruit additional thymocyte subsets that are distinguishable by CD4 or CD8 expression.

In summary, GM-CSF augments TCR-mediated thymocyte proliferation by acting early in the 4-day culture period. GM-CSF’s effect is mediated primarily, or completely, through an IL-2-dependent pathway. However, GM-CSF augmentation does not appear to be mediated by induction of IL-2 (Fig 1C), nor does GM-CSF induce the expression of IL-2R on thymocytes (Table 1). These results suggest that GM-CSF may not act directly on the T-lineage cells in the thymocyte population, but instead may act on ACs required for T-cell proliferation.

Effect of GM-CSF on thymic ACs. GM-CSF has been shown to induce effector functions of mature macrophages and dendritic cells. To determine whether GM-CSF-responsive ACs were present in the thymus, preincubation of AC-enriched populations was performed. The AC-enriched cells were cultured in GM-CSF or medium for 24 hours, then irradiated and cultured with freshly isolated thymocytes enriched for responder cells (La^-NW-nonadherent-thymocytes; a population that is not completely AC-depleted, but whose Con A response is enhanced by addition of exogenous AC cells). The Con A–induced proliferation of responders cocultured with GM-CSF–pretreated AC-enriched cells was much greater than that of responders cocultured with medium-pretreated AC-enriched cells (Fig 6). Thus, 24-hour GM-CSF pretreatment of an AC-enriched population augmented AC activity as measured in an AC-dependent proliferation assay.

These results indicated that it would be necessary to completely remove thymic ACs to determine if GM-CSF could also act directly on thymic T-lineage cells. The removal of the ACs was attempted using a rigorous depletion protocol that took advantage of the adherence properties, phagocytic properties, and La expression of the ACs. The AC-depleted populations were then tested for their ability to respond to an AC-dependent stimulus. We were unable to obtain a thymocyte population in which we had completely removed
GM-CSF AUGMENTS THYMOCYTE PROLIFERATION

In the presence of anti-CD3 MoAb and IL-2, GM-CSF did not augment thymocyte proliferation (Fig 7). This is in striking contrast to the AC-dependent bulk culture assays (Figs 1C and 2 through 5) in which GM-CSF augmented TCR-dependent thymocyte proliferation. These results, in conjunction with the AC pre-culture experiments (Fig 6), suggest that GM-CSF augmentation of TCR-dependent mature thymocyte proliferation is due to an augmenting effect of GM-CSF on thymic accessory cells, and not to a direct effect of GM-CSF on the mature single-positive T-lineage thymocytes themselves.

Effect of GM-CSF on SP-enriched thymocytes. Although GM-CSF does not appear to act directly on mature SP thymocytes, GM-CSF has been shown to act directly on an immature T-cell line, TALL-101. Consequently, we investigated whether GM-CSF is active on normal immature thymocytes. The single-cell assay was used to examine whether GM-CSF had an effect on the proliferation of the DN thymocyte subset. Most of the DN thymocytes are also negative for cell surface expression of TCR/CD3 complex. The proliferation of the DN thymocyte populations was therefore examined in the presence of GM-CSF without any anti-CD3 MoAb costimulus.

The first DN population examined was adult DN-enriched thymocytes. This population was greater than 95% the ability to respond in an AC-dependent manner, suggesting that there may be a population of thymic ACs that is very difficult to deplete. A candidate for this type of AC may be the dendritic cell. Only a few dendritic cells are required to enhance Con A-induced thymocyte proliferation, and the accessory function of these cells is enhanced by GM-CSF.

Effect of GM-CSF on SP-enriched thymocytes. To analyze the effect of GM-CSF on TCR-mediated proliferation of T-lineage thymocytes, an AC-independent single-cell assay was used. In this assay, cells were plated into the wells of Terasaki plates at a concentration that maximized the frequency of wells containing one cell, which were identified microscopically before culture. The wells were precoated with anti-CD3 MoAb to provide a multivalent matrix for TCR/CD3 cross-linking on SP thymocytes (the major TCR/CD3-responsive population), making the cultures AC-independent. The single-cell assay distributes SP thymocytes and ACs into separate wells, so that the TCR-stimulated proliferation of the SP thymocytes occurs in the absence of any ACs. The ACs themselves are TCR/CD3⁺ and would not be expected to proliferate in response to anti-CD3 MoAb.

From the AC-dependent comitogenic assays, we knew that GM-CSF augmentation was most readily seen when the comitogen was at a suboptimal concentration (data not shown), and that thymocyte proliferation induced at suboptimal concentrations of anti-CD3 MoAb required exogenous IL-2 (Fig 1C). In the single-cell AC-independent assay, 2 μg/mL of anti-CD3 MoAb and 10 U/mL of IL-2 were identified as appropriate suboptimal concentrations, at which SP thymocyte proliferation was low but consistent (data not shown).

When GM-CSF was added to single-cell cultures of enriched SP thymocytes containing these suboptimal concentrations of anti-CD3 MoAb and IL-2, GM-CSF did not augment thymocyte proliferation (Fig 7). This is in striking contrast to the AC-dependent bulk culture assays (Figs 1C and 2 through 5) in which GM-CSF augmented TCR-dependent thymocyte proliferation. These results, in conjunction with the AC pre-culture experiments (Fig 6), suggest that GM-CSF augmentation of TCR-dependent mature thymocyte proliferation is due to an augmenting effect of GM-CSF on thymic accessory cells, and not to a direct effect of GM-CSF on the mature single-positive T-lineage thymocytes themselves.

Effect of GM-CSF on SP-enriched thymocytes. Although GM-CSF does not appear to act directly on mature SP thymocytes, GM-CSF has been shown to act directly on an immature T-cell line, TALL-101. Consequently, we investigated whether GM-CSF is active on normal immature thymocytes. The single-cell assay was used to examine whether GM-CSF had an effect on the proliferation of the DN thymocyte subset. Most of the DN thymocytes are also negative for cell surface expression of TCR/CD3 complex. The proliferation of the DN thymocyte populations was therefore examined in the presence of GM-CSF without any anti-CD3 MoAb costimulus.

The first DN population examined was adult DN-enriched thymocytes. This population was greater than 95%
This population was greater than 96% DN cells (data not shown), with greater than 81% of these cells expressing intermediate to high levels of Thy-1 (data not shown), indicating that these cells also represent T-lineage cells. As shown in Fig 8B, 32% of the single-cell–containing wells exhibited proliferation in the presence of GM-CSF, compared with 23% in the absence of GM-CSF. These experiments suggest that GM-CSF can directly induce proliferation of immature DN T-lineage thymocytes in the absence of a co-stimulus.

This may be further supported by the fact that the Thy-1+ cells within the DN population are very heterogeneous and it is therefore unlikely that GM-CSF would be a stimulus for a significant fraction of the contaminating cell types. The Thy-1+ population contains B cells,42 natural killer (NK) cells,43 immature myeloid cells,44 and macrophages. B cells and NK cells have not been shown to respond to GM-CSF. The immature macrophages,44 the mature ACs,45 and the dendritic cells,40,41 which do express low levels of Thy-1, would not be expected to proliferate in the 2-day period of the assay. Although some proliferation of the myeloid cell types is not ruled out by our experiments, it is doubtful that myeloid cells could account for the augmented proliferation measured. Thus, the results presented in this study demonstrate that GM-CSF can stimulate mature SP thymocytes indirectly via augmentation of AC function, and suggest that GM-CSF can directly stimulate immature DN thymocytes.

**DISCUSSION**

GM-CSF augments TCR-mediated thymocyte proliferation. Augmentation by GM-CSF was readily seen when the comitogenic stimulus, either anti-CD3 plus IL-2 or Con A alone, was at a limiting concentration. A brief 24-hour exposure to GM-CSF at the initiation of culture was necessary and sufficient to induce augmented thymocyte proliferation. Thymic epithelial cell lines have been demonstrated to produce not only GM-CSF,5,11,12 but also GM-CSF4,10 and M-CSF.10 However, unlike GM-CSF, neither G-CSF nor M-CSF augmented Con A–induced thymocyte proliferation (data not shown). This suggests that among CSFs, GM-CSF specifically may play an important role in T-cell expansion.

The addition of anti-IL-2R MoAb inhibited the response induced by both Con A and by Con A plus GM-CSF, indicating that IL-2 is necessary for thymocyte proliferation in this system. However, it is unlikely that GM-CSF is merely stimulating the production of more IL-2 in the cultures, since GM-CSF did not augment anti-CD3 MoAb–induced proliferation in the absence of exogenously added IL-2 (Fig 1C), but GM-CSF did augment in the presence of optimal IL-2 concentrations (data not shown). IL-7 has been shown to induce IL-2R expression on thymocytes.36,42 However, we found no marked differences in IL-2R α chain expression between thymocytes stimulated with Con A and Con A plus GM-CSF, or between thymocytes stimulated with anti-CD3 plus IL-2 and anti-CD3 plus IL-2 plus GM-CSF as measured by flow cytometry (Table 1). These results suggest...
that GM-CSF does not mediate its effects through the upregulation of the IL-2Rα chain.

Addition of anti-IL-4 MoAb, anti-IFN-γ MoAb, or anti-TNF-α MoAb did not inhibit proliferation induced by Con A or by Con A plus GM-CSF. These results indicate that GM-CSF augmentation is not mediated through the action of IL-4, IFN-α, or TNF-γ in the cultures. These experiments do not rule out the possibility that other factors induced by GM-CSF may mediate the augmenting effect in thymocyte cultures stimulated with IL-1 in the absence of comitogens, augmentation by GM-CSF has been demonstrated. In that study, anti-IL-7 MoAb was able to significantly inhibit thymocyte proliferation induced by IL-1 and by IL-1 plus GM-CSF. However, the reduced proliferation was still above background, suggesting that IL-1 and GM-CSF may have effects other than the stimulation of IL-7 production.

To eliminate any influence of GM-CSF-activated AC on SP thymocytes, we examined SP thymocyte proliferation in an AC-independent TCR-mediated single-cell assay. In the single-cell assay, GM-CSF did not augment the proliferation of highly enriched SP cells cultured in the presence of limiting concentrations of anti-CD3 MoAb and IL-2 (Fig 7). These results suggest that GM-CSF cannot act directly on SP thymocytes and that GM-CSF augmentation of TCR-mediated thymocyte proliferation therefore occurs through the stimulation of ACs. Thus, one role of GM-CSF in the thymus may be to indirectly augment expansion of the T-lineage SP population.

A second role for GM-CSF in the thymus may be to directly induce the expansion of the T-lineage DN population. GM-CSF induced significant proliferation of highly enriched adult and fetal DN thymocytes in an AC-independent and TCR-independent single-cell assay (Fig 8). Both the adult and fetal DN populations in our studies contained greater than 75% Thy-1+ cells, indicating that the majority of the cells in these populations are T lineage. A small subset of the T-lineage DN population is CD3+ and expresses either γδ or αβ TCR; these cells appear to be functionally mature T cells that may represent an alternate differentiation pathway to the αβ TCR/CD3+ SP T cells. The majority of T-lineage DN thymocytes are immature CD3- cells that vary in phenotype and progenitor capacity. The T-lineage DN thymocytes that most closely resemble the bone marrow precursor are in a state of rapid proliferation, but the cytokine(s) responsible for inducing this proliferation are still unknown. The studies in this report suggest that GM-CSF should be considered as a candidate for this activity. With the recent cloning of the α chain of the murine GM-CSF receptor, it will be possible to determine which, if any, T-lineage DN thymocytes express the high-affinity GM-CSF receptor and might proliferate in response to GM-CSF.

The DN thymocyte population also contains cells capable of myeloid differentiation, but not proliferation in response to GM-CSF. Papiernik et al demonstrated that after a 4-day culture of a CD4+ CD8+ Ia+ Mac-1+ thymocyte population with GM-CSF, cells expressing Mac-1 could be detected, suggesting that macrophage differentiation had occurred. In other studies, dendritic cells cultured in GM-CSF underwent a maturation process in the absence of proliferative GM-CSF; the function of Ia+ dendritic dendritic cell precursors in thymocyte mitogenesis to lectin and lectin plus interleukin 1.

REFERENCES

2. Gore SD, Kastan MB, Cavin CI: Normal human bone marrow precursors that express terminal deoxynucleotidyl transferase include T-cell precursors and possible lymphoid stem cells. Blood 77:1681, 1991
cell development in murine fetal thymus organ cultures: interleukin
1 circumvents the block in T cell differentiation induced by mono-

15. Suda T, Zlotnik A: IL-7 maintains the T cell precursor poten-

16. Metcalf D: The granulocyte-macrophage colony-stimulating

17. Clark SC, Kamen R: The human hematopoietic colony-

2/interleukin 4-dependent T cell line induced by gran-
ulocyte-macrophage colony-stimulating factor (GM-CSF). J Im-
munol 138:4288, 1987

19. Park L, Friend D, Gillis S, Urdal DL: Characterization of the cell
surface receptor for granulocyte-macrophage colony-stimulating
factor J Biol Chem 261:177, 1986

20. Groopman JE, Mitsuysu RT, DeLeo MJ, Oette DH, Golde
DW: Effect of recombinant human granulocyte-macrophage colon-
y-stimulating factor on myelopoiesis in the acquired immuno-

21. Stewart-Akers AM, Cairns JS, Tweardy DJ, McCarthy SA:

22. Valtieri M, Santoli D, Caraccollo D, Kreuder BL, Altmann
SW, Tweardy DJ, Gemperlein I, Mavilio F, Lange B, Rovera G:
Establishment and characterization of an undifferentiated human
T-leukemia cell line which requires granulocyte-macrophage colo-

ting factor: comparison of the mouse and human genes. EMBO J
4:2561, 1985

24. Rennick D, Yang G, Gemmell L, Lee F: Control of hemo-
poiesis by a bone marrow stromal cell clone: Lipopolysaccharide
and interleukin-1-inducible production of colony stimulating fac-

25. Ceredig R, Lowenthal JW, Nabolz M, MacDonald HR: Expression of interleukin-2 receptors as a differentiation marker on

26. Malek TR, Robb RJ, Shevach EM: Identification and initial characteriza-
tion of a rat monoclonal antibody reactive with murine interleukin
2 receptor-ligand complex. Proc Natl Acad Sci USA 80: 1276, 1983

27. Ohara J, Paul WE: Production of a monoclonal antibody to
murine I-A antigens linked to the maturation of macrophage/dendritic cells within the isolation of functional thymus-derived murine lymphocytes. Thymus 12:s1. 1988

28. Symington FW, Sprent J: A monoclonal antibody detecting an
Ia specificity mapping in the I-A or I-E subregion. Immunogen-
etics 14:53, 1981


31. Lee O, Foo M, Sachs DH, Samelson LE, Bluestone JA: Identifi-
cation of a monoclonal antibody specific for murine T3. Proc
Natl Acad Sci USA 84:1374, 1987

32. Usunoff J: Characterization of a monoclonal antibody di-
rected against mouse macrophage and lymphocyte Fc receptors. J
Exp Med 150:580, 1979

33. Miyajima A, Otsu K, Schreurs J, Bond MW, Abrams JS,
Arai K: Expression of murine and human granulocyte-macrophage colon-
y-stimulating factors in S. cerevisiae: Mutagenesis of the po-
tential glycosylation sites. EMBO J 5:1193, 1986


35. Chen W-F, Ewing T, Scollay R, Shortman K: Growth of single
T cells and single thymocytes in a high cloning efficiency filler-
cell free micro culture system. Thymus 12:51, 1988


Muramatsu S: Macrophage factors which enhance the mixed leu-


Naito K, Muramatsu S: Accessory cell functions of dendritic cells
and macrophages in the thymic T-cell response to Con A. Immunol
62:393, 1987

40. Witmer-Pack MD, Olivier W, Valinsky J, Schuler G, Steim-
man RM: Granulocyte-macrophage colony-stimulating factor is es-
tential for the viability and function of cultured murine epidermal

41. Heuller C, Koch F, Schuler G: Granulocyte-macrophage colony-
stimulating factor and interleukin-1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimula-

42. Miyama-Inaba M, Kuma S-H, Inaba K, Ogata H, Iwai H,
Yasunizu R, Muramatsu S, Steimman RM, Ikehara S: Unusual phe-

43. Garnier-Wagner BA, Wittke PL, Tutt MM, Kuziel WA, Tucker
PW, Bennett M, Kumar V: Natural killer cells in the thymus: Studies in mice with severe combined immune deficiency. J Immunol
144:796, 1990

44. Papiernik M, Lepault F, Pontoux C: Synergistic effect of col-
y-stimulating factors and IL-2 on prothymocyte proliferation linked to the maturation of macrophage/dendritic cells within

45. Cheung DL, Hamilton JF: Regulation of human monocyte
dNA synthesis by colony-stimulating factors, cytokines, and cyclic

46. Armitage RJ, Namen A, Sassenfeld HM, Grabstein KH:
Regulation of human T cell proliferation by IL-7. J Immunol
144:938, 1990

47. Watson JD, Morrissey PJ, Namen AE, Conlon PJ, Widmer

48. Herbelin A, Machavoine F, Schneider E, Papiernik M, Dy
M: IL-7 is requisite for IL-1-induced thymocyte proliferation.
Involvement of IL-7 in the synergistic effects of granulocyte-macro-
phage colony-stimulating factor or tumor necrosis factor with IL-1.

49. Penit C, Vasseur F: Sequential events in thymocyte differenti-
ation and thymus regeneration revealed by a combination of bromodeoxyuridine DNA labeling and anti-mitotic drug treat-


Granulocyte-macrophage colony-stimulating factor augmentation of T-cell receptor-dependent and T-cell receptor-independent thymocyte proliferation

AM Stewart-Akers, JS Cairns, DJ Twardy and SA McCarthy