Ability of flt3 Ligand to Stimulate the In Vitro Growth of Primitive Murine Hematopoietic Progenitors Is Potently and Directly Inhibited by Transforming Growth Factor-β and Tumor Necrosis Factor-α

By Sten E.W. Jacobsen, Ole Petter Veiby, June Myklebust, Ceciie Okkenhaug, and Stewart D. Lyman

The recently cloned flt3 ligand (FL) stimulates the growth of primitive hematopoietic progenitor cells through synergistic interactions with multiple other cytokines. The present study is the first demonstrating cytokines capable of inhibiting FL-stimulated hematopoietic cell growth. Tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) potently inhibited the clonal growth of murine Lin- Sca-1+ bone marrow progenitors stimulated by FL alone or in combination with granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF), interleukin (IL)-3, IL-6, IL-11, or IL-12. TGF-β1 inhibited more than 96% of the myeloid colony formation in response to these cytokine combinations, whereas TNF-α reduced the number of colonies by 58% to 96% depending on the cytokine by which FL was combined. In addition, both TNF-α and TGF-β1 inhibited more than 90% of B220+ cell production from B220− bone marrow cells stimulated by FL + IL-7. The effects of TNF-α and TGF-β1 appeared to be due to a direct effect and on the early progenitors because the inhibition was observed at the single cell level, and because delayed addition of the two inhibitors for only 48 hours dramatically reduced their inhibitory effects. A neutralizing anti–TGF-β antibody showed the presence of endogenous TGF-β in the cultures and potently enhanced the ability of FL to stimulate progenitor cell growth in the absence of other cytokines. Agonistic antibodies specifically activating the p75 TNF receptors were more efficient than wild type murine TNF-α in signaling growth inhibition of Lin-Sca-1+ progenitor cells, whereas the p55 agonist had less effect than murine TNF-α. Finally, TGF-β1 increased the number of FL + IL-11–stimulated Lin-Sca-1+ cells in the G0 phase of the cell cycle with 76%, whereas TNF-α only had a marginal effect on cell cycle distribution. Thus, TGF-β, TNF-α, and p75 TNF receptor agonists are potent direct inhibitors of FL-stimulated progenitor cell growth in vitro.

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HEMATOPOIESIS is regulated, at least in part, through the opposing action of stimulatory and inhibitory cytokines.1-3 Cytokines with stimulatory effects on hematopoietic progenitor cell growth include the colony stimulating factors (CSFs), erythropoietin (Epo), multiple interleukins, leukemia inhibitory factor (LIF), stem cell factor (SCF; also called mast cell growth factor, kit ligand, and steel factor), and the recently cloned thrombopoietin.4-6 Transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and macrophage inflammatory protein-1α (MIP-1α) are cytokines with predominantly inhibitory effects on hematopoietic progenitors in vitro.7,11 However, under certain circumstances these cytokines can also directly stimulate the growth of hematopoietic progenitor cells.8,10,12,15

SCF has been identified as an essential regulator of hematopoiesis, and its relatively selective action on more primitive hematopoietic progenitor cells is well established.14-16 Thus, although SCF stimulates the growth of committed progenitors of the myeloid/erythroid and mast cell lineage as well,15,19 it has until recently been considered the most selective cytokine for early hematopoiesis. In particular, its potent ability to synergize with most other hematopoietic growth factors (HGFs) is unsurpassed.16-25 SCF signals through the c-kit tyrosine kinase receptor,26,27 which shows homology to c-fms28 and flt3/flk-2 receptors.29,30 In the hematopoietic system, flt3/flk-2 appears to be expressed selectively on primitive progenitors, although most long-term reconstituting stem cells appear not to express flt3.31,32 The flt3 ligand was recently cloned (FL)32,34 and although its range of activities have not yet been established in detail, it is clear that as SCF, it can synergize with multiple HGFs to enhance the growth and expansion of very primitive hematopoietic progenitor cells.31,37 In addition, FL can stimulate the growth of B- and T-cell progenitor cells.31,37,38

TGF-β is a bidirectional regulator of the growth of hematopoietic progenitor cells in that it can either inhibit or stimulate their growth.7,8,11 The most pronounced inhibitory effects of TGF-β are observed on primitive progenitors,7,8 and accordingly TGF-β is a potent inhibitor of SCF-stimulated progenitor cell growth.38

TNF-α is maybe the most pleiotropic cytokine isolated to date. Whereas the importance of TNF-α in normal steady state is unclear, it appears essential for nonspecific immunity.39-40 In addition, excessive production of TNF-α has been implicated to play a role in the pathogenesis of many acute and chronic diseases.41-48 One of them being the frequent anemia of chronic diseases (ACD).44 The effect of TNF-α on hematopoietic progenitor cell growth can either be inhibitory or stimulatory, depending on the cytokine stimulating growth.13,45-47 Specifically, TNF-α can enhance granulocyte macrophage (GM)-CSF–and interleukin (IL)-3–stimulated colony formation of normal human CD34+ bone marrow progenitor cells, and inhibit G-CSF–and CSF-1–stimulated growth.13,45-47 Furthermore, SCF-stimulated growth of normal progenitors is inhibited by TNF-α, whereas SCF-stimulated proliferation of leukemic progenitors can be enhanced.46,48

Recently two distinct TNF receptors, with molecular
weights of 55 kD (TNFR55) and 75 kD (TNFR75), have been cloned and demonstrated to be able to signal separately.\(^5\)\(^-\)\(^\text{33}\) Using agonistic antibodies or TNF-\(\alpha\) mutants, we have recently demonstrated that TNFR55 signals growth stimulatory and inhibitory effects on committed bone marrow progenitors, whereas activation of TNFR75 signals inhibition of CSF-stimulated growth of more primitive progenitors.\(^\text{34}\)\(^-\)\(^\text{36}\)

Whereas a number of studies have established the ability of FL to interact with multiple cytokines to synergistically enhance the growth of primitive hematopoietic progenitors, its interactions with inhibitory/bidirectional regulators such as TNF-\(\alpha\) and TGF-\(\beta\) have not yet been characterized. Accordingly, the present studies were designed to investigate the ability of TNF-\(\alpha\) and TGF-\(\beta\) to affect FL-stimulated myeloid and B-cell growth and differentiation from primitive murine hematopoietic progenitor cells.

**MATERIALS AND METHODS**

**Hematopoietic Growth Factors and TNF Receptor Agonistic Antibodies**

Recombinant murine flt3 ligand was cloned and purified as previously described.\(^\text{37}\) Purified recombinant murine (rm)IL-3 was from Peprotech Inc (Rocky Hill, NJ). Purified rhCSF-1 and recombinant rat (r)CSF were generously supplied by Dr Ian K. McNiece (Amgen Corp, Thousand Oaks, CA). rhCSF-1 was kindly provided by Dr Michael Geier (Cetus Corp, Emeryville, CA), and rhIL-6 and rhIL-11 was a gift from Genentech (Cambridge, MA). rmIL-12 was a generous gift from Dr Stan Wolf (Genetics Institute, Cambridge, MA), and rhIL-7 and rhIL-11 was a gift from Genentech (Genetics Institute, Cambridge, MA), and rhIL-7 from Immunex (Seattle, WA). rh-erythropoietin (Epo) was purchased from Cilag AG (Schaffhausen, Switzerland). rmTNF-\(\alpha\) was kindly supplied by Genentech (San Francisco, CA), and rhTNF-\(\alpha\) was a gift from Dr Werner Lesslauer (F. Hoffmann-La Roche AG, Basel, Switzerland). rhTGF-\(\beta\)-1 was a gift from Tony Purchio (Oncogene Corp, Seattle, WA). A mouse IgG, antibody neutralizes murine and human TGF-\(\beta\) (alpha and beta) as well as chicken TGF-\(\beta\), was kindly provided by Dr James R. Dash (Celtrix Laboratories, Palo Alto, CA). Another irrelevant mouse IgG, antibody was used as a control. Rabbit antimurine TNFR55 and TNFR75 polyclonal antibodies were generated against the soluble extracellular domains of the respective receptors as described previously.\(^\text{38}\) These antibodies have been demonstrated to act as specific agonists to the murine TNFR55 and TNFR75, respectively, and can mimic the activities of mTNF-\(\alpha\) on the respective receptors.\(^\text{39}\)\(^-\)\(^\text{41}\) The effect of the TNFR55 agonist was titrated as previously described on G-CSF-stimulated growth of Lin- bone marrow cells. Preimmune polyclonal rabbit IgG was used as a control. Unless otherwise indicated, all growth factors and antibodies were used at predetermined optimal concentrations; rhCSF-50 ng/mL, rmIL-3 20 ng/mL, rhCSF-1 50 ng/mL, rmCSF-100 ng/mL, rhIL-6 50 ng/mL, rhIL-7 100 ng/mL, rhIL-11 50 ng/mL, rmIL-12 50 ng/mL, rmIL-3 5 IU/mL, rmFL 250 ng/mL, rmTNF-\(\alpha\) 20 ng/mL, rhTNF-\(\alpha\) 20 ng/mL, rhTGF-\(\beta\)-1 20 ng/mL, TNFR55 and TNFR75 agonists at 1:2000 dilution, and the anti-TGF-\(\beta\)-1 antibody at 5 \(\mu\)g/mL.

**Isolation of Lin \(\mathrm{Sca}^+\) Bone Marrow Cells**

Lin- bone marrow cells were isolated from femurs of normal C57Bl/6 mice (5 to 8 weeks old), according to a previously described protocol.\(^\text{42}\) Briefly, low-density bone marrow cells were obtained using lymphocyte separation medium (Nycomed, Oslo, Norway). Cells were washed twice in Iscove's modified Dulbecco's medium (IMDM; Gibco, Paisley, UK) and resuspended in IMDM supplemented with 20% fetal calf serum (FCS) (Biowhittaker, Walkersville, Maryland), penicillin 100 U/mL, and 3 mg/mL L-glutamine (complete IMDM). The cells were incubated at 4°C for 30 minutes in a cocktail of predetermined optimal concentrations of antibodies; RA3-6B2 (B220 antigen; Pharmingen, San Diego, CA), BV6-RC5 (GR-1 antigen; Pharmingen), MA-C1 (Serotec, Oxfordshire, UK), Ly-2 (CD8; Becton Dickinson, Sunnyvale, CA), L3T4 (CD4; Pharmingen), and TER-119 (gift from Dr Tatsuo Kina, Kyoto University, Japan). Cells were washed twice and resuspended in complete IMDM. Sheep antirat IgG (Fc) conjugated immunomagnetic beads (Dynal, Oslo, Norway) were added at a cell to bead ratio of 1 to 20 and incubated on ice for 30 minutes. Labeled (Lin-) cells were removed by a magnetic particle concentrator (Dynal), and Lin- cells recovered from the supernatant. Lin- Sca-1+ cells were isolated as described by others.\(^\text{43}\)\(^-\)\(^\text{45}\) Briefly, 4 to 6 \(\times\) 10\(^5\) Lin- cells were resuspended per milliliter of complete IMDM, and incubated for 30 minutes on ice with either fluorescein isothiocyanate (FITC)-conjugated rat antimouse Sca-1 antibody (clone E13161-7; Pharmingen) or an isotype-matched control antibody. The cells were washed once, and Lin- Sca-1+ cells sorted on a Coulter Epics Elite Cell Sorter (Coulter Electronics, Hialeah, FL) equipped with a 488 nm tuned argon laser set to give a power of 15 mW, with a rate of 1,500 to 2,000 cells/second. Lin- cells falling in median right angle scatter and median to high forward scatter were analyzed for Sca-1 expression, and cells falling into both regions were selected. Light scatter was collected through a 488 nm band pass filter and FITC fluorescence was collected through a 488 nm long pass filter and a 525 nm band pass filter. The final recovery of Lin- Sca-1+ cells was 0.05% to 0.1% of the unfraccionated bone marrow.

**Single Cell Proliferation Assay**

Lin- Sca-1+ cells were seeded in microplate wells (Nunc, Kamstrup, Denmark) at a concentration of 1 cell per well in a volume of 20 \(\mu\)L IMDM (supplemented with 20% FCS, 100 U/mL penicillin, and 3 mg/mL L-glutamine), either manually or in some experiments by using a single cell deppositor coupled to the cell sorter. This method assures that >99% of the wells contain only one cell. Similar results were obtained with both methods. Wells were scored for colony growth (>50 cells) and clusters (10 to 50 cells) after 12 to 14 days incubation at 37°C and 5% CO\(_2\) in air.

**Cell Morphology**

Cells cultured in complete IMDM and different cytokine combinations were transferred to slides in a cytospin centrifuge. Following methanol fixation and Giemsa staining, morphology was examined under a light microscope.

**Cell Phenotyping by Flow Cytometry**

A total of 1 \(\times\) 10\(^6\) Lin- or Lin- Sca-1+ cells were plated in 400 \(\mu\)L complete IMDM with cytokines as indicated. Following a 7- to 9-day (Lin-) or 12-day (Lin- Sca-1+ colony) incubation, cells were counted, harvested, and analyzed for B220 and GR-1 expression by flow cytometry (FACSort; Becton Dickinson, San Jose, CA) after a 30-minute incubation on ice with phycoerythrin (PE)-conjugated rat antimouse B220 or FITC-conjugated rat antimouse GR-1 antibodies (Pharmingen). Control cells were stained with isotype-matched PE- or FITC-conjugated control antibodies (Pharmingen).

**Cell Cycle Analysis**

Lin- Sca-1+ cells were incubated for 40 hours in complete IMDM and FL + IL-11 as indicated. During the last 24 hours, cells were incubated with TNF-\(\alpha\), TGF-\(\beta\), or in the absence of both (control). A total of 150 \(\mu\)L of a DNA staining solution containing 0.1% Na-citrate, 0.1% TritonX-100, 50 U/mL RNaseA, and 10 \(\mu\)g/mL propidium iodide (all from Sigma, St Louis, MO) were added to the cells. Cells were then washed with an additional 150-\(\mu\)L DNA staining solution. Cells were analyzed for DNA content (PI-staining) in a FACSsort flow cytometer (Becton Dickinson, San Jose, CA). At least
potent growth stimulator of primitive bone marrow progenitors. To establish whether the growth of Lin-Sca-1 progenitor cells. Three hundred Lin-Sca-1 cells were plated at one cell per well in complete IMDM supplemented with predetermined optimal concentrations of cytokines (Materials and Methods). Colony formation was scored following 12 to 14 days incubation at 37°C and 5% CO2 in air. Scoring criteria: 1, 10 to 50 cells; 2, 50 cells to 10% of well covered cells; 3, cells covering 10% to 50% of well; 4, >50% of well covered by cells. The results represent the mean (SEM) of four experiments.

Table 1. Effect of Murine TNF-α on the Number and Size of Clones Formed by FL-Stimulated Lin– Sca-1 Progenitors

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Clone Size</th>
<th>Total Clones/300 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>7</td>
<td>2 0 0 9 (3)</td>
</tr>
<tr>
<td>IL-6</td>
<td>0</td>
<td>0 0 0 0 (0)</td>
</tr>
<tr>
<td>FL + IL-6</td>
<td>10</td>
<td>41 18 8 78 (8)</td>
</tr>
<tr>
<td>FL + IL-6 + TNF-α</td>
<td>13</td>
<td>6 1 0 20 (3)</td>
</tr>
<tr>
<td>G-CSF</td>
<td>3</td>
<td>0 0 0 3 (2)</td>
</tr>
<tr>
<td>FL + G-CSF</td>
<td>9</td>
<td>52 32 6 99 (5)</td>
</tr>
<tr>
<td>FL + G-CSF + TNF-α</td>
<td>14</td>
<td>6 0 0 19 (3)</td>
</tr>
<tr>
<td>IL-3</td>
<td>7</td>
<td>10 5 4 26 (6)</td>
</tr>
<tr>
<td>FL + IL-3</td>
<td>10</td>
<td>44 31 21 106 (12)</td>
</tr>
<tr>
<td>FL + IL-3 + TNF-α</td>
<td>26</td>
<td>8 5 2 42 (11)</td>
</tr>
</tbody>
</table>

Three hundred Lin–Sca-1 cells were plated at one cell per well in 20 µL complete IMDM as described in Materials and Methods and supplemented with predetermined optimal concentrations of cytokines (Materials and Methods). Wells were scored for cell growth following 12 to 14 days incubation at 37°C and 5% CO2 in air. Scoring criteria: 1, 10 to 50 cells; 2, 50 cells to 10% of well covered by cells; 3, cells covering 10% to 50% of well; 4, >50% of well covered by cells.

RESULTS

TGF-β1 and TNF-α Directly Inhibit FL-Stimulated Growth of Lin– Sca-1 Progenitor Cells in a Concentration-Dependent Manner

We and others have recently demonstrated that FL is a potent growth stimulator of primitive bone marrow progenitors in combination with the CSFs, IL-6, IL-11, and IL-12, whereas it has little stimulatory activity alone.31-33 In the present study, we investigated the ability of TGF-β1 and TNF-α to modulate FL-stimulated colony formation of Lin–Sca-1 progenitor cells plated at one cell per well to exclude indirect effects. A mean of 9 Lin–Sca-1 cells (per 300 cells plated) proliferated in response to FL (250 ng/mL) in the absence of other cytokines (Fig 1). These clones were without exception small and represented mostly clusters (10 to 50 cells) and a low number of colonies (>50 cells) (Table 1). The weak colony promoting activity of FL was completely blocked by rhTGF-β1 (20 ng/mL) and rmTNF-α (20 ng/mL) (Fig 1), suggesting that both TGF-β1 and TNF-α are inhibitors of the growth of Lin–Sca-1 progenitors responsive to FL alone. To establish whether the effects of TGF-β1 and TNF-α might be directly mediated on the progenitors and not on more differentiated cells produced in response to FL, a kinetic study was performed to determine the effects of delayed addition of TGF-β1 and TNF-α (Table 2). Whereas both TGF-β1 and TNF-α almost completely inhibited FL + IL-11–stimulated colony formation when added at the initiation of the culture, a 48-hour delay in the addition of TGF-β1 or TNF-α resulted in only a 36% reduction in the number of clones formed. However, the size of the clones formed were still dramatically reduced by the two inhibitors. If the addition of TGF-β1 and TNF-α were delayed 144 hours, no effect was observed on the number of clones formed, and only a small reduction in clone sizes was seen. Thus, to obtain full inhibitory effects on FL-stimulated growth of Lin–Sca-1 progenitors, TGF-β1 and TNF-α need to act at an early stage of proliferation.

Table 2. Kinetics of TGF-β and TNF-α Induced Inhibition of FL + IL-11–Stimulated Growth of Lin– Sca-1 Progenitor Cells

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Time (h)</th>
<th>Clone Size</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL + IL-11</td>
<td>0</td>
<td>15 38 10 5 67 (6)</td>
<td></td>
</tr>
<tr>
<td>FL + IL-11 + TGF-β</td>
<td>0</td>
<td>0 0 0 0 0 (0)</td>
<td></td>
</tr>
<tr>
<td>FL + IL-11 + TNF-α</td>
<td>0</td>
<td>5 2 0 7 (4)</td>
<td></td>
</tr>
<tr>
<td>FL + IL-11 + TGF-β</td>
<td>48</td>
<td>26 17 0 43 (6)</td>
<td></td>
</tr>
<tr>
<td>FL + IL-11 + TNF-α</td>
<td>48</td>
<td>28 14 0 43 (6)</td>
<td></td>
</tr>
<tr>
<td>FL + IL-11 + TGF-β</td>
<td>144</td>
<td>14 51 6 70 (8)</td>
<td></td>
</tr>
<tr>
<td>FL + IL-11 + TNF-α</td>
<td>144</td>
<td>16 53 7 76 (7)</td>
<td></td>
</tr>
</tbody>
</table>

Three hundred Lin–Sca-1 cells were plated at one cell per well in 10 µL complete IMDM and predetermined optimal concentrations of FL and IL-11 (Materials and Methods). At each time point (0, 48, or 144 hours following initiation of culture) an additional 10 µL of complete IMDM containing FL and IL-11 supplemented with either TGF-β (40 ng/mL; final concentration 20 ng/mL) or TNF-α (100 ng/mL; final concentration 50 ng/mL) were added to the cultures as indicated. Cultures were scored for clonal growth based on the size of individual clones following a total of 12 to 14 days incubation at 37°C and 5% CO2 in air according to the criteria described in Table 1. Results represent the mean (SEM) of three separate experiments.
Because both IL-11 and IL-12 have been demonstrated to be early acting and synergistic cytokines capable of stimulating the growth of primitive progenitor cells,\textsuperscript{2,3} and in that regard to potently synergize with FL,\textsuperscript{35} we next investigated the ability of TGF-\(\beta\)-1 and TNF-\(\alpha\) to affect FL + IL-11 and FL + IL-12-stimulated colony formation of Lin\(^-\)Sca-1\(^+\) bone marrow cells. Neither IL-11 or IL-12 alone stimulated colony formation of Lin Sca-1\(^+\) cells (data not shown). However, IL-11 and IL-12 potently synergized with FL to stimulate the formation of 80 and 51 clones, respectively, and TGF-\(\beta\)-1 (20 ng/mL) completely blocked the clonal growth of Lin Sca-1\(^+\) progenitors in response to both FL + IL-11 and FL + IL-12 (Fig 1). Similarly, TNF-\(\alpha\) reduced colony formation in response to FL + IL-11 and FL + IL-12 by 82% and 96%, respectively. TGF-\(\beta\)-1 at 2 ng/mL completely blocked all cluster and colony formation in response to FL + IL-12, whereas 20 ng/mL of TGF-\(\beta\)-1 was required to completely inhibit all clonal growth induced by FL + IL-11 (Fig 2A). Accordingly, the ED\(_{50}\) was 0.02 to 0.2 ng/mL for the inhibitory effect of TGF-\(\beta\)-1 on FL + IL-12-stimulated growth, and 0.2 ng/mL for progenitors stimulated by FL + IL-11 (Fig 2A).

Similarly, TNF-\(\alpha\) almost completely blocked FL + IL-12-stimulated colony formation at 20 ng/mL, whereas 13% of the FL + IL-11-stimulated Lin Sca-1\(^+\) progenitors formed colonies even at TNF-\(\alpha\) 200 ng/mL (Fig 2B). The ED\(_{50}\) for the inhibitory effect of TNF-\(\alpha\) on FL + IL-12 and FL + IL-11-stimulated colony formation was 0.2 to 2 ng/mL and 2 ng/mL, respectively.

Next, we examined the direct effects of TGF-\(\beta\)-1 and TNF-\(\alpha\) on the growth of single Lin Sca-1\(^+\) progenitors in response to FL in combination with other cytokines. mTNF-\(\alpha\) (20 ng/mL) reduced the number of colonies formed in response to FL + G-CSF, FL + IL-3, FL + IL-6, and FL + SCF by 79%, 63%, 76%, and 58%, respectively, whereas TGF-\(\beta\)-1 reduced the colony formation in response to all these cytokine combinations by more than 96% (Fig 1).

Because colony formation of Lin Sca-1\(^+\) progenitors in response to certain cytokine combinations was not completely inhibited by TNF-\(\alpha\), we also asked whether TNF-\(\alpha\) reduced the size of the remaining colonies (Table 1). Both in response to FL + IL-6, FL + G-CSF, and FL + IL-3, TNF-\(\alpha\) treatment resulted in a dramatic reduction in the number of big colonies, reflected in 65%, 74%, and 62%, respectively of the remaining clones being clusters (<50 cells) rather than colonies (>50 cells). In contrast, the cultures stimulated by FL + IL-6, FL + G-CSF, and FL + IL-3 in the absence of TNF-\(\alpha\), contained only 13%, 9%, and 9% clusters, respectively (Table 1). Thus, TNF-\(\alpha\) appears to reduce both the number of FL-stimulated proliferative clones, as well as the size of the remaining clones.

Effects of TNF-\(\alpha\) on the Differentiation of Lin Sca-1\(^+\) Progeny

The effects of TNF-\(\alpha\) on the differentiation of FL-stimulated progeny of Lin Sca-1\(^+\) cells was next addressed. This was of particular interest as the magnitude of inhibition by TNF-\(\alpha\) varied depending on the specific growth factor with which FL was combined, and this could be due to lineage restricted inhibition. As recently reported,\textsuperscript{35} FL in combination with IL-11, IL-6, IL-12, or G-CSF stimulated the production of 48% to 68% immature blast cells from 1,000 Lin Sca-1\(^+\) cells in liquid culture by day 12 of culture, 18% to 35% macrophages, and only 4% to 10% granulocytes. TNF-\(\alpha\) almost completely inhibited the cell production in response to these cytokines. However, because a high number of FL + SCF and FL + IL-3-stimulated progenitors appeared resistant to inhibition by TNF-\(\alpha\), it was of particular interest to investigate whether the partial inhibition was due to lineage-restricted effects of TNF-\(\alpha\) (Table 3). FL + IL-3 stimulated predominantly the production of macrophages (83%) and some granulocytes (13%) and undifferentiated blast cells (4%), and although TNF-\(\alpha\) reduced the cell number by more than 50%, a similar distribution of
Next, individual colonies formed in the presence and absence of TNF-α were investigated. FL were counted, and cytospin preparations were stained with Giemsa observed, in that only and a dramatic switch in the type of cells produced was cell production in response to FL treatment. Results are the mean (SEM) of four separate experiments.

Macrophages (71%), granulocytes (27%), and blast cells (2%) was observed. FL + SCF-stimulated cultures contained predominantly granulocytes (78%), as well as some macrophages (10%) and blast cells (12%), TNF-α inhibited cell production in response to FL + SCF by more than 80%, and a dramatic switch in the type of cells produced was observed, in that only 5% were granulocytes, whereas the macrophages were increased to 88% of the total population. Next, individual colonies formed in the presence and absence of TNF-α were investigated. FL + IL-3-stimulated colonies were predominantly of mixed GM type or contained almost exclusively macrophages, whereas few granulocyte or blast cell colonies were observed (Table 4). In agreement with the data on total cell production in liquid culture, the FL + IL-3-stimulated colonies resistant to TNF-α-induced inhibition showed similar frequencies of G, M, and blast cells as TNF-untreated cultures. Interestingly, and again in agreement with the data obtained on total cell production, FL + SCF-stimulated colonies were 57% and 4% of G and M type, respectively, whereas the TNF-resistant colonies were represented by 7% and 46% G and M colonies, respectively. Thus, TNF-α preferentially inhibits granulocyte production from FL + SCF-stimulated Lin-Sca-1+ progenitor cells.

### Table 3. Effects of TNF-α on the Differentiation of Lin-Sca-1+ Bone Marrow Cells

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Cells x 10⁶</th>
<th>Granulocytes (%)</th>
<th>Macrophages (%)</th>
<th>Blasts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL + IL-3</td>
<td>26, 5 (3, 6)</td>
<td>13 (5)</td>
<td>83 (7)</td>
<td>4 (2)</td>
</tr>
<tr>
<td>FL + IL-3 + TNF-α</td>
<td>12, 3 (2, 1)</td>
<td>27 (10)</td>
<td>71 (15)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>FL + SCF</td>
<td>4, 8 (0, 8)</td>
<td>78 (3)</td>
<td>10 (2)</td>
<td>12 (2)</td>
</tr>
<tr>
<td>FL + SCF + TNF-α</td>
<td>6, 9 (0, 4)</td>
<td>5 (2)</td>
<td>88 (6)</td>
<td>7 (2)</td>
</tr>
</tbody>
</table>

A total of 1,000 Lin-Sca-1+ cells were plated in 300 μL complete IMDM supplemented with predetermined optimal concentrations of cytokines (Materials and Methods) as indicated. Total cell numbers were counted, and cytospin preparations were stained with Giemsa after a 12 to 14-day incubation. Morphology was examined under a light microscope. At least 100 cells were examined per group in each treatment. Results are the mean (SEM) of four separate experiments.

### Table 4. Effects of TNF-α on Different Types of Colonies Formed in Response to FL + IL-3 or FL + SCF

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>G Colonies (%)</th>
<th>M Colonies (%)</th>
<th>GM Colonies (%)</th>
<th>Blast Colonies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL + IL-3</td>
<td>9 (4)</td>
<td>48 (10)</td>
<td>41 (6)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>FL + IL-3 + TNF-α</td>
<td>3 (2)</td>
<td>41 (9)</td>
<td>54 (12)</td>
<td>3 (2)</td>
</tr>
<tr>
<td>FL + SCF</td>
<td>57 (14)</td>
<td>4 (3)</td>
<td>29 (7)</td>
<td>11 (3)</td>
</tr>
<tr>
<td>FL + SCF + TNF-α</td>
<td>7 (4)</td>
<td>46 (11)</td>
<td>45 (9)</td>
<td>2 (1)</td>
</tr>
</tbody>
</table>

Lin-Sca-1+ cells were cultured at one cell per well as described in the Materials and Methods and supplemented with optimal cytokine concentrations. Colonies covering more than 5% of the well were individually sampled following 12 days of culture, and cytospins prepared and Giemsa stained as described in the Materials and Methods. Stained cytospin preparations were examined under a light microscope. Almost all colonies contained both granulocytes and macrophages, and scoring were based on the following criteria: G colonies contained >10% granulocytes, <10% macrophages, and <50% blast cells. M colonies contained >10% macrophages, <10% granulocytes, and <50% blast cells. GM colonies contained >10% macrophages, >10% granulocytes, and <50% blast cells. Blast colonies contained >50% blast cells. Results represent the mean (SEM) of four experiments with a total of at least 50 colonies scored in each group. The mean number (SEM) of colonies formed in response to FL + IL-3 were 39 (7) and 40 (6) in the absence and presence of TNF-α, respectively. In response to FL + SCF 65 (5) and 30 (4) colonies were formed in the absence and presence of TNF-α, respectively.

Abbreviations: G, granulocytes; M, macrophage.

TNF-α and TGF-β Potently Inhibit the Production of B220+ Cells From B220+ Bone Marrow Cells

Because FL in combination with IL-7 recently has been demonstrated to stimulate the growth of primitive B cell progenitors, we next investigated the ability of TGF-β and TNF-α to affect FL + IL-7-stimulated production of B220+ cells from Lin− cells.

FL + IL-7 potently stimulated the production of predominantly round lymphoid appearing cells (data not shown). A total of 62% ± 8% (mean of three experiments) of the cells produced in response to FL + IL-7 expressed B220, and TNF-α and TGF-β inhibited 92% and 98% of the B220 cell production, respectively (Fig 3). FL + IL-7 also stimulated the production of some macrophages, which was also inhibited by TNF-α and TGF-β (data not shown). Thus, TNF-α and TGF-β potently inhibit production of B220+ cells from B220+ B-cell progenitors in murine bone marrow.
Table 5. Effects of a Neutralizing Anti–TGF-β Antibody on FL-Stimulated Clonal Growth of Lin–Sca-1+ Progenitor Cells

<table>
<thead>
<tr>
<th>Clone size</th>
<th>Total Clones/300 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Medium</td>
<td>0 (0)</td>
</tr>
<tr>
<td>α-TGF-β</td>
<td>0 (0)</td>
</tr>
<tr>
<td>FL</td>
<td>5 (3)</td>
</tr>
<tr>
<td>FL + α-TGF-β</td>
<td>24 (7)</td>
</tr>
<tr>
<td>FL + control ab</td>
<td>7 (3)</td>
</tr>
</tbody>
</table>

Three hundred Lin–Sca-1+ bone marrow cells were plated at one cell per well in complete IMDM, supplemented with FL and antibodies at optimal concentrations (Materials and Methods), and cultured for 12 to 14 days at 37°C and 5% CO₂ in air. Cultures were scored for clonal growth according to the criteria described in Table 1. Results represent the means (SEM) of three separate experiments.

Endogenous TGF-β Inhibits the Clonal Growth of Lin–Sca-1+ Progenitor Cells

Autocrine production of TGF-β has been demonstrated to suppress the growth of hematopoietic progenitor cells. In the present study, a neutralizing anti–TGF-β antibody enhanced the clonal growth of Lin–Sca-1+ progenitors in response to FL alone, whereas an irrelevant control antibody had no effect (Table 5). The total number of clones was increased sevenfold, although most of them represented either clusters (<50 cells) or small colonies (Table 5). In contrast, a neutralizing antibody against mTNF-α had no effect on FL-stimulated growth (data not shown).

Interestingly, when Lin–Sca-1+ cells were cultured in liquid culture in the presence of FL and a neutralizing anti–TGF-β antibody, the resulting cells were a mixture of predominantly immature blast cells and mature macrophages (Table 6). Flow cytometry analysis of the cells demonstrated the presence of GR-1-positive, as well as B220-positive, cells (Table 6), suggesting that FL in the presence of anti–TGF-β stimulated the formation of cells of the myeloid, as well as B-cell, lineages.

Table 6. Effects of FL on the Differentiation of Lin–Sca-1+ Progeny in the Presence of a Neutralizing TGF-β Antibody

<table>
<thead>
<tr>
<th>%G</th>
<th>Morphology</th>
<th>%M</th>
<th>%B1</th>
<th>% GR-1</th>
<th>% B220</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 (4)</td>
<td>48 (11)</td>
<td>45 (10)</td>
<td>49 (6)</td>
<td>51 (7)</td>
<td></td>
</tr>
</tbody>
</table>

A total of 10,000 Lin–Sca-1+ cells isolated as described in Materials and Methods were plated in complete IMDM in the presence of 250 ng/mL rmFL and 5 μg/mL of a neutralizing anti–TGF-β antibody. Following 12 days incubation at 37°C and 5% CO₂ in air, cells were harvested and counted (mean of 5.8 x 10⁶ cells in three experiments). Cytospin preparations were Giemsa stained and examined under a light microscope for the percentage of granulocytes (G), macrophages (M) and undifferentiated blast (B1) cells. In each experiment, at least 200 cells were examined. Cells were also examined as described in Materials and Methods by flow cytometry for expression of GR-1 and B220. Results represent the mean (SEM) of three separate experiments.

Involvement of p55 and p75 TNF Receptors in TNF-α–Induced Inhibition of FL-Stimulated Growth of Lin–Sca-1+ Progenitors

Since the cloning of two distinct receptors capable of binding TNF-α, it has become increasingly clear that although most TNF-α responses are signaled through the 55 kD TNF receptor (TNFR55), the 75 kD TNF receptor (TNFR75) can also signal certain biological responses, and we have recently demonstrated that TNFR75 can signal growth inhibitory effects on primitive progenitors stimulated by CSFs.

Human TNF-α (hTNF-α) binds to the murine TNFR55 with similar efficiency as murine TNF-α (mTNF-α), but not to the murine TNFR75. To investigate the potential involvement of the two TNF receptors in signaling growth inhibition of FL-stimulated progenitor cell growth, we first took advantage of this restricted species cross-reactivity by comparing the effect of mTNF-α and hTNF-α on FL-stimulated colony formation of Lin–Sca-1+ progenitors plated at a concentration of one cell per well. FL-induced growth in combination with other cytokines (G-CSF, IL-3, IL-6, IL-11, IL-12, and SCF) was always inhibited most potently by mTNF-α (20 ng/mL). However, hTNF-α (20 ng/mL) also significantly inhibited colony formation in response to all FL combinations (Fig 4). The difference in the response elicited by murine and human TNF-α could not be due to the requirement for higher concentrations of hTNF-α than mTNF-α, as higher concentrations of hTNF-α (200 ng/mL) did not have more effect than 20 ng/mL (data not shown).

Next, we investigated the effects on FL-stimulated progenitor cell growth of specific agonistic antibodies against TNFR55 or TNFR75, capable of binding and signaling through their respective receptors. FL + IL-12–stimulated growth of Lin–Sca-1+ progenitors was completely blocked...
by an optimal titer (1:2000) on the TNFR75 agonist, whereas the TNFR55 agonist at an optimal titer (1:2000) resulted in a 29% inhibition, and preimmune serum (control) had no effect (Fig 5A). Furthermore, a higher concentration of the TNFR55 agonist (1:500) did not result in more inhibition than at 1:2000 (data not shown).

A similar pattern was observed for FL + IL-6-stimulated colony formation of Lin'Sca-1' progenitor cells, in that the TNFR75 agonist completely blocked colony formation, whereas the TNFR55 agonist inhibited 26% of the colony growth (Fig 5B). mTNF-α inhibited FL + IL-3-stimulated colony growth by 60% ± 5% (mean ± SEM of three experiments), the TNFR55 agonist resulted in 23% ± 5% inhibition, whereas the TNFR75 agonist inhibited colony formation by 81% ± 6%. Combined stimulation with the TNFR55 and TNFR75 inhibited 85% ± 7% of FL + IL-3-stimulated colony growth, suggesting that TNFR55 signaling does not interfere with the TNFR75 triggering. Finally, the TNFR55 agonist did not significantly affect the differentiation of Lin'Sca-1' cells stimulated by FL + IL-3 (data not shown). Thus, the in vitro growth of Lin'Sca-1' progenitor cells in response to FL is efficiently inhibited through selective activation of TNFR75.

**Cell Cycle Analysis of FL-Stimulated Lin'Sca-1' Cells Treated With TGF-β and TNF-α**

Previous studies have suggested that TGF-β can arrest human bone marrow progenitor cells in G1 of the cell cycle. Here, we examined whether this was also the case for TGF-β and TNF-α-induced inhibition of FL + IL-11-stimulated Lin'Sca-1' cells. To ensure that the cells to be analyzed were in fact responsive to FL + IL-11, Lin'Sca-1' cells were incubated for 40 hours in FL + IL-11, while adding TGF-β or TNF-α for the last 24 hours (Fig 6). PI-staining demonstrated that the fraction of cells in G1 was marginally increased from 38% to 43% in response to TNF-α, whereas the fraction of G1 cells in TGF-β-treated cultures increased to 67%. The fraction of cells in S-phase was reduced from 50% to 40% and 21% in response to TNF-α and TGF-β, respectively (Fig 6). Similarly, when FL + IL-11-stimulated Lin'Sca-1' cells were incubated for the full 40-hour incubation period in the presence of TNF-α, only a marginal increase in the number of G1 cells was observed (40% and 47% in the absence and presence of TNF-α, respectively; data not shown). Thus, TGF-β potently reduces the fraction of FL-stimulated cells in S-phase, while concomitantly increasing the fraction of cells in G1 whereas TNF-α has much less effect on the cell cycle distribution of FL + IL-11-stimulated Lin'Sca-1' cells.

**DISCUSSION**

The recently cloned FL has emerged as a potent and selective stimulator of primitive murine bone marrow progenitor cells in vitro. A study demonstrating that antisense oligonucleotides to STK-1, the human homolog of flt3, almost completely blocks human long-term bone marrow cultures, supports the notion of FL being a key regulator of hematopoiesis. The potential physiological role of flt3 was also supported by recent studies in flt3-deficient mice showing a deficiency in early B lymphopoiesis, as well as a defect in the capability of flt3 mutant stem cells to also reconstitute T cells and myeloid cells. Multiple cytokines (G-CSF, CSF-1, GM-CSF, IL-3, IL-6, IL-7, IL-11, IL-12, and SCF) have been demonstrated to synergistically enhance FL-stimulated progenitor cell growth in vitro. The present study is the first identifying inhibitors of FL-stimulated cell growth. Both TGF-β and TNF-α inhibited in a concentration-dependent manner myeloid colony formation of Lin'Sca-1' progenitor cells in response to FL alone, as well as in potent combinations with other hematopoietic growth factors. The growth inhibitory effects appeared to be mediated by a direct action and on the primitive progenitors and not through potentially contaminating accessory cells, as inhibition was observed when cells were plated at one cell per well, and as delayed addition of the inhibitors by 48 hours resulted in a much
TNF-α AND TGF-β: INHIBITORS OF FLT3 LIGAND

Fig 6. The effect of TNF-α and TGF-β on the cell cycle distribution of FL + IL-11-stimulated Lin−Sca-1+ bone marrow cells. Lin−Sca-1+ bone marrow cells were incubated in complete IMDM in the presence of optimal concentrations of FL and IL-11 for 40 hours (A), the last 24 hours in the presence of mTNF-α (50 ng/mL; B) or TGF-β (20 ng/mL; C). Cells were analyzed for cell cycle distribution following PI staining as described in Materials and Methods. Results are from one of three experiments with similar results.

less pronounced reduction in the number of clones formed (although the size of the clones were still dramatically reduced).

Interestingly, both TNF-α and TGF-β were potent inhibitors of FL + IL-7-stimulated production of B220+ cells from Lin− bone marrow cells, suggesting that they both might inhibit the ability of FL (in combination with IL-7) to stimulate the growth of primitive B-cell progenitor cells in the bone marrow, which might be a physiologically important role of FL. In the presence of a neutralizing TGF-β antibody, even FL alone stimulated the production of a low number of B220+ cells from Lin−Sca-1+ bone marrow cells, again supporting that FL might play a role in stimulating early B lymphopoiesis.

In agreement with previous studies, we found TGF-β to increase the number of FL-stimulated cells in the G1 phase of the cell cycle, whereas reducing the cells in S-phase, whereas TNF-α only slightly increased the number of cells in G1 of the cell cycle.

TGF-β and, in particular, TNF-α should be considered bidirectional regulators of hematopoiesis because they can either inhibit or stimulate the growth of bone marrow progenitor cells, depending on the maturity of the targeted progenitor cells, the specific cytokines by which they interact, and their concentration in culture. With regard to FL-stimulated growth of Lin−Sca-1+ progenitor cells, TGF-β almost completely inhibited colony formation regardless of the cytokines synergizing with FL. Inhibition of FL-stimulated colony formation was always observed in response to TNF-α as well, but the magnitude of inhibition varied depending on the specific cytokines by which FL was combined. In particular, a high number of FL + IL-3 and FL + SCF-responsive clones were resistant to TNF-inhibition. This differential effect of TNF-α could be due to a lineage-restricted inhibition. TNF-α did not significantly affect the types of colonies formed in response to FL + IL-3. However, whereas FL + SCF alone stimulated predominantly the formation of granulocyte-containing colonies, almost exclusively macrophage-containing colonies were formed when costimulated with TNF-α.

TGF-β is produced in many cell types, in particular in platelets and bone, and it has been suggested that TGF-β might play a key role in keeping primitive progenitors from actively cycling. Of particular interest are studies that have demonstrated that murine and human bone marrow progenitors can produce TGF-β in an autocrine manner, and that neutralization of this TGF-β enhances their in vitro clonogenic growth. Similarly, we demonstrate that the ability of FL to stimulate the growth of Lin−Sca-1+ progenitors is enhanced in the presence of a neutralizing anti-TGF-β antibody.

The physiological role of TNF-α in regulation of normal hematopoiesis is unclear. However, increased levels of TNF-α have been observed in acute and chronic diseases and have been implicated to play a role in many disorders, including suppression of hematopoiesis. It is possible that such suppression might result, at least in part, through the potent ability of TNF-α to inhibit the growth promoting activities of HGFs, in particular SCF and FL, which both appear to be important regulators of early hematopoiesis.

Based on the ability of TNF-α to synergize with GM-CSF and IL-3, but not G-CSF and Epo, it has been suggested that TNF-α should be considered a direct enhancer of early hematopoiesis. However, recent studies demonstrating the potent ability of TNF-α to inhibit SCF-supported hema-
tnopoietic progenitor cell growth\textsuperscript{46} and now FL-stimulated hematopoiesis do not support this, but rather suggest that the specific cytokine by which TNF-\(\alpha\) interacts is a critical determinant for the response (stimulatory or inhibitory) elicited.

TNF receptor signaling is complex,\textsuperscript{45} and although both TNF receptors have been demonstrated to be capable of signaling separately, for many TNF-responses optimal signaling through one of the two receptors can only be obtained through interaction with the other.\textsuperscript{46,47} In that regard, it has been suggested that TNFR75 might enhance TNFR55 responses through a ligand passing effect.\textsuperscript{48} We recently demonstrated that agonistic antibodies to TNFR75 can mimic the inhibitory effects of murine TNF-\(\alpha\) on the growth of Lin\-'Sca-1\-' progenitor cells, whereas human TNF-\(\alpha\) and TNFR55 agonists, which bind selectively to TNFR55, have little or no effect.\textsuperscript{49} In agreement with this, the TNFR75 agonist completely inhibited colony formation of Lin\-'Sca-1\-' progenitor cells in response to FL + IL-6 or FL + IL-11. However, at variance with our previous studies, some inhibition could also be obtained by selectively signaling through TNFR55. The different involvement of TNFR55 in our two studies could potentially be explained by TNFR55 being capable of signaling some inhibition of the growth response to FL, as opposed to the growth stimulated by combinations of CSFs.\textsuperscript{49} Alternatively, it might reflect the recruitment of distinct progenitor cell populations, which respond differently to TNFR55 agonists. Nevertheless, the present results further support a potentially important role of TNFR75 in signaling inhibition of primitive marine bone marrow progenitor cells.

It is worth noticing that the TNFR75 agonist used in the present studies was more efficient than wild type murine TNF-\(\alpha\) in signaling growth inhibition. Based on several observations, it seems unlikely that this is due to a nonspecific toxic effect of the TNFR75 agonist. First, the control (preimmune) showed no inhibition, and the TNFR55 agonist was less effective than the TNFR75 agonist. Second, the TNFR75 agonist does not inhibit the growth of more committed bone marrow progenitor cells.\textsuperscript{49} Finally, and most importantly, the TNFR75 agonist used in the present studies stimulates fivefold to 10-fold more proliferation (tritiated thymidine incorporation) than optimal concentrations of murine TNF-\(\alpha\) in the CT-6 cell line (Jacobsen et al, unpublished data, November 1994). This cell line has previously been demonstrated to proliferate through selective activation of TNFR75.\textsuperscript{46} Other recent studies do also suggest that some TNFR agonistic antibodies or TNF-\(\alpha\) mutants can signal more efficiently than wild type TNF-\(\alpha\).\textsuperscript{46,47} Although the reason for this is not clear, it could be explained by recent studies suggesting that the transmembrane form of TNF is superior to soluble TNF in activating TNFR75, and that TNFR75 agonistic antibodies are better than soluble TNF in mimicking effects of membrane TNF signaled through TNFR75.\textsuperscript{48} The reason for the superiority of membrane TNF and TNFR75 agonistic antibodies over soluble TNF in signaling through TNFR75 appears, at least in part, to be due to a more stable binding of membrane TNF and TNFR75 agonists to TNFR75 than of soluble TNF.\textsuperscript{48} Combined, this observation and the present studies suggest that membrane TNF might play a unique role in inducing growth suppression through TNFR75 on primitive murine hematopoietic progenitor cells.

TNFR75 agonists or antagonists might prove useful in the treatment of hematologic disorders. The present study along with others,\textsuperscript{45,46} suggests that TNFR75 agonists by keeping primitive progenitors quiescent might reduce the bone marrow toxicity associated with chemotherapeutic agents, whereas TNFR antagonists could be used to reverse the hematopoietic suppression observed in many chronic diseases.

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REFERENCES

ization of an inhibitor of hematopoietic stem cell proliferation. Na
ture 344:442, 1990


43. Jacob CO: Tumor necrosis factor \( \alpha \) in autoimmunity: Pretty girl or old witch? Immunol Today 13:122, 1992


Lewis M, Tartaglia LA, Lee A, Bennett GL, Rice GC, Wong GHW, Chen EY, Goeddel DV: Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. Proc Natl Acad Sci USA 88:2830, 1991


Ability of flt3 ligand to stimulate the in vitro growth of primitive murine hematopoietic progenitors is potently and directly inhibited by transforming growth factor-beta and tumor necrosis factor-alpha

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