Potentiation of Early Hematopoiesis by Tumor Necrosis Factor-α Is Followed by Inhibition of Granulopoietic Differentiation and Proliferation

By Christophe Caux, Catherine Favre, Sem Saeland, Valérie Duvert, Isabelle Durand, Patrice Mannoni, and Jacques Banchereau

We have previously shown that tumor necrosis factor-α (TNFα) strongly potentiates interleukin-3 (IL-3)-induced short-term proliferation of human CD34⁺ hematopoietic progenitor cells (HPC). Using longer term cultures of CD34⁺ HPC, we demonstrate here that this initial potentiation ceases after 10 to 12 days; whereupon TNFα displays inhibitory effects. Thus, TNFα was found to inhibit cells of granulocytic affiliation while it potentiates the development of maturing cells of the monocytic lineage both in liquid and semi-solid (day 14 colony-forming unit) cultures. TNFα was demonstrated to reversibly block granulocytic differentiation at the level of uncommitted CD13⁺, CD15⁺ blast cells that accumulate in IL-3 + TNFα cultures. Furthermore, growth of committed granulocytes (CD15⁺) from IL-3 cultures was also inhibited by TNFα through an arrest of cell cycle in G1/G0. Finally, the use of neutralizing anti-TNFα monoclonal antibody and limiting dilution studies indicate that the inhibitory effects of TNFα are direct. Taken together, our data demonstrate that, following a phase of potentiation of proliferation of early HPC, TNFα displays direct inhibitory effects due to negative interference with both granulocytic differentiation and proliferation of granulocytic cells.© 1991 by The American Society of Hematology.
CD34+ cells were seeded for expansion in 24-well culture plates (Linbro; Flow Laboratories, McLean, VA) at 1 x 10⁵ cells/mL. Optimal conditions were maintained by splitting these cultures every 4 to 5 days. At different time points as indicated, cells were collected for counting, phenotyping, and cytologic characterization, and establishment of secondary cultures (Fig 1A and C). For initiation of secondary cultures, cells were washed four times and further incubated for 3 hours in complete medium to eliminate growth factors. Subsequent cultures were established for cell viability determination, cell cycle analysis, and proliferation and clonogenic assays as described below (Fig 1A and C).

For proliferation assays, cells were distributed in 96-well round-bottom microtiter tissue-culture plates (Nunc, Roskilde, Denmark) between 5 x 10⁴ and 2 x 10⁵ cells/100 µL complete medium and incubated (37°C, 5% CO₂) with saturating concentrations of IL-3 with or without TNFα for 6 days. After incubation, cells were pulsed with 1 µCi of [3H]-Tdr for 6 hours, harvested, and counted. Tests were performed in triplicate, and results were expressed as mean counts per minute (cpm) ± standard deviation.

Progenitor assays in semisolid medium. Semisolid cultures of 1 mL were performed in methylcellulose by plating between 2 x 10³ to 10⁵ cells per tissue-culture grade 35-mm Petri dish (Corning Glass, Corning, NY), in the presence of 1 IU purified recombinant human erythropoietin (Epo; Amgen, Thousand Oaks, CA; specific activity > 7 x 10⁴ IU/mg) as previously described in detail.³ Duplicate dishes were plated in each experiment, and after 14 days of incubation at 37°C and 5% CO₂ colonies (≥50 cells) were counted using phase-contrast microscopy.

Cytoplogic characterization. Cells collected either from liquid or semi-solid cultures were cytocentrifuged for 7 minutes at 400 RPM in a Cytospin-2 (Shandon, Astmoor, UK) in medium containing 50% FBS. Cells were stained with May-Grünwald-Giemsma, and subsequently characterized microscopically.

Cytofluorimetric cell-surface phenotyping. Indirect immunofluorescence was performed according to standard techniques, using a panel of murine MoAbs (see below), revealed by fluorescein isothiocyanate (FITC)-conjugated sheep F(ab')₂ antiserum Ig (Bio-art, Meudon, France). Double-color fluorescence was performed by sequential incubation of the cells with unconjugated MoAbs, FITC-conjugated antimouse Ig, normal mouse serum, and MoAbs (see below) directly labeled with phycoerythrin (PE). MoAbs used were as follows: anti-LeuM3 and anti-LeuM3-PE (CD14), anti-LeuM1 (CD15), anti-HLA-DR, and anti-HLA-DR-PE (all obtained from Becton Dickinson), and anti-My7 (CD13) (Coulter Immunology, Hialeah, FL). Negative controls were performed with unrelated murine MoAbs. Fluorescence analysis was determined with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA).

Cytofluorimetric cell sorting. After 7 days of culture in IL-3 (10 ng/mL) + TNFα (25 ng/mL), cells were collected, washed several times, and labeled by sequential incubation with anti-My7 (CD13) and FITC-conjugated antimouse Ig. Using a FACS 440 (Becton Dickinson), the cells were separated on the basis of CD13 expression into a CD13⁺ fraction and a CD13⁻ fraction (Fig 1B).

Cell-cycle analysis. Cells from primary IL-3 cultures were incu-
bated for 48 to 72 hours in the presence of IL-3 with or without TNFα, and 0.1 μg/mL colcemid (GIBCO) was added during the last 16 hours. Cells were subsequently collected, washed twice in complete medium, and incubated with 10 μM/L Hoechst 33342 dye (Calbiochem, Behring Diagnostics, La Jolla, CA) at 10^6 cells/mL at 37°C for 45 minutes. In some experiments, cells were processed for double-color membrane fluorescence labeling as previously described. Using a FACS 440, cell-surface Ag expression and DNA content were analyzed with the argon laser tuned at 488 nm and the UV argon laser tuned at 354 to 361 nm, respectively (Fig 1C and D).

**Cell viability determination.** During primary culture in IL-3, cells were collected at the timepoints indicated and seeded in 96-well flat-bottom microculture plates (Falcon Labware, Oxnard, CA) at 5 x 10^3 cells/well in 100 μL in presence of IL-3 (10 ng/mL) with or without TNFα (25 ng/mL). After 48 hours of incubation, cells were pulsed with 50 μg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) at 37°C for 3 hours, as described. Acid-isopropanol (150 μL of 0.04 N HCl in isopropanol) was added to each well and mixed thoroughly to dissolve the crystals, and the plates were read on a Dynatech MR-580 Microelisa reader (Dynatech, Alexandria, VA), using a test wavelength of 570 nm and a reference wavelength of 630 nm (Fig 1C).

**Inhibitory activity of culture supernatants.** Cells precultured in IL-3 for 14 days were washed and recultured in presence of IL-3 with or without TNFα for 72 hours. Experiments were set up in duplicate wells, one well for cell cycle analysis and one for supernatant collection. Supernatants were stored at -20°C and subsequently tested for their inhibitory activity, in the presence of an anti-TNFα MoAb (1 μg/mL) or a control MoAb of same isotype (1 μg/mL), on the proliferation of CD34+ HPC precultured for 14 days in IL-3 (Fig 1E).

**Limiting dilution studies.** Cells precultured in IL-3 for 14 days were washed and recultured in limiting dilution conditions at one cell per well in 96-well round-bottom microtest tissue-culture plates (Nunc), as previously described, in Iscove’s modified Dulbecco’s medium (IMDM) containing 30% FBS, 10^-4 mol/L 2-mercaptoethanol, 0.5% wt/vol BSA neutralized with NaHCO₃, and L-glutamine and antibiotics as indicated above. For each factor tested, aliquots of 50 μL were seeded in 600 wells. After 6 days of culture (37°C, 5% CO₂), positive wells were scored and the number of cells in each clone was determined using an inverted microscope coupled to an automated image analysis system (Bio-com, Paris, France). Clone frequency was determined as the ratio of the total cell number enumerated in the 600 wells seeded to the number of responding cells present in the 600 wells observed (Fig 1F).

**RESULTS**

**Agonistic and antagonistic effects of TNFα on the response of CD34+ HPC to IL-3.** As we recently reported, TNFα enhances the early proliferative response of CD34+ HPC to IL-3 in liquid cultures, and consequently twofold to threefold more viable cells can be recovered after 5 to 10 days of culture in IL-3 + TNFα when compared with IL-3 alone (Fig 2). However, after 10 days of culture, cell proliferation halted in cultures containing IL-3 + TNFα, whereas proliferation persisted in IL-3 alone. Thus, after 32 to 34 days, two to six times more viable cells were generated in IL-3 cultures than in IL-3 + TNFα cultures (Fig 2).

When CD34+ HPC were plated in semi-solid medium, TNFα was found to enhance the generation of day 7 IL-3-dependent colonies, whereas at day 14 IL-3- and Epo-dependent colonies were inhibited (Table 1). TNFα totally blocked the generation of burst-forming unit-erythroid (BFU-e) and mixed erythroid (E-MIX) colonies, strongly inhibited the generation of GM colonies, and reduced the numbers of G colonies by 50%. In contrast, TNFα did not affect the generation of M colonies.

**Characterization of cells obtained in IL-3 + TNFα cultures.** To further analyze the modulatory effects of TNFα on IL-3-dependent proliferation of CD34+ HPC, we performed, at various time intervals, cytologic and phenotypic characterization of cells generated in the liquid cultures (Fig 1A). Cytologic analysis (Fig 3) demonstrated that more monocytes/macrophages (Fig 3A) and fewer granulocytes (Fig 3B) were observed in the presence of IL-3 + TNFα as compared with IL-3 alone. This effect was observed as early as 6 days of culture, and became more evident as cultures progressed. Thus, after 18 days, the proportions of monocytes/macrophages and of granulocytes were, respectively,
Table 1. TNFα Preferentially Inhibits Erythroid and Granulocytic Colonies

<table>
<thead>
<tr>
<th>Factors</th>
<th>Day 14 (mean ± SD)</th>
<th>GM</th>
<th>G</th>
<th>M</th>
<th>E-MIX</th>
<th>BFU-e</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>IL-3</td>
<td>212 ± 18</td>
<td>10 ± 4</td>
<td>150 ± 33</td>
<td>49 ± 17</td>
<td>11 ± 5</td>
<td>99 ± 38</td>
<td>319 ± 40</td>
</tr>
<tr>
<td>TNFα</td>
<td>4 ± 4</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>2 ± 2</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>IL-3 + TNFα</td>
<td>298 ± 35</td>
<td>2 ± 4</td>
<td>77 ± 40</td>
<td>43 ± 35</td>
<td>0 ± 0</td>
<td>1 ± 3</td>
<td>123 ± 56</td>
</tr>
<tr>
<td>Medium</td>
<td>7 ± 6</td>
<td>0 ± 0</td>
<td>2 ± 2</td>
<td>5 ± 2</td>
<td>0 ± 0</td>
<td>11 ± 11</td>
<td>18 ± 12</td>
</tr>
</tbody>
</table>

Values represent the mean ± SD of seven experiments. For each experiment, duplicate dishes, plated with 4 x 10^4 cells in presence of Epo (1 U/mL) and IL-3 or IL-3 + TNFα, were scored on day 14.

Fig 3. Cytologic characterization of cells from cultures of CD34+ HPC in the presence of IL-3 (■) or IL-3 + TNFα (□). For experimental procedure see Materials and Methods and Fig 1A. Data are expressed as percentage of cells and represent the mean of three experiments. (A) Monocytes/macrophages; (B) granulocytes; (C) blast cells.

Fig 5. Cytologic characterization of cells from cultures of CD34+ HPC in the presence of IL-3 (■) or IL-3 + TNFα (□). For experimental procedure see Materials and Methods and Fig 1A. Data are expressed as percentage of cells and represent the mean of three experiments. (A) Monocytes/macrophages; (B) granulocytes; (C) blast cells.

three times higher and three times lower in the presence of IL-3 + TNFα than in the presence of IL-3 alone. As shown in Fig 3C, the percentage of blast cells decreased steadily in both culture conditions and represented approximately 20% of cells after 18 days. Flow cytometric analysis (Fig 4) confirmed the cytologic observations in that TNFα induced an increase in the percentage of cells expressing CD14 (a monocyte/macrophage antigen) and a decrease in the percentage of cells expressing CD15 (a granulocyte antigen). Furthermore, fluorescence cell-sorting following culture of CD34+ HPC in the presence of IL-3 or IL-3 + TNFα confirmed that all CD14+ cells possess a monocytic morphology and that all CD15+ cells have a granulocytic morphology (not shown).

In addition, flow cytometry analysis demonstrated that, in the presence of IL-3 + TNFα, the percentage of cells expressing the myelomonocytic CD13 antigen was reduced as a consequence of an important expansion of CD13- cells (fourfold to sixfold the number obtained in IL-3 alone after 6 days of culture).

Cells precultured in IL-3 or IL-3 + TNFα were further assessed for colony-forming ability in semi-solid medium (Fig 1A). The frequency (Fig 5) and size (not shown) of myeloid colony-forming units (CFU) steadily decreased in presence of either IL-3 or IL-3 + TNFα. However, these events occurred earlier in the presence of TNFα, as illustrated by five times fewer CFU at day 12 (Fig 5). During the culture in the presence of IL-3, the proportion of CFU-M was nearly constant, representing 25% to 35% of the total CFU. In marked contrast, the combination of IL-3 + TNFα resulted in an increased proportion of CFU-M from 35% at day 0 to 90% of total CFU at day 18. CFU-GM, E-MIX, and BFU-e were no longer detected after 6 days of preculture in either IL-3 or IL-3 + TNFα (not shown).

When assessed in absolute numbers, 15 to 25 times more CD15+ granulocytic cells and 20 to 100 times more CFU-G were obtained after 18 days of culture in IL-3 alone when compared with IL-3 + TNFα. In contrast, while the CD14+ monocytic cells and the CFU-M were, respectively, 4 to 8 and 4 to 30 times more numerous in IL-3 + TNFα than in IL-3 alone at day 12, no significant quantitative differences could be observed at day 18 (not shown).

Thus, TNFα appears to simultaneously block granulocytic development, as defined by CFU-G generation and CD13/CD15 antigen expression, and to potentiate the development of monocytic-lineage cells.
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**Fig 4.** Phenotypic characterization of CD34' HPC cultured in the presence of IL-3 1.1 or 11-3 + TNFα (13). For experimental procedure see Materials and Methods and Fig 1A. Data are expressed as percentage of positive cells as determined by cytofluorimetric analysis and represent the mean of five experiments. (A) CD14 expression; (B) CD15 expression; (C) CD13 expression.

**TNFα inhibits IL-3-dependent differentiation of CD34' HPC towards granulocytes.** Next, we examined the CD13' cell population, which preferentially accumulated in IL-3 + TNFα cultures (see above). The CD13' cells failed to express the myelomonocytic-lineage markers CD14 or CD15 and were mostly composed of HLA-DR' blast cells. In contrast, CD13' cells obtained after culture in the presence of IL-3, when or without TNFα, represented granulocyte- and monocyte-committed cells expressing either CD14 or CD15 (not shown). Both CD13' and CD13' cell populations obtained after 7 days of culture of CD34' HPC in IL-3 + TNFα contained proliferating cells (not shown). CD13' and CD13' cells were FACS-sorted from day 7 cultures performed in IL-3 + TNFα, washed, and subsequently recultured for 7 more days with IL-3 or with IL-3 + TNFα (Fig 1B). Phenotypic analysis (Fig 6) indicated that a large percentage of the CD13' sorted cells acquired CD13 (30% to 60%; three separate experiments) and CD15 (25% to 40%) when recultured for 7 days with IL-3 alone. In contrast, when recultured with IL-3 + TNFα, the CD13' sorted cells were unable to express either CD13, CD14, or CD14, despite sustained proliferation. In parallel, the CD13' sorted cells remained CD13' when recultured with IL-3, expressing either CD15 or CD14, and addition of TNFα did not affect their phenotype.

Taken together, these data indicate that TNFα specifically blocks granulocyte differentiation at the level of CD13' cells, and that this effect is reversible because removal of TNFα from cultures results in subsequent IL-3-driven granulocytic differentiation.

**TNFα inhibits IL-3-dependent proliferation of granulocyte-committed cells.** Because TNFα has been characterized for its cytolytic activity on various cell types, we investigated whether such an effect could account for the TNFα-induced growth inhibition observed in later stages of IL-3-dependent cultures. Thus, CD34' HPC were cultured for up to 26 days with IL-3 alone. At various time intervals, cells were harvested and recultured with either IL-3 alone or IL-3 + TNFα and three biologic parameters were subsequently studied (Fig 1C): (1) viable cell numbers after 48 hours as determined by MTT assay and trypan blue exclusion; (2) H-TdR incorporation measured after 6 days; and (3) percentage of cells in S/G,M phases of cycle as measured by Hoechst 33342 staining after 48 hours of culture, including a final 16-hour colcemid pulse. Results illustrated in Fig 7 indicate that: (1) a 48-hour TNFα treatment did not alter cell viability; (2) TNFα enhanced IL-3-induced H-TdR incorporation at early time points (day 0 and 5) but inhibited it at later time points (days 12, 18, and 26); and (3) TNFα enhanced the percentage of cells entering into S and G,M in response to IL-3 at day 0 and 5 but decreased the percentage of cells entering into S and G,M in response to IL-3 from day 12 onward.

To further clarify the inhibitory effects of TNFα on the later stages of IL-3-dependent cell growth and differentiation, multicolor flow cytometric analysis was performed to correlate the phenotype (expression of two surface antigens) with cellular DNA content (as measured by Hoechst 33342 staining). Target cells were cultured for 12 days with IL-3, then recultured for 3 additional days with IL-3 with or without TNFα (Fig 1D). As illustrated in Table 2, TNFα reduced by approximately 20% the number of S/G,M cells (see column total). Half of the cultured cells were CD15' HLA-DR' (granulocyte-committed cells) and addition of TNFα strongly inhibited (mean inhibition 28.6%) the entry of these cells into S/G,M. Approximately one-third of the cultured cells were CD15' HLA-DR' (undefined maturing cells that lacked expression of CD14) with a relatively low proliferation rate (14.7% of cells in cycle), and were not significantly affected by TNFα. The minor population of blast cells expressing HLA-DR' but neither CD14 nor CD15 (11% of total cells) displayed an intense proliferative
capacity that could be further enhanced by TNFα. This observation is consistent with the stimulatory effects of TNFα on the IL-3–dependent growth of freshly isolated CD34+ cells. Finally, CD14+ of monocytic affiliation displayed the lowest proliferation rate and their IL-3–dependent entry into S/G2/M was only weakly inhibited by TNFα.

The present results indicate that the late inhibitory effects of TNFα are not due to direct cytotoxic effects but rather to an inhibition of IL-3–dependent proliferation of granulocyte-committed cells.

The inhibitory effects of TNFα are direct. Inasmuch, as we previously demonstrated the potentiating effects of TNFα on IL-3–induced CD34+ HPC proliferation to be direct, we wondered whether the inhibitory effects of TNFα observed in late cultures would be indirect as a consequence of novel cytokine synthesis.

Thus, cells cultured for 14 days with IL-3 were washed and recultured for 3 days in the presence of IL-3 with or without TNFα. As previously shown in Fig 7 and Table 2, addition of TNFα under such conditions resulted in inhibition of growth. To test whether the inhibitory activity was due to TNFα or to other inhibitors released in response to TNFα, cell supernatants were collected and assessed for their growth-inhibitory capacity in the presence of an
Table 2. TNFα Inhibits the IL-3–Dependent Proliferation of Granulocyte-Committed Cells

<table>
<thead>
<tr>
<th>Total</th>
<th>CD14+</th>
<th>CD14-</th>
<th>CD15+</th>
<th>CD15-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of Three Experiments</td>
<td>100 ± 0</td>
<td>11.1 ± 4.1</td>
<td>8.1 ± 1.8</td>
<td>48.5 ± 4.4</td>
</tr>
<tr>
<td>% Positive Cells*</td>
<td>19.5</td>
<td>15.0</td>
<td>14.3</td>
<td>15.0</td>
</tr>
<tr>
<td>% Cycling Cells†</td>
<td>32.4 ± 8.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values represent the percentage of each cell type and are expressed as the mean of three unrelated experiments without discrimination between incubation in IL-3 or in IL-3 + TNFα as no differences were observed.

†Values represent the percentage of cycling cells of each category after incubation in IL-3 or in IL-3 + TNFα (50,000 total cells were gated).

Table 3. TNFα Is Responsible for the Inhibitory Activity Contained in Supernatant of IL-3 + TNFα Cultured Cells

<table>
<thead>
<tr>
<th>Supernatants</th>
<th>Set No. 1</th>
<th>Set No. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3</td>
<td>IL-3 + TNFα</td>
<td>IL-3 + TNFα</td>
</tr>
<tr>
<td>MoAb control</td>
<td>39.8</td>
<td>35.1</td>
</tr>
<tr>
<td>MoAb anti-TNFα</td>
<td>3.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Exp 2</td>
<td>IL-3 + TNFα</td>
<td>IL-3 + TNFα</td>
</tr>
<tr>
<td>MoAb control</td>
<td>55.8</td>
<td>40.3</td>
</tr>
<tr>
<td>MoAb anti-TNFα</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Cells precultured in IL-3 for 14 days were washed and recultured in the presence of IL-3 with or without TNFα for 72 hours. Supernatants were collected and subsequently tested for their inhibitory activity, in the presence of a neutralizing anti-TNFα MoAb (1 μg/mL) or an unrelated control MoAb (1 μg/mL), on the proliferation of CD34+ HPC precultured for 14 days in IL-3 (see Materials and Methods and Fig 1E). Values represent percentage inhibition calculated as the ratio of [value in IL-3 + MoAb] – [value in test supernatant + MoAb]]/value in IL-3 + MoAb] × 100.

Discussion

In a previous study we demonstrated that TNFα strongly potentiates the early proliferative response of CD34+ HPC to IL-3 and GM-CSF. Here, we explored the effects of TNFα on longer term in vitro proliferation and differentiation.

Fig 8. TNFα retains growth-inhibitory activity at limiting dilution. Cells precultured for 14 days in IL-3 were washed and recultured at 1 cell/well in the presence of IL-3 (■), IL-3 + TNFα (■). Six hundred wells were seeded for each culture condition. After 6 days of culture, clone size was determined using an inverted microscope coupled to an image analyzer. TNFα or medium alone did not induce the development of any clones. Clones were classified according to number of cells/clone. For experimental procedure see Materials and Methods and Fig 1F. Clone frequencies obtained in this particular experiment were 16.5% (15.5% to 17.3%; 95% confidence limits) for IL-3 and 7.8% (7.4% to 8.2%; 95% confidence limits) for IL-3 + TNFα. The average clone size was the same for IL-3 and IL-3 + TNFα (11.9 cells/clone).
tion of CD34+ HPC. Within 8 days, the number of cells obtained in presence of IL-3 + TNFα was two to three times higher than in the presence of IL-3 alone. However, no further growth could be observed after 12 days and the number of cells eventually declined after 18 to 20 days. This result contrasted with cultures performed with IL-3 alone, in which cell growth persisted for at least 30 days. The inhibitory effects of TNFα on IL-3-dependent development of CD34+ HPC could also be observed in semisolid cultures, following an early potentiation of proliferation of clonogenic cells. TNFα strongly blocked the generation of day 14 IL-3-dependent BFU-e, G, and GM colonies, in accordance with previous studies using LD-BMC fractions and/or conditioned medium as source of CSFs.15-19,30 Thus, we observed a dual effect of TNFα in hematopoiesis, and in the present study we demonstrate that several mechanisms concur to TNFα-induced inhibition of granulocyte development.

First, TNFα was found to favor the development of a CD13+ subpopulation of HLA-DR+ blast cells lacking CD14 and CD15, apparently frozen at an early stage of differentiation. Upon removal of TNFα, this CD13+ cell population differentiated into granulocytic cells in response to IL-3, indicating that TNFα can block differentiation towards the granulocytic series and that this inhibition is irreversible.

Second, consistent with the inhibition of G colonies observed in semi-solid medium, TNFα blocked the development of cells of granulocytic lineage as determined by cytology and expression of the granulocyte-specific CD15 antigen and by monitoring of CFU-G generation. Accordingly, TNFα inhibited entry into cycle of granulocyte-committed cells, as previously reported in a case of chronic leukemia.20 Our data demonstrate an accumulation of cells in G1 and in G,M (as a consequence of colcemid block) strongly suggesting that TNFα blocks the proliferation of granulocyte-committed cells during G, phase of the cycle. Thus, TNFα inhibits granulocytic development via two mechanisms: (1) inhibition of differentiation of blast cells towards granulocytes; and (2) inhibition of proliferation of granulocyte-committed cells. These findings are in accordance with our previous description of an inhibitory role of TNFα on G-CSF-dependent proliferation of granulocyte-committed CD34+ HPC.21

Inhibition of proliferation of granulocyte-committed cells is not mediated by a direct short-term cytotoxic effect of TNFα on IL-3-precultured cells, as demonstrated by liquid culture studies. Nevertheless, TNFα induced a decrease in the number of IL-3-dependent colonies between day 7 and day 14, suggesting that cells whose differentiation into granulocytes is blocked may finally die, possibly from apoptosis, as demonstrated with IL-3-dependent cell lines weaned of IL-3.20

The present study also indicates that TNFα strongly inhibits IL-3-dependent erythropoiesis from CD34+ HPC, in agreement with results previously published with fractionated LD-BMC.20-26 However, the mechanisms involved in TNFα-mediated inhibition of erythropoiesis have not been explored. In contrast to the above mentioned effects, TNFα did not decrease the number of recruited CFU-M and even potentiated IL-3-dependent development of the monocytic lineage in liquid cultures of CD34+ HPC. These findings are in accordance with reports indicating a lack of inhibitory effect of TNFα on the development of CFU-M13,20 and a capacity of TNFα to enhance macrophage proliferation in synergy with M-CSF.24 However, the monocytic cells that acquire CD14 become slowly proliferating cells, therefore the TNFα-dependent early potentiation of monocytic development subsequently contributes to the arrest of growth progression observed in late cultures. In addition, CD14+ monocytes become sensitive to cycle inhibition by TNFα, which could be related to an induction of differentiation as described on promyelocytic cell lines.13,15,26

Our data indicate that the inhibitory effects of TNFα are direct, as previously demonstrated for the early potentiating activity.21 The mechanisms by which TNFα displays stimulatory effects on undifferentiated HPC and inhibitory effects on granulocyte-committed cells remain to be determined. In this context, TNFα has been shown to upregulate the expression of the IL-2 receptor on activated T cells27 and the TGFβ receptor on promyelocytic and monocytic leukemic cell lines,28 suggesting that the dual effects of TNFα could be due to differential modulation of CSF receptors in relation to lineage and differentiation stage of the cells. However, differential cell sensitivity to TNFα could involve the two distinct TNFα receptors29 for which specific CDNA receptors have recently been cloned.40-42 The availability of specific probes for the TNFα receptors will allow studies of their expression on cells generated in the presence of IL-3.

Finally, our studies on the agonistic and antagonistic effects of TNFα on in vitro hematopoiesis shed new light on the contrasting in vivo effects of TNFα, as reports have described both stimulatory43-45 and inhibitory46-48 effects on myelopoiesis. Our present results suggest that both, in vivo, stimulatory effects, previously ascribed to TNFα-mediated cytokine production,44,45 and inhibitory effects could be due to direct actions of TNFα.

TNFα, primarily identified as a cytotoxic factor, was later demonstrated to display growth stimulating properties on different cell types.35-45 In this context, TNFα has been shown to enhance GM-CSF and IL-3,56,57 but to inhibit G-CSF37 induced in vitro growth of acute myeloid leukemias. Such a capacity to display both proliferative and antiproliferative effects is indeed shared by many factors.58 In our studies, we have demonstrated that TNFα directly potentiates early normal hematopoiesis and inhibits subsequent granulocytic development, while it enhances monocytic expansion, suggesting a crucial and complex role for this cytokine in the regulation of hematopoiesis.

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