

Tumor Necrosis Factor α Directly and Indirectly Regulates Hematopoietic Progenitor Cell Proliferation: Role of Colony-stimulating Factor Receptor Modulation

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Summary

Tumor necrosis factor α (TNF- α) has been shown to both stimulate and inhibit the proliferation of hematopoietic progenitor cells (HPCs) in vitro, but its mechanisms of action are not known. We demonstrate that the direct effects of TNF- α on murine bone marrow progenitors are only inhibitory and mediated at least in part through downmodulation of colony-stimulating factor receptor (CSF-R) expression. The stimulatory effects of TNF- α are indirectly mediated through production of hematopoietic growth factors, which subsequently results in increased granulocyte-macrophage CSF and interleukin 3 receptor expression. In addition, the effects of TNF- α (stimulatory or inhibitory) are strictly dependent on the particular CSF stimulating growth as well as the concentration of TNF- α present in culture. A model is proposed to explain how TNF- α might directly and indirectly regulate HPC growth through modulation of CSF-R expression.

The survival, proliferation, and differentiation of hematopoietic progenitor cells (HPCs)¹ are controlled by positive and negative regulatory cytokines. Stimulatory hematopoietic growth factors (HGFs) include the CSFs (1, 2), the interleukins (3), erythropoietin (4), and stem cell factor (SCF) (5, 6). The CSFs include granulocyte CSF (G-CSF), macrophage CSF (M-CSF or CSF-1), granulocyte-macrophage CSF (GM-CSF), and IL-3 (2, 7). Presence of one or more of the CSFs is essential for the in vitro proliferation of bone marrow progenitor cells (2, 7). Biological actions of the CSFs are mediated through specific high-affinity cell surface receptors (2), and modulation of CSF-R expression has been proposed as a mechanism by which non-CSF HGFs can elicit inhibitory and/or stimulatory effects on CSF-stimulated hematopoiesis (8, 9).

In addition, a number of negative regulators of hematopoietic cell growth have been identified such as TGF- β (10), macrophage inflammatory protein-1 α (11), interferons (12), prostaglandins (13), and TNF- α (14, 15).

TNF- α is mainly a monocyte-derived cytokine (16), but its production can also be induced in other cell types such as T cells (17) and endothelial cells (18). TNF- α was originally characterized by its cytotoxic and cytostatic activity on tumor cells in vitro and in vivo (19, 20). However, TNF- α has subsequently been demonstrated to affect a wide range of biological activities of many normal cell types, including endothelium (21), fibroblasts (22), T cells (23), neutrophils (24), and monocytes (25). Conflicting results have been reported with regard to the effects of TNF- α on the growth and proliferation of HPCs, from potent inhibition (14, 15, 26–30) to stimulation (31–33). Furthermore, TNF- α can promote hematopoietic recovery from lethal radiation damage (34–36). It is not known whether the effects of TNF- α on HPCs are direct and/or indirect, or by which mechanism(s) TNF- α exerts its effects. However, TNF- α has been demonstrated to induce the production of a variety of HGFs, including IL-1, IL-6, M-CSF, G-CSF, and GM-CSF (37–42), and to modulate CSF-R expression on granulocytes, macrophages, and leukemic cells (43–47). Therefore, we addressed whether the stimulatory and inhibitory effects of TNF- α on hematopoietic cell growth were direct or indirect, and whether CSF-R modulation might represent a mechanism of TNF- α action. The present results demonstrate that TNF- α directly inhibits CSF-stimulated proliferation of murine bone marrow

¹ Abbreviations used in this paper: CM, conditioned medium; HGFs, hematopoietic growth factors; HPCs, hematopoietic progenitor cells; Hu, human; LDBM, light density bone marrow; Lin⁻, lineage negative; Mu, murine; PDGF, platelet-derived growth factor; SCF, stem cell factor.

progenitor cells, which directly correlates with CSF-R down-modulation. In addition, TNF- α has potent stimulatory effects that are indirect and mediated, at least in part, through production of HGFs and subsequent induction of CSF-Rs.

Materials and Methods

Growth Factors

Purified rMuGM-CSF and rHuman (rHuG-CSF) were generously supplied by Ian K. McNiece and Thomas Boone (Amgen Corporation, Thousand Oaks, CA). Recombinant murine (rMu) IL-3 was purchased from R and D Systems (Minneapolis, MN). rHuCSF-1 was a generous gift from Dr. Michael Geier (Cetus Corp., Emeryville, CA). rMuTNF- α was a gift from Mike Palladino (Genentech, San Francisco, CA).

Cytokine Antibodies

Rabbit anti-murine TNF- α , a polyvalent antiserum, was purchased from Genzyme Corp. (Boston, MA). It had a neutralizing activity of 10^6 U/ml. Goat anti-murine IL-6 was from R and D Systems, and, depending on the assay, 2–3 μ g/ml of this antibody neutralizes 1 ng/ml of murine IL-6. Goat anti-murine IL-1 β was purchased from R and D Systems. Rabbit anti-mouse IL-1 α polyclonal antibody (Genzyme Corp.) neutralizes 10 U of murine IL-1 α at a dilution of 1:50. Lyophilized rat anti-murine CSF-1 mAb was purchased from Oncogene Science (Manhasset, NY).

Preparation of CM. Light density bone marrow (LDBM) cells were incubated at 5×10^5 cells/ml in complete IMDM at 37°C, 5% CO₂ in the absence or presence of 2 ng/ml of rMuTNF- α . Cell-free supernatants were harvested after a 24-h incubation. To neutralize the effects of TNF- α in the CM, it was pretreated with a TNF- α antibody (Genzyme Corp.) at a concentration sufficient to neutralize 10 ng/ml of TNF- α .

Bone Marrow Cells

Normal murine bone marrow cells were obtained by aspirating femurs of normal BALB/c mice. LDBM cells were separated by centrifugation on lymphocyte separation medium (Organon Teknika Corp., Durham, NC). Cells were washed twice in IMDM and resuspended in IMDM supplemented with 10% FCS (Inovar, Gaithersburg, MD), 15 mg/liter gentamicin, and 3 mg/ml glutamine (complete IMDM).

Purification of Lin⁻ Bone Marrow Progenitors

Lin⁻ bone marrow progenitor cells were purified according to a previously described protocol (48). Briefly, LDBM cells were resuspended in complete IMDM and incubated at 4°C for 30 min with a cocktail of antibodies, RA3-6B2 (B220 antigen) and RB6-8C5 (GR-1 antigen) (gifts of R. Coffman, DNAX Corp., Palo Alto, CA); MAC-1 (purchased from Boehringer-Mannheim, Indianapolis, IN); Lyt-2 (CD8) and L3T4 (CD4) (purchased from Becton Dickinson & Co., Sunnyvale, CA). Cells were washed twice and resuspended in complete IMDM. Magnetic beads (Dynal, Great Neck, NY) were added at a ratio of 40:1 (beads/cells), and the mixture was incubated for 30 min at 4°C. Labeled (Lin⁺) cells were removed by a magnetic particle concentrator (Dynal), and Lin⁻ cells were recovered from the supernatant.

Purification and FACS Sorting of Lin⁻Thy-1⁺ Progenitors. Lin⁻ cells were indirectly labeled with the mAbs anti-Thy-1.2 (Becton Dickinson) or an isotype-matched control purchased from Phar-

mingen (San Diego, CA). Then, anti-rat Ig-FITC (Cappel Laboratories, Malvern, PA) were added at 1:20 dilution and incubated for 30 min at 4°C, and Thy-1⁺ cells were separated by FACS® (Becton Dickinson & Co.) as previously described (49).

Cell Lines

The 32DC13 progenitor cell line (50) was maintained in complete RPMI 1640 supplemented with 20% WEHI-3 conditioned medium. The NFS/N1.M6 (MC-6) mast cell line (51), a generous gift from Dr. Douglas E. Williams (Immunex Corp., Seattle, WA), was grown in complete RPMI 1640 with 20% WEHI-3 conditioned medium.

Soft Agar Colony Formation

A modification of the method of Stanley et al. (52) was used to measure colony formation of murine bone marrow progenitor cells in vitro. Briefly, 5×10^4 LDBM cells or 10^4 Lin⁻ cells in 1 ml of complete IMDM and 0.3% sea plaque agarose were plated in 35-mm Lux petri dishes (Miles Scientific, Naperville, IL) and incubated at 37°C in 5% CO₂ for 7 d and scored for colony growth (>50 cells).

[³H]Thymidine Incorporation Assays. LDBM cells were incubated in the presence of CSFs at 37°C, 5% CO₂ or TNF- α -conditioned medium (CM) in 96-well microtiter plates at a density of 5×10^4 cells in 100 μ l of complete IMDM. DNA synthesis was assessed with a pulse of 1 μ Ci of [³H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) for the last 6 h of the incubation period. Radioactivity was determined by liquid scintillation.

Single-Cell Proliferation Assay. Lin⁻ or Lin⁻Thy-1⁺ were seeded in Terasaki plates (Nunc, Kamstrup, Denmark) at a concentration of 1 cell per well in 20 μ l of complete IMDM. Wells were scored for proliferation (>10 cells) after 6–8-d incubation at 37°C, 5% CO₂.

Radioiodination of CSFs

The radioiodination of IL-3 was performed by a chloramine-T method as previously described (53), while G-CSF, CSF-1, and GM-CSF were labeled by a modified chloramine-T method. Briefly, 5–10 μ g of rMuGM-CSF, recombinant human (rHu)G-CSF, or rHuCSF-1 in 10 μ l of 0.1 mol/liter sodium phosphate buffer (pH 7.0), 10 μ l of 10% DMSO with 100 μ g/ μ l of polyethylene glycol, and 1 mCi of ¹²⁵I (Amersham, Arlington Heights, IL) were incubated at 4°C for 5 min in the presence of 10 μ l of 0.1 mg/ml chloramine-T. Then, 10 μ l of 0.3 mg/ml sodium metabisulfite and 10 μ l of 0.1 mol/liter potassium iodide was added. Iodinated CSFs were separated on a Sephadex G-10 or G-25 column equilibrated with PBS. The specific radioactivity was 7.2–10.2 $\times 10^6$, 0.9–2.0 $\times 10^6$ 1.7–2.8 $\times 10^6$, and 6.4–7.8 $\times 10^6$ cpm/pmol for radioiodinated GM-CSF, G-CSF, IL-3, and CSF-1, respectively. The biological activity of all CSFs were retained for at least 3–4 wk after radiolabeling, as determined by their ability to induce bone marrow proliferation.

Radioligand Binding Experiments. [¹²⁵I]CSF binding experiments were performed by a previously described phthalate oil separation method (54). LDBM cells or Lin⁻ cells were resuspended in 1 ml of 50 mM glycine-HCl (pH 3.0) for 1 min to release bound ligands. Cells were then washed twice in RPMI 1640 containing 1% BSA, 20 mmol/liter HEPES, and 0.1% sodium azide (binding medium). Cells were incubated with ¹²⁵I-cytokines in 200 μ l of binding medium. Specific binding of radiolabeled CSFs (20–300 pM) was

determined as the difference in CSF binding in the absence and presence of a 50-fold excess of unlabeled CSF following a 90-min incubation at 22°C (GM-CSF) or 37°C (G-CSF, CSF-1, and IL-3). Cell-bound [¹²⁵I]CSF was separated from unbound ligands by centrifugation through a 0.2-ml mixture of dibutylphthalate and 1 bis (2 ethyl-hexyl) phthalate oil (ratio, 1.5:1) (Eastman Kodak, Rochester, NY). Radioactivity was measured in a Biogamma 2 counter (Beckman Instruments, Fullerton, CA). Equilibrium binding data were analyzed according to Scatchard (55) and by computerized linear regression analysis.

Cell Surface Phenotyping

LDBM cells were labeled with anti-LFA-1, anti-MAC-1, and anti-Ly-5 (Boehringer-Mannheim), anti-Ly-17 (Dr. Margaret L. Hibbs, Melbourne, Australia), or RB6-8C5 (gift from Dr. Coffman),

and a goat anti-rat-FITC (Cappel Laboratories) as previously described (9). Cells were analyzed by FACS[®] on a EPIC-753 (Coulter Electronics, Hialeah, FL).

Results

Effects of TNF- α on CSF-induced Clonal Growth of Murine Bone Marrow Progenitor Cells In Vitro Previous studies had demonstrated considerable variability in the effects of TNF- α on the growth of HPCs in vitro, ranging from potent inhibition to stimulation (14, 15, 26–33). Because many of these studies used CM as a source of colony stimulating activity (CSA) as well as unfractionated bone marrow, we first assessed the effects of rMuTNF- α over a broad dose range on the growth of CFU cells (CFU-C) induced by purified rCSFs.

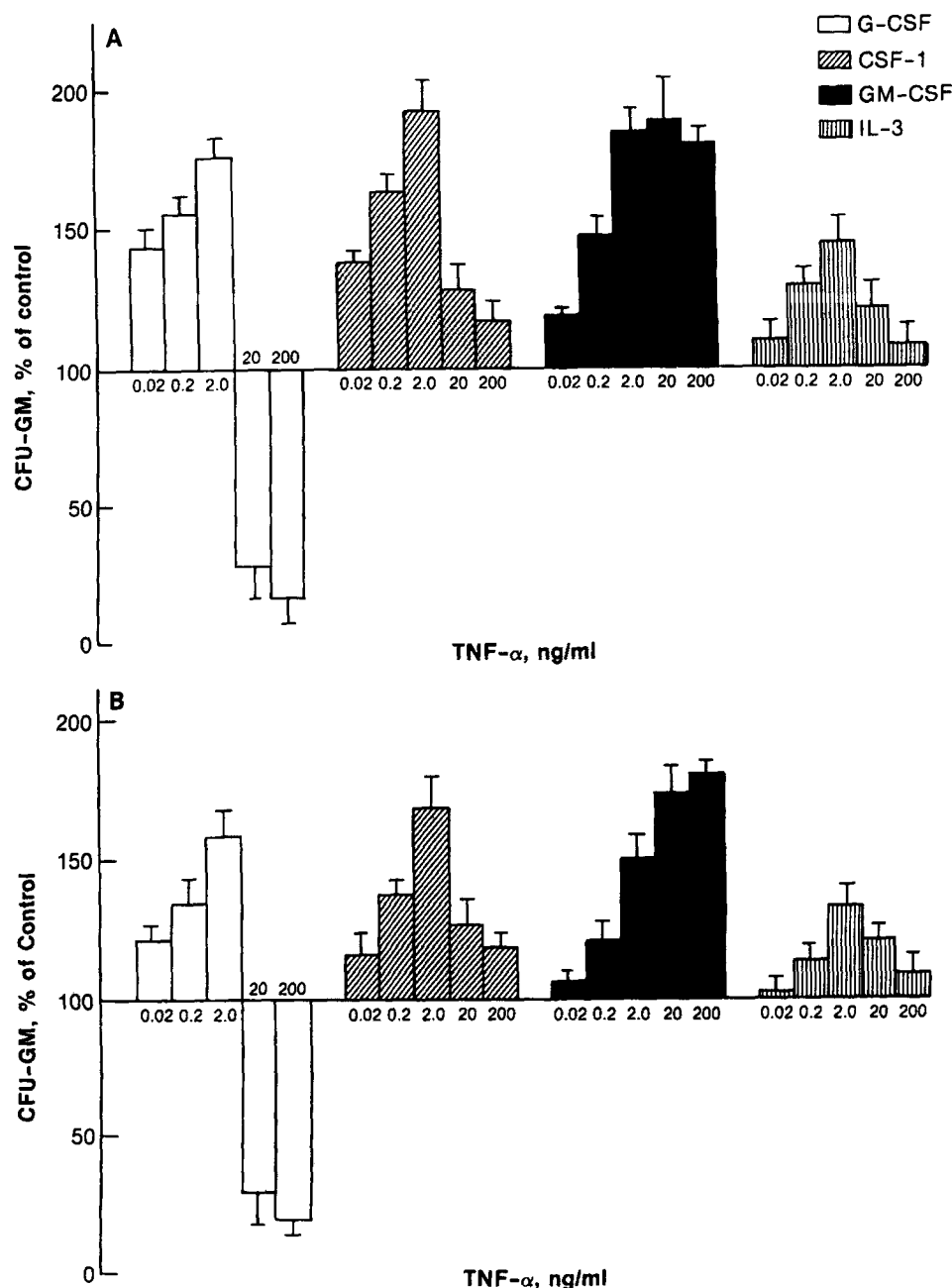


Figure 1. Effects of TNF- α on CSF-stimulated colony formation of LDBM cells and Lin⁻ progenitors. LDBM (A) and Lin⁻ cells (B), separated as described in Materials and Methods, were plated at 5×10^4 and 2×10^4 cells, respectively, in 1 ml of IMDM with 10% FCS and 0.35% sea plaque agarose. Cultures were supplemented with predetermined optimal concentrations of purified rMuGM-CSF (20 ng/ml), rHuG-CSF (20 ng/ml), rMuIL-3 (20 ng/ml), or rHuCSF-1 (50 ng/ml), and incubated at 37°C, 5% CO₂ for 7 d before being scored for colony formation (>50 cells). Results are presented as the mean \pm SD of duplicate determinations and are representative of at least four separate experiments.

In addition, we compared the effects of TNF- α on normal LDBM cells versus a highly enriched bone marrow progenitor cell population, designated lineage negative (Lin-) due to its absence of cell surface antigens characteristic of B and T lymphocytes, monocytes, and granulocytes (48).

Although TNF- α alone did not promote colony formation of LDBM cells (data not shown), it had a bidirectional effect on G-CSF-induced colony formation (Fig. 1 A). Specifically, concentrations of TNF- α at 2 ng/ml or below enhanced the number of G-CSF-stimulated CFU-C, with a 44% increase at 0.02 ng/ml and maximum enhancement of 75% at 2 ng/ml. In contrast, TNF- α at 20 and 200 ng/ml inhibited G-CSF-stimulated colony formation by 72% and 83%, respectively. A similar bidirectional response to TNF- α was observed on Lin- progenitors, in that G-CSF-stimulated CFU-C formation was maximally enhanced by 58% at 2 ng/ml, whereas a 70% and 80% inhibition was observed at 20 and 200 ng/ml, respectively (Fig. 1 B).

TNF- α also stimulated CSF-1-induced CFU-C formation of LDBM cells (Fig. 1 A). TNF- α increased the number of CSF-1-induced CFU-C by 37% at 0.02 ng/ml, and a maximum stimulation of 92% was observed at 2 ng/ml of TNF- α . In contrast to the effects observed on G-CSF-induced colony growth, higher concentrations of TNF- α did not inhibit CSF-1-induced colony formation, with a marginal increase in colony formation of 28% and 17% observed at 20 and 200

ng/ml, respectively. The same response profile to TNF- α was observed on CSF-1-stimulated Lin- progenitors (Fig. 1 B).

Similar to the results with CSF-1, IL-3-stimulated colony formation was enhanced at low concentrations of TNF- α (0.02–2.0 ng/ml), with a maximum of 43% and 33% for LDBM (Fig. 1, A) and Lin- cells (Fig. 1, B), respectively, whereas 20–200 ng/ml had less or no stimulatory effect.

Finally, and in contrast to the colony formation induced by the other three CSFs, GM-CSF-induced CFU-C in soft agar was increased at all concentrations of TNF- α tested (0.02–200 ng/ml). Maximum enhancement of 83% for LDBM (Fig. 1 A) and 74% for Lin- cells (Fig. 1 B) was observed at 20 ng/ml. Furthermore, the TNF- α -induced enhancement of GM-CSF-stimulated colony formation was specific and not due to contaminants such as LPS, since an antibody to TNF- α neutralized its stimulatory activity (data not shown).

Thus, TNF- α has bidirectional effects on murine bone marrow colony formation induced by purified rCSFs that is dependent on the particular CSF stimulating growth as well as the concentration of TNF- α in culture.

TNF- α Is a Bidirectional Modulator of CSF-R Expression on HPCs. Because TNF- α had been shown to downmodulate G-CSF and GM-CSF-Rs on human granulocytes (44, 45) and CSF-1R expression on murine monocytes (43, 47), we examined whether modulation of CSF-R expression could play

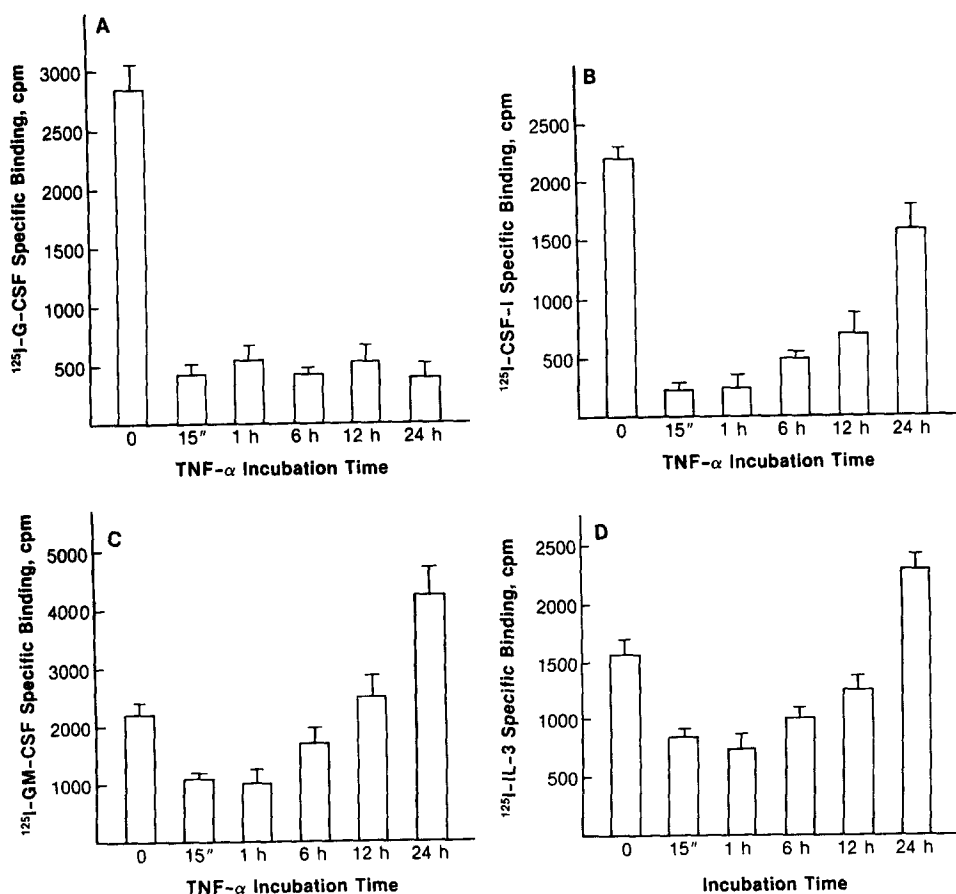


Figure 2. The effects of TNF- α on CSF binding to LDBM cells. LDBM cells (5×10^5 cells/ml) were incubated at 37°C in the presence of TNF- α 20 ng/ml. ^{125}I -G-CSF (A), ^{125}I -CSF-1 (B), ^{125}I -GM-CSF (C), and ^{125}I -IL-3 (D) specific binding were determined following various incubation periods as described in Materials and Methods. Results are presented as the mean \pm SD of duplicate determinations and are representative of at least four separate experiments.

a role in the bidirectional effects of TNF- α on bone marrow progenitor cell proliferation. TNF- α (20 ng/ml) reduced ^{125}I -G-CSF-specific binding to LDBM cells in a time-dependent manner (Fig. 2 A). Maximum downmodulation of G-CSF binding of 86% was observed by 15 min, which was sustained for at least 24 h (Fig. 2 A) and was reversed after removal of TNF- α (data not shown). CSF-1 binding was maximally reduced 87% by 15 min, remained suppressed for 12 h, but unlike G-CSF binding, recovered to 73% of control binding by 24 h (Fig. 2 B). GM-CSF (Fig. 2 C) and IL-3 binding (Fig. 2 D) were also rapidly downmodulated by TNF- α , with a maximum reduction by 1 h of 54% and 52%, respectively. However, in contrast to G-CSF and CSF-1 binding, both GM-CSF and IL-3 binding recovered to control levels within 12 h, and by 24 h an increase in GM-CSF

and IL-3 binding of 91% and 47%, respectively, was observed. This upregulation was prolonged for at least 48 h (data not shown). Furthermore, the specific binding of all four CSFs, were not significantly affected after a 24-h incubation in control medium (data not shown).

Equilibrium binding studies and subsequent Scatchard analysis revealed that untreated LDBM cells had 253 high-affinity ($K_D = 1.1 \times 10^{-10}$ M) G-CSF binding sites per cell and that TNF- α at 20 ng/ml by 1 h reduced the number of G-CSF-Rs to 80 without significantly affecting the receptor affinity (Fig. 3 A). Similarly, TNF- α 20 ng/ml reduced the number of both high- and low-affinity GM-CSF-Rs by 51% and 53%, respectively, after 1 h without affecting their affinities (Fig. 3 B).

TNF- α -induced modulation of CSF-Rs occurred in the

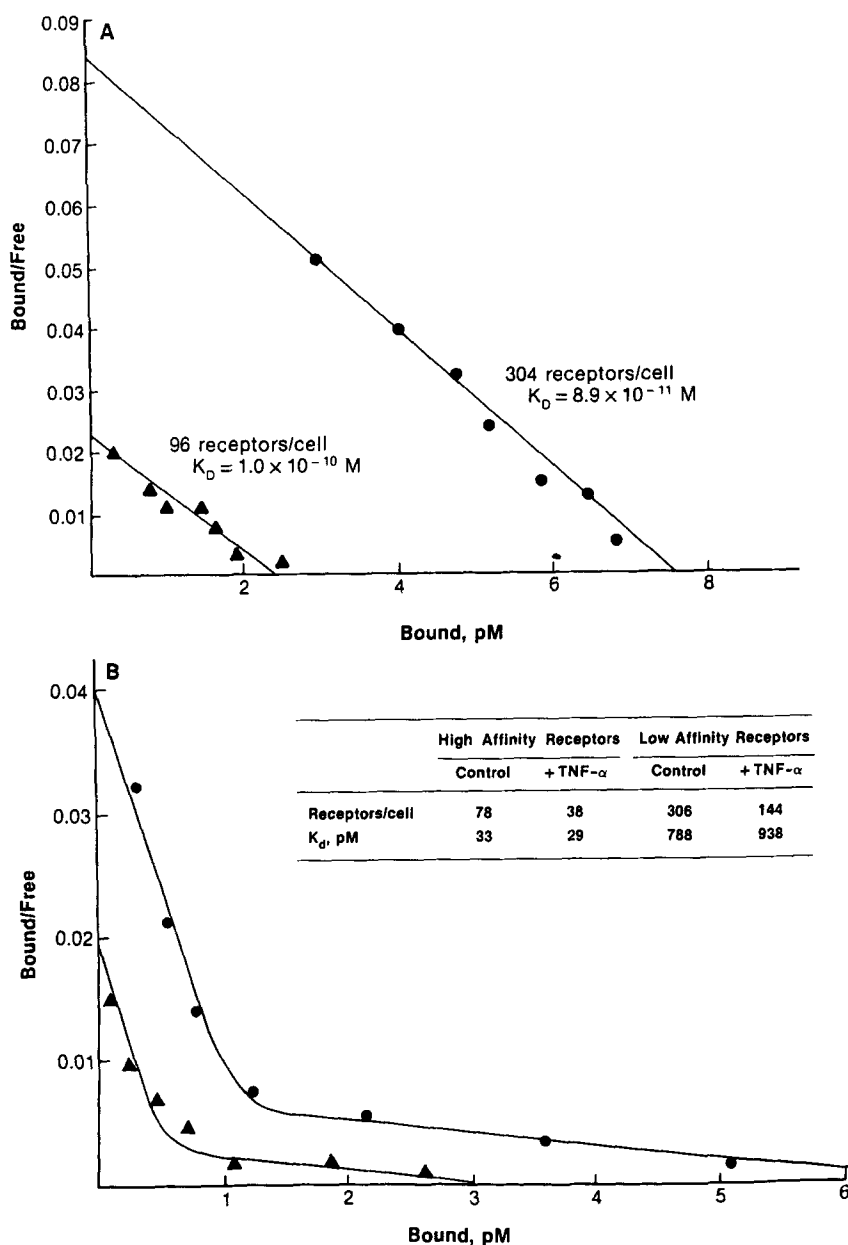


Figure 3. Scatchard analysis of TNF- α -induced downmodulation of G-CSF and GM-CSF-R expression on LDBM cells. LDBM cells (5×10^5 cells/ml) were incubated at 37°C for 1 h in the presence (\blacktriangle) or absence (\bullet) of TNF- α 20 ng/ml. Increasing concentrations of radiiodinated G-CSF (A) and GM-CSF (B) were added to 3.0×10^6 and 2.0×10^6 cells in 200 μl of binding medium, and G-CSF- and GM-CSF-specific binding was determined as described in Materials and Methods. Equilibrium binding data were analyzed according to Scatchard's method. Results are the mean of duplicate determinations and are representative of three separate experiments.

Table 1. Modulation of CSF Binding to Lin- Bone Marrow Progenitor Cells by TNF- α

Ligand	Incubation time	Specific binding	
		- TNF- α	+ TNF- α
	h	cpm	
G-CSF	1	494 \pm 42	85 \pm 23*
G-CSF	24	549 \pm 78	123 \pm 59*
CSF-1	1	2,348 \pm 213	389 \pm 67†
CSF-1	24	2,052 \pm 341	1,363 \pm 134*
GM-CSF	1	1,980 \pm 102	1,073 \pm 48*
GM-CSF	24	1,867 \pm 159	3,491 \pm 341*
IL-3	1	1,468 \pm 84	809 \pm 91†
IL-3	24	1,281 \pm 71	2,101 \pm 130†

Lin- bone marrow cells were separated as described in Materials and Methods, and incubated at 5×10^5 cells/ml at 37°C, 5% CO₂ in the presence or absence of TNF- α 20 ng/ml. CSF-specific binding was determined after a 1- and 24-h incubation as described in Materials and Methods. Data are presented as the mean of duplicate determinations \pm SD and are representative of at least three separate experiments. Results of three experiments were analyzed by *t* tests comparing - TNF- α vs. + TNF- α .

**p* \leq 0.01.

†*p* \leq 0.05.

absence of changes in cell numbers and viability (data not shown), and was not due to TNF- α binding to the CSF-Rs, because TNF- α did not compete for CSF binding sites at 4°C (data not shown).

Because bone marrow progenitor cells are in low frequency

Table 2. Effect of TNF- α on the Expression of Cell Surface Proteins on LDBM Cells

mAb	Percent positive cells		Mean fluorescence	
	- TNF- α	+ TNF- α	- TNF- α	+ TNF- α
Control	<5	<5	4	3
LFA-1	46	41	7	8
MAC-1	34	30	29	33
Ly-5	82	79	43	37
Ly-17	17	21	4	4
8C5	47	53	43	42

LDBM cells were incubated at 5×10^5 cells/ml at 37°C, 5% CO₂ for 1 h in the presence or absence of TNF- α 20 ng/ml. Cells were then incubated with primary mAbs and a secondary fluorescein conjugated Ab as described in Materials and Methods. An isotype-matched rat Ig was used as a control. Percent positive cells refers to the percentage of cells with a higher fluorescence intensity than 95% of the cells incubated with the control Ab. Results presented are representative for three separate experiments.

in LDBM, the effect of TNF- α on CSF-R expression was also investigated on Lin- bone marrow progenitor cells (Table 1). Their high proliferative potential is underscored by the finding that up to one in five Lin- cells proliferate under optimal conditions in single-cell cultures supplemented with multiple HGFs (Keller, J. R., S. E. W. Jacobsen, and F. W. Ruscetti, unpublished observations). As observed for LDBM cells, TNF- α reduced the binding of all four CSFs to Lin- cells by 1 h and upregulated GM-CSF and IL-3R expression by 187% and 64%, respectively, at 24 h (Table 1).

To address the specificity of TNF- α -induced modulation of CSF-Rs, the expression of other cell surface antigens on LDBM cells detected by mAb were examined by FACS[®] analysis after TNF- α treatment. TNF- α (20 ng/ml) did not affect the expression of LFA-1, MAC-1, Ly-5, Ly-17, or 8C5 by 1 h (Table 2) or 6 h (data not shown).

Differential Regulation of G-CSF-R Expression Depends on TNF- α Concentration. Because TNF- α optimally downmodulated the expression of all four CSF-Rs by 1 h, we chose to investigate the concentration-response relationship at this time point. TNF- α -induced downmodulation of G-CSF- and CSF-1-specific binding (Fig. 4), as well as GM-CSF- and IL-3-specific binding (data not shown), occurred in a concentration-dependent manner, with maximum inhibition at 20 ng/ml and an E₅₀ of 0.2-2.0 ng/ml.

In seeming conflict, TNF- α downmodulated G-CSF specific binding at low concentrations (0.2 ng/ml) by 1 h (Fig. 3), whereas G-CSF-stimulated colony formation was enhanced at the same concentration (Fig. 1). However, because colony formation occurs only after several days of incubation (56), we examined whether high concentrations of TNF- α (20-200 ng/ml), which inhibit colony formation, might correlate with prolonged downmodulation of G-CSF binding, whereas low

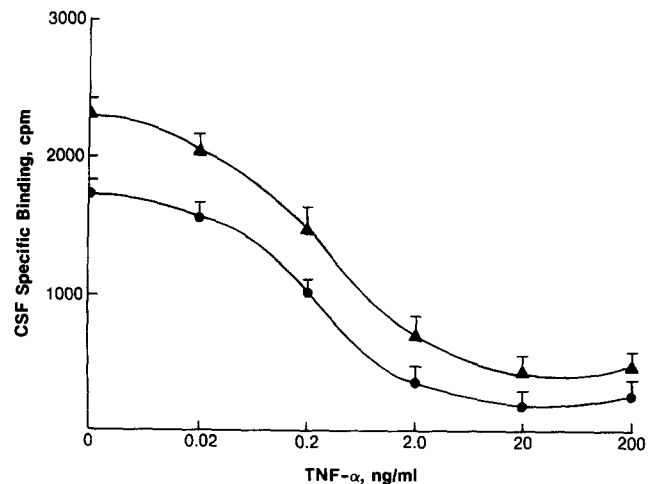


Figure 4. Dose-response of TNF- α -induced downmodulation of G-CSF and CSF-1 binding to LDBM cells. LDBM cells (5×10^5 cells/ml) were incubated at 37°C in the presence of increasing concentrations of TNF- α or in the absence of growth factors. ¹²⁵I-G-CSF (●) and ¹²⁵I-CSF-1 (▲) specific binding was determined as described in Materials and Methods after a 1-h incubation. Results are presented as the mean of triplicate determinations \pm SD and are representative of three separate experiments.

Table 3. Relationship between Dose- and Time-Dependence of TNF- α -induced Downmodulation of G-CSF Binding on LDBM Cells

TNF- α	¹²⁵ I-G-CSF specific binding	
	15 min	24 h
ng/ml		cpm
0	2,384 \pm 182	2,104 \pm 167
0.2	1,597 \pm 152*	2,013 \pm 87
2.0	691 \pm 54*	1,906 \pm 192
20	481 \pm 41*	452 \pm 103*
200	439 \pm 79*	513 \pm 110*

LDBM cells were incubated at 5×10^5 cells/ml at 37°C, 5% CO₂ in the absence or presence of TNF- α at indicated concentrations. G-CSF-specific binding was determined as described in Materials and Methods after 15-min and 24-h incubation. Data presented are the mean of duplicate determinations \pm SD and are representative of at least three separate experiments. Results of three experiments were analyzed by analysis of variance followed by *t* tests comparing - TNF- α vs. + TNF- α . * *p* \leq 0.01.

concentrations (0.2–2 ng/ml), which enhance colony formation, might correlate with recovery of G-CSF binding. Interestingly, TNF- α reduced G-CSF specific binding to LDBM cells by 33% at 0.2 ng/ml and by 71% at 2.0 ng/ml after 15 min, whereas no significant effect was observed by 24 h at the same concentrations of TNF- α (Table 3). In comparison, TNF- α at 20 and 200 ng/ml downmodulated G-CSF-specific binding by >75% at both 15 min and 24 h (Table 3). Thus, inhibition of G-CSF-stimulated colony formation as well as prolonged downmodulation of G-CSF-R expression on LDBM cells occurs at high (20–200 ng/ml) concentrations of TNF- α , whereas low concentrations of TNF- α induce both recovery of G-CSF-R expression and enhanced colony formation.

TNF- α -induced Downmodulation of CSF-R Expression Functionally Reduces Mitogenic CSF Responsiveness. Because TNF- α only transiently downmodulated the expression of receptors for all CSFs except G-CSF and because colony assays measure biological effects of cytokines after a 7-d incubation, a kinetic study of [³H]TdR incorporation on LDBM cells was performed to better correlate CSF-R modulation to CSF-induced proliferation. Significant proliferation of LDBM cells was observed in response to all CSFs by 6 h, at which time 20 ng/ml of TNF- α inhibited G-CSF-, CSF-1-, GM-CSF-, and IL-3-induced proliferation by 65%, 56%, 39%, and 44%, respectively (Fig. 5 A). This was correlated directly with downmodulation of all CSF-Rs at 6 h (Fig. 2). By 24 h (Fig. 5 B), TNF- α inhibited G-CSF-stimulated proliferation by 68%, whereas little or no effect was seen on [³H]TdR incorporation induced by the three other CSFs (Fig. 5 B). By 72 h, when only G-CSF-Rs were downmodulated, G-CSF-induced proliferation was inhibited by 79% by TNF- α , whereas CSF-1-, GM-CSF-, and IL-3-stimulated proliferations were

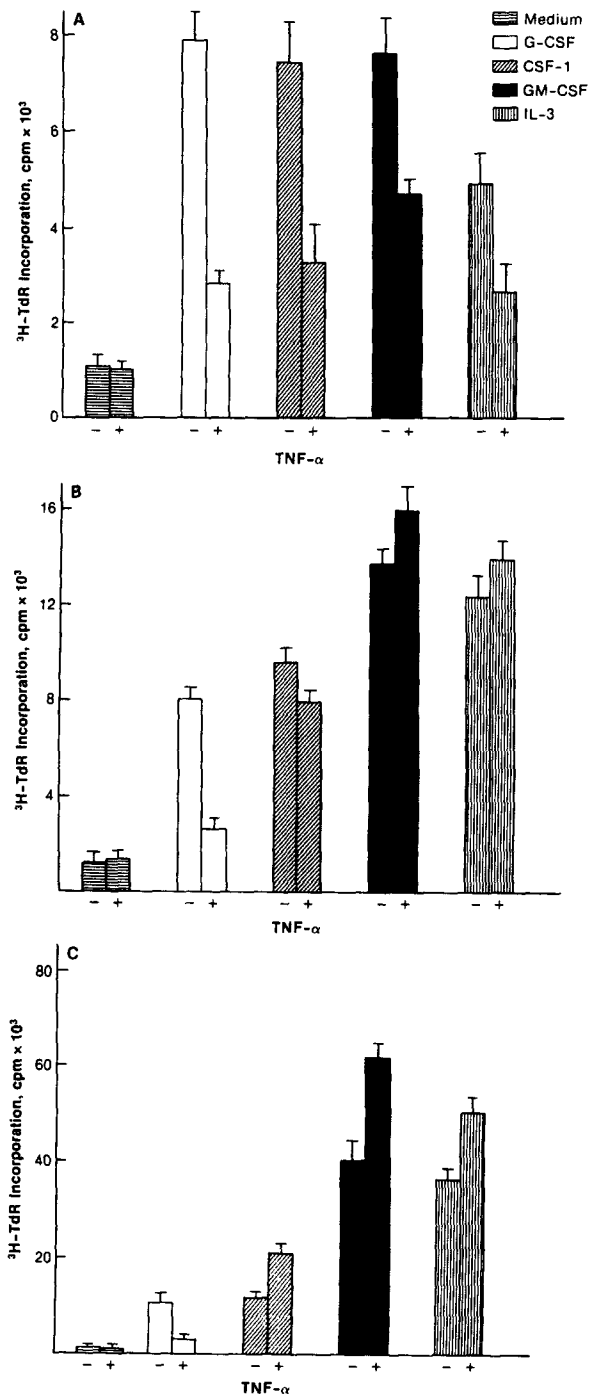


Figure 5. The effect of TNF- α on CSF-stimulated LDBM cell proliferation. LDBM cells (5×10^4 cells/well) were incubated with optimal concentrations of CSFs (as in Fig. 1) or without growth factors (medium). In the absence (-) or presence (+) of 20 ng/ml of TNF- α , [³H]TdR incorporation was determined as described in Materials and Methods after a 6-h (A), 24-h (B), and 72-h (C) incubation at 37°C, 5% CO₂. Data presented are the mean of triplicate determinations \pm SD and are representative of four separate experiments.

Table 4. Direct Effects of TNF- α on CSF-dependent Growth of Separated Bone Marrow Progenitor Populations

Cells	CSF	TNF- α	Growth in Terasaki plates	
			Frequency ⁻¹	Percent Inhibition
Lin -	-	-	0	-
Lin -	-	+	0	-
Lin -	G-CSF	-	75	-
Lin -	G-CSF	+	600	88*
Lin -	CSF-1	-	33	-
Lin -	CSF-1	+	60	45 [†]
Lin -	GM-CSF	-	22	-
Lin -	GM-CSF	+	39	44*
Lin -	IL-3	-	21	-
Lin -	IL-3	+	32	34*
Lin-Thy-1 +	G-CSF	-	0	0
Lin-Thy-1 +	G-CSF	+	0	0
Lin-Thy-1 +	CSF-1	-	75	-
Lin-Thy-1 +	CSF-1	+	125	40 [†]
Lin-Thy-1 +	GM-CSF	-	30	-
Lin-Thy-1 +	GM-CSF	+	50	40*
Lin-Thy-1 +	IL-3	-	8	-
Lin-Thy-1 +	IL-3	+	15	47 [†]

Lin - and Lin-Thy-1 + cells separated according to Materials and Methods were seeded as single cells in Terasaki plates as described. Cultures were incubated at 37°C, 5% CO₂ with predetermined optimal concentrations of CSFs as described in Fig. 1, in the presence (+) or absence (-) of 2 ng/ml of TNF- α . The frequency of responding progenitors (>10 cells/well) was determined after a 6-7-d incubation. Results presented are the mean of four separate experiments, with a minimum of 1,200 wells scored per group. Results of three experiments were analyzed by paired *t* tests comparing -TNF- α vs. +TNF- α .

* *p* < 0.01.

† *p* < 0.05.

enhanced by 78%, 54%, and 35%, respectively (Fig. 5 C). Thus, transient downmodulation of CSF-Rs (GM-CSF-R, CSF-1-R, and IL-3-R) correlates with a transient inhibition of thymidine uptake, whereas prolonged downmodulation (G-CSF-R) is accompanied by sustained inhibition. Conversely, TNF- α -induced upregulation of GM-CSF and IL-3Rs (by 24 h) and recovery of CSF-1Rs correlated with enhanced proliferative responsiveness.

TNF- α Directly Inhibits the Proliferation of Murine Bone Marrow Progenitors in Response to CSFs. Because indirect effects cannot be ruled out in soft agar colony assays, direct proliferative effects of TNF- α were assessed by examining the growth of single Lin - cells in Terasaki plates. In agreement with previously published data (57), the responding frequencies of Lin - cells were 1:75 for G-CSF, 1:33 for CSF-1, 1:22 for GM-CSF,

and 1:21 for IL-3 (Table 4). TNF- α at 2.0 ng/ml, a concentration that enhanced colony formation in response to all four CSFs (Fig. 1), reduced the frequency of responding single cells to 1:600 for G-CSF, 1:60 for CSF-1, 1:39 for GM-CSF, and 1:32 for IL-3-treated cultures, respectively (Table 4). Similar degrees of inhibition were also observed adding TNF- α at 20 ng/ml (data not shown). TNF- α was also examined for direct effects on a very primitive progenitor cell population within the Lin - cells, characterized by cell surface expression of the Thy-1 antigen (48). The proliferation of single Thy-1+/Lin - cells supplemented with CSF-1, GM-CSF, or IL-3 were also inhibited by TNF- α at 2.0 ng/ml (Table 4) and 20 ng/ml (data not shown).

Thus, in striking contrast to the stimulatory effects observed on colony formation in soft agar, TNF- α directly inhibits G-CSF-, CSF-1-, GM-CSF-, and IL-3-induced proliferation of murine bone marrow progenitor cells in single cell assays.

Indirect Stimulatory Effects of TNF- α : Proposed Mechanism of Action. Because TNF- α acted as a direct inhibitor of single bone marrow progenitors, it was possible that the stimulatory effects of TNF- α on CSF-induced colony formation as well as increased GM-CSF and IL-3R expression were indirectly mediated through induction of other cytokines. TNF- α has been demonstrated to induce the production of a number of cytokines, including the CSFs, IL-1, and IL-6 (37-42). Furthermore, synergistic effects on the growth of bone marrow progenitor cells have been shown to occur between different CSFs (58, 59) as well as between CSFs and IL-1 and IL-6 (8, 60, 61). Therefore, we first examined the CM of bone marrow cells exposed to TNF- α 2 ng/ml for 24 h (TNF- α -CM) for synergistic activity on GM-CSF-induced proliferation as well as GM-CSF-R expression on LDBM progenitor cells. TNF- α at 2 ng/ml stimulated GM-CSF-induced proliferation of LDBM cells by 57% after 48 h (Table 5). In comparison, TNF- α -CM enhanced GM-CSF-stimulated proliferation of LDBM cells by 39%, and this enhancement was also seen in the presence of a TNF- α antibody (Table 5). In addition, TNF- α -CM upregulated GM-CSF-specific binding after 24 h both in the presence (59%) and absence (70%) of anti-TNF- α (Table 5). Upregulation of the GM-CSF-R expression was also observed at 0.2 ng/ml and 20 ng/ml of TNF- α (data not shown). In contrast, the non-TNF- α -treated control CM had no such activity (Table 5). Thus, the stimulatory effects of TNF- α on GM-CSF-induced proliferation and on GM-CSF-R expression of murine bone marrow cells are indirect.

To determine whether the indirect effects of TNF- α were mediated through induction of other cytokines, mAbs against murine CSF-1, IL-3, IL-1 α , IL-1 β , and IL-6 were examined for neutralizing activity on colony formation stimulated by the combination of TNF- α and GM-CSF (Table 6). The CSF-1 antibody significantly (25-30%) inhibited the TNF- α stimulatory effect on GM-CSF-induced CFU-C, whereas the other antibodies had no significant effect alone (Table 6) or in combination (data not shown).

Because G-CSF has been demonstrated to possess potent

Table 5. Effect of CM of TNF- α -treated Bone Marrow Cells on GM-CSF-induced Proliferation and GM-CSF-R Expression on LDBM Progenitors

Stimulator*	Anti-TNF- α †	[³ H]TdR	¹²⁵ I-GM-CSF-
		incorporation‡	specific binding§
		cpm	cpm
GM-CSF	-	16,540 \pm 1,781	2,352 \pm 145
GM-CSF	+	15,265 \pm 1,524	2,384 \pm 173
GM-CSF + TNF- α	-	25,927 \pm 2,417†	4,825 \pm 287†
GM-CSF + TNF- α	+	15,627 \pm 1,739	2,194 \pm 149
GM-CSF + control CM	-	18,920 \pm 1,491	2,219 \pm 87
GM-CSF + control CM	+	16,793 \pm 1,034	2,109 \pm 107
GM-CSF + TNF- α -CM	-	22,994 \pm 2,139†	3,994 \pm 213†
GM-CSF + TNF- α -CM	+	23,739 \pm 1,805†	3,512 \pm 169†

* LDBM cells were incubated in 2 ng/ml of GM-CSF, 2 ng/ml of TNF- α , or in 50% CM from TNF- α -treated or -untreated (control) LDBM cells, prepared as described in Materials and Methods.

† The cultures were supplemented with a rabbit anti-mouse TNF- α antiserum (+) or a control serum (-) as described in Materials and Methods.

‡ [³H]TdR incorporation was determined as described in Materials and Methods, after a 48-h incubation at 37°C, 5% CO₂ in the presence of 2 ng/ml of GM-CSF. Results are the mean of triplicate determinations \pm SD and representative of three separate experiments.

§ ¹²⁵I-GM-CSF-specific binding to 2.0 \times 10⁶ LDBM cells was determined as described in Materials and Methods after a 24-h incubation. Data are the mean of duplicate determinations \pm SD and representative of three experiments. Results of this experiment were analyzed by *t* test using a pooled estimate of error.

† *p* < 0.01.

Table 6. Neutralizing Effects of Hematopoietic Growth Factor Antibodies on TNF- α -induced Enhancement of GM-CSF-stimulated Colony Formation

TNF- α	Antibody*	Colony formation (CFU-C)
-	-	45 \pm 4
+	-	81 \pm 5‡
+	TNF- α	48 \pm 7‡
-	CSF-1	42 \pm 3
+	CSF-1	69 \pm 3
-	IL-3	40 \pm 7
+	IL-3	87 \pm 9
-	IL-6	39 \pm 5
+	IL-6	76 \pm 6
-	IL-1 α	49 \pm 6
+	IL-1 α	73 \pm 8
-	IL-1 β	51 \pm 2
+	IL-1 β	92 \pm 10

Lin- bone marrow cells were plated at 2 \times 10⁴ cells in 1 ml of complete IMDM and 0.35% sea plaque agarose, supplemented with 20 ng/ml of rMuGM-CSF and incubated at 37°C, 5% CO₂ in the absence (-) or presence of 2 ng/ml of TNF- α . Colony growth (>50 cells) was scored after a 7-d incubation.

* Cultures were supplemented with optimal concentrations of anti-mouse cytokine antibodies described in Materials and Methods before addition of TNF- α . Results presented are the mean of duplicate determinations \pm SD and are representative of three separate experiments. Results of this experiment were analyzed by *t* tests using a pooled estimate of error.

‡ *p* < 0.01.

synergistic effects on CSF-1-, GM-CSF-, and IL-3-induced colony formation (58) and because a mAb against murine G-CSF was not available, we examined the ability of TNF- α -CM to stimulate the proliferation of the 32DC13 cell line. As previously demonstrated, this cell line proliferates in response to IL-3 and G-CSF (50), but not to other hematopoi-

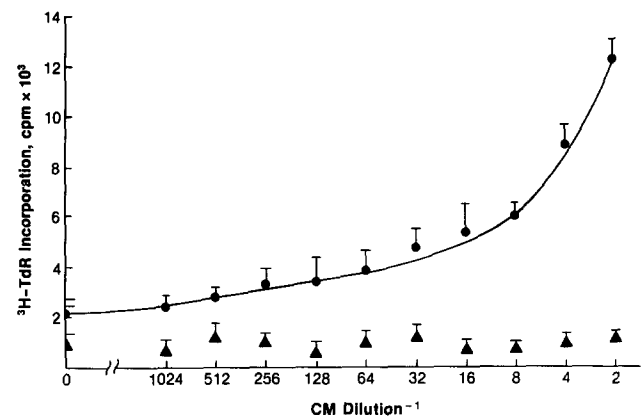


Figure 6. G-CSF activity in CM from TNF- α -stimulated LDBM cells. The 32DG13 cell line (●) and NFS/N1.M6 cell line (▲) were incubated at 10⁴ cells/well at 37°C, in the presence of increasing concentrations of CM from TNF- α -stimulated LDBM cells (prepared as described in Materials and Methods). [³H]TdR incorporation was determined as described in Materials and Methods after a 24-h incubation. Data presented are the mean of triplicate determinations \pm SD and are representative of three experiments. 32DC13 cells stimulated by 20 ng/ml of G-CSF and 20 ng/ml of IL-3 incorporated 40,609 \pm 5,737 and 47,717 \pm 6,198 cpm, respectively, whereas SCF- and IL-3-stimulated MC-6 cells incorporated 7,900 \pm 738 and 39,867 \pm 3,524 cpm, respectively.

etic growth factors such as SCF, CSF-1, GM-CSF, IL-1, IL-2, IL-4, IL-5, IL-6, and IL-7 (Keller, J. R., unpublished observations). TNF- α -CM stimulated the [^3H]TdR incorporation of 32DC13 cells in a dose-dependent manner in the presence of a TNF- α antibody (Fig. 6).

The recently identified SCF is also a potent synergistic factor (5, 6), and because a SCF antibody was not available, the CM of TNF- α -stimulated LDBM cells was treated for SCF activity on the NFS/N1.M6 (MC-6) mast cell line. This cell line proliferates in response to IL-3, IL-4, and SCF but not G-CSF (51). The TNF- α -CM did not promote the growth of MC-6 cells, indicating that the supernatants did not contain IL-3, IL-4, or SCF (Fig. 6).

G-CSF but Not CSF-1 Mimics TNF- α -induced Upregulation of GM-CSF and IL-3R Expression. To investigate whether TNF- α -induced upregulation of GM-CSF and IL-3R expression could be mediated by CSF production, Lin- progenitor cells were incubated in the presence or absence of 20 ng/ml of G-CSF or 50 ng/ml of CSF-1, and examined for GM-CSF and IL-3 binding after 24 h. G-CSF increased GM-CSF- and IL-3-specific binding by 137% and 49%, respectively (Table 7), without significantly affecting cell number or viability (data not shown). In contrast, CSF-1 did not significantly affect GM-CSF- or IL-3-specific binding to Lin- cells (Table 7). Furthermore, in agreement with the inability of TNF- α to increase CSF-1R expression (Fig. 2), G-CSF did not significantly affect CSF-1R expression by 24 h (Table 7).

Taken together, these data indicated that the stimulatory effects of TNF- α on GM-CSF- and IL-3-induced bone marrow progenitor cell proliferation are indirect and mediated, through HGF production, and subsequent upregulation of GM-CSF and IL-3R expression.

Table 7. G-CSF but Not CSF-1 Upregulates GM-CSF and IL-3R Expression on Lin- Progenitor Cells

Stimulator	^{125}I -CSF-specific binding		
	GM-CSF	IL-3	CSF-1
	<i>cpm</i>		
Medium	2,135 \pm 132	1,678 \pm 89	2,397 \pm 219
G-CSF	5,060 \pm 268*	2,501 \pm 192*	2,693 \pm 184
CSF-1	2,468 \pm 214	1,892 \pm 173	1,838 \pm 149*

Lin- cells (5×10^5 cells/ml) were incubated at 37°C, 5% CO₂ for 24 h in the presence of 20 ng/ml of G-CSF or 50 ng/ml of CSF-1, or in the absence of growth factors. ^{125}I -GM-CSF-, ^{125}I -IL-3-, and ^{125}I -CSF-1-specific binding was determined as described in Materials and Methods. Results presented are the mean \pm SD of duplicate determinations and representative of four separate experiments. The results of three experiments were analyzed by analysis of variance followed by *t* tests comparing stimulator vs. medium.

* $p < 0.01$.

Discussion

Although initial studies suggested that the effects of TNF- α on bone marrow colony growth in vitro were only inhibitory (14, 15, 26-33), more recent studies have proposed a stimulatory role for TNF- α in hematopoiesis (19-21). In the present study, we investigated the reason for this apparent discrepancy.

The data presented here confirm that the TNF- α is a bi-directional modulator of HPC proliferation, but demonstrate that direct effects of TNF- α on murine bone marrow cells and highly enriched multipotential progenitors are only inhibitory. These inhibitory effects of TNF- α were correlated with its ability to *trans*-downmodulate the receptor expression for the CSFs stimulating growth. In addition, TNF- α indirectly stimulates CSF-stimulated bone marrow colony formation through at least the induction of G-CSF and CSF-1. Some TNF- α -inducible cytokines, such as G-CSF, might mediate the observed synergistic response through upregulation of CSF-R expression. Fig. 7 is a schematic presentation of how these pathways might interact to mediate the pleiotropic effects of TNF- α .

TNF- α rapidly reduced the expression of all four CSF-Rs

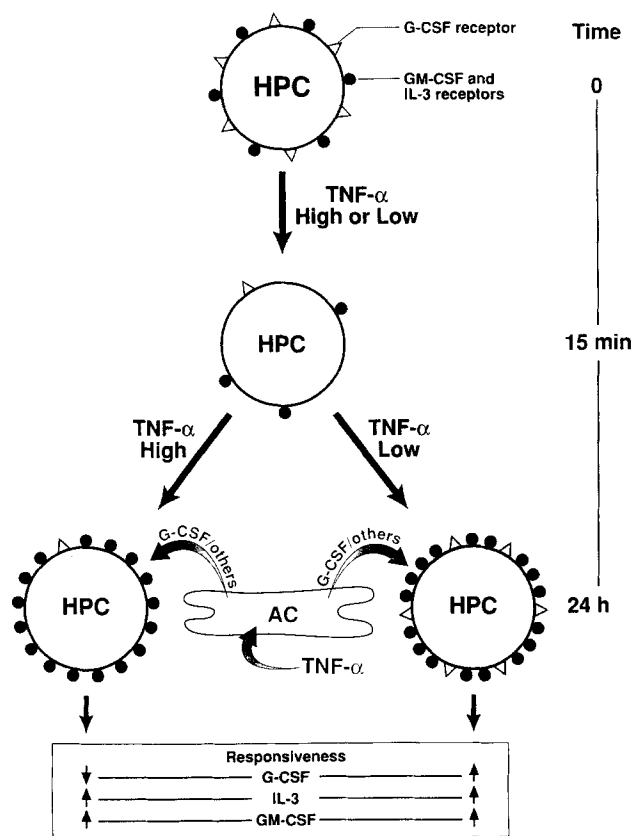


Figure 7. TNF- α directly and indirectly modulates hematopoietic progenitor cell (HPC) proliferation through CSF-R modulation. Production of G-CSF and other HGFs by accessory cells (AC) is induced at both high and low concentrations of TNF- α , resulting in increased expression of GM-CSF and IL-3Rs on HPC. Rapid *trans*-downmodulation of all CSF-Rs is observed at low and high concentrations of TNF- α . Only high concentrations of TNF- α results in prolonged downmodulation of G-CSF-Rs.

on LDBM cells as well as Lin⁻ progenitor cells. This correlated to reduced responsiveness to all four CSFs in the short-term [³H]TdR incorporation assay, as well as inhibition in the single-cell cloning experiments. The magnitude of inhibition was closely correlated to the magnitude of receptor downregulation. Furthermore, the downmodulation of CSF-Rs is specific since TNF- α neither affected the expression of a number of other cell surface proteins nor the expression of *c-kit*, another growth factor receptor on HPCs (Dubois, C. M., F. W. Ruscetti, and J. R. Keller, manuscript in preparation).

Previous studies demonstrated that TNF- α induced a transient reduction in the expression of CSF-1Rs on murine macrophages (43, 47). Because this correlated with synergistic growth stimulation, it was proposed that TNF- α might mimic the biological action of CSF-1 by downmodulating CSF-1Rs. However, in those studies, [³H]TdR incorporation was measured only after a 48-h incubation, at which time CSF-1R expression had returned to initial or even greater levels (43, 47). Here, we clearly demonstrate that the initial downmodulation of CSF-1Rs results in a transient inhibition of [³H]TdR incorporation. In addition, the effects of TNF- α on CSF-1-stimulated single bone marrow progenitor cells are only inhibitory. Additional support for the concept that CSF-1R *trans*-downmodulation results in reduced rather than enhanced CSF-1 responsiveness comes from a study demonstrating that IL-3- and GM-CSF-induced downmodulation of CSF-1R expression on a myeloid precursor cell line suppresses the biological responsiveness to CSF-1 (62).

In contrast to its direct inhibitory effects, the delayed stimulatory effects of TNF- α on CSF-1-, GM-CSF-, and IL-3-induced proliferation are indirect and mediated at least partially through production of CSF-1 and G-CSF, as detected by neutralizing antibodies and the ability of TNF- α -CM to induce proliferation of the G-CSF responsive 32DC13 cell line. This conclusion is also supported by previous work showing that TNF- α is an inducer of G-CSF production (38) and that potent synergistic interactions occur between G-CSF and the three other CSFs (58, 63, 64). It was further substantiated by the ability of G-CSF to mimic the delayed TNF- α -induced upregulation of GM-CSF and IL-3R expression. Furthermore, G-CSF did not affect CSF-1R levels significantly, suggesting that the synergistic activity of G-CSF on IL-3- and GM-CSF-, but not on CSF-1-stimulated colony formation could partially be mediated through receptor induction. In further support of such a mechanism, we found that the increase in GM-CSF and IL-3R expression preceded the synergistic activity observed on [³H]TdR incorporation.

Because TNF- α downmodulated G-CSF-R expression by 86%, it could be argued that the low level of G-CSF-Rs might be insufficient to mediate the G-CSF-induced upregulation of GM-CSF-Rs proposed in the present model. However, we have recently found that exogenously added G-CSF (20 ng/ml) can upregulate GM-CSF-R expression in the presence of high concentration of TNF- α (20 ng/ml) (Jacobsen, S. E. W., et al., unpublished observations). This suggests that lower levels of G-CSF-R occupancy are required to induce expression of

GM-CSF-Rs than to elicit proliferation of hematopoietic progenitor cells.

The use of Lin⁻ bone marrow cells was important to demonstrate that modulation of CSF-R expression occurred also on enriched progenitor cells. Lin⁻ selection of bone marrow progenitors removes T and B cells and macrophages; however, no selection for bone marrow stromal cells such as fibroblasts and endothelium was used. It was therefore not unexpected that TNF- α indirectly enhanced colony formation of Lin⁻ cells nearly as much as for LDBM cells, whereas they were directly inhibited in single cell assays. Adherence depletion can reduce but not eliminate these indirect effects (Jacobsen, S. E. W., unpublished observations). These findings underscore that negative selection of HPCs does not necessarily reduce the extent of indirect effects and that the question of indirect effects of biological modifiers on such progenitors can only be properly addressed by the use of single-cell cloning experiments. Although it is not possible to determine receptor expression on single cells, the present data suggest that TNF- α could directly induce prolonged downmodulation of all CSF-Rs.

It was possible that the TNF- α -induced downmodulation of CSF-Rs observed in the present study was mediated through induction of cytokines capable of reducing CSF-R expression. However, we have not been able to detect such activity in the supernatants after a 15–60-min incubation with TNF- α (S. E. W. Jacobsen, unpublished observations). It also seems unlikely that TNF- α -induced cell death or changes in proliferation could mediate the reduction in CSF binding. This is based on the observation that TNF- α -induced downmodulation of CSF-Rs occurred within minutes and that TNF- α alone did not affect the proliferation or viability of bone marrow progenitors as compared to untreated control cells, even after a 24-h incubation.

It has recently been demonstrated that TNF- α can stimulate the proliferation and upregulate the expression of GM-CSF and IL-3Rs on human acute myeloid leukemia cells (46). The mechanism of action was not investigated; however, the present data suggest that the induction of other cytokines could mediate the observed GM-CSF and IL-3R upregulation and enhanced responsiveness to these cytokines. In support of such an indirect mechanism, another study demonstrated that the synergistic activity of TNF- α on GM-CSF-stimulated AML proliferation could be partially neutralized by an antibody to IL-1 (65).

Bidirectional dose-dependent proliferative effects are not unique to TNF- α . In parallel to what we have observed for TNF- α , Battagay et al. (66) showed that TGF- β induces autocrine platelet-derived growth factor (PDGF) secretion of connective tissue cells at low concentrations, whereas it downmodulates the α -subunits of the PDGF-R at higher concentrations. The physiological relevance of such bidirectional concentration-dependent effects on HPCs are hard to assess since the local concentrations of these modulators in the bone marrow microenvironment are unknown.

The biological significance of growth factor receptor modulation as a mechanism for cell growth regulation has been

disputed, because it has been shown for hematopoietic (53, 67) as well as nonhematopoietic cells (68) that only a fraction of growth factor receptors need their ligand bound to elicit a maximum proliferative response (the spare receptor concept). A more recent study did however show that as many as 50% of the CSF-Rs must be occupied to induce optimal proliferation of hematopoietic progenitors (69). In addition, the spare receptor concept was established on cell lines with high levels of growth factor receptors, whereas bone marrow progenitors display profound heterogeneity and express an

average of only 100–300 of each CSF-R (2). In the present study, TNF- α reduced the number of G-CSF-Rs from 304 to 96, which could put many progenitors below threshold receptor level needed to induce a proliferative response.

Although it seems likely that also other pathways mediate the pleiotropic actions of TNF- α in hematopoiesis (16), the data presented here implicate a role of CSF-R modulation in the direct inhibitory as well as the indirect stimulatory effects of TNF- α on murine bone marrow progenitor cells (summarized in Fig. 7).

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