Brief Definitive Report

Tumor Necrosis Factor α Cooperates with Interleukin 3 in the Recruitment of a Primitive Subset of Human CD34+ Progenitors

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Summary

We have recently demonstrated that tumor necrosis factor α (TNF-α) potentiates interleukin 3 (IL-3)- and granulocyte/macrophage colony-stimulating factor–induced growth of CD34+ hematopoietic progenitor cells (HPC), and favors the generation of dendritic/Langerhans cells. The stimulatory effect of TNF-α was detailed in the present study. Thus, CD34+ HPC entering in cycle (S/G2M) after a 48-h pulse with IL-3 expressed the transferrin receptor (TfR), and fluorescence-activated cell sorter–separated TfR+ HPC, but not TfR− HPC, showed a high proliferative response to IL-3. In contrast, TfR− HPC were found to undergo strong proliferation in response to IL-3 + TNF-α. Limiting dilution experiments indicated that TNF-α increased both the frequency and the average size of clones generated from TfR− HPC as a result of the development of a higher number of large clones. In contrast, TNF-α did not enhance the IL-3-dependent proliferation of TfR+ HPC. Preculturing CD34+ HPC for 48 h with TNF-α enhanced the subsequent generation of IL-3-dependent colony-forming units. Precultures with TNF-α or cultures with suboptimal doses of TNF-α allowed the recruitment of cells with both granulocytic and monocytic differentiation potential. Taken together, our results indicate that TNF-α recruits a subpopulation of CD34+ HPC hyposensitive to IL-3, with high proliferative capacity and some features of multipotential progenitors, that are likely to be more primitive than those responding to IL-3 alone.

Models of in vitro hematopoiesis have revealed that regulation of development of human hematopoietic progenitor cells (HPC) depends upon so-called "growth stimulatory molecules" such as IL-3, GM-CSF, G-CSF, M-CSF, and stem cell factor, and so-called "growth inhibitory molecules" such as IFN, TGF-β, and TNF (1, 2). However, recent studies have shown that this concept of positive and negative regulators of myelopoiesis needs to be modulated. For instance, IFN-γ has recently been described to potentiate IL-3 and GM-CSF development of normal HPC (3, 4). Also, TGF-β has been shown to enhance the effects of GM-CSF on murine neutrophil (5) and macrophage (6) development. Moreover, we and others have demonstrated that TNF-α and -β stimulate IL-3- and GM-CSF-dependent proliferation of normal human CD34+ HPC (7, 8) and murine early progenitors (2).

We have recently described that the potentiating effect of TNF-α on early myelopoiesis was followed by an induction of the generation of dendritic/Langerhans cells (9) and an inhibition of erythroid and granulopoietic development (10). The present study was designed to further elucidate the mechanisms of the stimulatory effects of TNF-α on early myelopoiesis. It is shown that TNF-α potentiates early myelopoiesis through the recruitment of a population of cells hyporesponsive to IL-3 alone. The recruited progenitors display a high proliferative potential and likely represent a population more primitive than that responding to IL-3.

Materials and Methods

Collection and Purification of Cord Blood HPC. Umbilical cord blood samples were obtained according to institutional guidelines. Cells bearing CD34 antigen were isolated from nonadherent mononuclear fractions through positive selection by indirect immune "panning" using anti-My10 mAb (HPCA-1; Becton Dickinson & Co., Mountain View, CA), as reported elsewhere (11). A second purification step was performed using a cocktail of mAbs and magnetic beads (Dynabeads M450; Dynal, Oslo, Norway), as described (7). Thus, in all experiments the isolated cells were 95–99% CD34+.

Cell Cultures in Liquid Medium. Cells were precultured for 48 h either with 10 ng/ml IL-3 (sp act, 5×10^6 U/mg; Schering-Plough Research Institute, Kenilworth, NJ) before FACS® sorting (Becton Dickinson & Co.) according to transferrin receptor (TfR) expression, or with 25 ng/ml TNF-α (sp act, 2×10^7 U/mg; Genzyme Corp., Cambridge, MA) for preincubation experiments. The medium consisted of RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated FCS

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(Flow Laboratories, Irvine, UK), 10 mM Hepes, 2 mM L-glutamine, 5×10^{-5} M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (referred to as complete medium). For initiation of clonogenic assays after preincubation with cytokines, cells were washed four times, further incubated for 3 h (37°C) in complete medium, and washed again to eliminate the pulsing cytokine.

Progenitor Assays in Semisolid Medium. Semisolid cultures of 1 ml were performed in methylcellulose by plating 10^3 cells per tissue culture–grade 35-mm petri dish (Corning Glass Works, Corning, NY), without erythropoietin (Epo), as previously described in detail (11). Duplicate dishes were plated in each experiment, and after 7 and 14 d (37°C, 5% CO_2), colonies (≥50 cells) were counted using phase-contrast microscopy.

Limiting Dilution Studies. Limiting dilution was performed at one cell per well in 96-well round-bottomed microtest tissue culture plates (Nunc, Roskilde, Denmark), as previously described (7), in IMDM containing 30% FCS, 10^{-4} M 2-ME, 0.5% (wt/vol) BSA, and L-glutamine and antibiotics as indicated above. Each factor was tested in 540–2,000 wells. After 6 d of culture, positive wells were scored and the number of cells in each clone was determined. Clone frequency was determined as the log of the fraction of nonresponding wells, and average clone size was calculated as: the ratio of total cell number enumerated in the wells seeded/number of responding cells present in the wells observed. In some experiments, cultures were prolonged until day 20 and cells from individual clones ranging from 2,000 to 8,000 cells were processed for double-color fluorescence analysis (CD14 and CD15). The frequency of such clones was between 2 and 4% of total cells, and thus the probability that observed clones may derive from more than one cell was not significant.

Flow Cytometry Analysis and Cell Sorting. For cell surface phenotyping, double-color fluorescence was performed according to standard direct immunofluorescence techniques using FITC-conjugated anti-Leu-M1 (CD15) and PE-conjugated anti-Leu-M3 (CD14) (Becton Dickinson & Co.). Negative controls were performed with unrelated murine mAbs. Fluorescence analysis was determined with a FACScan® (Becton Dickinson & Co.).

For cell cycle analysis, cells were incubated for 48–72 h in the presence of 10 ng/ml IL-3, and 0.1 μg/ml colcemid (Gibco Laboratories) was added during the last 16 h. Cells were subsequently processed for membrane fluorescence and permeabilized (15 min) in 70% methanol. After washes, cells were incubated 15 min at 37°C with 2,000 U/ml ribonuclease A (sp act, 4,000 U/mg; Worthington Biochem. Corp., Freehold, NJ). 2 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) was added and cell surface Ag expression and DNA content were analyzed with a FACScan®.

For cell sorting, after 48 h of preincubation in IL-3 (10 ng/ml), cells were collected, washed several times, and labeled with FITC-conjugated anti-TfR (CD71) (Becton Dickinson & Co.). Using a FACStar Plus® (Becton Dickinson & Co.), the cells were separated according to TfR expression into a TfR^- fraction and a TfR^+ fraction. Reanalysis of the sorted populations showed a purity >97%.

Results

TNF-α Recruits a Subset of CD34⁺ HPC with High Proliferative Capacity, but Hyporesponsiveness to IL-3 Alone. We have previously shown that TNF-α stimulates early myelopoiesis through an enhancement of both the frequency and clone size of the responding $CD34^+$ HPC (7). Here we questioned whether the enhanced clone size was due to the specific recruitment of an IL-3-unresponsive population with high proliferative potential, or, alternatively, to an effect of TNF-α on the growth rate of IL-3-sensitive cells. Thus, we separated $CD34^+$ HPC according to their growth response to IL-3. Double-color fluorescence analysis showed that cells trig-

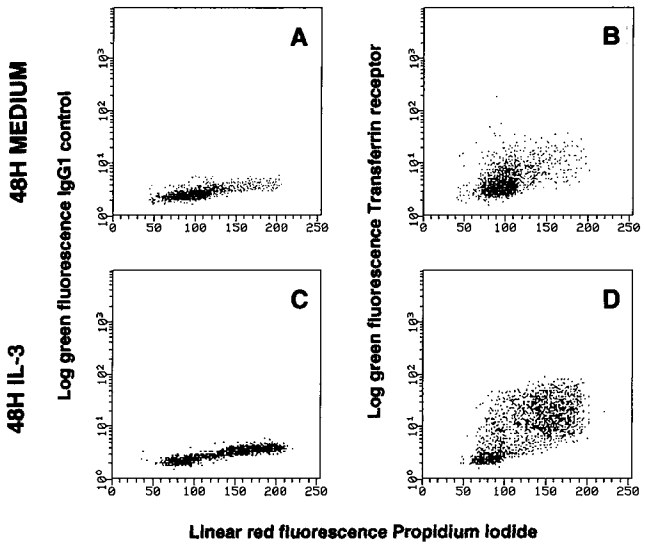

Figure 1. Expression of TfR on $CD34^+$ HPC in S/G2M after culture with IL-3. $CD34^+$ HPC cultured for 48 h in medium alone (*A* and *B*) or in the presence of 10 ng/ml IL-3 (*C* and *D*) were labeled with FITC anti-TfR (*B* and *D*) or a FITC control antibody (*A* and *C*) and stained with propidium iodide. Fluorescence was analyzed with a FACScan®.

Table 1. *$CD34^+TfR^-$ Cells Generate Day 7 CFU in Response to IL-3 + TNF-α*

	No. of day 7 colonies			
	TfR^+		TfR^-	
Exp.	IL-3	IL-3 + TNF-α	IL-3	IL-3 + TNF-α
1	37	36	16	70
	36	35	17	75
2	45	40	12	100
	55	32	11	109
3	38	35	13	82
	40	33	10	92

$CD34^+$ HPC were FACS®-sorted according to TfR expression after a 48-h preincubation in IL-3 (10 ng/ml) and assessed for their clonogenic capacity. Duplicate dishes, plated with 10^3 cells in presence of IL-3 (10 ng/ml) or IL-3 + TNF-α (25 ng/ml), were scored for numbers of colonies on day 7 (colonies ≥50 cells). Values are from three unrelated experiments.

gered by IL-3 to enter into cell cycle (S-G2M) express TfR (Fig. 1 *D*), whereas cells cultured in medium alone express only low levels of TfR and remain in the G1 phase (Fig. 1 *B*). After a 48-h culture with IL-3, the TfR^+ population (65–75% of total cells; range of six experiments) contained mostly cells in S-G2M whereas the TfR^- cells were mostly in G0-G1 (Fig. 1 *D*). As shown in Table 1, TfR^+ cells generated two to five times more CFU than TfR^- cells in response to IL-3 alone. However, while TNF-α did not alter IL-3-induced development of CFU from TfR^+ cells, it enhanced four- to eightfold the IL-3-dependent generation of CFU from TfR^- cells (ranges from three unrelated experiments). Thus, in response to IL-3 + TNF-α, TfR^- cells yielded approximately twice as many colonies as did TfR^+ cells. Limiting dilution experiments showed that, while TNF-α did not affect IL-3-dependent generation of clones from TfR^+ cells, it enhanced 2.5–3 times the frequency of responding clones from TfR^- cells (range from three independent experiments, one of which is shown in Fig. 2). Furthermore, TNF-α did not alter the size distribution of clones generated from TfR^+ cells. In contrast, TNF-α essentially stimulated the generation of large clones from TfR^- cells as observed by an average clone size increasing from 69 ± 13 cells/clone in IL-3 to 138 ± 10 cells/clone in IL-3 + TNF-α (means of three unrelated experiments, one of which is shown in Fig. 2). Taken together, these results demonstrate that TNF-α allows the recruitment of cells hyporesponsive to IL-3 alone that display a high proliferative potential.

A TNF-α Pulse Is Sufficient to Enhance the Proliferative Response to IL-3. As the potentiating effect of TNF-α reflects the recruitment of an IL-3-hyporesponsive population, we wondered whether the presence of TNF-α would be required during the whole course of proliferation or at the initiation of the process. Thus, $CD34^+$ HPC were precultured in the presence of TNF-α or medium alone, and, after extensive washes, tested for their proliferative response to IL-3, as determined by [^{3}H]TdR uptake and enumeration of day 7 CFU. Kinetics experiments, performed by [^{3}H]TdR uptake, indicated that a 48-h pulse in TNF-α was sufficient to allow subsequent levels of IL-3-dependent proliferation identical to those obtained when cells were continuously cultured with IL-3+TNF-α (not shown). This enhanced DNA replication was confirmed by enumeration of day 7 colonies. As shown in Fig. 3 *A*, cells pulsed with TNF-α and subsequently cultured in IL-3 generated virtually as many day 7 CFU as cells incubated in IL-3+TNF-α during the whole culture period. In addition, only TfR^- cells were sensitive to the enhancing effect of TNF-α preincubation (not shown). Taken together, these results demonstrate that, to permit recruitment of the IL-3-hyporesponsive population, TNF-α is required only at the initiation of the proliferative process.

TNF-α Recruits a Population of Cells with Both Monocytic and Granulocytic Differentiation Potential. We have recently shown that early potentiation of myelopoiesis by TNF-α is

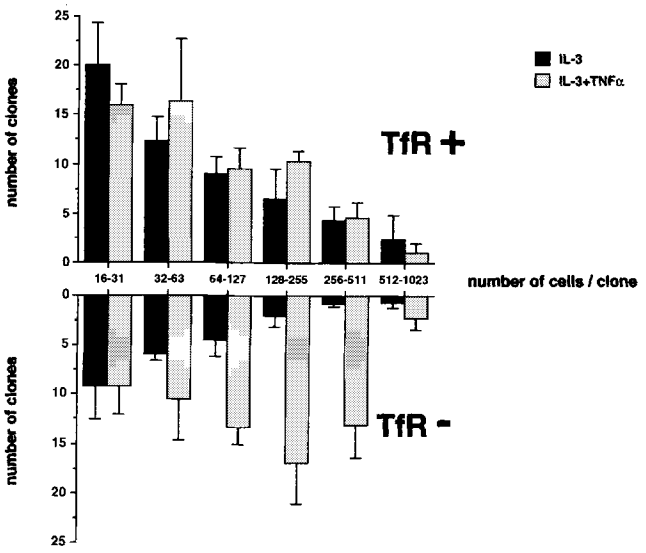

Figure 2. TNF-α recruits a population of cells with high proliferative capacity. After 48 h of preincubation in IL-3, and subsequent FACS®-sorting according to TfR expression, $CD34^+$ HPC were cultured at one cell/well in the presence of IL-3 (10 ng/ml) or IL-3 + TNF-α (25 ng/ml). 540 wells were seeded for each culture condition. After 7 d of culture, clone size was determined, by numeration, using an inverted microscope coupled to an image analyzer. TNF-α or medium alone did not induce the development of any clones. The clones were classified according to number of cells per clone. Numbers plotted correspond to the mean of triplicates of 180 wells observed. Clone frequency and average clone size were determined as described in Material and Methods. (*TfR*$^+$) Clone frequencies were 36.0% for IL-3 and 38.7% for IL-3 + TNF-α; average clone sizes (cells/clone) were 102 for IL-3 and 94 for IL-3 + TNF-α. (*TfR*$^-$) Clone frequencies were 13.6% for IL-3 and 44.8% for IL-3 + TNF-α; average clone sizes (cells/clone) were 84 for IL-3 and 148 for IL-3 + TNF-α. Data are from one experiment representative of three.

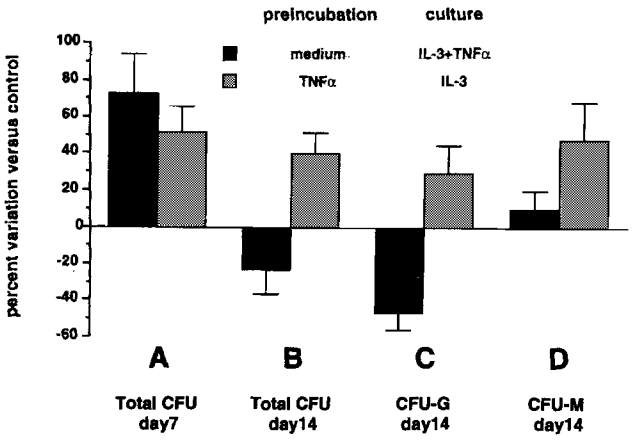

Figure 3. Upregulation of IL-3 responsiveness after TNF-α preincubation reflects the recruitment of a higher number of granulocytic and monocytic progenitors. $CD34^+$ HPC were preincubated for 48 h in either TNF-α (25 ng/ml) or medium alone, washed, and seeded (10^3 cells/plate) in duplicate dishes in the presence of IL-3 (10 ng/ml) or IL-3 + TNF-α (25 ng/ml). Colonies ≥50 cells were counted after 7 and 14 d. Data represent the mean of five separate experiments and are expressed as follows: 100× (number of CFU/control number of CFU in IL-3 after preincubation in medium) − 100.

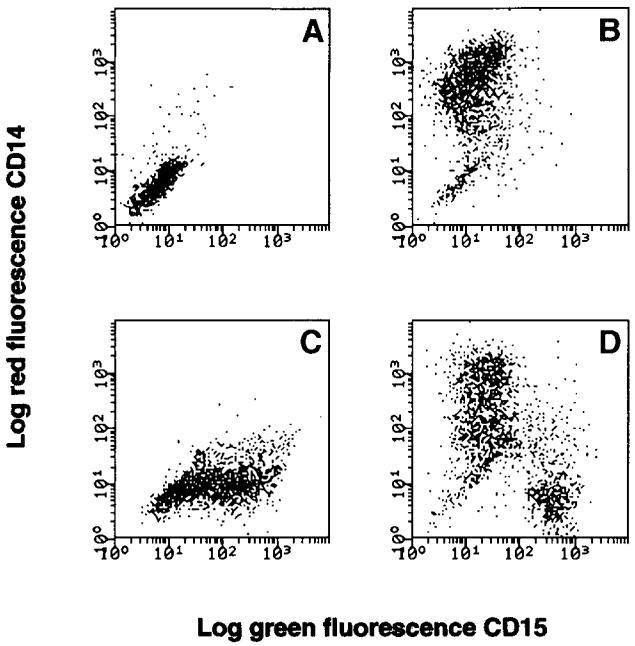

Figure 4. Expression of CD14 and CD15 on clones derived from CD34+ HPC. CD34+ HPC were seeded at one cell per well in presence of IL-3 with or without suboptimal concentration of TNF-α (1.25 ng/ml). After 20 d of culture, large clones (2,000–8,000 cells/well) were selected and analyzed by double-color fluorescence for expression of CD14 (monocytic cells) and CD15 (granulocytic cells). (*A*) Example of CD14−/CD15− clone (undetermined CFU); (*B*) example of CD14+/CD15− clone (CFU-M); (*C*) example of CD14−/CD15+ clone (CFU-G); (*D*) example of CD14+/CD15+ clone (CFU-GM).

followed by an inhibition of the development of granulopoietic cells (10), a phenomenon that interferes with the characterization of the progenitors initially recruited. Thus, we studied whether pulsing cells with TNF-α for 48 h would prevent this late inhibition of granulopoiesis. As described above, IL-3-dependent generation of day 7 colonies was equally enhanced by a 48-h pulse or a 7-d TNF-α coculture (Fig. 3 *A*). However, when CD34+ HPC were plated for 14 d, the IL-3 response differed strikingly between cells cultured with TNF-α for 14 d and those pulsed with TNF-α for only 48 h. Thus, preincubation in TNF-α enhanced total CFU numbers by 41% (Fig. 3 *B*) as a consequence of a 30 and 48% increase of CFU-G (Fig. 3 *C*) and CFU-M (Fig. 3 *D*), respectively. In contrast, the continuous presence of TNF-α resulted in a 23% inhibition of total CFU numbers (Fig. 3 *B*), mostly as a result of a 46% inhibition of the CFU-G numbers (Fig. 3 *C*) (10).

Interestingly, a low concentration of TNF-α (1.25 ng/ml) was able to provide virtually all the potentiating effect of TNF-α observed after 7 d of culture, without demonstrating inhibitory effects on day 14 CFU-G and CFU-M (not shown). This lack of inhibitory effects of low concentrations of TNF-α allowed us to study the effects of TNF-α on CFU-GM generation. Thus, CD34+ HPC were seeded at one cell per well in the presence of IL-3 with or without 1.25 ng/ml TNF-α, and after 20 d of culture large clones (2,000–8,000 cells/clone) were selected and analyzed by double-color fluorescence for expression of CD14 (monocytic cells) and CD15 (granulocytic cells). Fig. 4 shows FACS® profile examples of clones lacking both CD14+ and CD15+ cells (undefined CFU; Fig. 4 *A*); clones containing only CD14+ cells (CFU-M; Fig. 4 *B*); clones containing only CD15+ cells (CFU-G; Fig. 4 *C*); and clones containing CD14+ as well as CD15+ cells (CFU-GM; Fig. 4 *D*).

Table 2 shows the mean of results obtained from three separate single-cell experiments. Thus, addition of TNF-α to cultures did not modify the frequency of undetermined CFU (12.0 vs. 15.4%) nor that of CFU-M (10.0 vs. 12.1%). In contrast, addition of TNF-α to IL-3 cultures resulted in an increased frequency of CFU-GM clones from 9.4 to 47.5%. As a consequence, the proportion of CFU-G decreased from 61.6 to 30.6%.

These results demonstrate that TNF-α allows the recruitment of a population of CD34+ HPC with high proliferative capacity, and with both granulocytic and monocytic differentiation potential.

Discussion

Previous studies by us and others have shown that TNF-α potentiates the early proliferative response of CD34+ HPC to IL-3 and GM-CSF (7, 8, 12), and stimulates the generation of dendritic Langerhans cells (9, 13). Yet, in line with previous reports (14–17), we also demonstrated that, after this phase of potentiation, TNF-α inhibits erythroid and

Table 2. *TNF-α Recruits a Population of Cells with Both Monocytic and Granulocytic Differentiation Potential*

	CD14−CD15− clones (undetermined CFU)	CD14−CD15+ clones (CFU-G)	CD14+CD15− clones (CFU-M)	CD14+CD15+ clones (CFU-GM)
IL-3	15.4 (11.5–22.3)	61.6 (54.5–75.0)	12.1 (10.0–15.3)	9.4 (2.5–13.7)
IL-3 + TNF-α	12.0 (6.7–21.0)	30.6 (27.9–34.2)	10.0 (6.8–12.5)	47.5 (32.3–55.6)

CD34+ HPC were seeded at one cell per well (2,000 wells seeded per each culture condition) in the presence of IL-3 (10 ng/ml) with or without low concentration of TNF-α (1.25 ng/ml). After 20 d of culture, large clones (2,000–8,000 cells/clone) were selected and analyzed by double-color fluorescence for expression of CD14 (monocytic cells) and CD15 (granulocytic cells). Data represent mean and range (in parentheses) from three unrelated experiments and are expressed as percentage of total analyzed clones (40–50 per each culture condition for each experiment).

granulocytic development (10). Here, we show, based on three observations, that the enhancing effect of TNF-α on early myelopoiesis is due to the recruitment of primitive hematopoietic progenitor cells hyporesponsive to IL-3. First, by separating IL-3-sensitive and IL-3-hyposensitive $CD34^+$ HPC according to TfR expression, TNF-α was found to allow specifically the recruitment of TfR^- cells, which proliferate weakly in response to IL-3 alone. Second, preincubation experiments indicated that TNF-α is required only during the initiation of the proliferative process, thus demonstrating that TNF-α prepares cells to respond to IL-3. Third, morphologic and phenotypic characterization of clones generated in the presence of noninhibitory concentrations of TNF-α revealed that high proliferative potential cells selectively recruited by TNF-α are in fact granulocyte/monocyte bipotent progenitors.

Our claim considering TNF-α as an enhancer (7, and this study), rather than an inhibitor of early myelopoiesis, is substantiated by other recent reports (8, 12). In particular, TNF-α has been shown to potentiate the development of early murine progenitors with high proliferative potential that are dependent upon the combination of IL-1 + IL-3 or IL-1 + GM-CSF. In contrast, more committed progenitors, responsive to either IL-3 or GM-CSF, were not affected by TNF-α (2). In this context, another study has recently demonstrated that IL-3 receptor–negative progenitors are more primitive than those expressing IL-3 receptors (18). Finally, these in vitro observations, which identify TNF-α as an important molecule for the recruitment of early hematopoietic progenitors are supported by the demonstration of the radioprotective effect of TNF-α in vivo (19). Taken together, the critical role of TNF-α in early hematopoiesis is in accordance with the concept that development of the earliest progenitor cells necessitates the association of several factors (20, 21).

Elucidation of the molecular mechanisms through which TNF-α potentiates IL-3-dependent early myelopoiesis certainly deserves attention. In this context, we have observed (Sato, N., C. Caux, T. Kitamura, Y. Watanabe, K. Arai, J. Banchereau, and A. Miyajima, manuscript submitted for publication) that TNF-α is able, on $CD34^+$ HPC, to upregulate the expression of the β chain common to the IL-3/IL-5/GM-CSF receptor complexes (22). This finding affords a relatively simple explanation for the induction by TNF-α of an enhanced proliferative response of HPC to IL-3 and GM-CSF. A recent study has also shown that TNF-α can upregulate the expression of IL-3 and GM-CSF receptors on murine progenitors (12). However, at variance with our studies showing a direct effect of TNF-α on single cells, this concurrent study argues for an indirect role of G-CSF, which is produced by low density bone marrow cells in response to TNF-α. These contrasting conclusions may derive from species (mouse vs. human) or culture condition differences. In conjunction with the known stimulatory effect of a single in vivo injection of TNF-α on hematopoiesis (23), in vitro studies advocate for considering therapeutic pulses of TNF-α to boost early hematopoiesis. Together with GM-CSF or IL-3, such a treatment may allow a more efficient recovery from therapy-induced aplasia.

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