Tumor Necrosis Factor (TNF)-α Directly Inhibits Human Erythropoiesis In Vitro: Role of p55 and p75 TNF Receptors

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Two tumor necrosis factor receptors (TNFRs) with molecular weights of 55 kD (TNFR-p55) and 75 kD (TNFR-p75) have recently been identified and cloned. In previous studies, TNFR-p55 has been shown to exclusively mediate bidirectional effects of TNF-α on committed bone marrow granulocyte-macrophage progenitor cells, whereas both TNFR-p55 and TNFR-p75 can mediate inhibition of primitive progenitors requiring multiple cytokines to proliferate. We show here that TNF-α potently and directly inhibits the in vitro growth of committed erythroid progenitor cells in response to multiple cytokine combinations, and that TNF-α–induced inhibition of burst-forming unit-erythroid colony formation is mainly mediated through TNFR-p55, although TNFR-p75–mediated inhibition could be observed on progenitors responsive to erythropoietin alone. Moreover, at low TNF-α concentrations (2 ng/mL), TNF-α stimulates interleukin-3–dependent in vitro growth of committed granulocyte-macrophage progenitor cells, whereas it potently inhibits erythroid progenitor cell proliferation, showing that one concentration of TNF-α can simultaneously and bidirectionally modulate interleukin-3–dependent growth of committed granulocyte-macrophage (stimulation) and erythroid progenitor cells (inhibition).

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teins, and anti-TNFR antibodies were a kind gift from Drs W. Leslaurer and H. Loetscher (Hoffmann-LaRoche, Basel, Switzerland). HuTNF-α mutants specific for TNFR-p55 and TNFR-p75 have been prepared by site-directed mutagenesis. Solid-phase binding studies have shown that the Trp<sup>3</sup>Thr<sup>8</sup> TNF-α mutant protein binds with wild-type affinity to TNFR-p55 and does not bind at all to TNFR-p75, whereas the Asn<sup>13</sup>Arg<sup>14</sup> TNF-α mutant protein exclusively binds to TNFR-p75, although with a 5- to 10-fold lower affinity than wild-type TNF-α. Both mutant proteins were titrated and used at optimal concentrations as previously described. Rabbit antihuman TNFR-p75 polyclonal antibodies with TNF-α agonistic activity (paTNFR-p75) and a monoclonal antibody (MoAb) against the TNFR-p55 with TNF-α agonistic activity (htr-9) were raised and prepared as previously described. Htr-9 and paTNFR-p75 were initially titrated as described elsewhere and thereafter used at optimal concentrations of 10 µg/mL (htr-9) and 2 µg/mL (paTNFR-p75). Unless otherwise indicated, all growth factors were used at predetermined optimal concentrations: rHuIL-3 (20 ng/mL), rHuSCF (50 ng/mL), rHuIL-9 (100 IU/mL), and Epo (5 IU/mL).

**Cell separation.** Human BM cells were obtained by iliac crest aspiration from normal adult volunteers with informed consent and the approval of the Ethics Committee of The Norwegian Radium Hospital. Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway). Positive selection of CD34<sup>+</sup> cells was performed according to a previously described method. Briefly, BM mononuclear cells were resorted with Dynabeads M-450 directly coated with the CD34 MoAb Bi-3C5 (Product No. 111.10; Dynal, Oslo, Norway) for 45 minutes at 4°C on an apparatus that provided tilting and gentle rotation. The bead to total cell ratio was 1:1. Rosetted cells were attracted to a samarium cobalt magnet and nonrosetting cells were removed by pipetting and washed seven times. Detachment of beads from positively selected cells was performed by incubation with anti-Fab antibody (DETACHaBEAD; Dynal) at a concentration of 35 mg/mL for 1 hour at room temperature. Isolated cells, free of beads, were washed and counted. The purity of CD34<sup>+</sup> cells isolated by this method was reproducibly greater than 90% as determined by flow cytometric analysis.

To enrich and separate committed erythroid (burst-forming unit-erythroid [BFU-E]) and granulocyte-macrophage (colony-forming unit–granulocyte-macrophage [CFU-GM]) progenitors, we took advantage of the differential expression of CD45 isoforms previously described on these cells. Because all BFU-E are CD45R<sup>+</sup> and the majority of CFU-GM are CD45R<sup>-</sup>, CD34<sup>+</sup>CD45R<sup>+</sup> cells are enriched in committed erythroid progenitors, whereas committed granulocyte-macrophage, but not erythroid, progenitors reside in the CD34<sup>+</sup>CD45R<sup>-</sup> subpopulation. In addition, we wanted to deplete our test cell populations in primitive progenitors, which was obtained by selecting progenitors expressing CD38<sup>+</sup>. To isolate cells with the CD34<sup>+</sup>CD38<sup>+</sup>CD45R<sup>+</sup> phenotype by cell sorting, positively selected CD34<sup>+</sup> cells with a mean purity (n = 3) of 94% as assessed by flow cytometry were stained with anti-CD45R (UCHL-1; Becton Dickinson, San Jose, CA) directly conjugated to phycoerythrin (PE) and with anti-CD38 (IB06; Immunotech, Marseilles, France) directly conjugated to fluorescein isothiocyanate (FITC). Isotype-matched FITC- and PE-conjugated irrelevant mouse MoAbs served as controls. Cell sorting was performed on an Epics Elite cell sorter (Coulter Electronics, Hialeah, FL). A sort gate within a dual-parameter cytogram of forward light scatter against 90° side scatter was drawn. A second amorphous gate was drawn on two-color cytograms, and sort equations were set to positively sort cells satisfying both gates.

**Colony assay.** CD34<sup>+</sup> cells were plated in a volume of 1 mL Iscove's modified Dulbecco's medium (IMDM; GIBCO, Paisley, UK) containing 20% fetal calf serum (FCS; Sera-lab, Sussex, UK), 1.2% methylcellulose (Methocel 4000 mPa·s; Fluka AG, Buchs, Switzerland), 5 × 10<sup>-5</sup> mol/L 2-mercaptoethanol, 300 mg/L glutamine, 66 mg/L penicillin, 100 mg/L streptomycin, and recombinant human growth factors as indicated. After 2 weeks of incubation at 37°C and 5% CO<sub>2</sub> in air, colonies (>40 cells) derived from CFU-GM and BFU-E were assessed according to established criteria. Cultures were scored for BFU-E colony growth after 14 days of incubation at 37°C and 5% CO<sub>2</sub> in air. Results are presented as the mean number of colonies per 2 × 10<sup>5</sup> cells from at least four independent experiments with triplicate determinations; error bars show the SEM. No colony formation.

**Single-cell proliferation assay.** Test cells were seeded in Teraski plates (Nunc, Kamstrup, Denmark) at a concentration of 1 cell per well (300 wells per group) in 20 µL IMDM containing 20% FCS (Sera-lab), L-glutamine, penicillin, and streptomycin. Wells were scored for erythroid colony formation (>40 cells) after 2 weeks of incubation at 37°C and 5% CO<sub>2</sub> in air. To verify the erythroid composition of colonies induced by SCF + Epo, morphologic examination of randomly picked colonies was performed on May-Grünwald-Giemsa–stained cytocentrifuge cell preparations.

**Statistical analysis.** All results were expressed as the mean ± SEM of data obtained from three or more separate experiments. The statistical significance of differences between group means was determined using the Student's t-test.

**RESULTS**

**Effects of TNF-α on erythroid colony formation by human BM CD34<sup>+</sup> cells.** We and others have previously shown that TNF-α potently and directly can enhance granulocyte-macrophage colony formation of human CD34<sup>+</sup> progenitors stimulated by IL-3 or GM-CSF, whereas TNF-α inhibits the proliferative actions of other growth factors such as G-CSF, SCF, and CSF-1. TNF-α has been shown to both inhibit and stimulate erythroid colony growth depending on the growth factors used, as well as the concentration of TNF-α itself. In the present study, TNF-α invariably inhibited BFU-E colony formation of CD34<sup>+</sup> BM cells induced by multiple cytokine combinations (Fig 1). In agreement with previous reports, BFU-E colony growth induced by Epo
alone or Epo in combination with IL-3 was completely inhibited by TNF-α. In addition, and not previously shown, TNF-α inhibited BFU-E colony formation induced by SCF + Epo, IL-3 + SCF + Epo, and IL-9 + Epo by 76%, 70%, and 89%, respectively (Fig 1).

TNF-α inhibited BFU-E colony formation in a concentration-dependent fashion. Fifty percent inhibition of Epo-stimulated colony growth occurred at a TNF-α concentration of 0.02 ng/mL, whereas complete inhibition of erythroid colony-forming ability was observed at 2 ng/mL (Fig 2A). In comparison, 50% inhibition of SCF + Epo-induced BFU-E colony growth was observed at a TNF-α concentration of 2 to 20 ng/mL, with a maximum inhibition of 73% occurring at 200 ng/mL (Fig 2B). Finally, 50% inhibition of SCF-induced granulocyte-macrophage cluster formation was observed at a TNF-α concentration of 0.2 to 2 ng/mL (Fig 2C).

Whereas several previous studies have suggested that TNF-α can directly modulate the growth of granulocyte-macrophage progenitor cells,11,13,15 Means et al.27,28 recently reported that TNF-α-induced growth inhibition of colonies derived from human erythroid colony-forming units (CFU-E) was indirectly mediated through production of β-interferon from accessory cells. In contrast, we found that TNF-α inhibited the formation of SCF + Epo-induced erythroid colonies derived from individually plated CD34+ progenitors in a concentration-dependent fashion, suggesting a direct action of TNF-α on more primitive erythroid progenitors (Fig 3).

Differential effects of TNF-α on IL-3-stimulated granulocyte-macrophage and erythroid progenitors. Because it has been shown that low, but not high, concentrations of TNF-α can potently enhance GM-CSF- or IL-3–induced granulocyte-macrophage colony formation,11,15 we next examined the effects of TNF-α on the simultaneous development of CFU-GM and BFU-E colonies in cultures supplemented with IL-3 + Epo. As shown in Fig 4, IL-3 + Epo promoted the growth of 51 ± 5 BFU-E and 17 ± 2 CFU-GM colonies per 2 × 10^5 CD34+ cells plated. Interestingly, when adding TNF-α at 2 ng/mL, a 2.7-fold increase in the number of CFU-GM colonies was observed, whereas BFU-E colony formation was completely inhibited (Fig 4). At higher concentration of TNF-α (200 ng/mL), both CFU-GM and BFU-E colony formation was inhibited. Thus, at low concentrations, TNF-α has the ability of promoting the growth of granulocyte-macrophage colonies while concomitantly suppressing the growth of erythroid colonies.

To investigate whether the bifunctional effect of TNF-α on the growth of CFU-GM and BFU-E progenitors might be explained by TNF-α preferentially directing primitive multipotent or bipotent progenitors towards granulocyte-macrophage rather than erythroid development or, alternatively, by a differential effect of TNF-α on committed granulocyte-macrophage and erythroid progenitors, we next examined the effect of TNF-α on colony formation of CD34+CD38-CD45RO+ and CD34+CD38-CD45RO- cells. Whereas both these cell populations, lacking CD38 expression, are depleted in primitive progenitors,15,16 we found, in agreement with a previous study,16 that virtually all BFU-E (99%) resided in the CD45RO+ subset. In contrast, both
TNF-α, ng/ml

Fig 3. Effect of TNF-α on erythroid colony formation of CD34⁺ BM cells in single-cell assay. CD34⁺ cells were isolated and plated at 1 cell per well as described in the Materials and Methods in the presence of predetermined optimal concentrations of SCF + Epo and different concentrations of TNF-α as indicated. Erythroid colonies (≥ 40 cells) were assessed (Materials and Methods) and counted after 14 days of incubation at 37°C and 5% CO₂ in air. In some experiments, individual colonies were picked to determine that all colonies formed were in fact erythroid. Each group consisted of 300 wells. Results are presented as the mean number of positive wells in four separate experiments; error bars show the SEM.

subpopulations contained CFU-GM, although a majority (60%) were CD45RO⁻. As shown in Fig 5A, IL-3 + Epo induced the formation of 17 ± 1 CFU-GM colonies and 1.5 ± 1 BFU-E colonies of 2,000 CD34⁺CD38⁻CD45RO⁻ cells plated. Interestingly, CFU-GM colony formation of CD34⁺CD38⁻CD45RO⁻ cells increased about fourfold by adding TNF-α at 2 ng/mL. Thus, the depletion in primitive progenitors and the removal of progenitors with erythroid potential from the test cell population did not impair the ability of TNF-α to enhance IL-3-stimulated CFU-GM colony formation. Using CD34⁺CD38⁻CD45RO⁻ cells, IL-3 + Epo stimulated the growth of 23 ± 1 CFU-GM and 114 ± 13 BFU-E colonies of 2,000 cells plated (Fig 5B). The supplementation of TNF-α at 2 ng/mL almost completely blocked BFU-E colony formation, whereas CFU-GM colony numbers increased 2.5-fold. Thus, enriching progenitors capable of erythroid colony formation did not further enhance the ability of TNF-α to stimulate CFU-GM colony formation.

Role of p55 and p75 TNFRs in TNF-α-induced inhibition of erythropoiesis. In a previous study, we have shown that

Fig 4. Effects of TNF-α on IL-3-stimulated (□) CFU-GM and (●) BFU-E colony formation by human BM progenitor cells. CD34⁺ cells were isolated and plated in methylocellulose as described in the Materials and Methods at 2 × 10⁵ cells/plate. Individual cultures were supplemented with predetermined optimal concentrations of IL-3 + Epo and different concentrations of TNF-α as indicated. Cultures were scored for CFU-GM and BFU-E colony growth after 14 days of incubation at 37°C and 5% CO₂ in air. Results are presented as the mean number of colonies per 2 × 10⁵ cells from three independent experiments with duplicate determinations; error bars show the SEM. *No BFU-E colony formation.

Fig 5. Effects of TNF-α on IL-3-stimulated (□) CFU-GM and (●) BFU-E colony formation by (A) CD34⁺CD38⁻CD45RO⁻ cells and (B) CD34⁺CD38⁺CD45RO⁻ cells. Progenitors were isolated and plated in methylocellulose as described in the Materials and Methods at 2 x 10⁵ cells/plate. Individual cultures were supplemented with predetermined optimal concentrations of IL-3 + Epo and different concentrations of TNF-α as indicated. Cultures were scored for CFU-GM and BFU-E colony growth after 14 days of incubation at 37°C and 5% CO₂ in air. Results are presented as the mean number of colonies per 2 × 10⁵ cells from three independent experiments with duplicate determinations; error bars show the SEM. *No BFU-E colony formation.
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Fig 6. Relative role of p55 and p75 TNFRs in TNF-α-induced inhibition of BFU-E colony formation. CD34+ cells were isolated and plated in methylcellulose as described in the Materials and Methods at 2 x 10⁴ cells/plate in the presence of predetermined optimal concentrations of (A) Epo or (B) SCF + Epo. TNF-α (200 ng/mL), TNF-p55 agonists (TNF-α mutant at 200 ng/mL or htr-9 at 10 μg/mL), or TNF-p75 agonists (TNF-α mutant at 2 μg/mL or pTNF-p75 at 2 μg/mL) were added as indicated. Results are presented as the mean number of colonies per 2 x 10⁴ cells from six independent experiments with triplicate determinations, including three experiments using TNF-α mutants and three experiments with anti-TNF antibodies; error bars show the SEM. *No colony formation.

The effects of TNF-α can be mediated directly on target cells or indirectly by stimulating accessory cells to cytokine production. Whereas we and others have found direct effects of TNF-α on committed granulocyte-macrophage BM progenitor cells, recent studies by Means et al have suggested that TNF-α-induced inhibition of erythroid colony growth was indirect through stimulation of β-inter-
feron production from accessory cells. In contrast, the results of single-cell cloning experiments presented here suggest that TNF-α-induced inhibition of erythroid colony formation can be directly mediated on the progenitor cells. The divergent findings in the studies of Means et al.\(^7\) and in our study could be due to studying different erythroid progenitors, because our results were obtained assessing TNF-α-induced modulation of BFU-E colony formation stimulated by multiple cytokine combinations, whereas Means et al. evaluated TNF-α–induced inhibition of colony formation derived from more mature CFU-E progenitors stimulated by Epo alone. It is possible that TNFR expression and function varies among these two progenitor cell types. Alternatively, TNFR expression might differ on progenitors of the same type according to the conditions under which they have been generated.

It is well established that TNF-α can inhibit or stimulate the proliferation of hematopoietic progenitor cells, depending on the growth factor(s) it interacts with as well as the concentration of TNF-α in culture.\(^{15,16}\) The potent TNF-α–induced enhancement of CFU-GM colony growth and inhibition of erythroid colony formation stimulated by IL-3 + Epo is of particular interest, because it demonstrates that one concentration of TNF-α can simultaneously and bidirectionally modulate IL-3–dependent growth of granulocyte-macrophage (stimulation) and erythroid colonies (inhibition). The present study suggests that this can be explained by a differential effect of TNF-α on committed granulocyte-macrophage and erythroid progenitors and not by TNF-α switching more primitive multipotent or bipotent progenitors from erythroid towards granulocyte-macrophage development. This theory was supported by TNF-α retaining its ability to enhance IL-3–induced CFU-GM colony growth when the test cell population was depleted in primitive and erythroid progenitors, as well as by the fact that TNF-α–induced potentiation of IL-3–dependent CFU-GM colony formation was not further enhanced using a test cell population highly enriched in progenitors with erythroid potential.

Recent studies from our laboratory suggest that both TNFR-p55 and TNFR-p75 are involved in mediating TNF-α–induced inhibition of primitive hematopoietic progenitor cells requiring multiple cytokines to proliferate.\(^{15}\) In contrast, the TNFR-p55 exclusively mediates stimulatory effects on more mature GM-CSF– or IL-3–responsive granulocyte-macrophage progenitor cells, as well as potent inhibition of G-CSF–induced proliferation. The present study extends these findings to show that TNF-α–induced inhibition of BFU-E colony formation in response to multiple cytokine combinations is mediated predominantly through TNFR-p55. However, whereas signaling through TNFR-p55 had no effect on the proliferation of committed granulocyte-macrophage progenitor cells,\(^{15}\) we show here that this receptor type can mediate inhibition of committed erythroid progenitor cell growth. Although a small inhibition of SCF + Epo-induced BFU-E colony formation was observed, the TNFR-p75–mediated inhibition was more obvious on progenitors responding to Epo alone, indicating a predominant effect on more mature stages of erythroid progenitors. These findings might have several important clinical implications. First, TNFR-p55–specific mutant TNF-α has been shown to have less proinflammatory (toxic) effects than wild-type TNF-α,\(^{20}\) but as potent cytotoxic (antitumor) effects, and is thus being explored for the possible use as an antitumor agent. However, the present results suggest that the TNF-α mutant specific for TNFR-p55 is as potent as wild-type TNF-α in inhibiting erythropoiesis, and might therefore induce severe anemia. In contrast, administration of TNF-α mutants with selectivity for TNFR-p75 might be associated with little or no anemia. Second, antagonistic antibodies to TNFR-p55 could potentially be used to reverse the anemia observed in chronic diseases.

It has been shown that some TNFR-p55–mediated responses are much more efficiently elicited by wild-type TNF-α than TNFR-p55–selective TNF-α mutants, suggesting an accessory role of TNFR-p55 in TNFR-p55–sensed events.\(^{46,62}\) It is of interest that the data presented here, as well as in previous studies,\(^{15,20}\) suggest that this does not appear to be the case for TNFR-p55–mediated regulation of human hematopoietic progenitor cell growth.

In conclusion, we have demonstrated that TNF-α potently and directly inhibits the in vitro growth of committed erythroid progenitor cells in response to multiple cytokine combinations, and that TNF-α–induced inhibition of BFU-E colony formation is mainly mediated through TNFR-p55, although TNFR-p75–mediated inhibition could be observed on progenitors responsive to Epo alone. Ongoing studies on TNF receptor knock-out mice\(^{60}\) will help to further elucidate the role of TNF-α and its receptors in erythropoiesis.

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Tumor necrosis factor (TNF)-alpha directly inhibits human erythropoiesis in vitro: role of p55 and p75 TNF receptors

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