

Tumor Necrosis Factor α Is a Potent Synergistic Factor for the Proliferation of Primitive Human Hematopoietic Progenitor Cells and Induces Resistance to Transforming Growth Factor β but Not to Interferon γ

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Summary

Since tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and transforming growth factor (TGF)- β have all been shown to be specific inhibitors of early human hematopoiesis, we wanted to investigate the interactions of these three cytokines on very primitive human adult bone marrow CD34⁺⁺CD38⁻ hematopoietic progenitor cells, using a pre-colony-forming cell (pre-CFC) assay, which detects the effects of these cytokines on the initial phases of the differentiation of these primitive progenitors, which are unresponsive to interleukin (IL) 3 alone. Surprisingly, TNF- α was a very potent stimulator of the proliferation of CD34⁺⁺CD38⁻ cells and was the most potent synergistic factor for the IL-3-induced proliferation of these cells of all cytokines tested (IL-1, IL-6, granulocyte colony-stimulating factor, kit ligand). TNF- α was the only cytokine that, as a single added factor, induced substantial proliferation in CD34⁺⁺CD38⁻ cells in the presence of IL-3, except for kit ligand, which induced very limited proliferation. TNF- α , moreover, induced a high degree of resistance to the inhibitory effects of TGF- β in a dose-dependent way. The inhibitory effects of IFN- γ , however, were not affected by the presence of TNF- α . We hypothesize that in situations of hematopoietic stress, TNF- α may abrogate the inhibitory effect of ambient TGF- β in the bone marrow microenvironment to allow primitive stem cells to proliferate and differentiate in response to an increased demand for mature blood cells.

The proliferation and differentiation of primitive hematopoietic progenitor cells are regulated by direct interaction of these cells with bone marrow stroma and by the stimulatory and inhibitory effects of cytokines present in the microenvironment of the bone marrow (1).

Among cytokines inhibiting hematopoiesis, the action of TGF- β on progenitor cells has been characterized the best (2-5). The sensitivity of progenitor cells to the inhibitory effects of TGF- β is inversely correlated with their maturation stage: the proliferation and differentiation of a very primitive progenitor are profoundly inhibited by TGF- β (2-4), whereas more mature progenitors are even stimulated in some circumstances (5). We recently identified IFN- γ as a selective inhibitor of primitive human CD34⁺⁺CD38⁻ progenitor cells (6). TNF- α is a pleiotropic cytokine that is predominantly produced by macrophages (7) and has been shown by many investigators to be a bifunctional regulator of the proliferation and differentiation of more mature hematopoietic progenitor cells, depending on the cytokines present in the culture medium and on the degree of progenitor cell enrichment (8-10). A number of

reports from the same group have recently demonstrated that TNF- α is another direct and selective inhibitor of very primitive stem cells in the human (11-13) and murine (14) systems. Others, however, have demonstrated stimulatory effects of TNF- α on more primitive progenitors (10, 15).

The aim of this work is to characterize the interactions of the three aforementioned hematopoietic inhibitors on purified human CD34⁺⁺CD38⁻ cells, which have been shown to contain the most primitive identifiable human progenitors (6, 16). We used a two-stage pre-colony-forming cell (pre-CFC) assay, since this allows the characterization of the effects of these cytokines on the initial phases of the proliferation of CD34⁺⁺CD38⁻ cells (6, 17). Surprisingly, TNF- α turned out to be a very powerful stimulator of the proliferation of CD34⁺⁺CD38⁻ cells and to induce resistance to the inhibitory effects of TGF- β but not IFN- γ .

Materials and Methods

Bone Marrow Cells. Bone marrow samples were aspirated by sternal puncture from hematologically normal patients undergo-

ing cardiac surgery, after obtaining informed consent according to the regulations of the Ethics Committee of the University of Antwerp, in tubes containing 2 ml IMDM (Gibco Laboratories, Paisley, UK) and 5 U/ml preservative-free heparin (Novo Industries, Copenhagen, Denmark). Cells were separated on a lymphocyte separation medium (LSM; Boehringer Mannheim GmbH, Penzberg, Germany) density gradient and washed twice. Remaining RBC were lysed using an NH_4Cl containing lysing solution.

Cytokines and mAbs. Supernatant of the 43A1 hybridoma (IgG3, kindly donated by Dr. H.J. Bühring, University of Tübingen, Tübingen, Germany) was used as a source of anti-CD34 (18). FITC-conjugated rabbit anti-mouse Ig (ab')₂ fragments were purchased from Dako (Glostrup, Denmark), and PE-conjugated anti-CD38 (IgG1) antibodies as well as isotype-specific control antibodies were purchased from Becton Dickinson (Erembodegem, Belgium). Monoclonal neutralizing anti-TNF- α (IgG), irrelevant control antibody (IgG), and ultrapure natural TGF- β (10^6 U/mg) were purchased from Genzyme Corp. (Cambridge, MA). rhTNF- α (10^8 U/mg), IFN- γ (2.10^7 U/mg), IL-6 (10^8 U/mg), and IL-1 (5.10^7 U/mg) were obtained from Boehringer Mannheim GmbH (Penzberg, Germany). rhG-CSF, GM-CSF, and IL-3 were kind gifts of Dr. S. C. Clark (Genetics Institute, Cambridge, MA). Erythropoietin (epo 10^5 U/mg) was purchased from Cilag (Brussels, Belgium).

Cell Sorting and Pre-CFU Assay. Labeling of low density bone marrow cells for CD34 and CD38 and isolation of $\text{CD34}^{++}\text{CD38}^{-}$ cells by flow cytometric cell sorting were performed as described previously (6). Primary liquid cultures of $\text{CD34}^{++}\text{CD38}^{-}$ cells were performed in 96-well flat-bottom plates in duplicate at 100 cells/well in IMDM, 10% FCS, and combinations of the following recombinant human cytokines: 100 ng/ml IL-1, 200 U/ml IL-6, 30 U/ml IL-3, 100 ng/ml kit ligand (KL), and varying concentrations of TNF- α , TGF- β , and IFN- γ . After 14 d of primary culture, the number of cells in each well was counted using an inverted microscope at a magnification of 250, after which the cells were harvested, washed three times in IMDM with 10% FCS, and plated in secondary methylcellulose cultures (0.9%) supplemented with 20% FCS, 1% BSA, 10^{-5} M 2-ME, 30 U/ml IL-3, 100 ng/ml G-CSF, 100 ng/ml GM-CSF, and 2 U/ml epo, which were optimal concentrations for colony formation in preliminary experiments. These cultures were microscopically scored for colony formation after 14 d of culture at 37°C in 7.5% O_2 and 5% CO_2 in a fully humidified incubator. To ascertain that the effects of TNF- α in these experiments were direct, some experiments were performed at a single-cell level. $\text{CD34}^{++}\text{CD38}^{-}$ cells were sorted at 1 cell/well in 96-well V-bottomed plates (two plates per cytokine combination). In test sorts using fluorescent microbeads, on average <2% of the wells contained no beads, and no wells were detected that contained more than one bead. Each well contained 100 μl of culture medium consisting of IMDM, 10% FCS, IL-3, and KL (concentrations as in the primary cultures described above) and either no TNF- α or TNF- α at 1 ng/ml. After 14 d of culture (37°C, 5% O_2 , 5% CO_2 in a fully humidified incubator), the number of wells where growth had occurred was scored using an inverted microscope.

Statistics. In all experiments, Student's *t* test for paired samples was used.

Results

TNF- α Stimulates the Proliferation and Differentiation of $\text{CD34}^{++}\text{CD38}^{-}$ Cells. We have previously shown that $\text{CD34}^{++}\text{CD38}^{-}$ cells require at least the presence of IL-3,

KL, and either G-CSF, IL-1, or IL-6 to proliferate and generate secondary CFC. Optimal proliferation is obtained in the presence of IL-3, KL, and two of the other aforementioned synergistic factors (6). Since TNF- α has been reported to inhibit the proliferation of very primitive progenitor cells (11–14), we first investigated whether TNF- α would inhibit the proliferation and differentiation of optimally stimulated $\text{CD34}^{++}\text{CD38}^{-}$ cells in a two-stage pre-CFC assay. As shown in Fig. 1 A, addition of TNF- α to the liquid primary cultures of $\text{CD34}^{++}\text{CD38}^{-}$ cells supported by an optimal combination of cytokines, that is, IL-3, KL, IL-1, and IL-6, induced a very substantial in-

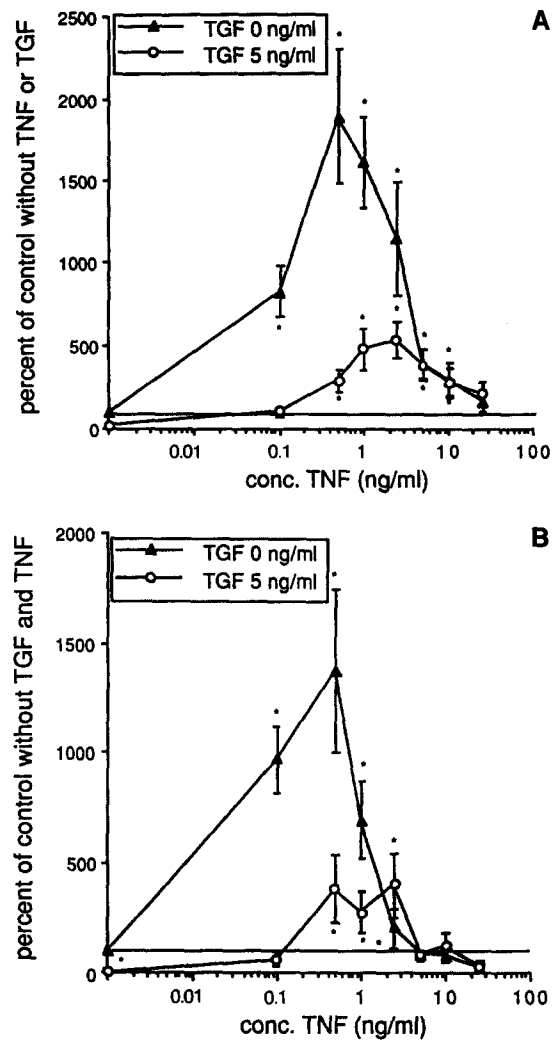


Figure 1. Dose response of the effect of TNF- α on the expansion of $\text{CD34}^{++}\text{CD38}^{-}$ human bone marrow cells (A) and on the generation of secondary CFC from $\text{CD34}^{++}\text{CD38}^{-}$ human bone marrow cells (B) after 14 d of liquid culture supported by IL-3, IL-1, IL-6, and KL in the absence (triangles) and presence (circles) of TGF- β (5 ng/ml). Results are expressed as the percentage of control cultures without TNF- α and TGF- β (horizontal line). Significant differences compared with control cultures are indicated with an asterisk. $n = 13$ except for TNF- α 1 ng/ml, where $n = 20$, for TNF- α 5 ng/ml, where $n = 24$, and for TNF- α 25 ng/ml, where $n = 7$ (A); $n = 7$ except for TNF- α 1 ng/ml, where $n = 12$, and for TNF- α 5 ng/ml, where $n = 16$ (B).

crease in the number of cells obtained after 14 d of liquid culture. The stimulatory action of TNF- α was maximal between 0.5 and 2.5 ng/ml and decreased at higher concentrations. Maximal cell expansion in the liquid cultures was on average increased 18-fold ($P = 0.0001$). This huge increase in cell number was paralleled by a somewhat less substantial increase in CFC output (Fig. 1 B). The maximal increase in the number of secondary CFC was on average 13-fold at 1 ng/ml of TNF- α ($P = 0.0001$). Secondary colonies consisted mostly of macrophage and mixed granulocyte-macrophage colonies, with a minority of pure granulocytic, erythroid, and mixed myeloid-erythroid colonies. No preferential production of any kind of CFC was noted after culture in the presence of TNF- α (not shown). At higher concentrations of TNF- α , no significant difference in CFC output was found compared with cultures without TNF- α , although cell expansion after 14 d of primary culture was significantly higher (fourfold at 5 ng/ml, $P < 0.05$). To test whether this was caused by a more rapid differentiation of primitive progenitor cells in the presence of TNF- α , secondary semisolid cultures were plated after 7 instead of 14 d of primary liquid culture in the presence of IL-3, KL, IL-1, IL-6, and 5 ng/ml TNF- α . Indeed, when the cells were plated in semisolid cultures after 7 d of liquid culture, a 16-fold increase in CFC output was seen in the presence of TNF- α compared with cultures without TNF- α , indicating that TNF- α promotes faster proliferation and differentiation of CD34⁺CD38⁻ cells (not shown).

Since TNF- α has such a potent stimulatory effect on optimally stimulated primitive progenitor cells, we next investigated whether TNF- α would be able to induce proliferation of CD34⁺CD38⁻ in the presence of IL-3 alone, to which these cells are normally unresponsive (Table 1). As we have previously shown (6), addition of single synergistic factors (IL-1, IL-6, G-CSF, KL) to cultures of CD34⁺CD38⁻ cells supported by IL-3 alone does not induce any proliferation or generation of secondary CFC, except for the combination of IL-3 plus KL, where very limited prolifera-

tion is seen. As shown in Table 1, TNF- α indeed induces substantial proliferation and generation of secondary CFC in cultures supported by IL-3 alone and by IL-3 and KL. It is clear from these data that TNF- α is much more potent as a single synergistic factor for IL-3-stimulated CD34⁺CD38⁻ cells than KL. Furthermore, as shown in Table 1, this effect was a direct one, since it could be reproduced in single-cell cultures of CD34⁺CD38⁻ cells. In the presence of neutralizing anti-TNF- α antibodies, no effect was seen (not shown). In the absence of IL-3, no proliferation is seen in cultures supported by TNF- α alone or by combinations of TNF- α with IL-1, IL-6, KL, and G-CSF (not shown). TNF- α is thus a potent direct synergistic factor for very primitive progenitor cells that are unresponsive to IL-3 alone.

TNF- α Reverses the Inhibitory Effects of TGF- β but Not IFN- γ . TGF- β is well known as a potent inhibitor of hematopoietic progenitor cells (2-4). Maximum inhibition is obtained at a concentration of 2.5-5 ng/ml (Fig. 2, A and B). As shown in Fig. 1 A (number of cells after 14 d of primary liquid culture) and Fig. 1 B (CFC output after 14 d of liquid culture), a concentration of 0.1 ng/ml of TNF- α was sufficient to restore cell proliferation and the output of CFC to baseline values of cultures without TGF- β and TNF- α . Moreover, at higher concentrations of TNF- α , that is, from 5 ng/ml on, the dose-response curves of TNF- α in the presence and absence of TGF- β , respectively, coincide. In the presence of an optimal inhibitory concentration of TGF- β , 1 ng/ml of TNF- α stimulates the proliferation of CD34⁺CD38⁻ cells supported by IL-3, IL-1, IL-6, and KL by ~50-fold. Although from Fig. 1, in which increases in cell number and CFC output were calculated as a percentage of control without TGF- β and TNF- α , one would be tempted to conclude that TNF- α induces complete resistance to the inhibitory effects of TGF- β , this is not the case when the relative inhibitions induced by different concentrations of TGF- β in the presence of 0, 1, and 5 ng/ml TNF- α are analyzed. As shown in Fig. 2 A (cell number after 14 d of primary liquid culture) and Fig. 2 B (CFC output after 14 d of primary liquid culture), there is an upward shift of the dose-response curve of TGF- β in the presence of increasing concentrations of TNF- α , but significant, albeit limited, relative inhibition still occurs at higher concentrations of TGF- β .

IFN- γ was recently identified by our group as a potent direct inhibitor of CD34⁺CD38⁻ cells (8). As with TGF- β , the inhibitory effect of IFN- γ is specific for more primitive progenitor cells. In contrast to TGF- β , however, an optimal concentration of IFN- γ (1,000 U/ml) induces the same relative inhibition of the proliferation and differentiation of CD34⁺CD38⁻ cells in the presence compared with the absence of TNF- α , respectively (Fig. 3).

Table 1. Effect of TNF- α (1 ng/ml) on the Proliferation and Differentiation of CD34⁺CD38⁻ Cells Supported by IL-3 and by IL-3 and KL

	Cell number*	CFC number*	Colonies/96 wells†
IL-3	19.3 ± 7	0.3 ± 0.3	0 ± 0
IL-3 + TNF	473 ± 95	25 ± 6	14.7 ± 1.4
<i>p</i>	0.0001	0.0001	0.0001
IL-3 + KL	58 ± 7	0.7 ± 0.3	1 ± 1
IL-3 + KL			
+ TNF	11,379 ± 1,035	88 ± 48	19.8 ± 3
<i>p</i>	0.0001	0.0001	0.0001

*Results expressed as number cells and CFC, respectively, obtained after 14 d of liquid culture per 100 input CD34⁺CD38⁻ cells; $n = 3$.

†Results expressed as number of positive wells per 96 wells in single-cell cultures of CD34⁺CD38⁻ after 14 d of culture; $n = 6$.

Discussion

This work demonstrates that TNF- α is a potent stimulator of the proliferation of very primitive adult hematopoie-

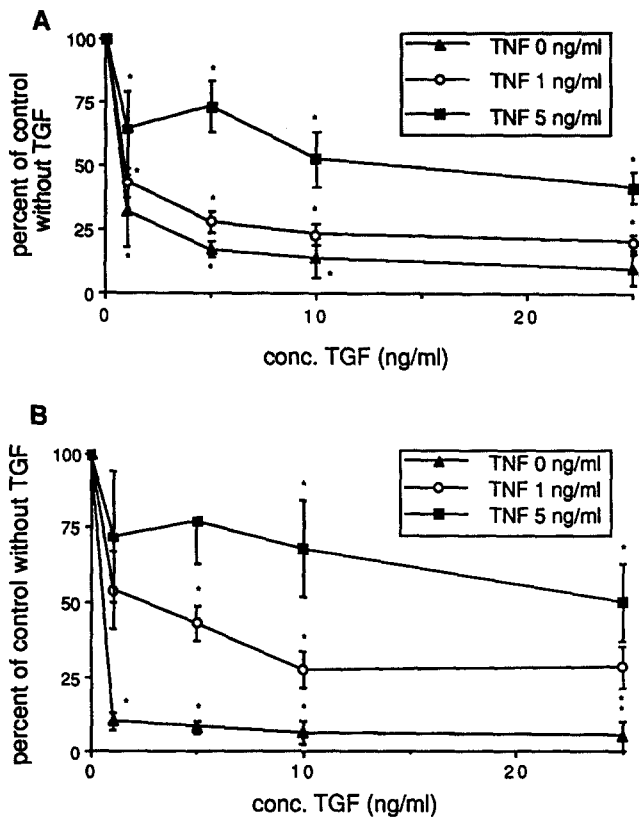


Figure 2. Dose response of the relative inhibitory effect of TGF- β on the expression of CD34⁺CD38⁻ cells (A) and on the generation of secondary CFC from CD34⁺CD38⁻ cells (B) after 14 d of liquid culture supported by IL-3, IL-1, IL-6, and KL in the absence (triangles) or presence of 1 ng/ml (circles) or 5 ng/ml (squares) of TNF- α , respectively. Results are expressed as the percentage of control cultures without TGF- β . Significant differences compared with control cultures are indicated with an asterisk. $n = 7$, except for TGF- β 5 ng/ml and TNF- α 0 ng/ml, where $n = 27$, TGF- β 5 ng/ml and TNF- α 1 ng/ml, where $n = 20$, and TGF- β 5 ng/ml and TNF- α 5 ng/ml, where $n = 24$ (A); $n = 7$, except for TGF- β 5 ng/ml and TNF- α 0 ng/ml, where $n = 27$, TGF- β 5 ng/ml and TNF- α 1 ng/ml, where $n = 20$, and TGF- β 5 ng/ml and TNF- α 5 ng/ml, where $n = 24$ (B).

tic progenitor cells, as defined by a high expression of CD34 and the absence of CD38 expression. As we and others have previously shown (6, 16), these cells represent 1–5% of bone marrow CD34⁺ cells, need multiple growth factors, including at least IL-3 and KL, for proliferation, and do not form colonies in classic semisolid assays, but give rise to secondary CFC after liquid culture in the presence of appropriate combinations of growth factors. The absence of growth in the presence of IL-3 and the very low level of growth in the presence of IL-3 plus KL indicates that they are indeed very primitive cells (19).

TNF- α is the most potent synergistic factor for the IL-3-induced proliferation of these cells of the cytokines tested (KL, IL-1, IL-6, and G-CSF) and is the only cytokine, next to KL, that as a single added agent can induce proliferation in CD34⁺CD38⁻ cells cultured in the presence of IL-3. Moreover, the combination of IL-3 plus TNF- α is much more potent than the combination of IL-3

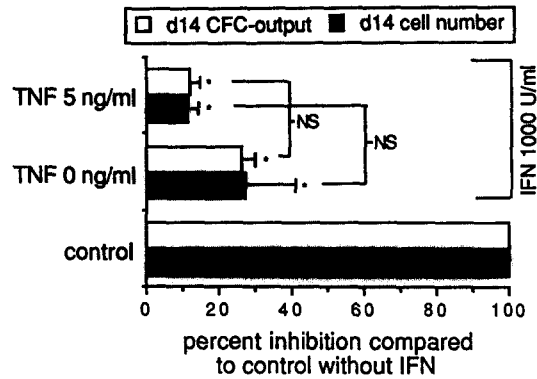


Figure 3. Relative inhibitory effect of IFN- γ (1,000 U/ml) on the expression and on the output of secondary CFC of CD34⁺CD38⁻ bone marrow cells after 14 d of liquid culture supported by IL-3, IL-1, IL-6, and KL, in the absence and presence of 5 ng/ml TNF- α . Results are expressed as a percentage compared with control cultures without IFN- γ . Significant differences compared with control cultures are indicated with an asterisk. $n = 6$.

plus KL. Quantitatively, this stimulatory effect is far greater than any stimulatory effect of TNF- α on hematopoietic progenitors reported in the literature thus far. According to the results of the single-cell culture experiments in this study, the synergistic effect of TNF- α on the proliferation of CD34⁺CD38⁻ cells is a direct one.

Stimulatory effects of TNF- α on very early hematopoietic progenitors have been reported before, but these reports dealt with populations that were still responsive to IL-3 alone (10, 15). On the other hand, although Rusten et al. (11) showed stimulatory effects of TNF- α on IL-3- and GM-CSF-responsive human progenitors, the same authors demonstrated an inhibitory effect of TNF- α mediated by the p75 TNF receptor on very primitive high proliferative potential (HPP) CFC in the murine (14) and human (11) system. Functionally, the cells used in the latter reports were similar to the cells described in this report, in that they have an HPP and are dependent on the presence of multiple growth factors for their proliferation. There is agreement, however, on the heterogeneity of the response of hematopoietic progenitor cells to TNF- α , depending on their differentiation stage, phenotype, and on the cytokines present in the culture medium. The fact that in our experiments only the effect on the initial phases of the proliferation of CD34⁺CD38⁻ cells were assessed might have played a role. This hypothesis is substantiated by existing evidence that TNF- α recruits a more primitive subset of progenitors (15) while inducing a differentiation block downstream (10, 20). Since the relative increase in the number of secondary CFC induced by TNF- α compared with cultures without TNF- α is larger after 7 d of liquid culture than after 14 d of liquid culture, it follows that TNF- α induces more rapid differentiation together with a stimulation of the proliferation of very primitive progenitors.

In this report, it is also shown for the first time that even very low concentrations (0.1 ng/ml) of TNF- α alleviate

the profound inhibitory effect of TGF- β on the proliferation of very primitive human progenitor cells. At these low concentrations of TNF- α , a profound inhibitory effect of TGF- β is still seen when compared with cultures without TGF- β , but, remarkably, at higher concentrations of TNF- α , a high degree of resistance against the effects of TGF- β is induced. Other cytokines, such as IL-1, IL-6, G-CSF, and basic fibroblast growth factor (21–23), have been shown to partially abrogate the inhibitory effects of TGF- β . In our experiments, however, TGF- β was still profoundly inhibitory, despite the presence of many of these factors. On the other hand, the inhibitory effects of IFN- γ (6) were unaffected by the presence of TNF- α .

Stem cells are known to be inhibited and “protected” from the stimulatory effects of ambient hematopoietic stimu-

lators by TGF- β , produced autocrinely or paracrinely by stem cells and the surrounding bone marrow stroma (24, 25). It is our hypothesis that in situations of increased demand for mature blood cells, TNF- α , a typical inflammatory cytokine, induces resistance to the inhibitory effects of TGF- β so that these cells become responsive to the proliferative effects of other cytokines. Exhaustion of the stem cells could be prevented by the fact that the selective inhibitory effect of another inflammatory cytokine, IFN- γ , is not opposed by TNF- α .

In conclusion, TNF- α is a very potent synergistic stimulator of very primitive human hematopoietic progenitor cells and induces resistance in these cells to the effect of TGF- β but not IFN- γ .

This work was supported by Kom op tegen Kanker under the auspices of the Nationaal Fonds voor Wetenschappelijk Onderzoek, grant 3.0133.91, from the Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Fund for Medical Scientific Research), grants from Sportvereniging tegen de Kanker and the United Fund of Belgium (1991), and by private charities organized by Jan Pszenicko, Veerle Van De Poel, Frederic Arts, Ronald Aka, Simone Goetschalckx, Friso Voerman, Kobe De Roeck, and the students of the Antwerp International School.

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Received for publication 6 July 1995 and in revised form 29 September 1995.

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