# Effect of TNF Expression by Stromal Cells on Hemopoietic and Stromal Precursor Cells in Long-Term Bone Marrow Cultures Derived from TNF-Deficient Mice

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The effect of TNF expression by wild type stromal sublayer on hemopoiesis was studied in cultures after second inoculation of TNF-deficient bone marrow cells. Long-term maintenance of hemopoiesis was determined solely by the absence of autocrine expression of TNF by hemopoietic cells. The production of hemopoietic precursors of all studied types in TNF-deficient cultures on a wild type sublayer was significantly higher than in cultures without TNF production. Presumably, initial expression of TNF in the sublayer promotes the survival of hemopoietic precursors with high proliferative potential in the culture. It is also obvious that TNF is required for normal functioning of stromal precursor cells.

**Key Words:** *long-term bone marrow culture; TNF-/- mice; hemopoietic precursor cells; stromal precursor cells*

No appreciable changes in the hemopoiesis are detected in TNF-deficient (TNF-/-) mice, but the structure of the spleen and lymphoid follicles is impaired [10]. However, hemopoiesis in long-term bone marrow cultures (LTBMC) derived from these mice is maintained during uncommonly long period (more than 200 weeks). It was previously shown that long-term hemopoiesis in a culture was not related to changed proliferation of precursor cells and their capacity to start apoptosis [4]. This phenomenon is not a result of neoplastic transformation of cells in TNF-/- LTBMC [6]. With time the incidence of early precursor cells gradually increases in TNF-/- LTBMC (in contrast to the wild type culture) and then the incidence of all detectable precursor cells sharply increases [5]. This phenomenon is observed in TNF-/- LTBMC during the first 100 weeks of culturing, after which the number of hemopoietic precursor cells decreases. It was also demonstrated that at about the same time undifferentiated stromal precursor cells appeared in the TNF-/- LTBMC suspension fraction [3].

Changes observed in LTBMC without TNF demonstrate the role of this factor in the regulation of hemopoietic and stromal precursor cells. However, an abnormally high level of hemopoiesis during at least the first 20 weeks was described for cultures with TNF-/- sublayer cells and wild type cells [2]. It remained unclear whether expression of TNF by the sublayer cells modified hemopoiesis and whether the absence of autocrine expression of this factor or absence of its expression by the sublayer cells was essential for the status of hemopoietic and stromal precursor cells. We studied the effect of TNF secreted by the stroma on hemopoietic and stromal precursor cells deficient by this cytokine.

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## MATERIALS AND METHODS

The study was carried out on 10-28-week-old C57BL/6 mice of both sexes with the wild type and TNF-/- genotypes [10]. The animals were obtained from vivarium of Affiliated Branch of Institute of Bioorganic Chemistry (Pushchino).

LTBMC were prepared as described previously [4,7]. These cultures were denoted as TNF-/-, *i.e.* only TNF-deficient cells were taken for creating the culture. Cultures consisting of preformed wild type sublayer and bone marrow cells from TNF-/- mice were prepared as follows: LTBMC were initially obtained by the above method; after 3 weeks of culturing, when adherent cell sublayer structure was established, the cultures were irradiated in a dose of 43.5 Gy on $^{137}$Cs IPK device, after which the second inoculation of TNF-/- mouse bone marrow cells was made (TNF-/- LTBMC on a wild type sublayer).

The incidence of hemopoietic precursor cells was evaluated by the count of cells forming cobblestone areas on days 7 and 28 (CAFC-7 and CAFC-28) as described previously [5], the count of granulocytic macrophagal CFU (CFU-GM) was determined by a previously described method [4], and the incidence of splenic CFU (CFU-S) by the method of Till and MacCulloch [1].

In order to evaluate the self-maintenance capacity of stromal cells released after long culturing into TNF-/- LTBMC suspension fraction, the cells of this fraction from the test cultures were inoculated into a 24-well plate (100,000 cells per well) for culturing in Fisher medium with 15% FCS and 10% WEHI 3B cell strain conditioned medium. Every 3-4 days 100,000 cells from the nonadherent fraction were transferred into the next well, while adherent cells were passaged. The procedure was repeated until complete exhaustion of the nonadherent fraction.

The immunophenotype of suspension fraction cells was identified using surface markers CD45 (common leukocytic marker) [9] and Sca-1 expressed on early hemopoietic precursors [12] and on stromal cells [8,11]. The cells were fixed in 4% paraformaldehyde (Sigma) and incubated first with rat antimurine antibodies to CD45 or Sca-1 (BD Pharmingen) and then with FITC-conjugated goat antirat Ig (BD Pharmingen). Stained cells were counted on a FACS (Calibur) or visually (per 200 cells) under a fluorescent microscope (Opton).

The genotype of sublayer cell after 83 weeks of TNF-/- LTBMC culturing on a wild type sublayer was identified by PCR [10].

The results were processed using Student's *t* test.

## RESULTS

The type and duration of hemopoiesis in TNF-/- LTBMC on a wild type sublayer did not differ from those for TNF-/- LTBMC (Fig. 1). It seems that the sublayer genotype and hence, TNF expression by the sublayer are not essential for manifestation of this phenomenon. Hence, the absence of autocrine expression of TNF by hemopoietic cells is responsible for the maintenance of hemopoiesis in the culture for 4 years. However, analysis of the incidence of hemopoietic precursor cells of different degree of maturity in suspension fractions of cultures of both types showed significant differences. The incidence of CFU-GM in TNF-/- LTBMC at late terms of culturing was virtually by two orders of magnitude lower than in TNF-/- LTBMC on a wild type sublayer during the same period (Fig. 2, *a*). The differences in less differentiated precursor cells (CFU-S) were significant: the incidences of these cells in LTBMC cultured on a wild type sublayer even after 90 weeks of culturing surpassed those for TNF-/- LTBMC one order of magnitude (Fig. 2, *b*). The concentration of CFU-S in these cultures significantly surpassed the standard concentration of these cells in the bone marrow (20-30/$10^5$ cells).

The count of CAFC-7 correlating with that of CFU-S 12 was beyond the method sensitivity limits chosen in our study and by one order of magnitude surpassed the content of CAFC-7 in TNF-/- LTBMC suspension fraction (Fig. 2, *c*). The count of CAFC-28 reflecting the incidence of cells with repopulating activity in LTBMC on a wild type sublayer varied from 0.1 to 10.0 per $10^5$ suspension fraction cells during the late periods of culturing

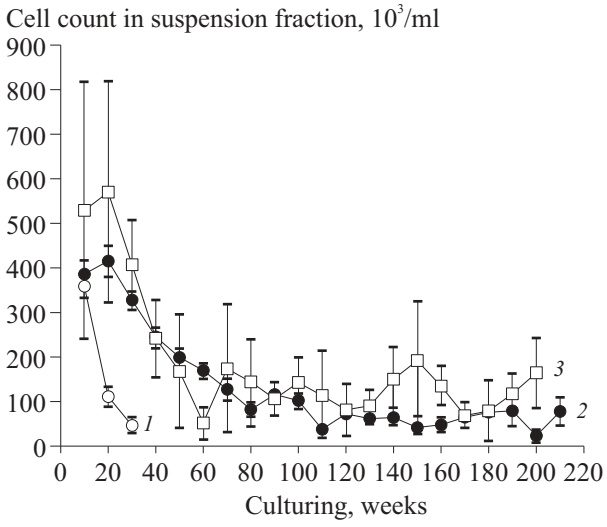


**Fig. 1.** Cell count in wild type (WT) LTBMC suspension fraction (*1*), TNF-/- (*2*), and TNF-/- on WT sublayer (*3*).

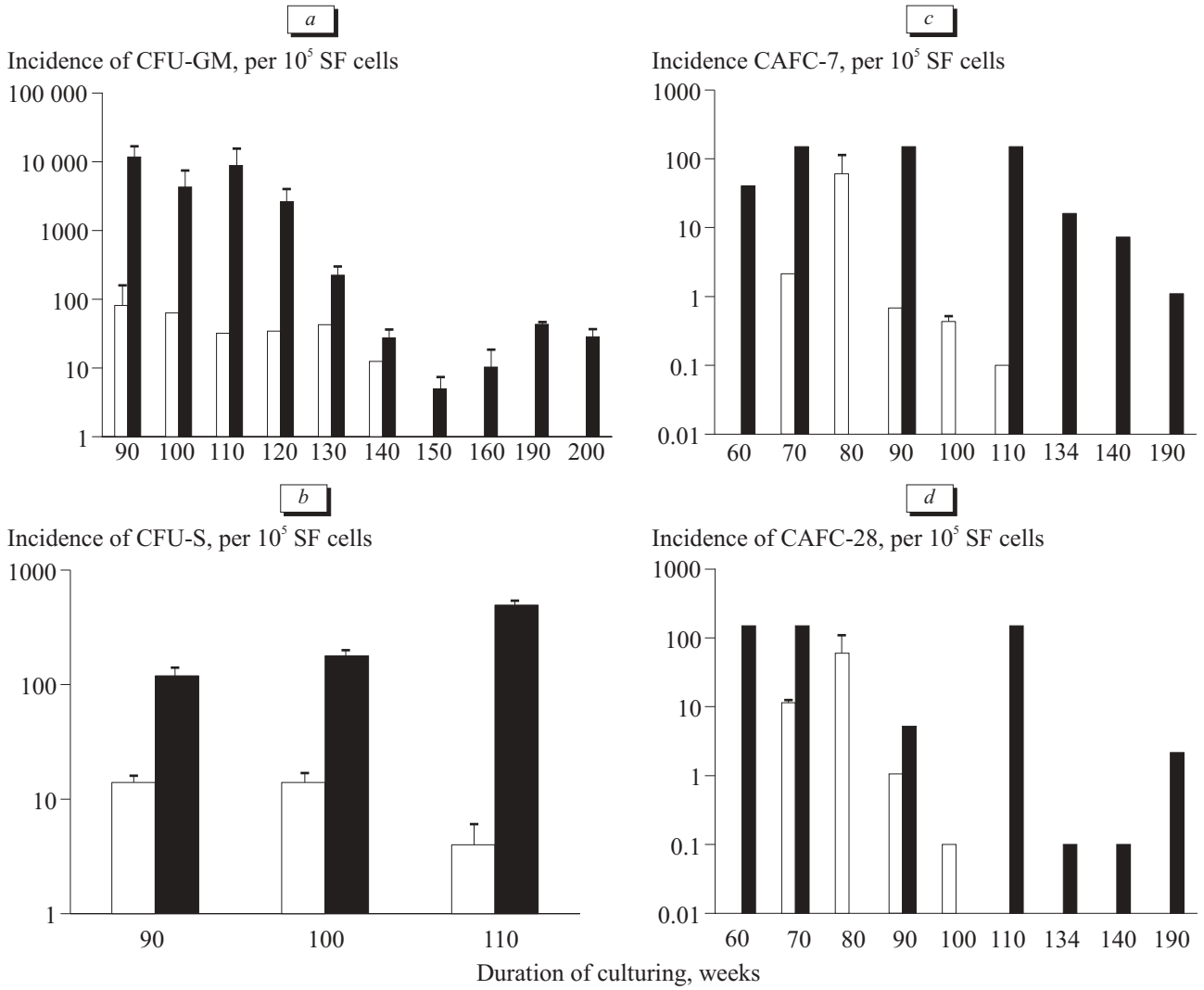


**Fig. 2.** Changes in the production of hemopoietic precursor cells of different maturity in a culture on WT sublayer. *a*) incidence of CFU-GM in suspension fractions (SF) of LTBMC of TNF-/- and TNF-/- on WT sublayer; *b*) incidence of CFU-S in SF of LTBMC of TNF-/- and TNF-/- on WT sublayer; *c*) incidence of CAFC-7 in SF of LTBMC of TNF-/- and TNF-/- on WT sublayer; *d*) incidence of CAFC-28 in SF of LTBMC of TNF-/- and TNF-/- on WT sublayer. Light bars: TNF-/-; dark bars: TNF-/- on WT sublayer.

and by one order of magnitude surpassed the corresponding parameter in TNF-/- culture (Fig. 2, *d*). It was previously shown that the incidences of all above-mentioned hemopoietic precursor cells in TNF-/- LTBMC were appreciably higher than in wild type cultures and these differences manifested after 30-40 weeks in culture, when hemopoiesis in wild type LTBMC was exhausted [5]. Taking into account the fact that TNF expression by the sublayer cells does not lead to cessation of hemopoiesis (as in wild type LTBMC), we conclude that autocrine expression of TNF by hemopoietic cells prevents long-term survival of hemopoietic precursors in the culture. TNF expression by the sublayer cells increases the number of hemopoietic precursor cells even in comparison with TNF-/- LTBMC. Total cell count in the culture does not increase in this case, and therefore the differentiation capacity of these cells is decreased, similarly as in TNF-/- culture [5].

PCR analysis of sublayer cell genotype detected no wild type cells after 80 weeks of culturing (data not presented).

Hence, stromal TNF-/- cells supersede irradiated wild type cells in culture, while all changes in the class of hemopoietic precursor cells developed as a result of short-term exposure to TNF.

Non-hemopoietic cells capable of forming adherent cell strains were detected in TNF-/- LTBMC suspension fraction after 70 weeks of culturing [5]. These cells appeared also during culturing on a wild type sublayer: 3 days after inoculation of 100,000 nonadherent cells into a well with 70-week culture we observed not only increased number of non-

**TABLE 1.** Self-Maintenance of Cells from LTBMC Suspension Fraction on Wild Type Sublayer during Derivation of Stromal Strains

| Passage No. | Number of nonadherent cells, $\times 10^3$ | |
|---|---|---|
| | 70-week LTBMC | 141-week LTBMC |
| 0 | 100 | 166.7 |
| 1 | 800 | 350 |
| 2 | 320 | 250 |
| 3 | 520 | 50 |
| 4 | 160 | 190 |
| 5 | 85 | 40 |

adherent cells, but also the formation of a confluent sublayer of adherent cells. Consecutive repeated transfers of nonadherent cells into new wells showed that they can survive no more than 4 passages and that adherent and nonadherent cells formed during each of these passages. After the 5th passage the number of cells in the suspension irreversibly decreased (Table 1).

In order to analyze the cells they were subcultured after attaining confluence. A fraction of nonadherent live cells always emerged in the culture growing during more than 15 passages. A total of $1.5\text{-}2.0\times10^6$ cells formed in the suspension fraction during the first 10 passages, the maximum number ($2.08$ and $2.03\times10^6$) appeared after passages of adherent cells taken during passages 2 and 3 of LTBMC suspension fraction cells, while the minimum number ($1.4\times10^6$) was obtained after passages of adherent cells taken after the last (4th) passage of suspension fraction cells. Evidently, stromal cells appearing in the TNF-/- LTBMC suspension fraction on a wild type sublayer after long culturing are characterized by significant, but limited self-maintenance capacity. On the other hand, 63.4% cells in the TNF-/- LTBMC suspension fraction on wild type sublayer are $CD45^+$ (hemopoietic cells) and 89.4% are Sca-$1^+$. Hence, 52.8% cells carry both markers and are presumably early hemopoietic precursors [12]. On the other hand, cells expressing only Sca-1 (of stromal nature) are also present in the suspension [8,11]. These very cells seem to give rise of adherent cell strains.

Hence, TNF plays a dual role of TNF in the regulation of hemopoiesis: this factor is essential for survival of early precursors with a high proliferative potential and for negative regulation of the number of hemopoietic precursor cells. For stromal cells TNF is a positive regulator essential for their normal functioning.

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## REFERENCES

1. N. I. Drize, M. S. Drutskaya, L. P. Gerasimova, *et al.*, *Byull. Eksp. Biol. Med.*, **130**, No. 7, 76-79 (2000).
2. M. S. Drutskaya, D. V. Kuprash, S. A. Nedospasov, *et al.*, *Ibid.*, **131**, No. 2, 184-187 (2001).
3. I. N. Nifontova, Yu. V. Ol'shanskaya, and N. I. Drize, *Ibid.*, **138**, 284-288 (2004).
4. I. N. Nifontova, M. A. Ershler, and N. I. Drize, *Ibid.*, **133**, No. 5, 537-540 (2002).
5. I. N. Nifontova, M. A. Ershler, and N. I. Drize, *Ibid.*, **135**, No. 3, 330-333 (2003).
6. M. A. Ershler, I. N. Nifontova, Yu. V. Ol'shanskaya, *et al.*, *Ibid.*, **133**, No. 5, 537-540 (2002).
7. T. M. Dexter, T. D. Allen, and L. G. Lajtha, *J. Cell. Physiol.*, **91**, 335-344 (1977).
8. T. P. Gumley, I. F. McKenzie, and M. S. Sandrin, *Immunol. Cell. Biol.*, **73**, 277-296 (1995).
9. J. A. Ledbetter and L. A. Herzenberg, *Immunol. Rev.*, **47**, 63-90 (1979).
10. M. Pasparakis, L. Alexopoulou, V. Episkopou, and G. Kollias, *J. Exp. Med.*, **184**, 1397-1411 (1996).
11. M. Satoh, H. Mioh, Y. Shiotsu, *et al.*, *Exp. Hematol.*, **25**, 972-979 (1997).
12. M. Trevisan and N. N. Iscove, *J. Exp. Med.*, **181**, 93-103 (1995).