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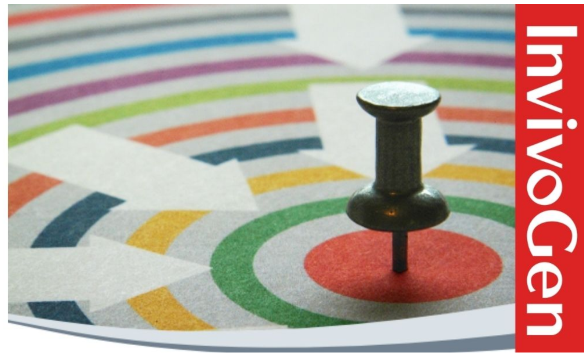


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# REGULATION OF HUMAN BONE MARROW LACTOFERRIN AND MYELOPEROXIDASE GENE EXPRESSION BY TUMOR NECROSIS FACTOR-α[1]

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**Lactoferrin (LF) and myeloperoxidase (MPO) are glycoproteins synthesized in early myeloid cells (promyelocytes, myelocytes) and stored in granules of polymorphonuclear neutrophilic granulocytes. Both proteins are involved in the host inflammatory response, and LF has been found to have myelosuppressive activity in vivo and in vitro. Little is known, however, about the regulation of their production. We investigated the stability of LF and MPO mRNA and the effects of purified recombinant human TNF-α on LF and MPO levels in normal human bone marrow. Low density human bone marrow cells were cultured in the presence or absence of actinomycin D (10 μg/ml) or TNF-α (200 U/ml). LF and MPO RNA levels were analyzed by Northern blots using, respectively, a 650-bp insert from the plasmid pHL41, and a 2.3-kb insert from the plasmid pMPO2 as probes. It was found that: 1) LF mRNA is a fairly stable molecule, with a half-life of between 8 and 9 h, whereas MPO is less stable, with a half-life of between 4 and 5 h; 2) TNF-α decreases both LF and MPO mRNA levels, an effect seen by 24 h with MPO mRNA and 48 h with LF mRNA; 3) nuclear run-on assays revealed that TNF decreases transcription of the LF gene by 70% and the MPO gene by 50%; and 4) the suppressive effect of TNF-α on LF and MPO mRNA levels is not due to cell killing or selective differentiation and is reversible.**

The mammalian hematopoietic system is a complex network of cells and the cytokines that they produce and that regulate them. These cells have multiple functions in the body, one of which is mediation of the host response to infection. A protein implicated in this response is LF.[3]

LF is an iron binding protein with a molecular mass of approximately 76,000 Da. It is found in secondary granules of neutrophils, among hematopoietic cells (1). The protein has been demonstrated to have bactericidal and bacteriostatic activity (2, 3). Patients with neutrophil specific granule deficiency, a congenital disorder characterized by recurrent bacterial and fungal infections, lack neutrophil LF mRNA and protein (4). LF has also been implicated as a regulator of myelopoiesis (5–13). Its synthesis appears to occur mainly during the myelocyte stage of granulocyte maturation, and the protein continues to accumulate until the band stage of maturation (14, 15). Because of this, the synthesis of LF can be used as a marker for granulocyte maturation and function. Presently, very little is known about the control of LF gene expression. A cDNA copy of LF mRNA has recently been cloned, and this can be used as a probe for LF mRNA, to evaluate factors regulating the expression of the LF gene (16).

The regulation of gene expression of MPO, a second protein made in cells of the granulocyte series and found in primary granules, has been studied more extensively. Its mRNA appears at an earlier stage of development than that of LF, mainly in the promyelocyte stage (14, 17–22). This protein is also involved in the inflammatory response in that it breaks down peroxide in the presence of halide ions, contributing to the bactericidal function of granulocyte (2, 3, 23). However, the mechanism by which myeloperoxidase production is regulated during the inflammatory response is not known. DNA probes are also available to study MPO regulation (24).

A cytokine potentially playing a role in the regulation of LF and MPO gene expression in normal human bone marrow cells is TNF-α, which is produced by activated monocytes and other cells. TNF-α has a central role in the inflammatory response. It stimulates degranulation of neutrophils and specifically stimulates release of LF (25–27). TNF-α also decreases the proliferation of hematopoietic progenitor cells (28–30). We demonstrate here that TNF-α shuts off transcription of the LF and MPO genes in normal human bone marrow cells, a first direct demonstration of repression of LF and MPO gene expression in normal human bone marrow cells.

## MATERIALS AND METHODS

*Cells.* Human bone marrow cells were obtained from normal donors with informed consent and separated by centrifugation through Ficoll-hypaque (Pharmacia, Piscataway, NJ). Low density (<1.077 g/cm$^3$) cells were cultured in McCoy's medium containing 10% FCS at 37°C in a 5% $CO_2$, 5% $O_2$ atmosphere, at a density of 5 × $10^6$ cells/5 ml culture. The low density cell fraction contains

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[2] Address correspondence and reprint requests to Carolyn Srivastava, Ph.D., Walther Oncology Center, Department of Medicine, Indiana University School of Medicine, 975 W. Walnut Street, IB 501, Indianapolis, IN 46202-5121.
[3] Abbreviations used in this paper: LF, lactoferrin: MPO, myeloperoxidase; G-CSF, granulocyte CSF; GM-CSF, granulocyte-macrophage CSF.



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mononuclear cells and is enriched for progenitor cells of various lineages: granulocyte monocyte, erythroid, lymphoid. It also contains monocytes and mature lymphocytes. Cytokines and actinomycin D (10 μg/ml; Sigma Chemical Co., St. Louis, MO) were added at the initiation of culture. Cycloheximide was used at 10 μg/ml.

*Cytokines.* Recombinant human TNF-α (sp. act., $5 \times 10^7$ U/mg protein) was a gift from Genentech (South San Francisco, CA). After redetermination of the units in the absence of actinomycin D (28), TNF-α was used at 200 U/ml. Recombinant human GM-CSF (used at 200 U/ml; sp. act., $10^8$ U/mg protein) was a gift from Immunex Corp. (Seattle, WA). Recombinant human G-CSF (used at 200 U/ml, 95% pure, sp. act. $>5 \times 10^7$ U/mg protein) was a kind gift from Cetus Corp. (Emeryville, CA).

*RNA analysis.* At the indicated times, cells were scraped into medium, pelleted, and washed once with PBS. RNA was then isolated using guanidinium isothiocyanate lysis and phenol extraction by the method of Chomczynski and Sacchi (31). Separation of the RNA (approximately 3 to 5 μg/sample) on 1.2% agarose gels, transfer to a nylon membrane, and hybridization to $^{32}$P-labeled probe were as described previously (32). Autoradiograms were scanned with a Bio-Rad model 620 densitometer. The LF probe used was a *PstI-EcoRI* insert from the plasmid pHL41 (16). The MPO probe used was a *HindIII-EcoRI* insert from the plasmid pMPO2 (24), kindly provided by Dr. H. P. Koeffler and Dr. C. Miller (University of California at Los Angeles). The human actin probe was an insert from the plasmid pHL1311, obtained from Dr. W. Salser (University of California at Los Angeles) (33).

*Nuclear run-on assays.* Nuclear run-ons were performed essentially as described by Weber et al. (34). Cells (approximately $2 \times 10^7$/point) were treated as indicated, scraped into medium, and pelleted. They were then washed twice with ice-cold PBS and lysed in a solution containing 10 mM Tri-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40. After a 5 min incubation on ice, the nuclei were pelleted at 500 × *g* for 5 min. The supernatant was removed and the nuclei were again treated with lysis solution. The nuclei were resuspended in 50 μl of glycerol buffer (50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, and 40% glycerol), frozen in liquid nitrogen, and stored at −80°C until use. Frozen nuclei were thawed on ice and an equal volume of transcription buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, and 5 mM dithiothreitol) containing 100 μCi of [α-$^{32}$P] UTP (>3000 Ci/mmol; Amersham, Clearbrook, IL) was added. Transcription was allowed to proceed for 30 min at room temperature, and was terminated by the addition of 100 μl of 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris, pH 7.5, 12 μg of DNase I, 15 U of placental RNase inhibitor (both from BRL, Gaithersburg, MD), and 12 μg of yeast transfer RNA. The reaction was then incubated for 20 min at 37°C. Proteinase K and sodium laurylsulfate were added to final concentrations of 750 μg/ml and 1%, respectively, and the reaction was additionally incubated at 37°C for 30 min. It was then extracted with phenol-chloroform, ethanol was precipitated three times, and the final pellets were counted. Equal numbers of cpm of labeled RNA were hybridized to nylon membranes containing 5 μg each of plasmids pUC 19, pHL41, pHL1311, and pMPO2 in a solution containing 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.5, 300 mM NaCl, 10 mM EDTA, 0.2% SDS, 0.1% sodium pyrophosphate, 1 × Denhardt solution (0.02% BSA, 0.02% Ficoll, and 0.02% polyvinylpyrolidone), and 200 μg/ml transfer RNA, at 65°C for 40 to 48 h. The filters were then washed and exposed to Kodak XAR film.

## RESULTS

*Stability of LF and MPO mRNA.* Stability of LF and MPO mRNA was measured by the addition of actinomycin D (final concentration, 10 μg/ml) to cultures of normal bone marrow cells. RNA was isolated and analyzed by Northern blots, using the pHL 41 and pMPO2 probes. As can be seen in Figure 1*A*, the LF RNA begins to decay at about 8 h after actinomycin D addition, and by 16 h it is drastically decreased to less than 2% of control. Densitometer scans of five such blots revealed that the half-life of LF mRNA is approximately 8 to 9 h. MPO mRNA, in contrast, is less stable than LF mRNA. The RNA begins to decay at about 4 h after actinomycin D addition, and its half-life is 4 to 5 h. Cycloheximide did not appear to stabilize these molecules (data not shown).

*TNF-α-induced decrease in LF and MPO mRNA levels.* Normal bone marrow cultures were incubated with 200 U/ml of TNF-α for the indicated times before harvest and RNA isolation (Fig. 1*B*). This concentration was chosen because it had previously been shown to decrease hematopoietic cell proliferation (28–30). Subsequently, a dose-response experiment showed that 200 U/ml was in excess of the amount of TNF-α needed for optimal shutoff of LF gene expression. As can be seen, treatment with TNF-α resulted in a decrease in LF mRNA by 48 h after the initiation of culture. This decrease, which was highly reproducible (at least eight experiments), was not seen in control, untreated cultures. TNF-α also decreased MPO mRNA levels (Fig. 1*B*). In contrast to LF mRNA, MPO mRNA levels decreased after 12 to 24 h of TNF-α treatment. The decrease was seen in at least seven experiments. Actin mRNA levels in these experiments were unaffected by TNF-α (data not shown). To determine whether TNF-α caused this decrease by directly destabilizing mRNA, cultures were treated with both TNF-α and actinomycin D for the times indicated in Figure 1*A*. The data demonstrate that TNF-α does not directly destabilize LF mRNA, but may have a destabilizing effect on MPO mRNA. The half-life of MPO mRNA decreased to approximately 3 h in the presence of TNF-α, as revealed by densitometer scans of the autoradiograms.

*Shutoff of transcription of the LF and MPO genes by TNF-α.* Since TNF-α appears not to directly destabilize LF mRNA, it is possible that LF transcription is decreased or shut off by TNF-α. To evaluate this possibility, nuclear run-on assays were performed (Fig. 2). Bone marrow cells (approximately $2 \times 10^7$/culture) were incubated for the indicated times, either with or without TNF-α, and the nuclei were incubated with $^{32}$P-labeled UTP in an in vitro transcription reaction. The labeled transcripts were then hybridized to filters containing complementary DNA to the indicated genes, and exposed to x-ray film. The densitometer scans of the autoradiogram shown in Figure 2 indicate that, at both 24 and 48 h, transcription of the LF gene was decreased in the TNF-treated samples relative to the control cultures, by about 70%. The data indicate that LF transcription had been shut off by 24 h of TNF-α treatment. MPO transcription was decreased by about 50% at 48 h. In contrast, actin transcription in the cultures was unaffected by TNF-α.

*Assessment of LF and MPO mRNA levels in the nonadherent population of TNF-α-treated low density bone marrow cells.* Since TNF-α has been shown to stimulate proliferation and differentiation of monocytes, and since our cultures contain many cell types including monocytes, it was possible that the decrease in LF mRNA was due to an increase in the number of monocytes, thus diluting out the cells containing LF mRNA. Therefore, total bone marrow cells were cultured in the presence or absence of TNF-α, and RNA was isolated from the nonadherent population and analyzed by Northern blot. This excluded >99% of the monocytes. As seen in Figure 3, TNF-α treatment resulted in a decrease in LF and MPO mRNA levels, similar to that seen with total bone marrow cells, demonstrating that the inhibition of LF and MPO mRNA by TNF-α was not due to dilution by the adherent cell population.

*Assay of LF and MPO mRNA levels in the presence of G-CSF and GM-CSF.* G-CSF and GM-CSF stimulate granulocyte colony formation and cause immature cells

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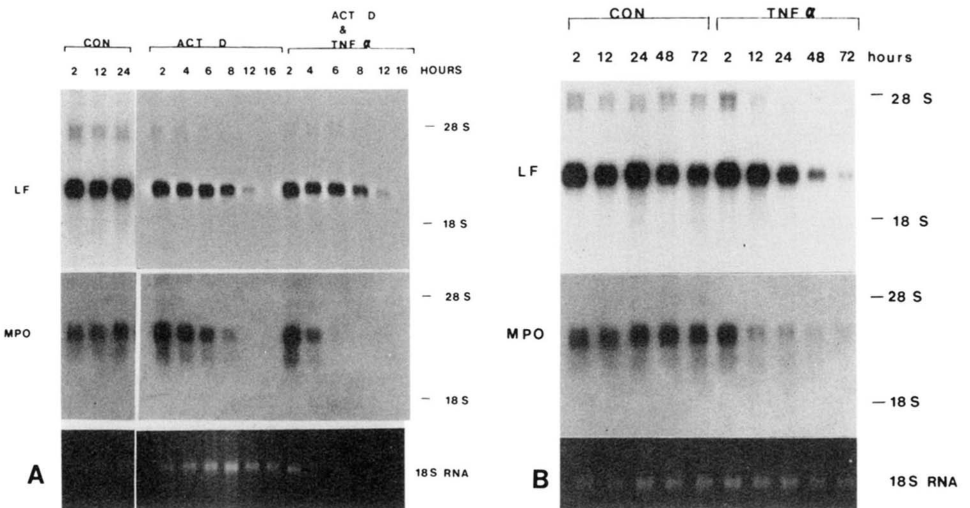


*Figure 1.* Stability and regulation by TNF-α of LF and MPO mRNA. Low density bone marrow cells were left untreated (*CON*) or treated with actinomycin D (10 μg/ml) (*A*), TNF-α (200 U/ml) (*B*), or a combination (*A*) for the indicated times. Cells were harvested and the RNA was isolated and analyzed as described in *Materials and Methods*. **The apparent inequalities in mRNA loading in *A* were due to uneven lighting of the gel when it was photographed. This experiment has been repeated three times with identical effects on the levels of LF and MPO mRNA when equivalent amounts of RNA were loaded as monitored by staining of 18 S RNA.

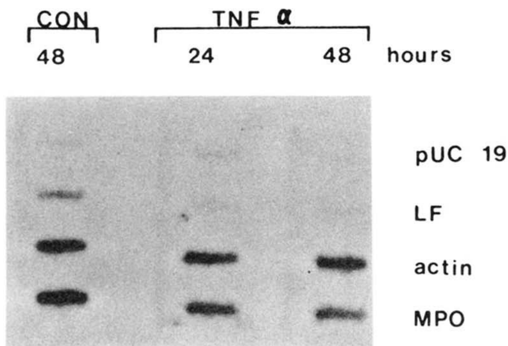


*Figure 2.* Effect of TNF-α on transcription rates of LF and MPO genes in bone marrow cells. Cells were left untreated (*CON*) or treated for 24 to 48 h with TNF-α. Nuclei were isolated and run-on assays performed as described. The $^{32}$P-nuclear RNA was hybridized to plasmid DNA on nylon filters.

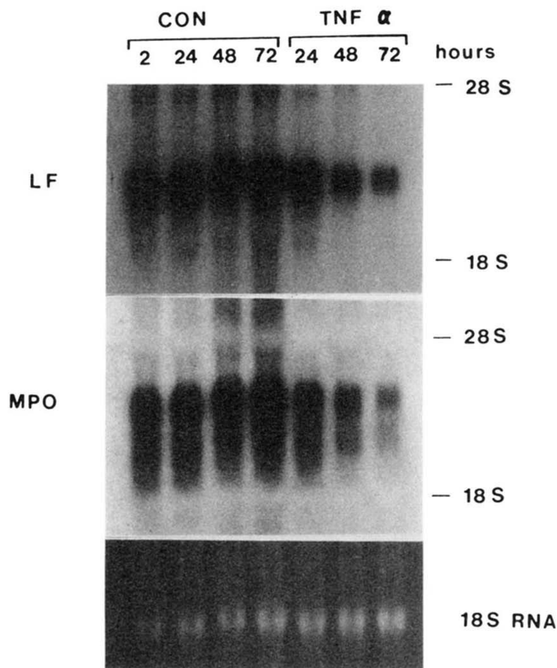


*Figure 3.* Assay of LF and MPO RNA in the nonadherent fraction of control or TNF-α-treated bone marrow cultures. Total low density bone marrow cultures were left untreated (*CON*) or treated with TNF-α for the times indicated. RNA was isolated from the nonadherent cells and analyzed by Northern blot.

of the granulocyte lineage to mature. In order to compare their effect on LF and MPO mRNA levels with that of TNF-α, and also to determine whether TNF-α influences this effect, the experiment shown in Figure 4 was conducted. Bone marrow cells were incubated in the presence or absence of G-CSF or GM-CSF, with or without the addition of TNF-α for 1 through 5 days. The cells were then harvested and the RNA analyzed. TNF-α decreased MPO mRNA levels, as seen previously. Although MPO mRNA levels increased slightly at 2 days in the TNF-α-treated samples, there was a large overall decrease relative to the controls. G-CSF and GM-CSF both increased the amount of LF mRNA slightly after 4 days. The effect of these cytokines on MPO mRNA levels was more dramatic. Both increased the amount of MPO mRNA over control. A densitometer scan of a shorter exposure of the blot of the G-CSF- and GM-CSF-treated samples revealed that, in the presence of G-CSF, MPO mRNA levels increased 2.5-fold from 1 day to 2 days, remained elevated for 3 days, and began to decline by 5 days. GM-CSF treatment resulted in a 2.4-fold increase in MPO mRNA levels from 1 to 2 days, and a decline thereafter. TNF-α reduced the MPO mRNA levels in G-CSF and GM-CSF-stimulated cells, but the pattern of increase and decrease remained the same. The TNF-α-induced decrease in LF mRNA, which had been seen previously, occurred in this experiment, even in the presence of G-CSF or GM-CSF. Actin levels, however, were unaffected by the treatments.

*Viability of TNF-α-treated low density bone marrow cells.* The shut off of LF transcription by TNF-α could be due to one or more factors: 1) TNF-α may kill the cells that produce LF and MPO mRNA; or 2) TNF may induce the cells to mature to a point at which they no longer synthesize LF or MPO mRNA. To assess these possibilities, cells were cultured in the presence or absence of 200 U/ml TNF-α as described previously (Tables I and II). In experiment 2 (Table II), the cells were separated at the end of the culture period into adherent and nonadherent fractions. In both experiments, the cells were counted, and viability was assessed by trypan blue exclusion. As can be seen, treatment with TNF-α did not decrease viability at 24, 48, or 72 h. In addition, the absolute numbers of differentials (including promyelocytes and myelocytes) of the total and nonadherent fractions were

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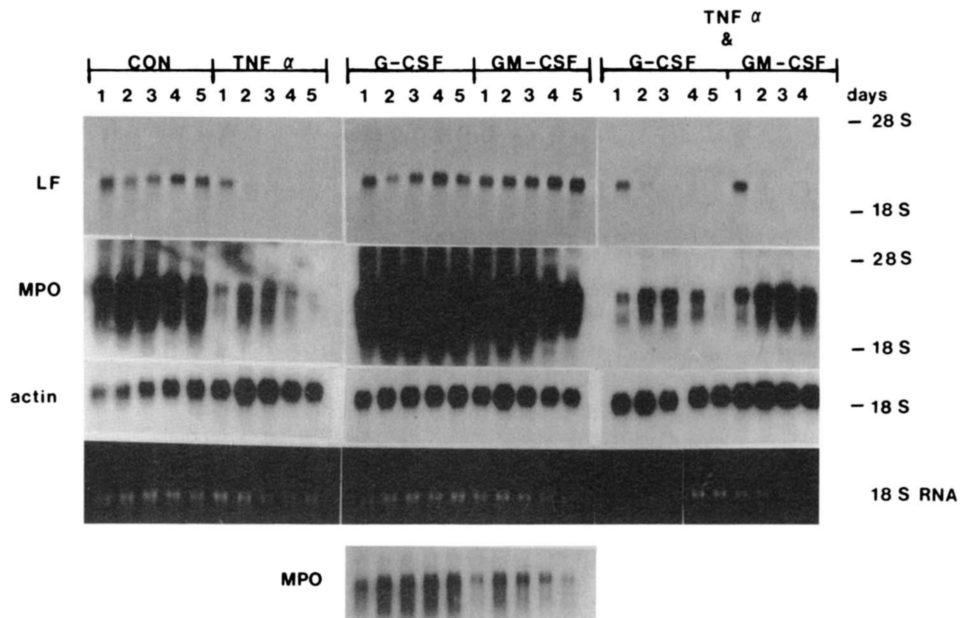


*Figure 4.* Effect of TNF-α on G-CSF and GM-CSF-treated bone marrow cells. Cells were left untreated (*CON*), or treated with TNF-α, G-CSF, GM-CSF, TNF-α + G-CSF, or TNF-α + GM-CSF for 1 through 5 days. The RNA was isolated and analyzed by Northern blots. All lanes for each probe were hybridized and exposed to x-ray film simultaneously. A shorter exposure of the blot of MPO RNA from G-CSF and GM-CSF cells is shown at the bottom.

TABLE I

*Effect of TNF-α on bone marrow cell number and viability in culture: experiment 1*

| Time (h) | Cell Number | Cell Viability (%) |
|---|---|---|
| Control | | |
| 0 | $1 \times 10^6$ | 98 |
| 24 | $6 \times 10^5$ | 98 |
| 48 | $1 \times 10^6$ | 87 |
| 72 | $7 \times 10^5$ | 90 |
| 96 | $7 \times 10^5$ | 86 |
| TNF-α[a] | | |
| 24 | $6 \times 10^5$ | 96 |
| 48 | $7 \times 10^5$ | 88 |
| 72 | $7 \times 10^5$ | 92 |
| 96 | $5 \times 10^5$ | 76 |

[a] TNF-α was used at a concentration of 200 U/ml. Total low density cells were harvested and counted.

TABLE II

*Effect of TNF-α on bone marrow cell number and viability in culture: experiment 2*

| Time (h) | Nonadherent | | Adherent | |
|---|---|---|---|---|
| | Cell number | Cell viability (%) | Cell number | Cell viability (%) |
| Control | | | | |
| 2 | $17.2 \times 10^6$ | 98 | $1.2 \times 10^6$ | 95 |
| 24 | $16.0 \times 10^6$ | 92 | $3.0 \times 10^6$ | 91 |
| 48 | $18.8 \times 10^6$ | 95 | $0.5 \times 10^6$ | 86 |
| 72 | $16.0 \times 10^6$ | 91 | $0.4 \times 10^6$ | 82 |
| TNF-α[a] | | | | |
| 24 | $12.8 \times 10^6$ | 95 | $5.2 \times 10^6$ | 95 |
| 48 | $20.4 \times 10^6$ | 99 | $1.6 \times 10^6$ | 96 |
| 72 | $16.4 \times 10^6$ | 90 | $3.4 \times 10^6$ | 90 |

[a] TNF-α was used at a concentration of 200 U/ml. Cells were separated at the end of the culture period into adherent and nonadherent fractions.

similar at 24 and 48 h whether they had been cultured in the presence of control medium or 200 U TNF-α/ml (data not shown). The effects of TNF-α on LF and MPO gene expression were apparent already by 24 to 48 h. Significant decreases in cell counts and increases in numbers of monocytes macrophages became apparent only by 96 h when cells were cultured with TNF-α.

*Reversibility of the TNF-induced decrease of LF and MPO mRNA.* To evaluate whether effects of TNF-α on gene expression were reversible, bone marrow cultures were exposed to TNF-α for 2, 24, or 48 h. The cells were then washed (three times) free of TNF-α and harvested immediately or further incubated for 24, 48, 72, or 96 h before harvesting. Alternatively, the cells were incubated in the presence or absence of TNF-α continuously for 24, 48, or 72 h, and the RNA was isolated and analyzed as described previously. Figure 5 shows the results of such an experiment. Treatment of cultures with TNF-α resulted in a decrease in LF and MPO mRNA, as demonstrated previously. The effect was prominent first at 48 h after addition of TNF-α, for LF, and 24 h for MPO, as seen previously, although a 2-h pulse of TNF-α resulted in a smaller decrease than continuous incubation for 48 h. The results indicate that 24 to 48 h after the removal of TNF-α, the LF mRNA levels increase, indicating that TNF-α is not killing the LF-synthesizing cells, or causing

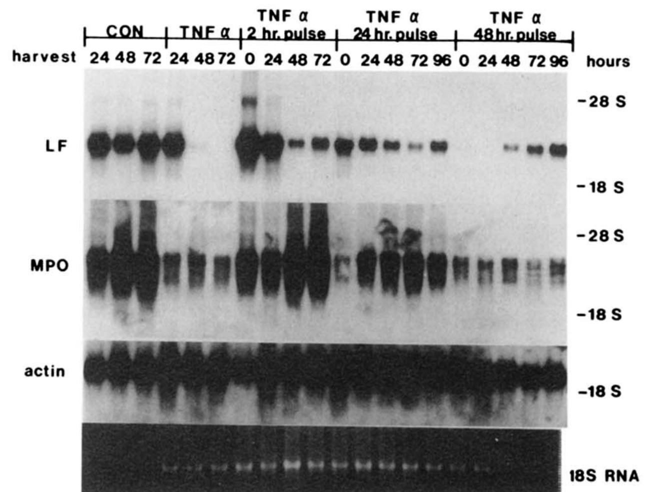


*Figure 5.* Effect of pulses of TNF-α on LF and MPO mRNA levels. Cells were left untreated (*CON*), treated continuously with TNF-α (*TNF α lanes*) before harvest, or treated for 2, 24, or 48 h with TNF-α. One aliquot of cells was harvested at the end of each pulse. The remaining cells in each group were washed three times to remove the TNF-α, and incubated for the indicated times. The harvest times are shown as hours after removal of TNF-α.

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them to mature past the point at which they could potentially express LF mRNA. A reversal of the shutoff of MPO gene expression was seen after 2-h and 24-h pulses, but not after the 48-h pulse.

## DISCUSSION

We have demonstrated that LF mRNA production is regulated in a hematopoietic system. The half-life of the RNA is approximately 8 to 9 h, so it is relatively stable. MPO mRNA is less stable, but does not turn over rapidly, with a half-life of 4 to 5 h. This is consistent with the results of Tobler et al. (22), who found that the half-life of MPO mRNA is 4.5 h in HL60 cells. As would be expected, neither RNA sequence contains a 3′ AU-rich region described by Shaw and Kamen (35) for rapidly turning over mRNA. Since LF and MPO are synthesized and stored during several stages of granulocyte maturation, their mRNA would be expected to remain stable.

TNF-α apparently decreases the levels of LF by shutting off transcription, rather than by directly destabilizing the mRNA, as evidenced by experiments with actinomycin D. The fact that the TNF-α effect is not seen until after 24 h of treatment may be due to the stability of the LF message. Alternatively, TNF-α may induce a secondary factor that directly destabilizes or shuts off synthesis of LF mRNA. MPO mRNA, in contrast, may be destabilized by TNF-α, in addition to a reduction of transcription. Therefore, the effect on MPO is seen more quickly, in less than 24 h.

Our experiments demonstrate that the negative regulatory effect of TNF-α on LF and MPO gene expression is not due to cell kill or differentiation and is reversible. TNF-α may generally shut down transcription in early granulocytic cells, since it decreases levels of mRNA for both LF and MPO. It would be of interest to determine whether levels of all RNA in these cells are decreased, or whether TNF-α acts on specific RNA. This may be helpful in gaining insight into the mechanism by which TNF-α inhibits proliferation of granulocyte-macrophage progenitor cells, since shut off of transcription may be involved.

In our experiments, GM-CSF and G-CSF increased the levels of LF mRNA only slightly, but increased MPO mRNA dramatically. Jaffe et al. (20) have shown that treatment of murine bone marrow cells with GM-CSF increases the proportion of promyelocytes and myelocytes, and causes an increase in MPO mRNA levels, to a maximum at 40 to 64 h. They found that LF protein synthesis, presumably representing LF mRNA levels, reached a maximum by 7 days. These results are similar to ours. It is possible that the slight increase that we saw in LF mRNA levels at 4 to 5 days of GM-CSF treatment may have been greater by 7 days, when more cells had matured to the myelocyte stage. Our data on MPO mRNA levels in GM-CSF-treated cells are very consistent with those of Jaffe et al. (20). We observed an increase at 2 days and a decline thereafter. Any potential stimulatory effect of G-CSF or GM-CSF on LF mRNA synthesis was completely abolished by TNF-α, suggesting that the repressive effect by TNF-α is stronger than stimulatory effects by G-CSF or GM-CSF. TNF-α also drastically reduced the stimulatory effect on MPO levels seen with G-CSF and GM-CSF. It could potentially induce the production of a DNA binding factor that represses transcription, such as that described by Kagiyama and Pastan (36). A transcriptional repressor may bind to *cis*-acting elements in the LF and MPO promoters, competing with transcriptional activators.

It is of interest that TNF-α stimulates release of LF from neutrophils during bacterial infection, while repressing the transcription of LF mRNA. One explanation may be that a negative feedback loop exists in this system, to prevent an overproduction and release of LF. Since LF has been shown to inhibit myelopoiesis (5–13, 37), an excess of the protein might be disadvantageous. TNF-α has been demonstrated to induce production of hematopoietic growth factors from a number of cell types (38–42) These growth factors may, as shown in in vivo mouse experiments (12, 37), also counteract some of the negative effects of excess LF.

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