

Activation of Neutrophils by Tumor Necrosis Factor- α During Sepsis

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Introduction

Endotoxin exposure of macrophages during the onset of bacterial infections leads to the sequential production of the cytokines tumor necrosis factor- α (TNF- α), interleukin-1 β and interleukin-6 [1]. These cytokines are thought to enhance the local anti-bacterial immune response. TNF- α , the most potent modulator of neutrophil function, induces in vitro the inhibition of neutrophil chemotaxis [2], the expression of CD11/CD18 integrins resulting in the adherence of neutrophils to matrix proteins with the release of oxidants [3] and specific granule constituents [4], extravasation [5, 6], and increased phagocytosis [7].

Topical TNF- α administration in vivo induces localized vascular hyperpermeability and extravasation [8]. Systemic TNF- α exposure, in contrast, is experimentally associated with neutrophil-mediated oxidative and proteolytic tissue destruction [9]. This deleterious role of systemic neutrophil activation is confirmed by the correlation of the initial plasma levels of TNF- α during infection with the incidence of the adult respiratory distress syndrome and with the mortality rate [10].

The significance of TNF- α as a determinant for the prolonged neutrophil-mediated oxidative and proteolytic tissue destruction during the sepsis syndrome is, however, uncertain due to rapidly decreasing TNF- α plasma levels [10] and the experimental observation of cellular deactivation towards TNF- α following repeated in vitro exposure of neutrophils to TNF- α or cross-deactivation following cellular contact with bacterial agents [11]. This cross-deactivation has been shown to include shedding of the TNF- α receptors leading to an increase in the plasma TNF- α binding capacity and to an inhibition of TNF- α actions on other cells. [12]. The data showing deactivation of neutrophils towards TNF- α were generated with neutrophils from healthy normal individuals. Neutrophils from septic patients are, however, in a different functional state as visible from the flow cytometric measurement of cell biochemical

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parameters of neutrophil function such as the activation of the oxidative burst cascade or the activation of the Na^+/H^+ -antiport leading to alkalization of the intracellular pH and an increase in cell volume [13, 14]. The goal of this study was, therefore, to characterize $\text{TNF-}\alpha$ effects on neutrophils from septic individuals in comparison with healthy normals. This was done by comparing in vitro effects of $\text{TNF-}\alpha$ on neutrophils from healthy normals with the functional state of in vivo activated neutrophils from septic patients, and their responses to addition of $\text{TNF-}\alpha$ or neutralizing anti- $\text{TNF-}\alpha$ antibodies.

Materials and Methods

Patients. Seventy-two venous blood samples were obtained from intensive care patients with or without bacterial infection or sepsis. Sepsis was assumed in the presence of a defined bacterial focus and secondary systemic effects of sepsis [15]. The 32 patients without infection had multiple organ failure scores, as defined by Goris et al. [16], of 1.87 ± 0.55 (mean \pm standard error), in contrast to a score of 2.81 ± 0.31 for the 26 bacterially infected patients, and 6.71 ± 0.19 for the 14 patients with sepsis.

Cells. A suspension of leukocytes in autologous plasma was prepared by overlaying heparinized blood samples from intensive care patients and seven healthy normals on an erythrocyte-aggregating medium (Histopaque-1077, Sigma, Deisenhofen, FRG) for 30 min at 22°C and 1 g [17]. The plasma supernatant is depleted of erythrocytes through accelerated sedimentation at the plasma-medium interface. This procedure induces no measurable prestimulation of the cells as contact of the neutrophils with the separation medium, centrifugation, or lysis of erythrocytes are avoided.

Staining. The cell suspension in autologous plasma ($20\text{ }\mu\text{l}$) was preincubated in 1 ml of Hank's balanced salt solution (HBSS, without phenol red or bicarbonate, Sigma; with 10 mM HEPES, pH 7.35) for 5 min at 37°C either with $1\text{ }\mu\text{l}$ of the fluorogenic substrate dihydrorhodamine 123 (DHR, Molecular Probes, Eugene, OR, USA; 1 mM in N,N-dimethylformamide, DMF) for the quantitation of the hydrogen peroxide production by the intracellular oxidation of nonfluorescent DHR to green fluorescent rhodamine 123 [17, 18] or with $1\text{ }\mu\text{l}$ of the fluorogenic pH indicator 1,4-diacetoxy-2,3-dicyanobenzene (ADB, Calbiochem, Frankfurt, FRG; 50 mM in DMF) for the measurement of the intracellular alkalization during cellular activation [13].

Stimulation. The cells loaded with the fluorogenic substrates were primed by a 5-min incubation with 10 ng/ml of human recombinant $\text{TNF-}\alpha$ (Sigma, produced in yeast, specific activity $2 \times 10^7\text{ U/mg protein}$) at 37°C or incubated without $\text{TNF-}\alpha$ as a control. Direct effects of $\text{TNF-}\alpha$ were measured after 15 min

of incubation without further stimulation, whereas effects of TNF- α on the response of neutrophils to stimulation were measured after additional 15 min of incubation with 10^{-7} M of the bacterial peptide N-formyl-Meth-Leu-Phe (FMLP, Sigma; stock 1 mM in DMF). Alternatively, samples were maximally stimulated with 10^{-7} M of the tumor promoter phorbol 12-myristate 13-acetate (PMA, Sigma; stock 1 mM in DMF) as a positive control.

Flow cytometry. 10 000 cells per sample were analyzed after counterstaining of dead cells with propidium iodide (PI, 30 μ M, stock 3 mM in HEPES-buffered saline). The rhodamine 123 green fluorescence (515–545 nm), the PI red fluorescence (> 650 nm) and the cellular forward and right-angle light scatter of the DHR-stained cell samples were measured with 488-nm argon laser excitation on a FACScan flow cytometer (Becton Dickinson, San José, CA, USA). The cellular blue (420–440 nm) and green and red fluorescence (> 500 nm) of the ADB-stained cell samples was measured with 365-nm excitation by a high-pressure mercury arc lamp together with electrical determination of the cell volume on a FLUVO-II flow cytometer (Heka-Elektronik, Lambrecht/Pfalz, FRG). The list mode data were evaluated by DIAGNOS1 software using automated batch-processing with fixed gates and windows [19].

Results

Oxidative Burst Activity

A low oxidative burst activity with only $11.4\% \pm 2.6\%$ of the neutrophils (mean \pm SEM, $n = 7$) showing a high oxidative burst response was induced by incubation of the blood samples from healthy normals with 10^{-7} M FMLP (Fig. 1a). A similar low response was seen when the cells were incubated with 10 ng/ml TNF- α alone. A 5-min priming of neutrophils with TNF- α , in contrast, led to a high all-or-none-response to FMLP in a subpopulation of $32.0\% \pm 2.5\%$ of the neutrophils with a mean fluorescence 23.6-fold (± 3.9) over the fluorescence of nonstimulated cells (Fig. 1b). The percentage of highly responsive neutrophils increased upon stimulation with increasing amounts of FMLP (up to 5×10^{-6} M) suggesting a wide heterogeneity of the thresholds controlling the FMLP-stimulated oxidative burst response. PMA stimulation by contrast induced a homogeneously high response in $96.8\% \pm 0.8\%$ of the neutrophils showing that all neutrophils can react with oxidative burst activity.

Neutrophils from septic patients following incubation under the same conditions showed a high oxidative burst response in a large subpopulation of neutrophils already in the presence of 10^{-7} M FMLP alone, indicating that these cells had been primed *in vivo* (Figs. 2a, 3). These cells also showed a higher response to exogenously added TNF- α suggesting that no deactivation towards TNF- α had occurred. Endogenous TNF- α was directly identified as a mediator

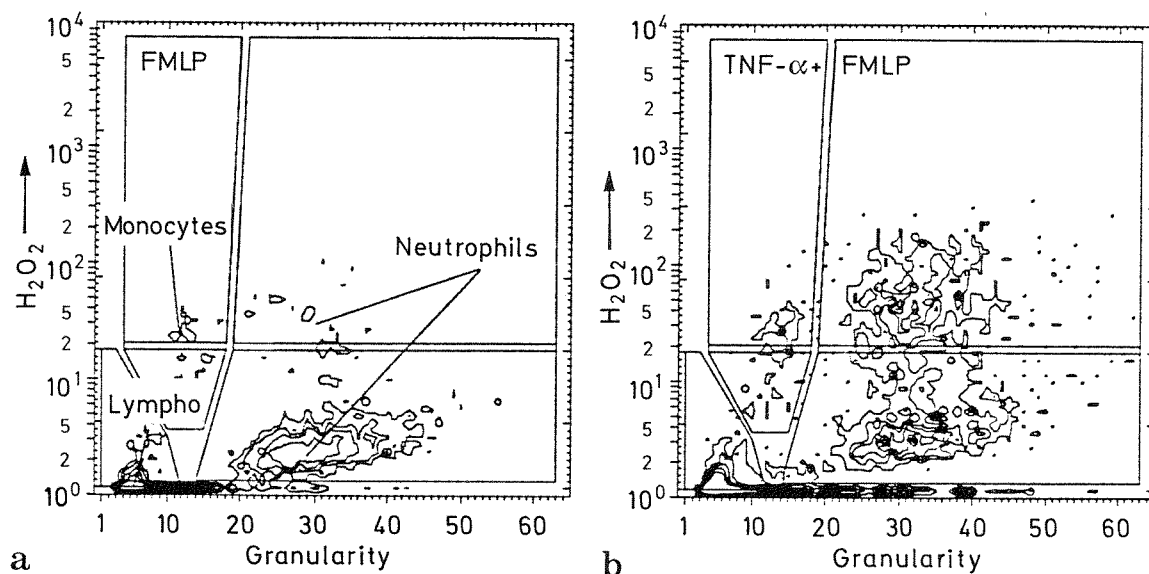


Fig. 1a, b. Effect of TNF- α on the oxidative burst response of neutrophils stimulated with the bacterial peptide FMLP. The intracellular production of hydrogen peroxide by peripheral blood leukocytes from a healthy individual was measured by flow cytometry by the intracellular oxidation of nonfluorescent DHR to the green fluorescent rhodamine 123 following stimulation with 10^{-7} M FMLP for 15 min **a** without or **b** with pretreatment of the cells with 10 ng/ml TNF- α for 5 min. Lymphocytes, monocytes and neutrophils are distinguished by the cellular side scatter

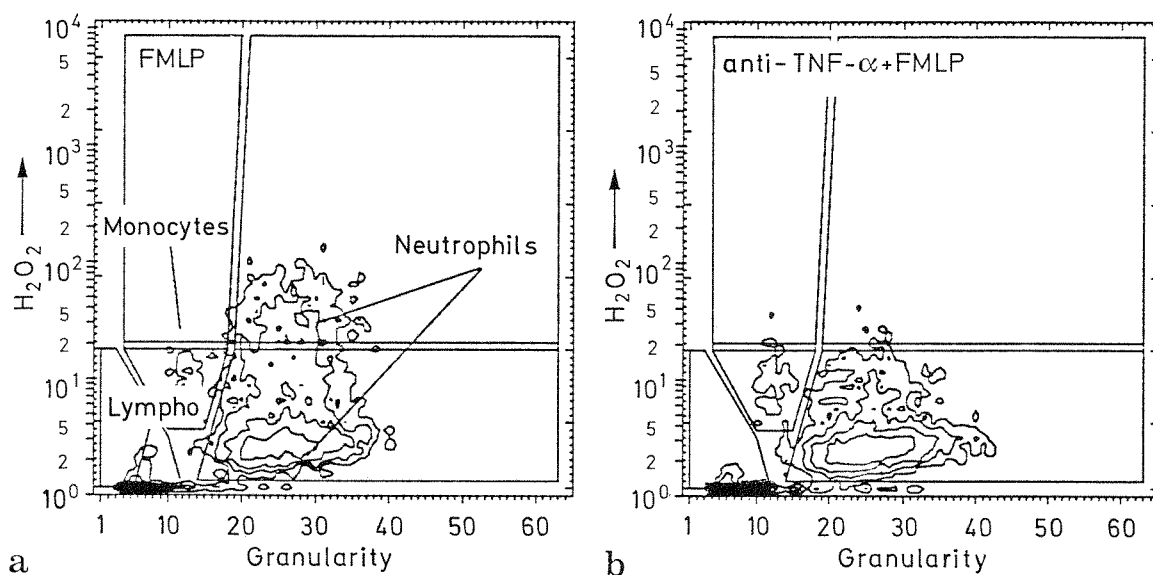


Fig. 2a, b. Effect of a neutralizing monoclonal anti-TNF- α antibody on the oxidative burst response of neutrophils to the bacterial peptide FMLP during sepsis. The intracellular production of hydrogen peroxide was measured as described in Fig. 1. Leukocytes from a patient with sepsis were stimulated with 10^{-7} M FMLP for 15 min **a** without or **b** with a 20-min preincubation in the presence of 200 ng/ml anti-TNF- α

of the in vivo priming of neutrophils in a subset of the blood samples obtained during sepsis by inhibition of the highly reactive subpopulation after a 20-min preincubation with a neutralizing anti-TNF- α antibody (clone 195, Boehringer Mannheim, Mannheim, FRG; Fig. 2b).

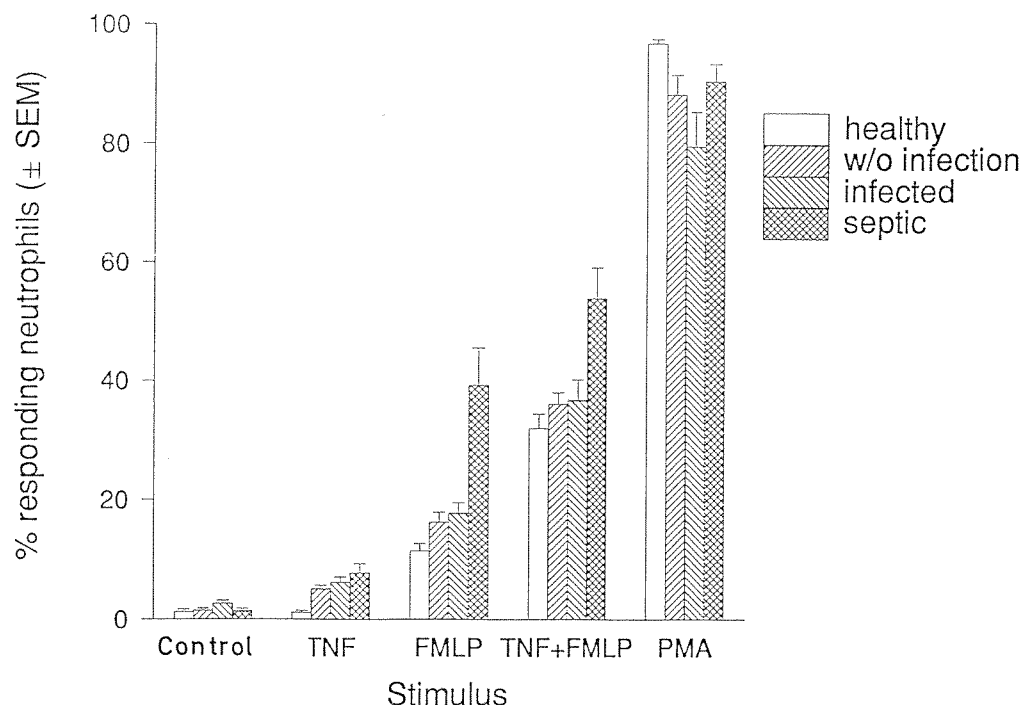


Fig. 3. Percentage of neutrophils responding with a high oxidative burst to stimulation with TNF- α or FMLP in blood samples from healthy normals ($n = 7$), and intensive care patients without ($n = 32$) or with bacterial infection ($n = 26$) or with sepsis ($n = 14$). The percentage of responsive neutrophils is calculated from the number of neutrophils in the upper right analysis window (Figs. 1 and 2), when related to the total number of neutrophils

Na⁺/H⁺-Antiport Activation

An increase of neutrophil cell volume, mediated by the activation of amiloride-sensitive Na⁺/H⁺ exchange, is already observed upon minor stimulation, in contrast to an oxidative burst response only upon stimulation above a certain threshold. Thus TNF- α alone induced a 11.2% (\pm 3.3%) increase in neutrophil cell volume (Fig. 4) compared to a 27.8% (\pm 2.9%) increase of cell volume

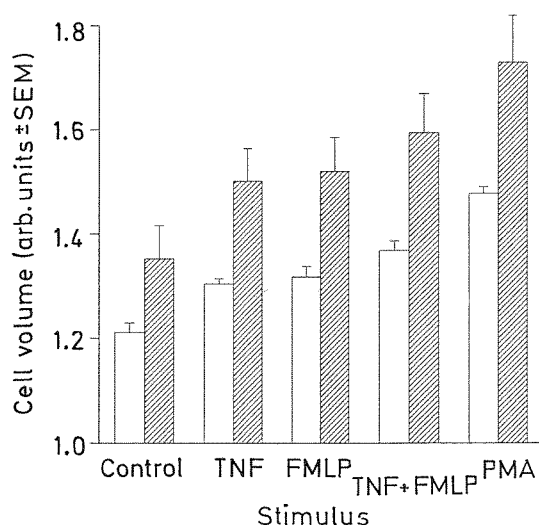


Fig. 4. Cell volume of neutrophils from healthy individuals ($n = 4$; no hatching) and patients with sepsis ($n = 4$; hatched). The leukocytes were stimulated for 15 min with 10^{-7} M FMLP or 10^{-7} M PMA with or without a 5-min pretreatment with 10 ng/ml TNF- α . The cell volume was measured by electrical sizing of cells hydrodynamically focused through the center of a cylindrical orifice (80 μ m in diameter)

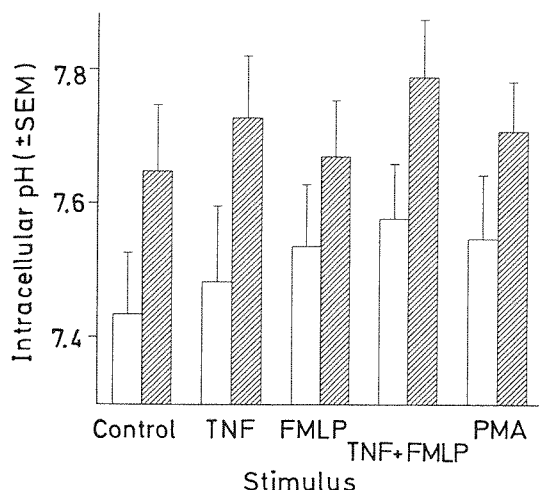


Fig. 5. Intracellular pH of neutrophils from healthy individuals ($n = 4$; no hatching) and patients with sepsis ($n = 4$; hatched). The leukocytes were stimulated for 15 min with 10^{-7} M FMLP or 10^{-7} M PMA with or without pretreatment with 10 ng/ml TNF- α for 5 min. The intracellular pH was measured by the ratio of the blue to green fluorescence of the pH indicator 2,3-dicyanohydroquinone loaded into the cells by the intracellular cleavage of ADB by cellular esterases

induced by maximal stimulation of the cells with PMA. Furthermore, the alkalinization of the intracellular pH was a sensitive indicator of TNF- α effects on neutrophils with a similarly high response to the combination of TNF- α and FMLP as to PMA alone (Fig. 5).

Neutrophils from patients during sepsis were in an activated state as shown by an increased cell volume (Fig. 4) and a more alkaline intracellular pH (Fig. 5). TNF- α alone and in combination with FMLP induced significant increases in neutrophil cell volume and pH confirming the high responsiveness of the neutrophils from septic patients to exogenous TNF- α observed in the oxidative burst measurements.

Discussion

This study shows that the *in vitro* priming of neutrophils by TNF- α leads to the graded recruitment of a previously unresponsive subpopulation of neutrophils to a high oxidative burst response towards FMLP. Neutrophils from patients with bacterial infections in combination with the systemic organ dysfunction of the sepsis syndrome were already highly stimulated *in vivo* as visible from a large FMLP-responsive subpopulation, in contrast to a low systemic neutrophil activation in patients with focal bacterial infection. This suggests that local neutrophil activation is sufficient to control a focal bacterial infection. When neutrophils are, however, systemically activated as shown by a primed oxidative burst response (Figs. 2, 3), this is tightly correlated to systemic and unspecific tissue destruction suggesting a deleterious role of the "activated" neutrophil in the peripheral circulation. The participation of endogenous TNF- α in neutrophil activation was demonstrable only in a subset of the septic samples by inhibition of the primed subpopulation of neutrophils with a neutralizing anti-TNF- α antibody. This is in agreement with the detection of increased plasma

levels of TNF- α only in a subset of septic patients [10]. Neutrophil activation during sepsis is, therefore, either due to additional priming factors or to a relative lack of inhibitory macromolecules like the acute phase protein serum amyloid A [20].

The modulation of the size of the FMLP-responsive subpopulation of neutrophils by TNF- α suggests a modulation of the thresholds for the response to stimulation of the specific neutrophil receptor for bacterial proteins in form of an all-or-none event. Such a heterogeneous all-or-none response to FMLP stimulation has been previously suggested by flow cytometric assays of membrane depolarization [21]. The mechanism of the interaction of TNF- α with the FMLP response is not understood. The magnitude of the FMLP response is tightly regulated by the expression of the FMLP receptor, which is low in unstimulated cells, but rapidly upregulated upon stimulation [22]. TNF- α may potentially affect the termination of the cellular response to FMLP by the lateral redistribution of the FMLP receptor on the plasma membrane leading to a decreased signal transduction [23] and by receptor internalization [24], or may enhance responses at a postreceptor level [25, 26].

Neutrophil activation, independently of the oxidative burst cascade, leads to an increase in the pH and cell volume mediated by the amiloride-sensitive Na^+/H^+ -antiport [27]. The extent of this alkalization modulates the respiratory burst response to stimulation of the FMLP receptor of neutrophils [28]. The alkalization of the intracellular pH and an increase of the neutrophil cell volume induced by TNF- α alone in this study was associated with an increased oxidative burst response upon further FMLP stimulation. Also samples from septic patients had a more alkaline pH and an increased cell volume when compared with samples from healthy controls confirming an earlier study in our laboratory, which included a total of 225 blood samples [13]. The increases in the intracellular pH and cell volume, therefore, seem to be indicators of an increased stimulation of the neutrophils which is associated with a decreased threshold for the induction of the all-or-none regulated oxidative burst response.

In conclusion, the correlation of the intravascular activation of neutrophils, visible from a highly FMLP-responsive subpopulation, with the systemic organ dysfunction of the sepsis syndrome, in contrast to no activation of circulating neutrophils in patients with focal bacterial infection, suggests a deleterious role of systemic neutrophil activation. A similar activation of neutrophils is inducible by a 5-min treatment of neutrophils from healthy normals with TNF- α . The activation of neutrophils from septic patients could be increased by *in vitro* exposure to TNF- α and partially reversed by anti-TNF- α antibodies in a subset of the samples. The pharmacological modulation of TNF- α effects on neutrophils by agents such as adenosine-analogs, which reverse the TNF- α -induced inhibition of neutrophil chemotaxis [29], should, therefore, be helpful in the inhibition of tissue destruction during sepsis. The flow cytometric parameters can be used for the detection of neutrophil activation predisposing to tissue destruction [13] as well as for the monitoring of the effects of therapeutic intervention.

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