Promotion of Neutrophil Apoptosis by TNF-α[1]

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We examined the ability of TNF-α to modulate human neutrophil apoptosis. Neutrophils cultured with TNF-α alone undergo a low but significant increase in the number of apoptotic cells. More interestingly, when neutrophils were pretreated with TNF-α for 1–2 min at 37°C and then were exposed to a variety of agents such as immobilized IgG, IgG-coated erythrocytes, complement-treated erythrocytes, zymosan, PMA, zymosan-activated serum, fMLP, *Escherichia coli*, and GM-CSF for 3 h at 37°C, a marked stimulation of apoptosis was observed. Similar results were obtained in neutrophils pretreated with TNF-α for 30 min, 1 h, 3 h, and 18 h. Dose-dependent studies showed that TNF-α enhances neutrophil apoptosis at concentrations ranging from 1 to 100 ng/ml. In contrast to the observations made in neutrophils pretreated with TNF-α, there was no stimulation of apoptosis when TNF-α was added to neutrophils previously activated by conventional agonists. Experiments performed to establish the mechanism through which TNF-α promotes neutrophil apoptosis showed that neither reactive oxygen intermediates nor the Fas/Fas ligand system appear to be involved. Our results suggest that TNF-α plays a critical role in the control of neutrophil survival by virtue of its ability to induce an apoptotic death program which could be triggered by a variety of conventional agonists. ***The Journal of Immunology,*** **2001, 166: 3476–3483.**

Polymorphonuclear neutrophils are short-lived cells. In the absence of appropriate stimuli, they rapidly undergo characteristic changes indicative of apoptosis, including cell shrinkage, nuclear chromatin condensation, DNA fragmentation into nucleosome-length fragments, and cell surface exposure of phosphatidylserine (PS)[3] (1, 2). Apoptosis, which represents an alternative fate to necrosis, not only determines neutrophil uptake by macrophages but also is associated with a loss of neutrophil functions, such as chemotaxis, phagocytosis, stimulated shape change, degranulation, and respiratory burst (3–5). For these reasons, neutrophil apoptosis may be considered a mechanism that contributes to the resolution of acute inflammation (1–5).

As a first line of defense against host insult, neutrophils are rapidly recruited to inflammatory sites, where the expression of their apoptotic program can be modified by a number of agents. In vitro studies have identified a variety of agents that modulate neutrophil apoptosis. GM-CSF, IL-2, leukotriene B_4, corticosteroids, and LPS inhibit neutrophil apoptosis (6–11), whereas proteolytic enzymes, immune complexes, bacteria and virus induce neutrophil apoptosis (12–15). Controversial results, on the other hand, have been reported regarding the effects of C5a, fMLP, G-CSF, and IL-6 (3, 5–9).

During the course of inflammatory processes, macrophages, lymphocytes, and/or mast cells produce TNF-α, a powerful priming agonist of neutrophils (16, 17). Previous in vitro studies have shown that TNF-α enhances the expression of CD11b/CD18 on neutrophils, increases neutrophil adhesion to endothelium, triggers adherent neutrophils to release large amounts of reactive oxygen intermediates (ROI), and promotes neutrophil degranulation, phagocytosis, and Ab-dependent cell-mediated cytotoxicity (18–22).

TNF-α has been variably reported either to induce, delay, or have no effect on neutrophil apoptosis (6, 23–25). These contrasting results could be explained, at least in part, by recent findings of Murray et al. (26), who showed that although prolonged incubation ($>$18 h) of human neutrophils with TNF-α indeed causes a decrease in the extent of apoptosis, TNF-α can induce apoptosis in a proportion of cells at earlier times ($<$8 h). In the current work, we re-examine the impact of TNF-α on neutrophil survival. We found that TNF-α plays a critical role in the control of neutrophil survival. This function is related not only to the recognized ability of TNF-α to induce the apoptosis in a proportion of neutrophils but also to its capacity, unique among other inflammatory mediators, to induce an apoptotic death program which could be rapidly triggered by a variety of conventional agonists.

Materials and Methods

Reagents

The following agents were used: human IgG, zymosan (Z), PMA, fMLP, LPS (*Escherichia coli* 055:B5), Con A, acridine orange, ethidium bromide, propidium iodide, catalase (from bovine liver, 50,000 U/mg protein) and superoxide dismutase (from bovine erythrocytes, 5,000 U/mg protein (Sigma, St. Louis, MO). Z-activated serum (ZAS), used as a source of C5a, was prepared by incubating 15 mg Z with 1 ml fresh serum with end-over-end rotation for 1 h at 37°C. Then serum was heat inactivated for 30 min at 56°C. After spinning at 1000 $\times$ *g* for 15 min at 4°C, the supernatant was collected and stored at -70°C. IgG-coated erythrocytes (IgG-E) were prepared using SRBC sensitized with subagglutinating amounts of rabbit IgG anti-SRBC and suspended (1% v/v) in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 1% heat-inactivated FCS

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[3] Abbreviations used in this paper: PS, phosphatidylserine; iIgG, immovilized human IgG; IgG-E, IgG-coated erythrocytes; CE, complement-treated erythrocytes; Z, zymosan; ZAS, Z-activated serum; ROI, reactive oxygen intermediate; MFSC, mean forward scatter; MFI, mean fluorescence intensity; SOD, superoxide dismutase; CGD, chronic granulomatous disease.

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(Life Technologies). Immobilized IgG (iIgG) was prepared by incubating microplates (96-well flat-botton) with IgG (1 mg/ml in saline) for 18 h at 37°C. Before use, plates were washed six times with saline. Complement-treated erythrocytes (CE) were prepared as follows. SRBC were sensitized with specific rabbit IgM Abs. Then they were treated with C5-deficient mouse serum for 30 min at 37°C in a shaking water bath. After washing, cells were suspended (1% v/v) in RPMI 1640 medium supplemented with 1% FCS. Recombinant human TNF-α, IL-2, IL-4, IL-6, IL-12, and IFN-γ were purchased from Sigma, and IL-1α, IL-8, IL-10, IL-18, and GM-CSF were purchased from R&D Systems (Minneapolis, MN). Anti-human TNF-α polyclonal neutralizing Ab was purchased from R&D Systems.

Blood samples

Blood samples were obtained from healthy donors who had taken no medication for at least 10 days before the day of sampling. Blood was obtained by venipuncture of the forearm vein, and it was drawn directly into heparinized plastic tubes.

Neutrophil isolation

Neutrophils were isolated by Ficoll-Hypaque gradient centrifugation (Ficoll; Pharmacia, Uppsala, Sweden; Hypaque, Winthrop Products, Buenos Aires, Argentina) and dextran sedimentation as described elsewhere (27). Contaminating erythrocytes were removed by hypotonic lysis. After washing, the cells (>96% of neutrophils on May-Grunwald-Giemsa-stained cytopreps) were resuspended in RPMI 1640 supplemented with 1% FCS.

Cell cultures

Aliquots of 0.10 ml of neutrophil suspensions (2.5×10^6/ml) were placed in 96-well flat-botton microplates. Unless otherwise stated, neutrophils were treated with TNF-α (1–100 ng/ml) for 1–2 min at 37°C. Then cells were stimulated with different agents. Apoptosis was evaluated after 3 h of culture at 37°C in 5% CO_2, as described below. In a separate set of experiments, the effect of TNF-α on neutrophil apoptosis was evaluated in whole-blood cultures. To this aim, whole blood was diluted (20% v/v) in RPMI 1640 medium and aliquots of 0.10 ml of this suspension were placed in 96-well flat-botton microplates. Diluted blood samples were treated with TNF-α (10 ng/ml) for 1–2 min at 37°C. Then cells were stimulated with different agents and apoptosis was evaluated after 3 h of culture by fluorescence microscopy, as described below.

Quantitation of cellular apoptosis and viability by fluorescence microscopy

Quantitation was performed as previously described (28) using the fluorescent DNA-binding dyes acridine orange (100 μg/ml) to determine the percentage of cells that had undergone apoptosis and ethidium bromide (100 μg/ml) to differentiate between viable and nonviable cells. With this method, nonapoptotic cell nuclei show "structure"; variations in fluorescence intensity that reflect the distribution of euchromatin and heterochromatin. By contrast, apoptotic nuclei exhibit highly condensed chromatin that is uniformly stained by acridine orange. In fact, the entire apoptotic nucleus is present as bright spherical beads. To assess the percentage of cells showing morphologic features of apoptosis, at least 200 cells were scored in each experiment. Previous observations have demonstrated that morphological assessment of neutrophil apoptosis closely correlates with results obtained using other methods to assay apoptosis, such as propidium iodide staining and annexin V binding (29).

Quantitation of neutrophil apoptosis by annexin-V binding and flow cytometry

Annexin-V binding to neutrophils was performed using an apoptosis detection kit (Immunotech, Marseille, France). Briefly, 2.5×10^5 neutrophils were incubated in the presence or absence of TNF-α (10 ng/ml) for 1–2 min, and then they were treated with iIgG, ZAS, or IgG-E for an additional period of 3 h. After this period, cells were labeled with annexin-V FITC and propidium iodide for 20 min at 4°C and analyzed by two-color flow cytometry (FACScan flow cytometer; Becton Dickinson, San Jose, CA) using CellQuest analysis software (Becton Dickinson), as previously described (30). Results are reported as percentage of annexin-V-positive cells.

Quantitation of neutrophil apoptosis by propidium iodide staining and flow cytometry

The proportion of neutrophils that display a hypodiploid DNA peak, i.e., apoptotic cells, was determined using a modification of the protocol of Nicoletti et al. (31). Briefly, cell pellets containing 2.5×10^6 neutrophils were suspended in 400 μl of hypotonic fluorochrome solution (propidium iodide, 50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100) and incubated for 2 h at 4°C. The red fluorescence of propidium iodide in individual nuclei was measured using a FACScan flow cytometer (Becton Dickinson). The forward scatter and side scatter of particles were simultaneously measured. Cell debris was excluded from analysis by appropriately raising the forward scatter threshold. The red fluorescence peak of neutrophils with normal (diploid) DNA content was set at channel 250 in the logarithmic mode. Apoptotic cell nuclei emitted fluorescence in channels 4–200.

Neutrophil shape change

Neutrophils (2.5×10^6/ml) suspended in RPMI 1640 medium with 1% FCS were incubated in a shaking water bath for 5 min at 37°C in the absence or presence of LPS (1 μg/ml) or Con A (10 μg/ml). Cells were then centrifuged, suspended in PBS, and fixed by the addition of an equal volume of 0.5% glutaraldehyde in PBS. Neutrophil shape change was measured by flow cytometry and results were expressed as mean forward scatter values.

CD18 expression

Neutrophils (2.5×10^6/ml) suspended in RPMI 1640 medium with 1% FCS were incubated in the absence or presence of LPS (1 μg/ml) or Con A (10 μg/ml) for 15 min at 37°C. Then cells were washed, fixed with 0.5% paraformaldehyde in PBS for 30 min at 4°C, and washed with PBS. Finally, cells were stained with FITC-conjugated anti-CD18 mAb. Fluorescence was recorded by flow cytometry. Results are expressed as the mean fluorescence intensity (MFI) in arbitrary fluorescence units.

Statistical analysis

Student's paired *t* test was used to determine the significance of differences between means, and $p < 0.05$ was taken as indicating statistical significance.

Results

Promotion of neutrophil apoptosis by TNF-α

In a first set of experiments, resting neutrophils were preincubated with TNF-α (10 ng/ml) for 1–2 min at 37°C and then were cultured in the presence or absence of different stimuli. After 3 h of incubation at 37°C, apoptosis was revealed by fluorescence microscopy using the fluorescent DNA-binding dye acridine orange. Fig. 1 shows that when neutrophils were cultured with TNF-α alone, a low but significant increase in the number of apoptotic cells was observed. By contrast, when neutrophils were pretreated with TNF-α for 1–2 min at 37°C and then were cultured in the presence of iIgG, IgG-E, CE, PMA, ZAS, Z, fMLP, *E. coli*, and GM-CSF, there was a marked increase in the apoptotic rate. In contrast, no promotion of apoptosis was observed using LPS, Con A, IL-1α, IL-2, IL-8, IL-10, and IFN-γ as stimuli. We then analyzed the specificity of action of TNF-α. Using iIgG, Z, IgG-E, and ZAS as apoptotic triggering agents, we observed that preincubation of TNF-α with a specific IgG polyclonal neutralizing Ab abolished the proapoptotic effect of TNF-α (data not shown).

During the course of apoptosis, PS, a negatively charged phospholipid, becomes exposed at the cell surface (30). The ability of TNF-α to induce apoptosis was then analyzed by flow cytometry using FITC-labeled annexin-V, which specifically binds to PS, in combination with propidium iodide staining. In agreement with the results described above, we found that when neutrophils were pretreated with TNF-α (10 ng/ml) for 1–2 min at 37°C and then were cultured in the presence of iIgG, a marked increase in the number of apoptotic cells was observed (Fig. 2). Comparable results were observed using ZAS and IgG-E as triggering stimuli, being the percentage of apoptotic cells of 5 ± 3, 4 ± 2, 3 ± 2, 9 ± 3, 37 ± 6, and 68 ± 5 for untreated cells and cells treated with ZAS (1/10), iIgG, TNF-α (10 ng/ml), TNF-α plus ZAS, and TNF-α plus iIgG, respectively (mean $\pm$ SEM, $n = 5$, $p < 0.01$, TNF-α treated cells vs TNF-α + ZAS or TNF-α + iIgG-treated cells). Similar results

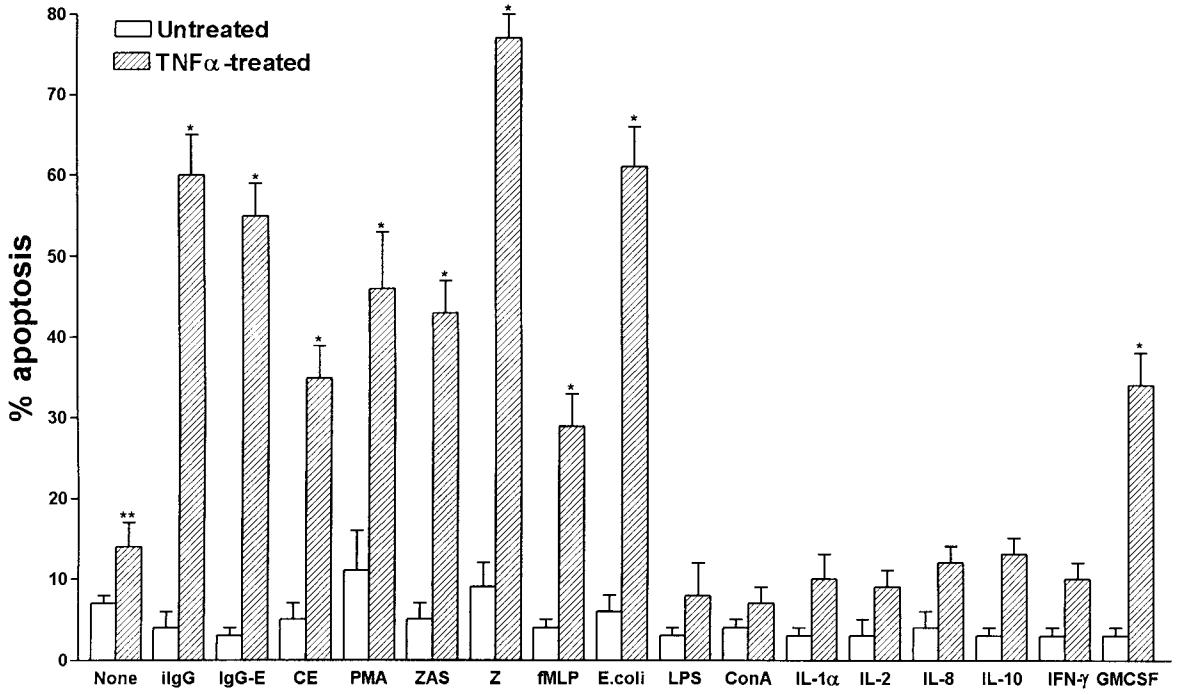

FIGURE 1. Promotion of neutrophil apoptosis by TNF-α. Neutrophils (2.5×10^6/ml) were cultured in the presence or absence of TNF-α (10 ng/ml) for 1–2 min at 37°C. Then cells were treated with iIgG, IgG-E (0.25% v/v), CE (0.25% v/v), PMA (0.5–2.5 ng/ml), ZAS (1/10), Z (50 μg/ml), fMLP (10 nM), *E. coli* (neutrophil:*E. coli* ratio, 1:25), LPS (1 μg/ml), Con A (10 μg/ml), or different cytokines: IL-1α, IL-2, IL-8, IL-10, IFN-γ, and GM-CSF (100 ng/ml). After 3 h, the percentage of apoptotic cells was determined by fluorescence microscopy. Results are expressed as the mean ± SEM of 5–10 experiments. *, Statistical significance ($p < 0.01$) compared with neutrophils cultured with TNF-α alone. **, Statistical significance ($p < 0.05$) compared with control neutrophils.

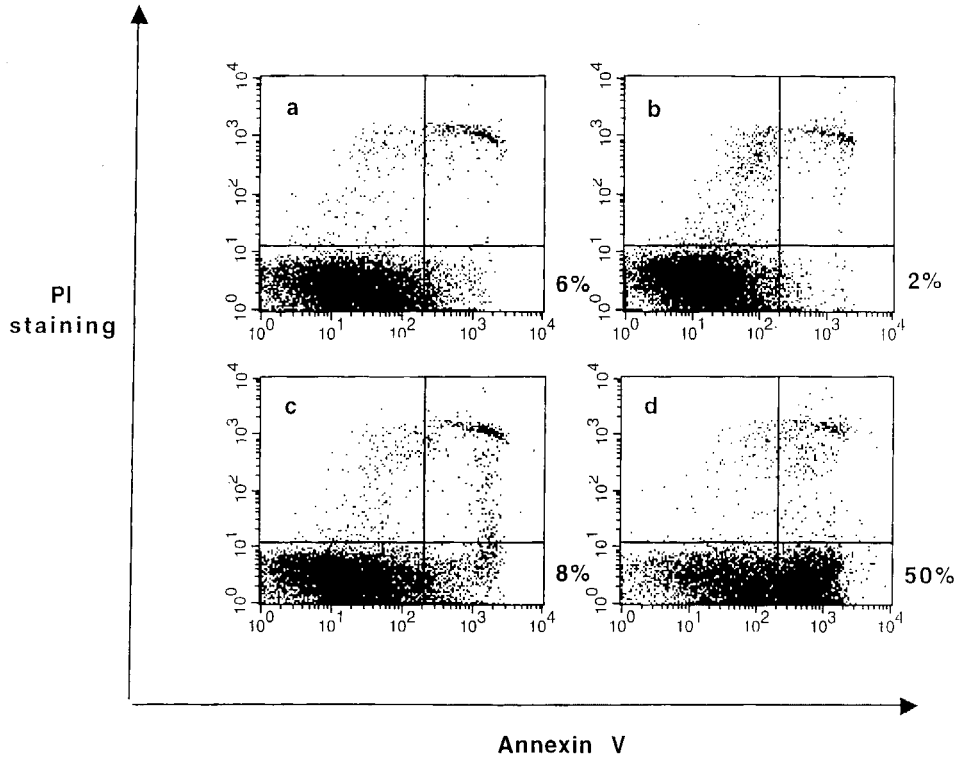

FIGURE 2. Promotion of neutrophil apoptosis by TNF-α analyzed by annexin-V-binding assay. Neutrophils (2.5×10^6/ml) were cultured in the presence (*c* and *d*) or absence (*a* and *b*) of TNF-α (10 ng/ml) for 1–2 min at 37°C. Then cells were cultured with (*b* and *d*) or without iIgG (*a* and *c*) for an additional period of 3 h, and apoptosis was analyzed by using FITC-labeled annexin-V in combination with propidium iodide staining. A representative experiment is shown ($n = 5$). Results are expressed as percentage of apoptotic cells.

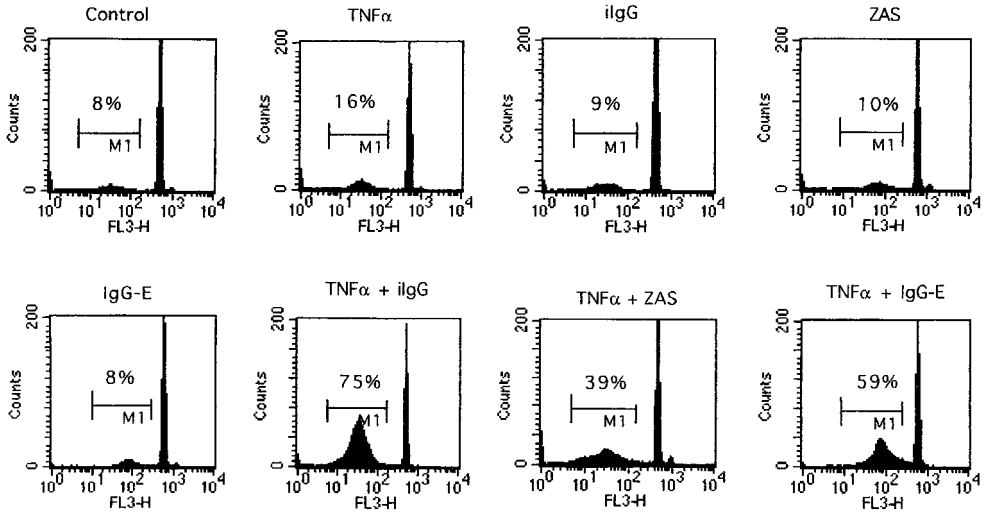

FIGURE 3. Promotion of neutrophil apoptosis by TNF-α analyzed by propidium iodide staining and flow cytometry. Neutrophils (2.5×10^6/ml) were cultured in the presence or absence of TNF-α (10 ng/ml) for 1–2 min at 37°C. Then cells were cultured with iIgG, ZAS (1/10), or IgG-E (0.25% v/v). After 3 h, the percentage of apoptotic cells was determined by flow cytometry as described in *Materials and Methods*. Histograms of a representative experiment ($n = 6$) showing the percentage (M1) of nuclei with hypodiploid DNA content.

were also observed when apoptosis was analyzed by propidium iodide staining and flow cytometry. Treatment with TNF-α enabled iIgG, ZAS, and IgG-E to trigger a strong apoptotic response, as measured by the increased proportion of neutrophils that display a hypodiploid DNA peak (Fig. 3).

We next examined the range of concentrations at which TNF-α enhanced neutrophil apoptosis. To this aim, cells were cultured for 1–2 min at 37°C with different concentrations of TNF-α. Then cells were exposed to ZAS or iIgG, and the percentage of apoptotic neutrophils was assessed after 3 h of incubation either by fluorescence microscopy or propidium iodide staining and flow cytometry. Fig. 4 shows that pretreatment with TNF-α caused a dose-dependent stimulation of apoptosis at concentrations ranging from 1 to 100 ng/ml.

Results depicted in Fig. 1 also showed that pretreatment with TNF-α was completely unable to increase apoptosis in neutrophils stimulated with either LPS or Con A. To confirm that under our experimental conditions LPS and Con A effectively triggered neutrophil activation, their ability to increase the expression of CD18 as well as to stimulate neutrophil shape change was evaluated by flow cytometry as described in *Materials and Methods*. As expected, treatment of neutrophils with either LPS (1 μg/ml) or Con A (10 μg/ml) increased the expression of CD18 (MFI = 175 ± 23, 312 ± 27, and 299 ± 16) and also induced neutrophil shape change (MFSC = 312 ± 31, 487 ± 29, and 415 ± 21, untreated, LPS, and Con A-treated neutrophils, respectively; mean ± SEM, $p < 0.01$, untreated vs LPS or Con A-treated cells).

Previous observations suggest that standard preparative techniques using Ficoll-Hypaque gradient and lysis of contaminating erythrocytes may result in neutrophil activation, loss of membrane integrity, and/or loss of cells during aging by clumping (32, 33). To rule out the possibility that our results could be related to the induction of cell injury or "priming" during purification, we performed experiments in whole-blood cultures as described in *Materials and Methods*. Diluted whole-blood samples (20% v/v in

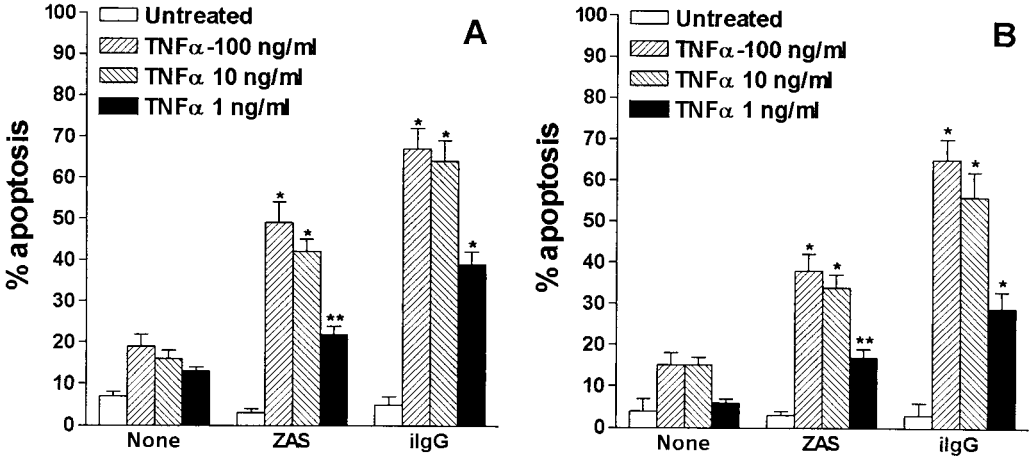

FIGURE 4. Dose-dependent stimulation of neutrophil apoptosis by TNF-α. Neutrophils (2.5×10^6/ml) were cultured with different concentrations of TNF-α for 1–2 min at 37°C. Then cells were stimulated by ZAS (1/10) or iIgG, and apoptosis was revealed after 3 h of culture by either fluorescence microscopy (*A*) or propidium iodide staining and flow cytometry (*B*). Results are expressed as the mean ± SEM of four to six experiments. * and **, Statistical significance ($p < 0.01$ and $p < 0.05$, respectively) compared with neutrophils cultured with TNF-α alone.

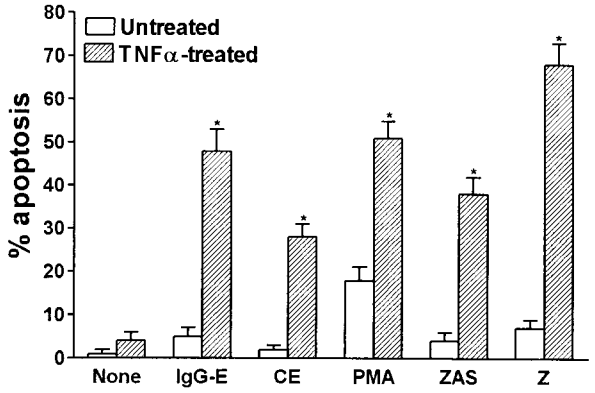

FIGURE 5. Promotion of neutrophils apoptosis by TNF-α in whole-blood cultures. Diluted blood samples (20% v/v) were treated with TNF-α (10 ng/ml) for 1–2 min at 37°C. Then samples were treated with IgG-E (1% v/v), CE (1% v/v), PMA (0.5–2.5 ng/ml), ZAS (1/10), or Z (50 μg/ml). After 3 h, the percentage of apoptotic cells was determined by fluorescence microscopy. Results are expressed as the mean ± SEM of five experiments. *, Statistical significance ($p < 0.01$) compared with neutrophils cultured with TNF-α alone.

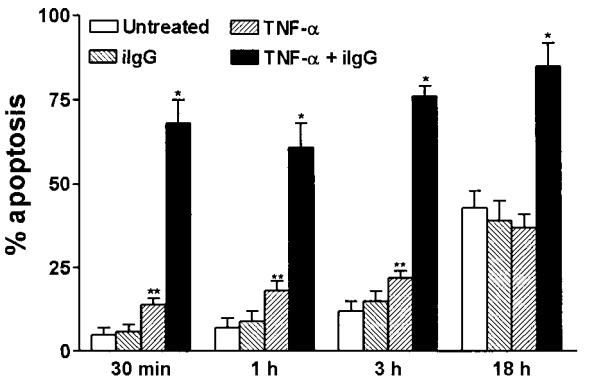

FIGURE 6. Analysis of apoptosis after incubation of neutrophils with TNF-α for different times. Neutrophils (2.5×10^6/ml) were cultured with or without TNF-α for different times (30 min, 1 h, 3 h, and 18 h). Then cells were cultured for an additional period of 3 h in the absence or presence of iIgG and apoptosis was revealed by fluorescence microscopy. Results are expressed as the mean ± SEM of four to five experiments. *, Statistical significance ($p < 0.01$) compared with neutrophils cultured with TNF-α alone at each time point. **, Statistical significance ($p < 0.05$) compared with untreated cells.

RPMI 1640 medium) were incubated with TNF-α (10 ng/ml) for 1–2 min at 37°C and then they were exposed to different agents. After 3 h of incubation at 37°C, apoptosis was revealed by fluorescence microscopy. Fig. 5 shows that, in agreement with the observations made in purified neutrophils (see Fig. 1), pretreatment of whole-blood samples with TNF-α induced a marked stimulation of apoptosis in neutrophils stimulated by either IgG-E, CE, PMA, ZAS, and Z.

Kinetic of promotion of apoptosis by TNF-α

The above-described experiments were all performed with neutrophils pretreated with TNF-α for 1–2 min at 37°C. To analyze whether the proapoptotic effect of TNF-α was also observed with longer pretreatments, neutrophils were cultured for different times with TNF-α and then were stimulated by iIgG. Fig. 6 shows that TNF-α markedly increased the proportion of apoptotic cells at all of the times assessed.

We next analyzed whether the action of TNF-α was reversible. To this end, we performed another set of experiments in which TNF-α was removed from the culture medium before the addition of the triggering stimulus. Neutrophils were cultured with TNF-α for 2 min at 37°C, washed three times, and resuspended in culture medium. Cells were exposed to Z (50 μg/ml) for an additional period of 3 h at 37°C, and apoptosis was then revealed by fluorescence microscopy. We found that removal of TNF-α from the culture medium did not impair its ability to promote apoptosis: percent apoptosis = 3 ± 2, 4 ± 2, 11 ± 2, and 61 ± 7, for untreated cells and cells treated with Z (50 μg/ml), TNF-α (10 ng/ml), and Z + TNF-α (mean ± SEM, $n = 6$, $p < 0.05$, untreated vs TNF-α-treated cells, and $p < 0.01$, TNF-α vs Z + TNF-α-treated cells).

The observations described above were made in experiments in which resting neutrophils were pretreated with TNF-α for different times before the addition of triggering stimuli. We then analyzed whether neutrophils first stimulated with iIgG, IgG-E, ZAS, or GM-CSF undergo an increase in their apoptotic rate as a consequence of the addition of TNF-α to the culture medium. Fig. 7 shows that, in contrast to the observations made in TNF-α-pretreated neutrophils, we observed no stimulation of apoptosis when TNF-α was added to activated neutrophils. These results could be explained, at least in part, considering previous findings which indicated that neutrophil activation by different stimuli induces the shedding of TNF-α receptors (34, 35). In agreement with these reports, we observed that neutrophil activation by iIgG, IgG-E, ZAS, or GM-CSF markedly decreased the expression of both TNF-α receptors (TNF-R55 and TNF-R75) from the cell surface (data not shown).

Promotion of apoptosis by TNF-α does not appear to involve the Fas/Fas ligand (FasL) system nor the production of ROI

Neutrophils express not only Fas but also FasL. The Fas/FasL system appears to be responsible, at least in part, for the rapid rate of spontaneous apoptosis observed for unstimulated neutrophils (36). We next examined whether promotion of apoptosis by TNF-α was associated with an increase in the expression of Fas and/or FasL. Neutrophils were pretreated for 1–2 min at 37°C with or without TNF-α and then they were cultured for 2 h with iIgG.

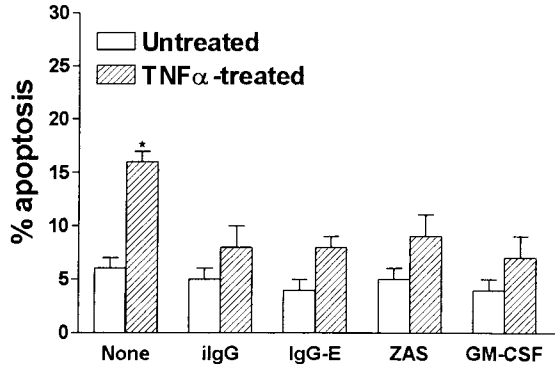

FIGURE 7. TNF-α does not increase apoptosis of neutrophils previously activated by conventional agonists. Neutrophils (2.5×10^6/ml) were cultured with or without iIgG, IgG-E (0.25% v/v), ZAS (1/10), or GM-CSF (100 ng/ml) for 15 min at 37°C. Then cells were cultured in the absence or presence of TNF-α (10 ng/ml) for 3 h and apoptosis was revealed by fluorescence microscopy. Results are expressed as the mean ± SEM of five experiments. *, Statistical significance ($p < 0.05$) compared with neutrophils cultured without TNF-α.

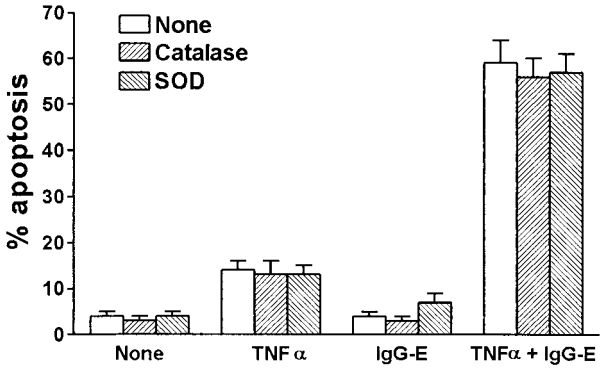

FIGURE 8. Neither catalase nor SOD prevent the acceleration of apoptosis induced by TNF-α. Neutrophils (2.5×10^6/ml) were cultured with or without TNF-α for 1–2 min at 37°C in the presence or absence of catalase or SOD (200 U/ml). Then IgG-E (0.25%) were added and apoptosis was revealed after 3 h of culture by fluorescence microscopy. Results are expressed as the mean ± SEM of five experiments.

Using the mAb CH-11 directed to Fas and the mAb G247–4 directed to FasL, we found no differences in the expression of Fas or FasL between untreated and TNF-α-treated cells. The MFI for Fas expression in control cells and cells treated with iIgG, TNF-α (10 ng/ml), and TNF-α plus iIgG was 28 ± 5, 29 ± 4, 24 ± 5, and 23 ± 5, respectively (mean ± SEM, $n = 5$). FasL expressión in cells treated as described above was 7 ± 3, 8 ± 2, 6 ± 3, and 8 ± 3, respectively (mean ± SEM, $n = 5$). These results suggest that the Fas/FasL system is not involved in the acceleration of apoptosis induced by TNF-α. This conclusion was further supported by the fact that pretreatment of neutrophils with a blocking mAb directed to FasL (NOK-1), used at concentrations 5-fold higher than those needed to saturate all binding sites as determined by FACS analysis, did not impair the acceleration of apoptosis induced by TNF-α: percent apoptosis after 3 h of culture, 74 ± 11 vs 68 ± 7 for TNF-α-treated neutrophils incubated with iIgG in the absence or presence of the mAb NOK-1, respectively ($n = 3$).

Previous work has shown that after activation by different stimuli, neutrophils undergo apoptosis through an oxygen-dependent pathway (13, 14, 37, 38). To analyze whether ROI were involved in the acceleration of apoptosis induced by TNF-α, we determined the effect of catalase and superoxide dismutase (SOD). Fig. 8 shows that neither catalase nor SOD impaired the acceleration of apoptosis induced by TNF-α in cells incubated for 3 h with either IgG-E or Z. These data contrast with the results obtained in neutrophils cultured for 18 h only with IgG-E in which, as we previously described (14), the promotion of apoptosis induced by IgG-E was not modified by SOD (data not shown) but was almost completely abrogated by catalase: percent apoptosis = 33 ± 6, 28 ± 5, 69 ± 11, and 34 ± 4, for untreated cells and cells treated with catalase (200 U/ml), IgG-E (0.25%), and IgG-E plus catalase, respectively (mean ± SEM, $n = 5$).

To further analyze whether the production of ROI was involved in the acceleration of neutrophil apoptosis by TNF-α, assays were performed using neutrophils isolated from three patients with chronic granulomatous disease (CGD), a rare hereditary disorder characterized by a diminished or absent production of ROI due to a defect in any one of the components of NADPH oxidase (39). Neutrophils from these patients produced, in response to fMLP or Z, O_2^- levels <1% those produced by normal neutrophils (data not shown). Our results showed that, as observed with normal neutrophils, there was a strong stimulation of apoptosis in CGD neutrophils cultured with TNF-α (2 min at 37°C) and then exposed to Z: percent apoptosis revealed at 3 h of culture by fluorescence microscopy = 4 ± 2, 11 ± 6, 2 ± 1, and 53 ± 8 for untreated cells and cells treated with TNF-α (10 ng/ml), Z (50 μg/ml), and TNF-α + Z, respectively ($n = 3$). These results suggest that promotion of neutrophil apoptosis by TNF-α does not involve an oxygen-dependent pathway.

Promotion of neutrophil apoptosis appears to be a selective action of TNF-α

We next analyzed whether other cytokines could reproduce the effects of TNF-α. The following cytokines were assessed: IL-1α, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, IFN-γ, and GM-CSF. Neutrophils were incubated with each of these cytokines for 1–2 min at 37°C and then were treated with Z or IgG-E. Apoptosis was revealed by fluorescence microscopy after 3 h of culture. Fig. 9 shows that in contrast to the observations made in TNF-α-treated neutrophils, none of these cytokines was able to increase apoptosis.

Discussion

The results presented here suggest that TNF-α plays a critical role in the control of neutrophil survival by inducing an apoptotic death program which can be rapidly triggered by a variety of stimuli. Thus, our data reveal that when neutrophils were pretreated with TNF-α and then were exposed to different inflammatory agents, there was a marked stimulation of apoptosis (range, 30–75%). By contrast, in agreement with previous reports (23–26), we found a

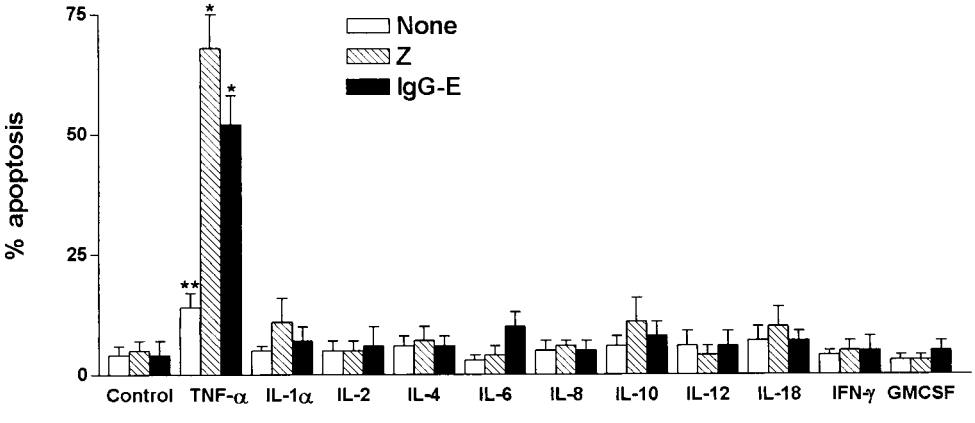

FIGURE 9. Analysis of the ability of different cytokines to increase neutrophil apoptosis. Neutrophils (2.5×10^6/ml) were cultured for 1–2 min at 37°C in the presence or absence of TNF-α, IL-1α, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, IFN-γ, and GM-CSF (100 ng/ml). Then cells were cultured with or without Z (50 μg/ml) or IgG-E (0.25% v/v) for 3 h and apoptosis was revealed by fluorescence microscopy. Results are expressed as the mean ± SEM of four to seven experiments. *, Statistical significance ($p < 0.01$) compared with neutrophils cultured with TNF-α alone. **, Statistical significance ($p < 0.05$) compared with control neutrophils.

slight increase in the number of apoptotic cells (range, 9–18%) when neutrophils were cultured only with TNF-α.

Using a broad panel of stimuli which includes immune complexes (IgG-E, iIgG), physiologic peptides (fMLP), bacteria (*E. coli*), yeast (Z from *Saccharomyces cerevisiae*), pharmacologic agents that activate protein kinase C (PMA), cytokines (IL-1α, IL-2, IL-8, IL-10, IFN-γ, and GM-CSF), and ZAS (used as a source of C5a), we found marked differences in their ability to trigger apoptosis of neutrophils treated with TNF-α. On the basis of these differences, these stimuli should be classified in three different groups: 1) potent inducers of apoptosis (range, 55–75%) such as IgG-E, iIgG, *E. coli*, and Z; 2) moderate and low inducers of apoptosis (range, 30–45%), which include fMLP, ZAS, CE, PMA, and GM-CSF; and 3) agents unable to enhance apoptosis, such as LPS, Con A, IL-1α, IL-2, IL-8, IL-10, and IFN-γ. Not only conventional agonists appear to be able to accelerate apoptosis of TNF-α primed neutrophils. Recent results published by Kettritz et al. (40) showed that extracellular matrix proteins, such as fibronectin, collagen I, collagen IV, and laminin, may also be able to induce a low but significant increase in the apoptotic rate of TNF-α-treated neutrophils.

The mechanisms through which TNF-α increases neutrophil apoptosis have not been defined yet. The observation that a blocking Ab directed to FasL was unable to prevent apoptosis supports the notion that the Fas/FasL system is not involved. Moreover, the inability of catalase and SOD to decrease apoptosis and, more importantly, the fact that TNF-α was able to promote apoptosis of CGD neutrophils argues against the participation of an oxidative-dependent pathway. In this regard, it is important to consider our previous results indicating that 1) IgG-E stimulates apoptosis of resting neutrophils, revealed at 12–18 h of culture (this effect was not evident in the current study since apoptosis was always revealed at 3 h of culture); and 2) the enhancement in the apoptotic rate induced by IgG-E is almost completely abrogated by catalase, and it was not observed in neutrophils from CGD patients (14). These data suggest that, in contrast to what was observed in TNF-α-primed neutrophils, stimulation of apoptosis of resting neutrophils by IgG-E involves an oxygen-dependent pathway. Similar results were obtained by using iIgG and Z as stimulus. Catalase markedly inhibited apoptosis of resting neutrophils induced by iIgG or Z (revealed at 18 h of incubation), whereas it did not exert any effect on apoptosis of TNF-α-primed neutrophils triggered by these stimuli (G. Salamone, unpublished results). Together, these data suggest that TNF-α not only enables different stimuli to trigger a strong apoptotic response, but also modifies the mechanisms through which these stimuli induce neutrophil apoptosis.

We have also observed that TNF-α does not increase apoptosis of neutrophils first activated by conventional agonists. This result could be explained, at least in part, considering previous findings from Porteu and Nathan (34) which indicated that neutrophil activation by a variety of agents results in the shedding of TNF-R from the cell surface. In agreement with these results, we observed that activation of neutrophils by iIgG, IgG-E, ZAS, or GM-CSF results in a rapid loss of both TNF-α receptors (TNF-R55 and TNF-R75; our unpublished results).

Having demonstrated that TNF-α promotes neutrophil apoptosis, we also sought to determine whether other cytokines such as IL-1α, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, GM-CSF, and IFN-γ could also exert a similar function. Using Z and IgG-E as triggering stimuli, we found no enhancing effects, suggesting that TNF-α has the unique ability, not shared by other cytokines, to induce an apoptotic death program which could be rapidly triggered by a variety of conventional agonists.

Although our results support a critical role for TNF-α in promoting neutrophil apoptosis, additional experiments are required to establish the relevance of this regulatory mechanism. In this regard, however, it is important to consider recent findings published by Skerrett et al. (41), who examined the role of TNF-R in lung acute inflammation. They found that neutrophil infiltration in the lungs after exposure to aerosolized *Pseudomonas aeruginosa*, but not after exposure to aerosolized LPS, was augmented in mice lacking TNF-R, in comparison with wild-type animals, despite diminished bronchoalveolar concentrations of chemokines. Since exposure to TNF-α enables Gram-negative bacteria, but not LPS, to trigger a strong apoptotic response, these observations could reflect the ability of *P. aeruginosa*-elicited, but not LPS-elicited neutrophils, to survive longer in lung airspaces from TNF-R-deficient animals.

In summary, we found that TNF-α plays an important role in the control of neutrophil survival by virtue of its ability to induce an apoptotic death program that can be triggered by a variety of conventional agonists. Additional experiments are required to elucidate the signaling pathways involved in TNF-α priming as well as to define the impact of this mechanism in the resolution of acute inflammatory processes.

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