

# Cytokine-activated Endothelial Cells Delay Neutrophil Apoptosis In Vitro and In Vivo: A Role for Granulocyte/Macrophage Colony-stimulating Factor

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## Summary

The activation of endothelium is important in recruiting neutrophils to sites of inflammation and in modulating their function. We demonstrate that conditioned medium from cultured, activated endothelial cells acts to significantly delay the constitutive apoptosis of neutrophils, resulting in their enhanced survival and increased phagocytic function. The antiapoptotic activity is, in part, attributable to granulocyte/macrophage colony-stimulating factor (GM-CSF) secreted by activated endothelial cells. The *in vivo* relevance of these findings was investigated in a cytokine-induced model of acute meningitis in mice. Peripheral blood neutrophils (PBNs) from mice with meningitis exhibited a delay in apoptosis compared with untreated mice. Furthermore, neutrophils recovered from the inflamed cerebrospinal fluid (CSF) exhibited enhanced survival compared with neutrophils isolated from the peripheral blood of the same animals. In unchallenged GM-CSF-deficient mice, the apoptosis of circulating PBNs was similar to wild-type animals; however, after cytokine-induced meningitis, the delay in neutrophil apoptosis typically observed in wild-type mice was attenuated. In contrast, the apoptosis of neutrophils recovered from the CSF of mice of both genotypes was comparable. Taken together, these studies suggest that neutrophil apoptosis is regulated during an inflammatory response, in both intravascular and extravascular compartments. GM-CSF released by activated endothelium can act to increase neutrophil survival and function in the peripheral blood, whereas other factor(s) appear to perform this function in the extravascular space.

**Key words:** neutrophil • apoptosis • endothelium • granulocyte/macrophage colony-stimulating factor • inflammation

Circulating neutrophils have a half-life of only 6–10 h and execute a constitutive program of cell death, after which they are cleared in the spleen (1, 2). The onset of apoptosis is associated with the loss of several important neutrophil functions, including adhesion and phagocytosis (3, 4). The programmed cell death of neutrophils can be delayed *in vitro* by inflammatory mediators such as GM-CSF, IL-6, and bacterial endotoxin (5–7). Indeed, experimental studies using labeled neutrophils suggest that the life span of extravasated neutrophils is considerably longer than blood neutrophils: 24 h after the onset of an inflammatory reaction, large numbers of neutrophils are still present at the inflamed site long after the cessation of neutrophil influx, typically noted at 1–4 h (1, 2). In humans, a delay in apoptosis of circulating neutrophils has been noted in pa-

tients with burns (8), sepsis (9), or the systemic inflammatory response syndrome (10) and may contribute to the enhanced neutrophil accumulation and inflammatory injury observed in these clinical settings. This enhancement in neutrophil survival presumably allows them to function more effectively at sites of inflammation. However, once they have completed their functions, an orderly elimination of neutrophils is essential for the resolution of inflammation. The latter process occurs by the apoptosis of neutrophils *in situ* followed by their rapid removal by macrophages and other phagocytes (11). We and others have shown that neutrophil phagocytosis of opsonized particles significantly accelerates apoptosis (12, 13). In particular, phagocytosis via the leukocyte adhesion receptor CD11b/CD18, a  $\beta$ 2-integrin, causes an acceleration of apoptosis which is dependent on oxygen radicals generated via NADPH oxidase (12). This represents an effective mechanism of removing extravasated neutrophils that have reached the end of their useful life span, thereby preventing the release of their potentially histotoxic contents.

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Part of this work was presented previously in abstract form (Coxon, A., T. Tang, and T.N. Mayadas. 1998. *FASEB J.* 12[No. 5, Pt. II]:4499).

Endothelial cells are strategically positioned to regulate neutrophil accumulation in inflamed tissues. This accumulation is a balance of neutrophil recruitment and clearance. Activated endothelial cells recruit neutrophils through the release of chemoattractants and by expressing leukocyte adhesion receptors, such as the selectins and IgG superfamily members on their surface (for a review, see reference 14). Endothelial cells have also been implicated in the delay of apoptosis of both neutrophils and lymphocytes, and thus potentially function to increase the number of these leukocytes at sites of inflammation as well as prolong their life span in the peripheral blood. *In vitro*, LPS-activated neutrophils that had transmigrated through an unactivated endothelial cell monolayer had a delay in apoptosis that was ascribed to a CD11b/CD18-mediated adhesion event (15). In another report, an endothelial-mediated delay in lymphocyte apoptosis was attributed to IL-6 secreted by LPS-activated endothelial cells (16).

In this study, we show that IL-1 $\beta$  and TNF- $\alpha$ -activated, but not unactivated, endothelial cells play a significant role in delaying neutrophil apoptosis *in vitro*, and that this delay results in the retention of an important neutrophil function, phagocytosis of opsonized particles. Furthermore, we demonstrate that GM-CSF released by the activated endothelium is responsible for the delay. We examined neutrophil apoptosis and the role of GM-CSF, *in vivo*, in a model of murine meningitis. This model is induced by the lumbar injection of the human cytokines IL-1 $\beta$  and TNF- $\alpha$  and leads to rapid neutrophil accumulation in the cerebrospinal fluid (CSF)<sup>1</sup> (17). The peripheral blood neutrophils (PBNs) of mice subjected to cytokine-induced meningitis exhibited a delay in apoptosis compared with uninjected mice. Furthermore, neutrophils that had accumulated in the CSF had a further delay in apoptosis compared with PBNs from the same animals. An antiapoptotic activity was detected in the CSF of mice subjected to cytokine-induced meningitis but not in the CSF of untreated mice, suggesting that it was a soluble, cytokine-inducible substance. Studies in GM-CSF-deficient mice demonstrated that GM-CSF contributed to the delay in apoptosis of PBNs but was not essential for the further delay in apoptosis of extravasated neutrophils, despite the presence of GM-CSF in the CSF compartment. Our *in vitro* studies, together with our *in vivo* observations, suggest that the activated endothelium which is at the blood-tissue interface plays a crucial role in delaying neutrophil apoptosis. Furthermore, we show that GM-CSF plays a role in delaying the apoptosis of circulating neutrophils, whereas other unidentified soluble factors are essential for the further delay of apoptosis observed in extravasated neutrophils.

<sup>1</sup>Abbreviations used in this paper: CSF, cerebrospinal fluid; HUVEC, human umbilical vein endothelial cell; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PBN, peripheral blood neutrophil; WCM, white cell medium.

## Materials and Methods

**Mice.** Age-matched wild-type and CD11b/CD18-deficient mice (12) of the pure 129Sv strain were bred and maintained in a virus antibody-free facility at the Longwood Medical Research Center, Harvard Medical School. GM-CSF-deficient mice of the 129Sv/C57Bl6 mixed background and their age-matched wild-type counterparts (18) were provided by Dr. Lloyd Old (Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, New York, NY). The GM-CSF-deficient mice used in our study were 6–8-wk-old males and showed no pulmonary pathology (18) or elevated PBN counts. These mice were housed in the specific pathogen-free facility at the Memorial Sloan-Kettering Cancer Center until they were shipped to the quarantine facility of Harvard Medical School, where they were housed briefly.

**Cytokine-induced Meningitis and Isolation of Murine Neutrophils.** Cytokine-induced meningitis was performed essentially as described by Tang et al. (17). Mice were injected with IL-1 $\beta$ /TNF- $\alpha$  by lumbar puncture at vertebrate level L-1 or L-2 and, after 4 h, exsanguinated, and CSF was retrieved from the posterior fossa by aspiration into glass capillaries, as described by Griffin (19). CSF from each mouse was placed directly into Poly-HEME (Sigma Chemical Co.)-coated wells containing IMDM and 5% FCS, since in our experience these cells rapidly adhere to plastic or glass.  $2 \times 10^5$  cells/well from each mouse were plated in duplicate and incubated at 37°C, 5% CO<sub>2</sub>. Peripheral blood samples were collected by retroorbital puncture in tubes containing a final concentration of 5 mM EDTA. PBNs were isolated from the anticoagulated blood pooled from two to three animals by density centrifugation using NIM2 gradients (Cardinal Associates), as described previously (12), and were >95% pure. Alternatively, 300  $\mu$ l of whole blood from individual mice was subjected to two rounds of RBC lysis with 0.2% NaCl and resuspended in IMDM, 5% FCS.  $2 \times 10^5$  cells/well were incubated at 37°C. Neutrophil apoptosis was assessed by morphological criteria, and viability was assessed by trypan blue exclusion. To determine if GM-CSF was present in the CSF of mice, CSF was isolated from untreated and cytokine-injected mice. The CSF was harvested 2 h after injection of IL-1 $\beta$ /TNF- $\alpha$  because very few leukocytes have transmigrated into the CSF at this time point (17). The retrieved CSF was centrifuged to remove any cells present, and the concentration of GM-CSF was determined by ELISA (Endogen).

**PMN Transendothelial Cell Migration Assay.** Human umbilical vein endothelial cells (HUVECs) were used at confluence on transwell inserts coated with human fibronectin (Becton Dickinson Labware). The bottom chamber was coated with polyheme to prevent adherence of PMNs to the plastic (12). Endothelial cells were treated with human recombinant IL-1 $\beta$  (10 U/ml; Genzyme) and TNF- $\alpha$  (50 ng/ml; Genzyme). The monolayer was washed four times with white cell medium (WCM: HBSS without Ca<sup>2+</sup> or Mg<sup>2+</sup>, 0.5% BSA, 25 mM Hepes, pH 7.35). Human PBNs, isolated from normal donors, were resuspended in WCM at  $5 \times 10^6$  PBNs/ml.  $2.5 \times 10^6$  PBNs were added to the top chamber of the transmigration chamber, and the chemoattractants FMLP (Sigma Chemical Co.) or leukotriene B<sub>4</sub> (LTB<sub>4</sub>; Biomol Research Labs, Inc.) or buffer alone was added to the bottom chamber. Transmigration of neutrophils was allowed to proceed for 1 h at 37°C. Control samples contained neutrophils in WCM or WCM plus the chemoattractant used in the transmigration assay, and were placed in polyheme-coated wells for 1 h at 37°C. Neutrophils that had transmigrated into the lower chamber and control samples were collected, spun down, resuspended in IMDM plus 5% FCS, plated in polyheme-coated wells

at  $2 \times 10^5$  PBNs/well, and placed at  $37^\circ\text{C}$ , 4%  $\text{CO}_2$  (time 0 h). All samples were plated in duplicate, and apoptosis was assessed (see below) after 6 and 8 h in culture.

**Neutrophil Coincubations with Endothelial Cells or Endothelial-derived Conditioned Medium.** HUVECs were plated in 24-well plates coated with gelatin and were used at confluence 48 h after plating. Actinomycin D (Calbiochem) and cycloheximide (Sigma Chemical Co.) were used at  $12 \mu\text{M}$  and  $50 \mu\text{g/ml}$ , respectively. The proteasome inhibitors MG132 and lactacystin (Calbiochem) were used at 20 and  $40 \mu\text{M}$ , respectively. Calpain Inhibitor II (Calbiochem) was used as a negative control peptide in these experiments. The HUVEC monolayer was pretreated with these reagents for 1 h before the addition of cytokines, and then washed four times with WCM. The endothelial cells were then incubated with  $5 \times 10^6$  isolated neutrophils in WCM or with WCM alone to collect factors released into the medium. In the latter case, the conditioned medium was removed and incubated with fresh  $2.5 \times 10^6$  neutrophils for an additional 1 h. Neutrophils were collected, spun down, and resuspended in IMDM, 5% FCS. In experiments where neutrophils were cultured in the continuous presence of conditioned medium, the medium was harvested in IMDM. The conditioned medium was then supplemented with 5% FCS and incubated with neutrophils at  $37^\circ\text{C}$  from 6 to 24 h. In all cases,  $2.0 \times 10^5$  cells in  $500 \mu\text{l}$  medium were plated in polyheme-coated wells, and 6 or 18 h after plating aliquots were assessed for apoptosis, as described below.

**Assessment of Apoptosis.** Small aliquots of each sample ( $100 \mu\text{l}$ ) were removed and cytospun onto slides. Cytospin preparations were fixed in methanol, stained with Wright-Giemsa, and examined by oil immersion light microscopy at a final magnification of  $1,000\times$ . The percentage of apoptotic neutrophils was determined by counting the number of cells showing features associated with apoptosis (chromatin condensation and fragmented nuclei). At least 200 cells per slide were counted without prior knowledge of the sample. We and others have previously demonstrated that morphological assessment of neutrophil apoptosis closely correlates with results obtained using other methods to assay apoptosis, such as propidium iodide staining, annexin V binding, and decreased surface CD16 expression (12, 20). The data were tabulated as percentage of control apoptosis (percentage of apoptosis occurring in PMNs that had been incubated with endothelial cells, divided by the percentage of apoptosis in control samples). Control refers to samples which were subjected to the same experimental treatment but without any contact with the endothelial cell or conditioned medium from activated endothelial cells. We found this necessary because the rate of spontaneous neutrophil apoptosis varies from donor to donor (10–60% at 6 h). Apoptosis was also assessed by examining the percentage of unfixed cells able to bind annexin V and exclude propidium iodide (Apoptosis Detection kit from R&D Systems, Inc.). Assays were performed as outlined in the manufacturer's instructions and analyzed by flow cytometry using a FACScan™ (Becton Dickinson).

**Neutrophil Phagocytosis Assay.** Phagocytosis of complement-opsonized Oil-red-O was measured as we have described previously (12). Serum opsonization of Oil-red-O particles was performed as described previously (21). In brief,  $4 \times 10^6$  cells were incubated with  $100 \mu\text{l}$  of the serum-opsonized particles in a total volume of  $500 \mu\text{l}$  of Dulbecco's PBS, with or without 1 mM N-ethyl-maleimide (NEM), an inhibitor of phagocytosis. Cells were washed, and the red dye was extracted using dioxane and quantified at  $\text{OD}_{525}$ . The rate of phagocytosis was calculated as micrograms of Oil-red-O per  $10^6$  PMNs per minute.

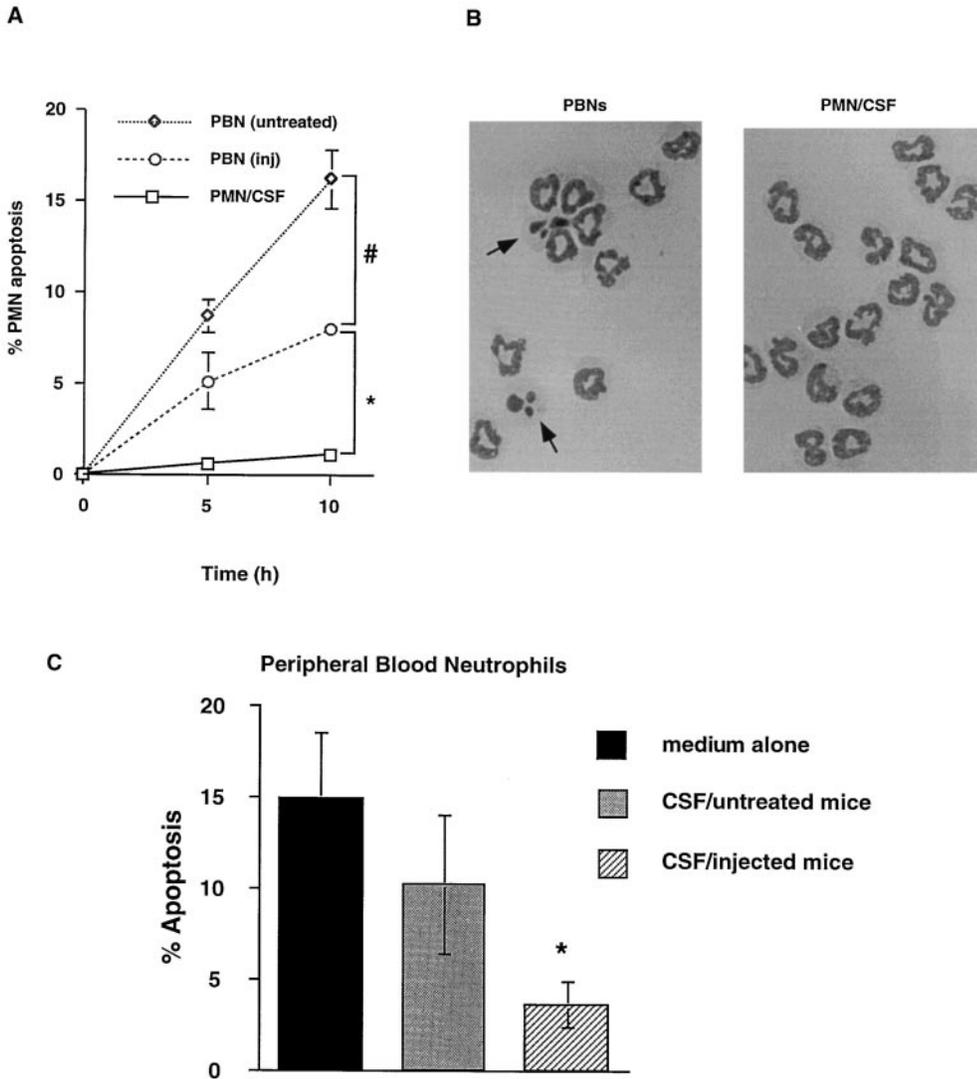
**Immunodepletion Experiments and Measurement of GM-CSF.** HUVECs were activated with IL- $1\beta$ /TNF- $\alpha$  for 90 min, the cells were washed four times to remove the cytokines, and conditioned medium was collected after an additional 90 min. A neutralizing polyclonal antibody directed against human GM-CSF (Genzyme) bound to Sepharose-protein A was added to the conditioned medium at  $100 \mu\text{g/ml}$ . A rabbit IgG, similarly coupled to Sepharose, was used as a negative control. The samples were incubated overnight at  $4^\circ\text{C}$ . The antibody-bound Sepharose was removed from the supernatant by centrifugation at  $12,000 g$  for 10 min. Human PMNs were incubated in the medium for 18 h, and the percentage of apoptotic PMNs was assessed. The concentration of GM-CSF present in conditioned medium was measured by ELISA (Genzyme) before and after immunoadsorption. Human recombinant GM-CSF (Genzyme) was used at concentrations of 20–500 pg/ml.

**Statistical Analysis.** Data are presented as average  $\pm$  SEM. Statistical significance was assessed by unpaired Student's *t* test.

## Results

**Neutrophils Transmigrated into the CSF of Mice after Cytokine-induced Meningitis Have a Significant Delay in Apoptosis Compared with Circulating PBNs.** We have previously developed a model of acute, cytokine-induced meningitis in mice. In this model, significant accumulation of leukocytes ( $>95\%$  neutrophils) in the CSF occurs 4 h after introduction of human IL- $1\beta$ /TNF- $\alpha$  into the subarachnoid space by lumbar puncture. Accumulation of leukocytes is dependent on neutrophil-endothelial interaction, as mice deficient in P- and E-selectins displayed an almost complete inhibition in CSF leukocyte influx (17). We used this model to assess apoptosis of neutrophils during inflammation *in vivo*. Neutrophils that had accumulated in the CSF 4 h after lumbar injection of IL- $1\beta$ /TNF- $\alpha$  were harvested, and PBNs were purified by density centrifugation from whole blood obtained from the same animals at the same time point. The neutrophils were placed in culture, and apoptosis was assessed at subsequent times. Apoptosis of PBNs in mice with cytokine-induced meningitis was significantly delayed compared with those isolated from untreated mice. Furthermore, in mice with meningitis, there was a significant reduction in the percentage of apoptotic neutrophils in the CSF compared with PBNs from the same animals (Fig. 1 A). Thus, cytokine-induced inflammation in the central nervous system leads to a delay in apoptosis of circulating neutrophils and a further delay in neutrophils that have migrated across the endothelial-blood interface into an inflammatory site.

In these experiments, we routinely used density gradient centrifugation to isolate neutrophils from murine whole blood. To rule out the possibility that this procedure might induce apoptosis or select for a subset of neutrophils, we compared apoptosis in blood neutrophils isolated either by our standard density centrifugation procedure or by two cycles of hypotonic lysis of RBCs in samples of anticoagulated whole blood. A 0.3-ml blood sample obtained from a mouse with meningitis yielded  $>80\%$  neutrophils compared with  $>95\%$  in neutrophils isolated by density gradi-



**Figure 1.** PMNs and neutrophils extravasated into the CSF of mice after cytokine-induced meningitis have delayed apoptosis, and an activity released into the CSF is responsible for this delay. 129Sv wild-type mice were subjected to cytokine-induced meningitis by injection of IL-1 $\beta$ /TNF- $\alpha$  by lumbar puncture or were left untreated (A). After 4 h, CSF containing leukocyte infiltrate was collected from each mouse ( $n = 6$ ). PMNs represented >90% of the cells present. Peripheral blood was pooled from two groups of two mice which had been subjected to meningitis, and two groups of two untreated mice. PMNs from each CSF (PMN/CSF) and peripheral blood neutrophil (PBNs) samples were cultured in duplicate, and neutrophil apoptosis was assessed at the indicated times ( $*P < 0.005$ ,  $\#P < 0.02$  at both time points). (B) Representative cytopins of the 5-h time point of PMNs from the CSF, and PBNs from cytokine-injected mice are shown. Apoptotic neutrophils are indicated by arrows. In a separate experiment, CSF was harvested from untreated mice and from mice 2 h after induction of meningitis (C). The isolated CSF was centrifuged to remove any cells that might be present; importantly, at this early time point very few neutrophils are present in the CSF. PBNs from untreated mice were cultured in the CSF or medium for 4 h. The neutrophils were then diluted with medium, and apoptosis was assessed after an additional 5 h.  $n = 3$  individual experiments;  $*P < 0.05$  compared with CSF/untreated mice.

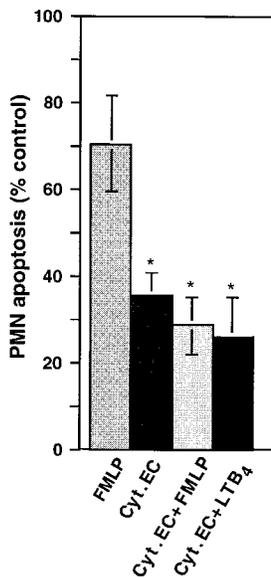
centrifugation. PBNs from mice with meningitis, isolated by either method, had similar levels of apoptosis after 5 h in culture (data not shown).

*CD11b/CD18-dependent Adhesion Is Not Responsible for the Observed Delay in Apoptosis of Transmigrated Neutrophils.* Previous studies have suggested that ligation of CD11b/CD18 with antibodies will modulate apoptosis in neutrophils (15, 22). To more definitively examine the potential role of CD11b/CD18-dependent adhesion in our model system, we subjected CD11b/CD18-deficient mice and their wild-type counterparts to cytokine-induced meningitis. Mice deficient in CD11b/CD18 showed no significant difference in the numbers of neutrophils that accumulated in the CSF during cytokine-induced meningitis (Tang, T., and T.N. Mayadas, unpublished data). Moreover, there was no significant difference in the percentage

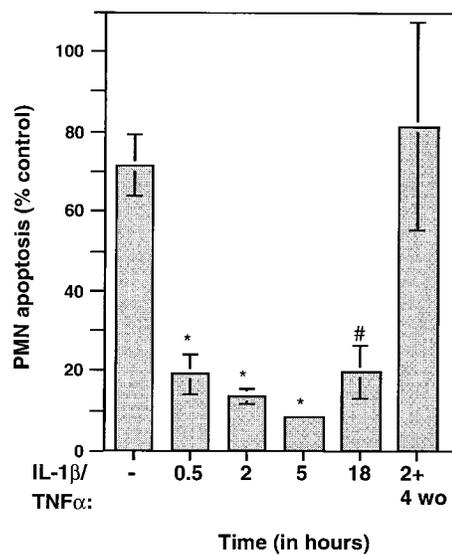
of spontaneous apoptotic neutrophils in samples harvested from the CSF of wild-type and CD11b/CD18-deficient mice and subsequently cultured for 10 h (wild-type,  $2.25 \pm 0.6\%$ ; and CD11b/CD18 $^{-/-}$ ,  $1.2 \pm 0.34\%$ ;  $P = 0.15$ ). Similarly, neutrophil apoptosis in PBNs was comparable between wild-type and CD11b/CD18-deficient mice (wild-type,  $12.83 \pm 2.13\%$ ; and CD11b/CD18 $^{-/-}$ ,  $12.4 \pm 2.67\%$ ;  $P = 0.91$ ). Thus CD11b/CD18-dependent adhesion does not appear to play a role in the delayed apoptosis observed in extravasated neutrophils, or PBNs, during this model of cytokine-induced meningitis.

*CSF Retrieved from Mice Subjected to Cytokine-induced Meningitis, but Not Untreated Mice, Exhibits Antiapoptotic Activity In Vitro.* To determine whether antiapoptotic factor(s) are released into the extravascular compartment in vivo, mouse PBNs were incubated with medium alone,

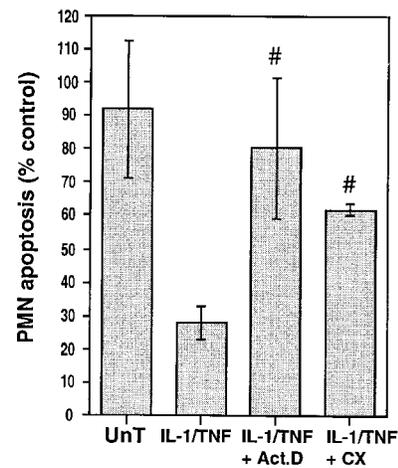
### A Transmigration



### B Co-culture



### C Co-culture



**Figure 2.** Cytokine-activated endothelial cells significantly delay neutrophil apoptosis, and inhibition of de novo synthesis abrogates expression of the antiapoptotic activity. (A) Neutrophils were allowed to transmigrate through untreated (–) or IL-1β/TNF-α-activated (Cyt.EC) HUVEC monolayers. Buffer alone or buffer plus the chemoattractants FMLP or LTB<sub>4</sub> were included in the bottom chamber of the transwell. Neutrophils incubated with untreated HUVECs transmigrated in response to FMLP in the bottom chamber. Control neutrophils were incubated in the presence or absence of the respective chemoattractant, without endothelial cells. (B) Neutrophils were cocultured with HUVEC monolayers for 1 h after HUVECs were stimulated with IL-1β/TNF-α for the indicated times. 2+4 wo indicates that 2 h cytokine-treated HUVECs were washed, incubated for 4 h without cytokines, and then incubated with neutrophils for 1 h. For A and B, \**P* < 0.005 and #*P* < 0.05 compared with untreated HUVEC monolayers. (C) Endothelial cells were pretreated with 12 μM actinomycin D (+ Act.D) or 50 μg/ml cycloheximide (+ CX) for 1 h, and IL-1β/TNF-α was added for an additional 1 h. The monolayer was washed, and neutrophils were added for 1 h. #*P* < 0.05 compared with IL-1β/TNF-α. For A, B, and C, neutrophils were recovered after transmigration or coculture, and apoptosis was assessed 6 h after culturing in fresh medium. Data are expressed as percentage of apoptosis of control neutrophils (% control) to account for the donor variability in the amount of spontaneous apoptosis after 6 h. *n* = 4 experiments.

CSF harvested from untreated mice, or CSF from mice 2 h after cytokine induction of meningitis. The 2-h time point was chosen because very few neutrophils are present in the CSF at this time point (17), thus diminishing the potential contribution of neutrophil-derived factors to the measured antiapoptotic activity. CSF from mice with meningitis caused a significant reduction in the spontaneous apoptosis of isolated PBNs in culture, whereas CSF from untreated mice had little effect (Fig. 1 C). These studies indicate that the normal constituents of the CSF do not delay apoptosis, but rather that cytokine activation leads to an accumulation of antiapoptotic factors into the CSF. The IL-1β/TNF-α injected into the CSF by lumbar puncture was not responsible for the observed delay in neutrophil apoptosis, since direct IL-1β/TNF-α treatment of PBNs in vitro led to slightly elevated levels of apoptosis compared with PBNs cultured in medium alone (data not shown).

*Cytokine-activated Endothelial Cell Monolayers Significantly Delay Apoptosis of Transmigrated Neutrophils: Endothelial Cell–Neutrophil Coculture Is Sufficient.* Endothelial cells are uniquely situated to regulate apoptosis of circulating neutrophils in the blood, as well as those transmigrated into the extravascular space. Therefore, we examined the ability of cytokine-activated endothelial cells to modulate neutrophil

apoptosis in an in vitro model of transmigration. Ca<sup>2+</sup> and Mg<sup>2+</sup> are omitted from the buffers so as to preserve the integrity of the activated endothelial cell monolayer during coculture with neutrophils (23, 24). Neutrophils were allowed to transmigrate across untreated or IL-1β/TNF-α-treated HUVECs plated in transwells, in the presence or absence of chemoattractants in the bottom chamber. Transmigrated neutrophils were removed, placed in fresh medium, and after 6 h incubation, apoptosis was assessed. Neutrophils that had transmigrated across cytokine-activated endothelial cells had a significant reduction in apoptosis compared with control neutrophils which were similarly treated but were not exposed to endothelial cells (Fig. 2 A). Transmigration across unactivated HUVECs in response to FMLP attenuated apoptosis to a much lesser extent than cytokine-activated HUVECs, suggesting that the endothelial cell-derived antiapoptotic activity is cytokine inducible.

To determine if transmigration was necessary for this effect or if contact with the endothelial cells was sufficient, neutrophils were cocultured for 1 h with HUVEC monolayers in tissue culture dishes, and then recovered and cultured for 6 h, as above. Neutrophils incubated with unactivated endothelial cells had a slight delay in apoptosis,

whereas IL-1 $\beta$ /TNF- $\alpha$ -treated endothelial cells resulted in a much greater delay in apoptosis (Fig. 2 B). A significant delay in neutrophil apoptosis was detectable after as little as 0.5 h of IL-1 $\beta$ /TNF- $\alpha$  activation and was manifested over an 18-h period of cytokine stimulation. If endothelial cells were cytokine treated for 2 h, followed by a 4-h "chase incubation" without cytokines, antiapoptotic activity was no longer detectable. Therefore, these endothelial-derived "antiapoptotic" factor(s) appear to be rapidly downregulated after cytokine withdrawal.

*Expression of Endothelial Antiapoptotic Factor(s) Is Dependent on De Novo Protein Synthesis and Activation of Nuclear Factor  $\kappa$ B and the Stimulus Used to Activate Endothelial Cells.* Cytokine-stimulated HUVECs pretreated with actinomycin D, an inhibitor of transcription, no longer delayed the apoptosis of neutrophils during coculture experiments. In addition, pretreating cytokine-stimulated HUVECs with cycloheximide, an inhibitor of protein synthesis, also attenuated the delay (Fig. 2 C). Therefore, the expression of the antiapoptotic factor(s) requires the transcriptional activation of one or more endothelial genes.

In cytokine-activated endothelial cells, nuclear factor  $\kappa$ B (NF- $\kappa$ B) plays a prominent role in the upregulation of genes encoding adhesion molecules and other proinflammatory products such as cytokines and growth factors (25). To determine the role of NF- $\kappa$ B in the expression of the endothelial-derived antiapoptotic factor(s), we used two structurally unrelated inhibitors of NF- $\kappa$ B, lactacystin and the peptide aldehyde MG132 (26, 27). Both of these inhibitors prevented translocation of NF- $\kappa$ B to the nucleus at the concentrations used, as assessed by gel shift analysis (data not shown). Treatment of IL-1 $\beta$ /TNF- $\alpha$ -stimulated endothelial cells with 40  $\mu$ M lactacystin decreased the delay in neutrophil apoptosis to levels that were closer to those seen with unstimulated endothelial cells (IL-1 $\beta$ /TNF- $\alpha$  + lactacystin, 45.9  $\pm$  4.5%;  $P$  < 0.005 compared with IL-1 $\beta$ /TNF- $\alpha$  alone, 10.5  $\pm$  5%; unstimulated, 76.5  $\pm$  12.5%). Similarly, treatment with MG132 attenuated the antiapoptotic effect but to a lesser extent than lactacystin (data not shown). These data suggest that NF- $\kappa$ B-dependent gene regulation may be involved in the elaboration of the antiapoptotic activity of cytokine-treated endothelial cells.

The production of this antiapoptotic activity also depends on the particular stimulus used to activate HUVECs (Table I). For example, IL-1 $\beta$  treatment alone was not as effective as TNF- $\alpha$  alone, and treatment with both together had a synergistic effect in delaying neutrophil cell death. IFN- $\gamma$  treatment of endothelial cells resulted in a delay of apoptosis comparable to that seen with IL-1 $\beta$  alone. Of the stimuli used to activate endothelial cells, a combination of TNF- $\alpha$  and IL-1 $\beta$  appeared to be most effective in inducing expression of the antiapoptotic activity.

*Conditioned Medium from Cytokine-activated Endothelial Cells Delays Neutrophil Apoptosis and Prevents the Associated Loss of Neutrophil Function.* To determine if the inhibition of neutrophil apoptosis was dependent on contact with the endothelial cells, or if the antiapoptotic factor(s) were re-

**Table I.** Effect of Different Regimens of Endothelial Cell Activation on PMN Apoptosis

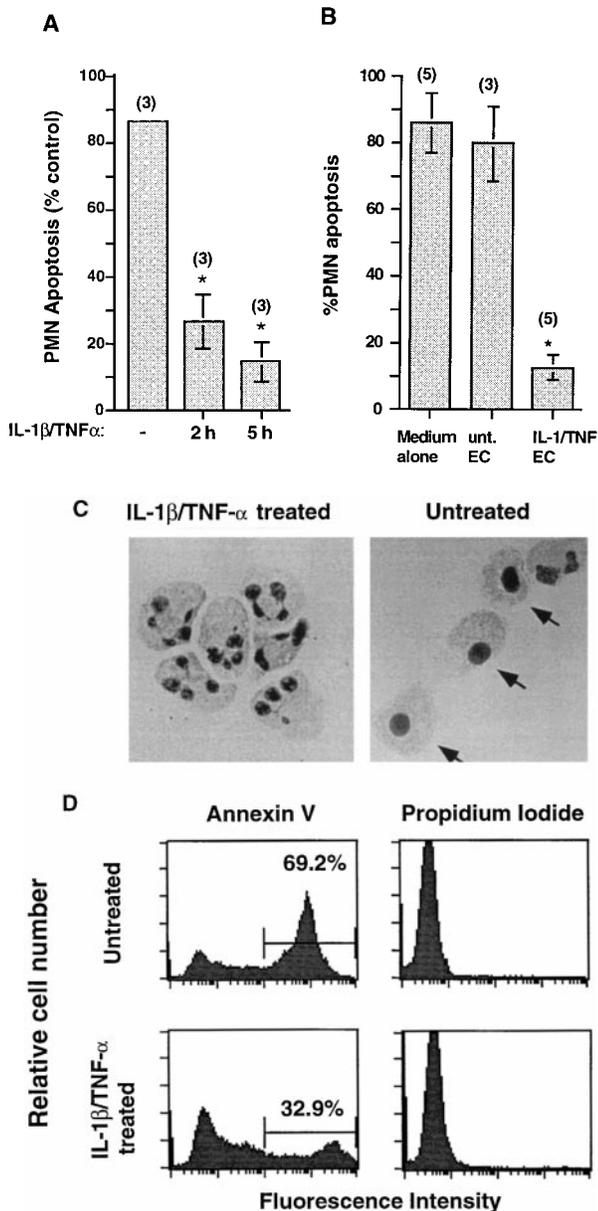
Endothelial cell treatment	Dose	PMN apoptosis
<i>n</i> = 3		% control $\pm$ SEM
Untreated	–	77.2 $\pm$ 14.5
IL-1 $\beta$	10 U/ml	47.3 $\pm$ 1.4
TNF- $\alpha$	50 ng/ml	28.3 $\pm$ 3.4*
IL-1 $\beta$ /TNF- $\alpha$	10 U/ml/50 ng/ml	17.6 $\pm$ 5.5*
IFN- $\gamma$	1,500 U/ml	52.9 $\pm$ 9.7
IL-3	30 ng/ml	86.2 $\pm$ 13.4

HUVECs were activated with various cytokines for 2 h, washed four times, and then cocultured with human PMNs for 1 h. The PMNs were then cultured alone for 6 h, and apoptosis was assessed.

\* $P$  < 0.05 compared with untreated.

leased by the cells, conditioned medium was harvested from untreated and IL-1 $\beta$ /TNF- $\alpha$ -treated endothelial cells. Conditioned medium from IL-1 $\beta$ /TNF- $\alpha$ -treated endothelial cells, but not untreated cells, significantly delayed neutrophil apoptosis (Fig. 3 A) to a level comparable to that observed after coculturing neutrophils with endothelial cells. Again, if cytokine-activated endothelial cells were pretreated with actinomycin D or cycloheximide, the observed antiapoptotic effect was attenuated; pretreatment with the NF- $\kappa$ B inhibitors, MG132 or lactacystin, also attenuated the delay in apoptosis to a similar extent, as seen in Fig. 4 B (data not shown). These data suggest that the antiapoptotic gene products are secreted and regulated at the level of transcription, and that this is partly due to activation of NF- $\kappa$ B.

Experiments described so far were designed to mimic the brief exposure of neutrophils to the endothelium during transmigration in vivo. That is, transmigration assays or cocultures were done for 1 h, after which the neutrophils were cultured in fresh medium and therefore removed from the endothelial cell-derived antiapoptotic stimuli. Under these conditions, neutrophil apoptosis was significantly delayed after 6 h, as shown in Fig. 2. However, after 8 h in culture the delay in apoptosis was not as striking as at 6 h (data not shown), suggesting that the endothelial cell-derived antiapoptotic effect was transient. To assess whether the continuous incubation of neutrophils with conditioned medium from activated endothelial cells would delay neutrophil apoptosis over a prolonged period, neutrophils were incubated with conditioned medium for 18 h, a time period when >90% of untreated neutrophils typically are apoptotic. Apoptosis was assessed by morphological criteria as in previous experiments, as well as by FACS<sup>®</sup> analysis of neutrophils stained with FITC-annexin V. Annexin V recognizes phosphatidylserine, which is increased on the surface of apoptotic cells (28; Fig. 3 C). After 18 h, neutrophils incubated with conditioned medium



**Figure 3.** Conditioned medium from cytokine-stimulated HUVECs significantly delays neutrophil apoptosis. (A) HUVECs were left untreated (–) or were treated with IL-1β/TNF-α for the indicated times, washed, and incubated with buffer alone for 1 h. The conditioned buffer was removed and incubated with neutrophils for 1 h. The neutrophils were then put into fresh medium, and apoptosis was assessed after 6 h. (B) Conditioned medium was collected from untreated (unt. EC) and 1.5-h cytokine-activated (IL-1/TNF EC) HUVECs, as described above. In contrast to conditions in A, the neutrophils were cultured for 18–24 h in the continuous presence of conditioned medium or medium alone, and percentage of apoptosis was assessed. The number of experiments performed is shown in parentheses. (C) Representative cytopins are shown, and apoptotic cells are indicated by arrows. (D) A representative FACS<sup>®</sup> analysis of samples in B that were stained with FITC–Annexin V, which preferentially binds to apoptotic cells. \**P* < 0.005 compared with IL-1β/TNF-α (A) or medium alone (B).

from IL-1β/TNF-α-stimulated endothelial cells had a dramatic delay in apoptosis compared with cells that were incubated with conditioned medium from untreated endothelial cells or fresh medium (Fig. 3 B).

**Table II.** Phagocytosis of Complement-opsonized Particles by PMNs Incubated with HUVEC-conditioned Medium

Time of incubation and treatment	μg Oil-red-O/10 <sup>6</sup> PMNs/min
1 h medium	31.3
18 h medium	9.10
18 h + CM/unstimulated EC	11.25
18 h + CM/cytokine-stimulated EC	22.2

PMNs were incubated for 1 or 18 h in complete medium or with conditioned medium (CM) harvested from unstimulated endothelial cells (EC) or endothelial cells stimulated with IL-1β/TNF-α for 1 h. The PMNs were then assessed for their ability to phagocytose complement-opsonized particles. Results from one representative experiment out of three performed are shown. Values represent the rate of phagocytosis.

Since apoptotic neutrophils lose their ability to phagocytose (3), we assessed whether the endothelial cell-mediated delay in apoptosis leads to a retention of this function. We assessed the ability of neutrophils to phagocytose complement-opsonized particles after they were incubated in the presence or absence of endothelial cell-derived conditioned medium for 18–21 h. Neutrophils incubated with conditioned medium derived from cytokine-treated endothelial cells had a similar level of phagocytosis as freshly isolated neutrophils, whereas neutrophils that were incubated with conditioned medium from untreated HUVECs or medium alone had lost the ability to phagocytose opsonized particles (Table II).

**GM-CSF Released from Activated Endothelial Cells Contributes to the Antiapoptotic Activity In Vitro.** GM-CSF has been previously shown to inhibit neutrophil apoptosis (5, 29) and is released by several different cell types, including cytokine-activated endothelial cells. GM-CSF has been reported in the conditioned medium of HUVECs after 4 h, with peak activity after 24 h of IL-1β stimulation (30). To test the hypothesis that GM-CSF was contributing to the observed delay in neutrophil apoptosis, we first assessed GM-CSF levels in the medium of endothelial cells under conditions that led to the expression of antiapoptotic activity (Table III). These studies revealed that the presence of GM-CSF correlated with the endothelial cell-derived antiapoptotic activity, with the exception of IL-3, which delayed neutrophil apoptosis but had no detectable GM-CSF, suggesting that antiapoptotic factor(s) other than GM-CSF were operative under this condition. GM-CSF was detected as early as 0.5 h after IL-1β/TNF-α treatment, a time point at which significant antiapoptotic activity was observed. In addition, removal of the cytokines for 4 h before collection of conditioned medium from 2-h cytokine-treated endothelial cells led to a loss of GM-CSF expression and loss of antiapoptotic activity. Furthermore, combined treatment of endothelial cells with IL-1β and TNF-α, which led to the highest expression of antiapoptotic activity, also led to the greatest release of GM-CSF activity.

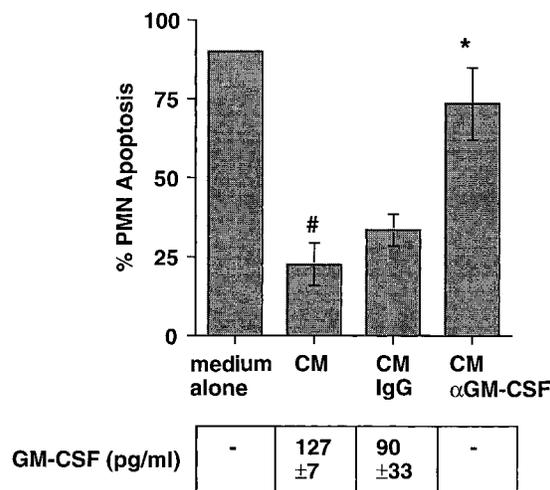
**Table III.** Expression of Antiapoptotic Activity Correlates with GM-CSF Production

Endothelial treatment	PMN apoptosis	GM-CSF
	% control $\pm$ SEM	pg/ml $\pm$ SEM
Untreated	77.2 $\pm$ 14.5	0
0.5 h IL-1 $\beta$ /TNF- $\alpha$	19.1 $\pm$ 4.8	52.9 $\pm$ 9.6
5 h IL-1 $\beta$ /TNF- $\alpha$	8.3 $\pm$ 0.6	190.2 $\pm$ 32.7
18 h IL-1 $\beta$ /TNF- $\alpha$	19.7 $\pm$ 6.7	297.9 $\pm$ 25.2
2 h IL-1 $\beta$ /TNF- $\alpha$ + 4 w/o	81.5 $\pm$ 26.1	5.7 $\pm$ 5.7
2 h IL-1 $\beta$	47.3 $\pm$ 1.4	23.0 $\pm$ 0.1
2 h TNF- $\alpha$	28.3 $\pm$ 3.4	13.4 $\pm$ 0.7
2 h IL-1 $\beta$ /TNF- $\alpha$	17.6 $\pm$ 5.5	93.7 $\pm$ 7.4
2 h IL-3	52.9 $\pm$ 9.7	0
2 h IFN- $\gamma$	86.2 $\pm$ 13.4	0

Conditions of endothelial treatment and the values for percentage of apoptosis are those described in Fig. 3 B and Table I. Levels of GM-CSF present in the conditioned medium were assessed by ELISA. Data represent the average of three independent experiments.

To directly determine whether GM-CSF in the endothelial-conditioned medium was contributing to the antiapoptotic activity, the conditioned medium from activated endothelial cells was immunodepleted using a polyclonal antibody to GM-CSF. Purified human PMNs were then incubated in the medium for 18 h, and apoptosis was assessed. Antiapoptotic activity was greatly diminished in conditioned medium that was immunodepleted of GM-CSF, as assessed by ELISA, whereas similar incubations with an isotype control IgG had no effect on GM-CSF levels or apoptosis (Fig. 4). Immunodepletion of GM-CSF with an mAb (BVD2-23B6) yielded similar results (data not shown). Together, these studies indicate that GM-CSF is present in the conditioned medium of activated endothelial cells under conditions that lead to the expression of antiapoptotic activity, and that removal of GM-CSF by immunodepletion leads to loss of this activity. Therefore, we conclude that GM-CSF is primarily responsible for the delayed apoptosis observed in our *in vitro* assay system.

*GM-CSF Is Required for the Apoptotic Delay Observed in PBNs.* To critically examine the contribution of GM-CSF to neutrophil apoptosis *in vivo*, we used GM-CSF-deficient mice and their wild-type counterparts. We first isolated PBNs from untreated animals of both genotypes and found no significant difference in the levels of apoptosis after culture (data not shown). We then performed cytokine-induced meningitis in these mice. GM-CSF-deficient mice had normal peripheral blood counts (18; data not shown), and mice of both genotypes responded with significant accumulation of neutrophils in the CSF (wild-type,  $1.1 \pm 2.7 \times 10^6$ ; GM-CSF<sup>-/-</sup>,  $2.5 \pm 5.47 \times 10^6$  cells recovered in CSF). Significantly, there was a 30–40% increase in the level of apoptosis in PBNs isolated from

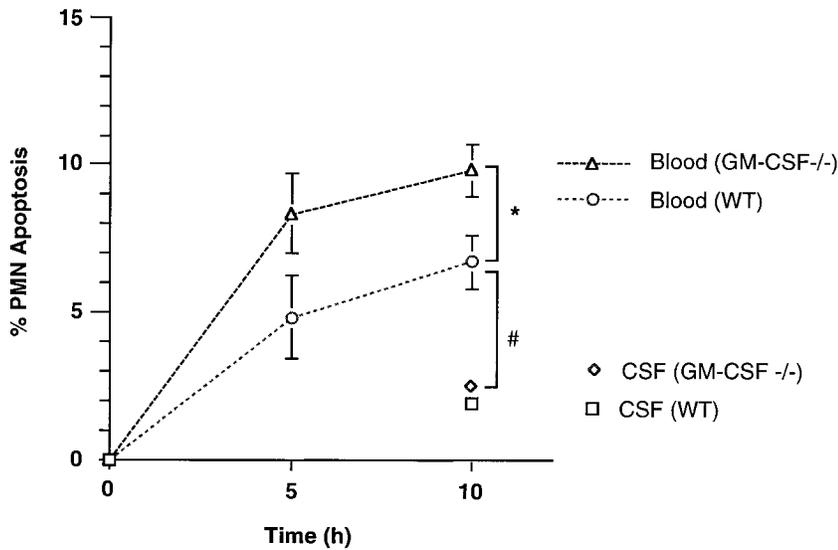


**Figure 4.** Effect of immunodepleting conditioned medium of GM-CSF on PMN apoptosis. Conditioned medium (CM) from IL-1 $\beta$ /TNF- $\alpha$ -treated HUVECs was immunodepleted of GM-CSF using neutralizing polyclonal antibodies (CM  $\alpha$ GM-CSF). A rabbit IgG was used as negative control (CM IgG). PMNs were incubated for 18 h in the conditioned medium, and the percentage of apoptotic PMNs was assessed. #*P* < 0.05 compared with medium alone, and \**P* < 0.05 compared with CM and CM/IgG. The amount of GM-CSF present in the conditioned medium before and after immunodepletion was assessed by ELISA, and the average (pg/ml) is shown below the respective conditions. *n* = 3 experiments.

GM-CSF-deficient mice compared with wild-type mice (Fig. 5). Therefore, GM-CSF plays an important role in the regulation of neutrophil apoptosis in the peripheral blood only during inflammation. We observed no difference in the percentage of apoptotic neutrophils retrieved from the CSF of GM-CSF-deficient and wild-type mice, despite the fact that GM-CSF was detected in the CSF of wild-type animals (naive mice, 0 pg/ml; mice subjected to meningitis for 4 h,  $135.1 \pm 42.2$  pg/ml). Thus, soluble factors other than GM-CSF are responsible for increasing neutrophil survival in the extravascular compartment.

## Discussion

The localized accumulation of functional neutrophils at sites of inflammation is pivotal in the host's defense against infection, and the orderly elimination of neutrophils is equally important in resolution of the inflammatory response. It is well recognized that the endothelium, upon activation by various proinflammatory stimuli including cytokines such as IL-1 $\beta$  and TNF- $\alpha$ , plays an active role in recruiting leukocytes via the expression of adhesion molecules and chemoattractants (31). The results reported here establish a previously unrecognized role for the activated endothelium in prolonging neutrophil survival and retaining neutrophil functional capabilities in the context of inflammation, one that may have important implications for the regulation of the acute inflammatory response. Our observations suggest two levels at which neutrophil apoptosis



**Figure 5.** Mice deficient in GM-CSF have less of a delay in PBN apoptosis after cytokine-induced meningitis than wild-type mice. Neutrophils were isolated from the CSF and peripheral blood of eight wild-type (WT) and eight GM-CSF-deficient (GM-CSF<sup>-/-</sup>) mice, 4 h after cytokine-induced meningitis. The harvested neutrophils were then placed in culture, and apoptosis was assessed on cytopins at the indicated time points. \**P* < 0.05, #*P* < 0.002.

is regulated during inflammation. The first is a delayed apoptosis in neutrophils in the peripheral circulation, and the second is a more substantial delay in apoptosis of neutrophils that have extravasated into a site of inflammation. The endothelium is positioned to directly affect the apoptosis of circulating neutrophils. It may also affect the apoptosis of extravasated neutrophils, although the role of other cell types in the tissue cannot be ruled out.

Our *in vitro* studies demonstrate that the antiapoptotic activity of endothelial cells is rapidly inducible and that it is sustained over 24 h in the presence of cytokines. However, the activity decays in the absence of ongoing stimuli. Thus, the antiapoptotic activity from endothelial cells may function to influence the survival of neutrophils during the earliest periods of their recruitment. Furthermore, only under conditions where neutrophil recruitment and inflammation were ongoing would neutrophil survival be extended, since the elaboration of the antiapoptotic factors is dependent on cytokine stimulation of the endothelial cells. NF- $\kappa$ B, which is a pleiotropic regulator of the proinflammatory activities of endothelial cells such as leukocyte adhesion receptor expression (25), is important in the expression of endothelial antiapoptotic activity. Thus, mechanisms regulating the recruitment of neutrophils to the endothelium also play a role in the survival of those neutrophils. CD11b/CD18-mediated adhesion is not required for the delay in neutrophil apoptosis *in vivo*, although another study *in vitro* suggested that this may be the case since antibody cross-linking of CD11b/CD18 or CD11a/CD18 attenuated neutrophil cell death (15).

We show that the antiapoptotic activity is secreted and that it is extremely potent. The number of neutrophils that are apoptotic after a 24-h period in culture is reduced by 80% in neutrophils that are incubated with conditioned medium from endothelial cells compared with those incubated in medium alone. The enhanced neutrophil survival in the presence of endothelial-derived factors is associated

with a retention in the ability of the neutrophil to phagocytose, a principal function of these phagocytes at sites of inflammation. Thus, endothelial cells promote the survival of neutrophils and their function in response to cytokine activation, thereby potentially enhancing the accumulation of functional neutrophils at the site of inflammation. Many recombinant forms of inflammatory mediators can inhibit apoptosis and prolong neutrophil survival *in vitro* (3, 5). Some of these, GM-CSF, IL-6, and IL-1 $\beta$ , are known to be released by the activated endothelium, although the role of these cytokines in a physiological context is not clear. Here, we demonstrate that GM-CSF released from cytokine-activated endothelial cells *in vitro* is responsible for the antiapoptotic activity. Two aspects of the regulation of GM-CSF expression observed in this study have not been previously described (30, 32, 33). GM-CSF was released into the medium of endothelial cells as early as 0.5 h after cytokine activation, and IL-1 $\beta$  and TNF- $\alpha$  had a synergistic effect in stimulating GM-CSF production in endothelial cells.

The role of GM-CSF in neutrophil apoptosis *in vivo* was critically examined by assessing apoptosis of neutrophils retrieved from both untreated GM-CSF knockout and wild-type mice, and mice subjected to cytokine-induced meningitis. These studies suggested that GM-CSF has no effect on the spontaneous programmed cell death of PBNs in untreated mice, but is responsible for delaying the spontaneous apoptosis of PBNs during an inflammatory response. On the other hand, the greater delay in apoptosis of extravasated neutrophils was found to be regulated by soluble factors other than GM-CSF. Thus, neutrophil apoptosis is differentially regulated in the peripheral blood and extravascular tissue, not only in terms of the extent of the delayed apoptosis but also in the soluble mediator(s) involved.

Data from this study, together with previous data demonstrating that phagocytosis promotes neutrophil apoptosis (12, 13), have led us to propose the following model for

the regulation of neutrophil apoptosis during an inflammatory response. The vascular endothelium actively recruits PBNs to sites of inflammation via expression of adhesion molecules and chemoattractant cytokines. The constitutive cell death program in the recruited neutrophils is delayed by antiapoptotic factors released from the endothelium, including GM-CSF. This delay in the programmed cell death of neutrophils during inflammation allows the neutrophil to retain its functional capabilities. Once neutrophils have completed their principal function, which is the phagocytosis of bacteria and cellular debris, the death program is re-engaged. It follows that defects in the regulation of neutrophil apoptosis could have severe consequences for the host.

Dysfunctional upregulation of the endothelial antiapoptotic factor(s) could result in increased accumulation of PMNs in the extravascular tissue and prolong the inflammatory response, culminating in tissue damage. Alternatively, a reduction in the antiapoptotic activity could lead to premature apoptosis of neutrophils in the tissue, thereby compromising host defense. Further studies of the regulation of expression of the key effectors of neutrophil apoptosis secreted by activated vascular endothelium, including GM-CSF and other as yet undefined mediators, should add to an understanding of the control potential of the inflammatory response and the disease-related consequences.

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