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Inhibition of meningitis-associated neutrophil apoptosis by TNF-α depends on functional PI3-kinase in monocytes

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ABSTRACT

In bacterial meningitis, neutrophils cope with bacterial infection but also lead to tissue damage. The balance of beneficial and harmful effects may depend on the lifespan of the neutrophils in the CNS. Here, we show that CSF of patients with meningococcal meningitis contains a neutrophil apoptosis-inhibiting capacity that correlates with TNF-α content. In vitro experiments show that *Neisseria meningitidis* as well as LPS derived from these bacteria regulated neutrophil apoptosis mainly by stimulating TNF-α production in monocytes. Whereas LPS-induced PI3K-dependent survival signals in monocytes are critical for neutrophil survival, PI3K signaling in granulocytes did not contribute to the increased lifespan of neutrophils. We conclude that LPS-driven PI3K signaling in monocytes regulates neutrophil apoptosis and thereby, may be crucial in the initiation of secondary brain damage in bacterial meningitis. *J. Leukoc. Biol.* 93: 259–266; 2013.

Introduction

Bacterial meningitis is a common disease worldwide that causes significant morbidity and mortality, despite the introduction of and continued improvement in antibiotics and the progress made on management of endotoxic shock [1]. In industrialized countries, the case fatality rates for the three most common etiologic agents of bacterial meningitis, *Haemophilus influenzae*, *N. meningitidis*, and *Streptococcus pneumoniae*, are still between 10% and 25% [1]. In bacterial meningitis, major pathological findings are characterized by (1) extensive infiltration of the leptomeninges and perivascular spaces with PMNs and (2) CSF changes, including polymorphonuclear pleocytosis and intracranial hypertension. The pathophysiological mechanisms leading to brain damage during bacterial meningitis involve bacterial components and host factors, such as cytokines, arachidonic-acid metabolites, platelet-activating factor, complement factors, NO, and reactive oxygen intermediates [2]. Whereas PMNs contribute to clearance of infectious pathogens, by their release of the aforementioned toxic factors, they are also directly involved in tissue damage. Thus, the outcome of meningitis may much depend on the regulation of the lifespan of PMNs, which invade from the blood to the site of infection in the meninges.

PMNs are constitutively short-lived, undergoing apoptosis in a few hours [3]. Activation of PMNs by LPS delays apoptosis of the cells [3]. In a mouse model of pneumococcal meningitis, inhibition of apoptosis by overexpression of the antiapoptotic molecule bcl-2 was associated with aggravated disease, as characterized by pronounced PMN infiltration with vasogenic edema, vasculitis, and intracerebral hemorrhagies [4]. In the same meningitis model, roscovitine, an inhibitor of cyclin-dependent kinases, has been shown to induce apoptosis of PMNs and to ameliorate the course of disease in mice [4].

Bacterial cell wall components, including LPS, bind to TLRs, which via MyD88-dependent or -independent pathways, lead to activation of neutrophils with up-regulation of adhesion molecules and production of cytokines [3]. LPS signals via CD14/TLR4, which are expressed on PMNs as well as on monocytes/macrophages, although TLR4 expression is higher in monocytes than in neutrophils [5]. As anti-LPS strategies have failed to ameliorate the clinical course of gram-negative sepsis [6], outer-membrane constituents besides LPS may contribute to the symptoms encountered in gram-negative septic shock. To study the role of bacterial cell-wall components in the host response to infection, viable *N. meningitidis* mutants have been constructed that are devoid of LPS but still contain all other outer-membrane constituents [7]. LPS -deficient meningococci are reported to induce

Abbreviations: CSF=cerebrospinal fluid, PMN=PMN neutrophil, WBC=white blood cell

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TABLE 1. Characterization of the Tested Meningococcal Isolates

Strain	Original designation	Description	Case/carriage	Serogroup	Sequence type	Clonal complex	PorA-VR1	PorA-VR2	FetA-VR
α510			Carriage	Y	23	23	5–2	10–1	F4–1
α530		lpxL1 mutation	Carriage	Y	23	23	5–2	10–1	F4–1
α696			Carriage	B	44	41/44	19	15–1	F1–5
WUE2120			Case	C	11	11	5	2	F3–9
WUE3022	H44/76		Case	B	32	32	7	16	F3–3
WUE3023	H44/76-pLAK33	lpxA mutation		B	32	32	7	16	F3–3
DE8383			Case	C	8	8	5	2	F5–8
DE9686			Case	B	42	41/44	7–2	4	F1–5

Description of the meningococcal isolates that were tested for their effects on human neutrophils.

substantial amounts of TNF-α, IL-1β, and IFN-γ in peripheral blood mononuclear cells [8]. Thus, the regulation of the lifespan of PMN is a critical issue in the outcome of bacterial meningitis.

Survival of neutrophils is not only regulated by LPS and other bacterial cell-wall components but also by cytokines. Whereas TNF-α and IL-8 have been shown to increase survival of neutrophils [9–11], IL-10 has been shown to shorten neutrophil lifespan in the presence of LPS [12, 13]. The LPS-induced effects on neutrophils can also be blocked by specific inhibitors of intracellular signaling pathways. Repeatedly, PI3K has been identified to be required for the LPS-induced lifespan changes in vitro and in vivo [12, 14]. Importantly, common neutrophil preparations contain a low percentage of contaminating monocytes. It has not been evaluated yet whether PI3K exerts its effect on neutrophils directly or indirectly by acting on mononuclear cells. Our data provide evidence for the dependence of neutrophil lifespan on TNF-α, executing the prolongation of neutrophil lifespan in monocytes in a PI3K-dependent way.

MATERIALS AND METHODS

Reagents

Neisseria LPS was prepared from strain B1940, according to the method described by Hammerschmidt et al. [15]. *Escherichia coli* O55:B5 LPS was pur-

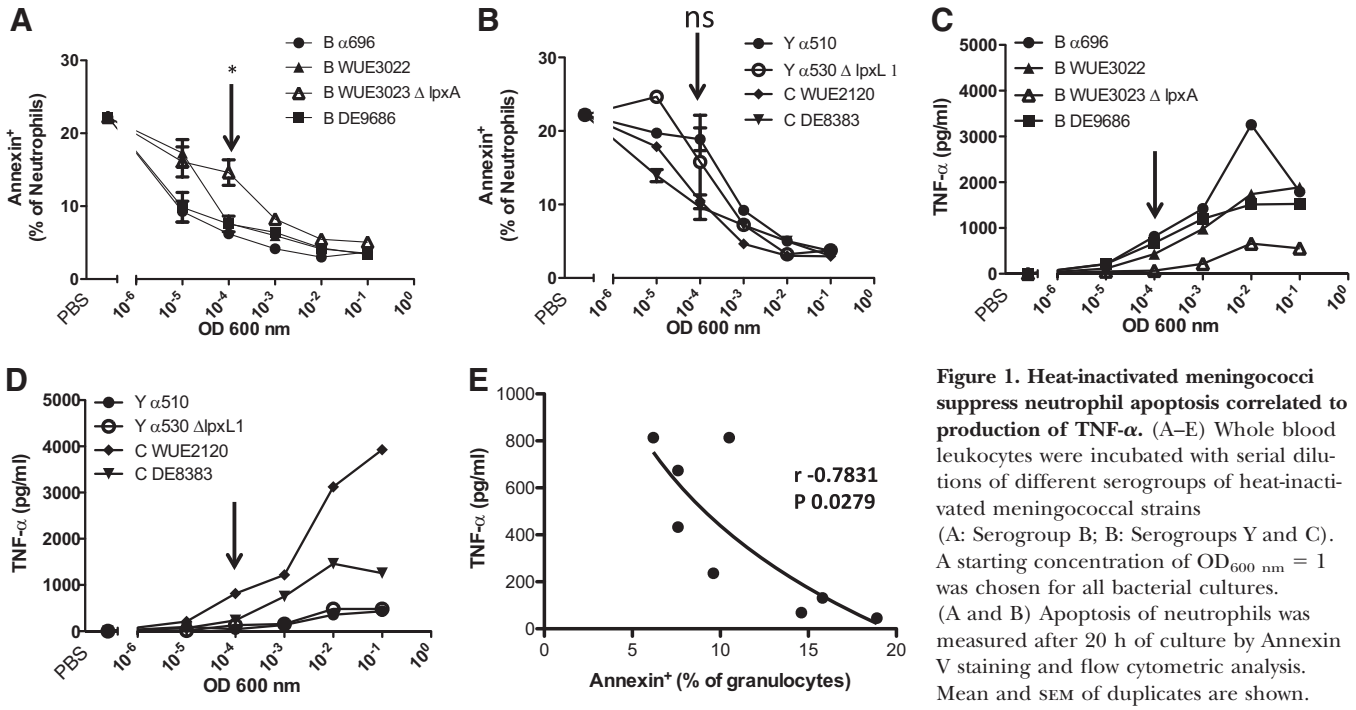

Figure 1. Heat-inactivated meningococci suppress neutrophil apoptosis correlated to production of TNF-α. (A–E) Whole blood leukocytes were incubated with serial dilutions of different serogroups of heat-inactivated meningococcal strains (A: Serogroup B; B: Serogroups Y and C). A starting concentration of $OD_{600\ nm} = 1$ was chosen for all bacterial cultures. (A and B) Apoptosis of neutrophils was measured after 20 h of culture by Annexin V staining and flow cytometric analysis. Mean and SEM of duplicates are shown. B WUE3023, the lpxA mutant of B WUE3022, protected signicantly less from apoptosis at intermediate concentrations indicated by an arrow (*$P<0.05$, Student's *t*-test). The Yα530 isolate carrying a lpxL1 mutation did not differ significantly (NS) in the capacity to protect neutrophils from apoptosis compared with the WT Yα510 isolate at the same OD tested (arrows; C and D). TNF-α was measured by ELISA in the culture supernatants after 20 h of culture. Supernatants of duplicates were pooled for analysis. The arrows indicate the intermediate *Neisseria* concentration (OD) mentioned above. As a result of the pooling of supernatants, no significances can be calculated (E). A correlation of neutrophil apoptosis and TNF-α production was assessed by plotting the results of various meningococcal serogroups tested at a concentration of $OD_{600\ nm} = 10^{-4}$. $P < 0.05$ (Spearman test).

chased from Sigma (St. Louis, MO, USA). LPS was diluted in PBS and used at the indicated concentrations. Etanercept was purchased from Amgen (Thousand Oaks, CA, USA), control human IgG-Fc antibody was purchased from R&D Systems (Minneapolis, MN, USA). Both were used at 50 μg/ml final concentrations. Ly294002 was purchased from Sigma and used at 10 μM final concentration.

N. meningitidis

The meningococcal isolates used are described in detail in **Table 1**. Sequence type and clonal complexes were determined by multilocus sequence typing, according to Jolley et al. [16]. WUE3022 was originally designated H44/76. WUE3023 is a lpxA mutant of WUE3022, initially designated H44/76-pLAK33, as described by Steeghs et al. [17] and has been provided by Liana Steeghs. lpxL1 mutations were described by van der Ley et al. [18]. The α-strains are part of a carriage strain collection as described by Claus et al. [19]. The DE strains are part of the invasive isolates collection of the German reference laboratory for meningococci at the Institute for Hygiene and Microbiology (Wuerzburg, Germany).

Neutrophil isolation and culture

Ficoll gradient centrifugation to purify neutrophils from EDTA blood of healthy volunteers was performed after informed consent. After removal of the plasma, the mononuclear cell layer, and most of the Ficoll, the erythrocyte layer with neutrophils on top was erythrocyte-lysed (Erythrocyte-lysis buffer: 8.26 g NH_4Cl, 1 g $KHCO_3$, 0.037 g EDTA/l H_2O) and neutrophils resuspended in RPMI with 10% FCS. To test whole blood leukocytes, EDTA blood was erythrocyte-lysed directly. In other control experiments, the Ficoll-neutrophil fraction was purified further by positive selection with anti-CD15 beads and magnetic cell separation (MACS), according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA, USA). Magnetic bead-mediated neutrophil purification was not associated with neutrophil activation, as MACS-purified neutrophils expressed low levels of CD16 similarly to Ficoll-purified neutrophils (Supplemental Fig. 1A). In contrast, LPS activation resulted in neutrophil activation and high levels of CD16 expression (Supplemental Fig. 1). MACS-purified neutrophil preparations reduced contamination of mononuclear cells from 2.5% to <0.5% (Supplememntal Fig. 1B). Alternatively, Ficoll-purified neutrophils were plated on 96-well flat-bottom, tissue-culture plates (Falcon) to allow mononuclear-contaminating cells to attach to the tissue-culture plates. After 30 min, the nonadherent plate-purified neutrophils were used for the indicated experiments. In some experiments, mononuclear cells were added back to MACS-purified neutrophils, resulting in a fraction 5–10% mononuclear cells of total cells when assessed by flow cytometry. In general, 2–5 $\times$ 10^5 neutrophils were seeded in 96-well plates and then stimulated with the indicated substances. Survival was assessed 20 h later by Annexin V staining and flow cytometric analysis.

Detection of apoptotic cells

After the indicated time of culture, cells were washed in Annexin buffer (eBioscience, San Diego, CA, USA) and stained with Annexin-allophycocyanin, Annexin-FITC, or Annexin-PE for 30 min at room temperature (eBioscience).

Prosurvival effect of LPS-stimulated, mononuclear cell-derived supernatants

MACS-purified neutrophils were cultured in serum-free X-Vivo15 medium (Lonza, Switzerland) containing 1/3 diluted supernatants of LPS (1 ng/ml)-stimulated mononuclear cells. After 20 h of culture in the presence or absence of the PI3K inhibitor Ly294002 (10 μM final concentration), neutrophil apoptosis was assessed by Annexin V staining and flow cytometric analysis.

Cytokine measurements

Cell culture supernatants, as indicated in the experiments, were tested for TNF-α production by cytokine bead array (Cytolab, Switzerland) or by ELISA (DuoSet, R&D Systems).

CSF samples

In all patients, lumbar puncture was performed for diagnostic purposes after informed consent. The use of the samples was approved by the ethics committee of the medical faculty of the University of Munich (Germany). Lumbar CSF samples were obtained from 10 patients with meningococcal meningitis and four control patients with noninflammatory neurologic diseases (hydrocephalus). Patients with meningococcal meningitis had clinical signs of meningitis (fever, headache, meningism), elevated CSF - WBC counts (11,826±10,673 cells/μl), and a positive CSF or blood culture for *N. meningitidis*. Control patients suffered from noninflammatory neurological diseases of the CNS (CSF-WBC, 3.5±1.3 cells/μl.) Lumbar punctures were performed for diagnostic purposes after informed consent. Samples were stored at −70°C until analysis.

Statistics

Statistical significance was tested by one-way ANOVA, followed by Bonferroni post-testing, Student's *t*-test, and correlation, determined by Spearman, as indicated in the figure legends.

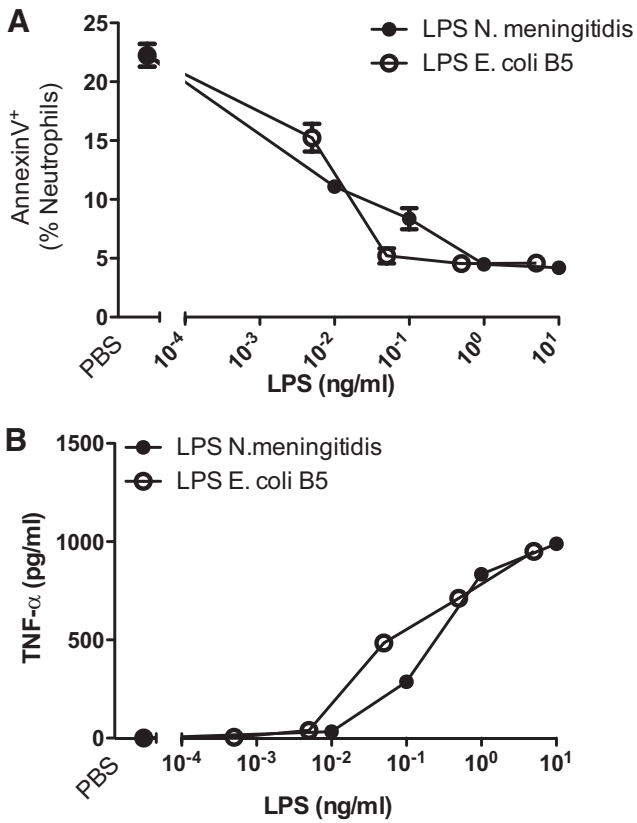

Figure 2. Meningococcal and *E. coli*-derived LPS regulate neutrophil apoptosis and TNF-α formation similarly. (A and B) Whole WBCs were incubated with titrated concentrations of LPS derived from meningococci or *E. coli* O55:B5. (A) Twenty hours later, neutrophil apoptosis was measured by Annexin V staining and flow cytometric analysis. Mean and SEM of triplicates are shown. (B) The concentration of TNF-α in the culture supernatants was measured at the same time-point by ELISA. One out of two experiments is shown.

RESULTS

Prolongation of neutrophil survival by different meningococcal serogroups correlates with their inducibility of TNF-α

We analyzed the kinetics of neutrophil apoptosis in the presence of *N. meningitidis*. When titrated doses of heat-inactivated *N. meningitidis* Serogroup B bacteria were incubated with peripheral blood leukocytes of healthy donors, we observed a dose-dependent suppression of neutrophil apoptosis (**Fig. 1A**). Associated was a dose-dependent increase of TNF-α in the culture supernatants (Fig. 1C). A similar finding of suppressed neutrophil apoptosis and increased TNF-α production was found when *N. meningitidis* Serogroups C and Y were tested (Fig. 1B and D). The number of Annexin V^+ apoptotic neutrophils correlated negatively with the amount of TNF-α in the culture supernatants, implying a direct relationship between these two readouts (Fig. 1E). To address the role of LPS in inducing the suppression of neutrophil apoptosis, we compared two *N. meningitidis* Serogroup B strains—WUE3022 and WUE3023. WUE3023 is a mutant of the paternal strain WUE3022 and lacks LPS in the outer cell wall. LPS-deficient WUE3023 suppressed neutrophil apoptosis significantly less than the LPS-competent B WUE3022 strain at intermediate doses, and this was associated with lower TNF-α formation induced by this strain (Fig. 1A and C, indicated by arrows).

Neutrophil apoptosis and TNF-α production are affected similarly by LPS derived from different Gram-negative bacteria

E. coli O55:B5-derived LPS has been used extensively to examine LPS effects on neutrophils in the literature. In contrast, *N.*

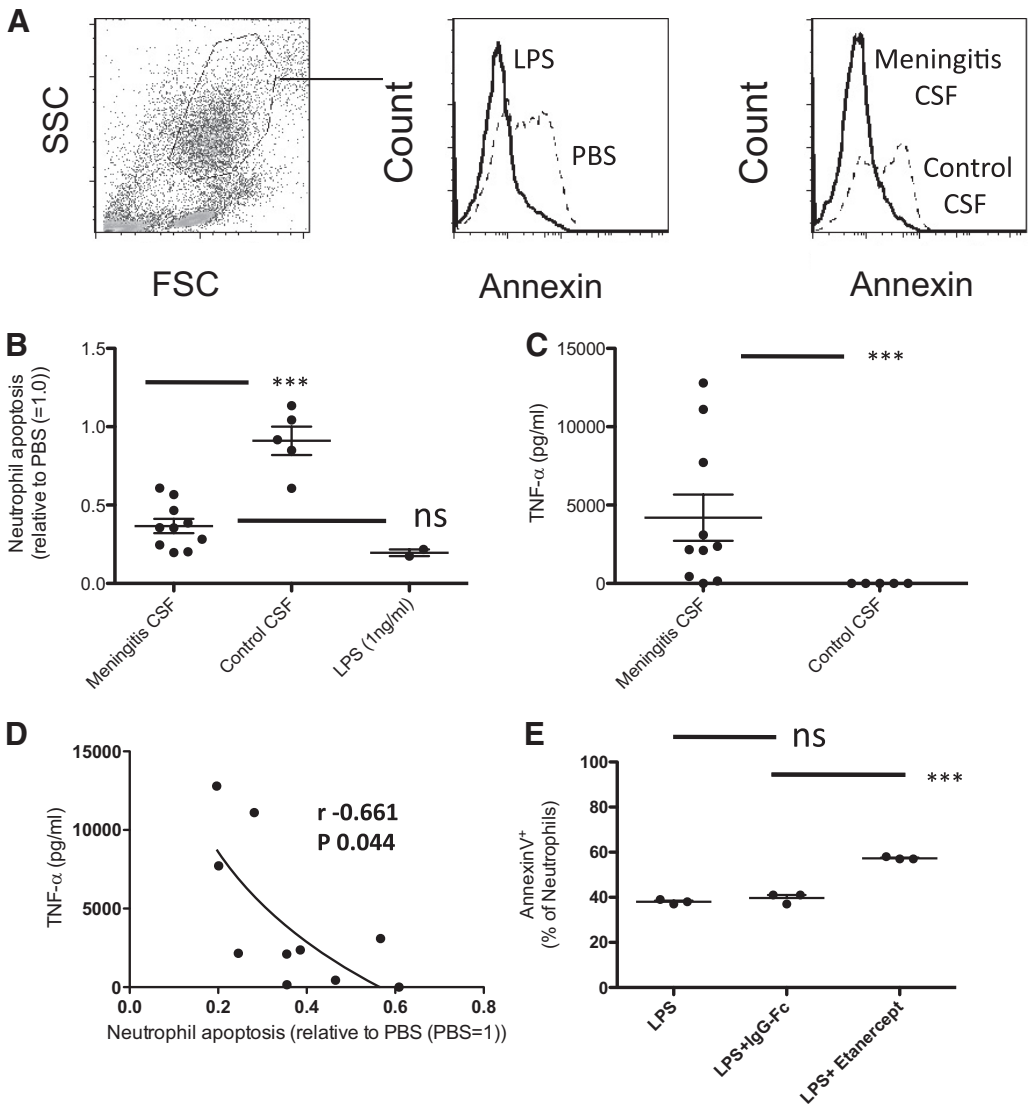

Figure 3. Inhibition of neutrophil apoptosis by CSF of patients with meningococcal meningitis is mediated by TNF-α. (A–E) Whole WBCs were incubated with CSF of meningococcal meningitis patients or control patients (noninflammatory CSF samples) for 20 h. PBS and *E. coli* O55:B5 LPS (1 ng/ml) were used as a negative and positive control. (A) Neutrophils were gated according to their high side-scatter (SSC) profile in flow cytometry. Whereas neutrophils normally undergo apoptosis (high Annexin V binding, dotted lines), LPS or CSF of meningitis patients inhibited this process (low Annexin V binding, solid lines). Representative flow cytometry plots are shown. FSC, Forward-scatter. (B) Significant differences in Annexin V^+ neutrophils of control (noninflammatory) CSF compared with meningococcal meningitis CSF are observed. (C and D) Whole blood leukocytes were incubated with CSF of meningococcal meningitis patients or control patients (noninflammatory CSF samples) for 20 h. TNF-α was measured in the culture supernatants by cytokine bead array. (C) TNF-α levels were significantly higher in meningococcal meningitis-derived CFS compared with control noninflammatory CSF ($P<0.001$, Student's *t*-test; D). Individual TNF-α levels correlated significantly with individual neutrophil apoptosis ($P<0.05$, Spearman's test). (E) Ficoll-isolated neutrophils were incubated with *E. coli* O55:B5 LPS in the absence or presence of the TNF-α-blocking biologic etanercept (soluble TNF-αR2 coupled to human IgG-Fc). Human IgG-Fc was used as a control. Neutrophil apoptosis was assessed by Annexin V staining and flow cytometric analysis 20 h later (***$P<0.001$, one-way ANOVA with Bonferroni post-test correction).

meningitidis-derived LPS is not commercially available. To address whether *E. coli* O55:B5-derived LPS could substitute for *N. meningitidis*-derived LPS for its functional effect to prolong neutrophil survival and to induce TNF-α production, we compared the two LPS preparations at titrated concentrations. TNF-α formation and suppression of neutrophil apoptosis were comparable for both sources of LPS tested (**Fig. 2A** and **B**).

Meningitis-derived CSF mediates neutrophil survival

To determine whether neutrophil apoptosis is antagonized also in *N. meningitidis* infection of the CNS in vivo, we tested CSF samples of 10 patients with proven meningococcal meningitis. There was a prolonged neutrophil lifespan in vitro in the presence of meningitis-derived CSF comparable with the effect of LPS (**Fig. 3A–C**). Of note, noninflammatory CSF samples from patients with hydrocephalus did not prolong neutrophil lifespan (Fig. 3B and C). As reported [20, 21], bacterial meningitis-derived CSF contained elevated cytokine levels of TNF-α (Fig. 3D), which again correlated significantly with suppression of neutrophil apoptosis on an individual level (Fig. 3D). To finally establish a causal relation of TNF-α formation and suppression of apoptosis, we treated LPS-stimulated Ficoll-purified neutrophil preparations with the TNF-α-blocking compound etanercept, which is a soluble TNFR2 coupled to human IgG-Fc. Etanercept but not control human IgG-Fc significantly reduced the LPS-mediated prolonged neutrophil survival (Fig. 3E). Thus, meningococcal meningitis in humans leads to a TNF-α-mediated increase of neutrophil survival in the CNS, an effect that is initiated by LPS cell-wall components.

LPS-induced TNF-α formation and suppression of neutrophil apoptosis are mediated by monocytes

Ficoll-gradient centrifugation-based isolation of neutrophils is the main technique used to study neutrophil function in vitro. However, the purity of these preparations is only 96–98%, which still leaves 2–3% of monocytes (Supplemental Fig. 1B). To determine whether this minor fraction of contaminating monocytes is responsible for protection from apoptosis and TNF-α production in response to LPS, we compared conventional Ficoll-purified neutrophil preparations with Ficoll-purified neutrophils depleted from monocytes by plating (see Materials and Methods). These highly purified neutrophils showed a significantly reduced TNF-α formation and were less protected from apoptosis when stimulated with LPS (**Fig. 4A** and **B**). Thus, monocytes, rather than neutrophils themselves, are the major determinants of TNF-α formation in response to LPS and are likely the main regulators of neutrophil apoptosis in meningococcal meningitis.

TNF-α-induced suppression of neutrophil apoptosis requires functional PI3K in monocytes but not in neutrophils

We next aimed at identifying signaling pathways involved in LPS-induced, TNF-α-mediated suppression of neutrophil survival. LPS stimulation of Ficoll-purified neutrophils suppressed neutrophil apoptosis, an effect that was blocked in the presence of the PI3K inhibitor Ly294002 (**Fig. 5A**). This effect was not explained by the solvent of the inhibitor (DMSO), as the same final concentrations of DMSO without the inhibitor did not affect neutrophil survival (data not shown). However, no effect of Ly294002 was found when MACS-purified neutrophils were LPS-stimulated (Fig. 5C). When mononuclear cells were added back to the MACS-purified neutrophils, again, a reduction of the LPS-stimulated neutrophil lifespan in the presence of Ly294002 was observed (Fig. 5C). Finally, when culture supernatants of LPS-stimulated mononuclear cells were incubated with fresh, highly purified neutrophils, their survival was prolonged (Fig. 5D), an effect that was not blocked in the presence of Ly294002 (Fig. 5D).

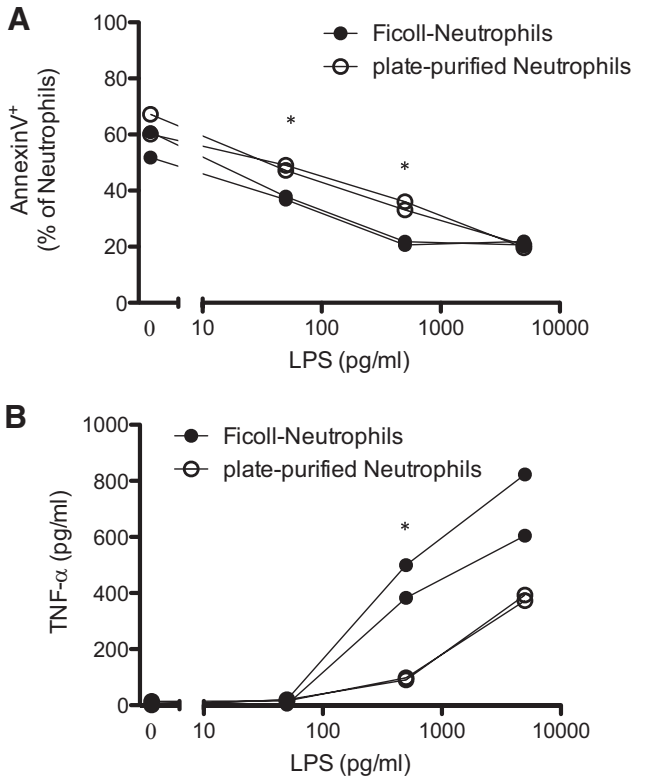

Figure 4. Neutrophil apoptosis and TNF-α formation are controlled by monocytes and not by neutrophils themselves. Neutrophils were isolated from whole blood by Ficoll gradient. To minimize monocyte contamination, Ficoll gradient-purified neutrophils were plated and monocytes allowed attaching to plates. After 30 min, nonadherent plate-purified neutrophils were then incubated with *E. coli* O55:B5 LPS at titrated doses, and neutrophil apoptosis (A) and TNF-α production in cultures (B) were compared after 20 h to cultures using conventional Ficoll-enriched neutrophils. (*$P<0.05$, two-way ANOVA). Individual data of duplicates are shown.

DISCUSSION

In summary, the data support that LPS induces TNF-α production by monocytes that suppress neutrophil apoptosis in a monocyte-intrinsic, PI3K-dependent process in bacterial meningitis in man. In contrast, neutrophils do not require func-

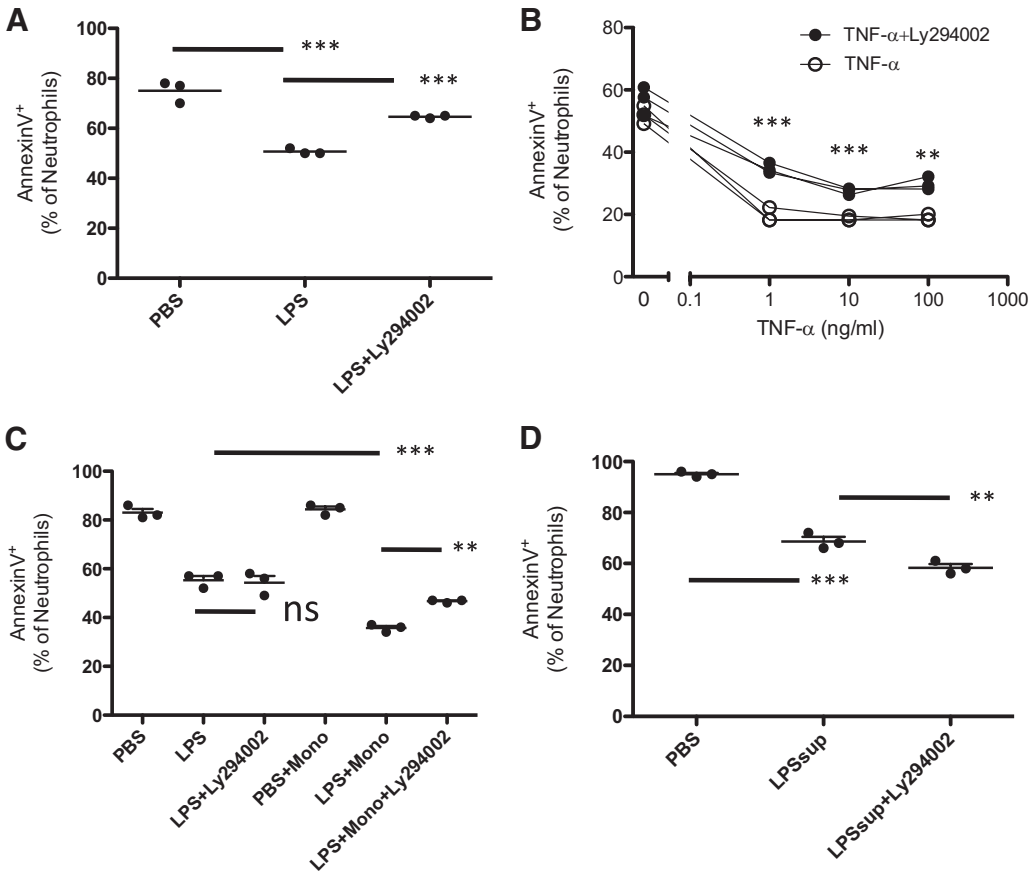

Figure 5. The suppressing effect on neutrophil apoptosis by TNF-α is mediated by PI3K signaling in monocytes. (A) Whole blood leukocytes were incubated with 1 ng/ml LPS of *E. coli* O55:B5 in the presence or absence of 10 μM Ly294002. Twenty hours later, neutrophil apoptosis was assessed by Annexin V staining and flow cytometric analysis. Individual results are shown. (B) Ficoll-enriched neutrophils were incubated with titrated amounts of rTNF-α in the presence or absence of 10 μM Ly294002. Twenty hours later, neutrophil apoptosis was assessed by Annexin V staining and flow cytometric analysis. Individual data of triplicates are depicted (C). Ficoll-isolated neutrophils were highly purified by MACS using the neutrophil-specific surface marker CD15. These MACS-purified neutrophils were then incubated in the presence or absence of 10 μM Ly294002 with 1 ng/ml LPS *E. coli* O55:B5. Neutrophil apoptosis was assessed by Annexin V staining and flow cytometric analysis 20 h later. As a control, MACS-purified neutrophils were supplemented with 5% mononuclear cells (+Mono) from the same blood donor before incubation with LPS in the presence or absence of Ly294002. Individual results are shown. (D) Ficoll-purified PBMCs were incubated with 1 ng/ml LPS *E. coli* O55:B5. Twenty hours later, the cell-free supernatants were collected and incubated with MACS-purified neutrophils in the presence or absence of 10 μM Ly294002. Twenty hours later, neutrophil apoptosis was measured by Annexin V staining and flow cytometric analysis. $^{**}P < 0.01$, one-way ANOVA; $^{***}P < 0.001$, one-way ANOVA.

tional PI3K to respond to TNF-α-induced antiapoptotic signals (**Fig. 6**). LPS-induced prolongation of the neutrophil lifespan has been well-described [3]. It seems advantageous to biologically couple neutrophil activation and the increased lifespan to result in an optimal and long-lasting antibacterial host response. On the other hand, this coupling may also result in decreased activation-induced neutrophil death that may be involved in immune pathology in sepsis and bacterial meningitis. Indeed, when counteracting the resolution of inflammation by interfering with apoptosis of neutrophils by overexpression of bcl-2, complications of pneumococcal meningitis, including vasculitis and intracerebral hemorrhage, become pronounced [4]. Likewise, blocking of receptors for leukocyte-endothelial adhesion interferes with transmigration of leukocytes into the brain, reduces neuronal apoptosis, and increases survival in experimental bacterial meningitis [22].

Neutrophil activation and attraction to the site of infection are mediated by cytokines and chemokines. In bacterial meningitis, high leukocyte values are associated with significant TNF-α concentrations in the CSF [21]. When added to neutrophils, TNF-α activates the oxidative burst and increases β2-integrin expression [23]. Antibodies to IL-8 and growth-related oncogene-α suppress the chemotactic effect of CSF on neutrophil chemotaxis in vitro [24]. Cytokines, such as TNF-α and IL-8, have been repeatedly described to reduce neutrophil apoptosis [10, 11, 13]. Our results confirm the presence of relevant levels of TNF-α in the CSF of patients with meningococcal meningitis and agree with the antiapoptotic property of TNF-α when cocultured with neutrophils. Moreover, our study shows for the first time that the CSF from patients with meningococcal meningitis but not from noninfectious controls protects neutrophils from apoptosis, the effect correlating with the CSF concentrations of TNF-α.

Based on numerous in vivo and in vitro studies, LPS is considered to play a pivotal role as a virulence factor in the pathophysiology of gram-negative septic shock [25]. Studies on mutants of Serogroup B meningococci, which are devoid of LPS, show that non-LPS components of the outer surface were able to induce production of TNF-α, IL-1β, and IFN-γ by PBMCs and to cause mortality in galactosamine -sensitized mice [8]. However, the doses of non-LPS meningococci required for effects on cytokines and on survival of mice were ten- and 100-fold higher, respectively [8]. When taking neutrophil survival

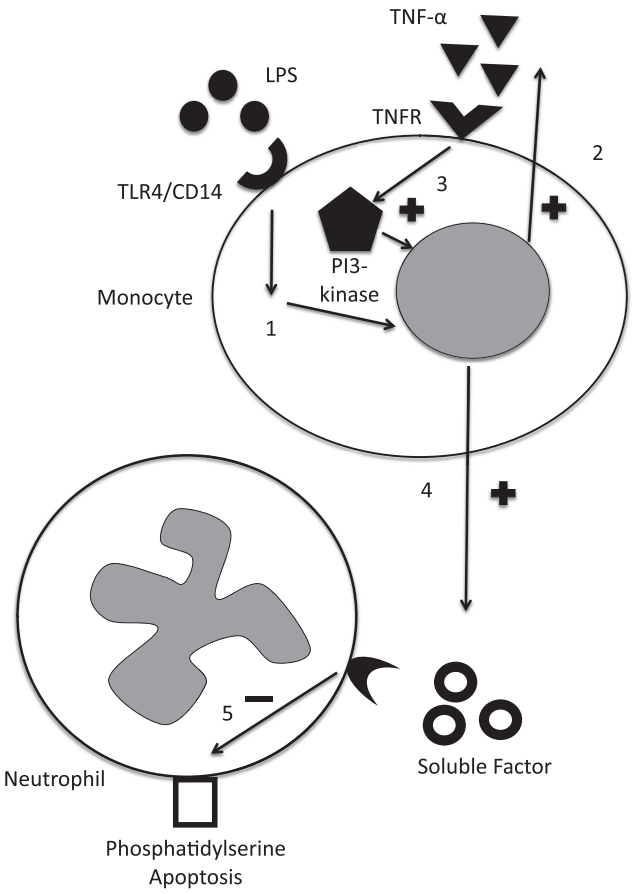

Figure 6. Schematic view of the LPS-mediated and TNF-α- and PI3K-dependent suppression of neutrophil apoptosis mediated by monocytes. LPS is binding to its receptor TLR4 and initiates MyD88-dependent signaling that (1) leads to induction of TNF-α (2). TNF-α binds to the TNFR (TNFRI and TNFRII) and induces a PI3K-dependent signaling cascade (3). PI3K-dependent signaling leads to the secretion of soluble factors (4) that then suppress apoptosis of neutrophils (5). PI3K is not required in neutrophils to inhibit apoptosis (5).

as a readout, the data presented here show that LPS-deficient WUE3023 meningococci were significantly less effective compared with WT Serogroup B meningococci, especially at intermediate bacterial concentrations. In agreement with reported lower TNF-α induction capacity of LPS-free meningococci, the lower effect observed on neutrophil survival was associated with less TNF-α induction. In studies of the *N. meningitidis* lpxA mutant, the non-LPS-mediated effects involve activation of the CD14 and TLR2 receptor complex rather then the CD14/TLR4 MD2 pathway used by WT meningococci [26]. Unlike LPS-free meningococci, the different *N. meningitidis* serogroups being tested for their effects on neutrophil survival, namely, Serogroups B, C and Y, did not differ from each other.

As it is an important concern with neutrophil experiments that neutrophil preparations obtained by conventional purification leave 2–3% of contaminating mononuclear cells, it seemed important to reconsider the site of production and effects of molecules that affect neutrophil survival [27]. The pathway worked out here shows LPS to activate primarily monocytes, which react with production of TNF-α. Thus, monocytes play a pivotal role in the protection of neutrophils from apoptosis. In keeping with our results, it has been shown in a model of experimental meningitis caused by *H. influenzae* that deactivation of macrophages results in reduction of the number of neutrophils in the brain, the effect being associated with protection against lethality [28]. Our results imply that the antiapoptotic effect on neutrophils induced by TNF-α depends on functional PI3K in monocytes, whereas PI3K is dispensable in neutrophils themselves. Numerous groups have reported that blockade of PI3K in monocytes increases rather than decreases TNF-α production in vitro and in vivo [29–31]. Thus, as we show here, PI3K is involved in converting the TNF-α signal in monocytes to IL-8 or other soluble factors known to suppress neutrophil apoptosis [9, 10]. Blocking PI3K in vivo might be considered in meningococcal meningitis, where increased neutrophil-mediated brain damage is expected.

AUTHORSHIP

M.R. designed research, performed research, analyzed data, and wrote the paper. U.M. performed research, analyzed data, and wrote the paper. D.J.S. performed experiments. U.K. and H-W.P. contributed clinical samples and wrote the paper. T.B. performed experiments. U.P. wrote the paper and analyzed data. H.C. contributed meningococcal strains and wrote the paper. H.G. designed research and wrote the paper. A.F. designed research, analyzed data, and wrote the paper.

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DISCLOSURES

The authors declare no conflict of interest.

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