

# In vivo-transmigrated human neutrophils are resistant to antiapoptotic stimulation

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## ABSTRACT

Neutrophils respond to microbial invasion or injury by transmigration from blood to tissue. Transmigration involves cellular activation and degranulation, resulting in altered levels of surface receptors and changed responsiveness to certain stimuli. Thus, fundamental functional changes are associated with neutrophil transmigration from blood to tissue. Neutrophils isolated from peripheral blood spontaneously enter apoptosis, a process that can be accelerated or delayed by different pro- or antiapoptotic factors. How tissue neutrophils that have transmigrated in vivo regulate cell death is poorly understood. In this study, in vivo-transmigrated neutrophils (tissue neutrophils) were collected using a skin chamber technique and compared with blood neutrophils from the same donors with respect to regulation of cell death. Skin chamber fluid contained a variety of cytokines known to activate neutrophils and regulate their lifespan. Freshly prepared tissue neutrophils had elevated activity of caspase 3/7 but were fully viable; spontaneous cell death after in vitro culture was also similar between blood and tissue neutrophils. Whereas apoptosis of cultured blood neutrophils was delayed by soluble antiapoptotic factors (e.g., TLR ligands), tissue neutrophils were completely resistant to antiapoptotic stimulation, even though receptors were present and functional. In vitro transmigration of blood neutrophils into skin chamber fluid did not fully confer resistance to antiapoptotic stimulation, indicating that a block of antiapoptotic signaling occurs specifically during in vivo transmigration. We describe a novel, functional alteration that takes place during in vivo transmigration and highlights the fact that life and death of neutrophils may be regulated differently in blood and tissue. *J. Leukoc. Biol.* 90: 1055–1063; 2011.

Abbreviations: 7-AAD=7-amino-actinomycin D, CD62L=CD62 ligand, CGD=chronic granulomatous disease, CR=complement receptor, FasL=Fas ligand, IP-10=IFN-inducible protein 10, KRG=Krebs-Ringer glucose buffer, LTA=lipotechoic acid, P<sub>3</sub>C=palmitoyl-3-cysteine-serine-lysine-4, PS=phosphatidylserine, rSAA=recombinant serum amyloid A

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## Introduction

Neutrophil granulocytes constitute an important part of innate immunity. These cells are the most common leukocytes in the bloodstream and circulate in a resting state waiting to be alerted to the tissue upon microbial invasion or tissue damage. To leave circulation, neutrophils adhere to the endothelial cell lining by interaction of adhesion molecules on both cell types. Transmigration occurs through or between the endothelial cells, and the migrating cells are directed toward the inflammatory site by a chemotactic gradient of endogenous and/or microbial chemoattractants [1]. Neutrophils are richly granulated, and molecules stored in granule membranes can be mobilized during activation to supply the cells with, e.g., adhesion molecules and chemoattractant receptors [2]. Degranulation occurs during transmigration, and in this way, tissue neutrophils become functionally different from resting neutrophils in circulation. At the inflammatory site, neutrophils are activated to engulf and eradicate invading microbes, using a battery of toxic substances such as ROS and antimicrobial peptides [3, 4]. The cells can also produce various inflammatory mediators that help fine-tune immune responses [5]. Neutrophils have a short lifespan and are destined to enter apoptosis, after which, the dead cells are removed rapidly by other phagocytes, e.g., macrophages [6]. Apoptosis preserves cell membrane integrity and prevents leakage of toxic contents and damage of surrounding tissues. Apoptosis and subsequent removal of the dead cells are thus extremely important for resolution of inflammation [7].

During in vitro culture, neutrophils spontaneously enter apoptosis, and the process can be accelerated or delayed by a variety of factors of microbial and endogenous origin [8]. Typically, danger signals such as TLR ligands are potent antiapoptotic factors that extend neutrophil viability [9–11], as are certain proinflammatory cytokines, e.g., GM-CSF [12]. Cell death can also be actively induced by signaling from the death receptor Fas, which accelerates the apoptotic rate [13]. A family of

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cysteine-aspartic proteases, caspases, is central for carrying out the apoptotic program [14]. Caspases are divided into initiator and effector caspases with members of the former activating members of the latter, which can then cleave a multitude of cellular substrates resulting in cell death. Apoptosis involves typical morphological changes, e.g., nuclear condensation, cleavage, and fragmentation of DNA, changes in the mitochondrial membrane potential, and exposure of PS on the cell surface [15, 16]. The latter is often used for quantification of apoptosis in assays using the PS-binding protein Annexin V.

Most studies about human neutrophils are conducted using cells isolated from peripheral blood (whole blood or buffy-coats). This is particularly true for investigations regarding apoptosis and the regulation of death processes. Given that neutrophil apoptosis and clearance to large extents take place in tissues, there is a surprising dearth of information describing how apoptosis is regulated in human neutrophils that have left the bloodstream and transmigrated into tissue. Tissue neutrophils can be obtained by an aseptic skin chamber technique, whereby blisters are induced, and the nonbleeding lesions, exposed after removal of the blister roofs, are covered by collection chambers filled with autologous serum [17]. These in vivo-transmigrated cells display alterations in gene transcription [18] and behave differently from neutrophils collected from peripheral blood. For example, transmigrated neutrophils have mobilized a subset of intracellular granules during transmigration and express different levels of surface receptors compared with neutrophils from blood and thus, differ also in responsiveness to subsequent stimulation [19], [20].

In this study, we set out to investigate how transmigrated neutrophils, extracted with the skin chamber technique (from here on referred to as tissue neutrophils), behave with regard to regulation of cell death. Our data show that skin chamber fluid, in addition to chemoattractants, contained a variety of cytokines known to modulate neutrophil apoptosis. Freshly prepared tissue neutrophils were morphologically as viable as blood neutrophils from the same donor but had elevated caspase activity. Despite this, spontaneous apoptosis of blood neutrophils and tissue neutrophils was similar after in vitro culture. However, a wide variety of antiapoptotic factors (e.g., TLR ligands and GM-CSF) was completely unable to delay apoptosis in tissue neutrophils, which is in striking contrast to blood neutrophils, where apoptosis was easily delayed by this agonists. The resistance to antiapoptotic stimulation of tissue neutrophils was not a result of absent receptors and could not fully be mimicked by in vitro transmigration of blood neutrophils into skin chamber fluid. Our findings support the view that tissue neutrophils are functionally different from blood neutrophils and that in vivo transmigration induces important changes in the way neutrophil cell death is regulated.

## MATERIALS AND METHODS

Ficoll-Paque was purchased from Fischer Scientific GTF AB (Gothenburg, Sweden) and Dextran from Pharmacosmos (Holbaek, Denmark). PE-conjugated CD62L and CD11b antibodies were from Becton Dickinson AB (San Jose, CA, USA). The CD35 antibody and the secondary FITC-conjugated mouse antibody were from Dako (Stockholm, Sweden) and CD95 (Fas)

mAb (functional grade) from Nordic BioSite (Täby, Sweden), as were the FITC-conjugated CD116 (GM-CSFR $\alpha$ ) antibody and a mAb directed to TLR2. Human rGM-CSF (endotoxin level  $\leq 0.1$  ng/ $\mu$ g) and LPS from *Escherichia coli* (serotype O111:B4) were from Sigma-Aldrich (St Louis, MO, USA); PGN and LTA, both from *Staphylococcus aureus*, were from Invivogen (San Diego, CA, USA), as were P<sub>3</sub>C. Human apo-rSAA (a chimeric protein comprised of human SAA1 with 3 aa replacements/additions from human SAA2) was purchased from PeproTech (Rocky Hill, NJ, USA). Caspase inhibitor VI (Z-VAD-FMK) was purchased from EMD Chemicals (La Jolla, CA, USA). Annexin V-Fluos was from Roche Diagnostics (Mannheim, Germany), and 7-AAD was from BD Biosciences (Stockholm, Sweden).

## Cell separation and preparation of serum

Neutrophils from peripheral freshly drawn blood or in one set of experiments, from buffy-coats stored for 18–24 h in room temperature, were separated using a standard technique [21, 22] based on Dextran sedimentation and Ficoll-Paque gradient centrifugation. After hypotonic lysis of remaining erythrocytes, the cells were washed repeatedly, and the pellet, containing  $\sim 95\%$  PMN, was resuspended in KRG phosphate buffer, supplemented with Ca<sup>2+</sup> (1 mM) and Mg<sup>2+</sup> (1.5 mM) and stored on ice.

Autologous serum was prepared as follows: blood was drawn into Venosafe plastic tubes (containing clot activator) and left to sediment for 2 h. The serum was then collected after centrifugation at 335 g for 10 min at 4°C and used in the skin chamber experiment or saved for multiplex analysis.

In vivo-transmigrated neutrophils (tissue neutrophils) were obtained using a skin chamber technique as described previously [17]. In short, blisters on the forearm of healthy volunteers were formed under negative pressure for 2 h. After removal of the blister roofs, autologous serum was added to collection chambers that were sealed and left for 24 h, after which, the neutrophil-containing chamber fluid was collected. The cells were pelleted and resuspended in KRG and stored on ice until use. Cell-free chamber fluid was saved for multiplex analysis. To minimize age differences between blood neutrophils and tissue neutrophils, blood was drawn  $\sim 18$  h after blister formation. All donors were healthy volunteers enrolled in the study after informed consent; the study was approved by the Ethical Board at the University of Gothenburg (Sweden; No. 543-07).

Peripheral blood was also drawn from healthy adults or from two patients with CGD, and the neutrophils were separated as described above. The blood was drawn after informed consent was obtained, as approved by the Research Ethics Board of the University of British Columbia (Canada). Further information about the patients can be found in Bylund et al. [23].

## Neutrophil culture

Neutrophils were cultured in RPMI 1640, supplemented with 10% FCS and 1% penicillin/streptomycin at a density of  $5 \times 10^6$  cells/ml. Cells were incubated at 37°C in 5% CO<sub>2</sub> for 30 min before addition of stimuli or medium and thereafter, reincubated for indicated times.

## Microscopy

Freshly prepared neutrophils (blood neutrophils and tissue neutrophils) were attached to glass slides by cytospin and stained with Giemsa and May Grünwald solution (Sigma-Aldrich), according to the manufacturer's description.

## Caspase 3/7 activity

Freshly prepared neutrophils from blood and tissue or cells cultured in the presence or absence of Z-VAD-FMK (100  $\mu$ M) for 4 h were counted and diluted in KRG in white 96-well plates ( $1 \times 10^4$  cells/well), together with Caspase-Glo 3/7 (Promega, Madison, WI, USA). Freshly prepared neutrophils from blood were also cultured in the presence of FasL (10  $\mu$ g/ml), LPS (100 ng/ml), or GM-CSF (100 ng/ml) for 4 h before dilution in KRG and addition of Caspase-Glo 3/7 as above. The luminescence assay was per-

formed according to the manufacturer's description using a multilabel reader LB940 Mithras (Berthold Technologies, Bad Wildbad, Germany).

### Assessment of cell death

Cell death was monitored essentially as described in Christenson et al. [24, 25]. In short, cell samples were washed in Annexin buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM  $\text{CaCl}_2$ , pH 7.4) and were then resuspended in 100  $\mu\text{l}$  buffer with addition of Annexin V-Fluos and 7-AAD. Samples were incubated in the dark for 10 min, and another 400  $\mu\text{l}$  buffer was added before analysis using a FACScan (Becton Dickinson, Mountain View, CA, USA) or an Accuri C6 (Accuri, UK). Apoptosis was assessed on the basis of Annexin V-Fluos fluorescence and necrosis on the basis of membrane permeability to 7-AAD (excitation 488 nm; emission 650 nm). All data were analyzed using WinMDI 2.8 software or CFlow Plus.

### Expression of surface receptors

Freshly prepared cells were incubated on ice for 30 min with either of the following antibodies: L-selectin (CD62L), CR3 (CD11b), CR1 (CD35), GM-CSFR (CD116), TLR2, TLR4, or isotype controls. For detection of TLR2 and CR1, cells were washed, and a FITC-labeled secondary antibody was added for 30 min on ice. After fixation with paraformaldehyde (2%), cells were washed twice and resuspended in PBS before FACS analysis; data were analyzed with WinMDI 2.8 or CFlow Plus.

### Quantification of IL-8

Freshly prepared cells ( $1.5 \times 10^6$ ) or cell cultures (incubated 20 h in the presence of absence of stimuli) were lysed with Triton-X 100 (0.1%) in the presence of Pefa-block (1 mM). Lysates were analyzed for IL-8 content using the human IL-8 DuoSet ELISA development kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instruction.

### Multiplex analysis

Control serum or skin chamber fluid (60  $\mu\text{l}$ , 1:3 dilution with sample buffer) was evaluated for cytokine content (IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12, IL-17, TNF- $\alpha$ , IFN- $\gamma$ , GM-CSF, and IP-10) with a Bio-plex Pro cytokine assay, 10-Plex Group 1 (Bio-Rad Laboratories AB, Sundbyberg, Sweden), according to the manufacturer's description.

### In vitro transmigration

Freshly prepared blood neutrophils ( $1 \times 10^6$ ) were put directly in skin chamber fluid or added on top of a membrane (Transwell-permeable support, 3  $\mu\text{m}$  polyester membranes, Corning, NY, USA), which was fitted in a cut-off polypropylene tube containing skin chamber fluid. Incubation/transmigration was allowed for 1.5 h, and cells in skin chamber fluid were collected, washed, and cultured as above in the presence or absence of LPS (100 ng/ml) or GM-CSF (100 ng/ml) for 20 h.

## RESULTS

### Analyses of in vivo-transmigrated cells

Transmigrated cells (tissue neutrophils) were collected from skin chambers after 24 h and compared with neutrophils separated from peripheral blood from the same donor. Flow cytometry showed that the majority of cells (mean  $86.8\% \pm \text{SEM } 4.4\%$ ;  $n=13$ ) in the skin chambers were neutrophils on the basis of size and granularity (Fig. 1A) with monocytes occasionally present ( $2.51\% \pm \text{SEM } 1.5\%$ ;  $n=13$ ). Microscopic examination also showed mainly viable neutrophils with characteristic lobular nuclei in both preparations (Fig. 1B), which were supported by flow cytometry analysis of cell viability with Annexin V and 7-AAD (Fig. 1C).

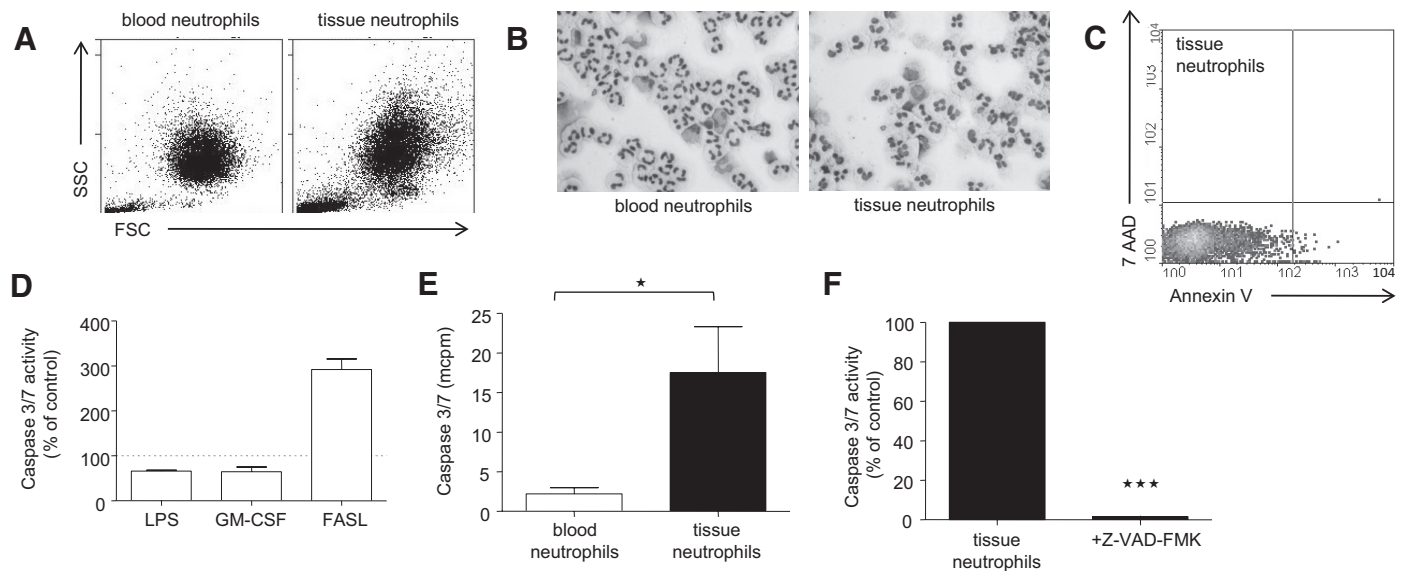
Visible signs of cell death, e.g., morphological changes and PS exposure, are preceded by activation of the caspase cascade. Culture of blood neutrophils for 4 h in the presence of proapoptotic anti-Fas antibody resulted in increased activity of caspase 3/7, and presence of the antiapoptotic factors LPS or GM-CSF during culture decreased the activity of caspase 3/7 (Fig. 1D). We next compared the activity of caspase 3/7 in blood and tissue neutrophils. Tissue neutrophils displayed increased activity of caspase 3/7 compared with blood neutrophils (Fig. 1E), suggesting that in vivo transmigration induces caspase activation. Cellular caspase 3/7 activity was totally blocked by incubation with the general caspase inhibitor Z-VAD-FMK (Fig. 1F). These data imply that despite having transmigrated into an inflammatory milieu, tissue neutrophils have activated caspase 3/7 without showing later signs of apoptosis, e.g., nuclear condensation and PS exposure.

### Apoptosis after in vitro culture

As tissue neutrophils showed increased caspase 3/7 activity to start with, we expected to find high levels of apoptosis as compared with blood cells after subsequent in vitro culture. However, levels of spontaneous cell death (apoptosis and necrosis) in blood neutrophils (mean  $71.0\% \pm \text{SEM } 4.63$ ;  $n=8$ ) and tissue neutrophils (mean  $65.8\% \pm \text{SEM } 3.62$ ;  $n=8$ ) did not differ significantly after 20 h in culture. A more detailed examination of spontaneous cell death showed slightly decreased apoptosis and increased necrosis in tissue neutrophils compared with blood neutrophils (Fig. 2A). The rate of apoptosis in in vitro-cultured neutrophils from peripheral blood can be manipulated, delayed or accelerated, by addition of anti- or proapoptotic factors, respectively. We wanted to see if the rate of cell death was susceptible to in vitro manipulation also in tissue neutrophils from skin chambers. Samples of blood neutrophils and tissue neutrophils were incubated for 20 h in the presence of antiapoptotic LPS or proapoptotic anti-Fas antibody capable of cross-linking the death receptor Fas. Whereas Fas cross-linking significantly enhanced cell death, tissue neutrophils were completely unaffected by LPS regarding apoptosis and necrosis (Fig. 2A). To investigate whether the inability to increase survival in response to LPS was specific for this particular stimulus, we next cultured cells in the presence of a wide variety of factors known to enhance viability of blood neutrophils. Whereas PGN, LTA,  $\text{P}_3\text{C}$ , GM-CSF and rSAA all significantly increased viability of blood neutrophils, tissue neutrophils were completely unaffected (Fig. 2B), indicating that the resistance of tissue neutrophils to antiapoptotic stimulation is not specific for LPS.

Neutrophils accumulate in the skin chambers over a 12-h period [26], and it is impossible to obtain blood neutrophils of identical age as the tissue neutrophils. We tested the ability to regulate apoptosis of blood neutrophils separated from 18- to 24 h old buffy-coats. In an identical manner as neutrophils from freshly drawn blood, neutrophils from aged buffy-coats responded to pro- and antiapoptotic stimulation (Fig. 2C). These data indicate that a difference in age between the blood and tissue neutrophils used in our study is not likely to explain the functional disparities between the two cell types.





**Figure 1. Characterization of tissue neutrophils.** Freshly prepared neutrophils from peripheral blood and skin chambers (tissue neutrophils) of the same donor were examined by flow cytometry. SSC, Side-scatter; FSC, forward-scatter (A). Cell preparations were also cytopun and stained with Giemsa/May Grünwald solutions for microscopic evaluation; a majority of cells were neutrophils with typical viable morphology (B). Viability of fresh tissue neutrophils was also monitored by Annexin V/7-AAD staining (C). Representative experiments from 13 different donors are shown. Blood neutrophils were incubated with buffer, anti-Fas antibody (FASL; 10  $\mu$ g/ml), LPS (100 ng/ml), or GM-CSF (100 ng/ml) for 4 h, washed, and evaluated for activity of caspase 3/7. Treatment with FASL resulted in elevated activity of these caspases in the cells, and LPS and GM-CSF decreased the caspase activity compared with buffer (dotted line, D;  $n=2-3$ ; shown are mean+SEM). Activation of caspase 3/7 was evaluated in freshly prepared blood and tissue neutrophils. Tissue neutrophils (black bar) showed increased activity of caspase 3/7 compared with almost undetectable activity of these caspases in blood neutrophils (white bar, E;  $n=6$ ). The tissue neutrophils were incubated for 4 h, with or without addition of the nonselective caspase inhibitor Z-VAD-FMK (100  $\mu$ M), washed, and thereafter, evaluated for caspase activity. Complete inhibition of caspase activity was seen in Z-VAD-FMK-treated cells (F;  $n=3$ ). Shown are mean + SEM, and statistical analysis was performed using a Student's *t* test (\* $P<0.05$ ; \*\*\* $P<0.0001$ ).

Our data show that tissue neutrophils from skin chambers die at a similar rate as blood neutrophils when cultured *in vitro* without additional stimulation. We also found that viability of blood and tissue neutrophils could be increased by Z-VAD-FMK (Fig. 2D), indicating that cell death was largely caspase-dependent. The most surprising finding, however, was that tissue neutrophils were completely resistant to the action of a wide variety of factors that significantly enhanced the lifespan of blood neutrophils.

### Transmigration involves alteration of cell surface receptors

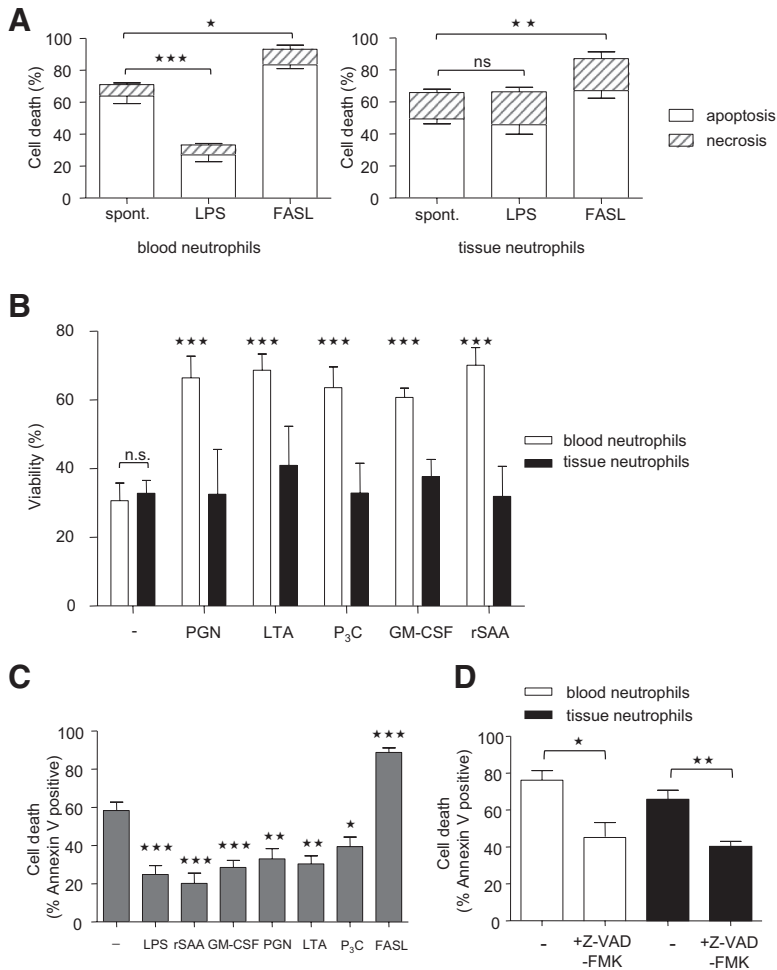
Earlier studies have shown that neutrophils from skin chambers have mobilized intracellular granules and thereby, altered their expression of cell surface receptors [19]. We corroborated these findings; tissue neutrophils displayed markedly increased surface expression of adhesion molecules CR1 and CR3 compared with blood neutrophils (Fig. 3A), indicating that mobilization of secretory vesicles and gelatinase granules has occurred during transmigration. Another activation feature on neutrophils is the shedding of L-selectin from the surface; whereas blood neutrophils expressed high levels of L-selectin, tissue neutrophils lacked this molecule (Fig. 3A).

One explanation to why tissue neutrophils did not respond to antiapoptotic stimulation could be that cell surface recep-

tors for antiapoptotic factors are removed as a consequence of transmigration. As PGN, LTA,  $P_3C$ , and LPS all are ligands for TLRs, we evaluated the expression levels of TLR2 and TLR4, as well as the expression level of GM-CSFR on tissue neutrophils and blood neutrophils. Surface expression of TLR2 and GM-CSFR was elevated on tissue neutrophils compared with blood neutrophils (Fig. 3B). TLR4 was undetectable on both neutrophil populations (data not shown). We concluded that receptors for antiapoptotic stimuli were present on the surface of tissue neutrophils and that lack of receptors did not explain the resistance to antiapoptotic stimulation displayed by these cells.

### Transmigration involves alterations in IL-8 production

TLRs also mediate other proinflammatory events in neutrophils besides delayed apoptosis, e.g., stimulation of IL-8 production [27]. To investigate whether TLR2 on tissue neutrophils was still functional with regard to IL-8 production, we cultured cells with  $P_3C$  and quantified IL-8 in cell lysates. Unstimulated tissue neutrophils contained much more IL-8 compared with blood neutrophils, and  $P_3C$  stimulation significantly increased IL-8 production even more (Fig. 4A). IL-8 production of blood and tissue neutrophils was also enhanced by stimulation with the TLR4 agonist LPS (Fig. 4B). These results indicate that receptors for antiapoptotic factors are available on tissue neutrophils and capable of



**Figure 2. In vitro modulations of apoptosis.** Neutrophils (blood neutrophils, left; tissue neutrophils, right) were incubated with LPS or FASL for 20 h at 37°C in 5% CO<sub>2</sub>. Cell death was thereafter assessed as Annexin V-positive and/or 7-AAD-positive events (apoptosis, white bars; necrosis, hatched bars) using flow cytometry (A;  $n=6-8$ ). Neutrophils (blood neutrophils, white bars; tissue neutrophils, black bars) were incubated with buffer, PGN (10  $\mu$ g/ml), LTA (10  $\mu$ g/ml), P<sub>3</sub>C (100 ng/ml), GM-CSF (100 ng/ml), or rSAA (2  $\mu$ M), for 20 h before viability (events negative for Annexin V and 7-AAD), was assessed by flow cytometry (B;  $n=3-6$ ). Also, neutrophils derived from buffy-coats that were stored in room temperature for 18–24 h prior to cell isolation were stimulated with buffer, LPS, rSAA, GM-CSF, PGN, LTA, P<sub>3</sub>C, or FASL, incubated overnight, and subjected to subsequent evaluation of cell death (Annexin V-positive cells) using flow cytometry (C;  $n=3-8$ ). In a separate set of experiments, neutrophils (blood neutrophils, white bars; tissue neutrophils, black bars) were also cultured with or without addition of Z-VAD-FMK (100  $\mu$ M) for 20 h before evaluation of cell death (D;  $n=3-4$ ). Shown are mean apoptosis ( $\pm$ SEM) and necrosis ( $\pm$ SEM; A) and mean  $\pm$  SEM (B–D) with statistical analyses performed using one-way ANOVA, followed by Dunnett's multiple comparison test ( $*P<0.05$ ;  $**P<0.005$ ;  $***P<0.0001$ ) compared with unstimulated control for respective neutrophil type (A–C) or a Student's  $t$  test ( $*P<0.05$ ;  $**P<0.005$ , D).

transmitting the signals leading to IL-8 production. The inability of tissue neutrophils to slow down the apoptotic rate is likely a result of events occurring along specific cell death pathways.

We next sought to explain the increased IL-8 levels in unstimulated tissue neutrophils and evaluated the content of IL-8 in freshly prepared cells. Compared with blood neutrophils, tissue neutrophils contained significantly more IL-8 already to start with (Fig. 4C), which could explain the higher IL-8 levels after stimulation and in vitro culture (Fig. 4A and B).

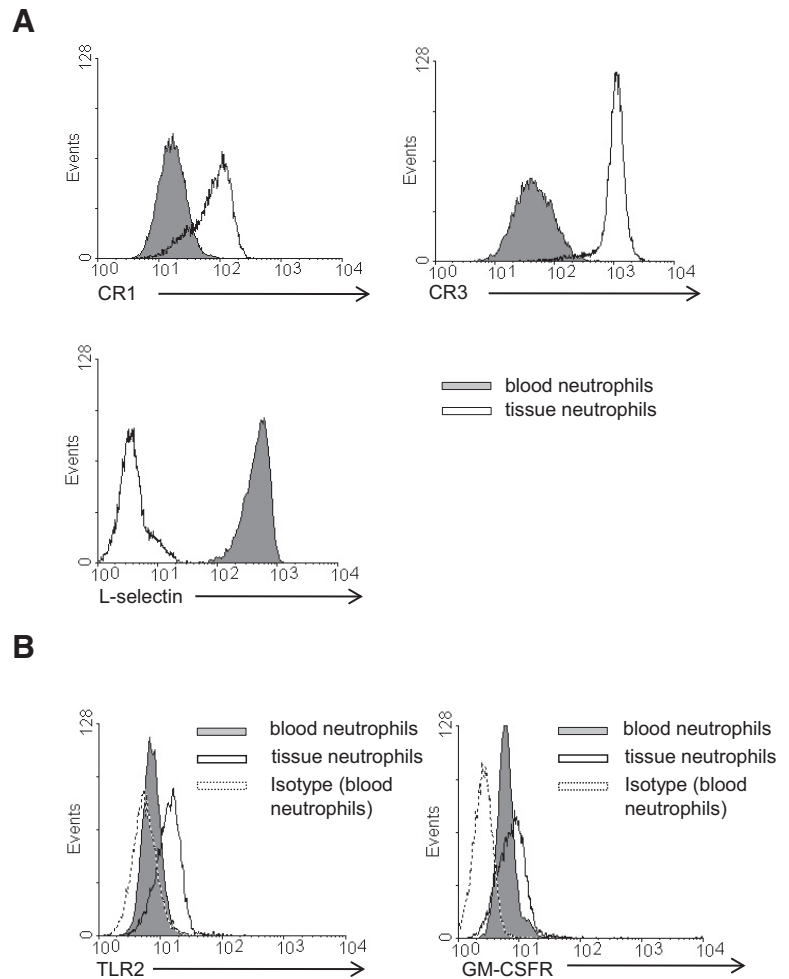
### Analysis of skin chamber fluid

The chemoattractants mainly responsible for attracting neutrophils to the skin chambers are C5a and IL-8 [28, 29], but several additional factors are also present in the chamber fluid. We subjected the skin chamber fluid to a multiplex analysis of cytokines, measuring levels of IL-8, IL-6, IL-1 $\beta$ , TNF- $\alpha$ , IL-10, IL-12, IL-17, GM-CSF, IFN- $\gamma$  and IP-10. All cytokines except IP-10 were elevated in skin chamber fluid compared with sera; the proinflammatory cytokines IL-8, IL-6, IL-1 $\beta$ , and TNF- $\alpha$  especially showed substantial increases (Fig. 5 and Supplemental Fig. 1). Many of the cytokines present in skin chamber

fluid, not least IL-8, have the ability to attract and activate neutrophils but also to affect the apoptotic process of these cells.

### Regulation of apoptosis after in vitro transmigration

One possible explanation for the resistance to antiapoptotic stimulation of tissue neutrophils is that these cells are exposed to the cytokines in the chamber fluid that somehow induce a block in antiapoptotic signaling. We incubated freshly prepared blood neutrophils in skin chamber fluid for 1.5 h, after which, the cells were washed and recultured for 20 h in the presence or absence of LPS or GM-CSF. Cells that had been preincubated in chamber fluid were still able to delay cell death in response to GM-CSF (Fig. 6), indicating that contact with soluble inflammatory mediators could not fully explain the resistance to antiapoptotic stimulation by tissue neutrophils. We also tried more closely to mimic tissue neutrophils by including an in vitro transmigration step to this experimental set-up. We allowed freshly prepared blood neutrophils to migrate through a polyester membrane into chamber fluid before the transmigrated cells were washed and cultured in the presence or absence of LPS or GM-CSF. Also, these cells responded to GM-CSF by delayed apoptosis (Fig. 6), indicating



**Figure 3 Surface alterations of tissue neutrophils.** Cellular activation and degranulation were evaluated by measuring surface expression of L-selectin, CR1, and CR3 on tissue neutrophils (open histograms) and blood neutrophils (filled histograms; A). Freshly prepared neutrophils (blood neutrophils, filled histograms; tissue neutrophils, open histograms) were stained for surface expression of TLR2 ( $n=4$ ) or GM-CSFR ( $n=3$ ). Isotype controls (dotted histograms) for blood neutrophils are used as control (B). Shown are representative experiments out of three to five independent donors.

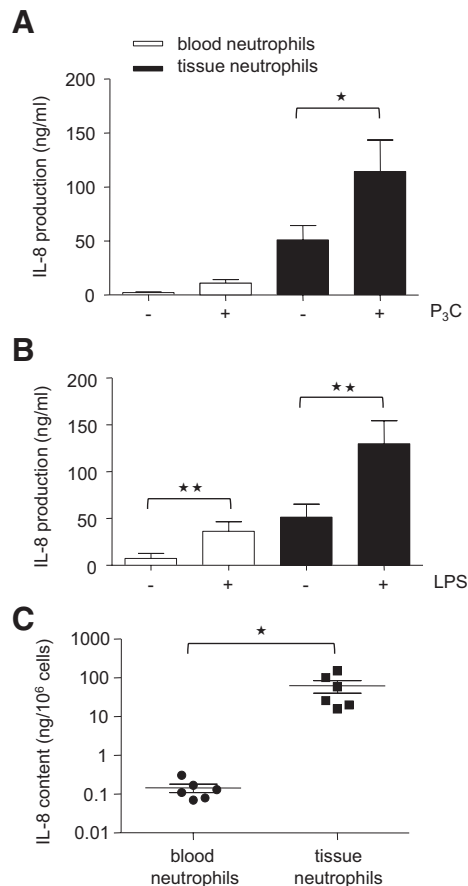
that in vitro transmigration did not fully confer resistance to antiapoptotic stimulation.

## DISCUSSION

Neutrophils are short-lived cells, and the way apoptosis is regulated in these cells has almost exclusively been studied using cells obtained from peripheral blood. We set out to explore the way cell death is regulated in neutrophils after they have left the circulation and migrated to the tissue. For this purpose, we used a skin chamber technique, where cells migrate to a moderate aseptic inflammation, driven primarily by complement factors and endogenous chemokines [26]. Our analysis of chamber fluid confirms that this is in fact a potentially proinflammatory milieu with high levels of multiple cytokines known to drive inflammation. Tissue neutrophils are drastically changed with regard to gene transcription [18], and it is not unlikely that additional changes occur during the actual transmigration processes, and many different mechanisms probably contribute to making tissue neutrophils functionally different from blood neutrophils.

Direct viability of skin chamber tissue neutrophils has previously only been determined using rather crude methods

(Trypan blue exclusion) [30, 31] and LDH release [32], which only pick up membrane permeability indicative of necrotic cell death; these reports as well as our data indicate very low levels of initial necrosis in skin chambers. We also found very low levels of apoptosis, as measured by Annexin V binding, in fresh tissue neutrophils. However, these cells displayed significantly higher caspase 3/7 activity compared with blood neutrophils. The skin chambers were in place for 24 h, and although steps were taken to minimize age differences between blood and tissue neutrophils, it is impossible to obtain preparations of identical age. The majority of neutrophils migrates between 12 h and 24 h after addition of sera to the chambers [26], and it is possible that the increased caspase activity stems from the oldest (first to transmigrate) subpopulation of cells. Regardless of caspase activation, blood neutrophils and tissue neutrophils displayed similar levels of spontaneous cell death when cultured in vitro in the absence of stimulation. A previous transcriptional profiling of tissue neutrophils demonstrated up-regulation of antiapoptotic genes and down-regulation of proapoptotic genes, compared with blood neutrophils, suggestive of a "transient antiapoptotic state" [18]. The fact that tissue neutrophils in our study were no different from



**Figure 4. IL-8 content in blood and tissue neutrophils.** IL-8 content was analyzed in cell lysates (blood neutrophils, white bars; tissue neutrophils, black bars) after overnight culture, with or without addition of P<sub>3</sub>C (100 ng/ml; A) or LPS (100 ng/ml; B). Also, IL-8 content of freshly prepared cells was measured (C). Shown are mean + SEM with statistical analyses performed using paired *t* tests (A and B, *n*=6; \**P*<0.05; \*\**P*<0.005) and mean ± SD with statistical analyses performed using a Student's *t* tests (C, *n*=6; \**P*<0.05).

blood neutrophils with regard to spontaneous cell death indicates that viability is not solely regulated on a transcriptional level.

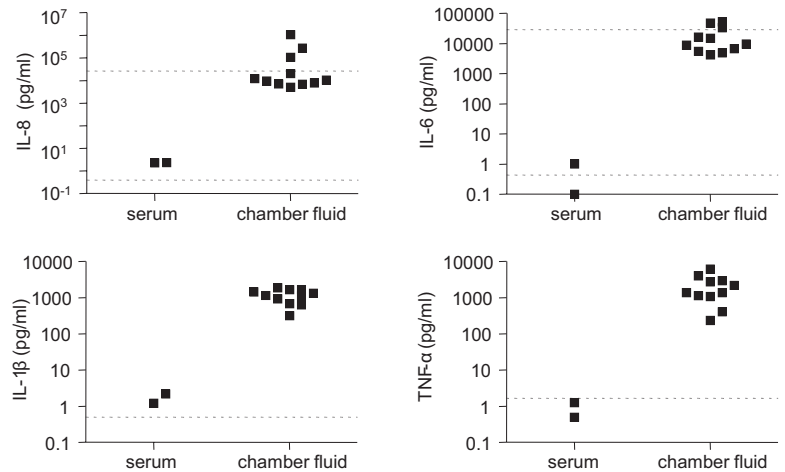
The main difference between blood and tissue neutrophils with regard to viability was that tissue neutrophils failed to delay cell death upon antiapoptotic stimulation, which markedly curbed caspase 3/7 activity of blood neutrophils, and preliminary data indicate that suppression of caspase 3/7 activity in tissue neutrophils is much harder to achieve. It could be that the heightened caspase activity of tissue neutrophils represents a commitment to death and that attempts to delay apoptosis need to precede caspase activation to be effective. The tissue neutrophils are likely to be slightly older than the corresponding blood neutrophils in our study. However, age alone could not explain why tissue neutrophils were resistant to antiapoptotic stimulation, as neutrophils from aged buffy-coats responded to antiapoptotic stimulation in an identical manner to neutrophils prepared from freshly drawn blood.

As mentioned, tissue neutrophils differ from blood neutrophils with respect to the expression of cell surface receptors, and we monitored expression of receptors capable of mediating neutrophil survival. We found TLR2 and GM-CSFR to be expressed on tissue neutrophils. The expression of TLR4 on human neutrophils is debated but is in all likelihood very low; with our TLR4 antibody, we could not detect this particular receptor on the surface of blood neutrophils or tissue neutrophils. Regardless, neutrophils respond well to LPS, and lipoproteins present in this nonrepurified preparation could well contribute to the activity through binding to TLR2 [33]. We also found that tissue neutrophils responded to LPS and P<sub>3</sub>C by increasing IL-8 production, showing that TLRs are functional after *in vivo* transmigration. The IL-8 content of tissue neutrophils was elevated compared with blood neutrophils already before culturing, which is probably the result of *de novo* synthesis [18, 27] and internalization of IL-8 present in the skin chamber fluid. The signals going from, e.g., TLR2 to IL-8 production are fairly well understood [34], and although the details about exactly where in this signal transduction cascade the antiapoptotic machinery is triggered are obscure, our data indicate a block along this specific antiapoptotic pathway in tissue neutrophils. The fact that tissue neutrophils were resistant to a wide variety of antiapoptotic factors of endogenous and microbial origin implies that the block is general and distant from the responsible surface receptors.

Using skin chamber technique and activation of the NADPH oxidase as a read out, it has been shown previously that tissue neutrophils behave radically different from blood neutrophils and are, for instance, primed (hyper-responsive) for subsequent activation by certain chemoattractants (e.g., formylated peptides), as the degranulation associated with transmigration results in up-regulation of chemoattractant receptors to the cell surface [35]. On the other hand, tissue neutrophils are nonresponsive to other chemoattractants (e.g., IL-8 and C5a) as a result of desensitization of certain receptors brought about by the abundance of ligands in the skin chamber fluid [29]. ROS derived from the NADPH oxidase could have a bearing on the apoptotic process, and it has been shown that blood neutrophils from patients with CGD, characterized by a nonfunctional NADPH oxidase, display lower levels of spontaneous apoptosis compared with ROS-competent neutrophils [36, 37]. That tissue neutrophils display an altered ability to generate ROS [29, 38] suggests that ROS could somehow be involved in the regulation of cell death. However, by using blood neutrophils from CGD patients [23], we clearly showed that these ROS-deficient cells are fully responsive to a proapoptotic anti-Fas antibody as well as antiapoptotic LPS (Supplemental Fig. 2). Although this indicates that regulation of apoptosis occurs independently of NADPH oxidase-derived ROS, it is not known how tissue neutrophils from CGD patients behave in this respect.

It is possible that the cytokine-rich chamber fluid somehow makes tissue neutrophils resistant to antiapoptotic stimulation. Among the cytokines found in chamber fluid (Fig. 5 and Supplemental Fig. 1), several have been described as

**Figure 5. Analysis of skin chamber fluid.** Cytokine content in sera ( $n=2$ ) and skin chamber fluid ( $n=11$ ) was assessed by a multiplex assay. The concentrations of the proinflammatory cytokines IL-8, IL-6, IL-1 $\beta$ , and TNF- $\alpha$  were all 1000-fold elevated in skin chamber fluid compared with sera. Dotted lines represent detection limits. Extended analysis of skin chamber fluid can be found in Supplemental figure.



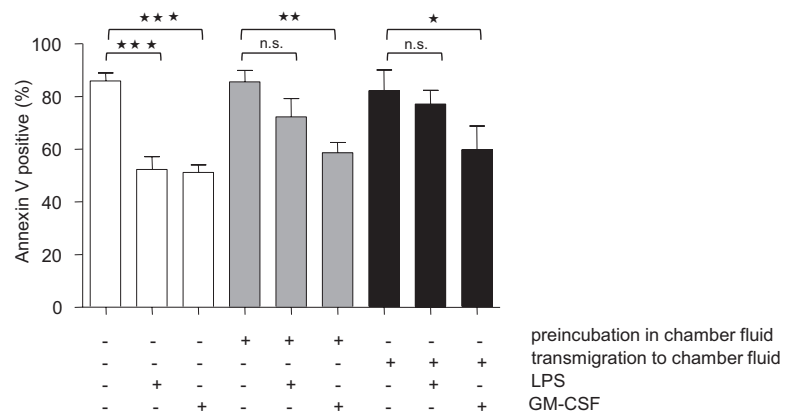
modulators of neutrophil apoptosis: IL-6 and TNF- $\alpha$  have been ascribed pro- and antiapoptotic activities, depending on dose and time [8], whereas GM-CSF, IL-1 $\beta$ , and IFN- $\gamma$  are potently antiapoptotic [39]. To test directly if the skin chamber fluid per se induced resistance to subsequent antiapoptotic stimulation, we incubated blood neutrophils in chamber fluid before culturing in the presence or absence of GM-CSF or LPS. This treatment made cells respond poorly to LPS, whereas the responsiveness to GM-CSF was intact. Similar data were obtained when an in vitro transmigration step was added; blood neutrophils that had crossed a polyester membrane into skin chamber fluid were also not fully resistant to antiapoptotic stimulation. We were thus unable to completely mimic the phenotype of tissue neutrophils by in vitro manipulation of blood neutrophils, suggesting that in vivo transmigration uniquely triggers the shift in antiapoptotic responsiveness.

The skin chamber technique used in this study is a relatively well-controlled and reproducible method to obtain large numbers of in vivo-transmigrated neutrophils. It is however possible that these tissue neutrophils differ from tissue neutrophils found during other inflammatory episodes, where neutrophils are abundant. Analysis of in vivo-transmigrated neutrophils from human lungs demonstrated an antiapoptotic transcription profile, accompanied by delayed rates of spontaneous ap-

optosis, compared with blood neutrophils [40]. However, the model used to obtain lung neutrophils depends on instillation of LPS into the lung segment prior to collecting the cells by an extensive negative-selection strategy, and the results obtained are thus not directly comparable with those of our study. Furthermore, the ability of lung neutrophils to respond to antiapoptotic stimulation in vitro was not tested. Future investigations will hopefully determine whether our results are specific for tissue neutrophils from skin chambers or if neutrophils that have transmigrated to other inflamed organs are equally resistant to antiapoptotic stimulation.

In conclusion, this study solidifies the notion that human neutrophils that have transmigrated in vivo into skin chambers are different from blood neutrophils in that they have undergone degranulation and thereby changed their surface flora of molecules and contain elevated levels of IL-8. We also add to the list of differences that tissue neutrophils display increased caspase activity and are resistant to subsequent antiapoptotic stimulation. The main sites where neutrophils are of importance are tissues that the cells reach after transmigration from blood. Thus, increased knowledge about the behavior of transmigrated neutrophils would be very helpful for our overall understanding of how these cells live and die during healthy, as well as in pathological, settings.

**Figure 6. Modulation of apoptosis after in vitro transmigration.** Freshly prepared blood neutrophils were incubated in skin chamber fluid or were allowed to transmigrate through a transwell filter with skin chamber fluid as chemoattractant. The cells were harvested after 1.5 h. Cells in the skin chamber fluid (transmigrated, black bars; directly incubated, gray bars) were washed and cultured with or without addition of LPS (100 ng/ml) or GM-CSF (100 ng/ml). Blood neutrophils cultured directly, without any contact with skin chamber fluid (white bars), were used as controls. Shown are mean  $\pm$  SEM ( $n=6$ ) with statistical analysis performed using one-way ANOVA, followed by Dunnett's multiple comparison test ( $*P<0.05$ ;  $**P<0.005$ ;  $***P<0.0001$  compared with unstimulated samples with the same pretreatment).





## AUTHORSHIP

K.C. and J.B. wrote the paper, designed the experiments, and analyzed data. K.C., L.B., and C.M. performed experiments with assistance from J.K., M.S., and C.D. regarding the skin chamber technique. Experiments and analysis of CGD data were performed by J.B. and D.P.S. All authors revised and approved the manuscript.

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## KEY WORDS:

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