

## Letter to the Editor

**Impaired microbial killing by neutrophils from patients with protein kinase C delta deficiency**

To the Editor:

Neutrophils are of critical importance in the host defense against bacterial and fungal infections. Upon microbial phagocytosis, neutrophils use at least 2 major and well-established intracellular killing mechanisms, that is, the production of reactive oxygen species (ROS) by the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, and the release of antimicrobial components from intracellular granules.<sup>1</sup> Protein kinase C delta (PKC $\delta$ ) is a serine and threonine kinase and a member of the so-called novel PKC isoenzyme family.<sup>2</sup> PKC $\delta$  is expressed in neutrophils<sup>3</sup> and can mediate the phosphorylation of the p47<sup>phox</sup> subunit of NADPH oxidase, suggesting a regulatory function in ROS production.<sup>4</sup> However, in the absence of specific inhibitors of PKC $\delta$  activity, a direct functional relevance for PKC $\delta$  in human neutrophils has not been established<sup>2</sup> and genetic manipulation of human neutrophils is essentially impossible due to the very short life span of these cells.

Here, we have studied the role of PKC $\delta$  by using neutrophils from recently reported patients with an inherited PKC $\delta$  deficiency.<sup>5,6</sup> These patients suffer from systemic autoimmunity and recurrent infections. Although the immunosuppressive medication to treat the autoimmune disease is a confounding factor, the patients may be intrinsically susceptible to recurrent infections.<sup>6</sup> Our findings illustrate that PKC $\delta$ -deficient neutrophils have a markedly decreased capacity to kill bacterial and fungal pathogens. This is particularly remarkable because their ability to phagocytose, produce ROS through NADPH oxidase, as well as their capacity to release granule-derived components is virtually unimpaired. Our work provides direct evidence for the involvement of PKC $\delta$  in neutrophil microbial killing through a previously unreported nonoxidative cytotoxic mechanism.

To evaluate the function of PKC $\delta$  in human neutrophils, we used purified neutrophils from 3 patients with 2 distinct mutations in the *PRKCD* gene (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Patient A, suffering from recurrent infections and autoimmunity, has a splice-site mutation in the *PRKCD* gene (c.1352+1G>A), leading to the lack of expression of PKC $\delta$ .<sup>6</sup> Similarly, a complete absence of PKC $\delta$  in neutrophils from this patient was observed (Fig 1, A). In addition, we used neutrophils from 2 other patients, both carrying a homozygous missense mutation in the *PRKCD* gene (siblings; c.1528G>A; p.G510S), with systemic lupus erythematosus<sup>5</sup> whose B cells showed minimal expression of PKC $\delta$ .<sup>5</sup> The neutrophils of these patients expressed residual levels (~20%-30% of normal controls) of PKC $\delta$  protein (Fig 1, A). Levels of PKC $\alpha$  and PKC $\beta$ I/II were comparable to those in controls (see Fig E1, A and B, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

In neutrophils, different PKC isoforms become activated on ligation of, for instance, G-protein-coupled receptors<sup>7</sup> or directly by the generic PKC agonist phorbol-12-myristate

13-acetate.<sup>4</sup> The ability of neutrophils from PKC $\delta$ -deficient patients to activate NADPH oxidase was tested by measuring extracellular H<sub>2</sub>O<sub>2</sub> production. PKC $\delta$ -deficient neutrophils displayed only a minor reduction in the phorbol-12-myristate 13-acetate-induced production of extracellular H<sub>2</sub>O<sub>2</sub> (Fig 1, B) and responded completely normally to more physiologically soluble (ie, platelet-activating factor/formyl-Met-Leu-Phe) and particulate stimuli, including (opsonized) zymosan and relevant gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria and yeast (*Candida albicans*) (Fig 1, B and C). For comparison, neutrophils isolated from patients with chronic granulomatous disease (see Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) carrying mutations in any one of the NADPH oxidase subunits show no detectable NADPH oxidase activity whatsoever (Fig 1, B). We also measured intracellular ROS production using dihydrorhodamine-labeled microbes, and this again showed normal intracellular ROS production (see Fig E2, A and B, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Collectively, these findings demonstrate that PKC $\delta$  does not have a generic role in triggering the respiratory burst in human neutrophils. Next, the ability of PKC $\delta$ -deficient neutrophils to kill microbial pathogens *in vitro* was investigated. Intracellular microbial killing by neutrophils involves at least 2 principal effector mechanisms, that is, ROS generated by NADPH oxidase and mobilization of various antimicrobial (poly)peptides from stored granules. Killing of gram-positive *S aureus* bacteria is primarily mediated by NADPH oxidase, whereas killing of gram-negative *E coli* involves a combination of both these mechanisms<sup>8</sup> (Fig 2, B and D). Neutrophils of the PKC $\delta$ -null patient A displayed severely impaired killing of *S aureus*, whereas no defect was seen with the other patients (Fig 2, B and C) who exhibit residual PKC $\delta$  expression (Fig 2, A and B). Killing of *E coli* was significantly reduced in all the 3 patients (Fig 2, C and D). These findings suggest that in spite of an apparently normal NADPH oxidase function, PKC $\delta$ -deficient neutrophils have a substantially impaired bacterial-killing capacity.

Subsequently, a role for PKC $\delta$  in fungal killing was explored. Neutrophils are known to play a major role in the host defense against fungal infections, including those caused by *Candida*, and mice lacking PKC $\delta$  are highly susceptible to *Candida* infections.<sup>9</sup> To investigate the role of PKC $\delta$  in fungal infections in a human context, the capacity of PKC $\delta$ -deficient neutrophils to kill *C albicans* conidia was studied. Neutrophils of PKC $\delta$ -deficient patients showed substantially reduced killing of *C albicans* conidia (Fig 2, E). These findings highlight the importance of PKC $\delta$  in neutrophil fungal killing.

PKC $\delta$ -deficient neutrophils have a normal ability to phagocytize pathogens (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), which is in agreement with earlier observations in PKC $\delta$ <sup>-/-</sup> mice.<sup>9</sup> We also tested whether neutrophils from PKC $\delta$ -deficient patients display impaired granule mobilization and release of active serine proteases from granules. There was no apparent defect in the exocytosis of either azurophilic or specific granules (see Fig E4, A and B, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). We also

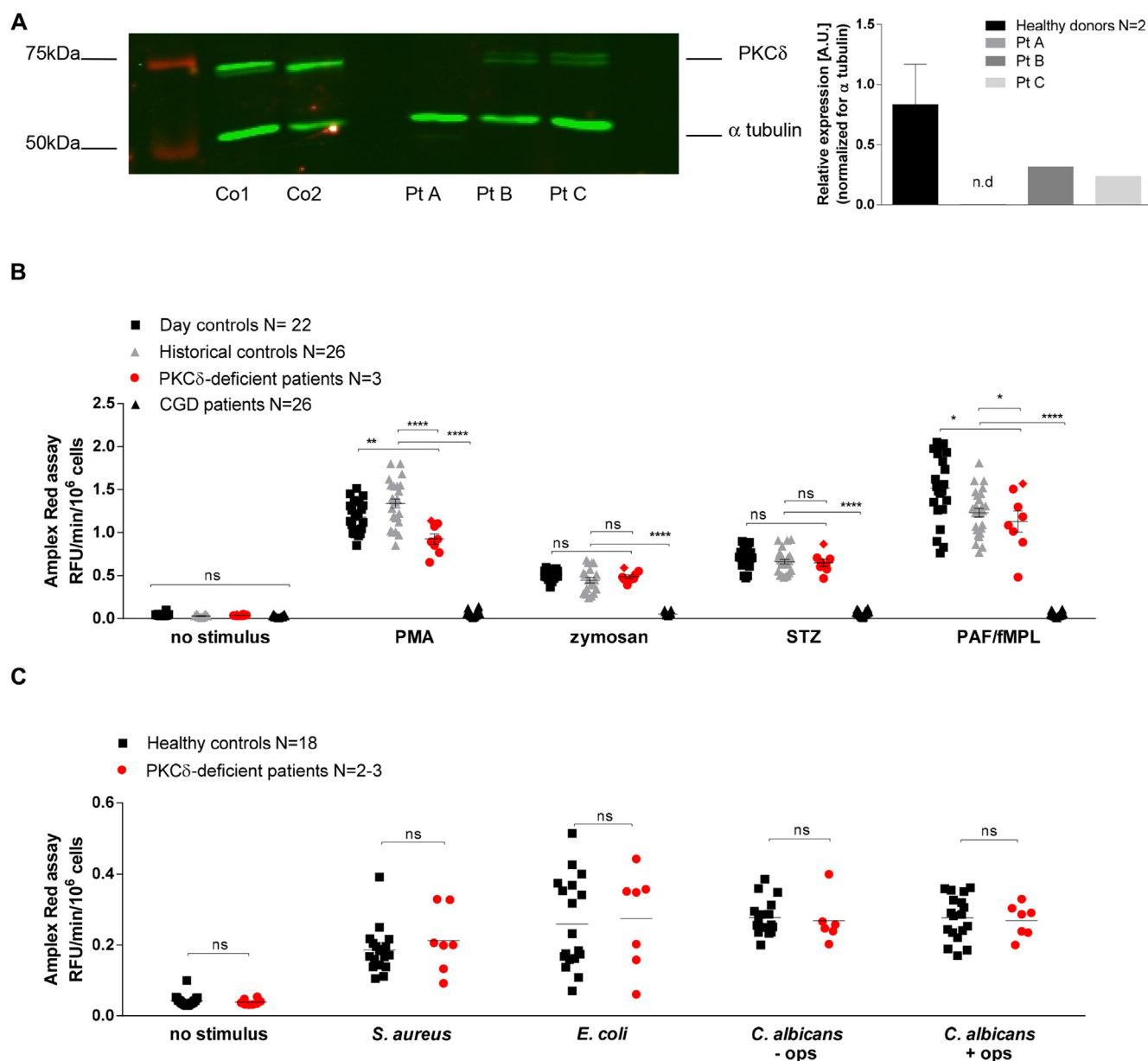


**FIG 1.** Neutrophils of PKC $\delta$ -deficient patients show normal extracellular release of H<sub>2</sub>O<sub>2</sub>. **A**, Western blot analysis performed on cell lysates of neutrophils from healthy controls and patients. Freshly isolated neutrophils of healthy donors and patients were stimulated with soluble (**B**) or bacterial (**C**) stimuli, and release of H<sub>2</sub>O<sub>2</sub> was measured by Amplex Red assay as described in the [Methods](http://www.jacionline.org) section of this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). CGD, Chronic granulomatous disease; fMPL, formyl-Met-Leu-Phe; nd, not determined; ns, not significant; ops, opsonized; PAF, platelet-activating factor; Pt, patient; RFU, relative fluorescence unit; STZ, serum-treated zymosan. \* $P < .05$ , \*\* $P < .01$ , and \*\*\*\* $P < .0001$ .

evaluated the intracellular delivery of lactoferrin and myeloperoxidase from granules to phagosomes containing *E. coli* bacteria by immuno-electron microscopy. The presence of both components in phagosomes of the PKC $\delta$ -deficient neutrophils was clearly demonstrated (see [Fig E5](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Taken together, these data essentially exclude a major defect in granule-phagosome fusion. Finally, we evaluated the expression of epitopes and the adhesive and migratory properties of the patients' neutrophils (see [Fig E6](#) in this article's Online

Repository at [www.jacionline.org](http://www.jacionline.org)). This did not reveal any notable differences with cells from healthy controls, suggesting normal neutrophil development and other functions relevant in the context of infection.

Collectively, our findings reveal a previously undescribed role for PKC $\delta$  in human neutrophils in the killing of microbes. Intriguingly, this pathway appears unrelated to the NADPH oxidase activity and granule mobilization. The observed defects in microbial killing may contribute to the recurrent infections associated with PKC $\delta$  deficiency.

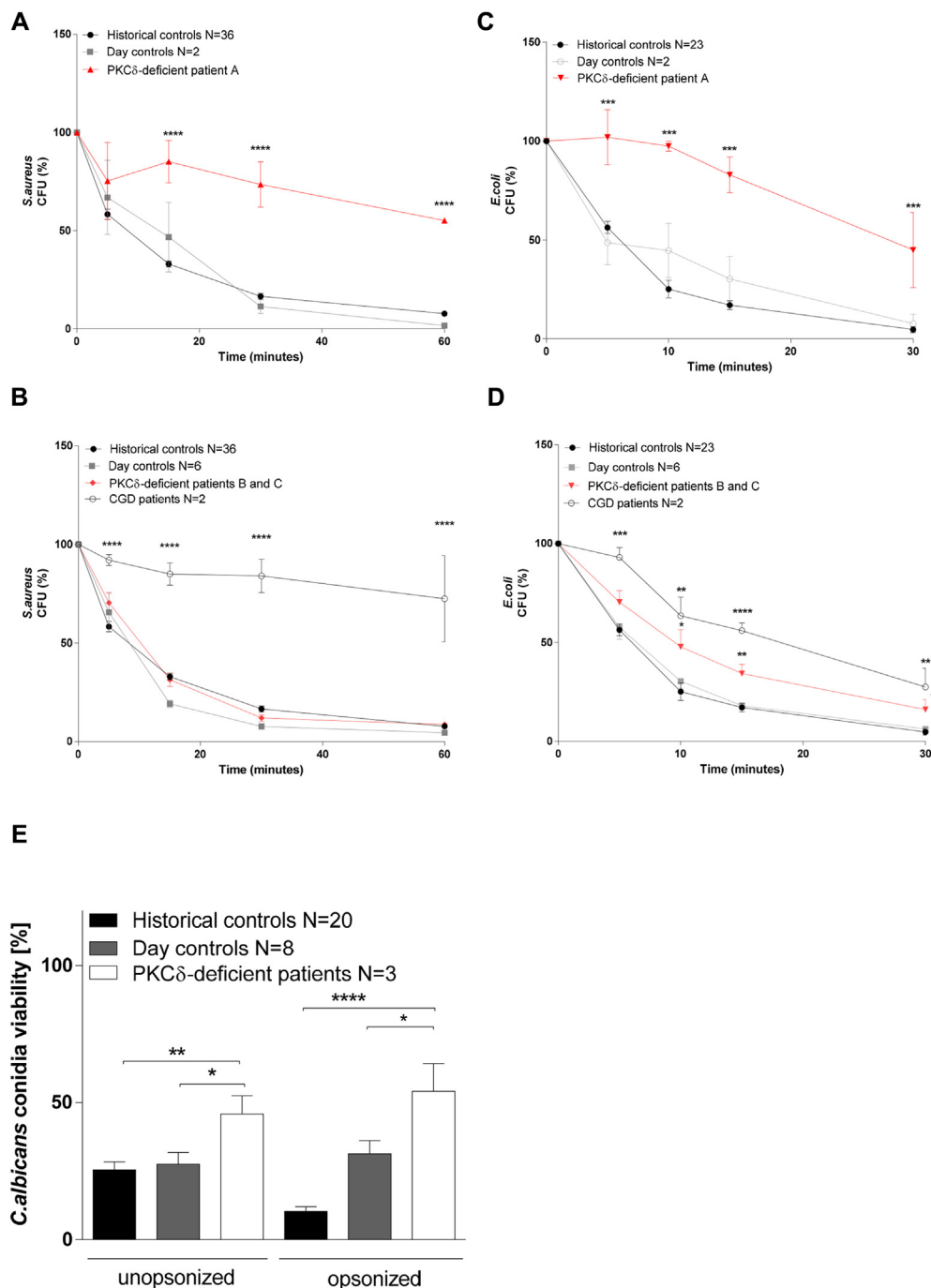


**FIG 2.** Neutrophils deficient in PKC $\delta$  have significantly decreased capacity to kill bacterial and fungal pathogens. The bacterial-killing capacity of freshly isolated neutrophils from patient A was assessed with *S. aureus* (A) and *E. coli* (C). The same killing assay was performed with freshly isolated neutrophils from patients B and C and patients with CGD with *S. aureus* (B) or *E. coli* (D). Killing of fungal pathogen by neutrophils of all PKC $\delta$ -deficient patients was determined with *C. albicans* conidia (E). Experiments were performed as described in detail in the [Methods](#) section of this article's Online Repository. CFU, Colony-forming unit; CGD, chronic granulomatous disease. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , and \*\*\*\* $P < .0001$ .

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## METHODS

### Purification of neutrophils from patients and healthy individuals

Heparinized blood was collected from healthy individuals or patients with a mutation in the *PRKCD* gene (for details, see Table E1) or from patients with mutations in the *NCF1* gene, resulting in chronic granulomatous disease (for details, see Table E2). The studies were approved by the local ethical committee of Sanquin Blood Supply, Amsterdam, The Netherlands (for healthy individuals); the Medical University of Vienna, Vienna, Austria (for patient A) and Comité de Protection des Personnes Sud-EST IV, France (for patients B and C); and Academic Medical Center, Amsterdam, The Netherlands (for patients with chronic granulomatous disease). The studies were performed after obtaining informed consent of the patients. Patients' characteristics have been described previously.<sup>E1,E2</sup> Neutrophils were purified by gradient centrifugation over Percoll from overnight transported blood (Pharmacia, Uppsala, Sweden),<sup>E3</sup> washed, and resuspended in HEPES-buffered saline solution.<sup>E4</sup> Historical controls refer to healthy donor neutrophils used in the killing assays in our laboratory during several years of unrelated experiments (for *S aureus*, *N* = 36; for *E coli*, *N* = 23; for *C albicans* conidia, *N* = 20). As day controls, neutrophils of healthy individuals were used along with patients' neutrophils (for *S aureus* and *E coli*, *N* = 4; for *C albicans* conidia, *N* = 4).

### NADPH oxidase activity

Activation of the respiratory burst was measured with the Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) Hydrogen Peroxidase Assay kit (Molecular Probes, Eugene, Ore) as described previously.<sup>E3</sup> Neutrophils were incubated with phorbol 12-myristate 13-acetate (100 ng/mL; Sigma-Aldrich, St Louis, Mo), zymosan (1 mg/mL; Sigma-Aldrich), serum-treated zymosan (STZ, 1 mg/mL), platelet-activating factor (PAF, 1  $\mu$ M; Sigma-Aldrich) followed by formyl-Met-Leu-Phe (fMLP, 1  $\mu$ M; Sigma-Aldrich), and opsonized *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* (all pathogens were added in a final concentration of  $0.25 \times 10^9$ /mL). Fluorescence was measured at 30-second intervals for 20 minutes with the Infinite 200 PRO (Tecan, Mannedorf, Switzerland). Maximal slope of  $H_2O_2$  release was assessed over a 2-minute interval to determine the activity of NADPH oxidase. Measurement of intracellular release of  $H_2O_2$  was performed with dihydrorhodamine-labeled microbes as described in detail previously.<sup>E5</sup>

### Bacterial and fungal killing

Bactericidal activity of neutrophils was assessed with the *E coli* strain ML-35 and the *S aureus* strain 502A. Survival of bacteria and *C albicans* (strain SC5314) was measured by determining colony-forming units as described previously.<sup>E6,E7</sup>

### Western blot analysis

Freshly purified neutrophils of healthy donors and patients were used to prepare total cell lysates. The samples were subjected to 7.5% sodium dodecyl-sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes were washed and blocked with 5% nonfat milk (Elk Campina, Zaltbommel, The Netherlands). The following primary antibodies were used: PKC $\delta$  (Invitrogen, Camarillo, Calif) and PKC $\alpha$  and PKC $\beta$ II (both Santa Cruz, San Diego, Calif). Membranes were incubated with antibodies in 1% nonfat milk followed by incubation with goat anti-mouse-IgG IRDye 700CW or goat anti-rabbit-IgG IRDye 800CW (LI-COR Biosciences, Lincoln, Neb). Quantification of bound antibodies was performed on an Odyssey Infrared Imaging system (LI-COR Biosciences). Immunostaining for alpha-tubulin (mouse anti-alpha-tubulin, Abcam, Cambridge, United Kingdom) followed by secondary goat anti-mouse-IgG IRDye 700CW (LI-COR Biosciences) served as a control for equal loading and electrophoretic transfer of each lane in the gels.

### Microbial phagocytosis

The capacity of neutrophils to phagocytose human serum-opsonized *E coli* and *S aureus* and unopsonized/opsonized *C albicans* conidia was determined

by flow cytometry. Fluorescently (fluorescein isothiocyanate [FITC])-labeled microbes were incubated with neutrophils, and samples were measured at different time points by using the LSRII flow cytometer equipped with FACS-Diva software (BD Biosciences, Bedford, Mass) at 37°C. Cells were gated on the basis of their forward and side scatter, and 10,000 gated events were collected per sample. Based on the neutrophils' FITC-mean fluorescence intensity and the percentage of FITC-positive neutrophils, phagocytosis was calculated relative to control immunoglobulin-treated or untreated neutrophils.

### Degranulation and release of proteases

Degranulation of azurophilic and specific granules from control and patients' neutrophils was measured by monitoring the cell surface upregulation of CD63 and CD66b, which are azurophilic and specific granules markers, respectively. Neutrophils were first incubated in HEPES-buffered saline solution at 37°C in a shaking water bath before adding the (priming) agents PAF (1 mM; Sigma-Aldrich, 5 minutes) or cytochalasin B (5 mg/mL; Sigma-Aldrich, 5 minutes) and were subsequently stimulated with fMLP (1  $\mu$ M; Sigma-Aldrich, 15 minutes). After stimulation, cells were put on ice, washed with HEPES-buffer saline solution once, and subsequently stained with antibodies against neutrophil-granule markers CD63-phycoerythrin (IgG<sub>1</sub>, 435) and CD66b-FITC (IgG<sub>1</sub>, CLB-B13.9). Extracellular release of neutrophil-granule proteases was triggered by cytoB/fMLP and measured by using the DQ-Green BSA assay, in which proteases cause an increase in fluorescence, expressed in relative fluorescence units, on the loss of auto-quenching of the substrate. In particular, the release of neutrophil-granule-derived protease activity was determined by incubating with DQ-Green BSA (10  $\mu$ g/mL; Molecular Probes, Eugene, Ore). On stimulation with cytoB/fMLP, the fluorescence was monitored at 120-second intervals for 1 hour by Infinite 200 PRO plate reader (Emission 535 nm).

### Immuno-electron microscopy

Immuno-electron microscopic identification of myeloperoxidase- and lactoferrin-containing granules after *E coli* phagocytosis was performed as described previously.<sup>E8</sup>

### Analysis of expression of surface epitopes by flow cytometry

Freshly isolated neutrophils ( $0.25 \times 10^6$ ) were stained with antibodies against EMR3, CD55, CD59, CD9, CD11b, CD66b, L-selectin, CD16, CD32, 7D5, and CD63 (all directly conjugated to FITC or phycoerythrin fluorochrome), and the intensity of fluorescence was measured on a LSRII flow cytometer equipped with FACSDiva software (BD Biosciences).

### Migration and adhesion of neutrophils

Chemotaxis in response to C5a, IL-8, or PAF and adhesion to several stimuli were measured with calcein-labeled neutrophils as described previously.<sup>E7,E9</sup>

### Statistical analysis

Statistical analysis was performed with Prism 6.01 (GraphPad Software). Data were evaluated by using the 2-tailed multiple student *t* test with the Holm-Sidak method, in which each variable was analyzed individually, without assuming a consistent SD. Each patient sample was analyzed at least twice at different occasions. The results are presented as mean  $\pm$  SEM, and a significant difference was assessed as a *P* value of less than .05.

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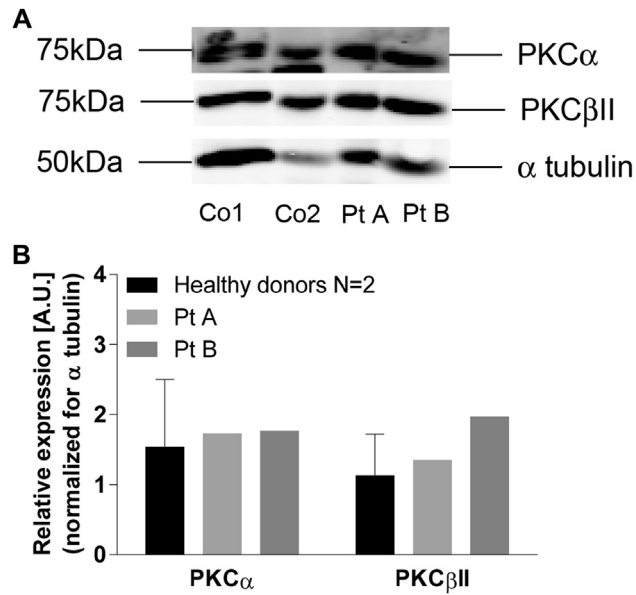


**FIG E1.** Western blot analysis of PKC $\alpha$  and  $\beta$  expression by human neutrophils. Whole-cell lysates were prepared as described in the [Methods](#) section, and the expression of PKC $\alpha$  and  $\beta$ II was detected with specific antibodies (**A**). Quantification of protein expression is depicted relative to  $\alpha$  tubulin as a loading control (**B**). *Pt*, Patient.

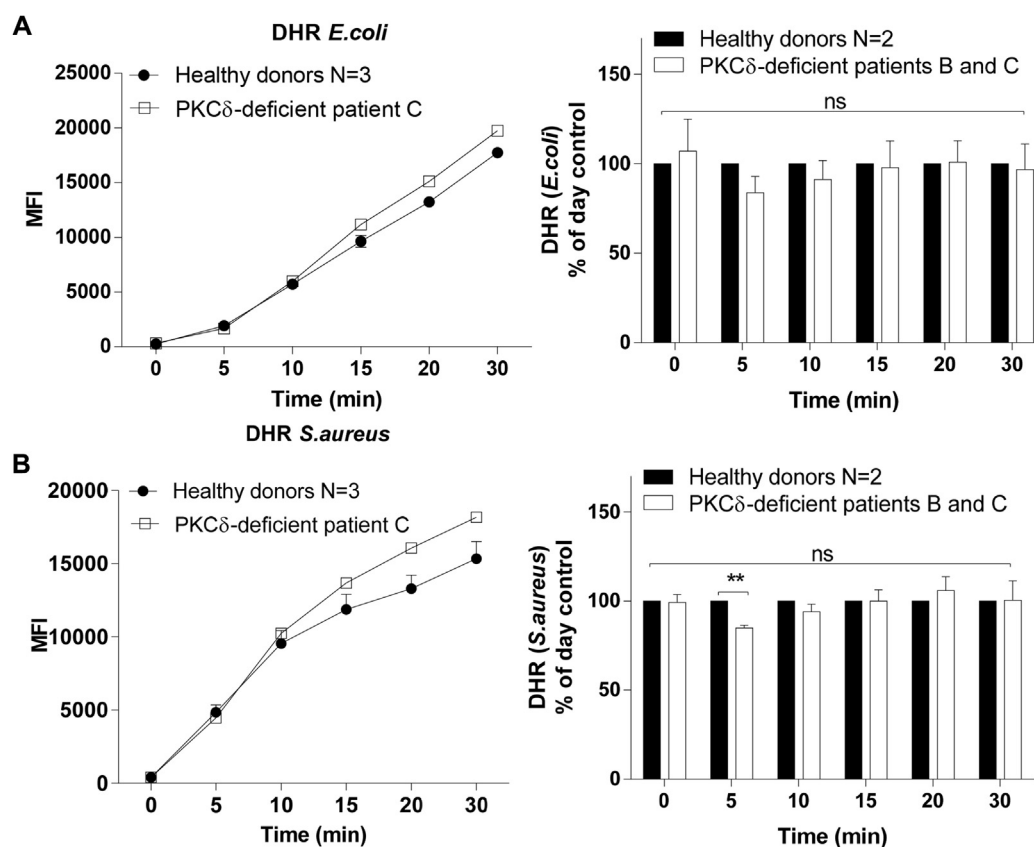
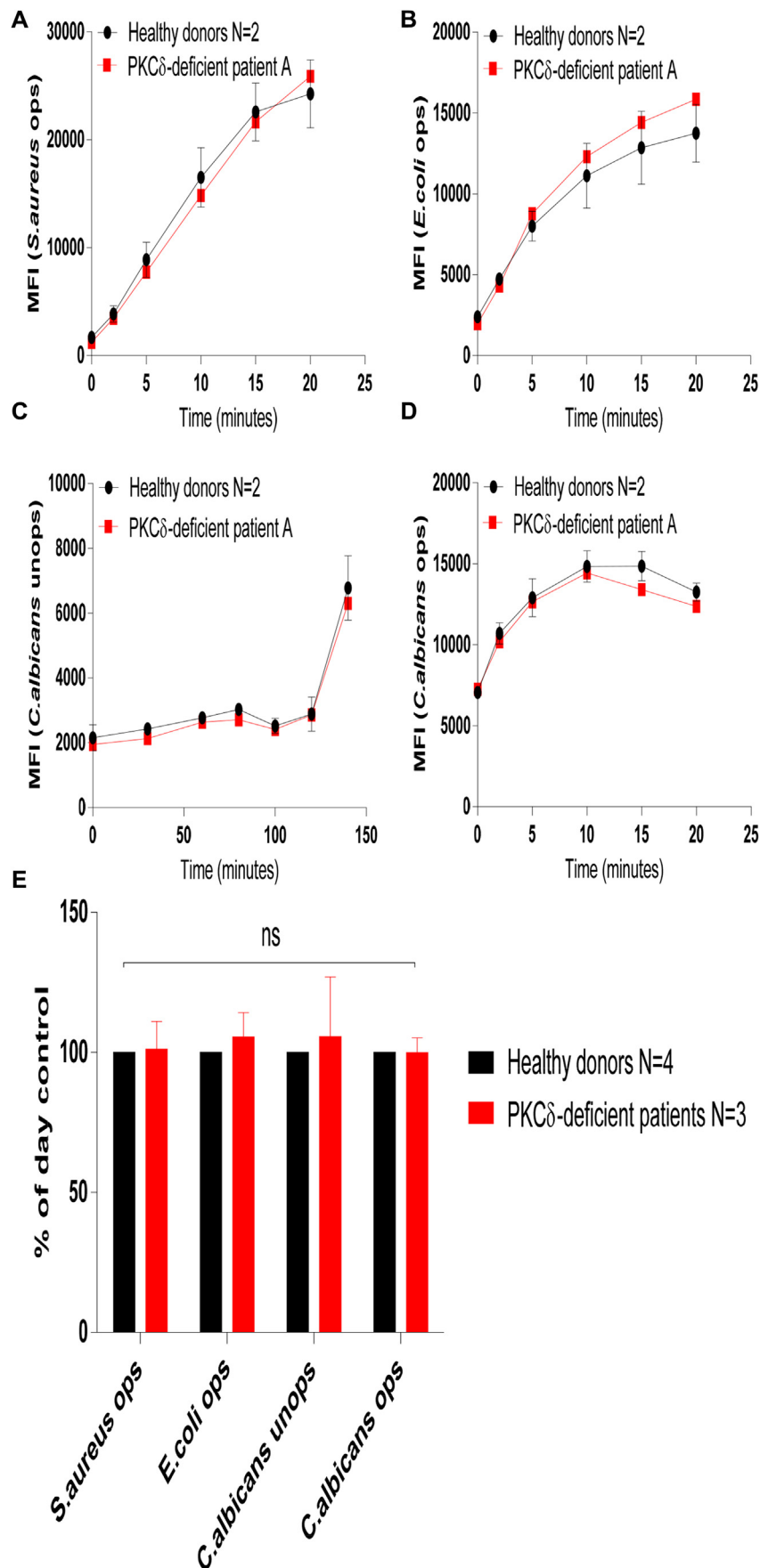


**FIG E2.** Intracellular ROS production as determined by the dihydrorhodamine (DHR) assay is not affected in PKC $\delta$ -deficient neutrophils. Intracellular production of ROS was measured after purified neutrophils were stimulated with DHR-labeled opsonized *E. coli* (A) or *S. aureus* (B). Figures on the left show examples of kinetics of the fluorescence measurement by flow cytometry. Figures on the right show average ROS production measured by DHR oxidation on the surface of bacteria by freshly isolated neutrophils of healthy donors and PKC $\delta$ -deficient patients. Because of high interday variation, values obtained from neutrophils of healthy donors were set at 100% and measurements from neutrophils from patients were calculated as a percentage of a healthy control. Statistics were performed by using the multiple *t* test with the Holm-Sidak method, in which  $\alpha = 5.000\%$ . MFI, Mean fluorescence intensity. \*\**P* < .01.



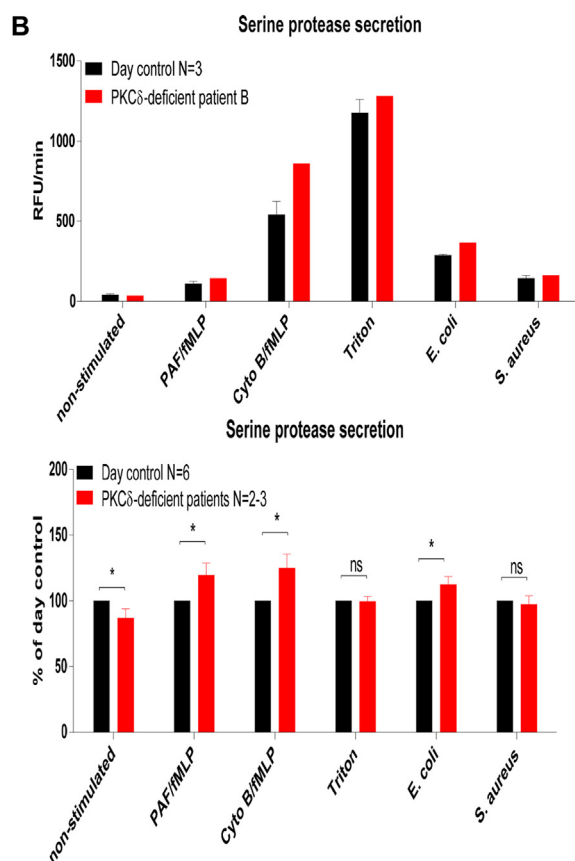
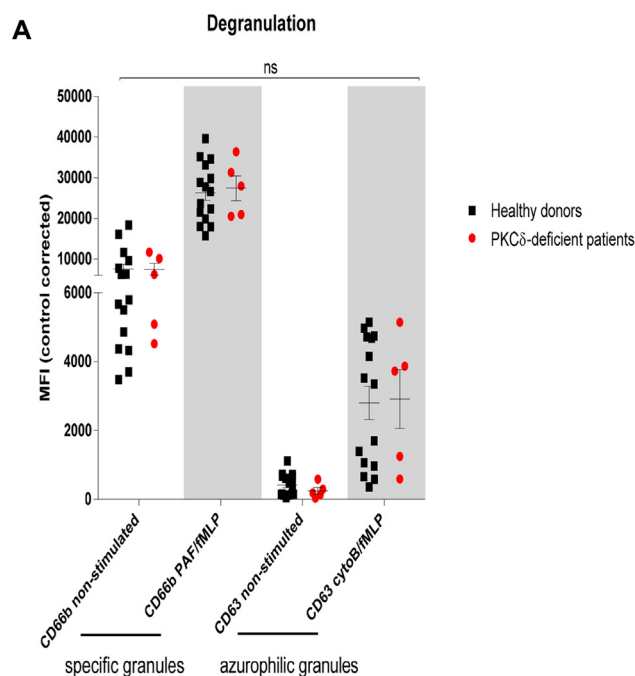


**FIG E4.** Degranulation and release of proteases by PKC $\delta$ -deficient neutrophils show no defect. **A**, Surface expression of CD66b (specific granules marker) and CD63 (azurophilic granules marker) was detected by flow cytometry after neutrophils of healthy donors and PKC $\delta$ -deficient patients had been triggered to degranulate with various stimuli. Values depicted are corrected for isotype control fluorescence. **B**, Secretion of proteases from granules of healthy donors and PKC $\delta$ -deficient patients was determined using DQ-Green BSA assay as described in the [Methods](#) section. Figures show overview of protease release by healthy donors and all PKC $\delta$ -deficient patients, depicted first as an example of measurement in patient B in comparison to day control showing measured RFUs and second as a percentage of day controls. Statistical analyses were performed by using the multiple *t* test with the Holm-Sidak method, in which  $\alpha = 5.000\%$ . MFI, Mean fluorescence intensity; RFU, relative fluorescence unit. \**P* < .05.

**FIG E3.** PKC $\delta$ -deficient neutrophils show normal phagocytosis of microbial pathogens. Purified neutrophils of healthy donors and PKC $\delta$ -deficient patients were incubated with fluorescently-labeled pathogens and their uptake was quantified by flow cytometry. **A-D**, Kinetics of the uptake of opsonized *S aureus*, opsonized *E coli*, nonopsonized *C albicans*, and opsonized *C albicans*, respectively, by neutrophils of patient A and of healthy donors. **E**, Overview of pathogen uptake at its maximum, in which 100% represents the uptake of healthy donors' neutrophils and the uptake of neutrophils of each patient is expressed as a percentage of day control. Statistical analyses were performed by using the multiple *t* test with the Holm-Sidak method, in which  $\alpha = 5.000\%$ . MFI, Mean fluorescence intensity; ns, not significant.

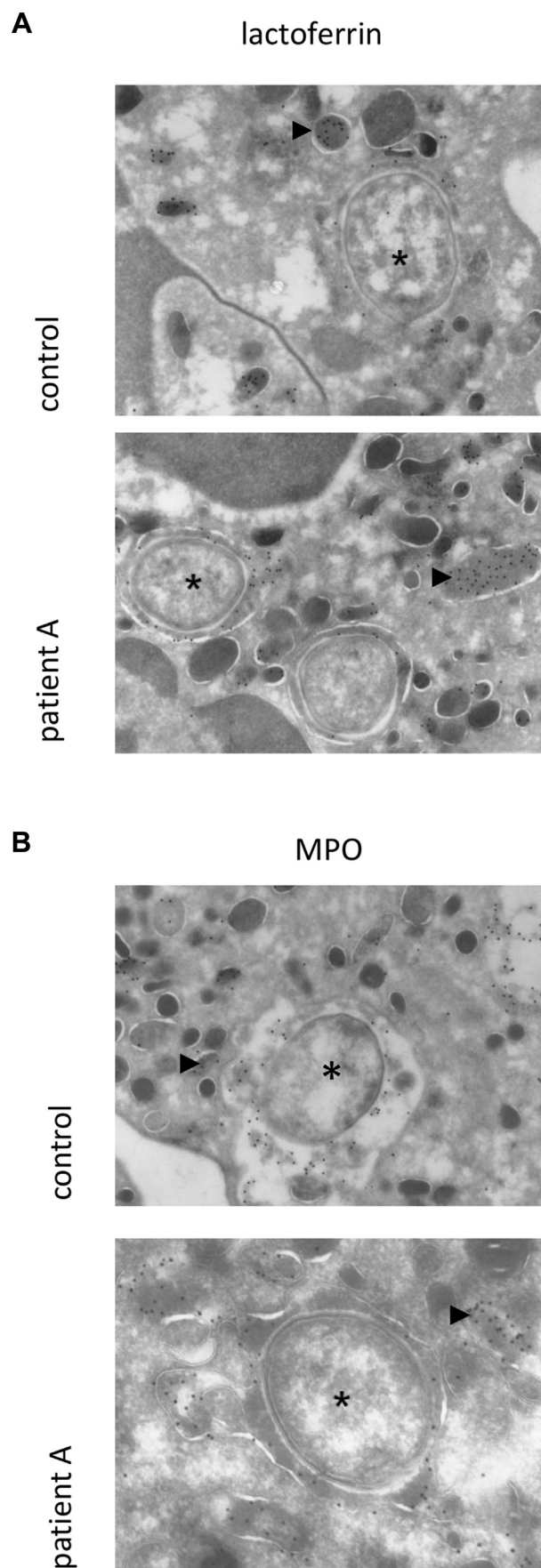


**FIG E5.** Phagosomes of PKC $\delta$ -deficient neutrophils show normal fusion and contain both lactoferrin and myeloperoxidase after *E coli* phagocytosis. Neutrophils of a healthy control and patient A were incubated for 30 minutes to allow phagocytosis and fusion with lysosomes. Immunoelectron microscopy was used to detect lactoferrin (**A**) and myeloperoxidase (MPO) (**B**) in phagolysosomes. Phagolysosomes containing *E coli* with lactoferrin/MPO are marked with an asterisk, and lactoferrin/MPO-positive granules are marked with ►.

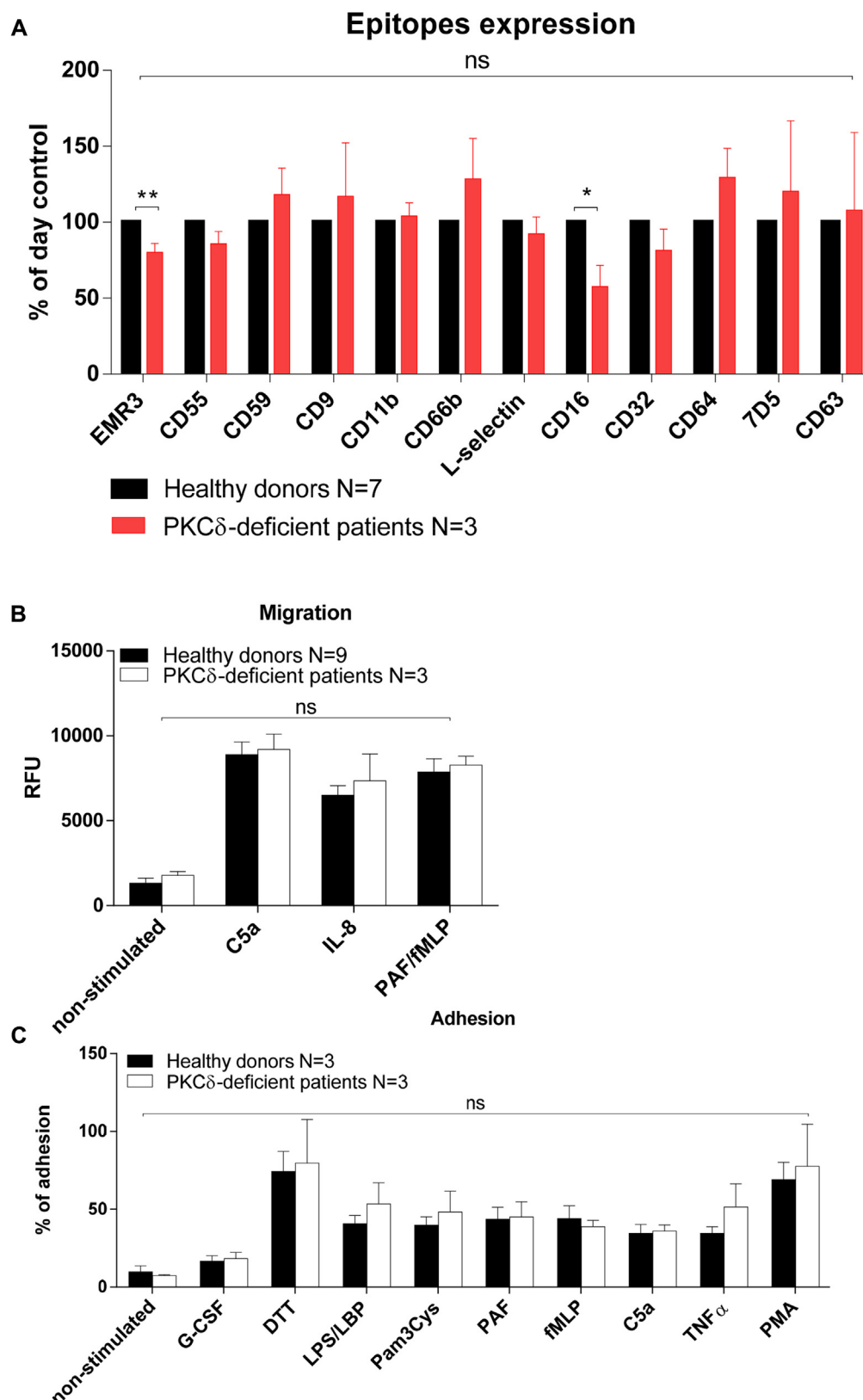


**FIG E6.** Neutrophils of PKC $\delta$ -deficient patients show normal expression of surface epitopes, chemotaxis, and adhesion. **A**, Flow cytometry analysis of purified neutrophils showed normal phenotype of PKC $\delta$ -deficient neutrophils compared with healthy donors. **B**, Neutrophils of PKC $\delta$  patients and healthy donors were used in a transwell migration assay as described in the [Methods](#) section showing normal chemotaxis. **C**, Adhesion of neutrophils to a plastic surface after stimulation with various stimuli was performed as described in the [Methods](#) section. Statistical analyses were performed by using the multiple *t* test with the Holm-Sidak method, in which  $\alpha = 5.000\%$ . DTT, Dithiothreitol; LBP, LPS-binding protein; ns, not significant; PMA, phorbol 12-myristate 13-acetate; RFU, relative fluorescence unit. \**P* < .05 and \*\**P* < .01.

**TABLE E1.** *PRKCD* gene mutations and characteristics of patients A, B, and C

| Patient | Age (y) | Sex    | Mutation                          | Protein expression<br>in neutrophils* | Clinical symptoms                                                                            |
|---------|---------|--------|-----------------------------------|---------------------------------------|----------------------------------------------------------------------------------------------|
| A       | 13-14   | Male   | c.1352+1G>A (both alleles)        | Not detectable                        | Autoimmunity, recurrent infections                                                           |
| B       | 15-16   | Male   | c.1528G>A; p.G510S (both alleles) | Yes, 26% of control                   | SLE, history of pleuritis and pneumonia                                                      |
| C       | 27-28   | Female | c.1528G>A; p.G510S (both alleles) | Yes, 20% of control                   | SLE, history of severe <i>Legionella pneumophilla</i><br>lung infection, recurrent pneumonia |

*SLE*, Systemic lupus erythematosus.

\*As determined by Western blot (see also [Fig 1, A](#)).

**TABLE E2.** Mutations and characteristics of patients D and E with chronic granulomatous disease

| Patient | Age (y) | Sex  | Mutation                         | Protein expression<br>in neutrophils* | Clinical symptoms                         |
|---------|---------|------|----------------------------------|---------------------------------------|-------------------------------------------|
| D       | 17      | Male | c.75_76delGT, <i>NCF1</i>        | Not detectable                        | Recurrent bacterial and fungal infections |
| E       | 12      | Male | c.417_451+650del685, <i>NCF1</i> | Not detectable                        | Recurrent bacterial and fungal infections |

\*As determined by Western blot.