ARTICLE

Impaired killing of *Candida albicans* by granulocytes mobilized for transfusion purposes: a role for granule components

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ABSTRACT

Granulocyte transfusions are used to treat neutropenic patients with life-threatening bacterial or fungal infections that do not respond to anti-microbial drugs. Donor neutrophils that have been mobilized with granulocyte-colony stimulating factor (G-CSF) and dexamethasone are functional in terms of antibacterial activity, but less is known about their fungal killing capacity. We investigated the neutrophil-mediated cytotoxic response against *C. albicans* and *A. fumigatus* in detail. Whereas G-CSF/dexamethasone-mobilized neutrophils appeared less mature as compared to neutrophils from untreated controls, these cells exhibited normal ROS production by the NADPH oxidase system and an unaltered granule mobilization capacity upon stimulation. G-CSF/dexamethasone-mobilized neutrophils efficiently inhibited *A. fumigatus* germination and killed *Aspergillus* and *Candida* hyphae, but the killing of *C. albicans* yeasts was distinctly impaired. Following normal *Candida* phagocytosis, analysis by mass spectrometry of purified phagosomes after fusion with granules demonstrated that major constituents of the antimicrobial granule components, including major basic protein (MBP), were reduced. Purified MBP showed candidacidal activity, and neutrophil-like Crisp-Cas9 NB4-KO-MBP differentiated into phagocytes were impaired in *Candida* killing. Together, these findings indicate that G-CSF/dexamethasone-mobilized neutrophils for transfusion purposes have a selectively impaired capacity to kill *Candida* yeasts, as a consequence of an altered neutrophil granular content.

Introduction

The intensified use of chemotherapy and immunosuppressive treatment modalities and related neutropenia results in increased morbidity and mortality due to bacterial and fungal infections.[1,2] Invasive fungal infections in particular are characterized by mortality rates of up to 90%, and this is in a large part due to the growing resistance to antifungals.[1-3] Granulocyte transfusions are administered to critically ill patients with neutropenia or neutrophil dysfunction and infections that do not respond to antimicrobial therapy.[4,5] Granulocyte-colony stimulating factor (G-CSF) and dexamethasone treatment of donors increases the yield of granulocytes for transfusion (GTX), but it also recruits a distinct pool of neutrophils from the bone marrow with an altered gene expression profile.[6] We previously found that certain genes known to be involved in the antifungal immune response were downregulated in G-CSF/dexamethasone-mobilized neutrophils.[6] However, it is not known whether this altered gene expression profile also impacts the cytotoxic response

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against the clinically relevant fungal pathogens, *Aspergillus fumigatus* and *Candida albicans.*

In general, human neutrophil killing mechanisms include reactive oxygen species (ROS) production by the NADPH oxidase system and non-oxidative cytotoxic mechanisms.[7,8] G-CSF has been shown *in vitro* to enhance neutrophil chemotaxis, phagocytosis and NADPH oxidase activation,[9,10] whereas dexamethasone exerts immunosuppressive effects on human and murine neutrophil function.[11,12] We and others have shown that neutrophils from G-CSF/dexamethasone-treated donors display prolonged survival rates, intact NADPH oxidase activation and a normal antimicrobial response against gram-positive and gram-negative bacteria.[13-16] Nevertheless, G-CSF-mobilized donor neutrophils have been reported to contain reduced levels of lactoferrin for example, derived from the specific granules, as compared to neutrophils from untreated controls.[17] During granulopoiesis granular proteins are synthesized, and when released by the mature neutrophil these proteins employ cytotoxic activity or limit the availibility of nutrients for the pathogen.[7,18] These granule-dependent cytotoxic mechanisms are pivotal in the host defense against fungal pathogens. It has, for instance, been shown that the human neutrophil inhibition of *A. fumigatus* germination depends on specific granule-derived lactoferrin, which mediates the sequestration of iron.[19] Granular extracts from human neutrophils, containing in particular cathepsin G and major basic protein (MBP), but also azurocidin and defensins, demonstrated candidacidal activity.[20,21] Previously, we found that genes involved in the antifungal response, including the gene that encodes for CARD9, were downregulated in the G-CSF/dexamethasone-mobilized neutrophils.[6] Human CARD9 deficiency is characterized by invasive fungal infection and impaired neutrophil candidacidal activity.[22]

In the present study we have investigated the killing of fungi by G-CSF/dexamethasone-mobilized neutrophils in detail. Our results demonstrate that G-CSF/dexamethasone-mobilized neutrophils have immature characteristics, produce normal amounts of ROS, efficiently inhibit *A. fumigatus* germination and kill their hyphae. However, the killing of *C. albicans* was substantially impaired in G-CSF/dexamethasone-mobilized neutrophils relative to their normal counterparts. Analyses of the phagosomes after fusion with granules revealed reduced levels of antimicrobial proteases, including MBP, in G-CSF/dexamethasone-mobilized neutrophils. Interestingly, MBP is required for the killing of *Candida* and contributes to the observed killing defect in G-CSF/dexamethasone-mobilized neutrophils.

Table 1. Distinct composition of the G-CSF/dexamethasone-mobilized phagosomes after fusion with granules.

Protein	Function
Major Basic Protein Homolog (MBPH)	C-type lectin, cytotoxin
Resistin (RETN)	Pro-inflammatory
Poly(rC)-binding protein 1 (PCBP1)	RNA binding
Serine/arginine-rich splicing factor 4 (SRSF4)	RNA binding
Adenylyl cyclase-associated protein 1 (CAP1)	Receptor resistin, filament dynamics
Lipocalin-2 (LCN2)	Ferric siderophore, metalloprotease
Major Basic Protein (MBP)	C-type lectin, cytotoxin
Eosinophil peroxidase (EPX)	Peroxidase activity
Peptidoglycan recognition protein 1 (PGLYRP1)	Peptidoglycan receptor
Vesicle-associated membrane protein 8 (VAMP8)	Vesicular fusion
Grancalcin (GCA)	Pro-inflammatory

Neutrophils from healthy controls and G-CSF/dexamethasone-treated donors were stimulated with C. albicans for 45 minutes; subsequently, the phagosomes were isolated and analyzed by Mass Spectrometry. The proteins that were significantly decreased in the neutrophil phagosomes from the G-CSF/dexamethasone-treated donors as compared to untreated controls are shown. N=5, FDR = 0.05 and S = 0.6

Methods

Cell isolation and study approval

Heparinized venous blood was collected from healthy granulocyte donors, with or without G-CSF/dexamethasone treatment. Donors received G-CSF (600 μg subcutaneously) and dexamethasone (8 mg orally), 16 to 20 hours before blood donation. The study was approved by the Sanquin Research Ethical Medical Committee (Amsterdam, The Netherlands) and in accordance with the Declaration of Helsinki (version Seoul 2008).

The granulocytes were isolated by centrifugation of heparin blood over isotonic Percoll with a specific density of 1.076 g/ml and after lysis of the erythrocytes with isotonic NH_4Cl-$KHCO_3$-EDTA solution resuspended in Hepes-buffered saline solution (Hepes-buffer).[22]

Killing of microorganisms

The microbicidal activity of granulocytes was assessed for *Candida albicans* (strain SC5314) and a clinical isolate of *Aspergillus fumigatus*. The microorganisms were grown under aerobic conditions at 30°C for 7 days on potato dextrose agar (*Aspergillus*) (Neogen, Lansing, Michigan, USA) or overnight in Luria-Bertani broth (LB) (*Candida*).

Hereafter, the *Aspergillus* yeasts were collected by centrifugation, washed twice in PBS and resuspended in RPMI 1640 medium (Life Technologies, Bleiswijk, The Netherlands). Opsonization was performed with 10% v/v human pooled serum for 15 minutes, at 37°C. For the neutrophil-mediated inhibition of germination, the same number of *Aspergillus* yeast cells were incubated with an increasing number of neutrophils (0.25, 0.5, 1.0 or 1.5 $*10^5$ cells/ml, E:T 1:2000, 1:1000, 1:500 or 1:350, respectively) in a 96-well plate overnight at 37°C in RPMI 1640 medium containing L-glutamine and 10% (v/v) FCS (Life Technolgies). Subsequently, the neutrophils were lysed in water/NaOH, pH 11.0 and incubated with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; thiazolyl blue) (Sigma). After the addition of acidic isopropanol (0.04 M HCl) the optical density was measured in the plate reader at 570 nm (Tecan, Männedorf, Switzerland) and the *A. fumigatus* hyphae viability was calculated as compared to the incubation without neutrophils (i.e. 100%). To assess the *A. fumigatus* and the *C. albicans* hyphae killing, neutrophils ($0^{-1}x10^5$ cells) were cultured for one hour (*Aspergillus*) or 2 hours (*Candida*) on a preformed monolayer at 37 °C. Hereafter, the cells were lysed in water/NaOH, pH 11.0 and incubated with MTT. The absorbance of the acidic isopropanol-diluted samples was measured on the plate reader (Tecan) and the viability calculated as a percentage of the viability after incubation without neutrophils.

To determine the neutrophil killing of *Candida*, the yeasts were collected by centrifugation, washed twice in PBS and resuspended

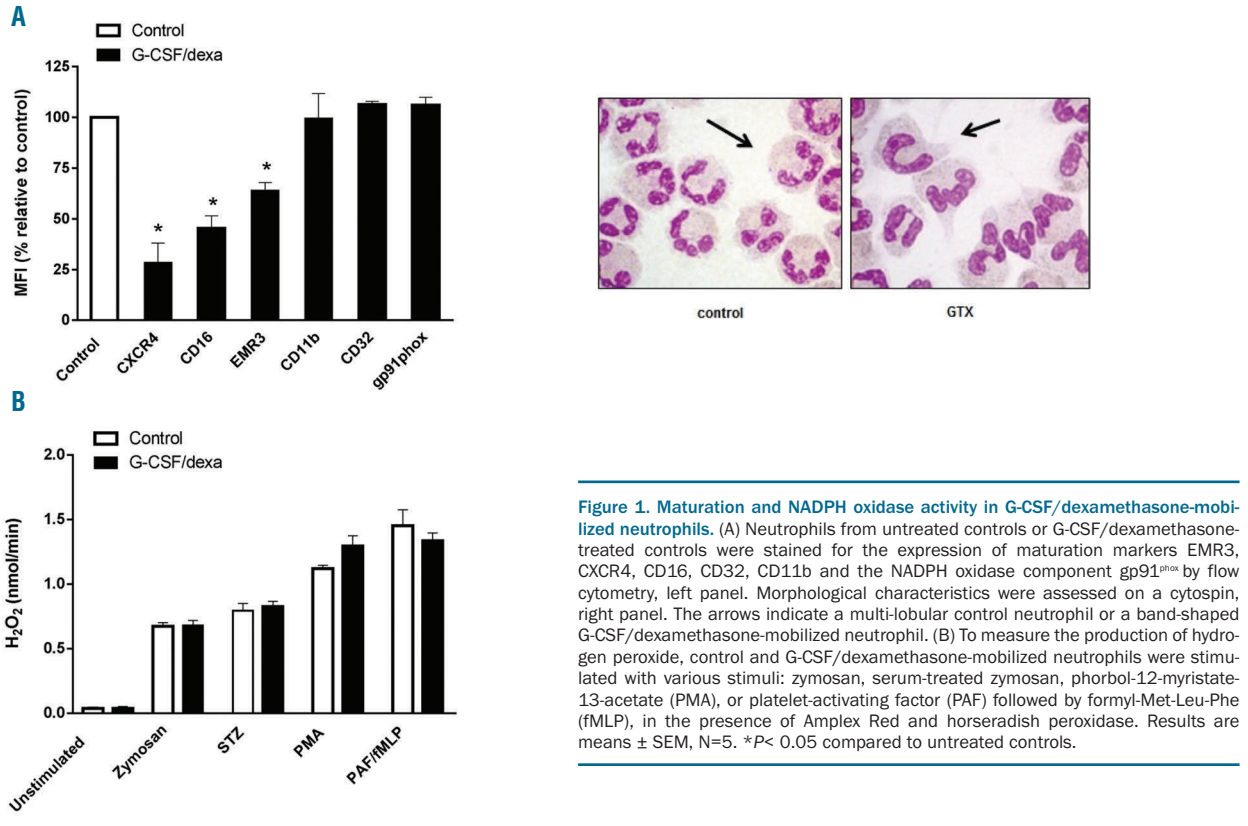

Figure 1. Maturation and NADPH oxidase activity in G-CSF/dexamethasone-mobilized neutrophils. (A) Neutrophils from untreated controls or G-CSF/dexamethasone-treated controls were stained for the expression of maturation markers EMR3, CXCR4, CD16, CD32, CD11b and the NADPH oxidase component gp91^{phox} by flow cytometry, left panel. Morphological characteristics were assessed on a cytospin, right panel. The arrows indicate a multi-lobular control neutrophil or a band-shaped G-CSF/dexamethasone-mobilized neutrophil. (B) To measure the production of hydrogen peroxide, control and G-CSF/dexamethasone-mobilized neutrophils were stimulated with various stimuli: zymosan, serum-treated zymosan, phorbol-12-myristate-13-acetate (PMA), or platelet-activating factor (PAF) followed by formyl-Met-Leu-Phe (fMLP), in the presence of Amplex Red and horseradish peroxidase. Results are means ± SEM, N=5. *P< 0.05 compared to untreated controls.

in Hepes-medium. After opsonization with 10% (v/v) pooled serum for 15 min, at 37°C, the *Candida* was added at a ratio of 4 : 1 neutrophil (5x10^6 cells/ml). At the desired time points, 100-µl samples were diluted in 2.5 ml of water/NaOH, pH 11.0. At the end of the incubation period, the number of viable microorganisms in each sample was determined by the pourplate method in LB agar. The colony-forming units (CFU) were determined after overnight incubation at 37°C, and the percentage of killing was calculated as described.[22]

The recombinant proteins for the candidacidal experiments were major basic protein (MBP) (kind gift from prof. G.J. Gleich, Utah, USA, recombinant protein produced in our lab) and major basic protein homologue (recombinant protein produced in our lab, detailed methodology in the *Online Supplementary Appendix)*.

Immunostaining and FACS analysis

The expression of surface-bound receptors on granulocytes was assayed in total leukocyte samples by flow cytometry (FACS), with the commercially available antibodies against human-CD11b (clone 44A, ATCC, Rockville, MD, USA), CD32 (clone AT10, AbD Serotec, Oxford, UK), CD16 (clone 3G8, BD Pharmingen, Breda, the Netherlands), EMR3 (clone 3D7, AbD, Puchheim, Germany), CXCR4 (Clone 44717, R&D systems, Oxford, UK) and gp91^{phox} (clone 7D5, MBL, Woburn, MA, USA). As a secondary antibody, Alexa488 rabbit anti-mouse-IgG (Molecular Probes, Bleiswijk, the Netherlands) was used. Samples were analyzed on an LSRII flow cytometer equipped with FACSDiva software (BD Biosciences). Cells were gated based on their forward and side scatter, and 10,000 gated events were collected per sample.

Degranulation assays

Neutrophils (2×10^6/ml) were incubated in Hepes buffer at 37°C in a shaking water-bath before adding the (priming) agents PAF (1 µM, 5 minutes, Sigma, Steinheim, Germany) or cytochalasin B (5 µg/ml, 5 minutes, Sigma) were added. Subsequently, the cells were stimulated with fMLP (1 µM, Sigma, 15 minutes). After stimulation, the cells were put on ice, washed with Hepes buffer once, and subsequently stained with antibodies against neutrophil granule markers: CD63-PE (IgG1, 435); CD66b-FITC (IgG1, CLB-B13.9). Data are expressed as mean fluorescence intensities (MFI). The cells were analyzed on an LSRII flow cytometer equipped with FACSDiva software (BD Bioscience). The release of elastase and lactoferrin was evaluated with ELISA kits (HyCult Biotech) according to the manufacturer's instructions. The proteolytic activity was determined by incubating neutrophils (2.5×10^6/ml in Hepes buffer) with DQ-Green BSA (10 µg/ml, Molecular Probes). Upon stimulation with cytochalasin B (5 µg/ml, Sigma)/ fMLP (1 µM) the fluorescence was monitored at 30-second intervals for 1 hour by infinitiPRO2000 plate reader (Excitation 485 nm; Emission 535 nm) (Tecan).

Statistics

Statistical analysis was performed with GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA). MS data were analyzed with Proteome Discoverer Software (Thermo Scientific, version 1.4), Scaffold (Proteome Software, version 4.0) and MaxQuant (FDR set at 0.05 and S0.6, version 1.4.1.2). Data were evaluated by paired, two-tailed student's *t*-test, two-way ANOVA with post hoc Bonferroni test and by the Mann-Whitney test. The results are presented as the mean ± SEM, as indicated. Data were considered significant when P<0.05.

Supplemental methods

Detailed methodology of the *Online Supplementary Figures* is described in the *Online Supplementary Appendix*.

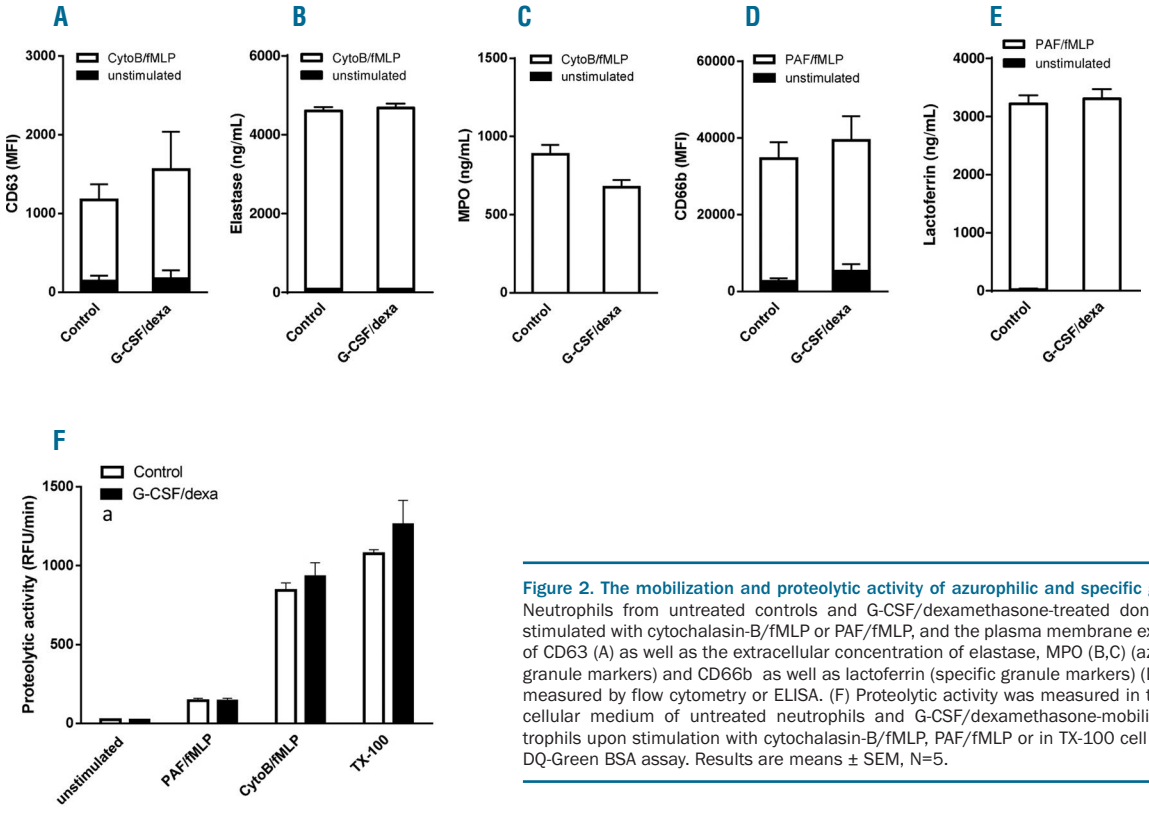

Figure 2. The mobilization and proteolytic activity of azurophilic and specific granules. Neutrophils from untreated controls and G-CSF/dexamethasone-treated donors were stimulated with cytochalasin-B/fMLP or PAF/fMLP, and the plasma membrane expression of CD63 (A) as well as the extracellular concentration of elastase, MPO (B,C) (azurophilic granule markers) and CD66b as well as lactoferrin (specific granule markers) (D,E) were measured by flow cytometry or ELISA. (F) Proteolytic activity was measured in the extracellular medium of untreated neutrophils and G-CSF/dexamethasone-mobilized neutrophils upon stimulation with cytochalasin-B/fMLP, PAF/fMLP or in TX-100 cell lysate by DQ-Green BSA assay. Results are means ± SEM, N=5.

Results

G-CSF/dexamethasone treatment recruits immature neutrophils with normal NADPH oxidase activity and granule mobilization capacity

Previously, we found that the G-CSF/dexamethasone-mobilized neutrophils demonstrated an altered gene expression profile, and this could either be due to the recruitment of a relatively immature population of neutrophils or direct gene-regulatory effects of G-CSF/dexamethasone. A single administration of subcutaneous G-CSF is combined with an oral dose of dexamethasone to obtain an optimal number of neutrophil mobilization for transfusion.[23]

We isolated neutrophils from healthy donors treated with G-CSF and dexamethasone, which resulted in a ~10-fold increase in circulating neutrophils (*Online Supplementary Figure S1*). The chemokine receptor CXCR4 involved in neutrophil retention in the bone marrow was reduced on the surface of G-CSF/dexamethasone-mobilized neutrophils as compared to control neutrophils (Figure 1A, left panel).[24] The G-CSF/dexamethasone-mobilized neutrophils demonstrated band-shaped nuclei as compared to the multilobular nuclei observed in neutrophils from healthy controls (Figure 1A, right panel). The G-CSF/dexamethasone-mobilized neutrophils also showed low surface expression of the late neutrophil maturation markers EMR3 and CD16, but normal levels of the early myeloid maturation markers CD11b and CD32, when compared to expression levels on circulating neutrophils from untreated controls (Figure 1A, left panel). Given the fact that the proteins involved in the antimicrobial functions of neutrophils, including the NADPH oxidase and the different intracellular granules, are gradually formed during granulopoiesis,[18] it was of interest to assess these in G-CSF/dexamethasone-mobilized neutrophils. Surface expression of gp91^{phox}, i.e. the catalytic plasma membrane component of the NADPH oxidase enzyme complex, was normal when detected with the mAb 7D5 (Figure 1A). The functional NADPH oxidase activity upon cell activation was also comparable between control and G-CSF/dexamethasone-mobilized neutrophils (Figure 1B).[14]

Furthermore, the mobilization of azurophilic granules was measured by the membrane expression of CD63 and the release of elastase and MPO upon stimulation with cytochalasin-B/fMLP (Figure 2A-C). The mobilization of specific granules was evaluated by the membrane expression of CD66b and the release of lactoferrin upon stimulation with PAF/fMLP (Figure 2D,E). The overall serine protease activity in the extracellular medium was determined (i.e. DQ BSA fluorescence upon proteolytic cleavage) (Figure 2F). All were found to be intact in G-CSF/dexamethasone-mobilized neutrophils as compared to normal neutrophils. Finally, immuno-EM analysis demonstrated the normal appearance and frequency of myeloperoxidase (MPO)-positive azurophilic granules in the G-CSF/dexamethasone-mobilized neutrophils (*Online Supplementary Figure S2*). Therefore, it appears that although the G-CSF/dexamethasone-mobilized neutrophils show signs of immaturity with respect to their nuclear morphology and the expression of certain surface markers, both the NADPH oxidase activity and the presence and mobilization of azurophilic and specific granule markers appeared to be unaltered.

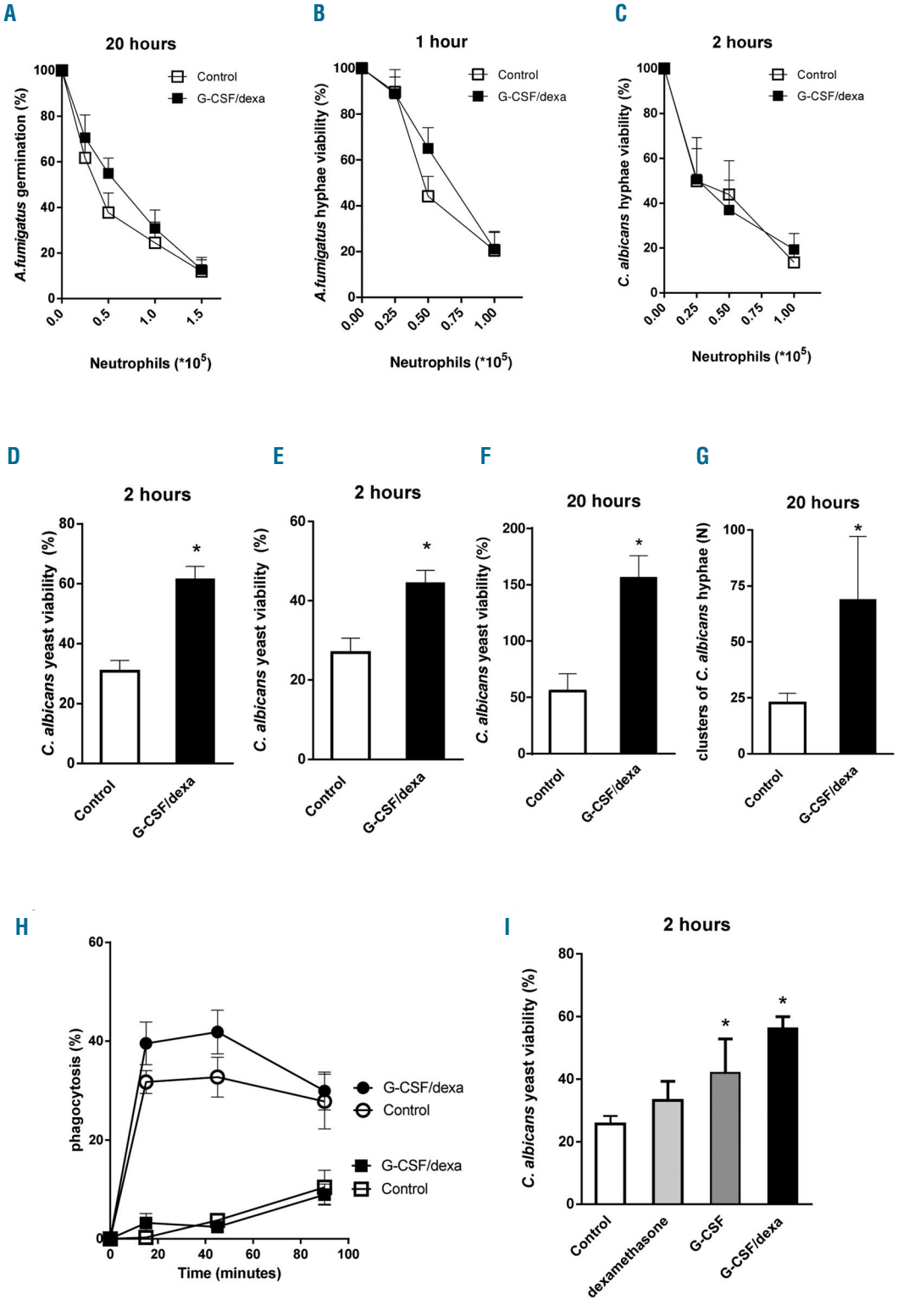

Figure 3. The killing of *A. fumigatus* and *C. albicans* by mobilized neutrophils. Untreated neutrophils from healthy controls or G-CSF/dexamethasone-treated donors were co-cultured overnight with *Aspergillus fumigatus* yeasts (A) or with a preformed hyphae monolayer (B), and the viability was assessed with the MTT assay and calculated as a percentage of the viability after incubation without neutrophils. (C) Control and G-CSF/dexamethasone-mobilized neutrophils were incubated with a *C. albicans* preformed hyphae monolayer, and the viability was assessed with the MTT assay. Neutrophils from healthy controls or G-CSF/dexamethasone-treated donors were incubated with serum-opsonized (D) or unopsonized (E) *C. albicans* yeast for 2 hours, and the long-term (20 hours) (F) killing was determined as the percentage of viable *Candida* yeasts relative to incubation without neutrophils by a colony-forming unit assay. (G) Control and G-CSF/dexamethason-mobilized neutrophils were incubated overnight with *Candida* yeasts and the clusters of hyphae were quantified by confocal microscopy. (H) Control neutrophils and G-CSF/dexamethasone-mobilized neutrophils were incubated with unopsonized (□) or serum-opsonized (○) *C. albicans* yeasts -FITC, and the phagocytosis was determined by confocal microscopy. The percentage of phagocytosis is the number of FITC-positive neutrophils relative to the total number of neutrophils. (I) Neutrophils from untreated controls and donors treated with G-CSF, dexamethasone or both were incubated with *C. albicans* yeasts, and the short-term (2 hours) killing was determined. Results are means ± SEM, N=3-12 * $P<0.05$ compared to untreated controls.

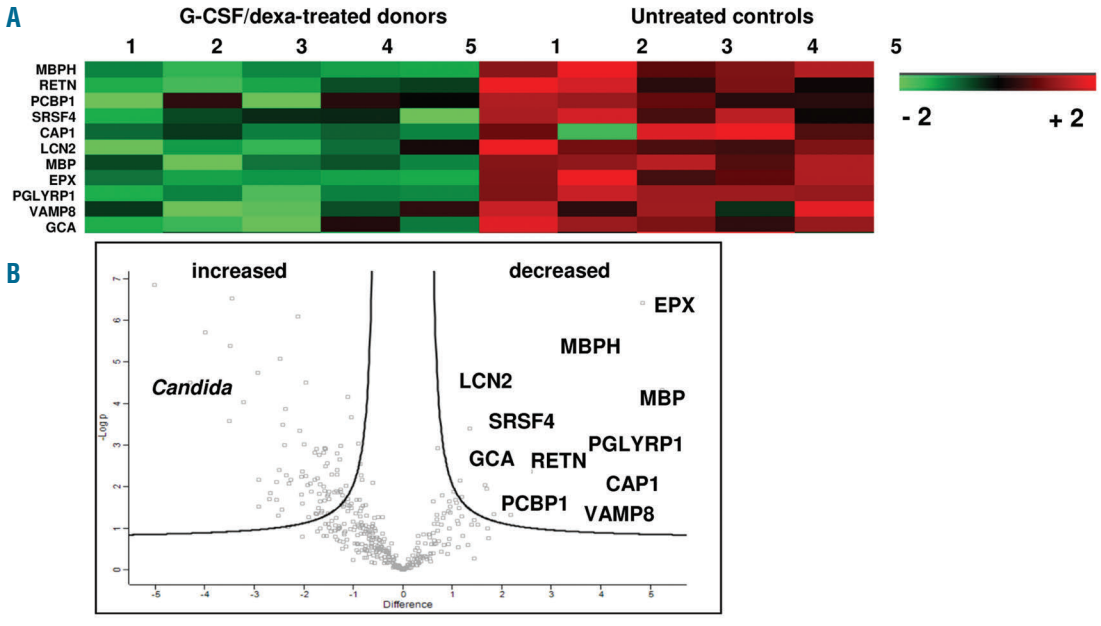

Figure 4. Distinct composition of the G-CSF/dexamethasone-mobilized neutrophil phagosomes after fusion with granules. (A) Neutrophils from healthy controls and G-CSF/dexamethasone-treated donors were stimulated with *C. albicans* for 45 minutes, the phagosomes were isolated and analyzed by mass spectrometry. The proteins that were significantly decreased in the phagosomes from the G-CSF/dexamethasone-treated donors as compared to untreated controls are shown in the heat map. The red boxes show upregulated and green downregulated proteins in G-CSF/dexamethasone-mobilized phagosomes compared to controls. (B) The differentially expressed proteins between the control and G-CSF/dexamethasone-mobilized phagosomes are depicted in a volcano plot. N=5, FDR = 0.05 and S = 0.6

Antifungal activity by G-CSF/dexamethasone-mobilized neutrophils

Next, we determined directly the cytotoxic capacity against *A. fumigatus* and *C. albicans*. Invasive infections start with the germination of yeasts into hyphae that enables them to invade tissues and spread via the bloodstream, which forms the basis for their pathogenicity.[25] Therefore we assessed both the intracellular killing of yeasts by neutrophils, which functions to prevent germination, as well as the extracellular destruction of preformed hyphae.

The neutrophils from G-CSF/dexamethasone-treated donors normally inhibited the *A. fumigatus* germination after overnight incubation with the yeasts as compared to untreated controls (Figure 3A). The G-CSF/dexamethasone-mobilized neutrophils also efficiently degraded a monolayer of preformed *A. fumigatus* hyphae (Figure 3B). A preformed monolayer of *C. albicans* hyphae was also as effectively degraded by the G-CSF/dexamethasone-mobilized neutrophils as by control neutrophils (Figure 3C). However, we observed that G-CSF/dexamethasone-mobilized neutrophils showed a clear and distinctive defect in both the short-term (2 hours) and long-term (20 hours) killing of the *C. albicans* yeasts as compared to the neutrophils from untreated controls (Figure 3D-F). In addition, the G-CSF/dexamethasone-mobilized neutrophils were less able to inhibit the *C. albicans* yeast germination in an overnight assay (Figure 3G). This defect in yeast killing could not be explained by changes in the phagocytic capacity, since the phagocytosis and killing of both unopsonized and serum-opsonized *C. albicans* yeasts was completely normal (Figure 3H).

We assessed whether the *in vivo* treatment with G-CSF or dexamethasone seperately could be held responsible for the *Candida* killing defect of the G-CSF/dexamethasone-mobilized neutrophils. Neutrophils were isolated from healthy donors treated with G-CSF or dexamethasone separately, each of which resulted in a ~7- or ~2-fold increase in circulating neutrophils, respectively (*Online Supplementary Figure S1*). When compared to the neutrophils from untreated controls, we observed that the dexamethasone-mobilized neutrophils were not impaired in the killing of *C. albicans* yeasts or any of the other fungal killing tests performed (Figure 3I), whereas the neutrophils from G-CSF-treated donors showed a significant *C. albicans* killing defect (Figure 3I), although not exactly to the same extent as in the case of donor-derived neutrophils mobilized with both G-CSF and dexamethasone (Figure 3I).

Taken together, a selective *C. albicans* yeast killing defect was observed for G-CSF/dexamethasone-mobilized neutrophils, whereas these neutrophils showed a normal cytotoxic response against the *Aspergillus* yeasts and hyphae, as well as against preformed *Candida* hyphae.

Candida-induced phagosome formation

To obtain further insight into the *Candida* yeast killing defect of G-CSF/dexamethasone-mobilized neutrophils upon normal phagocytosis, we decided to explore the contents of the *Candida* phagosome in more detail. Under normal conditions of phagocytosis the granules fuse with the phagosome containing internalized pathogens, thereby creating a cytotoxic environment for the degradation of microbes.[26,27] To determine the cytotoxic composition of the phagosome after fusion with granules, we magnetically labeled *Candida* yeast, and - after synchronized phagocytosis and lysis of the neutrophils - we isolated the phagosomes and measured their composition by mass

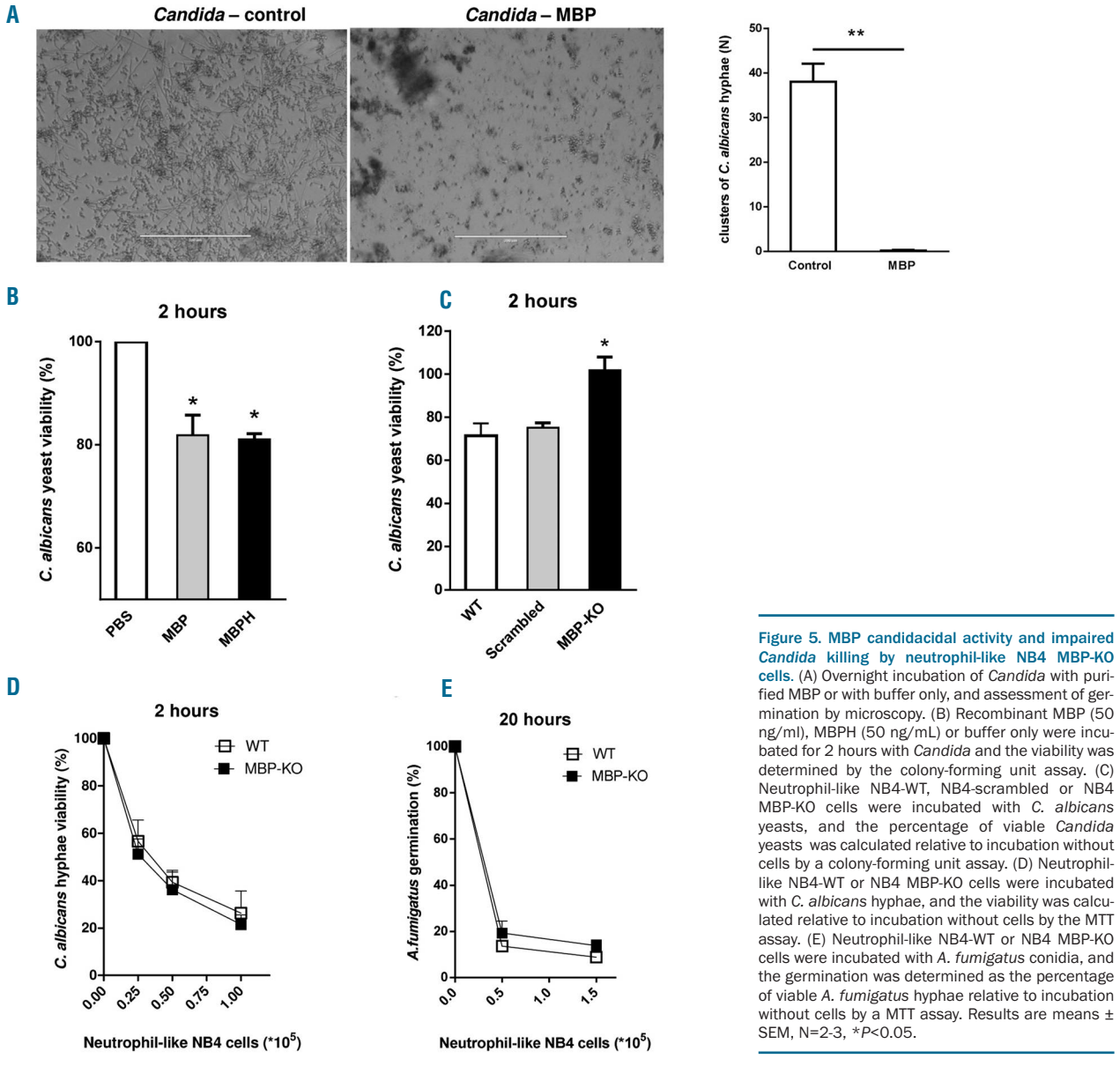

Figure 5. MBP candidacidal activity and impaired *Candida* killing by neutrophil-like NB4 MBP-KO cells. (A) Overnight incubation of *Candida* with purified MBP or with buffer only, and assessment of germination by microscopy. (B) Recombinant MBP (50 ng/ml), MBPH (50 ng/mL) or buffer only were incubated for 2 hours with *Candida* and the viability was determined by the colony-forming unit assay. (C) Neutrophil-like NB4-WT, NB4-scrambled or NB4 MBP-KO cells were incubated with *C. albicans* yeasts, and the percentage of viable *Candida* yeasts was calculated relative to incubation without cells by a colony-forming unit assay. (D) Neutrophil-like NB4-WT or NB4 MBP-KO cells were incubated with *C. albicans* hyphae, and the viability was calculated relative to incubation without cells by the MTT assay. (E) Neutrophil-like NB4-WT or NB4 MBP-KO cells were incubated with *A. fumigatus* conidia, and the germination was determined as the percentage of viable *A. fumigatus* hyphae relative to incubation without cells by a MTT assay. Results are means ± SEM, N=2-3, **P*<0.05.

spectrometry, according to a previously reported method.[28]

First, confocal analyses confirmed that the isolated phagosomes after granule fusion were highly positive for *Candida* (green), MPO (red) and elastase (yellow) (*Online Supplementary Figure S3A*). Secondly, kinetic analyses showed that the number of elastase peptides in *Candida* phagosomes similarly increased with time, which confirmed the normal phagocytosis by G-CSF/dexamethasone-mobilized neutrophils and indicates phagosomal maturation (Figure 3H, *Online Supplementary Figure S3B,C*).

Comparison of the G-CSF/dexamethasone-mobilized and control neutrophil *Candida*-phagosomes after fusion with granules for some of the known components showed a similar expression of e.g. the membrane-expressed integrin CR3 (CD11b/CD18, αMβ2) (*Online Supplementary Figure S3C*), which is critically involved in the recognition, uptake and killing of *C. albicans*.[8] This is clearly consistent with the comparable phagocytosis of *Candida* yeasts by G-CSF/dexamethasone-mobilized and control neutrophils (see above). In addition, cytochrome b558 of the NAPDH oxidase system was identified (*Online Supplementary Figure S3C*), which reinforces the normal ROS production upon uptake of *Candida* yeasts. Finally, the phagosomes after fusion with granules also contained a variety of components that were derived from the various granules in neutrophils, i.e. MPO (azurophilic), elastase (azurophilic), lactoferrin (specific) and MMP9 (gelatinase) (*Online Supplementary Figure S3C*), and there appeared to be no differences in the fusion of these granules with the phagosome upon comparison of G-CSF/dexamethasone-mobilized and control neutrophil phagosomes.

We subsequently evaluated whether there were differences in the phagosomal composition between

G-CSF/dexamethasone-treated donors and untreated controls that could potentially explain the observed killing defect in G-CSF/dexamethasone-mobilized neutrophils. In total 11 neutrophil-derived proteins were identified to be significantly decreased in the G-CSF/dexamethasone-mobilized phagosomes after fusion with granules (Figure 4). The neutrophil-derived proteins that we observed to be decreased in the G-CSF/dexamethasone-mobilized phagosomes have been described to be involved in various aspects of cellular innate immunity, including cytotoxic activity, vesicular fusion, pro-inflammatory activation and actin-filament rearrangement (Table 1).[20,29-32] In the G-CSF/dexamethasone-mobilized phagosomes after fusion with granules, 79 proteins were signficantly upregulated, including 65 *Candida*-derived proteins and 14 proteins of human origin. Several of these host proteins are known to be involved in vesicular trafficking and as a negative regulator of phagosomal formation, e.g. Rap1A and Rab27A (*Online Supplementary Table S1*).[33,34]

We focused on the most pronounced differences between the G-CSF/dexamethasone-mobilized and control phagosomes after fusion with granules. The proteins major basic protein (MBP, PRG2) and major basic protein homolog (MBPH, PRG3) were virtually absent in the G-CSF/dexamethasone-mobilized phagosomes after fusion with granules (Figure 4). MS analyses of whole cell neutrophil lysates demonstrated that MBP, MBPH and EPX were also significantly reduced in neutrophils from G-CSF/dexamethasone-treated donors as compared to healthy controls (*Online Supplementary Figure S4*). Interestingly, MBP has a C-type lectin domain, and upon cleavage of the propeptide, becomes cytotoxic.[35,36] Upon testing the candidacidal effect of MBP and MBPH in the absence of neutrophils, we found that incubation for 2 hours or overnight of purified MBP or MBPH with *Candida* yeast resulted in strongly decreased yeast viability and germination (Figure 5A,B). The addition of MBP or MBPH did not affect the viability of *A. fumigatus* (*Online Supplementary Figure S5*). We used the Crispr-Cas9 technique to generate MBP knock-outs in NB4 cells (NB4-MBP-KO), which become neutrophil-like upon stimulation with ATRA (*Online Supplementary Figure S6*).[37] Both the Crispr technique and the knock-out of the protein MBP in particular did not interfere with important cytotoxic responses, including the ROS production by the NADPH oxidase system and *Candida* phagocytosis (*Online Supplementary Figure S6*). The neutrophil-like NB4-MBP-KO cells demonstrated a complete *Candida* killing defect when compared to neutrophil-like NB4-WT or NB4 cells that were transfected with a scrambled construct against a non-mammalian protein (Figure 5C). The neutrophil-like MBP knock-out cells normally killed *Candida* hyphae and inhibited the Aspergillus conidia germination, as also did the wild-type neutrophil-like NB4 cells (Figure 5D,E). These experiments further indicate that the killing of *Candida* depends on the presence of MBP and MBPH in the phagosome to contribute to the cytotoxic activity.

Discussion

In the present study we determined the cytotoxic activity against *Candida albicans* and *Aspergillus fumigatus* by neutrophils mobilized with G-CSF and dexamethasone for transfusion purposes. G-CSF/dexamethasone-mobilized neutrophils efficiently inhibited *A. fumigatus* germination and killed both the *Aspergillus* and *Candida* hyphae. However, the early and late killing of *C. albicans* yeasts were impaired by G-CSF/dexamethasone-mobilized neutrophils relative to normal neutrophils. Analyses of the phagosomes after fusion with granules revealed reduced levels of antimicrobial proteases, including MBP, in G-CSF/dexamethasone-mobilized neutrophils. Interestingly, MBP was required for the killing of *Candida* and contributes to the observed killing defect in G-CSF/dexamethasone-mobilized neutrophils.

G-CSF has been shown *in vitro* to enhance neutrophil functions in terms of chemotaxis, phagocytosis and NADPH oxidase activation,[9,10] whereas dexamethasone has immunosuppressive effects.[11,12] The incubation of neutrophils with dexamethasone prevents *A. fumigatus* hyphae killing and the addition of G-CSF restores the defect.[12] We found that the neutrophils from the G-CSF/dexamethasone-treated donors normally killed a monolayer of *Aspergillus* hyphae. An explanation for this discrepancy in results could be that Roilodes *et al*. added the dexamethasone *in vitro*, whereas the donors in our study were treated with a single dose of dexamethasone and/or G-CSF overnight *in vivo*. It has been described that neutrophils from donors treated for 5 consecutive days with G-CSF demonstrated normal MPO levels but decreased lactoferrin levels.[17] The neutrophil-mediated inhibition of *Aspergillus* yeasts germination depends on iron-sequestration by lactoferrin.[19] After one day of donor pretreatment we found normal levels of both MPO and lactoferrin in the G-CSF/dexamethasone-mobilized neutrophil phagosomes. In line with this observation the G-CSF/dexamethasone-mobilized neutrophils were completely able to inhibit the germination of *A. fumigatus*. The neutrophil killing of *Aspergillus* hyphae depends on ROS production by the NADPH oxidase system.[38] Both the ROS production and *A. fumigatus* hyphae killing was normal by the G-CSF/dexamethasone-mobilized neutrophils. The G-CSF/dexamethasone-mobilized neutrophils showed an effective cytotoxic response in the inhibition of *A. fumigatus* germination and the killing of the hyphae.

The G-CSF/dexamethasone-mobilized neutrophils were able to phagocytose *C.albicans*, but showed a clear defect in the intracellular killing. Analyses of the *Candida*-phagosomes revealed that several proteins were reduced in the G-CSF/dexamethasone-mobilized cells, whereas the aforementioned granule markers lactoferrin, MPO and elastase were found in comparable levels to controls. The most significant differences were MBP and MBP homolog (MBPH), present in controls and virtually absent in G-CSF/dexamethasone-mobilized phagosomes after fusion with granules and in whole G-CSF/dexamethasone-mobilized neutrophils. Since G-CSF/dexamethasone treatment recruits an immature pool of neutrophils, some granule components, including MBP, may not have been fully synthesized. MBP is mostly known as a marker for eosinophils. Borregaard *et al*. have already reported that MBPH is also present in various granules of neutrophils,[39] while we have now confirmed by Immuno-EM analysis that neutrophil granules do contain MBP (*Online Supplementary Figure S7*). Both MBP and MBPH have been demonstrated to desintegrate membranes and exert antimicrobial activity.[35,36] Gabay *et al*. investigated the antimicrobial properties of purified granule-derived proteins and found that MBP is one of the most potent candi-

dacidal proteins, e.g. it is 70-fold more toxic than defensins.[20] Purified human MBP also displayed strong *in vitro* inhibition of *Candida* germination under our conditions, which confirmed its fungicidal activity. Moreover, in a knock-out cell model to support the role of MBP, the neutrophil-like NB4-MBP-KO cells were found to be completely impaired in *Candida* killing without any effect on phagocytosis and ROS production. The results in this neutrophil-like cell model confirmed that MBP is involved in *Candida* killing.

In addition to MBPH, the analyses of the phagosomes identified several other proteins that were significantly decreased in the G-CSF/dexamethasone-mobilized phagosomes after fusion with granules. Eosinophil peroxidase (EP) is not strictly eosinophil specific[39] and found to be differentially expressed between G-CSF/dexamethasone-mobilized and control neutrophils, as well as in their phagosomes after granule fusion (Figure 4). Peroxidase activity is important, as neutrophils from MPO-deficient patients fail to kill *Candida*.[21] Although no difference in the major azurophil granule protein MPO was detected, we cannot exclude a contribution of EPO to the observed *Candida* killing defect in the G-CSF/dexamethasone-mobilized cells. The hormone resistin and its receptor adenylyl cyclase-associated protein 1 (CAP1) were also decreased in the G-CSF/dexamethasone-mobilized phagosomes after fusion with granules. Resistin is produced by granulocytes upon activation and has pro-inflammatory effects.[29] However, *Candida* killing improved only slightly when resistin was added, and was observed in both control and G-CSF/dexamethasone-mobilized neutrophils (*Online Supplementary Figure S8*). The G-CSF/dexamethasone-mobilized phagosomes after fusion with granules also showed reduced levels of the calcium-binding protein grancalcin and lipocalin-2. Although little is known about their exact role in humans, neutrophils from the respective knock-out mice showed normal candidacidal responses.[40,41]

The number of defensin-1 peptides were slightly decreased in the G-CSF/dexamethasone-mobilized phagosomes after fusion with granules (*Online Supplementary Figure S3C*). Defensins are derived from azurophilic granules and have been described to be cytotoxic for *Candida albicans*.[20] Although MBP proteins (PRG2) contributes to a very large extent, it may be the combined reduction of granule-derived antimicrobial proteins in the G-CSF/dexamethasone-mobilized neutrophil phagosomes that aggravates the *Candida* killing defect. It would be a relevant topic of future investigations to determine whether G-CSF or dexamethasone administration results in decreased expression of these granule-derived antimicrobial proteins.

Furthermore, the killing of *Candida* hyphae by the G-CSF/dexamethasone-mobilized neutrophils was normal. We have investigated the neutrophil-mediated killing mechanisms of *Candida* yeasts and hyphae. It appeared that both the NADPH oxidase system and the phagosomal maturation are required for the neutrophil-mediated killing of *Candida* yeasts, whereas these toxic mechanisms are redundant in the killing of *Candida* hyphae (*data not shown*).[8] This may explain why G-CSF/dexamethasone-mobilized neutrophils show a selective killing defect for *Candida* yeasts but not hyphae.

In conclusion, we have investigated the killing of *A. fumigatus* and *C. albicans* by G-CSF/dexamethasone-mobilized neutrophils in detail. Our results demonstrate that G-CSF/dexamethasone-mobilized neutrophils produce normal amounts of ROS, efficiently inhibit *A. fumigatus* germination and kill their hyphae. However, the killing of *C. albicans* yeasts was substantially impaired in G-CSF/dexamethasone-mobilized neutrophils relative to their normal counterpart. Analyses of the phagosomes after fusion with granules revealed reduced levels of antimicrobial proteins and in particular MBP in G-CSF/dexamethasone-mobilized neutrophil phagosomes, which contribute to the observed *Candida* killing defect. On some occasions, the *Candida* yeast form also plays a critical role in fungal dissemination, e.g. *Candida glabrata* yeasts do not form hyphae but cause severe infections.[42] In critically ill neutropenic patients with a *Candida* sepsis, the indications for G-CSF/dexamethasone neutrophil transfusions may not alter, because these neutrophils are still capable to help kill the invasive *Candida* hyphae when antifungals seem ineffective.

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References

1. Denning DW, Bromley MJ. Infectious Disease. How to bolster the antifungal pipeline. Science. 2015;347(6229):1414-1416.
2. Gudlaugsson O, Gillespie S, Lee K, et al. Attributable mortality of nosocomial candidemia, revisited. Clin Infect Dis. 2003;37(9):1172-1177.
3. Johnston DL, Lewis V, Yanofsky R, et al. Invasive fungal infections in paediatric acute myeloid leukaemia. Mycoses. 2013;56(4):482-487.
4. Drewniak A, Kuijpers TW. Granulocyte transfusion therapy: randomization after all? Haematologica. 2009;94(12):1644-1648.
5. Marfin AA, Price TH. Granulocyte Transfusion Therapy. J Intensive Care Med. 2013;30(2):79-88.
6. Drewniak A, van Raam BJ, Geissler J, et al. Changes in gene expression of granulocytes during in vivo granulocyte colony-stimulating factor/dexamethasone mobilization for transfusion purposes. Blood. 2009;113(23):5979-5998.
7. Brown GD. Innate antifungal immunity: the key role of phagocytes. Annu Rev Immunol. 2011;29:1-21.
8. Gazendam RP, van Hamme JL, Tool AT, et al. Two independent killing mechanisms of Candida albicans by human neutrophils: evidence from innate immunity defects. Blood. 2014;124(4):590-597.
9. Kitagawa S, Yuo A, Souza LM, Saito M, Miura Y, Takaku F. Recombinant human granulocyte colony-stimulating factor enhances superoxide release in human granulocytes stimulated by the chemotactic peptide. Biochem Biophys Res Commun. 1987;144(3):1143-1146.
10. Roilides E, Walsh TJ, Pizzo PA, Rubin M. Granulocyte colony-stimulating factor enhances the phagocytic and bactericidal activity of normal and defective human neutrophils. J Infect Dis. 1991;163(3):579-583.
11. Nohmi T, Abe S, Tansho S, Yamaguchi H. Suppression of anti-Candida activity of murine and human neutrophils by glucocorticoids. Microbiol Immunol. 1994;38(12):977-982.
12. Roilides E, Uhlig K, Venzon D, Pizzo PA, Walsh TJ. Prevention of corticosteroid-induced suppression of human polymorphonuclear leukocyte-induced damage of Aspergillus fumigatus hyphae by granulocyte colony-stimulating factor and gamma interferon. Infect Immun. 1993;61(11):4870-4877.
13. Dale DC, Liles WC, Llewellyn C, Rodger E,

Price TH. Neutrophil transfusions: kinetics and functions of neutrophils mobilized with granulocyte-colony-stimulating factor and dexamethasone. Transfusion. 1998; 38(8):713-721.
14. Drewniak A, Boelens JJ, Vrielink H, et al. Granulocyte concentrates: prolonged functional capacity during storage in the presence of phenotypic changes. Haematologica. 2008;93(7):1058-1067.
15. Mochizuki K, Kikuta A, Ohto H, et al. Extended storage of granulocyte concentrates mobilized by G-CSF with/without dexamethasone and collected by bag separation method. Transfus Med. 2007; 17(4):296-303.
16. van Raam BJ, Drewniak A, Groenewold V, van den Berg TK, Kuijpers TW. Granulocyte colony-stimulating factor delays neutrophil apoptosis by inhibition of calpains upstream of caspase-3. Blood. 2008;112(5):2046-2054.
17. Leavey PJ, Sellins KS, Thurman G, et al. In vivo treatment with granulocyte colony-stimulating factor results in divergent effects on neutrophil functions measured in vitro. Blood. 1998;92(11):4366-4374.
18. Borregaard N, Sehested M, Nielsen BS, Sengelov H, Kjeldsen L. Biosynthesis of granule proteins in normal human bone marrow cells. Gelatinase is a marker of terminal neutrophil differentiation. Blood. 1995;85(3):812-817.
19. Zarember KA, Sugui JA, Chang YC, Kwon-Chung KJ, Gallin JI. Human polymorphonuclear leukocytes inhibit Aspergillus fumigatus conidial growth by lactoferrin-mediated iron depletion. J Immunol. 2007;178(10):6367-6373.
20. Gabay JE, Scott RW, Campanelli D, et al. Antibiotic proteins of human polymorphonuclear leukocytes. Proc Natl Acad Sci USA. 1989;86(14):5610-5614.
21. Lehrer RI. Functional aspects of a second mechanism of candidacidal activity by human neutrophils. J Clin Invest. 1972;51 (10):2566-2572.
22. Drewniak A, Gazendam RP, Tool AT, et al. Invasive fungal infection and impaired neutrophil killing in human CARD9 deficiency. Blood. 2013;121(13):2385-2392.
23. Liles WC, Rodger E, Dale DC. Combined administration of G-CSF and dexamethasone for the mobilization of granulocytes in normal donors: optimization of dosing. Transfusion. 2000;40(6):642-644.
24. Devi S, Wang Y, Chew WK, et al. Neutrophil mobilization via plerixafor-mediated CXCR4 inhibition arises from lung demargination and blockade of neutrophil homing to the bone marrow. J Exp Med. 2013;210(11):2321-2336.
25. Kumamoto CA, Vinces MD. Contributions of hyphae and hypha-co-regulated genes to Candida albicans virulence. Cell Microbiol. 2005;7(11):1546-1554.
26. Kinchen JM, Ravichandran KS. Phagosome maturation: going through the acid test. Nat Rev Mol Cell Biol. 2008;9(10):781-795.
27. Zhao XW, Gazendam RP, Drewniak A, et al. Defects in neutrophil granule mobilization and bactericidal activity in familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) syndrome caused by STXBP2/Munc18-2 mutations. Blood. 2013;122(1):109-111.
28. Lonnbro P, Nordenfelt P, Tapper H. Isolation of bacteria-containing phagosomes by magnetic selection. BMC Cell Biol. 2008;9:35.
29. Jiang S, Park DW, Tadie JM, et al. Human resistin promotes neutrophil proinflammatory activation and neutrophil extracellular trap formation and increases severity of acute lung injury. J Immunol. 2014;192(10): 4795-4803.
30. Jones LC, Moussa L, Fulcher ML, et al. VAMP8 is a vesicle SNARE that regulates mucin secretion in airway goblet cells. J Physiol. 2012;590(Pt 3):545-562.
31. van EM, van Roomen CP, Renkema GH, et al. Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. Int Immunol. 2005;17 (11):1505-1512.
32. Xu P, Roes J, Segal AW, Radulovic M. The role of grancalcin in adhesion of neutrophils. Cell Immunol. 2006;240(2):116-121.
33. Pizon V, Desjardins M, Bucci C, Parton RG, Zerial M. Association of Rap1a and Rap1b proteins with late endocytic/phagocytic compartments and Rap2a with the Golgi complex. J Cell Sci. 1994;107(Pt 6):1661-1670.
34. Yokoyama K, Kaji H, He J, et al. Rab27a negatively regulates phagocytosis by prolongation of the actin-coating stage around phagosomes. J Biol Chem. 2011;286(7): 5375-5382.
35. Lehrer RI, Szklarek D, Barton A, Ganz T, Hamann KJ, Gleich GJ. Antibacterial properties of eosinophil major basic protein and eosinophil cationic protein. J Immunol. 1989;142(12):4428-4434.
36. Plager DA, Loegering DA, Weiler DA, et al. A novel and highly divergent homolog of human eosinophil granule major basic protein. J Biol Chem. 1999;274(20):14464-14473.
37. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods. 2014;11 (8):783-784.
38. Rex JH, Bennett JE, Gallin JI, Malech HL, Melnick DA. Normal and deficient neutrophils can cooperate to damage Aspergillus fumigatus hyphae. J Infect Dis. 1990;162(2):523-528.
39. Rorvig S, Ostergaard O, Heegaard NH, Borregaard N. Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors. J Leukoc Biol. 2013;94(4):711-721.
40. Ferreira MC, Whibley N, Mamo AJ, Siebenlist U, Chan YR, Gaffen SL. Interleukin-17-induced protein lipocalin 2 is dispensable for immunity to oral candidiasis. Infect Immun. 2014;82(3):1030-1035.
41. Roes J, Choi BK, Power D, Xu P, Segal AW. Granulocyte function in grancalcin-deficient mice. Mol Cell Biol. 2003;23(3):826-830.
42. Zaoutis TE, Greves HM, Lautenbach E, Bilker WB, Coffin SE. Risk factors for disseminated candidiasis in children with candidemia. Pediatr Infect Dis J. 2004; 23(7): 635-641.