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


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

Running title: Mobilized neutrophils and impaired *Candida* killing

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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 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 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 e.g. lactoferrin, 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 availability 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 \textit{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 \textit{A. fumigatus} germination and kill their hyphae. However, the killing of \textit{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.
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 NH₄Cl-KHCO₃-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 microorganism 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, Bleiswijk, The Netherlands). Opsonisation was performed with 10% v/v human pooled serum for 15 min, at 37°C. For the neutrophil-mediated inhibition of germination, the same number of Aspergillus yeast cells were incubated with an increasing number neutrophils (0.25, 0.5, 1.0 or 1.5 *10⁵...
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). 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 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 in Hepes-medium. After opsonisation 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 experminents 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 supplemental data).

**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, Oxon, UK) and gp91phox (clone 7D5, MBL, Woborn, MA, USA). As a secondary antibody, Alexa488-rabbit-anti-mouse-Ig (Molecular Probes, Bleiswijk, Netherlands) was used. Samples were analyzed on an LSRII flow cytometer equipped with FACSDiva software (BD). Cells were gated based on their forward and side scatter, and 10,000 gated events were collected per sample.

**Degranulation assays**

Neutrophils (2×10⁶/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). The release of elastase and lactoferrin was evaluated with ELISA kits (HyCult Biotechnology) according to the manufacturer’s instructions. The proteolytic activity was determined by incubating neutrophils (2.5×10^6/ml in Hapes-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 (Exitation 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 supplemental figures is described in the Supplemental Materials.
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 (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 gp91phox, i.e. the catalytic plasmamembrane component of the NADPH oxidase enzyme complex, was normal when detected with the mAb 7D5 (Figure 1A). Also the functional NADPH oxidase activity upon cell activation was 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 (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 granules-markers appeared to be unaltered.
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 control neutrophils did (**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 (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 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 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) (Supplementary Figure S3A). Secondly, kinetic analyses showed that 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, 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, \(\alpha_M\beta_2\)) (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 \(b_{558}\) of the NAPDH oxidase system was identified (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) (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 significantly 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 (Supplementary Table 1)\(^{(33;34)}\).

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 (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 (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 (Supplementary Figure S6)(37). Both the CRISPR technique and the knock-out of the protein MBP more in particular did not interfere with important cytotoxic responses, including the ROS production by the NADPH oxidase system and Candida phagocytosis (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 well as 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\(^{11;12}\). 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 \textit{A. fumigatus}. The neutrophil killing of \textit{Aspergillus} hyphae depends on ROS production by the NADPH oxidase system\(^{(38)}\). Both the ROS production and \textit{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 \textit{A. fumigatus} germination and the killing of the hyphae.

The G-CSF/dexamethasone-mobilized neutrophils were able to phagocytose \textit{C. albicans}, but showed a clear defect in the intracellular killing. Analyses of the \textit{Candida}-phagosomes revealed that several proteins were reduced in the G-CSF/dexamethasone-mobilized cells, whereas the afore-mentioned 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. already reported that MBP(H) is also present in various granules of neutrophils\(^{(39)}\), while we have now confirmed by Immuno-EM analysis that neutrophil granules do contain MBP (\textbf{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 candidacidal proteins, e.g. it is 70-fold more toxic as defensins are\(^{(20)}\). Purified human MBP also displayed strong \textit{in vitro} inhibition of \textit{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 \textit{Candida} killing without any effect on phagocytosis and ROS production. The results in this neutrophil-like cell model confirmed that MBP is involved in \textit{Candida} killing.

In addition to MBP(H), 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 (\textbf{Figure 4}). Peroxidase activity is important, as neutrophils from MPO-deficient patients fail to kill \textit{Candida}\(^{(21)}\). Although no difference in the major azurophil granule protein MPO was detected, we cannot exclude a contribution of EPO to the observed \textit{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, \textit{Candida} killing improved only slightly when resistin was added, and was observed in both control and G-CSF/dexamethasone-mobilized neutrophils (\textbf{Supplementary Figure})
S8). The G-CSF/dexamethasone-mobilized phagosomes after fusion with granules showed also 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 (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 most in particular MBP in G-CSF/dexamethasone-mobilized neutrophil phagosomes, which contribute to the observed *Candida* killing defect. In some occasions, the *Candida* yeast form plays also 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.
**Disclosures**

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**Authorship**

T.W.K. is the principal investigator who conceived and designed the study. R.P.G., J.H., D.v.R., A v.d. G, A.T.J.T, H.J., A.B.M., F.A., M.v.d.B. and C.E.M.A. performed the experiments. T.K.B and D.R. contributed to the design of the study. R.P.G. devised and performed the analyses and wrote the manuscript together with T.W.K. All authors approved the final manuscript revisions and declare that there are no potential conflicts (financial, professional, or personal) of relevance to the manuscript.
References


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

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
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<tbody>
<tr>
<td>Major Basic Protein Homolog (MBPH)</td>
<td>C-type lectin, cytotoxin</td>
</tr>
<tr>
<td>Resistin (RETN)</td>
<td>Pro-inflammatory</td>
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<td>Poly(rC)-binding protein 1 (PCBP1)</td>
<td>RNA binding</td>
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<tr>
<td>Serine/arginine-rich splicing factor 4 (SRSF4)</td>
<td>RNA binding</td>
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<tr>
<td>Adenylyl cyclase-associated protein 1 (CAP1)</td>
<td>Receptor resistin, filament dynamics</td>
</tr>
<tr>
<td>Lipocalin-2 (LCN2)</td>
<td>Ferric siderophore, metalloprotease</td>
</tr>
<tr>
<td>Major Basic Protein (MBP)</td>
<td>C-type lectin, cytotoxin</td>
</tr>
<tr>
<td>Eosinophil peroxidase (EPX)</td>
<td>Peroxidase activity</td>
</tr>
<tr>
<td>Peptidoglycan recognition protein 1 (PGLYRP1)</td>
<td>Peptidoglycan receptor</td>
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Table legend. 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

<table>
<thead>
<tr>
<th>Vesicle-associated membrane protein 8 (VAMP8)</th>
<th>Vesicular fusion</th>
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</thead>
<tbody>
<tr>
<td>Grancalcin (GCA)</td>
<td>Pro-inflammatory</td>
</tr>
</tbody>
</table>

Figure legends

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<sup>phox</sup> by flow cytometry, left panel.

Morphological characteristics were assessed on a cytopsin, 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.

**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.

**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/dexamethasone-mobilized neutrophils were overnight incubated 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
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.
Figure 4A

G-CSF/dexa-treated donors vs. untreated controls

Figure 4B

increased

Candida

decreased

EPX

MBPH

LCN2

SRSF4

GCA

RETN

PCBP1

CAP1

MBP

EPX

PGLYRP1

VAMP8

Y-axis: Difference

X-axis: Log2 Fold Change

-2 to +2
### Table S1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
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<tr>
<td>Cytoskeleton-associated protein 4 (CKAP4)</td>
<td>Receptor for antiproliferative factor (AVF)</td>
</tr>
<tr>
<td>Apolipoprotein B receptor (APOBR)</td>
<td>Receptor involved in lipid transport</td>
</tr>
<tr>
<td>Torsin-1A-interacting protein 1 (TOR1AIP1)</td>
<td>Required for nuclear membrane integrity</td>
</tr>
<tr>
<td>Non-specific lipid-transfer protein (SCP2)</td>
<td>Lipid binding and transport</td>
</tr>
<tr>
<td>Ras-related protein Rap-1A (RAP1A)</td>
<td>Signaling, vesicular trafficking</td>
</tr>
<tr>
<td>S-adenosylmethionine synthase isoform type-2 (MAT2A)</td>
<td>Metabolic processes</td>
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<td>Signal transducer and activator of transcription 5A/5B (STAT5A/5B)</td>
<td>Signaling, transcription factor</td>
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<td>40S ribosomal protein S8 (RPS8)</td>
<td>RNA binding</td>
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<tr>
<td>Protein S100-P (S100P)</td>
<td>Cellular Calcium signaling</td>
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<td>Ras GTPase-activating-like protein (IQGAP1)</td>
<td>Calmodulin binding, regulator cytoskeleton</td>
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<td>F-actin-capping protein subunit alpha-1 (CAPZA1)</td>
<td>Binds Actin, regulator cytoskeleton</td>
</tr>
<tr>
<td>Heat shock protein HSP 90-alpha (HSP90AA1)</td>
<td>Binds ATP, mediates LPS-induced inflammatory response</td>
</tr>
<tr>
<td>Ras-related protein Rab-27A (RAB27A)</td>
<td>Required for granule docking, maturation and exocytosis</td>
</tr>
<tr>
<td>60S ribosomal protein L8 (RPL8)</td>
<td>RNA binding</td>
</tr>
</tbody>
</table>
Figure S2.

control

G-CSF/dexa
Figure S3.

A. C. albicans

B. Elastase (AG)
Figure S3.

C. 

*Candida* phagolysosome

- CD11b
- CD18
- Elastase (AG)
- MPO (AG)
- Lactoferrin (SG)
- MMP9 (GG)
- Cytochrome b559
- Defensin-1 (AG)
Figure S5.
Figure S6.

A. GAPDH (37 kDa)  
MBP (25 kDa)  
WT KO  
unstimulated PMA

B. C. albicans yeast phagocytosis (%)

C. 

WT MBP-KO

Time (min)

(%)

C. albicans yeast phagocytosis (%)

WT 
MBP-KO

PMA

unstimulated
Figure S8.

2 hours

C. albicans yeast viability (%)
Supplemental Table and Figure legends

Table S1. Distinct composition of the G-CSF/dexamethasone-mobilized phagosomes

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 human-derived proteins that were significantly increased in the phagosomes from the G-CSF/dexamethasone-treated donors as compared to untreated controls are listed in the table. Data from 5 GTX donors compared to 5 healthy controls.

Figure S1. G-CSF/dexamethasone treatment increased neutrophil counts

The peripheral blood neutrophil counts from healthy controls, G-CSF/dexamethasone-treated donors (N=5), G-CSF-treated donors (N=2) and dexamethasone-treated donors (N=2).

Figure S2. MPO-positive azurophilic granules in mobilized neutrophils

Neutrophils from healthy controls and GTX donors were stained for the expression of MPO and assessed by immuno- Electron Microscopy. The cytoplasmatic part is enlarged, as indicated with the black rectangle in the original image.

Figure S3. *Candida*-induced phagosomal formation

(A) The expression of *Candida* (green), elastase (yellow) and MPO (red) in the phagolysosomes was visualized by confocal imaging. (B) The number of elastase peptides identified by MS in the phagolysosomes from controls at 15, 45 and 90 minutes of incubation with *Candida*. (C) The numbers of peptides for the integrin CR3
(CD11b/CD18), elastase (azurophilic granule), MPO (azurophilic granule), lactoferrin (specific granule), MMP9 (gelatinase granule), cytochrome b558 and defensin-1,-3 (azurophilic granule) identified in the control and GTX phagolysosomes by Mass Spectrometry. Results are means ± SEM, N=5. * P < 0.05.

Figure S4. Decreased MBP(H) and EPX levels in G-CSF/dexamethasone-mobilized neutrophils

Neutrophils from healthy controls and G-CSF/dexamethasone-treated donors were isolated and analyzed by Mass Spectrometry. MBP, MBPH and EPX were significantly decreased in G-CSF/dexamethasone-mobilized neutrophils as compared to control neutrophils, depicted in a volcano plot and graph. N=5

Figure S5. MBP(H) and the viability of Aspergillus fumigatus

Incubation of Aspergillus hyphae for 2 hours or overnight incubation of Aspergillus conidia with the purified MBP (20-50 ng/ml), MBPH (20-50 ng/ml) or with the buffer only, and assessment of the viability relative to incubation without the buffer or recombinant proteins by a MTT assay. Results are means ± SEM, N=2, *P < 0.05

Figure S6. Neutrophil-like NB4 MBP-KO cells and NAPDH oxidase activation

(A) Protein MBP or GAPDH expression of the neutrophil-like NB4 WT and NB4 MBP-KO cells analyzed by Western blot. (B) The neutrophil-like NB4 WT, NB4 scrambled or NB4 MBP-KO cells were stimulated with PMA and the ROS production by the NAPDH oxidase system was measured in the Amplex Red assay. (C) Neutrophil-like NB4 WT or
NB4 MBP-KO were incubated with *C. albicans* yeast-FITC, and the phagocytosis was determined by flow cytometry. The percentage of phagocytosis is the number of internalised FITC-positive neutrophils relative to the total number of neutrophils. Results are means ± SEM, N=3.

**Figure S7. MBP-positive granules in neutrophils**

Neutrophils from healthy controls were stained for the expression of MBP and assessed by immuno-Electron Microscopy. The cytoplasmatic part is enlarged indicated with the black rectangle.

**Figure S8. Effect of Resistin on neutrophil-mediated *Candida* killing**

Neutrophils from healthy controls or G-CSF/dexamethasone-treated donors were primed with Resistin (25 ng/ml), incubated with *Candida* yeast and the killing was calculated relative to incubation without neutrophils and assessed by the colony forming unit assay. Results are means ± SEM, N=3.
Supplemental materials and methods

NADPH oxidase activity

NADPH-oxidase activity was assessed as the release of hydrogen peroxide, determined by the Amplex Red method (Molecular Probes) by neutrophils stimulated with various stimuli: zymosan (1 mg/ml), serum-treated zymosan (STZ, 1 mg/ml), phorbol-12-myristate-13-acetate (PMA, 100 ng/ml), platelet-activating factor (PAF) followed by formyl-Met-Leu-Phe (fMLP), in the presence of Amplex Red (0.5 μM) and horseradish peroxidase (1 U/ml). Fluorescence was measured at 30-second intervals for 20 minutes with the infinitiPRO2000 plate reader (Tecan, Mannesdorf, Switzerland). Maximal slope of H2O2 release was assessed over a 2-minute interval.

Neutrophil phagocytosis of Candida conidia

The neutrophil phagocytosis of unopsonizes and serum-opsonized Candida conidia was assessed by confocal microscopy. During a 90-min incubation at 37°C of neutrophils and FITC-labeled Candida conidia, every 5 to 10 minutes a sample was measured on the EVOS Fluorescence Cell imaging system (Life Technologies, Bleiswijk, The Netherlands). Per sample 5 pictures were taken and the percentage of phagocytosis was determined based on the neutrophils with internalised Candida-FITC relative to the total number of neutrophils per field.

Isolation of Candida phagolysosomes and neutrophils for Mass Spectrometry

For the isolation of Candida phagolysosomes we adapted a previously described method. After the overnight culture of Candida under aerobic conditions at 30°C in Lysogeny Broth (LB), the conidia were labeled with biotin for 30 minutes at 37 °C (2 mg/ml Sulfo-
NHS-LC-Biotin Thermo Scientific, Waltham, MA, USA). The biotinylated *Candida* conidia were washed with MACS buffer (2 mM EDTA, 0.5% albumin in PBS) and resuspended with Streptavidin MicroBeads (Miltenyi Biotec, Leiden, The Netherlands). The magnetically labeled *Candida* conidia were selected with a MACS Separation column (Miltenyi Biotec) and resuspended in Hepes buffer. Synchronized phagocytosis was induced after incubation with neutrophils (5x10⁶ cells/ml) on melting ice for 20 minutes in round-bottom tubes (BD). This was followed by incubation in a 37 °C water bath (600 rpm) and samples were taken at t = 15, t = 45 and t = 90 minutes, washed twice with PBS and resuspended in Mitobuffer (0.2 M EDTA, 0.25 M sucrose (Sigma), 10 mM TRIS (Life) supplemented with one tablet Protease Inhibitor Mix (PIM, Roche Applied Science, Indianapolis, IN, USA), 1 mM Pefablock (Roche Applied Science) and 1 mM di-isopropyl fluorophosphate (DFP, Sigma). Samples were frozen for 10 seconds in liquid nitrogen and thawed at room temperature. 10 µl of 1M triethanolamine (Sigma) and 10 mg/ml digitonin were added, and samples were kept on ice for 10 minutes. The cell wall was then disrupted with a potter homogenizer and the *Candida* phagolysosomes were isolated with a MACS column and resuspended in PBS. The *Candida* phagolysomes and percoll-isolated neutrophil cell pellets were frozen in liquid nitrogen and stored until use.

**Sample preparation for Mass Spectrometry**

The neutrophil cell pellets (5-10 x 10⁶) were lysed in 4% SDS, 100 mM DTT, 100 mM Tris.HCl pH 7.5 supplemented with HALT protease and phosphatase inhibitor cocktail (Thermo Scientific) and processed into tryptic peptides using the Filter Aided Sample Preparation method². Peptides were desalted and concentrated using Empore-C18 StageTips³ and eluted with 0.5% (v/v) acetic acid, 80 % (v/v) acetonitrile. Sample volume was reduced by SpeedVac and supplemented with 2 % acetonitrile, 0.1% TFA to a final
volume of 12 µl. For each sample, 3 technical replicates were analyzed by injecting 3 µl of the sample.

To analyse the phagolysosomes by Mass Spectrometry, the samples were concentrated in a SpeedVac (Thermo Scientific, Waltham, MA, USA) and were lysed by passing the volume through an insulin syringe (BD). With the BCA (bicinchoninic acid) protein assay kit (Thermo Scientific), the protein concentration was determined and 5 µg of protein was used for sample preparation for mass spectrometry. Samples were sonified with a Branson Sonifier with double-stepped microtip and urea (Life Technologies) in 100 mM TrisHCl (pH = 8.5) was added to a final concentration of 8M to ensure complete disruption of the phagolysosomes. The urea concentration was lowered with 50 mM ammonium bicarbonate (ABC, Sigma) and together with 100 mM dithiothreitol (DTT, Thermo Scientific) incubated for 60 minutes at 25 °C. Then, 2.5 µl of 550 mM iodoacetamide (IAM, Thermo Scientific) was added for 30 minutes at 25 °C in the dark. Finally, trypsin (Promega) was added overnight at 25°C in a ratio of 1:20 to digest the proteins. Trifluoroacetic acid (TFA, Thermo Scientific) was added to acidify the samples (pH ≈ 2) and inactivate the trypsin.

The peptides were subsequently purified by solid phase extraction on octadecyl (C18) disks (3 M Empore, St. Paul, MN, USA). The eluate was concentrated by SpeedVac and formic acid (Thermo Scientific) was added to reach a concentration of 200 ng/µl. Three µl was injected in the Mass Spectrometer (Thermo Scientific, Orbitrap Fusion).

Mass spectrometry data acquisition

Tryptic peptides were separated by nanoscale C18 reverse chromatography coupled on line to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) via a nanoelectrospray ion source (Nanospray Flex Ion Source, Thermo Scientific). Peptides were loaded on a 20 cm 75–360 µm inner-outer diameter fused silica emitter (New
Objective) packed in-house with ReproSil-Pur C18-AQ, 1.9 µm resin (Dr Maisch GmbH). The column was installed on a Dionex Ultimate3000 RSLC nanoSystem (Thermo Scientific) using a MicroTee union formatted for 360 µm outer diameter columns (IDEX) and a liquid junction. The spray voltage was set to 2.15 kV. Buffer A was composed of 0.5% acetic acid and buffer B of 0.5% acetic acid, 80% acetonitrile. Peptides were loaded for 17 min at 300 nl/min at 5% buffer B, equilibrated for 5 minutes at 5% buffer B (17-22 min) and eluted by increasing buffer B from 5-15% (22-87 min) and 15-38% (87-147 min), followed by a 10 minute wash to 90% and a 5 min regeneration to 5%. Survey scans of peptide precursors from 400 to 1500 m/z were performed at 120K resolution (at 200 m/z) with a $1.5 \times 10^5$ ion count target. Tandem mass spectrometry was performed by isolation with the quadrupole with isolation window 1.6, HCD fragmentation with normalized collision energy of 30, and rapid scan mass spectrometry analysis in the ion trap. The MS2 ion count target was set to 104 and the max injection time was 35 ms. Only those precursors with charge state 2–7 were sampled for MS2. The dynamic exclusion duration was set to 60 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 3 s cycles. All data were acquired with Xcalibur software.

**Mass spectrometry data analysis**

The RAW mass spectrometry files were processed with the MaxQuant computational platform, 1.5.2.8. Proteins and peptides were identified using the Andromeda search engine by querying the human Uniprot database (downloaded February 2015). Standard settings with the additional options match between runs, Label Free Quantification (LFQ), and unique peptides for quantification were selected. The generated ‘proteingroups.txt’ table was filtered for potential contaminants, reverse hits and ‘only identified by site’ using
Perseus 1.5.1.6. The LFQ values were transformed in log2 scale, the three technical replicates per experimental condition grouped and averaged based on the median, and proteins were filtered for at least three valid values in one of the experimental groups. Missing values were imputed by normal distribution (width=0.3, shift = 1.8), assuming these proteins were close to the detection limit. Quantitative significance was performed using an adapted permutation-based false discovery rate (FDR) t test in Perseus 1.5.1.6 software using FDR 0.02 and S0 2.5.

Confocal microscopy

Neutrophils were incubated with *Candida* on cover glasses (Ø 12 mm, Thermo Scientific) for 45 minutes at 37 °C and 5% CO₂, after which the glasses were resuspended in 3.7% paraformaldehyde (PFA, Merck, Darmstadt, Germany) in PBS. For staining, the cells were permeabilized with a IntraPrep kit (Beckman Coulter), stained with Hoechst (Sigma) and with monoclonal antibodies against human Elastase (Abcam), Myeloperoxidase (MPO) (Abcam) and MBP (Monosan, Uden, The Netherlands). Alexa Fluor F(ab’)₂ fragments of IgG (633 nm or 563 nm, Life) were used as secondary antibodies. Hereafter, the cover slides were placed on microscope slides (76x26mm, Thermo Scientific). Confocal images were made on the Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany).

Western blot analysis.

Analysis of protein expression was performed by Western blot. The following antibodies were used for detection: polyclonal rabbit anti-human MBP (Abcam, Cambridge, UK) and monoclonal mouse anti human GAPDH (Merck).
**Electron Microscopy**

Purified neutrophils were fixed in 2% (w/v) paraformaldehyde with 0.2% (w/v) glutaraldehyde and then processed for ultrathin cryosectioning. Cryosections (50 nm thick) were cut at −120°C with diamond knives (diatome) in a cryo-ultramicrotome (Leica, Vienna, Austria) and transferred onto carbon/formvar-coated copper grids. For immunolabelling, the sections were incubated for 10 min with 0.15 M glycine in PBS and for 10 min with 1% BSA in PBS to block free aldehyde groups and prevent aspecific antibody binding, respectively. Sections were incubated with anti-human MPO (DAKO) and 10 nm protein-A conjugated colloidal gold (EMlab, University of Utrecht) all in 1% BSA in PBS, and finally embedded in methylcellulose with 0.6% uranyl acetate, and examined with a CM10 electron microscope (Philips, Eindhoven, The Netherlands).

**CRISPR knockout NB4 cells and differentiation into neutrophil-like cells**

To generate NB4 cells knock out for MBP, we used the Zhang lab Optimized CRISP Design tool (http://crispr.mit.edu/) to determine the Cas9 target sites present in the coding sequence of MBP. A ds oligo (Invitrogen) was generated of several target sequences, with an additional G 5' of the target sequence and specific BsmBI overhang. The oligo was then cloned into the BsmBI sites of pLentiCRISPR v2. The constructs were grown in E.coli Stbl3 and sequence verified. Lentiviral particles were generated by transient cotransfection of 293T cells with pLentiCRISPRv2 – MBP ko, psPAX2 and pCMV-VSVg. The day after transfection, the cells were put on NB4 medium. Virus-containing supernatant was harvested on day 2 and 3 after transfection and pressed through an 0.45 um filter, and 1 ml was used on 5*10^5 NB4 cells on two successive days. Transduced NB4 cells were selected with 1 µg/ml puromycin (Invivogen). Surviving cells were put on limiting dilution in a 96-wells plate at 0.5 cell / 150 µl, and growing clones were routinely maintained. Expression of MBP in these
clones was determined by Western blot. Transduction of NB4 cells with pLentiCRISPR v2 containing target sequences 5’-tccacctttgagaccccttt -3’ or 5’-gggtgctaagacgctgcctg-3’ resulted in clones that lacked expression of MBP. LentiCRISPR v2 was a gift from Feng Zhang (MIT, Cambridge, MA, USA, Addgene plasmid # 52961). NB4 scrambled (control cells) were generated by lentiviral transduction. Lentiviral particles were generated by transient cotransfection of 293T cells with pLKO.1puro scrambled (Sigma MISSION control SHC002), pMDLgp, pRSV-rev and pCMV-VSVg. The day after transfection, the cells were put on NB4 medium. Virus-containing supernatant was harvested on day 2 and 3 after transfection and pressed through an 0.45 μM filter, and 1 ml was used on 5*10^5 NB4 cells on two successive days. Transduced NB4 cells were selected with 1 μg/ml puromycin (Invivogen).

For the differentiation into neutrophil-like cells, the NB4 cells (final concentration 1*10^6/mL) were cultured for 7 days at 37°C in IMDM medium containing L-glutamine, Penicillin/Streptomycin and 20% FCS (Life, Bleiswijk, The Netherlands) and in the presence of 0.2 mM ATRA (Sigma). The differentiation into neutrophil-like cells was tested by the determination of the ROS production by the NADPH oxidase system in the Amplex Red assay (as described above).

*Production of recombinant MBP and MBPH*

Human MBP and MBPH DNA sequences with a 3’ HPC4 tag (EDQVDPRPLIDGK) were synthesized by Invitrogen. In addition, a thrombin cleavage site (SLVPR) was created in MBP between Q105 and T106. The final sequence was cloned into pCDNA3.1+.

Recombinant protein was produced in Freestyle HEK cells (Invitrogen) by transient cotransfection of pCDNA3.1+ - MBP/MBPH, pORF-p21, pORF-p27 and pSVLT, using 293Fectin (Invitrogen). Supernatant was harvested 3 days after transfection and filtered.
through 0.45 µM. Recombinant HPC4 tagged protein was purified using Anti-Protein C Affinity Matrix (Roche), elution fractions containing protein were dialysed against PBS.

Reference List

1 Lonnbro,P. et al. (2008) Isolation of bacteria-containing phagosomes by magnetic selection. BMC. Cell Biol. 9, 35


4 Sanjana,N.E. et al. (2014) Improved vectors and genome-wide libraries for CRISPR screening. Nat. Methods 11, 783-784