SUPPLEMENTARY APPENDIX

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

Roel P. Gazendam,[1] Annemarie van de Geer,[1] John L. van Hamme,[1] Anton T.J. Tool,[1] Dieke J. van Rees,[1] Cathelijn E.M. Aarts,[1] Maartje van den Biggelaar,[1] Floris van Alphen,[1] Paul Verkuijlen,[1] Alexander B. Meijer,[1] Hans Janssen,[2] Dirk Roos,[1] Timo K. van den Berg[1] and Taco W. Kuijpers[1,3]

[1]Sanquin Research, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam; [2]The Netherlands Netherlands Cancer Institute, Division of Cell Biology, Amsterdam; [3]Emma Children's Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

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Correspondence: r.gazendam@sanquin.nl

Table S1.

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

Figure S1.

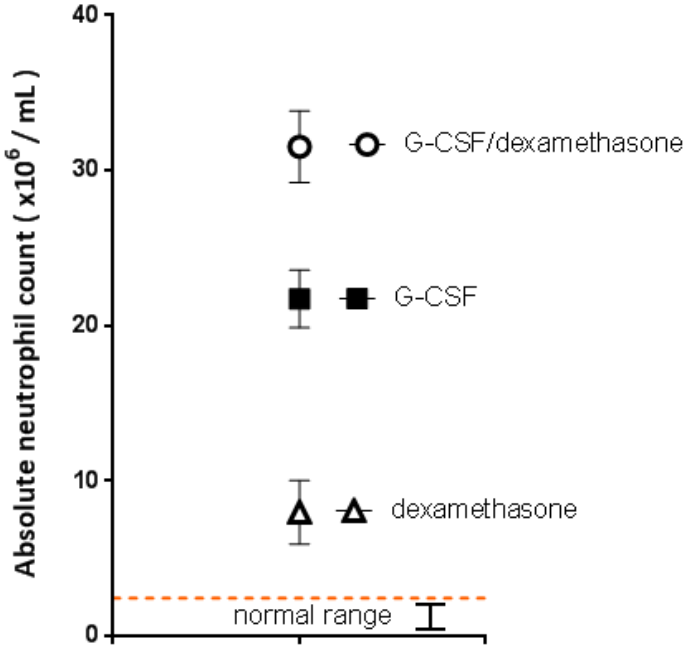
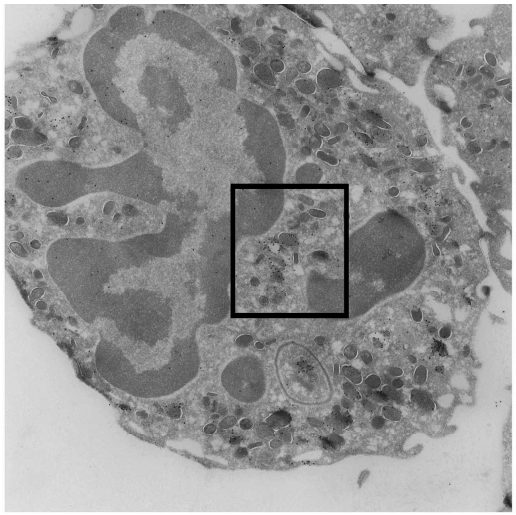
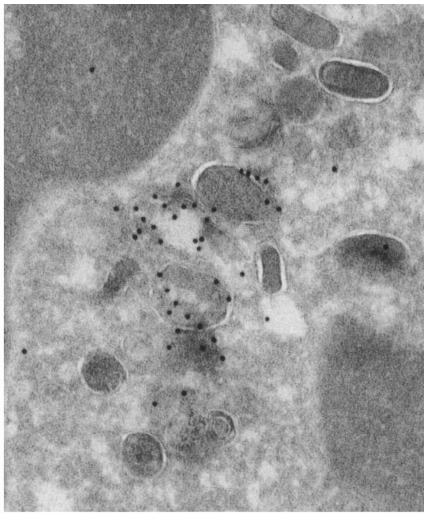

Absolute neutrophil count (x10^6 / mL)
40
30
20
10
0
G-CSF/dexamethasone
G-CSF
dexamethasone
normal range

Figure S2.

control

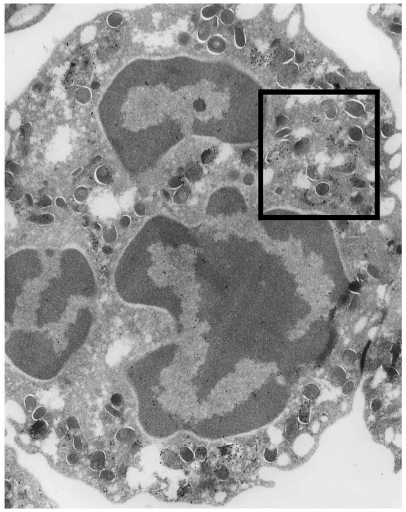


500 nm

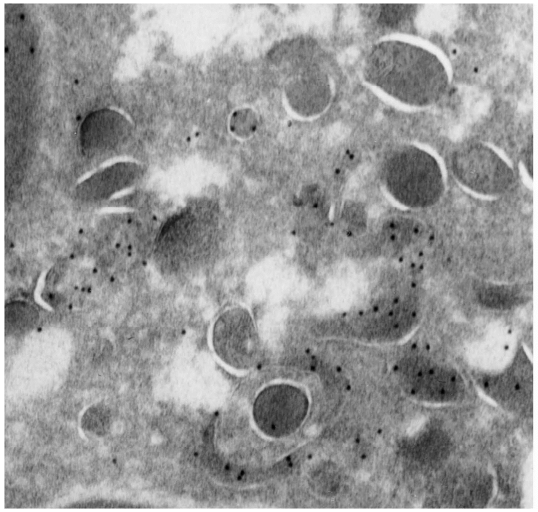


250 nm

G-CSF/dexa



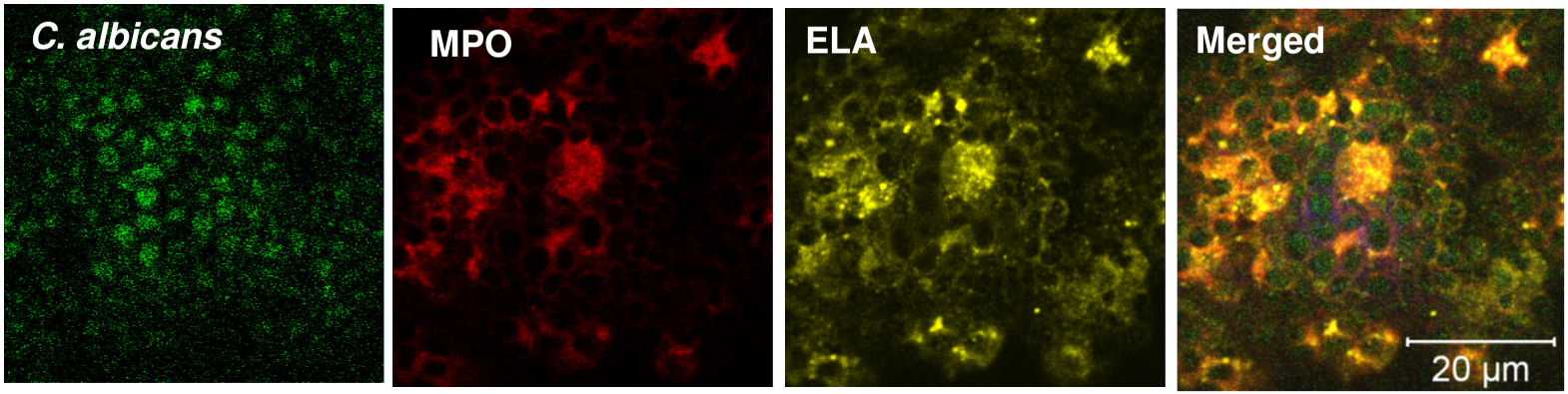
500 nm



250 nm

Figure S3.

A.


C. albicans
MPO
ELA
Merged
20 µm

B.

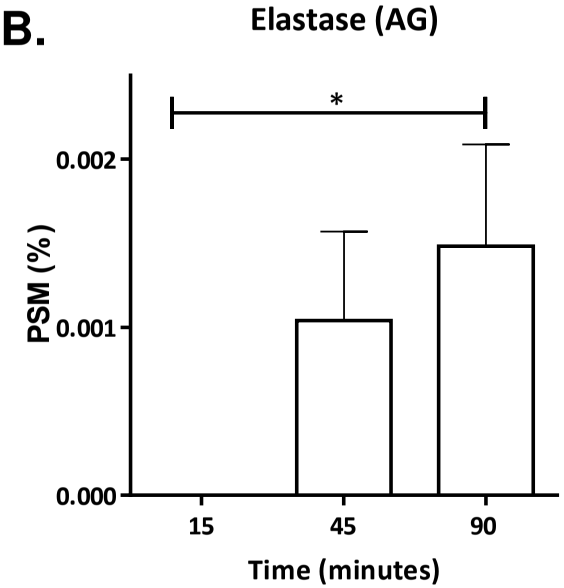

Elastase (AG)
*
0.002
0.001
0.000
PSM (%)
15
45
90
Time (minutes)

Figure S3.

C.

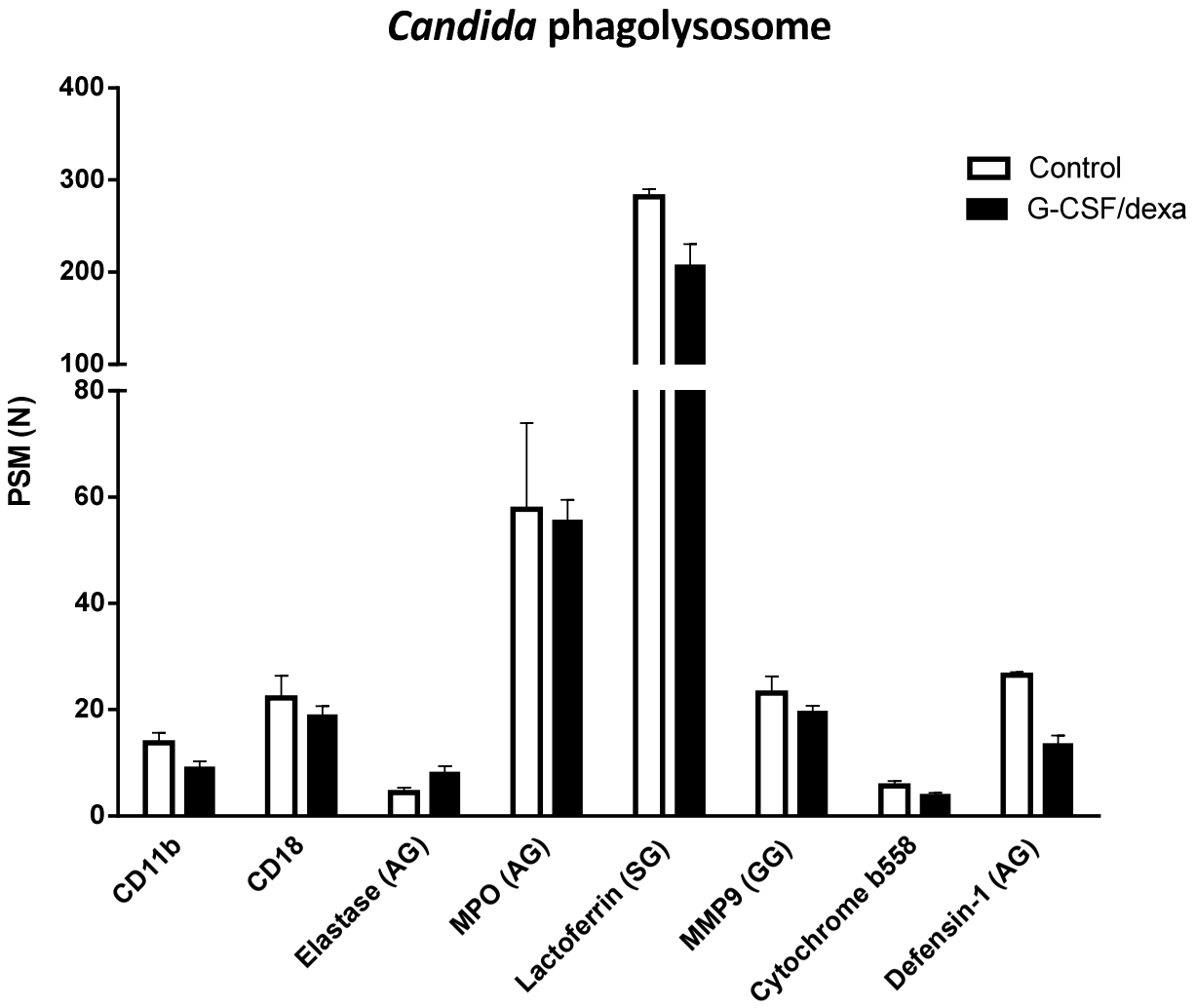
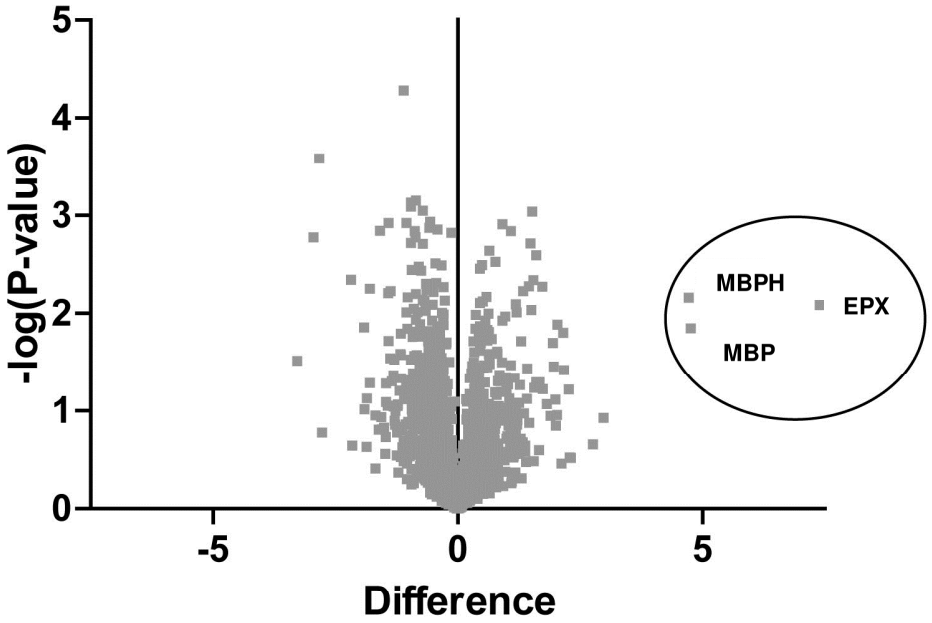

Candida phagolysosome
Control
G-CSF/dexa
PSM (N)
400
300
200
100
80
60
40
20
0
CD11b
CD18
Elastase (AG)
MPO (AG)
Lactoferrin (SG)
MMP9 (GG)
Cytochrome b558
Defensin-1 (AG)

Figure S4.


-log(P-value)
5
4
3
2
1
0
-5
0
5
Difference
MBPH
EPX
MBP

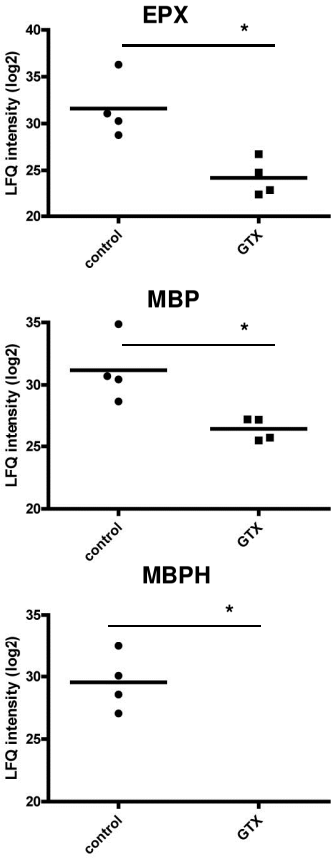

EPX
*
LFQ intensity (log2)
40
35
30
25
20
control
GTX
MBP
*
LFQ intensity (log2)
35
30
25
20
control
GTX
MBPH
*
LFQ intensity (log2)
35
30
25
20
control
GTX

Figure S5.

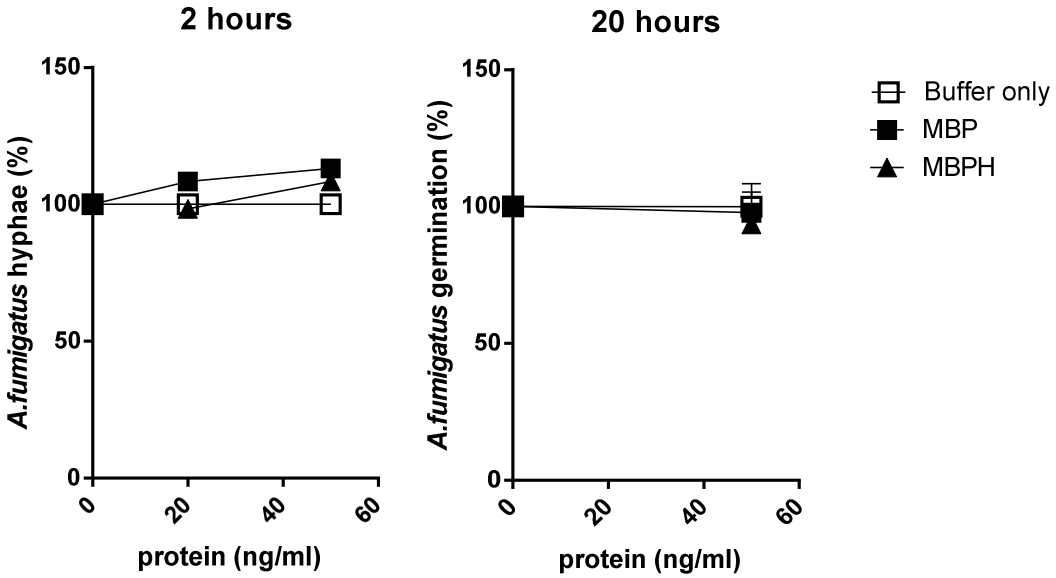

2 hours
20 hours
A.fumigatus hyphae (%)
A.fumigatus germination (%)
150
100
50
0
0
20
40
60
protein (ng/ml)
protein (ng/ml)
Buffer only
MBP
MBPH

Figure S6.

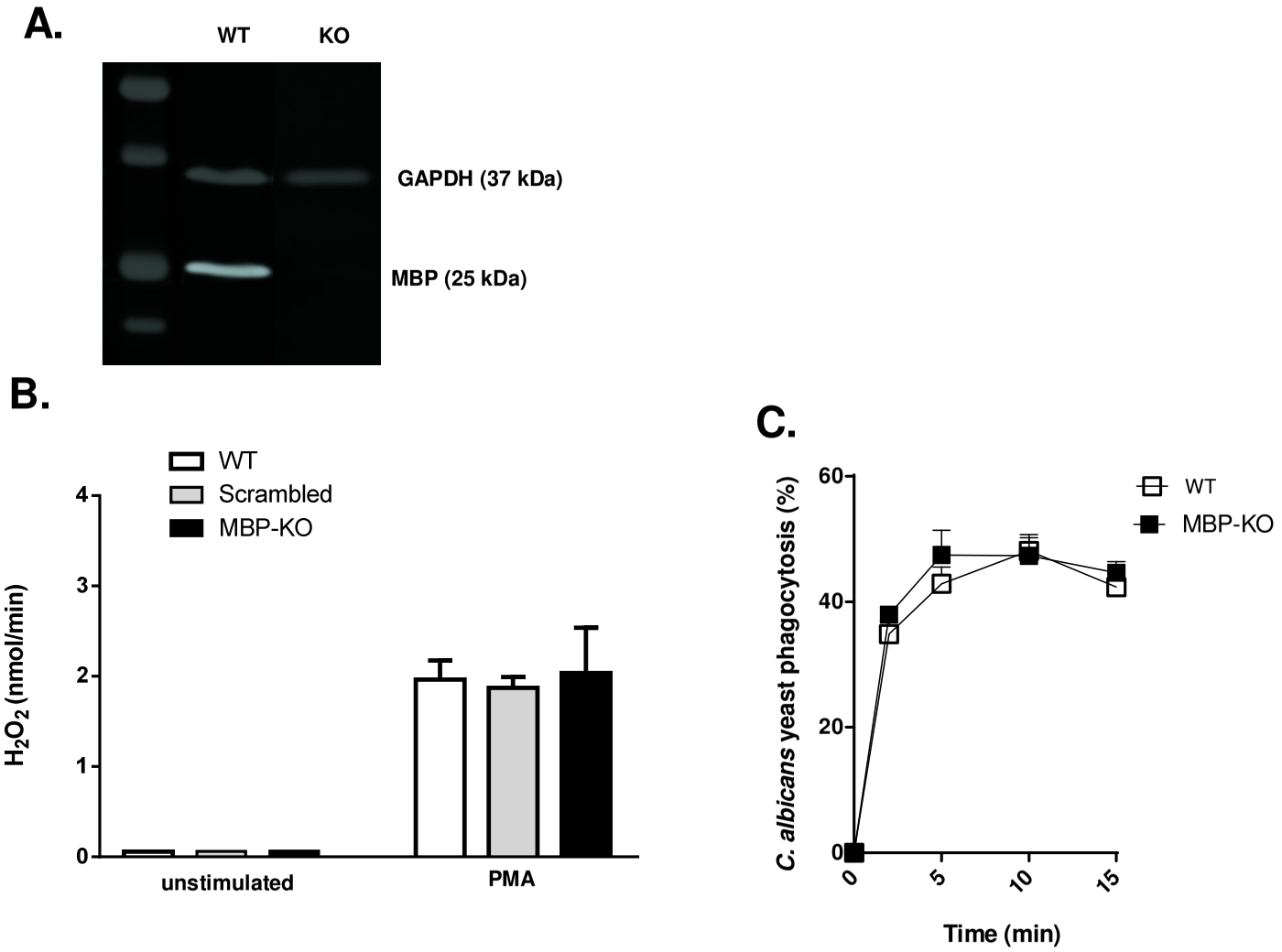
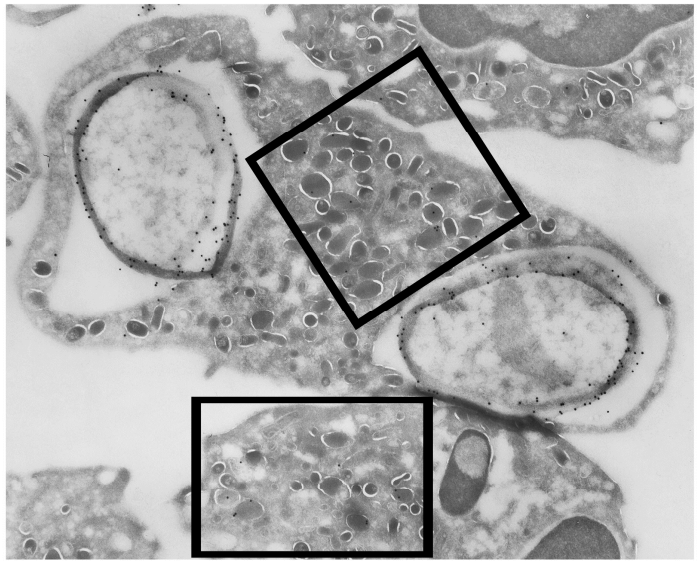
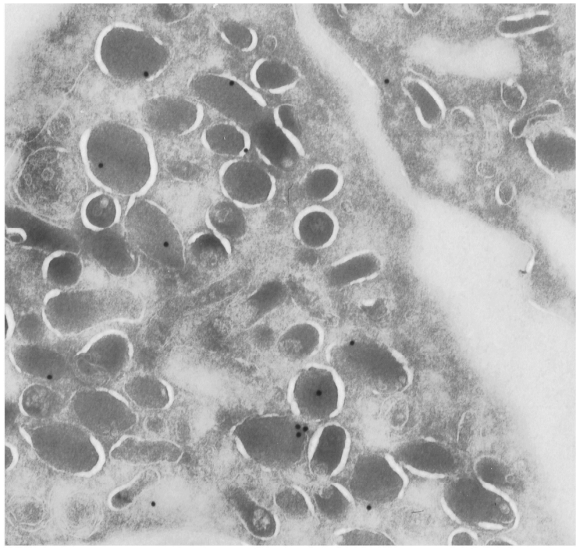

A.
WT
KO
GAPDH (37 kDa)
MBP (25 kDa)
B.
WT
Scrambled
MBP-KO
H_2O_2 (nmol/min)
0
1
2
3
4
unstimulated
PMA
C.
WT
MBP-KO
C. albicans yeast phagocytosis (%)
0
20
40
60
0
5
10
15
Time (min)

Figure S7.





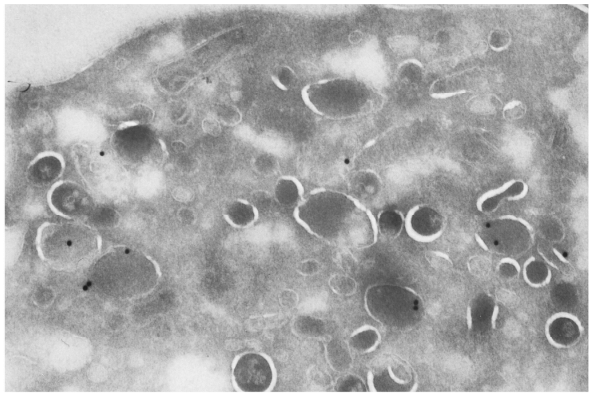
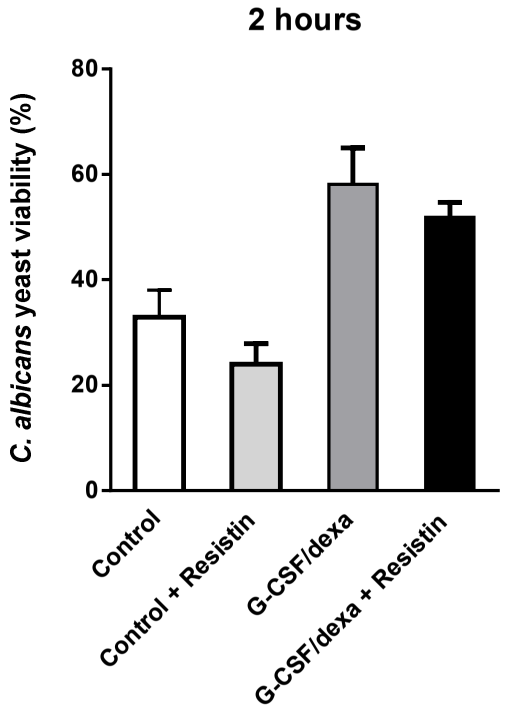


Figure S8.


2 hours
C. albicans yeast viability (%)
80
60
40
20
0
Control
Control + Resistin
G-CSF/dexa
G-CSF/dexa + Resistin

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 b_{558} 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[1]. 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, Indianopolis, 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 $\times 10^6$) 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 method[2]. Peptides were desalted and concentrated using Empore-C18 StageTips[3] 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 8Mto 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 × 105 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% CO_2, 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 targetsequences 5'-tccacctttgagacccctttt -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).[4] 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 uM, 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 37C 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 decribed above).

Production of recombinant MBP and MBPH

Human MBP and MBPH DNA sequences with a 3' HPC4 tag (EDQVDPRLIDGK) 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

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