

Restoration of anti-*Aspergillus* defense by neutrophil extracellular traps in human chronic granulomatous disease after gene therapy is calprotectin-dependent

Matteo Bianchi, MSc,^{a*} Maria J. Niemiec, MSc,^{b*} Ulrich Siler, PhD,^a Constantin F. Urban, PhD,^{b,‡} and Janine Reichenbach, MD^{a,‡} Zurich, Switzerland, and Umeå, Sweden

Background: *Aspergillus* spp infection is a potentially lethal disease in patients with neutropenia or impaired neutrophil function. We showed previously that *Aspergillus* hyphae, too large for neutrophil phagocytosis, are inhibited by reactive oxygen species-dependent neutrophil extracellular trap (NET) formation. This process is defective in chronic granulomatous disease (CGD) because of impaired phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase function. **Objective:** To determine the antifungal agent and mechanism responsible for reconstitution of *Aspergillus* growth inhibition within NETs after complementation of NADPH oxidase function by gene therapy (GT) for CGD.

Methods: Antifungal activity of free and NET-released calprotectin was assessed by incubation of *Aspergillus nidulans* with purified calprotectin, induced NETs from human controls, and CGD neutrophils after GT in the presence or absence of Zn^{2+} or α -S100A9 antibody, and with induced NETs from wild-type or *S100A9*^{-/-} mouse neutrophils.

Results: We identified the host Zn^{2+} chelator calprotectin as a neutrophil-associated antifungal agent expressed within NETs, reversibly preventing *A. nidulans* growth at low concentrations,

and leading to irreversible fungal starvation at higher concentrations. Specific antibody-blocking and Zn^{2+} addition abolished calprotectin-mediated inhibition of *A. nidulans* proliferation *in vitro*. The role of calprotectin in anti-*Aspergillus* defense was confirmed in calprotectin knockout mice.

Conclusion: Reconstituted NET formation by GT for human CGD was associated with rapid cure of pre-existing therapy-refractory invasive pulmonary aspergillosis *in vivo*, underlining the role of functional NADPH oxidase in NET formation and calprotectin release for antifungal activity. These results demonstrate the critical role of calprotectin in human innate immune defense against *Aspergillus* infection. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Chronic granulomatous disease, gene therapy, neutrophil extracellular trap, calprotectin, *Aspergillus* infection

From ^athe Division of Immunology/Hematology/BMT, University Children's Hospital Zurich; and ^bthe Antifungal Immunity Group, Molecular Biology Department, Laboratory for Molecular Infection Medicine Sweden, Umeå University.

*These authors contributed equally to this work.

‡These authors contributed equally to this work.

Supported by a grant of the Chronic Granulomatous Disorder Research Trust, United Kingdom (grant no. J4G/08/01 to J.R. and M.B.), a grant of GEBERT RÜF STIFTUNG, Switzerland (grant number GRS-046/10 to J.R.), a grant of the EU FP7 CELL-PID programme (J.R. and U.S.), and a grant of the Swiss National Science Foundation (grant no. 320000-121983 to U.S.). M.J.N. and C.F.U. were supported by a starting grant from MIMS, a stipend from Stiftelsen J. C. Kempes Minnes Stipendiefond, and a travel financing grant from Medicinska Fakulteten Umeå Universitet. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

Disclosure of potential conflict of interest: M. Bianchi and J. Reichenbach have received research support from the CGD Research Trust. M. J. Niemiec has received research support from the Stiftelsen J. C. Kempes Minnes. U. Siler has received research support from the Swiss National Science Foundation. C. F. Urban has received research support from Molecular Infection Medicine Sweden, Stiftelsen J. C. Kempes Minnes, and Med/Cinska Fakulteten Umeå University.

Received for publication October 28, 2010; revised December 26, 2010; accepted for publication January 10, 2011.

Reprint requests: Janine Reichenbach, MD, Division of Immunology/Hematology/BMT, University Children's Hospital Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland. E-mail: janine.reichenbach@kispi.uzh.ch. Constantin F. Urban, PhD, Antifungal Immunity Group, Molecular Biology Department, Laboratory for Molecular Infection Medicine Sweden, Umeå University, 90187 Umeå, Sweden. E-mail: constantin.urban@molbiol.umu.se.

0091-6749/\$36.00

© 2011 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2011.01.021

Neutrophils kill microbes by distinct reactive oxygen species (ROS)-dependent processes: intracellularly after phagocytosis and by extracellular mechanisms including neutrophil extracellular traps (NETs), which capture and kill bacteria,¹ parasites,² and fungi.^{3,4} NETs are composed of chromatin (histones and DNA), decorated with at least 20 other proteins.^{1,4,5} In naive neutrophils, 8 of these proteins localize to granules, such as neutrophil elastase, myeloperoxidase, or azurocidin, and 11 to the cytoplasm, such as catalase and the calprotectin complex.⁴ This heteroduplex formed by subunits S100A8 and S100A9 belongs to the calcium-binding protein family of S100 proteins and exerts strong microbistatic activity against a variety of microorganisms *in vitro*.⁶

Defective nicotinamide adenine dinucleotide phosphate (NADPH) oxidase function accounts for impaired phagocyte ROS production and poor microbial killing in chronic granulomatous disease (CGD), leading to recurrent life-threatening bacterial and fungal infections. *Aspergillus* spp infections cause pneumonia and disseminated disease and are the leading cause of death in these patients.⁷⁻¹⁰ ROS production by the NADPH oxidase is indispensable for microbe-triggered NET formation,^{5,11,12} whereas their mechanism of action in this process is unknown. After release from granules, neutrophil elastase (NE) and myeloperoxidase synergize to drive decondensation of chromatin, an early event preceding the release of NETs.^{13,14} ROS might contribute to the release of NE and myeloperoxidase from granules. We previously reported impaired NET formation in CGD *in vitro*, resulting in defective clearance of *Aspergillus* infection *in vivo*. NADPH oxidase-dependent ROS production and NET inhibition of *Aspergillus* conidia and hyphae were restored early after gene therapy (GT) in a patient with X-linked

Abbreviations used

CGD:	Chronic granulomatous disease
DAPI:	4'-6-diamidino-2-phenylindole, dihydrochloride
GT:	Gene therapy
LTR:	Long terminal repeat
NADPH:	Nicotinamide adenine dinucleotide phosphate
NE:	Neutrophil elastase
NET:	Neutrophil extracellular trap
phox:	Phagocyte oxidase
PMA:	Phorbol 12-myristate 13-acetate
ROS:	Reactive oxygen species

gp91^{phox}-deficient CGD, paralleled by clearance of therapy-refractory *Aspergillus nidulans* lung infection.¹¹

The antifungal agent responsible for *A nidulans* growth inhibition within NETs has not been characterized. Here we show that among the 24 NET-associated proteins, the major antifungal agent inhibiting *Aspergillus* growth within NETs is the cytoplasmic calprotectin protein complex (S100A8/A9 heteroduplex). This is in good agreement with our previous report showing that calprotectin is the major NET component against *Candida albicans*.⁴

METHODS

We treated a boy 8 years 7 months old with X-linked gp91^{phox} (phox, phagocyte oxidase)-deficient CGD and multifocal therapy-refractory *A nidulans* lung infection with a monocistronic long terminal repeat-driven γ -retroviral SF71gp91^{phox} vector (see reference¹¹ for details; additional information in this article's Methods section in the Online Repository at www.jacionline.org) under a protocol approved by the ethics review board of the University Children's Hospital Zurich and the Swiss Expert Committee for Bio-Safety after written informed consent of the parents.

NET induction and quantification

Neutrophil extracellular trap formation was quantified as described¹¹ after stimulation of 5×10^4 neutrophils (control, CGD unsorted, CGD gp91^{phox}-negative [gp91^{phox}-], and gp91^{phox}-positive [gp91^{phox}+]) sorted neutrophils) for 4 hours with 40 nmol/L phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Taufkirchen, Germany) and staining the NET-DNA with 1 μ mol/L Sytox green (Invitrogen, Molecular Probes, Leiden, The Netherlands) in a black 96-well plate. Nonstimulated neutrophils were used as negative control. The plates were read in a fluorescence microplate reader (Victor 3, PerkinElmer, Waltham, Mass) with a filter setting of 485/535 nm (excitation/emission).

***A nidulans* growth inhibition**

Antifungal activity of purified calprotectin was assessed by incubating *A nidulans* conidia or hyphae with 0.5 to 128 μ g/mL recombinant S100A8 and S100A9 (ProtEra, Florence, Italy) $\pm$ 1 μ mol/L ZnSO₄ (Sigma-Aldrich) or 15 μ g/mL rabbit polyclonal α -S100A9 antibody (H00006280-D01P, Abnova, Taipei, Taiwan). Antifungal activity of NETs was determined by incubating *A nidulans* conidia or hyphae (strain isolated as described¹⁵ from bronchoalveolar lavage fluid of the patient) with human and murine NETs or human NET extracts $\pm$ 0 to 2 μ mol/L ZnSO₄ or 15 μ g/mL rabbit polyclonal α -S100A9 antibody, respectively (additional information in the Methods section in the Online Repository).

NET induction with *A nidulans*

In a 24-well plate, 2×10^5 control, CGD gp91^{phox}- and gp91^{phox}+ neutrophils were incubated with *A nidulans* hyphae obtained from culture of 6×10^4

A nidulans conidia on polylysine-coated cover slips for 5 hours at 37°C, then fixed in 4% paraformaldehyde for bright field microscopy and immunostaining/confocal microscopy, or 2.5% glutaraldehyde for scanning electron microscopy. For confocal microscopy (SP5; Leica, Wetzlar, Germany), specimens were blocked with 5% donkey serum (Jackson ImmunoResearch, Suffolk, United Kingdom), 1% BSA, 3% cold water fish gelatin (Sigma-Aldrich), and 0.25% Tween 20 in PBS, incubated with primary antibodies directed against S100A8/A9 (BM4029 and BM4027; Acris, Herford, Germany), common *Aspergillus* spp soluble proteins (NB100-65026, Novus Biologicals, Cambridge, United Kingdom), and species-specific secondary antibodies coupled to Alexa Fluor 488 and 568 (Invitrogen, Molecular Probes). DNA was stained with 4'-6-diamidino-2-phenylindole, dihydrochloride (DAPI; Sigma-Aldrich). For scanning electron microscopy (CM208; Philips, Eindhoven, The Netherlands), specimens were postfixed with 2% osmium tetroxide, dehydrated with graded ethanol series (50% to 100%), critical point-dried, and coated with 10 nm platinum.

Release of calprotectin

In a 96-well plate, 5×10^4 control, CGD gp91^{phox}- and gp91^{phox}+ neutrophils were stimulated in duplicate with 40 nmol/L PMA at 37°C and 5% CO₂ for 4 hours to form NETs. After 30 minutes NET digestion with 5 U/mL Micrococcal nuclease (Worthington Biochemicals, Lakewood, NJ) + 1 U/mL Deoxyribonuclease-1 (Sigma-Aldrich), samples were centrifuged for 10 minutes at 10,000g to remove debris. Heterodimeric calprotectin (S100A8/A9) concentration was measured by ELISA (Hycult, Uden, The Netherlands; detection limit 1.6 ng/mL) with 1/10, 1/40, and 1/80 dilutions. As control, total cytoplasmic calprotectin was quantified after neutrophil lysis.

RESULTS**NET quantification in CGD neutrophils after GT**

We aimed to identify factors that limit *A nidulans* growth in NADPH oxidase-dependent NETs. Therefore, we first analyzed whether NET formation was restored 2.6 years after GT in gp91^{phox}+ (transduced) compared with gp91^{phox}- (nontransduced) neutrophils from the reported patient with CGD.¹¹ Staining with α -gp91^{phox} antibody showed 22.8% gp91^{phox}+ neutrophils. Sorting of gp91^{phox}- and gp91^{phox}+ neutrophils by autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) resulted in purity of 98.3% and 92.5% (Fig 1, A). Quantification of NET formation in CGD neutrophils after GT correlated well with the percentage of gp91^{phox}+ cells, 20.5% of unsorted, 3.2% of gp91^{phox}-, and 87.6% of gp91^{phox}+ neutrophils releasing NETs (Fig 1, B). The O₂⁻ production of individual gp91^{phox}+ neutrophils was 27.8% (compared with control), measured by reduction of cytochrome c (data not shown),¹⁶ indicating this threshold as enough for substantial, albeit not 100%, NET formation.

Calprotectin inhibits *A nidulans* growth efficiently

We previously identified the cytoplasmic protein complex calprotectin as major antifungal effector in NETs preventing growth of *C albicans*⁴ and therefore reasoned that calprotectin might also be responsible for NET-mediated growth inhibition of *A nidulans*. There is currently no information available whether purified calprotectin is able to inhibit *Aspergillus* spp. To evaluate the inhibitory activity of each calprotectin subunit on *A nidulans* growth, we incubated conidia and hyphae with purified subunits S100A8 and S100A9.¹⁷ Addition of S100A8 did not inhibit fungal growth, whereas S100A9 had a small effect on conidia germination but not on hyphae growth (Fig 2, A and B; see this article's Fig E1 in the Online Repository at www.jacionline.org). Only

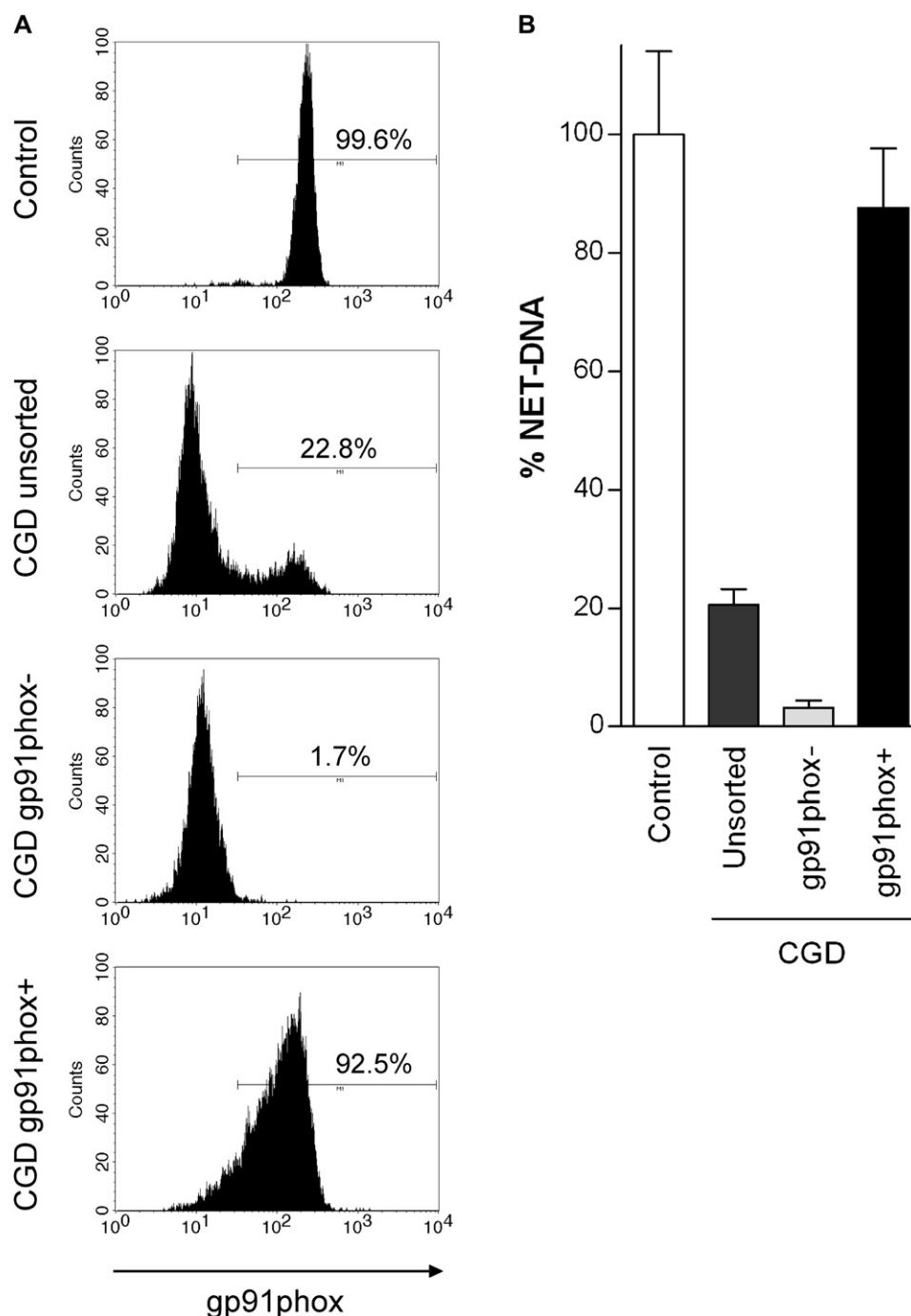


FIG 1. NET quantification in CGD gp91^{phox+} and gp91^{phox-} sorted neutrophils. **A**, Neutrophils were sorted by magnetic beads and autoMACS. **B**, NET formation was quantified after PMA activation of neutrophils by staining of released NET-DNA with Sytox green. Data are means ± SDs of representative triplicate experiments. The difference between control and gp91^{phox+} neutrophils was nonsignificant ($P > .05$).

both subunits together strongly inhibited *A nidulans* growth, indicating that the heteroduplex is required for full inhibition of *Aspergillus* spp, consistent with a report on its anticandidal activity.¹⁸ In all cases, addition of 1 $\mu\text{mol/L}$ zinc (Zn^{2+}) restored fungal growth, confirming the putative role of Zn^{2+} chelation by calprotectin in suppressing fungal growth.¹⁸⁻²⁰ In addition, we preincubated combined S100A9 and S100A8 with a polyclonal α -S100A9 antibody. *A nidulans* growth of conidia and hyphae

was restored in the presence of specific antibody (Fig 2, C and D; see this article's Fig E2 in the Online Repository at www.jacionline.org), confirming the requirement for interaction of both calprotectin subunits for antifungal activity. Unspecific control antibody did not have any effect. Here we used an excess amount of 5 $\mu\text{g/mL}$ purified S100A8 and S100A9, but similar inhibitory effects were obtained with as low as 0.5 $\mu\text{g/mL}$, a calprotectin amount found in NETs from about 4 million neutrophils.⁴

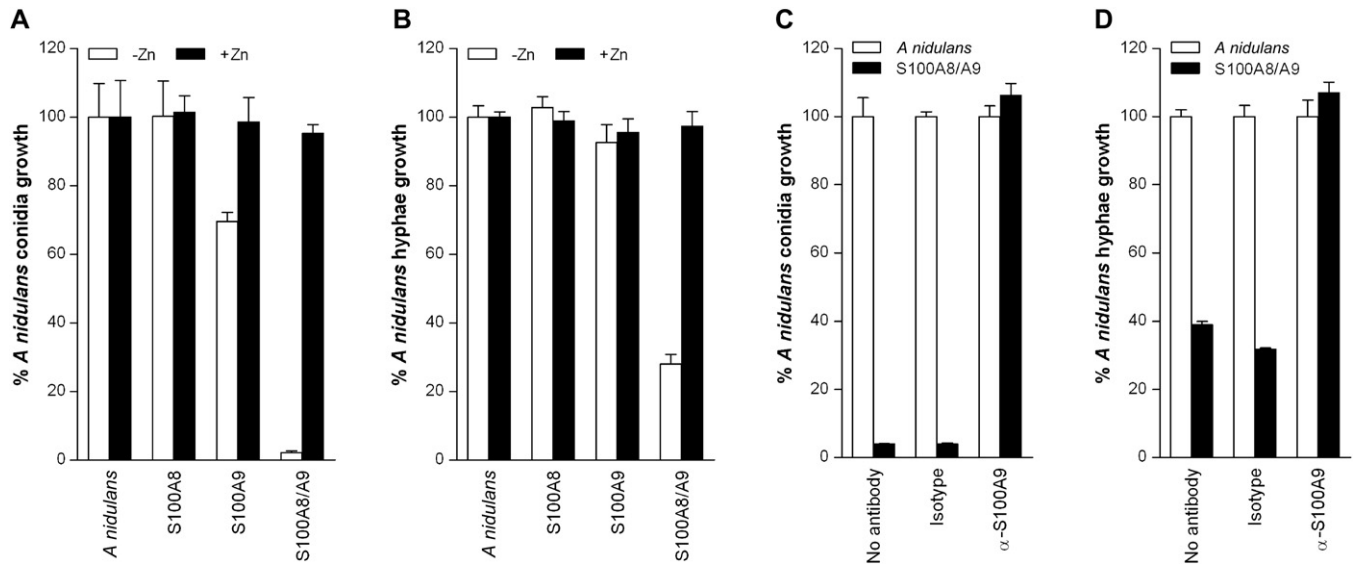


FIG 2. Inhibition of *A. nidulans* growth by purified calprotectin. **A and B**, Conidia and hyphae incubated with 5 μ g/mL S100A8 and/or S100A9 $\pm$ 1 μ mol/L Zn^{2+} . **C and D**, Conidia and hyphae incubated with 5 μ g/mL S100A8 and S100A9 $\pm$ 15 μ g/mL polyclonal α -S100A9 antibody or unspecific control antibody. Data are means $\pm$ SDs of representative triplicate experiments.

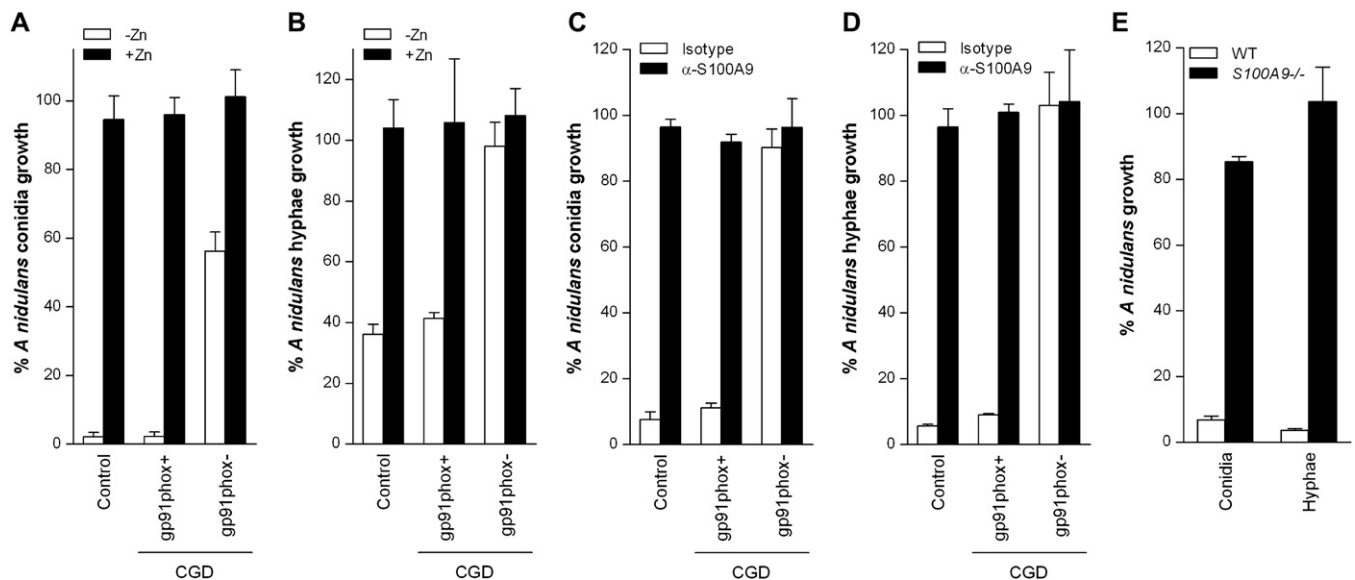


FIG 3. Calprotectin-dependent antifungal activity of CGD gp91^{phox+} neutrophils. *A. nidulans* conidia or hyphae incubated with NETs (**A and B**) or NET supernatant (**C and D**) from control and CGD-sorted neutrophils $\pm$ 1 μ mol/L Zn^{2+} or 15 μ g/mL α -S100A9, respectively. **E**, Wild-type (WT) and S100A9^{-/-} mouse NETs infected with *A. nidulans* conidia or hyphae. Data are means $\pm$ SDs of representative triplicate experiments.

Antifungal activity of CGD gp91^{phox+} cells is calprotectin-dependent

To study whether restored antifungal activity of CGD gp91^{phox+} neutrophils after GT was attributable to calprotectin, we PMA-stimulated gp91^{phox+} and gp91^{phox-} neutrophils to induce NETs, and coincubated with *A. nidulans* conidia and hyphae $\pm$ Zn^{2+} . Only gp91^{phox+} neutrophils showed strong antifungal activity, equivalent to control (Fig 3, A and B; see this article's Fig E3 in the Online Repository at www.jacionline.org). gp91^{phox-} neutrophils did not form NETs (Fig 1, B), yet partially inhibited conidia germination, probably by phagocytosis,²¹ but

were inefficient against hyphae. Full restoration of *A. nidulans* growth by addition of Zn^{2+} supports the assumption that NET antifungal activity was calprotectin-dependent (at calprotectin concentrations of 450-500 ng/mL, measured by ELISA).

Restoration of *A. nidulans* conidia and hyphae growth by Zn^{2+} addition was dose-dependent (see this article's Fig E4 in the Online Repository at www.jacionline.org). Addition of Mn^{2+} , Fe^{2+} , and Cu^{2+} (0-2.5 μ mol/L), however, did not restore fungal growth (not shown), indicating that Zn^{2+} binding by calprotectin has a major NET anti-*Aspergillus* activity. Because addition of metal ions can have pleiotropic effects, we applied an α -S100A9

antibody to block calprotectin-mediated inhibition in concentrated NET extracts, subsequently incubated with *A nidulans* conidia or hyphae. NET extracts from gp91^{phox+} neutrophils in the presence of unspecific control antibody showed strong antifungal activity, similar to control (Fig 3, C and D; see this article's Fig E5 in the Online Repository at www.jacionline.org). Supernatants from non-NET-forming gp91^{phox-} neutrophils in the presence of unspecific control antibody did not have antifungal activity, even against conidia germination, supporting the hypothesis that their low antifungal activity observed against conidia (Fig 3, A) was a result of phagocytosis.

We then tested whether NET-mediated *A nidulans* inhibition was calprotectin-dependent comparing wild-type and calprotectin-deficient mouse neutrophils. NETs were PMA-induced in mature neutrophils from both mouse strains and incubated with *A nidulans* conidia and hyphae. Indeed, NETs from calprotectin-deficient neutrophils did not prevent *A nidulans* growth, whereas NETs from wild-type neutrophils did (Fig 3, E), although the amount of NET formation was similar in calprotectin-deficient and wild-type neutrophils.⁴ Complete restoration of fungal growth in the presence of α -S100A9 antibody in human gp91^{phox+} and control NET extracts and the inability of calprotectin-deficient murine NETs to block fungal growth confirmed the essential antifungal role of NET-associated calprotectin against *A nidulans*.

Antifungal effect of calprotectin

To determine whether calprotectin-mediated inhibition of *A nidulans* growth within NETs was reversible or irreversible, we incubated conidia and hyphae on NETs for 4 days to 4 weeks at neutral pH, then added Zn²⁺ to restore fungal growth. NETs strongly inhibited *A nidulans* conidia germination and hyphae growth even after 4 weeks (Fig 4, A-P), indicating strong stability of calprotectin/Zn²⁺ complexes, agreeing well with their reported stability against proteases.²² Surprisingly, addition of Zn²⁺ restored fungal growth even after 4 weeks, demonstrating that calprotectin has fungistatic but not fungicidal activity on *A nidulans* at low concentrations (450–500 ng/mL, measured by ELISA).

We next tested whether antifungal activity was still reversible at higher calprotectin concentrations that might occur *in vivo*. We incubated conidia and hyphae for 4 days with increasing concentrations of purified calprotectin S100A8 and S100A9, then added Zn²⁺ to restore fungal growth. Conidia germination was inhibited at any concentration (Fig 4, Q, white bars), whereas hyphae were slightly more resistant, showing a dose-dependent increase of growth arrest between 0.5 and 4 μ g/mL S100A8/A9 (Fig 4, R, white bars). Fungal growth was not restored by Zn²⁺ when the S100A8/A9 concentration was higher than 16 μ g/mL (Fig 4, Q-R, black bars). Light microscopy showed intact hyphae at low S100A8/A9 concentrations (Fig 4, S), whereas in the presence of >16 μ g/mL S100A8/A9, hyphae had a translucent cell wall and a fragmented internal structure (Fig 4, T). Thus, we demonstrate a previously unknown microbicidal effect against *A nidulans* at high calprotectin concentrations.

A nidulans-induced NET formation is strictly dependent on NADPH oxidase

We investigated whether the induction of NETs by *A nidulans* is dependent on functional NADPH oxidase and is restored after

CGD GT. CGD gp91^{phox+} and gp91^{phox-} neutrophils were incubated with *A nidulans* hyphae and observed for NET formation by light microscopy, scanning electron microscopy, and immunostaining with confocal microscopy. Hyphae induced NETs in control and gp91^{phox+} neutrophils but, as expected, less strongly compared with PMA stimulation. We observed that NET formation mainly occurred when neutrophils were in direct contact with hyphae (Fig 5, A and B, black arrows). This might suggest that close pathogen-neutrophil interaction promotes induction of NETs. The molecular details behind this trigger mechanism will be subject to further investigation. In contrast, gp91^{phox-} neutrophils did not form NETs when in contact with hyphae (Fig 5, C, white arrowheads), underlining the role of functional NADPH oxidase in NET formation and antifungal defense. Analysis by scanning electron microscopy showed that *A nidulans* hyphae were entangled by NETs from control and gp91^{phox+} neutrophils (Fig 5, D, G, and E, H), whereas gp91^{phox-} neutrophils surrounded and wrapped around hyphae but did not make NETs (Fig 5, F, I).

Calprotectin is NET-associated after *A nidulans* incubation

The association of calprotectin and *A nidulans*-induced NETs was studied by immunofluorescence and confocal microscopy, staining DNA with DAPI and calprotectin and hyphae with primary antibodies. Colocalization of DNA (blue) and calprotectin (red) demonstrates that calprotectin was released and associated with NETs when control (Fig 5, J, M, P) and gp91^{phox+} (Fig 5, K, N, Q) neutrophils were incubated with *A nidulans* hyphae (green). Moreover, we demonstrate that gp91^{phox-} neutrophils showed neither NET formation nor extracellular calprotectin on *A nidulans* incubation (Fig 5, L, O, R), although they were able to recognize *A nidulans*, as judged by migration toward and wrapping around hyphae (Fig 5, R). DNA was confined to the nucleus (Fig 5, L) and calprotectin to the cytoplasmic compartment of these cells (Fig 5, O).

Notably, the quantification of calprotectin by ELISA confirmed our microscopic findings. Control, gp91^{phox+} and gp91^{phox-} neutrophils were activated with PMA for 4 hours, and calprotectin release was determined by ELISA after NET formation and subsequent digestion of NETs. Control and gp91^{phox+} neutrophils released approximately 4 times the calprotectin concentration compared with gp91^{phox-} neutrophils (Fig 6), supporting the previous results. Differences in total cytoplasmic calprotectin concentration among control, gp91^{phox+}, and gp91^{phox-} neutrophils were not statistically significant (data not shown). Thus, we conclude that NADPH oxidase is required not only for the release of NETs but also for the release of calprotectin, the crucial component for the inhibition of *A nidulans* hyphae.

DISCUSSION

Recent work suggests NET contribution to elimination of fungal infections in healthy subjects because hyphae are too large to be phagocytosed.^{23,24} Patients with CGD are unable to make NETs^{5,11} and are susceptible to therapy-refractory infection with *Aspergillus* spp.¹⁰ Especially infections with *A nidulans* are a major threat to these patients because of higher virulence and frequent resistance to antifungal treatment compared to *Aspergillus fumigatus*.⁷ Here we show that genetic complementation

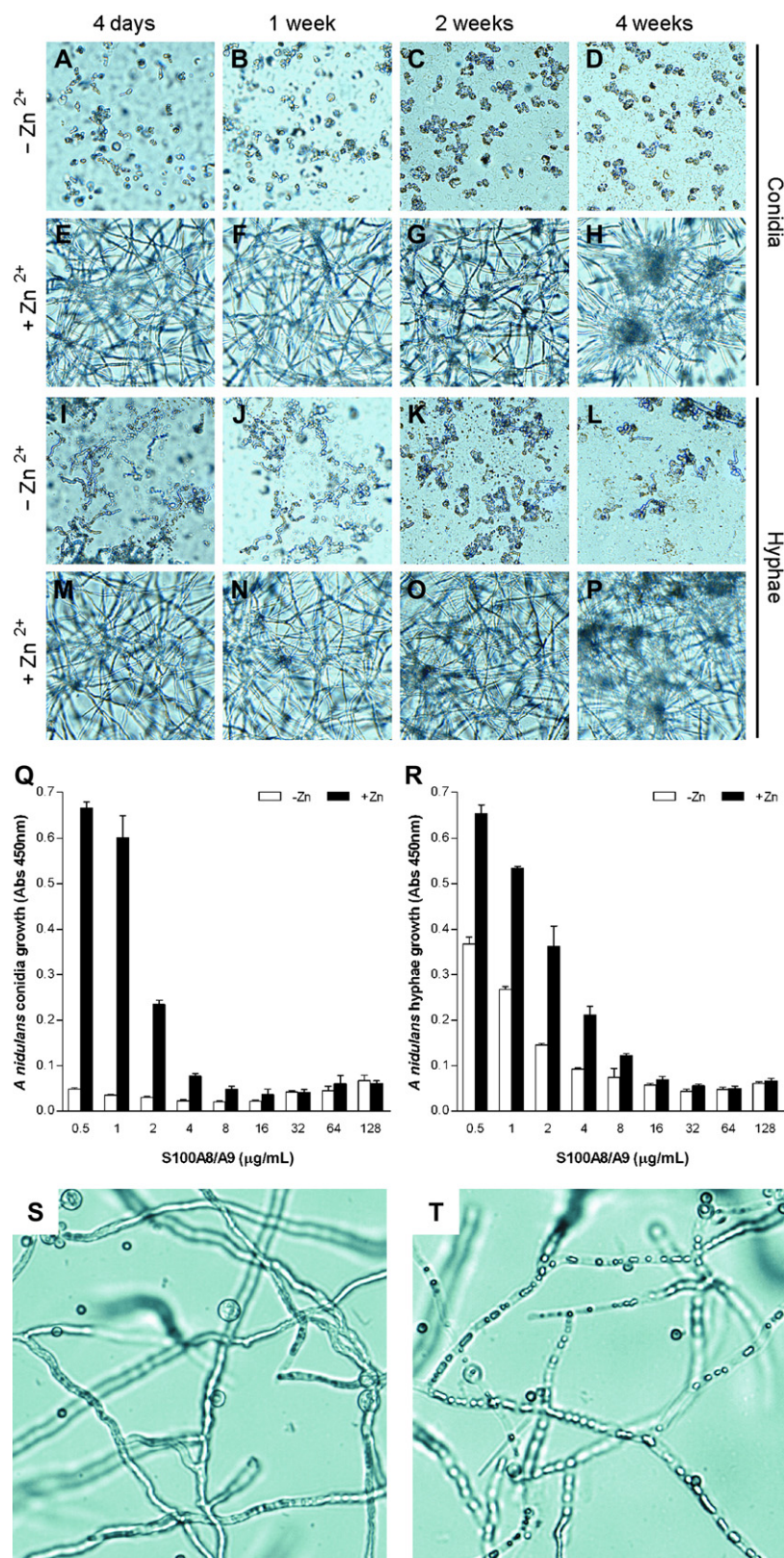


FIG 4. Antifungal effect of calprotectin. **A-P**, Fungistatic activity: *A. nidulans* conidia or hyphae incubated with NETs for 4 days to 4 weeks, after which 1 $\mu\text{mol/L}$ Zn^{2+} was added overnight. **Q and R**, Fungicidal activity: *A. nidulans* conidia or hyphae incubated with 0.5 (**S**) to 128 (**T**) $\mu\text{g/mL}$ purified S100A8/A9 for 4 days, after which 50 $\mu\text{mol/L}$ Zn^{2+} was added overnight.

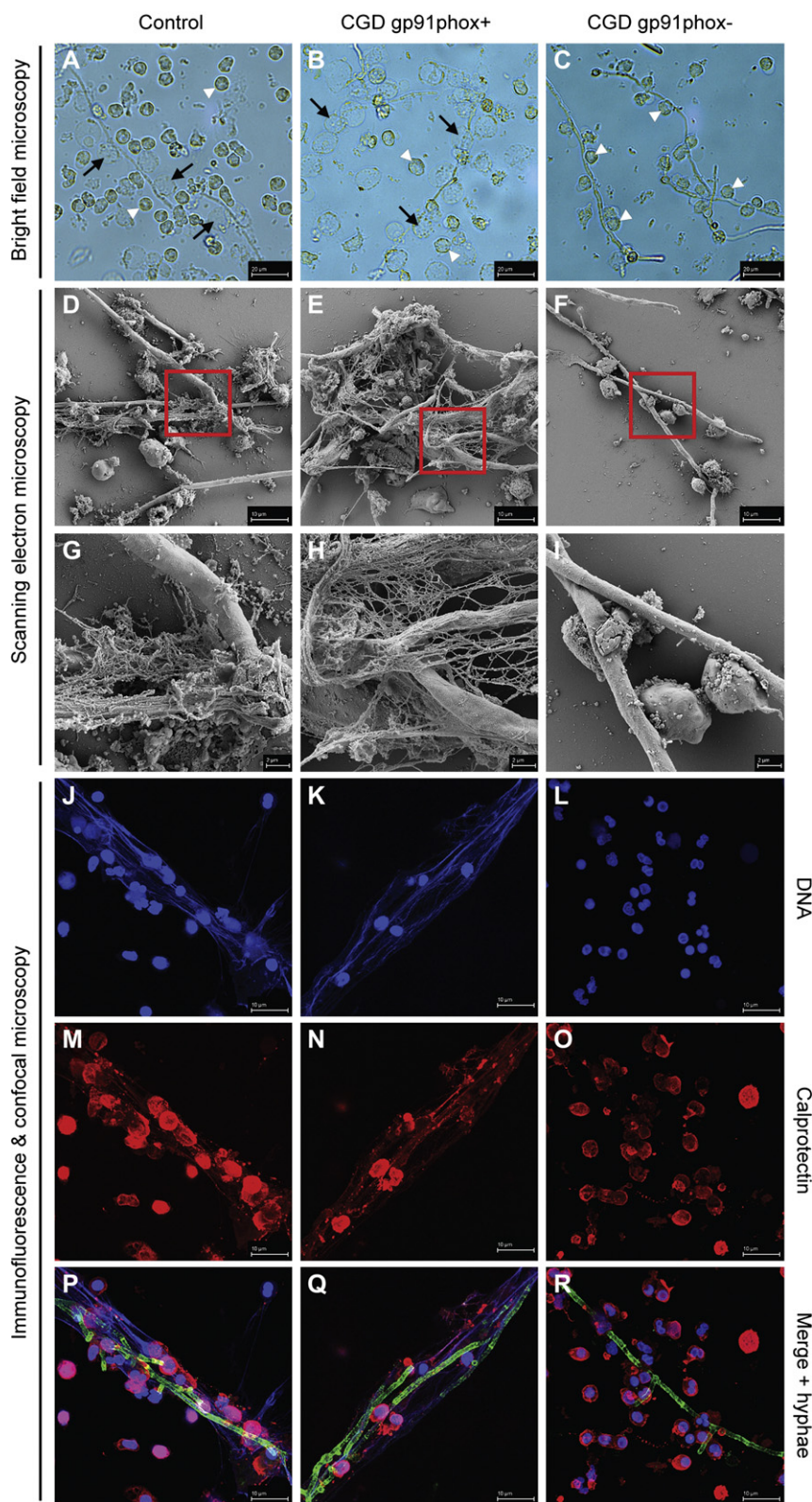


FIG 5. *A. nidulans*-induced NETs contain calprotectin. Control and CGD sorted neutrophils were infected with hyphae, and NET formation was determined by bright field microscopy (A-C), scanning electron microscopy (D-I), and immunofluorescence and confocal microscopy (J-R). NET formation was determined by DAPI (blue), calprotectin by staining with antibodies against S100A8/A9 (red), and hyphae with antibodies against *Aspergillus* soluble proteins (green).

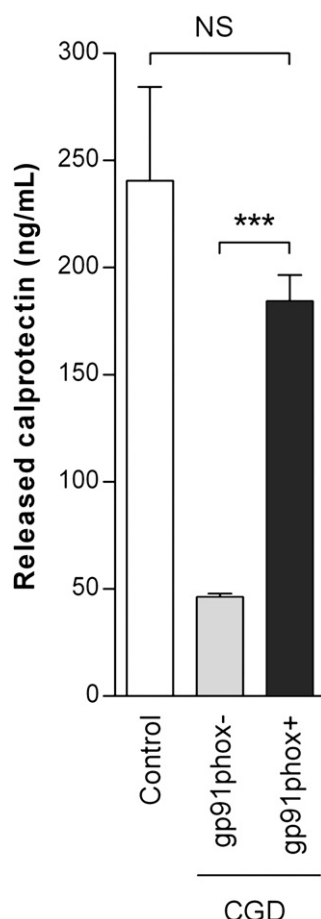


FIG 6. Calprotectin release by gp91^{phox+} and gp91^{phox-} neutrophils. Calprotectin release was quantified by ELISA after neutrophil activation by PMA inducing NET formation. NETs were digested by MNase/DNase-1 to quantify NET-associated calprotectin. Significance was assessed by the Student *t* test: NS, not significant (*P* > .05); ****P* < .001.

of NADPH oxidase by human CGD GT restored the ability of neutrophils to release NETs and NET-associated calprotectin, which is responsible for growth inhibition of *A. nidulans* by Zn²⁺ sequestration. A threshold of 27.8% of O₂⁻ production per individual gp91^{phox+} neutrophil and a chimerism of 22.8% of oxidase-positive cells was enough for sufficient NET formation *in vitro* and clinical clearance of multifocal therapy-refractory *A. nidulans* lung infection in a patient with CGD *in vivo*.

The S100 Ca²⁺-binding protein calprotectin is a heteroduplex of subunits S100A8 and S100A9 that is expressed by granulocytes, monocytes, and early differentiation stages of macrophages.²⁵ It represents about 40 % of neutrophil cytoplasm,²⁶ accumulates at high concentrations (1–20 mg/mL) in abscess fluid supernatants,²⁷ and has been shown to bind Zn²⁺ *in vitro*.²⁸ Its growth-inhibitory effect on fungi is most likely mediated by metal chelation and is reversible by micromolar quantities of Zn²⁺.^{18,27,28} We set out to study the role of calprotectin in innate defense against CGD-relevant *A. nidulans*. Growth of *A. nidulans* was strongly inhibited when coincubated with both purified calprotectin subunits, which is in accordance with a report by Sohnle et al¹⁷ and with crystal structure²⁹ as well as mass spectrometric analyses³⁰ suggesting that the Ca²⁺-dependent formation of S100A8/A9 heterotetramers would lead to enhanced

Zn²⁺-binding capacity, whereas none of the homodimers should display significant affinity for Zn²⁺.²⁹

Our study demonstrates the major role of NET-associated calprotectin in suppressing growth of *A. nidulans* conidia and hyphae and shows the presence of calprotectin in NETs induced by *A. nidulans* hyphae. *A. fumigatus* has been reported recently to trigger NET release,^{31,32} resulting in growth inhibition of *A. fumigatus* that could be prevented by Zn²⁺.³² However, the mechanism of inhibition was not clearly shown in these reports. Here we show that blocking of calprotectin by specific antibodies and the absence of calprotectin in NETs from calprotectin-deficient mouse neutrophils completely abrogated the inhibition of *A. nidulans* conidia and hyphae growth. This is a more precise analysis because NETs also contain other Zn²⁺-binding proteins, such as S100A12,³³ which is involved in antiparasite responses.

According to our findings, calprotectin has to be presented extracellularly for antifungal activity against *A. nidulans*. To the best of our knowledge, leakage of a cytoplasmic protein into phagolysosomes has not been described. We previously showed that neutrophil degranulation stimulated by the bacterial peptide formyl-Methionyl-Leucyl-Phenylalanine does not induce secretion of calprotectin, indicating nonvesicular localization.⁴ However, we cannot entirely exclude that minor amounts of calprotectin are found in phagolysosomes and contribute to the slight inhibitory effect we reported on CGD neutrophils infected with *A. nidulans* conidia.

We show that the presence of functional NADPH oxidase in human neutrophils is an absolute requirement for *A. nidulans* growth inhibition by NET-associated calprotectin, because only gp91^{phox+} and not gp91^{phox-} neutrophils made NETs containing calprotectin and inhibited *A. nidulans* growth efficiently. GT of human CGD restored the amount of released calprotectin during NET formation to wild-type levels, and NET-associated calprotectin colocalized with *A. nidulans* hyphae. Analysis of gp91^{phox-} neutrophils enabled us to establish the role of NADPH oxidase for the release of calprotectin during NET formation, which is an advantage over chemical inhibition of NADPH oxidase using diphenylene iodonium chloride. We propose that NET formation is an NADPH oxidase-dependent mechanism by which neutrophils secrete calprotectin into the extracellular compartment on infection with *Aspergillus* hyphae, resulting in both NET-bound and free calprotectin. NET-associated calprotectin is concentrated with trapped *Aspergillus* for antifungal function. Unbound calprotectin may in addition have distinct functions, such as chemotaxis or inflammation.

The fact that gp91^{phox-} neutrophils also partially inhibited conidia germination suggests that NADPH oxidase-independent mechanisms linked to phagocytosis, such as activity of the granule serine proteases NE and cathepsin G^{34–36} (although the mechanism has been recently object of controversy^{37–39}), might contribute to early defense against *Aspergillus*. The need of NE for NET formation¹⁴ could explain the susceptibility to infection of NE-deficient mice described by Reeves et al³⁵ as an indirect action. Other mechanisms such as the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase, considered critical for regulating immune responses and suppression of inflammation in mice,⁴⁰ do not play a role in human CGD as we previously reported.^{41,42}

Fungi have a high Zn²⁺ requirement (10⁻⁷–10⁻⁵ mol/L) for growth⁴³ with a low minimum inhibitory concentration of calprotectin.⁴⁴ Because of the lack of a well developed extracellular

Zn²⁺-scavenging system, *A. fumigatus* is extremely sensitive to Zn²⁺ deprivation.⁴⁵ The Zn²⁺ requirement for *A. nidulans* in our study was in the same range as described for other fungi. In contrast with *Staphylococcus aureus*, which is more sensitive to calprotectin-mediated Mn²⁺ deprivation,¹⁹ we found that *A. nidulans* could resume growth only after Zn²⁺ supplementation, but not after Mn²⁺, Fe²⁺, or Cu²⁺ addition. This supports the central role of Zn²⁺ for *A. nidulans* growth.

Interestingly, *A. nidulans* hyphae were not killed by NETs at low calprotectin concentrations *in vitro*, although hyphal structures seemed to be severely deranged by NETs. This corresponded to the range of fungistatic calprotectin concentrations described for *Candida*.⁴⁴ *A. nidulans* growth was restored after addition of Zn²⁺, even after a month of inhibition by NETs. Because much higher calprotectin concentrations have been observed in human abscesses,²⁷ *Aspergillus* hyphae might still be killed by NET-associated calprotectin *in vivo*. Killing of *Candida* spp or *Cryptococcus neoformans* was demonstrated at calprotectin concentrations of 3 to 5 µg/mL.⁴⁴ We show for the first time that calprotectin concentrations ≥16 µg/mL lead to irreversible growth arrest of *A. nidulans* conidia and hyphae, most likely by Zn²⁺ starvation. In contrast with our findings, a previous publication reported only minor antifungal activity of NETs against *A. fumigatus*,³¹ probably because of a shorter incubation period. Alternatively, complete *in vivo* killing might also be achieved by other antimicrobial peptides⁴⁶ or by so far unknown mechanisms: in addition to their antimicrobial properties, S100A8, S100A9, and S100A8/A9 are chemotactic for neutrophils and monocytes.⁴⁷⁻⁴⁹ Notably, for this function, calprotectin needs to be extracellular, and thus *Aspergillus*-induced NET formation supports it as well.

Here we establish that calprotectin released in NETs displays concentration-dependent reversible and irreversible growth inhibitory activity against virulent *A. nidulans*. NETs may be involved in disarming *A. nidulans* and may prevent further spreading by providing high local concentrations of calprotectin. Definitive *in vivo* proof in human beings is obviously ethically impossible. In conclusion, we show that calprotectin is a critical factor in the innate immune defense of human neutrophils to *Aspergillus* infection and adds to the concept of metal chelation as a strategy for inhibiting microbial growth at the sites of infection. The severe immunodeficient phenotype and the high susceptibility to *Aspergillus* infection of patients with CGD might be linked to absence of NETs, and restoration of NADPH oxidase function and NET formation by GT leads to rapid cure of refractory invasive aspergillosis in X-linked CGD.

We thank the patient and his family for their trust. We are indebted to the medical and nursing staff of the bone marrow transplantation unit of University Children's Hospital Zurich. We thank Reinhard Seger for his support and helpful discussions, and Manuel Grez and Klaus Kühlke for developing and providing the SF71gp91^{phox} vector, respectively. We are grateful to Klaus Marquardt for electron microscopy, to Andres Kaech for help with confocal microscopy, and to Alex Imhof for isolating *A. nidulans* conidia. Furthermore, we acknowledge Thomas Vogl and Johannes Roth for supplying S100A9 knockout mice.

Clinical implications: Gene therapy for CGD restores NET formation and calprotectin release by neutrophils, leading to efficient *A. nidulans* inhibition. Calprotectin is a critical factor in human innate immune defense against *Aspergillus* infection.

REFERENCES

- Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science* 2004;303:1532-5.
- Guimaraes-Costa AB, Nascimento MT, Froment GS, Soares RP, Morgado FN, Conceicao-Silva F, et al. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. *Proc Natl Acad Sci U S A* 2009;106:6748-53.
- Urban CF, Reichard U, Brinkmann V, Zychlinsky A. Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cell Microbiol* 2006;8:668-76.
- Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. *PLoS Pathog* 2009;5:e1000639.
- Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* 2007;176:231-41.
- Hsu K, Champaiboon C, Guenther BD, Sorenson BS, Khammanivong A, Ross KF, et al. Anti-infective protective properties of S100 calgranulins. *Antiinflamm Antiallergy Agents Med Chem* 2009;8:290-305.
- Segal BH, DeCarlo ES, Kwon-Chung KJ, Malech HL, Gallin JI, Holland SM. *Aspergillus nidulans* infection in chronic granulomatous disease. *Medicine (Baltimore)* 1998;77:345-54.
- Seger RA. Modern management of chronic granulomatous disease. *Br J Haematol* 2008;140:255-66.
- Winkelstein JA, Marino MC, Johnston RB Jr, Boyle J, Curnutte J, Gallin JI, et al. Chronic granulomatous disease: report on a national registry of 368 patients. *Medicine (Baltimore)* 2000;79:155-69.
- Segal BH, Romani LR. Invasive aspergillosis in chronic granulomatous disease. *Med Mycol* 2009;47(suppl 1):S282-90.
- Bianchi M, Hakkim A, Brinkmann V, Siler U, Seger RA, Zychlinsky A, et al. Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood* 2009;114:2619-22.
- Ermert D, Urban CF, Laube B, Goosmann C, Zychlinsky A, Brinkmann V. Mouse neutrophil extracellular traps in microbial infections. *J Innate Immun* 2009;1:181-93.
- Metzler KD, Fuchs TA, Nauseef WM, Reumaux D, Roesler J, Schulze I, et al. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood* 2010;117:953-9.
- Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol* 2010;191:677-91.
- Bonnett CR, Cornish EJ, Harnsen AG, Burritt JB. Early neutrophil recruitment and aggregation in the murine lung inhibit germination of *Aspergillus fumigatus* Conidia. *Infect Immun* 2006;74:6528-39.
- Mayo LA, Curnutte JT. Kinetic microplate assay for superoxide production by neutrophils and other phagocytic cells. *Methods Enzymol* 1990;186:567-75.
- Sohnle PG, Hunter MJ, Hahn B, Chazin WJ. Zinc-reversible antimicrobial activity of recombinant calprotectin (migration inhibitory factor-related proteins 8 and 14). *J Infect Dis* 2000;182:1272-5.
- Murthy AR, Lehrer RI, Harwig SS, Miyasaki KT. In vitro candidastatic properties of the human neutrophil calprotectin complex. *J Immunol* 1993;151:6291-301.
- Corbin BD, Seeley EH, Raab A, Feldmann J, Miller MR, Torres VJ, et al. Metal chelation and inhibition of bacterial growth in tissue abscesses. *Science* 2008;319:962-5.
- Sohnle PG, Hahn BL, Santhanagopalan V. Inhibition of *Candida albicans* growth by calprotectin in the absence of direct contact with the organisms. *J Infect Dis* 1996;174:1369-72.
- Behnsen J, Narang P, Hasenberg M, Gunzer F, Bilitewski U, Klippel N, et al. Environmental dimensionality controls the interaction of phagocytes with the pathogenic fungi *Aspergillus fumigatus* and *Candida albicans*. *PLoS Pathog* 2007;3:e13.
- Nacken W, Kerkhoff C. The hetero-oligomeric complex of the S100A8/S100A9 protein is extremely protease resistant. *FEBS Lett* 2007;581:5127-30.
- Latge JP. *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* 1999;12:310-50.
- Mizgerd JP. Acute lower respiratory tract infection. *N Engl J Med* 2008;358:716-27.
- Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *J Leukoc Biol* 2009;86:557-66.
- Edgeworth J, Gorman M, Bennett R, Freemont P, Hogg N. Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. *J Biol Chem* 1991;266:7706-13.
- Clohesy PA, Golden BE. Calprotectin-mediated zinc chelation as a biostatic mechanism in host defence. *Scand J Immunol* 1995;42:551-6.
- Sohnle PG, Collins-Lech C, Wiessner JH. The zinc-reversible antimicrobial activity of neutrophil lysates and abscess fluid supernatants. *J Infect Dis* 1991;164:137-42.

29. Korndorfer IP, Brueckner F, Skerra A. The crystal structure of the human (S100A8/S100A9)₂ heterotetramer, calprotectin, illustrates how conformational changes of interacting alpha-helices can determine specific association of two EF-hand proteins. *J Mol Biol* 2007;370:887-98.
30. Vogl T, Leukert N, Barczyk K, Strupat K, Roth J. Biophysical characterization of S100A8 and S100A9 in the absence and presence of bivalent cations. *Biochim Biophys Acta* 2006;1763:1298-306.
31. Bruns S, Kniemeyer O, Hasenberg M, Aimanianda V, Nietzsche S, Thywissen A, et al. Production of extracellular traps against *Aspergillus fumigatus* in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. *PLoS Pathog* 2010;6:e1000873.
32. McCormick A, Heesemann L, Wagener J, Marcos V, Hartl D, Loeffler J, et al. NETs formed by human neutrophils inhibit growth of the pathogenic mold *Aspergillus fumigatus*. *Microbes Infect* 2010;12:928-36.
33. Moroz OV, Blagova EV, Wilkinson AJ, Wilson KS, Bronstein IB. The crystal structures of human S100A12 in apo form and in complex with zinc: new insights into S100A12 oligomerisation. *J Mol Biol* 2009;391:536-51.
34. Decleva E, Menegazzi R, Busetto S, Patriarca P, Dri P. Common methodology is inadequate for studies on the microbicidal activity of neutrophils. *J Leukoc Biol* 2006;79:87-94.
35. Reeves EP, Lu H, Jacobs HL, Messina CG, Bolsover S, Gabella G, et al. Killing activity of neutrophils is mediated through activation of proteases by K⁺ flux. *Nature* 2002;416:291-7.
36. Tkalecivic J, Novelli M, Phylactides M, Iredale JP, Segal AW, Roes J. Impaired immunity and enhanced resistance to endotoxin in the absence of neutrophil elastase and cathepsin G. *Immunity* 2000;12:201-10.
37. Ahluwalia J, Tinker A, Clapp LH, Duchon MR, Abramov AY, Pope S, et al. The large-conductance Ca²⁺-activated K⁺ channel is essential for innate immunity. *Nature* 2004;427:853-8.
38. Essin K, Salanova B, Kettritz R, Sausbier M, Luft FC, Kraus D, et al. Large-conductance calcium-activated potassium channel activity is absent in human and mouse neutrophils and is not required for innate immunity. *Am J Physiol Cell Physiol* 2007;293:C45-54.
39. Femling JK, Cherny VV, Morgan D, Rada B, Davis AP, Czizjak G, et al. The antibacterial activity of human neutrophils and eosinophils requires proton channels but not BK channels. *J Gen Physiol* 2006;127:659-72.
40. Romani L, Fallarino F, De Luca A, Montagnoli C, D'Angelo C, Zelante T, et al. Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. *Nature* 2008;451:211-5.
41. Hakkim A, Hurwitz R, Bianchi M, Brinkmann V, Siler U, Seger R, et al. Protecting against *Aspergillus* infection in CGD. *Blood* 2009;114:3498.
42. Jurgens B, Fuchs D, Reichenbach J, Heitger A. Intact indoleamine 2,3-dioxygenase activity in human chronic granulomatous disease. *Clin Immunol* 2010;137:1-4.
43. Sugarman B. Zinc and infection. *Rev Infect Dis* 1983;5:137-47.
44. Steinbakk M, Naess-Andresen CF, Lingaas E, Dale I, Brandtzaeg P, Fagerhol MK. Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. *Lancet* 1990;336:763-5.
45. Lulloff SJ, Hahn BL, Sohnle PG. Fungal susceptibility to zinc deprivation. *J Lab Clin Med* 2004;144:208-14.
46. Levitz SM, Selsted ME, Ganz T, Lehrer RI, Diamond RD. In vitro killing of spores and hyphae of *Aspergillus fumigatus* and *Rhizopus oryzae* by rabbit neutrophil cationic peptides and bronchoalveolar macrophages. *J Infect Dis* 1986;154:483-9.
47. Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *J Immunol* 2003;170:3233-42.
48. Vandal K, Rouleau P, Boivin A, Ryckman C, Talbot M, Tessier PA. Blockade of S100A8 and S100A9 suppresses neutrophil migration in response to lipopolysaccharide. *J Immunol* 2003;171:2602-9.
49. Raquil MA, Anceriz N, Rouleau P, Tessier PA. Blockade of antimicrobial proteins S100A8 and S100A9 inhibits phagocyte migration to the alveoli in streptococcal pneumonia. *J Immunol* 2008;180:3366-74.

METHODS

Patient description and GT

We treated a boy 8 years 7 months old with X-linked gp91^{phox}-deficient CGD and multifocal therapy-refractory *A nidulans* lung infection with a monocistronic long terminal repeat-driven γ -retroviral SF71gp91^{phox} vector (see reference^{E1} for details) under a protocol approved by the ethics review board of the University Children's Hospital Zurich and the Swiss Expert Committee for Bio-Safety after written informed consent of the parents. Collection of CD34⁺ cells, transduction, conditioning with low-dose intravenous busulfan (8.8 mg/kg), and clinical follow-up were performed as described.^{E2} The *A nidulans* lung infection was completely cleared 6 weeks after GT. Therapy/prophylaxis with oral voriconazole was continued throughout and has not yet been tapered 2.6 years after GT. To avoid interactions, voriconazole was stopped 4 days before *Aspergillus* experiments and continued thereafter. At the time of analysis, the patient was free of any infection, and 22.8% of neutrophils expressed gp91^{phox}, with an O₂⁻ production of 27.8% per gp91^{phox}-expressing cell (relative to healthy controls).^{E3} Clinical and molecular follow-up of GT in this patient will be described elsewhere.

A nidulans strain

The *A nidulans* strain used was isolated from bronchoalveolar lavage fluid of the patient; conidia were grown and collected as described.^{E4} For all experiments, conidia and hyphae were grown in serum-free RPMI medium (phenol red-free) supplemented with 10 mmol/L HEPES. If not stated otherwise, fungal growth was quantified in all assays with the tetrazolium dye 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Invitrogen, Molecular Probes, Leiden, The Netherlands) as described.^{E5} This method, unlike colony-forming unit enumeration, does not require dispersion of the hyphae.

Isolation of human and murine neutrophils

Human neutrophils were isolated from peripheral blood of healthy donors or the patient with CGD by using dextran-Ficoll (GE Healthcare, Munich, Germany)^{E6} for all NET assays with *A nidulans* and for autoMACS sorting, whereas Percoll (GE Healthcare) density gradient separation^{E7} was used for NET quantification assays. For all experiments, neutrophils were resuspended in serum-free RPMI medium (phenol red-free) supplemented with 10 mmol/L HEPES and used within 1 hour after isolation.

Murine neutrophils were isolated from *S100A9*^{-/-} mice backcrossed 10 times into C57 BL/6. These mice are deficient in both calprotectin subunits S100A8 and S100A9 protein.^{E8} Mice were bred in our animal facility according to regulations of the Jordbruksverket Sweden (Dnr A29-69). Mature murine neutrophils were isolated from bone marrow as previously described.^{E9} Briefly, bone marrow cells from tibia and femur were singularized by using a 70- μ m cell strainer and separated by centrifugation for 30 minutes at 1500g on a discontinuous Percoll gradient with 52% (vol/vol), 69% (vol/vol), and 78% (vol/vol). Neutrophils harvested from the distinct layer between 69% and 78% were resuspended in HBSS without Ca²⁺ and Mg²⁺.

Sorting of CGD gp91^{phox} and gp91^{phox}-neutrophils

Control and patient neutrophils were first stained for 30 minutes at room temperature with 10 μ g/mL mouse α -gp91^{phox}-fluorescein isothiocyanate antibody (clone 7D5; MBL, Woburn, Md), then 30 minutes at 4°C with anti-fluorescein isothiocyanate MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for magnetic separation by autoMACS (Miltenyi Biotec), according to the manufacturer's instructions. Purity of the resulting gp91^{phox}- and gp91^{phox}+ populations was directly measured by the fluorescent-activated cell sorter.

A nidulans growth inhibition by purified calprotectin

In a 96-well plate, 10⁴ *A nidulans* conidia or hyphae obtained from culture of 10⁴ conidia were incubated 24 hours at 37°C with 5 μ g/mL recombinant

S100A8 and/or 5 μ g/mL recombinant S100A9 (ProtEra, Florence, Italy) $\pm$ 1 μ mol/L ZnSO₄ (Sigma-Aldrich, Taufkirchen, Germany) or 15 μ g/mL rabbit polyclonal α -S100A9 antibody (H00006280-D01P; Abnova, Taipei, Taiwan) versus unspecific control antibody (Sigma-Aldrich).^{E10} This α -S100A9 antibody recognizes the whole S100A9 subunit alone or the S100A9 subunit complexed with S100A8. Fungal growth is expressed as percentage of control values (*A nidulans* conidia or hyphae incubated in media without neutrophils $\pm$ ZnSO₄, S100A8/A9, or antibodies).

A nidulans growth inhibition by NETs

In a 96-well plate, 10⁵ control, CGD gp91^{phox}-, and gp91^{phox}+ sorted neutrophils were activated with 40 nmol/L PMA (Sigma-Aldrich) at 37°C and 5% CO₂ for 4 hours until NET formation was complete. Zero to 2 μ mol/L ZnSO₄ (Sigma-Aldrich) and *A nidulans* conidia or hyphae (hyphae previously grown overnight in a 24-well plate, collected by pipetting and scraping the wells with a cell scraper) with a multiplicity of infection *A nidulans*/neutrophils of 0.1 were then added, in a final volume of 160 μ L. Afterward, the plates were centrifuged for 5 minutes at 400g and incubated for 24 hours at 37°C, allowing conidia germination and hyphal outgrowth. Fungal growth is expressed as percentage of control values (*A nidulans* conidia or hyphae incubated in media $\pm$ ZnSO₄).

A nidulans growth inhibition by NET extracts

Neutrophil extracellular trap formation was induced in 8 $\times$ 10⁵ control, CGD gp91^{phox}-, and gp91^{phox}+ neutrophils by activation with 40 nmol/L PMA at 37°C and 5% CO₂ for 5 hours in a 24-well plate (500 μ L/well; 4 wells each; 3.2 $\times$ 10⁶ cells total). For maximal DNA fragmentation, NETs were then digested for 30 minutes with 5 U/mL MNase (Worthington Biochemical, Lakewood, NJ) + 1 U/mL DNase-1 (Sigma-Aldrich) and pooled supernatant concentrated $\sim$ 10-fold by centrifugation on filter columns with a 3-kD cutoff (Amicon; Millipore, Zug, Switzerland), following the manufacturer's instructions. In a 96-well plate, 10⁴ *A nidulans* conidia or hyphae obtained from culture of 6 $\times$ 10⁴ *A nidulans* conidia were then incubated for 24 hours at 37°C with the concentrated NET extracts (diluted 1/3) with or without 15 μ g/mL α -S100A9 or unspecific control antibody in 100 μ L final volume. Fungal growth is expressed as percentage of control values (*A nidulans* conidia or hyphae incubated in media $\pm$ antibodies).

A nidulans growth inhibition by murine *S100A9*^{-/-} NETs

In a 24-well plate, 5 $\times$ 10⁵ mouse neutrophils in 500 μ L RPMI with 1% (vol/vol) mouse serum were stimulated with 100 nmol/L PMA for 20 hours at 37°C with 5% CO₂ to induce NET formation. The supernatant was discarded; NETs were washed once with RPMI and incubated with 500 μ L RPMI containing *A nidulans* conidia or hyphae at multiplicities of infection 0.1 and 0.01, respectively. Afterward, plates were centrifuged for 5 minutes at 300g and incubated for 20 hours at 37°C. Fungal growth is expressed as percentage of control values (*A nidulans* conidia or hyphae incubated in media).

Fungistatic and fungicidal effect of calprotectin

In a 96-well plate, 10⁴ *A nidulans* conidia or hyphae obtained from culture of 10⁴ conidia were incubated on previously formed NETs (from 10⁵ neutrophils activated with 40 nmol/L PMA at 37°C and 5% CO₂ for 4 hours) or with 0.5 to 128 μ g/mL purified S100A8/A9. After 4 days to 4 weeks, RPMI or ZnSO₄ (final concentration, 1 μ mol/L for NETs and 50 μ mol/L for purified S100A8/A9) were added to the wells and incubated overnight. Growth of *A nidulans* conidia or hyphae was assessed by using an inverted light microscope (Leica DM IL HC Fluo, Wetzlar, Germany) coupled to a CCD camera (Leica DFC 480 R2).

Statistical analysis

A 2-tailed Student *t* test was used for analysis of 2 groups. Differences were considered statistically significant when *P* < .05. All statistical tests were performed by using GraphPad Prism.

REFERENCES

- E1. Bianchi M, Hakkim A, Brinkmann V, Siler U, Seger RA, Zychlinsky A, et al. Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood* 2009;114:2619-22.
- E2. Ott MG, Schmidt M, Schwarzwaelder K, Stein S, Siler U, Koehl U, et al. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med* 2006;12:401-9.
- E3. Mayo LA, Curnutte JT. Kinetic microplate assay for superoxide production by neutrophils and other phagocytic cells. *Methods Enzymol* 1990;186:567-75.
- E4. Bonnett CR, Cornish EJ, Harmsen AG, Burritt JB. Early neutrophil recruitment and aggregation in the murine lung inhibit germination of *Aspergillus fumigatus* conidia. *Infect Immun* 2006;74:6528-39.
- E5. Meshulam T, Levitz SM, Christin L, Diamond RD. A simplified new assay for assessment of fungal cell damage with the tetrazolium dye, (2,3)-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanil ide (XTT). *J Infect Dis* 1995;172:1153-6.
- E6. Weiss J, Kao L, Victor M, Elsbach P. Oxygen-independent intracellular and oxygen-dependent extracellular killing of *Escherichia coli* S15 by human polymorphonuclear leukocytes. *J Clin Invest* 1985;76:206-12.
- E7. Aga E, Katschinski DM, van Zandbergen G, Laufs H, Hansen B, Muller K, et al. Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite *Leishmania major*. *J Immunol* 2002;169:898-905.
- E8. Manitz MP, Horst B, Seeliger S, Strey A, Skryabin BV, Gunzer M, et al. Loss of S100A9 (MRP14) results in reduced interleukin-8-induced CD11b surface expression, a polarized microfilament system, and diminished responsiveness to chemoattractants in vitro. *Mol Cell Biol* 2003;23:1034-43.
- E9. Ermer D, Urban CF, Laube B, Goosmann C, Zychlinsky A, Brinkmann V. Mouse neutrophil extracellular traps in microbial infections. *J Innate Immun* 2009;1:181-93.
- E10. Sohnle PG, Hunter MJ, Hahn B, Chazin WJ. Zinc-reversible antimicrobial activity of recombinant calprotectin (migration inhibitory factor-related proteins 8 and 14). *J Infect Dis* 2000;182:1272-5.

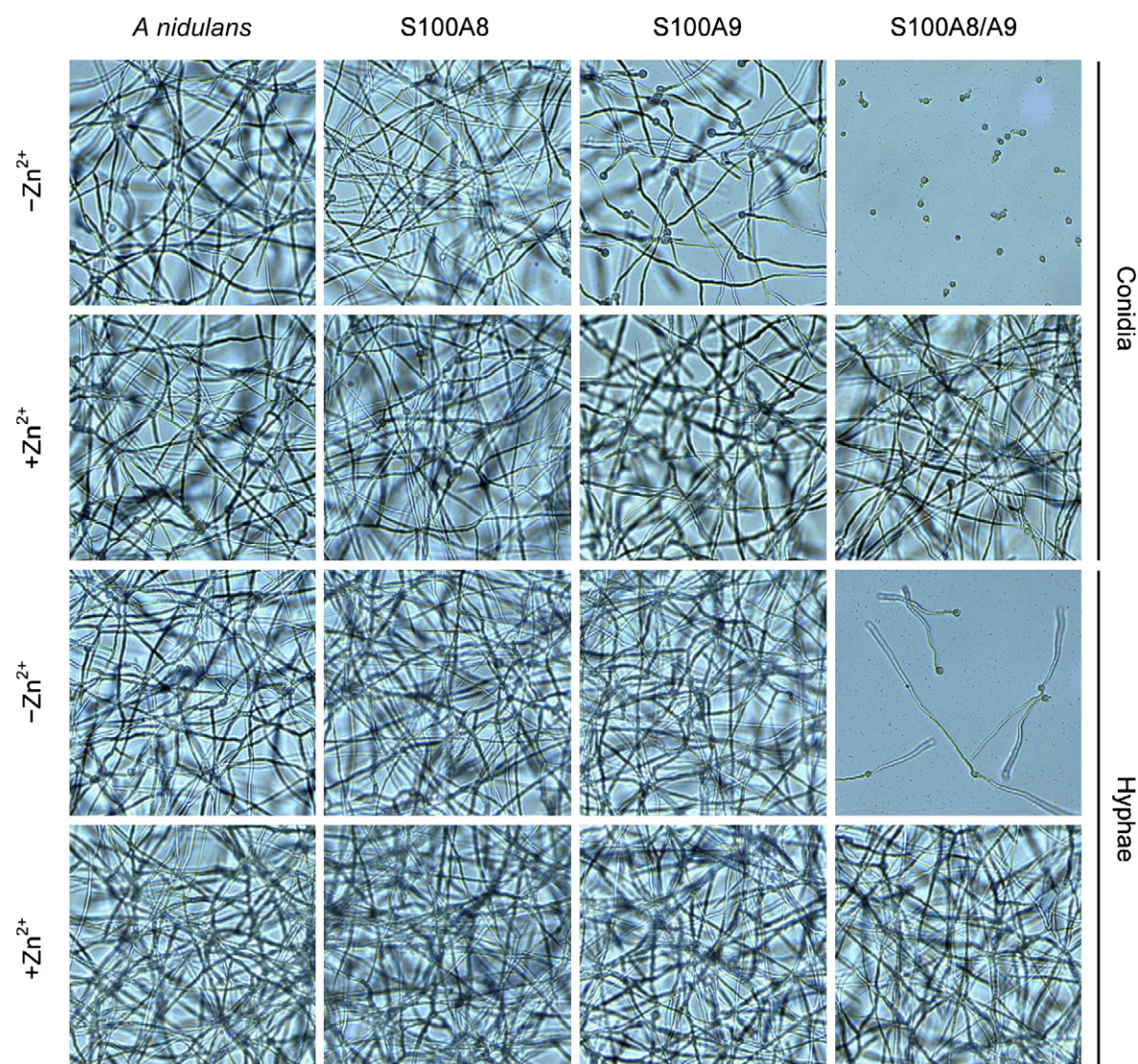


FIG E1. Inhibition of *A. nidulans* growth by purified calprotectin is abolished by Zn^{2+} addition. Incubation with the S100A8 (5 $\mu\text{g/mL}$) subunit did not prevent *A. nidulans* conidia or hyphae growth, whereas incubation with S100A9 (5 $\mu\text{g/mL}$) partially inhibited conidia germination. Coincubation with both subunits strongly inhibited conidia germination and hyphae growth. In all cases, antifungal activity was completely abolished in presence of 1 $\mu\text{mol/L}$ Zn^{2+} .

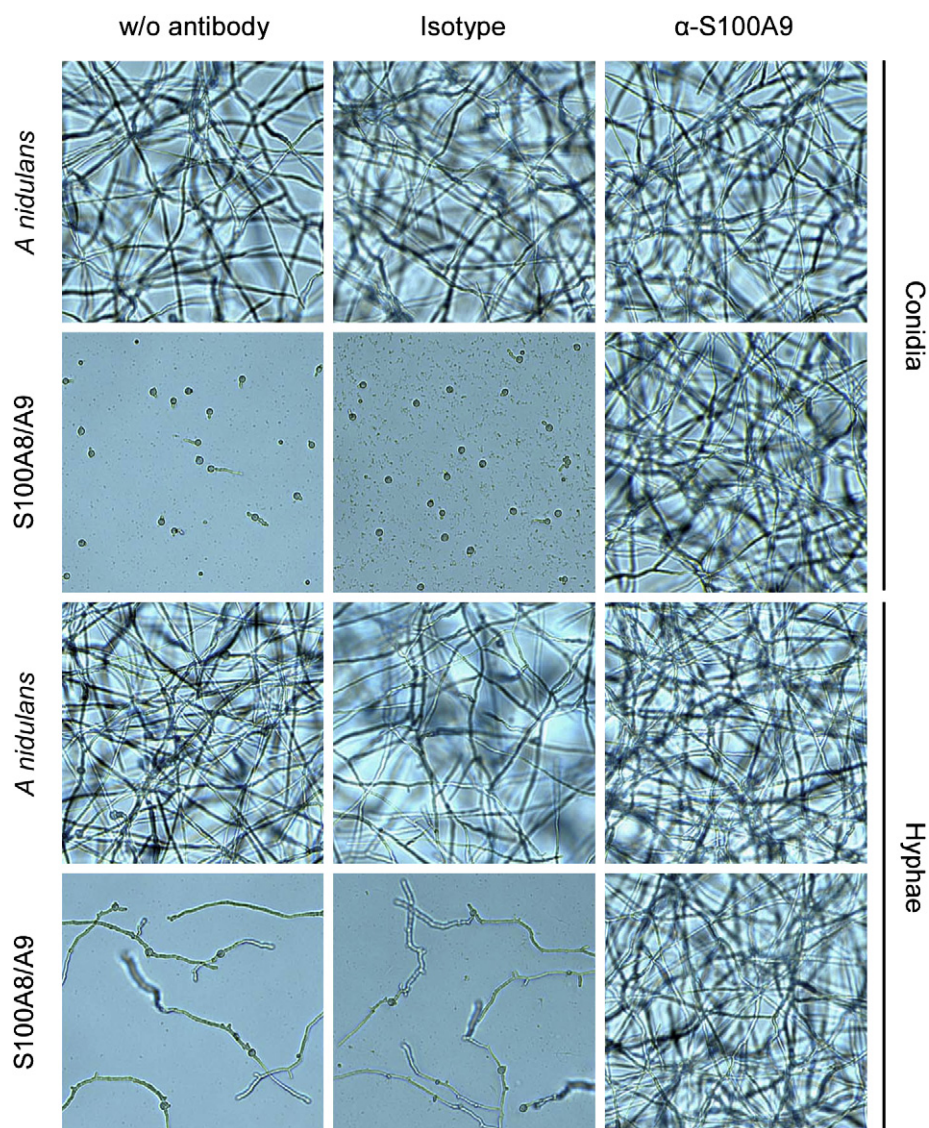


FIG E2. Inhibition of *A. nidulans* growth by purified calprotectin is abolished by α -S100A9 antibody. A total of 15 μ g/mL polyclonal α -S100A9 antibody fully inhibited antifungal activity of the S100A8/A9 calprotectin complex on conidia or hyphae. As control, S100A8/A9 was incubated with an unspecific control antibody, showing no effect on antifungal activity of the S100A8/A9 calprotectin complex. *w/o*, Without.

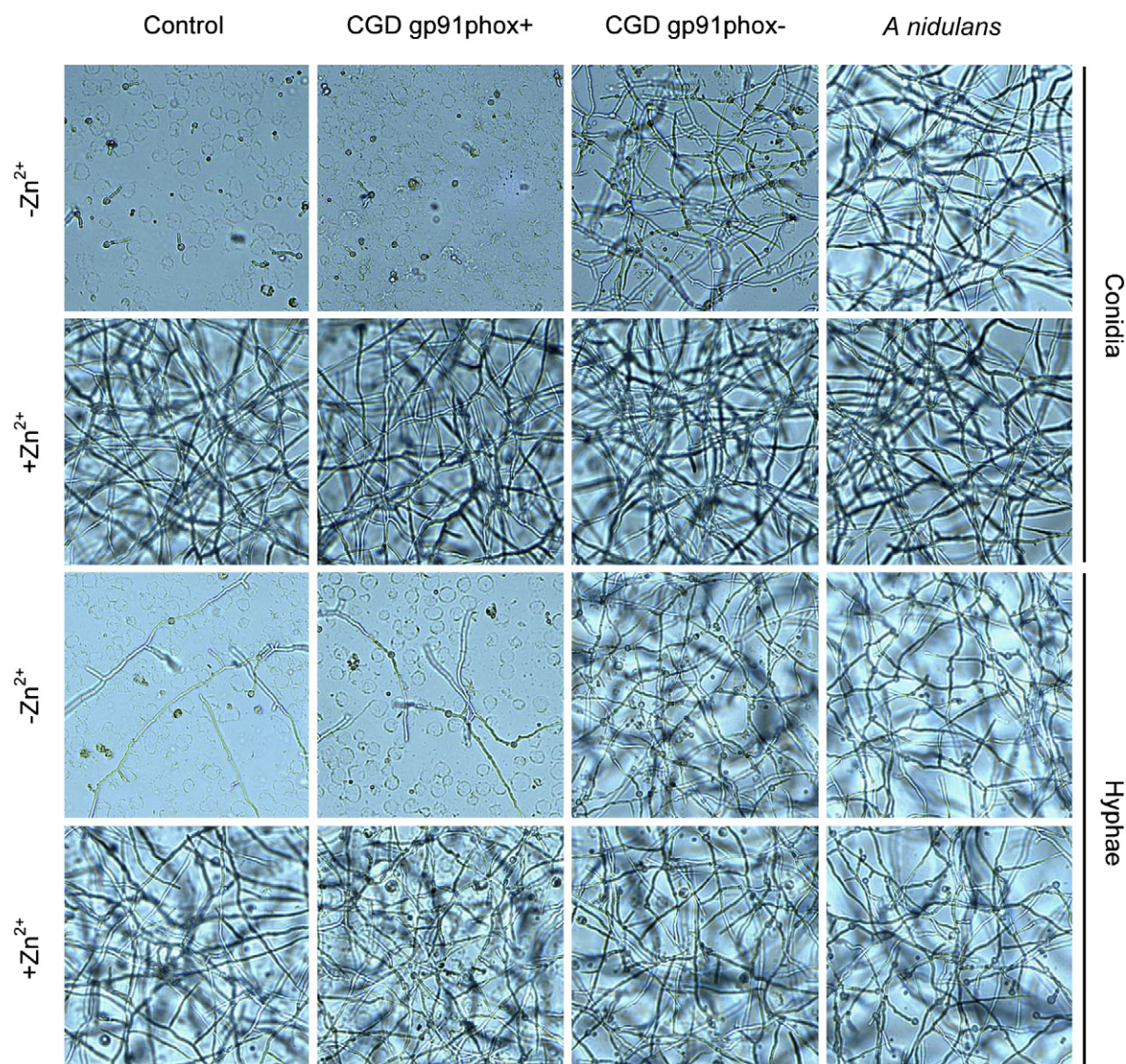


FIG E3. Calprotectin-dependent antifungal activity of CGD gp91^{phox+} neutrophils is abolished by Zn²⁺ addition. Control and CGD sorted neutrophils were stimulated with PMA for NET formation and incubated overnight with *A. nidulans* conidia or hyphae in presence or absence of 1 $\mu\text{mol/L}$ Zn²⁺. gp91^{phox+} neutrophils showed strong antifungal activity against conidia or hyphae, comparable to control. gp91^{phox-} neutrophils were inefficient in controlling fungal growth, with only partial inhibition of conidia germination. In all cases, antifungal activity was completely abolished in the presence of 1 $\mu\text{mol/L}$ Zn²⁺.

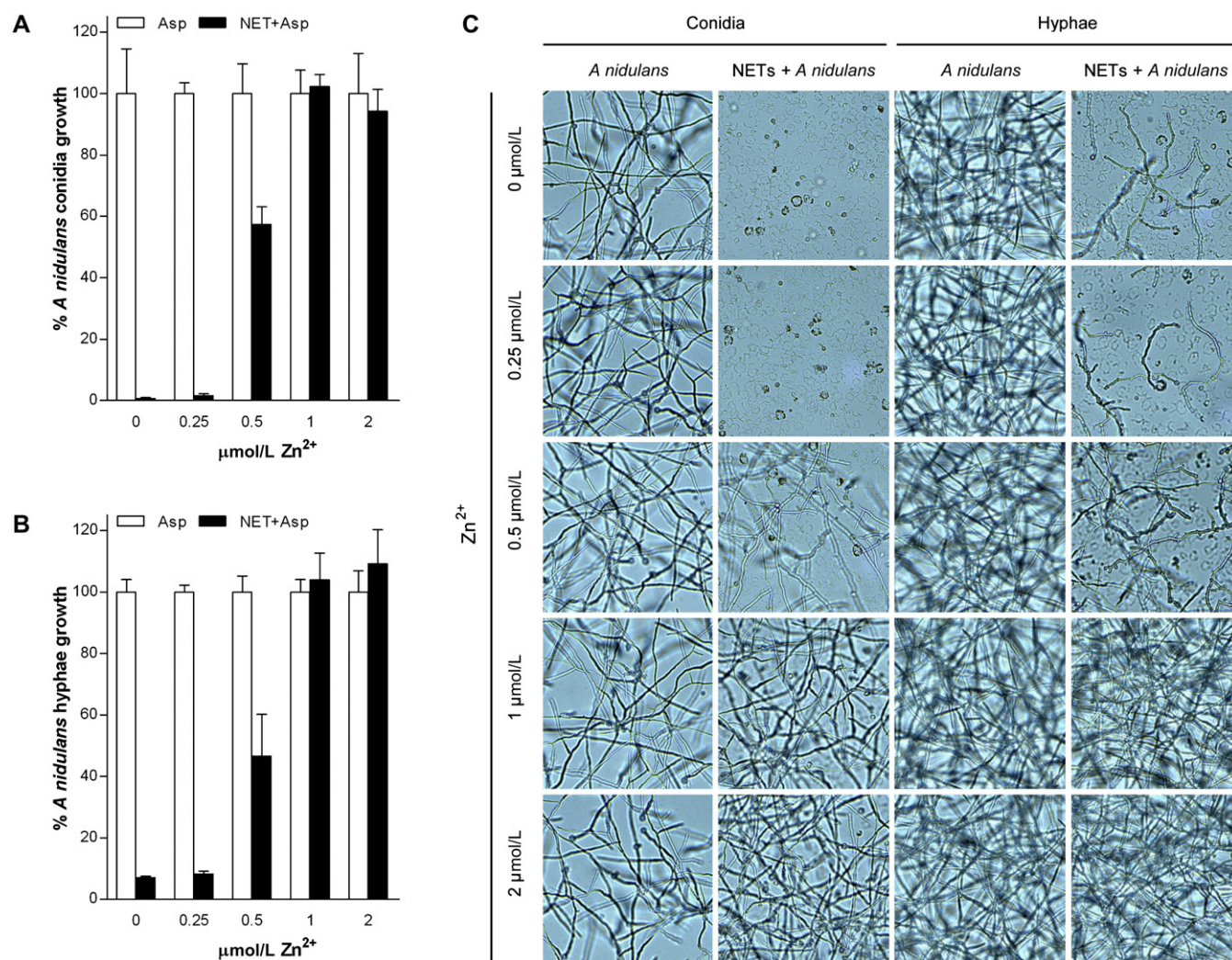


FIG E4. NET inhibition of *A. nidulans* growth is abolished by Zn^{2+} addition. Human neutrophils were stimulated with PMA for NET formation and subsequently infected overnight with *A. nidulans* conidia or hyphae $\pm \text{Zn}^{2+}$. Increasing concentrations of Zn^{2+} abolished the antifungal activity of NETs against conidia (**A**) or hyphae (**B**). **C**, Representative pictures of data shown in **A** and **B**.

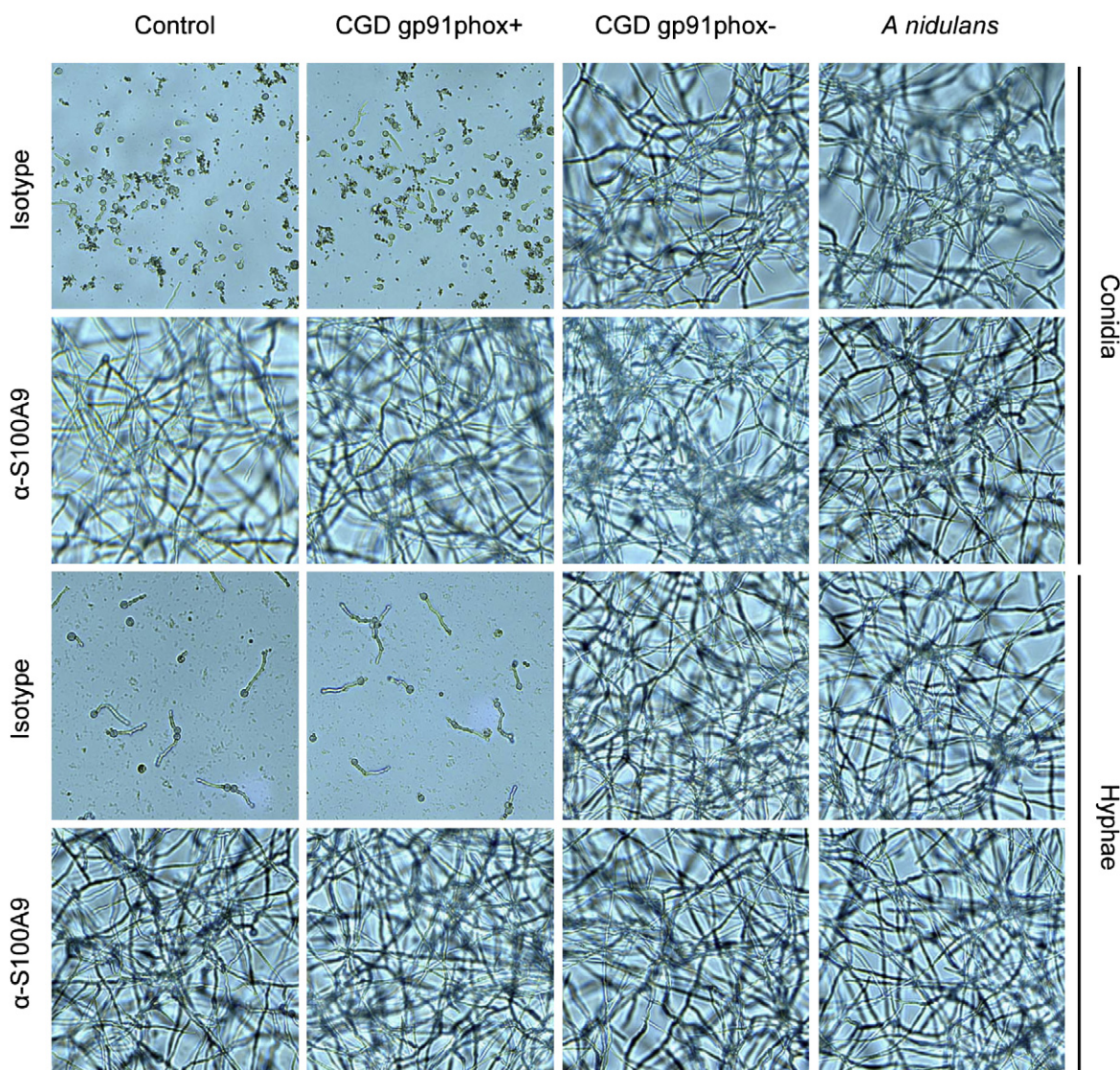


FIG E5. Calprotectin-dependent antifungal activity of CGD gp91^{phox} neutrophils is abolished by α -S100A9 antibody. Control and CGD sorted neutrophils were stimulated with PMA for NET formation; NET supernatant was digested by DNase/MNase, concentrated, and incubated with *A. nidulans* conidia or hyphae after previous incubation with 15 μ g/mL polyclonal α -S100A9 or unspecific control antibody. NET extracts from control and gp91^{phox} neutrophils showed strong antifungal activity against conidia or hyphae. Supernatant from gp91^{phox} neutrophils had no inhibitory activity on fungal growth. In all cases, fungal growth was completely restored by addition of α -S100A9 antibody, proving that the restored antifungal activity of gp91^{phox} neutrophils after GT was calprotectin-dependent.