Brief report

Restoration of NET formation by gene therapy in CGD controls aspergillosis

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Chronic granulomatous disease (CGD) patients have impaired nicotinamide adenine dinucleotide phosphate (NADPH) oxidase function, resulting in poor antimicrobial activity of neutrophils, including the inability to generate neutrophil extracellular traps (NETs). Invasive aspergillosis is the leading cause of death in patients with CGD; it is unclear how neutrophils control *Aspergillus* species in healthy persons. The aim of this study was to determine whether gene therapy restores NET formation in CGD by complementation of NADPH oxidase function, and whether NETs have antimicrobial activity against *Aspergillus nidulans*. Here we show that reconstitution of NET formation by gene therapy in a patient with CGD restores neutrophil elimination of *A nidulans* conidia and hyphae and is associated with rapid cure of preexisting therapy refractory invasive pulmonary aspergillosis, underlining the role of functional NADPH oxidase in NET formation and antifungal activity. (Blood. 2009;114: 2619-2622)

Introduction

Activated neutrophils kill microbes intracellularly after phagocytosis and by extracellular mechanisms, including neutrophil extracellular traps (NETs), which are composed of chromatin decorated with granular proteins.[1] NETs bind bacteria[1] and fungi[2] and expose antimicrobial molecules. Generation of NETs requires reactive oxygen species produced by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.[3]

Chronic granulomatous disease (CGD) is caused by mutations in genes encoding NADPH oxidase subunits. CGD patients do not produce reactive oxygen species, kill microbes poorly, and are susceptible to recurrent life-threatening infections.[4] *Aspergillus spp* infections cause pneumonia and disseminated disease and are the leading cause of death in these patients.[4-6]

It is unclear how *Aspergillus* infections are controlled in healthy persons.[7-13] In CGD patients, these infections are frequently refractory to antifungal therapy, treatment with interferon-γ, or granulocyte transfusions.[5] Here we show that the recently discovered NADPH oxidase-dependent microbicidal pathway through NETs[1-3] is efficient against *Aspergillus nidulans* conidia and hyphae in vitro and that restoration of NET formation by GT of X-CGD aided clearing severe invasive *A nidulans* infection in vivo.

Methods

Gene therapy

We treated an 8.5-year-old boy with X-linked gp91^{phox}-deficient CGD and therapy refractory *A nidulans* lung infection with a monocistronic long terminal repeat-driven gamma-retroviral SF71gp91^{phox} vector (see supplemental data, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). The protocol for the patient's treatment was 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 from his parents in accordance with the Declaration of Helsinki. For follow-up monitoring, gp91^{phox} expression was measured by fluorescence-activated cell sorter (FACS) on peripheral neutrophils after 30 minutes of staining at room temperature with 10 μg/mL gp91^{phox}-fluorescein isothiocyanate (FITC) antibody (Anti-Flavocytochrome b558, clone 7D5, MBL). NADPH oxidase activity was measured by standard dihydrorhodamine and nitroblue tetrazolium tests (supplemental materials). Bone marrow colony assays and determination of proviral gp91^{phox} sequences in genomic DNA were performed as described.[14]

NET induction

NET formation was visualized as described (supplemental materials) and quantified after stimulation of 5×10^4 neutrophils for 3 hours with 40 nM phorbol 12-myristate 13-acetate (PMA) and staining the NET-DNA with 1 μM Sytox green (Invitrogen) in a black 96-well plate (BD Biosciences). The plates were read in a fluorescence microplate reader (Victor,[3] PerkinElmer Life and Analytical Sciences) with a filter setting of 485 nm/535 nm (excitation/emission).

NET antifungal activity

The *A nidulans* strain used was isolated from bronchoalveolar lavage fluid of the patient; conidia were grown and collected as described.[7] Neutrophils after GT were stained with gp91^{phox}-FITC antibody and sorted by FACS (FACSAria, BD Biosciences) into gp91^{phox}-negative (gp91^{phox-}) and -positive (gp91^{phox+}) populations. A total of 10^5 neutrophils were activated with PMA (40 nM) at 37°C for 4 hours in a 96-well plate, and then infected with conidia (multiplicity of infection *A nidulans*/neutrophils = 0.5) plus or minus prior digestion of NETs with 10 U/mL of Micrococcal Nuclease (MNase, Worthington Biochemical) for 30 minutes. Afterward the plates were centrifuged for 5 minutes at 400*g* and incubated for 16 hours at 37°C,

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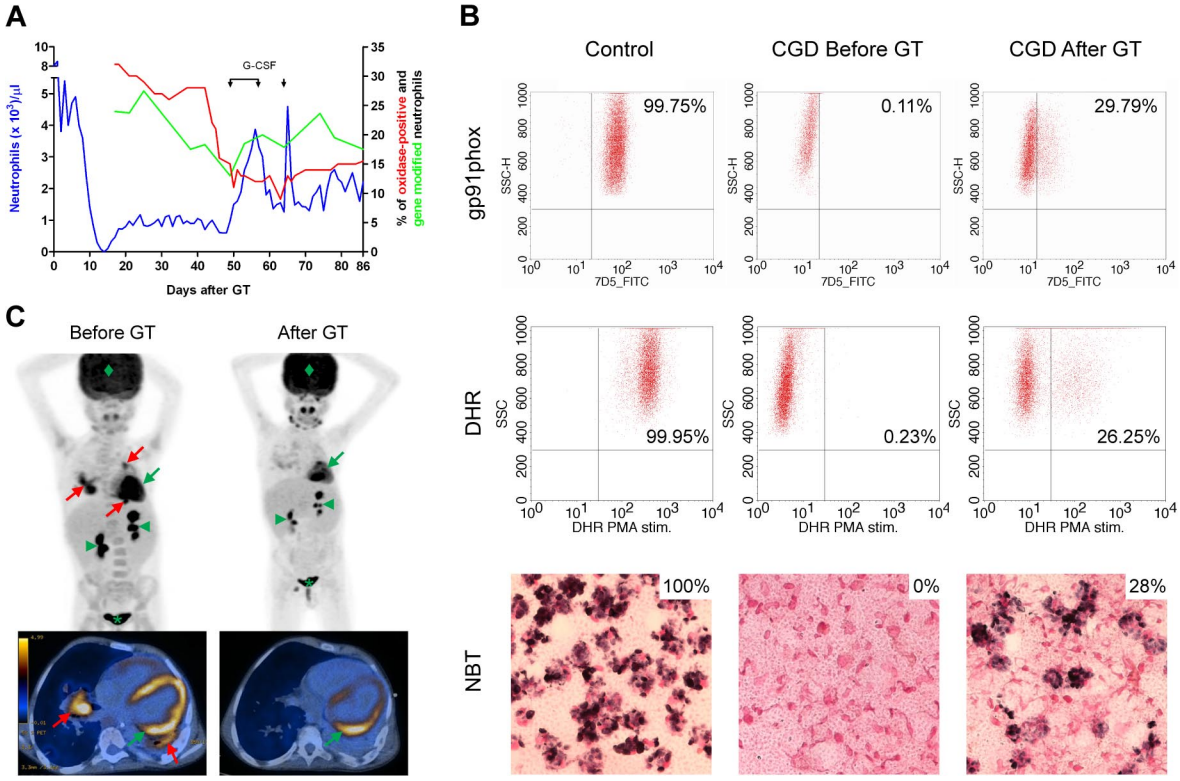

Figure 1. Restoration of NADPH oxidase function. (A) Hematopoietic reconstitution and gene marking after GT. Absolute neutrophil counts (left y-axis), quantification of gene-modified cells in peripheral neutrophils by quantitative polymerase chain reaction and quantification of neutrophils with NADPH oxidase activity by DHR test (right y-axis) are shown. When the percentage of transduced neutrophils decreased, granulocyte colony-stimulating factor (5 μg/kg per day subcutaneously) was administered on days 49 to 57 and on day 64. (B) Reconstitution of NADPH oxidase activity. Before and 6 weeks after GT, gp91^{phox} protein expression was measured by FACS analysis after 30 minutes of staining with 10 μg/mL gp91^{phox}-FITC antibody. Superoxide production was assessed by oxidation of DHR on stimulation with PMA and by reduction of nitroblue tetrazolium to formazan (dark precipitate) after stimulation with opsonized zymosan. The thresholds were determined using unstained (FACS) or unstimulated (DHR) cells for each experiment. (C) PET-CT scan. Before GT, PET-CT scan showed several active infectious foci with fluorine-18-fluoro-2-deoxy-D-glucose uptake in both lungs of the patient (red arrows); the infection cleared 6 weeks after administration of gene-corrected cells. In green, physiologic FDG uptake in heart (arrow), kidneys (arrowheads), bladder (*), and brain (diamond) are indicated for reference.

allowing germination and hyphal outgrowth. Alternatively, 5×10^4 conidia were incubated for 12 hours at 37°C to allow hyphal outgrowth; then 10^5 neutrophils were added, centrifuged for 5 minutes at 400*g*, and incubated for 2 and 5 hours with PMA (40 nM) to induce NET formation, plus or minus 10 U/mL MNase. Fungal growth was quantified with XTT (Invitrogen) as described.[15]

Results and discussion

In healthy subjects, NETs might be essential to eliminate fungi because hyphae are too large to be phagocytosed.[7,9,12,13,16-21] CGD patients are unable to make NETs.[3] Indeed, neutrophils of our X-CGD patient could only make NETs and inhibit growth of *A nidulans* after genetic complementation by GT. Neutrophils expressing functional gp91^{phox} increased from 0% to 26% to 29% 6 weeks after GT (Figure 1A-B), then decreased and leveled at approximately 16% for up to 3 months. The *A nidulans* infection completely cleared 6 weeks after GT (Figure 1C), correlating with the rise in neutrophils with NADPH oxidase activity.

The patient's neutrophils did not make NETs before GT as analyzed by fluorescence (not shown), immunofluorescence, and scanning electron microscopy (Figure 2A-B, D-E).[3] After GT, the patient's neutrophils made NETs (Figure 2C,F), the percentage of cells releasing NET-DNA (28%; Figure 2G) correlating with the level of oxidase chimerism (Figure 1B). Activation of sorted neutrophils showed that reconstitution with functional NADPH oxidase allowed corrected CGD neutrophils to make NETs (Figure 2H).

To test whether efficient eradication of the patient's infection was the result of the recovered ability to make NETs, neutrophils were infected with the *A nidulans* strain isolated from the patient. Approximately 80% of conidia germination (Figure 2I) and 45% of hyphal growth (Figure 2J) were inhibited by CGD gp91^{phox+} neutrophils, comparable with the antimicrobial activity of control neutrophils. CGD gp91^{phox-} neutrophils were inefficient in controlling fungal growth (Figure 2I-J, supplemental Figure 1). When NETs were dismantled with MNase before infection, fungicidal activity was abrogated to that of CGD gp91^{phox-} neutrophils.

In the absence of NETs, control of hyphal growth was independent of NADPH oxidase activity: when neutrophils were infected after 2-hour PMA stimulation, before NETs had been made, CGD gp91^{phox+}, CGD gp91^{phox-}, and neutrophils from healthy donor controlled growth of *A nidulans* with similar modest efficiency (Figure S2) in a NET-independent fashion because antimicrobial activity was not affected by MNase. This limited NET-independent antimicrobial activity, presumably by conidia phagocytosis, degranulation, or unknown mechanisms, suggests an NADPH oxidase-independent antifungal mechanism, clinically ineffective before GT. These data propose that the patient's clearance of fungal infection after GT was controlled by NETs. Definitive in vivo proof is obviously technically impossible.

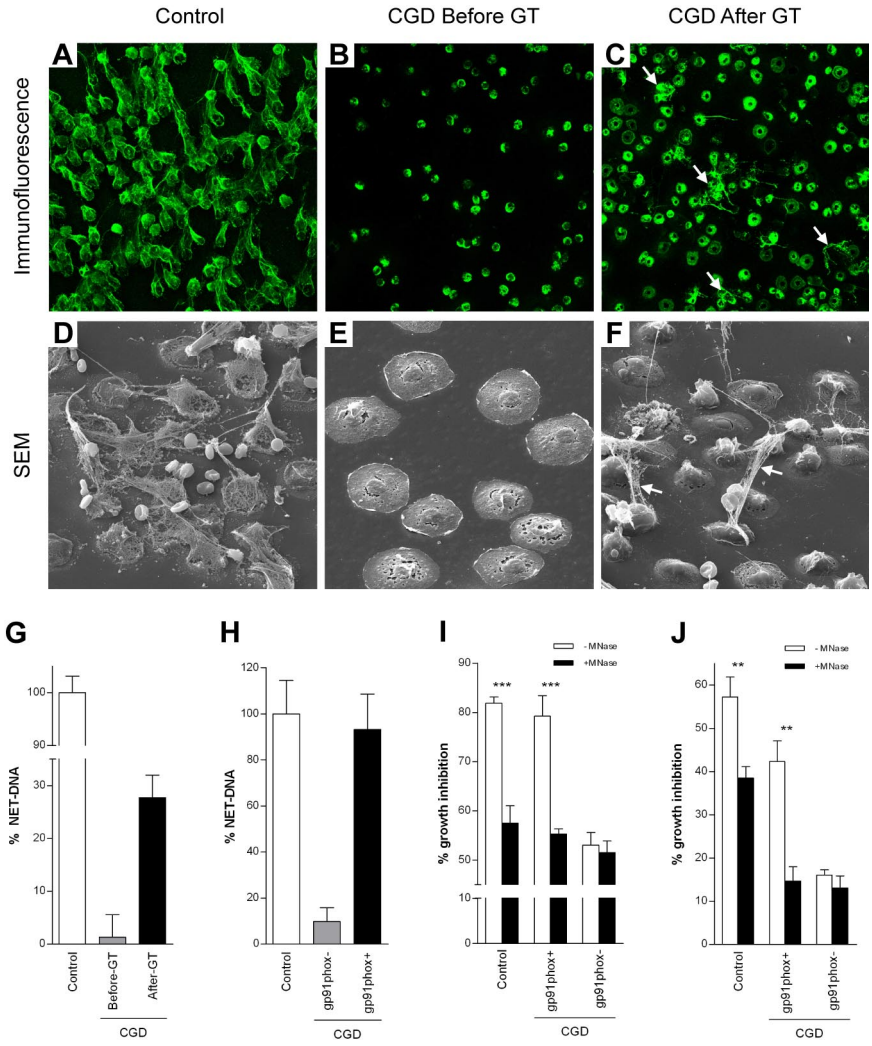

Figure 2. NET formation and inhibition of *A nidulans* growth. Control (A,D), but not CGD (B,E), neutrophils made NETs on 3-hour PMA stimulation. For immunofluorescence, NETs were stained with an antibody that recognizes neutrophil elastase (green; A-C). NETs were clearly visible also by scanning electron microscopy (SEM; D-F). Neutrophils isolated from the CGD patient before GT could be activated because they flattened out (E) but did not make NETs. The ability to form NETs was partially restored by GT 6 weeks after GT (C,F white arrows). (G) Quantification of NET-DNA released after 3 hours of PMA stimulation of control neutrophils, CGD neutrophils before and 6 weeks after GT or (H) after stimulation of CGD gp91^{phox+} and CGD gp91^{phox-} FACS-sorted neutrophils. CGD gp91^{phox+} neutrophils showed normal NET formation, whereas CGD gp91^{phox-} neutrophils showed only residual NET formation. FACS-sorting efficiency was 90% to 92% for CGD gp91^{phox-} and 95% to 96% for CGD gp91^{phox+} cells. (I-J) NET inhibition of *A nidulans* conidia and hyphae. (I) Conidia were plated on FACS-sorted neutrophils prestimulated with PMA plus or minus MNAse (ie, when NET formation was complete, cells were dead and therefore incapable of phagocytosis). Hyphal outgrowth was measured after 16 hours. (J) Hyphae were coincubated with FACS-sorted neutrophils, and PMA plus or minus MNAse and hyphal viability was assessed after 5 hours. (G-J) Data are mean ± SD of a representative triplicate experiment. Inhibition of fungal growth is expressed as percentage of control values (*A nidulans* conidia or hyphae incubated in media). The differences between −MNase and +MNase were significant (for control and CGD gp91^{phox+} cells) by Student *t* test: $^{**}P < .01$; $^{***}P < .001$.

Alveolar macrophages probably constitute the first line of defense to conidia that escape mucociliary clearance in healthy persons.[13,16] Whether reconstituted NADPH oxidase function in alveolar macrophages also contributed to microbial killing in the patient presented is difficult to assess. In neutrophils, however, conidia resist intracellular killing because of their relative tolerance to reactive oxygen species.[7,9,12,19-22] Our results suggest that conidia are killed mainly extracellularly rather than after phagocytosis. Both conidia and hyphae get ensnared by neutrophils and probably killed within NETs by concentrated antimicrobials. Cooperation of gp91^{phox-} and gp91^{phox+} neutrophils in NET antifungal activity is doubtful because we showed that gp91^{phox-} neutrophils do not make NETs when coincubated with gp91^{phox+} neutrophils (unsorted cells in Figure 2F-G),[10] suggesting that the amount of H_2O_2 released by gp91^{phox+} neutrophils is insufficient to induce NETs in gp91^{phox-} cells.[3]

A GT approach to treat CGD may be used to overcome recalcitrant, life-threatening infections but is currently limited as salvage therapy to experimental studies in selected patients with very poor performance status and lacking an human leukocyte antigen-identical hematopoietic stem cell donor.[4] GT was rapidly beneficial to our CGD patient who had suffered from an otherwise incurable fungal infection. Until day 86 after GT, there was no clonal dominance in bone marrow culture-derived CD34$^+$ cells (not shown) or expansion of gene-corrected cells in blood (Figure 1A). There is a risk, however, of insertional mutagenesis by transactivating retroviral vector insertions into proto-oncogenes, as shown in a recent GT trial with 2 adult CGD patients who developed monosomy 7 and myelodysplastic syndrome (M. Grez, Institute of Biomedical Research, Georg-Speyer-Haus, Frankfurt, Germany, oral communication, April 2009) using the same gamma-retroviral SF71gp91^{phox} vector.[14] In addition, 5 patients developed leukemia in 2 GT trials in children with severe combined immunodeficiency.[23,24] These experiences mandate the careful follow-up of patients.

In conclusion, we show that the severe immunodeficient phenotype and the high susceptibility to *Aspergillus* infection of CGD patients might be linked to the absence of NETs, and that restoration of NADPH oxidase function and NET formation by GT leads to rapid cure of refractory invasive aspergillosis in X-linked CGD.

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Authorship

Contribution: M.B. and A.H. performed the experiments, analyzed data, and contributed to the writing of the manuscript; V.B. did the immunofluorescence image acquisition and contributed to data analysis; U.S. did the bone marrow cultures and contributed to data analysis; R.A.S. designed the clinical gene therapy protocol, attended the patient together with J.R., and contributed to writing of the manuscript; and A.Z. and J.R. designed and directed the study and contributed to the writing of the manuscript.

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References

1. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science.* 2004;303:1532-1535.
2. Urban CF, Reichard U, Brinkmann V, Zychlinsky A. Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. *Cell Microbiol.* 2006;8:668-676.
3. Fuchs TA, Abed U, Goosmann C, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol.* 2007;176:231-241.
4. Seger RA. Modern management of chronic granulomatous disease. *Br J Haematol.* 2008; 140:255-266.
5. Segal BH, DeCarlo ES, Kwon-Chung KJ, Malech HL, Gallin JI, Holland SM. Aspergillus nidulans infection in chronic granulomatous disease. *Medicine (Baltimore).* 1998;77:345-354.
6. Winkelstein JA, Marino MC, Johnston RB Jr, et al. Chronic granulomatous disease: report on a national registry of 368 patients. *Medicine (Baltimore).* 2000;79:155-169.
7. 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;74:6528-6539.
8. Diamond RD, Clark RA. Damage to Aspergillus fumigatus and Rhizopus oryzae hyphae by oxidative and nonoxidative microbicidal products of human neutrophils in vitro. *Infect Immun.* 1982; 38:487-495.
9. Morgenstern DE, Gifford MA, Li LL, Doerschuk CM, Dinauer MC. Absence of respiratory burst in X-linked chronic granulomatous disease mice leads to abnormalities in both host defense and inflammatory response to Aspergillus fumigatus. *J Exp Med.* 1997;185:207-218.
10. Rex JH, Bennett JE, Gallin JI, Malech HL, Melnick DA. Normal and deficient neutrophils can cooperate to damage Aspergillus fumigatus hyphae. *J Infect Dis.* 1990;162:523-528.
11. Schaffner A, Douglas H, Braude A. Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to Aspergillus: observations on these two lines of defense in vivo and in vitro with human and mouse phagocytes. *J Clin Invest.* 1982;69:617-631.
12. Zarember KA, Sugui JA, Chang YC, Kwon-Chung KJ, Gallin JI. Human polymorphonuclear leukocytes inhibit Aspergillus fumigatus conidial growth by lactoferrin-mediated iron depletion. *J Immunol.* 2007;178:6367-6373.
13. Latgé JP. Aspergillus fumigatus and aspergillosis. *Clin Microbiol Rev.* 1999;12:310-350.
14. Ott MG, Schmidt M, Schwarzwaelder K, 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;12:401-409.
15. 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;172:1153-1156.
16. Mizgerd JP. Acute lower respiratory tract infection. *N Engl J Med.* 2008;358:716-727.
17. Ibrahim-Granet O, Philippe B, Boleti H, et al. Phagocytosis and intracellular fate of Aspergillus fumigatus conidia in alveolar macrophages. *Infect Immun.* 2003;71:891-903.
18. Philippe B, Ibrahim-Granet O, Prevost MC, et al. Killing of Aspergillus fumigatus by alveolar macrophages is mediated by reactive oxidant intermediates. *Infect Immun.* 2003;71:3034-3042.
19. Cornish EJ, Hurtgen BJ, McInnerney K, et al. Reduced nicotinamide adenine dinucleotide phosphate oxidase-independent resistance to Aspergillus fumigatus in alveolar macrophages. *J Immunol.* 2008;180:6854-6867.
20. Levitz SM, Farrell TP. Human neutrophil degranulation stimulated by Aspergillus fumigatus. *J Leukoc Biol.* 1990;47:170-175.
21. Segal AW. How neutrophils kill microbes. *Annu Rev Immunol.* 2005;23:197-223.
22. Lehrer RI, Jan RG. Interaction of Aspergillus fumigatus spores with human leukocytes and serum. *Infect Immun.* 1970;1:345-350.
23. Hacein-Bey-Abina S, Garrigue A, Wang GP, et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest.* 2008;118:3132-3142.
24. Howe SJ, Mansour MR, Schwarzwaelder K, et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest.* 2008;118:3143-3150.