



Olfm4 deletion enhances defense against *Staphylococcus aureus* in chronic granulomatous disease

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Chronic granulomatous disease (CGD) patients have recurrent life-threatening bacterial and fungal infections. Olfactomedin 4 (OLFM4) is a neutrophil granule protein that negatively regulates host defense against bacterial infection. The goal of this study was to evaluate the impact of *Olfm4* deletion on host defense against *Staphylococcus aureus* and *Aspergillus fumigatus* in a murine X-linked *gp91phox*-deficiency CGD model. We found that intracellular killing and in vivo clearance of *S. aureus*, as well as resistance to *S. aureus* sepsis, were significantly increased in *gp91phox* and *Olfm4* double-deficient mice compared with CGD mice. The activities of cathepsin C and its downstream proteases (neutrophil elastase and cathepsin G) and serum levels of IL-1 β , IL-6, IL-12p40, CXCL2, G-CSF, and GM-CSF in *Olfm4*-deficient as well as *gp91phox* and *Olfm4* double-deficient mice were significantly higher than those in WT and CGD mice after challenge with *S. aureus*. We did not observe enhanced defense against *A. fumigatus* in *Olfm4*-deficient mice using a lung infection model. These results show that *Olfm4* deletion can successfully enhance immune defense against *S. aureus*, but not *A. fumigatus*, in CGD mice. These data suggest that OLFM4 may be an important target in CGD patients for the augmentation of host defense against bacterial infection.

Introduction

Chronic granulomatous disease (CGD) comprises a group of recessive inherited disorders characterized by recurrent life-threatening bacterial and fungal infections with granuloma formation (1). The disease results from mutations in any one of four subunits of the respiratory burst oxidase (NADPH oxidase) found in phagocytic leukocytes. Two-thirds of CGD cases are due to X-linked *gp91phox* deficiency (2). NADPH oxidase generates superoxide, the precursor to hydrogen peroxide and other reactive oxygen derivatives with microbicidal activity (3). The relative resistance of microbes to nonoxidative killing mechanisms, such as defensins, bactericidal/permeability-increasing factor, lactoferrin (4), and other lysosomal proteases, may also play a role in the distinctive pattern of microbial infection seen in CGD (5). The delivery of superoxide by the NADPH oxidase complex into the phagosome is also thought to indirectly activate several classes of proteases by increasing K⁺ influx and pH (6).

Olfactomedin 4 (OLFM4) is a member of the olfactomedin domain-containing glycoprotein family whose expression is known to be regulated by G-CSF, PU.1, and NF- κ B (7). OLFM4 is stored in the granules of mature neutrophils (8). Recent function studies revealed that OLFM4 is an important modifier of neutrophil bacterial killing and host innate immunity against Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Escherichia coli* (8), and *Helicobacter pylori* (9). These features are mediated through its restriction on neutrophil cathepsin C-mediated protease activities (8) and nucleotide-binding oligomerization domain-mediated (NOD-mediated) NF- κ B acti-

vation (9). NADPH oxidase-mediated bacterial killing is totally abolished in CGD patients. Here, we hypothesize that deletion of *Olfm4* in CGD neutrophils may restore microbe-killing activities through nonoxidative mechanisms.

Results and Discussion

In normal neutrophils, microorganisms are killed by the combined action of oxygen-dependent and oxygen-independent mechanisms. CGD neutrophils possess only oxygen-independent mechanisms, and killing mainly depends on the microbicidal effect of the neutrophil granule proteins (5). In this study, we first investigated whether deletion of *Olfm4*, a negative modulator of host defense against bacterial infection, could boost X-linked CGD mouse immunity against *S. aureus*, a commonly encountered pathogen in CGD patients. X-CGD mice have a null allele for *gp91phox* and are similar to patients with CGD in that they manifest impaired resistance to infections with *S. aureus* and *Aspergillus fumigatus* (10). Nitroblue tetrazolium (NBT) reduction assays (Figure 1A) and superoxide burst assays (Figure 1B) confirmed the lack of NADPH oxidase activity in *gp91phox*-deficient mice.

We first crossed *gp91phox*-deficient mice with *Olfm4*-deficient mice to create *gp91phox* and *Olfm4* double-deficient mice. These double-deficient mice demonstrated normal development and growth without symptoms of colitis or skin disease. We then analyzed neutrophils derived from the bone marrow of mice with different genotypes for their intracellular killing of two strains of *S. aureus*: *Rosenbach* and *USA300/LAC*, the latter of which is a methicillin-resistant *S. aureus* (MRSA). In both strains, the neutrophils from *gp91phox*^{+/+}*Olfm4*^{-/-} (*Olfm4*-deficient) mice demonstrated increased capability to kill intracellular *S. aureus*, while neutrophils from *gp91phox*^{-/-}*Olfm4*^{+/+} (CGD) mice had decreased bacterial-kill-

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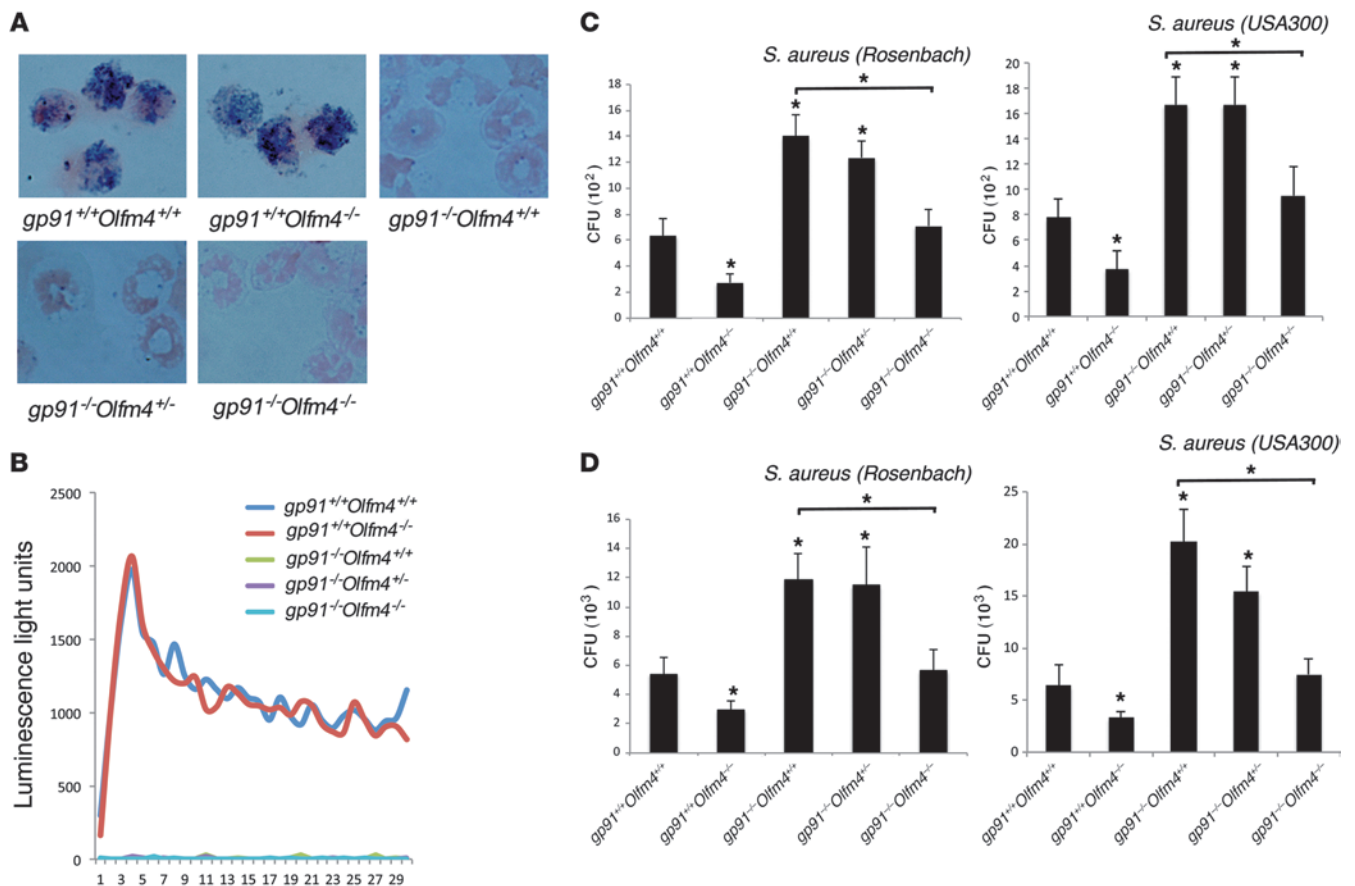


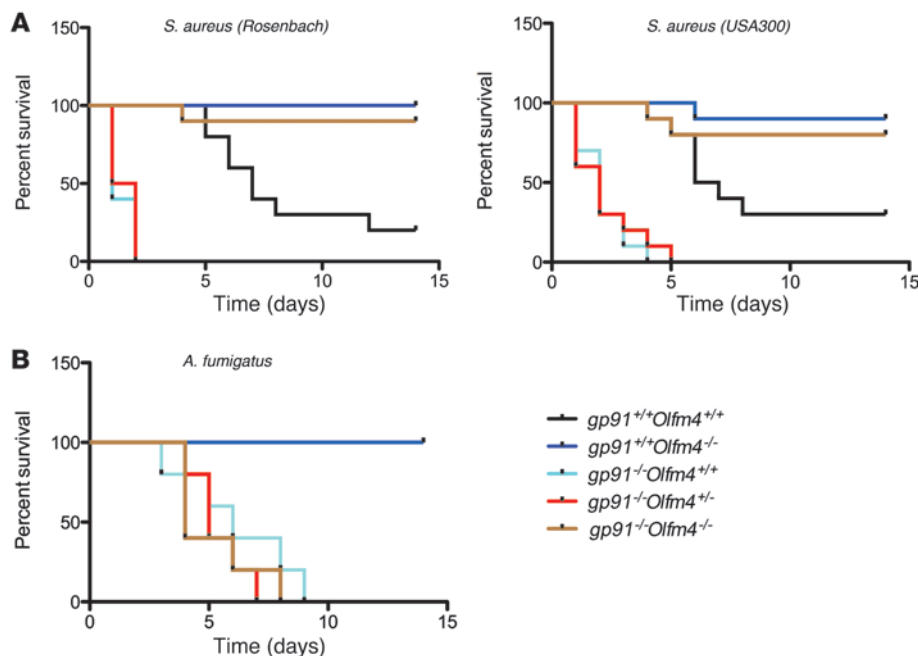
Figure 1 Intracellular killing and in vivo peritoneal clearance of *S. aureus* in *Olfm4*- and *gp91phox*-deficient mice. (A) NBT assays and (B) superoxide burst assays were performed to confirm the loss of NADPH oxidase activity in different genotypes of mice. Original magnification, $\times 200$ in NBT assays. (C) Neutrophils derived from the bone marrow of *Olfm4*- and *gp91phox*-deficient mice were incubated with preopsonized *S. aureus* (*Rosenbach* or *USA300*). The number of viable bacteria (CFU) after the cells were treated with gentamicin (50 μ g/ml) was determined on plates. Data are expressed as the mean \pm SD ($n = 5$). (D) Mice were challenged i.p. with *S. aureus* (*Rosenbach* or *USA300*). After 6 hours, the peritoneal cavity was lavaged, and the number of viable bacteria (CFU) was determined on plates. Data are expressed as the mean \pm SD ($n = 5$). * $P < 0.05$ when compared with WT (*gp91phox*^{+/+}*Olfm4*^{+/+}) mice or as indicated.

ing ability compared with those from *gp91phox*^{+/+}*Olfm4*^{+/+} (WT) mice (Figure 1C). The bacterial-killing abilities in *gp91phox*^{-/-}*Olfm4*^{-/-} (double-deficient) neutrophils were restored to the levels of those from WT mice (Figure 1C). Neutrophils from *gp91phox*^{-/-}*Olfm4*^{+/+} and *gp91phox*^{-/-}*Olfm4*^{-/-} (CGD) mice demonstrated a similar level of bacterial killing (Figure 1C). We then performed in vivo peritoneal *S. aureus* clearance assays for the two strains among different genotypes of mice. Similarly, *Olfm4*^{-/-} (*Olfm4*-deficient) mice had increased in vivo peritoneal *S. aureus* clearance, and *gp91phox*^{-/-} *Olfm4*^{+/+} (CGD) mice had decreased bacterial-clearance ability. In addition, double-deficient mice had enhanced in vivo bacterial clearance abilities compared with CGD mice and demonstrated a similar level of bacterial clearance ability to that of WT mice (Figure 1D). These results indicate that a homozygous deletion of the *Olfm4* gene in CGD mice could enhance CGD mouse *S. aureus* killing and in vivo clearance.

Next, we investigated whether deletion of *Olfm4* in CGD mice could increase the host defense against systemic infections of *S. aureus* and *A. fumigatus*, the latter of which is another common

pathogen in CGD patients. Mice of various genotypes were infected with *S. aureus* of *Rosenbach* or *USA300* by i.p. injection, and their survival was monitored. All CGD mice and *gp91phox*^{-/-}*Olfm4*^{-/-} mice died within 2 to 5 days (Figure 2A). Most WT mice died gradually during a 2-week observation period. In contrast, almost all the *Olfm4*-deficient and *gp91phox* and *Olfm4* double-deficient mice survived. Consistent with our earlier observations, these results suggest that a homozygous, but not a heterozygous, deletion of *Olfm4* could increase host system defense against *S. aureus* infection and prevent *S. aureus* sepsis-induced mortality in CGD mice.

Pulmonary aspergillus infection is the most common microbial cause of death in CGD patients, and NADPH oxidase-deficient mice are also susceptible to experimental aspergillus challenge (11). Therefore, we wanted to determine whether OLFM4 is involved in the mouse host defense against *A. fumigatus* using a lung infection model. Following oropharyngeal challenge with a moderate inoculum (5×10^5 conidia per mouse), CGD, double-deficient, and *gp91phox*^{-/-}*Olfm4*^{-/-} mice all died within 9 days, whereas WT and *Olfm4*-deficient mice all survived (Figure 2B).

**Figure 2**

Susceptibility of *Olfm4*- and *gp91phox*-deficient mice to *S. aureus* or *A. fumigatus* infection. (A) Survival probability plots (Kaplan-Meier) of experimental groups infected i.p. with *S. aureus* (Rosenbach or USA300) (2×10^8 CFU per mouse; $n = 10$). Survival was monitored every 6 hours. (B) Survival probability plots (Kaplan-Meier) of experimental groups infected with oropharyngeal aspiration of *A. fumigatus* (5×10^5 CFU per mouse; $n = 5$). Survival was monitored twice a day.

These results showed that *Olfm4* deletion did not prevent pulmonary *A. fumigatus* infection-induced death in CGD mice. We also evaluated host immune response in *Olfm4*^{-/-} mice compared with WT mice 24 hours after a high *A. fumigatus* inoculum (1×10^7 conidia per mouse). Bronchoalveolar fluid leukocytosis was not significantly different between the two genotypes (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI68453DS1). No significant difference between the two genotypes was observed in the quantitative fungal burden in the lungs (Supplemental Figure 1B) or in the Grocott-Gomori methenamine-silver staining in lung sections (Supplemental Figure 1C). Lung histology showed a similar level of inflammation characterized by peribronchial and perivascular inflammatory cell infiltration, including neutrophils and mononuclear cells, in *Olfm4*^{-/-} and WT mice (Supplemental Figure 1D). Taken together, these results suggest that OLFM4 may not play a role in the mouse host defense against pulmonary aspergillosis.

Neutrophils from *gp91phox*-deficient mice or CGD patients have impaired fungicidal activity, and the generation of ROS by neutrophils is considered to be the major antifungal mechanism lacking in CGD (12). Although evidence suggests that neutrophils can mediate antifungal activities via nonoxidative mechanisms, including defensins (13), lactoferrin (4), and pentraxins (14), neutrophils from CGD patients do not cause substantial damage to hyphae in vitro (15), suggesting that cellular nonoxidative mechanisms alone are insufficient to kill hyphae under physiologic conditions. It has been reported that *A. fumigatus* was markedly resistant to neutrophil granule extract (5). Neutrophils use oxygen-dependent mechanisms to attack hyphae germinating from conidia that escape alveolar macrophage surveillance (16). A recent study demonstrated that neutrophil proteases and cathepsin C, which are important for bacterial innate immunity (17, 18), do not play a role in mouse host defense against pulmonary aspergillosis (11). Because OLFM4 regulates bacterial killing largely through modulation of cathepsin C and neutrophil proteases in the granules (8), this may explain why

Olfm4 deletion did not alter the mice's defense against *A. fumigatus*, but led to enhanced host defense against *S. aureus*. These results support the conclusion that NADPH oxidase and neutrophil proteases have distinct antibacterial and antifungal functions.

To explore the mechanism through which *Olfm4* deletion enhanced the bactericidal activities of CGD neutrophils, we analyzed the activities of cathepsin C and its downstream proteases (neutrophil elastase and cathepsin G) in the neutrophils from mice after *S. aureus* challenge. Cathepsin C activity in the neutrophils of *Olfm4*-deficient mice and double-deficient mice was significantly higher than that in WT mouse neutrophils, while cathepsin C activity in CGD (*gp91phox*^{-/-}*Olfm4*^{+/+}) and *gp91phox*^{-/-}*Olfm4*^{-/-} mice was similar to that in WT mice (Figure 3A). Accordingly, the neutrophil elastase (Figure 3B) and cathepsin G (Figure 3C) activities in *Olfm4*-deficient and double-deficient mice were also substantially higher than those in WT mice as well as in CGD and *gp91phox*^{-/-}*Olfm4*^{-/-} mice. These results suggest that the increase in serine protease activities observed with *Olfm4* deletion is NADPH independent. The compromised neutrophil bacterial killing and host innate immunity against *S. aureus* in CGD mice due to oxidative mechanism deficiency could be successfully restored by the enhancement of serine protease activities in neutrophils subsequent to *Olfm4* deletion. Serine proteases are stored in granules in their active form until they are released following neutrophil exposure to inflammatory stimuli. Once released, neutrophil serine proteases are potentially fully active and have broad biological effects, including intracellular microbial killing and modulation of inflammatory cell recruitment (19).

A previous study showed that OLFM4 binds NOD1 and NOD2 and inhibits NOD-mediated NF- κ B signaling (9). Here, we analyzed cytokine and chemokine levels in the serum of mice following challenge with *S. aureus*. The levels of IL-1 β , IL-6, IL-12p40, CXCL2, G-CSF, and GM-CSF were significantly higher in the *Olfm4*-deficient mice as well as in the *gp91phox* and *Olfm4* double-deficient mice than the levels in WT and CGD mice (Figure 3D). All of these cytokines

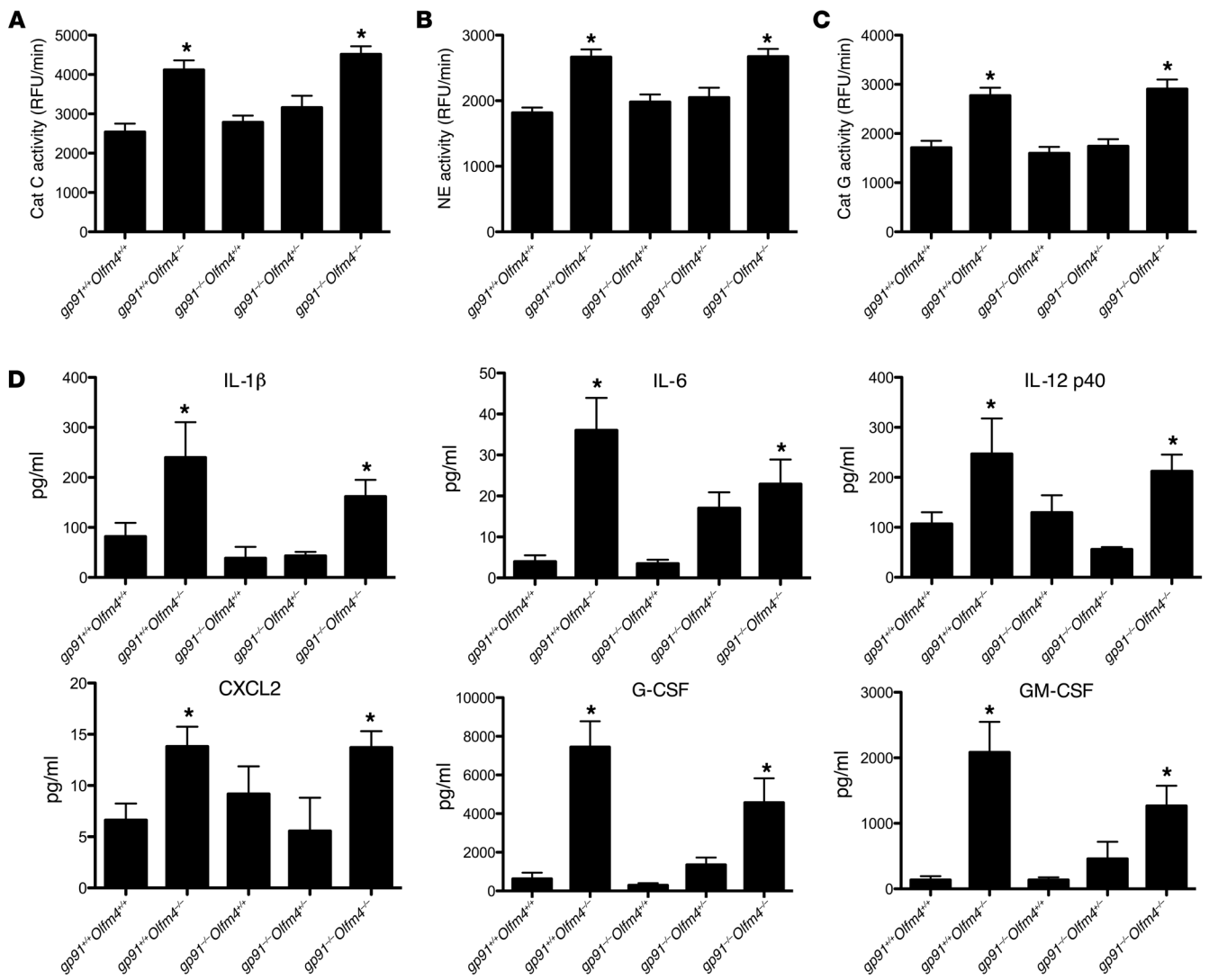


Figure 3 Cathepsin C and serine protease activities and cytokine/chemokine serum levels in *Olfm4*- and *gp91phox*-deficient mice. (A–C) Neutrophils (5×10^6) derived from the bone marrow of mice with different genotypes 6 hours after i.p. infection with *S. aureus* (5×10^7 CFU per mouse) were lysed, and an equal amount of lysate was used for assays of cathepsin C (Cat C) (A), neutrophil elastase (B), and cathepsin G (Cat G) (C) activity using the corresponding AMC-labeled substrate. * $P < 0.05$ when compared with WT (*gp91phox^{+/+}Olfm4^{+/+}*) mice. Data are expressed as the mean \pm SD ($n = 5$). RFU, relative fluorescence unit. (D) Cytokine and chemokine levels in the serum of mice with different genotypes 6 hours after i.p. infection with *S. aureus* (5×10^7 CFU per mouse) were determined by high-throughput immunoassay. Data are expressed as the mean \pm SD for each experimental group ($n = 5$). * $P < 0.05$ versus WT (*gp91phox^{+/+}Olfm4^{+/+}*) mice.

and chemokines are known target genes of the NF- κ B pathway. We consistently found that NF- κ B activity in the neutrophils from *Olfm4*-deficient as well as *gp91phox* and *Olfm4* double-deficient mice was significantly higher than that in WT and CGD mice (Supplemental Figure 2). The enhanced cytokine levels in *Olfm4*-deficient mice could be caused by the upregulated NF- κ B signaling as well as by increased serine protease activities. Neutrophil serine proteases might not only regulate the activity of chemokines and cytokines by proteolysis, but might also modulate their release through cellular activation (19). NOD2 is critical for innate recognition and antibacterial defense against *S. aureus* (20, 21). Therefore, the enhanced NOD-mediated defense pathways caused by *Olfm4* deletion may also contribute to the enhanced host defense against *S. aureus* in CGD.

NOD signaling works synergistically with TLR signaling to recognize *S. aureus* infection and induce inflammatory responses (22). The TLR2-MyD88 pathway has been recognized to play important roles in mouse innate immunity against *S. aureus* infection (23). To determine whether the effect of *Olfm4* deletion on *S. aureus* immune defense is mediated through the TLR-MyD88 pathway, we investigated the host defense of *MyD88* and *Olfm4* double-deficient mice against *S. aureus* systemic infection. While the *MyD88*-deficient mice were highly susceptible to *S. aureus* infection, additional *Olfm4* elimination remarkably improved the survival of *MyD88*-deficient mice and enhanced the serum levels of some NF- κ B-targeted pro-inflammatory cytokines and chemokines (Supplemental Figure 3). These results suggest that enhanced mouse innate



immunity due to *Olfm4* deletion is TLR-MyD88 signal independent and that NOD-mediated NF- κ B may play an important role. The mechanism of OLFM4 regulation of host immune defense against *S. aureus* is summarized in Supplemental Figure 4.

Taken together, we found that deletion of *Olfm4*, a critical negative regulator of neutrophil protease activities and the NOD-mediated pathway, could enhance the immune defense against *S. aureus* infection in CGD mice. This finding provides a rationale for enhancing CGD patient defense against bacterial infections potentially through the modulation of OLFM4 levels.

Methods

Further information can be found in Supplemental Methods.

Mice, bacteria, and fungi. X-linked *gp91phox*-deficient mice on a C57BL/6 background were purchased from The Jackson Laboratory. *Olfm4*-deficient mice backcrossed six generations onto a C57BL/6 background were described previously (8). *Olfm4* and *gp91phox*-double-deficient mice were derived from crossing *Olfm4*-deficient and *gp91phox*-deficient mice. Animals were kept in a specific pathogen-free facility at the NIH. All experiments were conducted with 8- to 10-week-old male animals. *S. aureus* (Rosenbach) ATCC 10390 was purchased from ATCC, and *S. aureus* (USA300/LAC) was provided by Frank R. DeLeo of the NIH. Bacteria were grown in tryptic soy broth or on tryptic soy agar plates (Teknova). *A. fumigatus* strain B-5233, a pulmonary clinical isolate from a leukemic patient, was maintained on *Aspergillus* minimal medium (4). Conidia were harvested from 1-week-old cultures with PBS and quantitated on a hemacytometer.

Intracellular bacterial-killing assay. Purification of bone marrow-derived mouse neutrophils and the bacterial-killing assay were performed as described previously (8).

Protease activity assays. The assays were performed as previously described (8) with minor modifications. Cathepsin C activity was assayed in 25 mM 2-ethanesulfonic acid (MES), pH 6.0, 50 mM NaCl, 5 mM DTT, and 0.1% polyethylene glycol (PEG) 3350 using Gly-Arg-AMC (Bachem) at 10 μ M. Neutrophil elastase (NE) activity was assayed in 100 mM Tris-HCl, pH 7.5,

and 50 mM NaCl using Ala-Ala-Pro-Val-AMC (Bachem) as a substrate (100 μ M). Cathepsin G activity was assayed in 100 mM Tris-HCl, pH 7.5, and 50 mM NaCl using Ala-Ala-Pro-Phe-AMC (Bachem) as a substrate (100 μ M). Reaction progress was monitored continuously with product (AMC) on a FLUOstar OPTIMA Fluorimeter (BMG LABTECH) with 380-nm excitation and 460-nm emission wavelength filters.

Statistics. Kaplan-Meier curves were generated with GraphPad Prism 5.0 software (GraphPad) and analyzed using the log-rank method. The Mann-Whitney *U* test was used to compare the quantitation of CFU, fungal burden, and cytokine levels. A *P* value of less than 0.05 was considered statistically significant.

Study approval. All animal procedures were conducted according to protocols approved by the National Heart, Lung, and Blood Institute and the National Institute of Allergy and Infectious Diseases Animal Care and Use committees.

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