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

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**Introduction**

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-1β, 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.

**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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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 NAPDH oxidase activity in different genotypes of mice. Original magnification, ×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 μg/ml) was determined on plates. Data are expressed as the mean ± 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 ± SD (n = 5). *P < 0.05 when compared with WT (gp91phox+/+Olfm4+/+) mice or as indicated.

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).
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 × 10⁷ 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 (neutrophil elastase and cathepsin G) in the neutrophils from mice after A. fumigatus challenge. Cathepsin G activity in the neutrophils of Olfm4−/− and double-deficient mice was also significantly higher than 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−/− 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 A. 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-κB signaling (9). Here, we analyzed cytokine and chemokine levels in the serum of mice following challenge with A. aureus. The levels of IL-1β, 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 2
Susceptibility of Olfm4−/− and gp91phox−/− 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 × 10⁸ 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 × 10⁴ CFU per mouse; n = 5). Survival was monitored twice a day.
and chemokines are known target genes of the NF-κB pathway. We consistently found that NF-κB 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-κB 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-κB–targeted pro-inflammatory cytokines and chemokines (Supplemental Figure 3). These results suggest that enhanced mouse innate

**Figure 3**

Cathepsin C and serine protease activities and cytokine/chemokine serum levels in Olfm4- and gp91phox-deficient mice. (A–C) Neutrophils (5 × 10⁶) derived from the bone marrow of mice with different genotypes 6 hours after i.p. infection with S. aureus (5 × 10⁷ 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 ± 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 × 10⁷ CFU per mouse) were determined by high-throughput immunoassay. Data are expressed as the mean ± SD for each experimental group (n = 5). *P < 0.05 versus WT (gp91phox+/+Olfm4+/+) mice.
immunity due to *Olfm4* deletion is TLR-MyD88 signal independent and that NOD-mediated NF-κB 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*–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 hemocytometer.

*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 HEPES, pH 7.5, 5 mM DTT, and 0.1% 3-ethylthiosemicarbazide (ETSB). Cathepsin G activity was assayed in 100 mM Tris-HCl, pH 7.5, and 50 mM NaN[3] using Ala-Ala-Pro-Val-AMC (Bachem) as a substrate (100 μM). 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 μM). 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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