NOD2 contributes to cutaneous defense against
Staphylococcus aureus through α-toxin-dependent
innate immune activation

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Staphylococcus aureus is a major cause of community-acquired and nosocomial infections including the life-threatening conditions endocarditis, necrotizing pneumonia, necrotizing fasciitis, and septicemia. Toll-like receptor (TLR)-2, a membrane-bound microbial sensor, detects staphylococcal components, but macrophages lacking TLR2 or both TLR2 and TLR4 remain S. aureus responsive, suggesting that an alternative microbial recognition receptor might be involved. The cytoplasmic sensor nucleotide-binding oligomerization domain containing (NOD)-2/caspase recruitment domain (CARD) 15 detects muramyl dipeptide from bacterial peptidoglycans and mediates cytokine responses to S. aureus in vitro, but the physiological significance of these observations is not well defined. Here we show that NOD2-deficient mice exhibit a delayed but ultimately exacerbated ulcerative response and impaired bacterial clearance after s.c. infection with S. aureus. NOD2-dependent recognition of S. aureus and muramyl dipeptide is facilitated by α-toxin (α-hemolysin), a pore-forming toxin and virulence factor of the pathogen. The action of NOD2 is dependent on IL-1β-amplified production of IL-6, which promotes rapid bacterial killing by neutrophils. These results significantly broaden the physiological importance of NOD2 in innate immune from the recognition of bacteria that primarily enter the cytoplasm to the detection of bacteria that typically reside extracellularly and demonstrate that this microbial sensor contributes to the discrimination between commensal bacteria and bacterial pathogens that elaborate pore-forming toxins.

Innate immunity | interleukin-1 | interleukin-6 | microbial pathogenesis

S. aureus is the major cause of human skin and wound infections acquired in hospitals and in the community (1). The increasing prevalence of virulent strains of methicillin-resistant S. aureus is a paramount clinical challenge and underlines the importance of understanding the mechanisms of invasive disease and normal host defense. After S. aureus penetration of the skin and entry into the s.c. tissues, often facilitated by cutaneous injury, surgery, or medical devices, local innate immune responses are crucial in limiting the establishment of an infectious focus and reducing disease severity. Microbial sensing by the innate immune system is mediated by pattern recognition receptors, such as Toll-like receptors (TLRs) and the more recently described group of nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (2, 3). TLRs recognize diverse bacterial ligands at the cell surface or within endosomes (2). By contrast, many NLRs detect their microbial ligands in the cytoplasm. For example, the prototypic NLR family member NOD2/caspase recruitment domain (CARD) 15 senses muramyl dipeptide (MDP), a ubiquitous building block of the cell wall peptidoglycan in most gram-positive and gram-negative bacteria, in the cytosol. Activation of NOD2 initiates an inflammatory response through NF-κB and MAPK signaling pathways. NOD2 has a role in host defense against the invasive intracellular pathogen Listeria monocytogenes (4), but little is known about the physiological functions of the phylogenetically conserved NOD2 in host defense against a broader range of bacteria.

Although TLR2 is involved in the detection of staphylococcal cell wall components (5, 6), macrophages of TLR2/TLR4 double-deficient mice continue to respond to S. aureus with proinflammatory cytokine secretion, suggesting that an alternative microbial receptor might be involved in bacterial recognition (7–9). NOD2 mediates cytokine responses to S. aureus in transfected cells and seems to contribute to host defense against systemic infection with the pathogen (10–12), but the underlying recognition mechanisms and their physiological significance in the skin, the predominant infection site, are not understood. Here we report that NOD2 is critical for innate recognition of S. aureus and for effective antibacterial defense in a murine skin infection model. IL-1β and IL-6 production, as well as the surrounding α-hemolysin toxin of the pathogen, play key roles in the NOD2-mediated defense pathway.

Results

Delayed and Exacerbated Local Inflammation in NOD2-Deficient Mice.

To determine the function of NOD2 in cutaneous host defense against S. aureus, we used WT (Nod2+/+) and NOD2-deficient (Nod2−/−) mice on the C57BL/6j genetic background and infected them s.c. with S. aureus (5 × 10⁷ cfu). WT mice developed skin ulcerations by 2 days that peaked in size by 5 days and had largely healed by 21 days (Fig. 1 A and C). In contrast, Nod2−/− mice developed ulcerations more slowly during the first 3 days after infection but then experienced significantly larger and more persistent ulcerations starting at 6 days (Fig. 1 A and C). In parallel with the delayed but ultimately exacerbated ulcerative response, Nod2−/− mice had 5- to 10-fold higher bacterial cfu in the skin than the WT mice from day 4 onward, indicating that NOD2 is required for normal bacterial clearance (Fig. 1 B). The defect in bacterial clearance was not related to an inability to recruit neutrophils, which are critical innate effector cells against S. aureus (13), because no differences were observed in neutrophil numbers at the site of infection in Nod2−/− compared with WT mice by histological assessment or measurement of myeloperoxidase in skin homogenates (Fig. 1 C and supporting information (SI) Fig. S1). Similarly, serum IgG titers...
against *S. aureus* were not significantly different in WT and *Nod2*^{-/-} mice (Fig. S2), suggesting that NOD2 is dispensable for normal induction of an adaptive immune response against the bacteria.

**Impaired Cytokine Response in NOD2-Deficient Mice.** We next asked whether differences in cytokine responses to *S. aureus* infection may account for the different pattern of disease progression observed in the absence of NOD2. Real-time PCR analysis of several key cytokines implicated in innate immune defense revealed that mRNA levels for IL-6 (Fig. 2A), C-X-C Ligand (CXCL) 1, CXCL2, and TNFα (Fig. S3) were markedly (≥5-fold) reduced in the skin of *Nod2*^{-/-} mice compared with WT mice 4 days after s.c. infection with *S. aureus*. Analysis of IL-6 in skin homogenates showed significantly decreased levels in *Nod2*^{-/-} mice early after infection (Fig. 2A). In parallel, serum IL-6 levels were lower in *Nod2*^{-/-} mice 8 h after s.c. infection and 4 h after a systemic challenge (Fig. 2B). Furthermore, ex vivo incubation of freshly isolated skin tissue with *S. aureus* revealed an almost complete loss of IL-6 induction in skin from *Nod2*^{-/-} mice compared with WT mice (Fig. 2C), indicating that NOD2-dependent recognition by resident skin cells is critical for the innate IL-6 response to *S. aureus*. Keratinocytes can secrete IL-6 after *S. aureus* infection (14). Furthermore, bone marrow-derived macrophages from *Nod2*^{-/-} mice released significantly less IL-6 after 24-h incubation with *S. aureus* than did cells from WT mice (Fig. 2C), indicating that macrophages also can be important IL-6 producers in a NOD2-dependent manner. In contrast, no significant differences in TNFα were found in skin homogenates of the different genotypes in the first 4 days after infection (Fig. S3).

**Pore-Forming α-Toxin Facilitates NOD2-Dependent Recognition of *S. aureus* and Muramyl Dipeptide** Based on the observation that IL-6 induction was a NOD2-dependent innate response to *S. aureus*, we next turned to the question of how the cytoplasmic sensor NOD2 can contribute to recognition of *S. aureus*. HEK293T cells were transfected with NOD2 cDNA and an NF-κB-dependent luciferase reporter, because IL-6 is an NF-κB target gene. Infected cell cultures with *S. aureus* stimulated NF-κB activity in a NOD2-dependent manner, whereas infection with a commensal member of the skin microflora, *Staphylococcus epidermidis*, had minimal impact on NF-κB activity (Fig. 3A). Consistent with the increase in NF-κB activity in transfected HEK293T cells, the expression of the 2 prototypic NF-κB target genes, IL-6 and IL-8, were increased after *S. aureus* infection of THP-1 monocytes (Fig. 3B and C), which express NOD2 consti-
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Fig. 3. Role of α-hemolysin in NOD2-mediated cell responses to S. aureus and MDP. (A) NOD2-transfected HEK293T cells were incubated for 8 h with WT or α-hemolysin-deficient (ΔS) S. aureus or with S. epidermidis (S. epi) at a MOI of 5. NF-κB induction was determined by luciferase reporter assay and is expressed as ratio relative to uninfected controls. Data from 1 representative of 4 independent experiments are shown as mean (SD); n = 3. (B and C) Human THP1 monocytes were incubated for 24 h with WT (closed bars) or Δhla (open bar) S. aureus, and IL-8 and IL-6 levels in the supernatants were assayed by ELISA. Data shown are mean (SE), n = 6, of 3 independent experiments. (D) THP1 cells were incubated for 24 h with S. epidermidis at the indicated MOI in the presence (+ HL, closed bar) or absence (– HL, open bar) of staphylococcal α-hemolysin (100 ng/mL). Data are mean (SE), n ≥ 6, of 3 independent experiments. (E) THP1 cells were incubated for 24 h with WT or Δhla S. aureus (S.a.) at a MOI of 2 in the presence or absence of α-hemolysin (HL) (100 ng/mL). Data are mean (SD) of 1 representative of 4 independent experiments; n = 3. (F) THP1 cells were treated for 8 h with the indicated combinations of active MDP isomer (MDP-LD, 1 μg/mL), inactive MDP isomer (MDP-DD, 10 μg/mL), and α-hemolysin (HL, 100 ng/mL). IL-8 levels in the supernatants were determined by ELISA. Data are mean (SE) of 3 independent experiments. *, P < 0.05.

Importance of α-hemolysin in the early host response to S. aureus infection. (A) Skin lesions were measured after 1 day. Cfu in skin homogenates were determined on day 4. (B) IL-6 levels were determined by ELISA in skin homogenates after 4 days or in serum after 4 h. All data are mean (SE) of 3 or 4 independent experiments. *, P < 0.05; ns, not significant.

Interdependence of NOD2 and α-Toxin for Cutaneous Defense Against S. aureus. Given the importance of α-hemolysin in facilitating cytokine induction in response to S. aureus in vitro, we next determined its physiologic role in early innate defense against the bacteria in vivo. Infection of WT mice with the Δhla mutant caused smaller acute lesions (day 1), resembling those seen after infection of Nod2−/− mice with WT S. aureus (Fig. 4). Furthermore, on day 4 bacterial numbers in the skin were significantly higher in WT mice infected with Δhla than in mice infected with WT S. aureus (Fig. 4A), again reminiscent of the findings in Nod2−/− mice infected with WT bacteria (Fig. 1B). Consistent with the in vitro data, the Δhla mutant also induced significantly lower IL-6 levels in the serum and in the skin of WT mice after s.c. infection (Fig. 4B). The attenuation in IL-6 induction after infection of WT mice with the mutant was comparable to that observed after infection of Nod2−/− mice with the WT and Δhla mutant of S. aureus, underlining the interdependence of NOD2 and α-hemolysin in innate activation of host defense in vivo. Together, these results indicate that α-hemolysin-dependent recognition by NOD2 is important for inducing an early innate inflammatory response to cutaneous infection with S. aureus.

NOD2-Dependent Release of IL-1β Promotes IL-6 Response to S. aureus. To understand better the host factors that govern NOD2-dependent IL-6 induction, we focused on the role of IL-1β, a potent proinflammatory mediator that can be released upon NOD2 activation (15). Incubation of transfected HEK293T cells with WT S. aureus resulted in the release of mature, bioactive IL-1β in a NOD2-dependent manner (Fig. 5A and Fig. S4). The α-hemolysin-deficient S. aureus mutant (Δhla) did not stimulate IL-1β secretion, further supporting the conclusion that the α-hemolysin of S. aureus is required for NOD2 activation upon S. aureus infection.

In confirmation of the in vitro findings, Nod2−/− mice displayed decreased IL-1β mRNA expression and IL-1β levels in skin homogenates after s.c. infection with WT S. aureus (Fig. 5B). Importantly, IL-1β was a strong amplifier of the IL-6 response to S. aureus, because IL-6 levels were markedly attenuated in skin homogenates of S. aureus-infected mice deficient.
Skin

**HEK293T cells were transfected with expression plasmids for NOD2, pro-IL-1β, and caspase-1 and were incubated for 8 h with WT or α-hemolysin-deficient (Δ) S. aureus at a MOI of 5. Secretion of IL-1β into the supernatants was determined by ELISA. Data are mean (SD) from 1 representative of 3 independent experiments. (A and C) Nod2−/−, IL-1R−/−, Caspase-1−/−, and WT mice were infected s.c. with 5 × 10⁵ cfu of WT S. aureus. IL-1β mRNA levels (normalized to GAPDH) in the infected skin were determined by real-time PCR and are expressed relative to the levels in uninfected WT mice (n = 5/group). IL-1β and IL-6 levels were assayed by ELISA in skin homogenates (n = 5/group) or serum (n = 5/group). All data are mean (SE); *, P < 0.05.

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**IL-6-Deficient Mice Mimic the Cutaneous Phenotype of NOD2-Deficient Mice upon S. aureus Infection.** Because IL-6 was markedly attenuated in Nod2−/− mice after S. aureus infection, we next determined the function of this cytokine in host defense against the bacteria. Mice deficient for IL-6 (Il6−/−) were infected s.c. with S. aureus and examined for lesion development and bacterial load. Similar to Nod2−/− mice, Il6−/− mice showed delayed appearance of ulcerated skin lesions relative to WT mice (Fig. 6A). In parallel, Il6−/− mice had significantly increased bacterial numbers in the skin, indicating that they could not control the infection normally (Fig. 6A). Thus, loss of NOD2 or IL-6 caused a similar phenotype in acute skin defense against S. aureus, suggesting that IL-6 may, at least in part, mediate the functions of NOD2 in this model.

**NOD2 Mediates Its Effects Through IL-6 Secretion and Neutrophil Activation.** To explain how IL-6 can contribute to antibacterial defense, we tested its effect on neutrophil killing of S. aureus. The addition of IL-6 to neutrophils freshly obtained from the bone marrow of either WT or Nod2−/− mice significantly enhanced bacterial killing within 30 min (Fig. 6B). This activity may be related to the ability of IL-6 to stimulate intracellular calcium fluxes in neutrophils, leading to the release of bactericidal oxygen radicals (16), and to induce inducible NOS expression in phagocytes (17). No difference in S. aureus killing was observed by unstimulated Nod2−/− vs. WT neutrophils. Thus, although neutrophils express NOD2 constitutively, these data suggest that NOD2 activity is not simply intrinsic to the sensing neutrophil but rather triggers a paracrine mechanism of IL-6 secretion and activation of accumulating neutrophils at the site of infection.

Finally, to validate pharmacologically the role of IL-6 in the host defense defect observed in Nod2−/− mice, we infected the mice s.c. with S. aureus and treated them daily with recombinant IL-6 by local s.c. injection. IL-6 treatment of Nod2−/− mice completely reversed the bacterial clearance defect in the mice, with bacterial numbers that were comparable to those in untreated WT mice (Fig. 6C). Thus, IL-6 complementation could functionally overcome the loss of NOD2, strongly suggesting that IL-6 is responsible for mediating the activity of NOD2 in cutaneous defense against S. aureus.

**Discussion**

The data we report significantly broaden the physiological importance of the cytoplasmic microbial sensor NOD2, from the limited recognition of bacteria, such as L. monocytogenes, that for the critical IL-1β processing enzyme, caspase-1 (Fig. 5C). Furthermore, decreased serum IL-6 levels were observed in mice deficient for caspase-1 or IL-1 receptor 4 h after s.c. infection with S. aureus (Fig. 5C). In contrast, IL-1β levels in skin homogenates were not affected significantly by IL-6 deficiency [mean (SD), 18.2 (4.2) ng in IL-6-deficient mice vs. 10.2 (4.8) ng in control mice; n = 3, P = not significant]. Together, these results indicate that IL-1β plays a critical role in promoting the IL-6 response to S. aureus infection.

**Fig. 5.** IL-1β mediates induction of IL-6 upon infection with S. aureus. (A) HEK293T cells were transfected with expression plasmids for NOD2, pro-IL-1β, and caspase-1 and were incubated for 8 h with WT or α-hemolysin-deficient (Δ) S. aureus at a MOI of 5. Secretion of IL-1β into the supernatants was determined by ELISA. Data are mean (SD) from 1 representative of 3 independent experiments. (B and C) Nod2−/−, IL-1R−/−, Caspase-1−/−, and WT mice were infected s.c. with 5 × 10⁵ cfu of WT S. aureus. IL-1β mRNA levels (normalized to GAPDH) in the infected skin were determined by real-time PCR and are expressed relative to the levels in uninfected WT mice (n = 5/group). IL-1β and IL-6 levels were assayed by ELISA in skin homogenates (n = 5/group) or serum (n = 5/group). All data are mean (SE); *, P < 0.05.

**Fig. 6.** Contribution of IL-6 to antibacterial defense. (A) Il6−/− mice (open circles) and WT mice (filled circles) were infected s.c. with 5 × 10⁵ cfu of WT S. aureus and were examined daily for lesion sizes and for bacterial load on day 4. Data are mean (SD); n = 3/group. A representative photograph of the lesions on day 2 is shown in the inset. (B) Freshly prepared bone marrow neutrophils from Nod2−/− mice (open bars) and WT mice (closed bars) were incubated with S. aureus (MOI = 1) with and without IL-6 (50 ng/mL) for 30 min, and surviving bacteria were determined by cfu assay. Data are mean (SE) of 3 independent experiments. (C) Nod2−/− mice (open bar) and WT mice (closed bar) were infected s.c. with 5 × 10⁵ cfu of WT S. aureus. Data are mean (SE); n = 4/group. The indicated group of Nod2−/− mice was treated once daily s.c. with 400 ng of recombinant IL-6. *, P < 0.05.
cytoplasmic access of whole bacteria, because it is required for
neutrophils of patients with the disease (30, 31).

IL-1 (25).

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amplifiers of the NOD2-initiated innate immune response. In
results show that caspase-1 and IL-1 are important physiologic

increased expression of its target genes (12), and it activates
caspase-1 and thereby promotes the release of mature IL-1β
(15).

The relative importance of these 2 pathways for mediating the
physiologic functions of NOD2 is poorly understood. Our
results show that caspase-1 and IL-1 are important physiologic
amplifiers of the NOD2-initiated innate immune response.
In the absence of this amplification pathway, the NOD2-dependent
IL-6 response to S. aureus was diminished, and host defense was
compromised. Furthermore, our data suggest a new mechanism
by which IL-1β exerts its acute host defense function against
S. aureus (23), identifying IL-6 as an important downstream
mediator of IL-1 signaling. IL-6, in turn, can activate the
killing capacity of neutrophils as critical early effectors against
the bacteria (24), a function that may be further augmented by
IL-1β (25).

Mutations of NOD2 are associated with the development of
several clinically important chronic inflammatory diseases, most
notably intestinal Crohn’s disease (26, 27). The respective NOD2
mutants are widely believed to exhibit loss-of-function (28, 29),
but a general explanation for how such loss promotes inflam-
matory responses has not yet emerged. Our results suggest a
model mechanism by which loss of NOD2 function exacerbates
local inflammation. Delayed and ineffective recognition of a
localized bacterial infection in the absence of NOD2 can lead to
an increase in bacterial load and secondarily to a delayed but
more severe local inflammatory response. In this model, NOD2
acts indirectly through local production of factors, such as IL-6,
which stimulate innate immune defense, particularly the ability
of neutrophils to kill bacteria effectively. These concepts may
have implications for understanding the mechanisms by which
mutation NOD2 promotes Crohn’s disease, where intestinal bac-
teria are considered to be a key driving force in disease patho-
genesis, and increased survival of S. aureus has been reported in
neutrophils of patients with the disease (30, 31).

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5. Lien E, et al. (1999) Toll-like receptor 2 functions as a pattern recognition receptor for

Materials and Methods

Bacterial Strains. S. aureus (ATCC 29213) and S. epidermidis (ATCC 12228) were
obtained from the American Type Culture Collection (ATCC). S. aureus 8325 K
and the Δhla mutant DK 1090 were kindly provided by A. Cheung (Dartmouth
Medical School) and A. Bayer (UCLA).

Mouse Infection Model. The murine model of necrotizing skin infection has been
described elsewhere (32, 33). Briefly, log-phase S. aureus were resus-
cinded in PBS, mixed 1:1 with sterile Cytodex beads (Sigma), and an inoculum of
5 × 10^7 cfu of S. aureus was injected s.c. into the flank of 6- to 12-week-old
mice. Nod2−/− mice, Il6−/− mice, Caspase-1−/− mice, and Il-1R−/− mice have
been described previously (34–37). CS7Bl/6J mice were used as WT controls.
For IL-6 treatment, 400 ng of human recombinant IL-6 per mouse was injected
s.c. daily for 4 days. Lesion size was monitored daily. Mice were killed at
different times after infection, and the affected skin regions were fixed in
Bouin’s solution and examined on H&E-stained paraffin sections. In parallel,
skin homogenates were prepared and used to determine bacterial numbers by
cfu assay, cytokine levels by ELISA, and mRNA expression by real-time PCR.
Real-time PCR was performed as previously described (38). Primer sequences
are available upon request. Myeloperoxidase activity was determined by
enzymatic assay as previously described (38).

Neutrophil-Killing Assays. Neutrophils were isolated by density gradient cen-
trifugation from single-cell suspensions of the bone marrow of Nod2−/− and
Nod2+/+ mice. Log-phase bacteria were mixed with neutrophils at a 1:1 ratio
in the absence or presence of 50 ng/ml human recombinant IL-6. Cultures
were incubated for 30 min at 37 °C, and surviving bacteria were determined
cytochemically by cfu assay.

Dual Luciferase Assay. HEK293T cells were grown in 12-well plates in low-
glucose DMEM supplemented with 10% FBS and 10 mM Hepes. Subconfluent
cells were transfected with the aid of FUGENE 6 reagent (Roche) with 1 ng
NOD2 expression plasmid (WT hNOD2 DNA in a pBKVCMV backbone), 10 ng
NF-κB-dependent firefly luciferase reporter plasmid (pNFκB-luc, Stratagene),
10 ng pRL SV40 Renilla luciferase reporter plasmid (Promega), 50 ng each of
expression plasmids for caspase-1 and pro-IL-1β, and pBluescript II KS (Strat-
agene) to adjust for DNA content (250 ng total DNA/well). Luciferase activity
was assayed with a Dual Luciferase reporter assay (Promega). Results are
expressed as relative induction of stimulated compared with untreated
controls, normalized for Renilla luminescence.

Infection Studies. THP-1 cells, grown in RPMI medium (Cellgro) supplemented
with 10% FBS, 2 mM glutamine, 10 mM Hepes, 1 mM sodium pyruvate, and 50
μM β-mercaptoethanol, were co-cultured with live bacteria at different mul-
tiplicities of infection (MOIs) as well as combinations of MDP (either active
MDP or inactive MDP-DD [InvivoGen]) and NF-κB. CFU assay, cytokine levels by
ELISA, and mRNA expression by real-time PCR. Differences between groups
were evaluated by Mann–Whitney rank sum test or Student’s
P < 0.05 was considered as significant.

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