Critical Role of NOD2 in Regulating the Immune Response to *Staphylococcus aureus*[▽]

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NOD2 (the nucleotide-binding oligomerization domain containing protein 2) is known to be involved in host recognition of bacteria, although its role in the host response to *Staphylococcus aureus* infection is unknown. NOD2-deficient (*Nod2*^{−/−}) mice and wild-type (WT) littermate controls were injected intraperitoneally with *S. aureus* suspension (10⁷ bacteria/g of body weight), and their survival was monitored. Cultured bone marrow-derived neutrophils were harvested from *Nod2*^{−/−} and WT mice and tested for cytokine production and phagocytosis. Compared to WT mice, *Nod2*^{−/−} mice were significantly more susceptible to *S. aureus* infection (median survival of 1.5 days versus >5 days; *P* = 0.003) and had a significantly higher bacterial tissue burden. Cultured bone marrow-derived neutrophils from *Nod2*^{−/−} and WT mice had similar levels of peritoneal neutrophil recruitment and intracellular killing, but bone marrow-derived neutrophils from *Nod2*^{−/−} mice had significantly reduced ability to internalize fluorescein-labeled *S. aureus*. *Nod2*^{−/−} mice had significantly higher levels of Th1-derived cytokines in serum (tumor necrosis factor alpha, gamma interferon, and interleukin-2 [IL-2]) compared to WT mice, whereas the levels of Th2-derived cytokines (IL-1β, IL-4, IL-6, and IL-10) were similar in *Nod2*^{−/−} and WT mice. Thus, mice deficient in NOD2 are more susceptible to *S. aureus*. Increased susceptibility is due in part to defective neutrophil phagocytosis, elevated serum levels of Th1 cytokines, and a higher bacterial tissue burden.

Staphylococcus aureus infection in humans is a serious, common, and costly medical concern. *S. aureus* is cleared from the bloodstream via phagocytic killing by the effector cells of the innate immune system, neutrophils, and macrophages and by opsonizing antibodies directed against capsule and peptidoglycan (17, 28). Some *S. aureus* escape killing and subsequently replicate in infected tissues, which generates a proinflammatory response mediated by the release of cytokines and chemokines from macrophages, neutrophils, and other cells of innate immune systems (33). Thus, the proper innate immune response limits the establishment of infectious foci and decreases the severity of *S. aureus* infections.

The innate immune response must recognize the *S. aureus* and respond to limit the infection. While Toll-like receptor 2 (TLR2) is known to be an essential extracellular receptor of *S. aureus* (32), additional host proteins are also likely to play key roles in host recognition of this pathogen (18). NOD2 (the nucleotide-binding oligomerization domain-containing protein 2) is a potential intracellular sensor for a variety of pathogens and microbe associated molecular patterns (14, 19, 34), including *Listeria monocytogenes* (20), *Mycobacterium tuberculosis* (2, 7), and *Bacillus anthracis* (13). NOD2 has also been proposed to serve as a potential intracellular sensor for *S. aureus*, possibly by recognition of peptidoglycan fragments from intracellular bacteria (10).

Polymorphisms within the gene for NOD2 may influence its putative role in innate immunity and inflammation. For exam-

ple, a frameshift mutation in the NOD2 gene is associated with Crohn's disease, an autoimmune disease associated with severe inflammation of the bowel mucosa (14, 27). Evidence suggests that lack of bacterial sensing by the mutant NOD2 in individuals with Crohn's disease contributes to the pathology of disease (36, 39). A loss of surveillance activity by NOD2 may result in the inability of local responses in the intestinal mucosa to control bacterial infection, thereby initiating systemic responses and leading to aberrant inflammation.

The role of NOD2 in *S. aureus* infection is unclear. We sought to understand the role of this cytosolic sensor protein in *S. aureus* infection by using mice deficient in NOD2 (*Nod2*^{−/−} mice). Interestingly, unlike *Nod2*^{−/−} mice intraperitoneally (i.p.) challenged with another gram-positive bacterium, *Listeria monocytogenes* (19), *S. aureus*-challenged *Nod2*^{−/−} mice were more susceptible to infection than the wild-type (WT) control mice. The increased susceptibility was associated with higher bacterial load in the various organs, defective neutrophil phagocytosis, and an exaggerated TH-1 derived cytokine response after *S. aureus* infection.

MATERIALS AND METHODS

S. aureus strains Sanger 476 and MW2 were used in mortality studies. An overnight bacterial culture of *S. aureus* was diluted with fresh tryptic soy broth and incubated (37°C) with aeration to log-phase growth (i.e., an optical density at 600 nm of ~1.0) (30). *S. aureus* was harvested by centrifugation, rinsed, and resuspended in saline. To mimic the disseminated *S. aureus* infection in humans, which typically arises from a primary focus of infection and spreads to other sites, we used an i.p. route of infection in our animal model (16).

Mice deficient in NOD2 (*Nod2*^{−/−}) or TLR2 (*Tlr2*^{−/−}) or WT littermate control mice were obtained from Jackson laboratories (Bar Harbor, ME) and housed in pathogen-free facilities. All animal studies were approved by the Institutional Animal Care and Use Committee. *Nod2*^{−/−}, *Tlr2*^{−/−}, and WT mice were injected i.p. with 10⁷ CFU/g and observed every 8 h for mortality. For some studies,

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FIG. 1. Increased susceptibility of mice deficient in NOD2 (*Nod2*^{-/-}) to *S. aureus* infection. (A) Survival curves for *Nod2*^{-/-}, *Tlr2*^{-/-}, and WT mice after *S. aureus* injection. *Nod2*^{-/-} and *Tlr2*^{-/-} mice were injected i.p. with *S. aureus* strain Sanger 476 (10⁷ CFU/g) and monitored for survival every 8 h (*n* = 8). (B) Survival curves for *Nod2*^{-/-} and WT mice after *S. aureus* injection. *Nod2*^{-/-} and WT control *Nod2*^{+/+} mice were injected i.p. with *S. aureus* strain MW2 or Sanger 476 (10⁷ CFU/g) and monitored for survival every 8 h (*n* = 8).

mice euthanized at 6, 12, 18, and 24 h after injection. Blood was collected by intracardiac puncture. Peritoneal lavage was performed as previously described (1).

Bacterial load and leukocyte counts. Kidneys, spleens, and mesenteric lymph nodes were harvested and either frozen (-80°C), fixed in 10% buffered formalin for subsequent analysis, or homogenized in phosphate-buffered saline (PBS) and diluted 10-fold serially. Heparinized blood or peritoneal lavage (50 µl) was serially diluted in PBS. The serial dilutions (50 µl) were plated in tryptic soy agar plates and incubated (37°C, overnight) to count the number of CFU of *S. aureus*. The total number of white blood cells in the peritoneal fluid and peripheral blood was determined by using a hemacytometer. Leukocytes were typed by evaluating a Wright's stained smear of blood and peritoneal fluid.

Isolation of bone marrow-derived neutrophils. Bone marrow neutrophils were isolated as described previously (5). Briefly, the femurs and tibias were removed and stripped of all muscle. The marrow was flushed from each bone with Hanks balanced salt solution, and the cell aggregates were disrupted by trituration to generate a unicellular suspension. The cells were pelleted, and erythrocytes were removed by hypotonic lysis. Neutrophils were separated from the remaining cells by centrifugation over discontinuous Percoll (Amersham Biosciences) gradients at 500 × *g* for 30 min at 4°C, consisting of 55% (vol/vol), 65% (vol/vol), and 75% (vol/vol) Percoll in PBS. Neutrophils were recovered at the interface of the 65 and 75% fractions and were >90% pure and >95% viable in the neutrophil-rich fraction as determined by Wright-Giemsa staining (6) and cultured (37°C, 10% CO₂) in RPMI 1640 supplemented with 10% fetal calf serum (both from the American Type Culture Collection).

Phagocytosis assay. Phagocytosis of heat-killed fluorescein isothiocyanate-labeled *S. aureus* (Molecular Probes) was evaluated as described previously (29). Briefly, naive bone marrow-derived neutrophils or peritoneal macrophages were plated in 96-well plates at 2 × 10⁴ cells/well. The cells were incubated over ice (4°C, 20 min) with 2 × 10⁵ bacteria and then further incubated (37°C, 30 min) to allow internalization. Cells were placed on ice to stop internalization and were maintained on ice for the remainder of the assay. Total cell-associated fluorescence (binding and uptake) was measured by flow cytometry (10,000 events) using the CellQuest program (Becton Dickinson). Bacterial internalization was measured by quenching the extracellular fluorescence with 0.2% trypan blue shortly before analysis. Binding was calculated as the difference between total cell-associated fluorescence and intracellular fluorescence. The amount of phagocytosis (phagocytic index) was expressed as the percentage of fluorescence-positive cells multiplied by the mean fluorescence of these cells.

Microscopy. Bone marrow-derived neutrophils were plated (10⁴) on glass coverslips and incubated over ice (4°C, 20 min) with heat-killed fluorescein isothiocyanate-labeled *S. aureus* or zymosan (10⁵ bacteria or zymosan particles). The cells were further incubated (37°C, 30 min) to allow internalization. The cells were washed three times with PBS, fixed using formaldehyde and mounted with Vectashield fluorescent mounting medium (Vector Laboratories). Slides were imaged on an inverted fluorescence microscope (TE 2000-U; Nikon) equipped with a Nikon camera using Openlab software (version 4.0.2; original magnification, ×40; NA 0.60) at room temperature.

Measurement of cytokine production. Serum was separated from blood obtained by intracardiac puncture. Protein concentrations were determined by

bicinchoninic acid method kit (Pierce). Equal amounts of protein for each sample were used to measure the levels of interleukin-1β (IL-1β), IL-2, IL-4, IL-6, IL-10, tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), macrophage chemotactic peptide, and macrophage inhibitory peptide were determined by enzyme-linked immunosorbent assay (Duo kit; Invitrogen) according to the manufacturer's instructions. The serum levels of cytokines were expressed as ng or pg per ml of serum.

Measurement of reactive oxygen radical production. Bone marrow-derived neutrophils were plated at 10⁵ cells/well in 24-well tissue culture plates (Becton Dickinson) in PBS and incubated (37°C, 60 min) with 10⁶ CFU/well of *S. aureus* (Sanger 476). Production of reactive oxygen radicals was measured by a commercially available assay (Luminox; Stratagene) according to the manufacturer's instructions.

Statistical analysis. Values are expressed as means ± the standard errors of the means (SEM) and median values, as appropriate. Statistical analyses were performed using a Student *t* test, the nonparametric Mann-Whitney U test, or the Wilcoxon signed-rank test. A *P* value of <0.05 was considered significant.

RESULTS

Mice deficient in NOD2 are susceptible to *S. aureus*. We evaluated the importance of NOD2 and TLR2 in host response to *S. aureus* using Sanger 476 (a methicillin-susceptible *S. aureus* strain) and MW2 (a methicillin-resistant *S. aureus* strain). *Nod2*^{-/-} mice were more susceptible (*P* < 0.05) to *S. aureus* (Sanger 476; median survival, 24 h) compared to *Tlr2*^{-/-} mice (median survival, 66 h) (Fig. 1A). Furthermore, the susceptibility of *Nod2*^{-/-} mice to *S. aureus* compared to WT control mice was consistent when injected with either Sanger 476 (median survival, 24 h versus >120 h; *P* = 0.0043) or MW2 (median survival, 32 h versus >120 h; *P* = 0.0039) (Fig. 1B).

To evaluate correlates for increased mortality in *Nod2*^{-/-} mice, we analyzed the extent of tissue invasion of *S. aureus* after infection. The animals were euthanized at 6, 12, and 18 h, and bacterial loads were determined in the kidney, mesenteric lymph nodes, spleen, peripheral blood, and peritoneal fluid. The bacterial load in these tissues increased over these time points in the *Nod2*^{-/-} mice and were significantly higher at 18 h in the kidney (0.071 × 10³ CFU/g in WT mice versus 3.57 × 10³ CFU/g in *Nod2*^{-/-} mice), peritoneal fluid (3.4 × 10⁹ CFU/ml in WT mice versus 35.21 × 10⁹ CFU/ml in *Nod2*^{-/-} mice), blood (33 × 10⁴ CFU/ml in WT mice versus 309 × 10⁴ CFU/ml in *Nod2*^{-/-} mice), and mesenteric lymph

FIG. 2. Increased bacterial load in mice deficient in NOD2 (*Nod2*^{-/-}) after *S. aureus* infection. (A to E) Bacterial loads in various organs of *Nod2*^{-/-} and WT mice after *S. aureus* injection. *Nod2*^{-/-} and WT control were injected i.p. with *S. aureus* strain Sanger 476 (10⁷ CFU/g), and the bacterial loads in the kidneys, mesenteric lymph nodes, spleens, and the peritoneal fluid were determined at 0, 6, 12, and 18 h. The results are expressed as means $\pm$ the SEM. *, Significantly different from the bacterial load at the respective time points in *Nod2*^{-/-} mice compared to WT mice ($P \leq 0.05$, $n = 5$).

nodes (4.8 CFU/g in WT mice versus 31 CFU/g in *Nod2*^{-/-} mice). (Fig. 2A to D).

Defective neutrophil function in mice lacking NOD2. The ability of neutrophils from *Nod2*^{-/-} mice to perform each step associated with neutrophil function, i.e., recruitment to the infection site, detection and phagocytosis of *S. aureus*, and intracellular killing by oxidative burst pathway components, was evaluated. The leukocyte count in the peripheral blood (Fig. 3A) was significantly higher in *Nod2*^{-/-} mice at 6, 12, and 18 h postinfection ($P < 0.005$) than in WT mice. The neutrophil count in the peritoneal fluid (Fig. 3B) was also significantly higher in *Nod2*^{-/-} mice at 12 and 18 h postinfection ($P < 0.005$) than in WT mice.

The ability of neutrophils from *Nod2*^{-/-} mice to phagocytose *S. aureus* was assessed. Naive bone marrow-derived neutrophils from *Nod2*^{-/-} and control mice were incubated with fluorescein-labeled *S. aureus*. The neutrophils from WT control mice had higher number of internalized *S. aureus* than the neutrophils from *Nod2*^{-/-} mice (Fig. 3C). To quantitatively assess phagocytosis, we determined the ability of naive bone marrow-derived neutrophils or bone marrow-derived macrophages from *Nod2*^{-/-} mice and control mice to phagocytose Alexa 344-labeled *S. aureus* in the presence or absence of an opsonizing agent. The phagocytic index was significantly lower ($P \leq 0.05$) in the neutrophils or the macrophages ($P \leq 0.05$) from *Nod2*^{-/-} mice than in the control mice when incubated with Alexa 344-labeled *S. aureus*, indicating defective phagocytic ability of neutrophils and macrophages from *Nod2*^{-/-} mice (Fig. 3D). Thus, neutrophils from *Nod2*^{-/-} mice have a reduced ability to phagocytose *S. aureus*.

The internalized *S. aureus* in the phagosome fuses with

the lysosome to form the phagolysosome. This is followed by production of reactive oxygen intermediates by the activity of nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase. We measured the ability of neutrophils from *Nod2*^{-/-} mice and WT control mice to produce oxygen radicals by NADPH monitoring. The level of reactive oxygen radicals increased over 60 min after incubating the bone marrow-derived neutrophils from *Nod2*^{-/-} and WT mice with *S. aureus*. There was no significant difference ($P = 0.091$) in the level of reactive oxygen radical production between *Nod2*^{-/-} mice [(46.52 $\pm$ 3.33)-fold] and WT mice [(39.53 $\pm$ 6.15)-fold] (Fig. 3E).

Altered inflammatory cytokine and chemokine response in *Nod2*^{-/-} mice. We analyzed the production of several Th1 and Th2 derived cytokines in serum of *Nod2*^{-/-} or control mice after *S. aureus* infection. The levels of Th1-derived cytokines (TNF- α , IFN- γ , and IL-2) were significantly elevated in *Nod2*^{-/-} mice compared to WT controls [TNF- α , (3.21 $\pm$ 0.7)-fold; IFN- γ , (2.79 $\pm$ 0.73)-fold; IL-2, (2.60 $\pm$ 0.14)-fold] (Fig. 4). In contrast, the levels of Th2-derived cytokines (IL- β , IL-4, IL-6, and IL-10) did not differ significantly in *Nod2*^{-/-} and WT control mice.

DISCUSSION

The human protein NOD2 plays a key role in host innate immunity by recognizing specific microbial components in the cytosol (14). Mutations in the *NOD2* gene have been associated with susceptibility to Crohn's disease (24), Blau's syndrome (23), early-onset sarcoidosis (16), graft versus host disease (12), tuberculosis (2), and *Bacillus anthracis* infection

FIG. 3. Altered neutrophil function in mice deficient in NOD2 protein (*Nod2*^{-/-}) after *S. aureus* infection. (A and B) Decreased neutrophil recruitment in peripheral blood and peritoneal fluid of mice deficient in *Nod2*^{-/-} mice after *S. aureus* infection. *Nod2*^{-/-} and WT mice were injected i.p. with *S. aureus* (10⁷ CFU/g), and the numbers of neutrophils in peritoneal fluid and blood were determined at 0, 6, 12, and 18 h. The results are expressed as means $\pm$ the SEM. *, Significantly different from the neutrophil count in *Nod2*^{-/-} mice compared to WT mice at the respective time points ($P \leq 0.05$, $n = 5$). (C) Decreased phagocytosis of fluorescein-labeled *S. aureus* in bone marrow derived neutrophils from *Nod2*^{-/-} and WT mice. Bone marrow-derived neutrophils from *Nod2*^{-/-} and WT mice were treated with 10⁶ fluorescein-labeled *S. aureus* (10⁶ CFU/ml). Phagocytosed bacteria were visualized by fluorescence microscopy. Representative images (magnification, $\times 400$) of the results from two separate experiments are shown. (D) Bone marrow-derived neutrophils or bone marrow-derived macrophages from *Nod2*^{-/-} and WT mice were treated with 10⁶ Alexa 344-labeled *S. aureus* or zymosan. The total cell-associated fluorescence (binding and uptake) was measured by flow cytometry. The phagocytic index was expressed as the percentage of fluorescence-positive cells multiplied by the mean fluorescence of these cells. The results are expressed as means $\pm$ the SEM ($P \leq 0.05$, $n = 5$). (E) Reactive oxygen species production by naive neutrophils from *Nod2*^{-/-} and WT mice. Naive bone marrow-derived neutrophils from *Nod2*^{-/-} and WT mice were treated with heat-killed *S. aureus* (10⁷ CFU), and reactive oxygen species production was measured by a bioluminescence assay. The results are expressed as the fold increase in the production of reactive oxygen species over the control (untreated cells) at different time intervals. The results are expressed as means $\pm$ the SEM. *, Significantly different in *Nod2*^{-/-} mice compared to WT mice ($P \leq 0.05$, $n = 5$).

(13), The role of NOD2 in controlling and monitoring *S. aureus* infections is still under debate (15, 26). In the present study, we have shown that *Nod2*^{-/-} mice are more susceptible to *S. aureus* infection than *Tlr2*^{-/-} mice (Fig. 1A). Compared

to WT mice, the NOD2-deficient mice have defective neutrophil phagocytosis (Fig. 3), elevated serum levels of neutrophil derived cytokines (Fig. 4), and higher bacterial tissue burdens (Fig. 1).

FIG. 4. Altered Th1/Th2 cytokine response in mice deficient in NOD2 (*Nod2*^{-/-}) after *S. aureus* infection. (A to H) Altered serum Th1/Th2 cytokine response and chemokine levels in *Nod2*^{-/-} mice. *Nod2*^{-/-} and WT mice were injected i.p. with *S. aureus* (10^7 CFU/g), and blood was collected by intracardiac puncture at 18 h. The serum was separated, and the levels of IL-1β, IL-2, IL-4, IL-10, TNF-α, IFN-γ, macrophage chemotactic peptide (MCP), and macrophage inhibitory peptide (MIP) were determined by ELISA. The levels of Th1-derived cytokines (TNF-α, IFN-γ, and IL-2) were significantly elevated in *Nod2*^{-/-} mice compared to WT mice. Th2-derived cytokines (IL-1β, IL-4, IL-6, and IL-10) were not significantly different in *Nod2*^{-/-} mice compared to WT mice. The results are mean ± the SEM. *, Significantly different from control mice; †, significantly different in *Nod2*^{-/-} mice injected with *S. aureus* compared to WT mice ($P \leq 0.05$, $n = 6$).

Previous studies using TLR2-deficient mice have highlighted the importance of TLR2 in initiating the innate immune response toward *S. aureus* (11, 12, 15, 32). Knuefermann et al. found that, in TLR2-deficient mice sacrificed 12 h after *S. aureus* infection, the heart was protected against cardiac dysfunction compared to WT controls and that the myocardial tissue from TLR2-deficient mice expressed lower levels of TNF-α and IL-1β (18). Thus, depending on the infectious model, deficiency of TLR2 can be either protective or detrimental to the host organism, which suggested that the host innate immune response to *S. aureus* is complex and may

involve additional pattern recognition receptors. Similarly, macrophages and astrocytes from TLR2-deficient mice still produced inflammatory mediators when challenged by *S. aureus* (8, 23). In addition, induction of TNF-α synthesis by *S. aureus* cell wall components such as muramyl dipeptide is independent of TLR2 (37, 38). In the present study, we observed higher bacterial loads and increased mortality in *Nod2*^{-/-} mice (median survival, 24 h) compared to *Tlr2*^{-/-} mice (median survival, 66 h) after *S. aureus* infection. This result underscores the role of NOD2, in addition to TLR2, in regulating innate immune response to *S. aureus*. Taken together, these previous

reports and our current findings suggest that other pattern recognition receptors in addition to TLR2 are likely to be involved in regulating innate immune response to *S. aureus*. In the present investigation, we demonstrate that NOD2 is likely to be one of these additional receptors for *S. aureus* in particular and potentially gram-positive pathogens in general.

The results of this investigation suggest that the basis of susceptibility to *S. aureus* in NOD2-deficient mice involves defective phagocytosis by neutrophils and macrophages. Neutrophils from *Nod2*^{-/-} mice exhibited normal neutrophil recruitment, NADPH-dependent oxidative response, and *S. aureus* killing but defective phagocytosis, as assessed by both flow cytometry and fluorescence microscopy. It is unclear whether an inability to detect intracellular *S. aureus* in NOD2-deficient mice contributes to defective phagocytosis or if NOD2 regulates the process of phagocytosis by an unknown mechanism. Several studies have confirmed the association between NOD2 and different cytoskeletal proteins, including actin (22, 31). The cytoskeletal complexes driving the polymerization of branched actin filaments at bacterial invasion sites are similar to those recruited in membrane ruffles or in phagocytic pseudopodia (25). Since NOD2 is sequestered with actin in cytoskeletal structures such as lamellipodia and membrane ruffles in association with the small RhoGTPase, Rac1, they might play the role of a scaffold ensuring a close proximity between NOD2 and muramyl dipeptide at bacterial invasion sites (25). Thus, it is tempting to speculate that NOD2 may regulate phagocytosis through as-yet-unknown mechanism due to its association with the cytoskeletal machinery of the cell. Recent data by Kufer et al. demonstrating recruitment of NOD2 at the entry foci of *Shigella flexneri* in HeLa cells (21) support this hypothesis. An experiment to transfuse neutrophils from a WT mouse to rescue the susceptible *Nod2*^{-/-} mouse would provide a comprehensive evidence for the role of NOD2 in clearing *S. aureus*, and we plan to pursue this approach in future studies.

In addition, it is possible that NOD2-deficient mice have additional deficiencies in innate immunity that also contribute to the observed susceptibility to *S. aureus*. For example, *Nod2*^{-/-} mice have decreased expression of α -defensins within the intestinal mucosa, making them susceptible to pathogens crossing the intestinal barrier (19). However, the parenteral route of infection in our model makes gut mucosal α -defensins a less likely explanation for our findings.

Although the Th1-derived proinflammatory cytokines TNF- α , IFN- γ , and IL-2 were significantly elevated in *Nod2*^{-/-} mice compared to the controls, Th2-derived cytokines (IL- β , IL-4, IL-6, and IL-10) exhibited similar levels. The increase in Th1-derived cytokines in *Nod2*^{-/-} mice after *S. aureus* infection correlates well with increased levels of Th1 cytokines in many individuals with Crohn's disease (3). It is unclear whether the increase in Th1-derived cytokines is compensating for reduced phagocytosis. TLR2-induced activation of NF- κ B and subsequent production of Th1-derived cytokines TNF- α and IFN- γ is negatively regulated by the activation of NOD2 (35, 36). Thus, in the absence of this regulation, peptidoglycan from the *S. aureus* cell wall can elicit an excessive NF- κ B-dependent Th1-cell response by the effector cells of innate immune system (35).

Although increased serum levels of IL-2 and IFN- γ in the *Nod2*^{-/-} mice are unlikely to completely explain the increased

susceptibility, they may contribute in part to increased mortality of the *Nod2*^{-/-} mice, underscoring the complex nature of host immune response to *S. aureus*. Interestingly, an excessive production of proinflammatory cytokines can lead to multiple organ dysfunction syndrome and death (4). This excessive production of proinflammatory cytokines may be another potential contributing factor to the increased susceptibility to *S. aureus* exhibited by the NOD2-deficient mice.

It is tempting to speculate that NOD2 serves as a key receptor for *S. aureus* components in the intracellular environment in much the same way as TLR2 does in the extracellular compartment. Entry of *S. aureus* associated pattern recognition molecules into neutrophils and other effectors cells may represent a condition where the neutrophils effectively phagocytose the bacteria and activate cytosolic host defense. Delivery of *S. aureus* products into the neutrophils may serve as an "alarm signal" for host innate immunity, providing an additional mechanism by which to identify *S. aureus*.

In conclusion, NOD2 constitutes a primary intracellular microbial-recognition system that is parallel to the extracellular TLR microbial-recognition system. NOD2 is largely independent of the TLR system and, as such, can complement this system to potentiate the innate immune response (9). In the present investigation, we showed that mice deficient in NOD2 are more susceptible to *S. aureus* and that this increased susceptibility is due in part to defective neutrophil phagocytosis, elevated serum levels of neutrophil derived cytokines, and higher bacterial tissue burden. Further studies are under way to increase our understanding of this complex process.

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