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Cutting Edge: Myeloid Differentiation Factor 88 Is Essential for Pulmonary Host Defense against *Pseudomonas aeruginosa* but Not *Staphylococcus aureus*¹

Shawn J. Skerrett,²* H. Denny Liggitt, † Adeline M. Hajjar,‡ and Christopher B. Wilson‡

Myeloid differentiation factor 88 (MyD88) is an adapter molecule required for signal transduction via Toll-like receptors (TLRs) and receptors of the IL-1 family. Consequently, MyD88-deficient mice are highly susceptible to bacterial infections, including systemic infection with *Staphylococcus aureus*. To determine the role of MyD88 in innate immunity to bacterial pneumonia, we exposed MyD88-deficient and wild-type mice to aerosolized *Pseudomonas aeruginosa* or *S. aureus*. As predicted, MyD88-deficient mice failed to mount an early cytokine or inflammatory response or to control bacterial replication after infection with *P. aeruginosa*, which resulted in necrotizing pneumonia and death. By contrast, MyD88-deficient mice controlled *S. aureus* infection despite blunted local cytokine and inflammatory responses. Thus, whereas MyD88-dependent signaling is integral to the initiation of cytokine and inflammatory responses to both pathogens following infection of the lower respiratory tract, MyD88 is essential for innate immunity to *P. aeruginosa* but not *S. aureus*. The Journal of Immunology, 2004, 172: 3377–3381.

Toll-like receptors (TLRs)³ are a family of phylogenetically conserved transmembrane proteins that recognize diverse microbial patterns and signal the initiation of innate immune responses to infection (1). Individual TLRs differ in their ligand specificities and patterns of cellular distribution (1). In the case of bacterial infection, multiple TLRs may be involved in the activation of host defenses depending on the nature of the bacterium. Unique cellular distributions of individual TLRs may contribute to tissue-specific responses to particular microbial stimuli (1).

Ligand binding to TLRs leads to the transcription of proinflammatory cytokines and other genes involved in innate immunity through signaling cascades that involve myeloid differentiation factor 88 (MyD88) (1). Consequently, macrophages from mice lacking MyD88 do not produce proinflammatory cytokines in response to bacterial components that are known TLR ligands (1), although TLR4 and TLR3 can link with other adapter proteins to mediate signal transduction in the absence of MyD88 (1, 2). In addition to transducing most TLR-dependent signals, MyD88 is required for signaling via members of the IL-1 receptor family, including receptors for IL-1 and IL-18 (3).

Despite the critical functions of TLRs and MyD88 in activating cellular responses to microbial stimuli, relatively few studies have examined the roles of individual TLRs and MyD88 in response to live bacteria in vivo. Mice with targeted deletions of TLR2 are more susceptible to i.v. challenge with *Staphylococcus aureus* (4), but are not more susceptible to polymicrobial or *Listeria monocytogenes* peritonitis than wild-type mice (5, 6). Mice with mutations of TLR4 are less resistant to bacterial infection than wild-type controls in some models (7–10) but not others (5, 11, 12). Likewise, mice deficient in MyD88 are markedly susceptible to systemic challenge with *S. aureus* or *L. monocytogenes* (4, 6, 13, 14), but are partially protected from polymicrobial peritonitis, possibly because of attenuation of an injurious inflammatory response (5).

To determine the role of MyD88-dependent signaling in host defense against bacterial pneumonia, we compared the responses of MyD88-deficient and wild-type mice to pulmonary infection with *Pseudomonas aeruginosa* or *S. aureus*. *P. aeruginosa* is a Gram-negative bacterium that is an important cause of acute pneumonia in critically ill and immunocompromised individuals and also causes chronic airway infection in the setting of cystic fibrosis. *S. aureus* is a Gram-positive bacterium and a leading agent of nosocomial pneumonia. We found that MyD88-deficient responses were essential for effective lung defenses against *P. aeruginosa*. By contrast to the increased susceptibility of MyD88-deficient mice to pulmonary infection with *P. aeruginosa* and to systemic infection with *S. aureus* (4),
MyD88 was not required for control of lung infection with *S. aureus*.

Materials and Methods

**Mice**

MyD88-deficient (MyD88<sup>−/−</sup>) mice were kindly provided by Dr. S. Akira (Osaka, Japan) (3) and were backcrossed to C57BL/6 mice for six generations, then intercrossed to generate MyD88<sup>−/−</sup> mice. Controls were MyD88<sup>+/+</sup> mice of the same genetic background, with the exception of one experiment with *S. aureus* in which C57BL/6 mice bred in house were used as controls with similar results. Mice were housed under specific pathogen-free conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Washington (Seattle, WA).

Exposure to aerosolized bacteria and bacterial enumeration

The PAK strain of *P. aeruginosa*, a gift from S. Lory (Harvard University), and *S. aureus*, a human blood isolate from the microbiology laboratory of the Veterans Affairs Medical Center in Seattle, WA, were prepared as described previously (15). Mice were exposed to aerosolized bacteria in a whole-animal chamber for ~30 min, as previously described (15). Immediately, 4 h, and 24 h after infection, left lungs and spleens were homogenized in 1 ml of PBS, serially diluted, and quantitatively cultured on Luria-Bertani agar. Colonies were counted after 24- to 48-h incubation at 37°C.

Bronchoalveolar lavage (BAL) cell counts and scoring of cell-associated bacteria

BAL was performed as described elsewhere (15). The lavage fluid was centrifuged at 300 x g and supernatants were stored at ~80°C. The cell pellets were resuspended in RPMI 1640 containing 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT) and cells were counted in a hemacytometer. Differential counts, the proportion of cells harboring bacteria, and the number of bacteria per cell were determined after staining of cytospin specimens with a modified Wright-Giemsa technique (Diff-Quik, Dade Behring, Dudingen, Switzerland).

Histopathology

Right lungs were inflated in situ to ~20 cm of pressure with 4% paraformaldehyde, then removed and stored at 4°C in the same fixative. The tissue was embedded in paraffin, sectioned, and stained with H&E. A veterinary pathologist examined two to four sections from individual mice in a manner blinded to genotype and time after infection.

Measurement of total protein and cytokines

The total protein content of BAL fluid was measured using the bicinchoninic acid assay (Pierce, Rockford, IL). Immunoreactive murine TNF-α, IL-1β, macrophage inflammatory protein 2 (MIP-2), and KC were measured in BAL fluid and in lung homogenates by sandwich ELISA using Ab pairs and recombinant standards purchased from R&D Systems (Minneapolis, MN). Homogenized lung tissue was diluted 1/1 in lysis buffer containing 2X protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany). After a 30-min incubation on ice, the mixture was centrifuged at 1500 x g and supernatants were stored at ~80°C until assayed. The lower limits of detection were 10 pg/ml.

Data analysis

Data are expressed as mean ± SE. Statistical comparisons between the two groups of mice were made using Student’s *t* test. A *p* < 0.05 was considered to be significant.

Results

**Marked susceptibility of MyD88<sup>−/−</sup> mice to lung infection with *P. aeruginosa* but not *S. aureus***

There were dramatic differences in the outcome of the two groups of mice after inhalation of *P. aeruginosa*. Wild-type controls exhibited lethargy, piloerection, and labored breathing 4 h following aerosol administration of a high (~2 x 10<sup>8</sup> CFU/lung) or low (~5 x 10<sup>5</sup> CFU/lung) inoculum of *P. aeruginosa*. By 24 h, control mice effected a 10-fold reduction in the number of bacteria in their lungs and signs of illness largely resolved (Fig. 1A). No control mice died after either inoculum. Unlike controls, MyD88<sup>−/−</sup> mice showed no signs of illness at 4 h, but by 24 h after exposure to the low inoculum they were lethargic and in respiratory distress. *P. aeruginosa* replicated exponentially in the lungs of MyD88<sup>−/−</sup> mice, and greater numbers of bacteria also were recovered from their spleens (Fig. 1, A and C). The high inoculum was uniformly fatal for MyD88<sup>−/−</sup> mice within 24 h (data not shown). In sharp contrast to the results with *P. aeruginosa*, no mortality was observed in either group of mice after inhalation of a high inoculum of *S. aureus* (~3 x 10<sup>6</sup> CFU/lung). By 24 h, both control and MyD88<sup>−/−</sup> mice had achieved a net reduction in the number of viable *S. aureus* (Fig. 1, B and D). Thus, MyD88 is essential for the pulmonary containment of *P. aeruginosa* but not *S. aureus*.

**Impaired proinflammatory cytokine production and neutrophil recruitment in MyD88<sup>−/−</sup> mice**

Rapid production of proinflammatory cytokines and recruitment of neutrophils in response to *P. aeruginosa* were almost completely abolished in MyD88<sup>−/−</sup> mice, while the response to *S. aureus* was considerably blunted. In the case of *P. aeruginosa* pneumonia, 4-h lung homogenate concentrations of TNF-α in MyD88<sup>−/−</sup> mice (Fig. 2A) were not different from concentrations in uninfected MyD88<sup>−/−</sup> mice (48.0 ± 7.6 pg/ml) after either the low inoculum (Fig. 2A) or the high inoculum (data not shown). Levels of IL-1β, MIP-2, and KC in 4-h lung homogenates from MyD88<sup>−/−</sup> mice were measurably above the concentrations in uninfected animals (37.0 ± 2.7, 24.8 ± 4.5, and 47.6 ± 11.5 pg/ml, respectively), but dramatically lower than corresponding levels in lungs of infected wild-type mice. After inhalation of *S. aureus*, 4-h lung homogenate concentrations of each of these cytokines were significantly higher in MyD88<sup>−/−</sup> mice than in uninfected mice, although concentrations were still much lower than in infected controls (Fig. 2B). Similar results were obtained from cytokine assays of BAL fluid (data not shown).

The BAL cell populations of uninfected MyD88<sup>−/−</sup> and control mice did not differ in number (~0.2 x 10<sup>6</sup>/lung) or...
composition (>99% macrophages). However, neutrophils accounted for ~90% of the BAL cell population by 4 h of infection with either inoculum of *P. aeruginosa* in control mice, but were ≤1% of cells in MyD88−/− mice after infection with a low inoculum (Fig. 2C) and only 4.5 ± 0.3% of BAL cells after infection with a high inoculum (data not shown). By 24 h, neutrophils accounted for >90% of BAL cells in both groups of mice, but the total number of BAL neutrophils remained significantly reduced in the MyD88−/− mice. A similar pattern was observed after *S. aureus* infection (Fig. 2D), although neutrophilic inflammation was less intense than after *P. aeruginosa* infection. Thus, rapid production of proinflammatory cytokines and early but not late neutrophil recruitment to the lungs in response to acute bacterial pneumonia is largely MyD88-dependent, particularly in response to *P. aeruginosa*.

The capacity of alveolar macrophages (AM) to ingest bacteria appeared to be preserved in MyD88−/− mice. Cell-associated bacteria were evident in 16.4 ± 5.2% of AM lavaged from MyD88−/− mice 4 h after inhalation of *P. aeruginosa* vs 22.2 ± 6.8% of AM from control animals (*n* = 4–5, NS); >99% of these cells contained <10 organisms. After inhalation of *S. aureus*, cell-associated bacteria were observed in 90.3 ± 1.3% of AM lavaged from MyD88−/− mice vs 86.3 ± 1.9% of wild-type AM (*n* = 7–8, NS). More than 10 cocci/cell were counted in 62.7 ± 3.7% of the AM from MyD88−/− mice vs 52.3% of wild-type AM (*p < 0.5*).

Epithelial necrosis and altered inflammatory response to *P. aeruginosa* infection in MyD88−/− mice

The lung tissues of uninfected MyD88−/− and wild-type mice were histologically normal (data not shown). However, the histologic patterns of lung inflammation and injury after inhalation of *P. aeruginosa* differed markedly between MyD88−/− and control animals. The lung tissues of uninfected MyD88−/− and wild-type mice were histologically normal (data not shown). However, the histologic patterns of lung inflammation and injury after inhalation of *P. aeruginosa* differed markedly between MyD88−/− and control animals.

**FIGURE 3.** Aberrant lung inflammation and injury in MyD88−/− mice. Wild-type and MyD88−/− mice were infected by aerosol with *P. aeruginosa* (PA) or *S. aureus* (SA) as in Fig. 1, and lung tissues were harvested 4 and 24 h later. Original magnification, ×10 or ×40 as indicated. Sections are representative of four mice studied at each time point from each infection.
and control mice. At 4 h, the lungs of controls revealed predominantly perivascular but also peribronchial and alveolar neutrophilic infiltrates associated with prominent edema and hemorrhage (Fig. 3). By contrast, lungs of MyD88−/− mice exhibited diffuse congestion, scant inflammation, and no alveolar inflammation (Fig. 3). At 24 h, controls showed less intense perivascular inflammation, edema, and hemorrhage, but more prominent neutrophilic alveolitis, whereas MyD88−/− mice demonstrated widespread peribronchial and bronchiolar neutrophilic infiltrates, necrosis of bronchial epithelium, extensive neutrophilic alveolitis, and alveolar hemorrhage. Consistent with these differences, at 24 h the BAL fluid of MyD88−/− but not control mice contained many RBC, and concentrations of protein were higher in MyD88−/− compared with control mice (2056 ± 399 vs 599 ± 95 µg/ml, respectively, p < 0.01).

After inhalation of S. aureus, histologic lesions in wild-type mice were similar, albeit milder, to those observed in wild-type mice infected with P. aeruginosa. Sections from MyD88−/− mice harvested 4 h after inhalation of S. aureus demonstrated little or no perivascular or peribronchial inflammation and no alveolitis (Fig. 3). Sections harvested 24 h after infection showed more perivascular and peribronchial infiltrates and scattered alveolar inflammation (Fig. 3). There was no evidence of epithelial necrosis in MyD88−/− mice infected with S. aureus, and no hemorrhage was observed in either group of mice.

Discussion
The major findings of these studies are that MyD88-deficient mice fail to mount an effective defense against intrapulmonary challenge with P. aeruginosa but control lung infection with S. aureus. MyD88−/− mice exposed to aerosolized P. aeruginosa did not initiate early cytokine and inflammatory responses to infection and were unable to contain intrapulmonary bacterial replication, leading to extensive necrosis of the respiratory epithelium, dissemination of infection, and death. In comparison, MyD88-deficient mice controlled S. aureus infection without lung epithelial injury or mortality despite blunted cytokine and inflammatory responses. These observations indicate that MyD88-deficient signaling is integral to the initiation of early cytokine and inflammatory responses to both Gram-negative and Gram-positive bacterial infections of the lower respiratory tract. However, whereas MyD88 is essential for the control of lung infection with P. aeruginosa, it is not required for pulmonary defense against S. aureus.

Live bacteria such as P. aeruginosa and S. aureus present diverse ligands for TLRs, including LPS (TLR4), peptidoglycan and lipopeptides (TLR2), flagellin (TLR5) and CpG DNA (TLR9) (1). In addition to TLRs, MyD88 also links the IL-1 and IL-18 receptors to signaling pathways that lead to inflammatory mediator production (1, 3). However, the defects in host defense in MyD88-deficient mice likely result primarily from the loss of signaling downstream of TLRs: After intranasal inoculation of P. aeruginosa, mice deficient in the agonistic type I IL-1R or IL-18 and mice treated with a soluble IL-1R antagonist have modest reductions in lung inflammation but enhanced bacterial clearance compared with controls (16, 17). In any case, the near absence of early local proinflammatory cytokines and neutrophils in MyD88−/− mice infected with P. aeruginosa and the markedly reduced response to S. aureus indicate that MyD88-dependent pathways are required for the initial response to these pathogens in the lower respiratory tract. Furthermore, these observations imply that non-TLR-dependent proinflammatory signals in acute bacterial pneumonia, such as formyl peptides and complement fragments (18), are unable to elicit rapid neutrophil recruitment in the absence of MyD88. The delayed inflammatory response observed in MyD88−/− mice 24 h after infection might reflect TLR-dependent but MyD88-independent responses. For example, LPS can induce signal transduction with delayed kinetics by a TLR4-dependent pathway in the absence of MyD88, making use of alternative adapter proteins (1, 2). However, to date TLR-dependent but MyD88-independent responses to molecules from S. aureus have not been described, suggesting that TLR-independent recognition of microbial ligands, such as through nucleotide-binding oligomerization domain proteins (19), also may contribute to the delayed response. Alternatively, the late inflammation may have been initiated by epithelial injury.

MyD88 was essential for clearance of P. aeruginosa but not S. aureus, from the lungs. This difference may reflect the different roles for specific phagocytes in resistance to these pathogens. Host defense against P. aeruginosa pneumonia is dependent on early neutrophil recruitment (20, 21). In contrast, pulmonary defense against S. aureus largely relies on alveolar macrophages, which efficiently clear S. aureus in vivo (20, 22). We found the capacity of alveolar macrophages to ingest S. aureus in vivo to be undiminished in MyD88−/− mice. Neutrophils do contribute to pulmonary defense against S. aureus at high bacterial inocula (20, 23), and we cannot exclude that MyD88−/− mice might exhibit impaired resistance to higher doses of staphylococci. The ability of MyD88−/− mice to control lung infection with S. aureus contrasts sharply with their marked susceptibility to i.v. infection with this organism (4). The notion that MyD88 may be dispensable in certain contexts does not appear to be restricted to Gram-positive bacteria, since MyD88-deficient mice are protected from polymicrobial peritonitis (5), a process caused predominantly by Gram-negative pathogens.

Thus, while MyD88 has a role in the activation of innate immunity to both P. aeruginosa and S. aureus, the consequences of MyD88 deficiency appear to be both pathogen and tissue specific. These findings reveal substantial complexity in innate defenses, whereby essential or redundant roles for specific components are dependent both on the nature of the microbe and the site(s) of infection.

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References


