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Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades

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Abstract

MyD88 is an adaptor molecule essential for signaling via the Toll-like receptor (TLR)/IL-1 receptor family. TLR4 is a member of the TLR family and a point mutation in the *Tlr4* gene causes hyporesponsiveness to lipopolysaccharide (LPS) in C3H/HeJ mice. We have previously shown that both TLR4- and MyD88-deficient mice are hyporesponsive to LPS. In this study we examined the responsiveness of these two knockout mice to various bacterial cell wall components. Cells from TLR4-deficient mice responded to several kinds of LPS, peptidoglycan and crude cell wall preparation from Gram-positive bacteria and mycobacterial lysates. In contrast, macrophages and splenocytes from MyD88-deficient mice did not respond to any of the bacterial components we tested. These results show that MyD88 is essential for the cellular response to bacterial cell wall components.

The innate immune system is characterized by the use of germline-encoded receptors for pathogen recognition. *Drosophila* depend entirely on the innate response for their host defense (1). Regulation of the antifungal immune response in adult flies has been shown to involve the Toll receptor (2), which was originally identified as a receptor essential for dorsoventral patterning during early embryonic development (3). Toll is a type I transmembrane receptor whose extracellular domain contains a leucine-rich repeat and whose cytoplasmic domain is analogous to that of the mammalian IL-1 receptor (IL-1R) family (4). Similar to IL-1 signaling, binding of Toll by the extracellular ligand Spatzle leads to activation of Dorsal, an NFκB-like transcription factor (4). In mutants deficient in the Toll pathway, induction of the antifungal peptide drosomycin is dramatically affected (2). Mutation in 18-wheeler, another Toll family member, results in a defect in the antibacterial host defense but does not affect the antifungal response (5). Thus, particular pathogens induce the production of specific antimicrobial peptides in *Drosophila* through the selective activation of the Toll pathway (6).

Innate immunity in vertebrates plays a similar role in the detection of invading infectious organisms, and subsequently instructs adaptive immune system by producing proinflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-1 and IL-6, as well as co-stimulatory molecules on the cell surfaces of immune cells (1). Recently, six human homologues of *Drosophila* Toll, designated Toll-like receptors (TLR) 1–6, have been reported (7–9). TLR2 has been shown to be a signaling receptor that is activated by lipopolysaccharide (LPS), a part of the outer membrane of Gram-negative bacteria (10,11). Further, it has recently been shown that LPS hyporesponsiveness in the mouse strain C3H/HeJ is due to a missense point mutation in the *Tlr4* gene (12–14). Macrophages and B cells from TLR4-deficient mice are hyporesponsive to LPS, indicating that TLR4 is required for LPS signaling (14).

MyD88 is an adaptor molecule essential for IL-1R family signaling (15–17). Triggering of the intracellular IL-1R family signaling cascade requires the recruitment of MyD88 to the receptor complex, which then relays a signal to NFκB via IL-1R-associated kinase (IRAK). TLR2 and TLR4 have been reported to also utilize MyD88 as an adaptor molecule *in vitro* (11,18,19). Other TLR family members that contain cyto-

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plasmic domains homologous to that of IL-1R might also share MyD88. We have recently shown that MyD88-deficient mice are highly resistant to LPS-induced shock, and that both macrophages and B cells from MyD88-deficient mice displayed no biological responses to LPS (20). Recently, it has been shown that overexpression of TLR2 conferred responsiveness to several Gram-positive bacterial components such as peptidoglycan (PGN) (21,22), lipoteichoic acid (LTA) (21) and bacterial lipoproteins (23,24) *in vitro*. To investigate the roles of MyD88 in the recognition of specific pathogen components, we examined and compared the responsiveness of TLR4- and MyD88-deficient mice to various bacterial cell wall components.

We first examined the responsiveness of mouse cells to LPS derived from *Salmonella minnesota* Re-595. Peritoneal macrophages from wild-type, TLR4-deficient and MyD88-deficient mice were cultured in the presence of various concentrations of Re-595 LPS, and the production of TNF-α was measured. Secretions of TNF-α from wild-type macrophages increased in a dose-dependent manner. In contrast, macrophages from TLR4-deficient or MyD88-deficient mice did not produce any detectable amounts of TNF-α in response to LPS, even when added to a concentration of 100 μg/ml (Fig. 1A). We next examined the responsiveness of splenocytes to Re-595 LPS. Splenocytes were cultured in the presence of various concentrations of LPS. Whereas this stimulation elicited a dose-dependent mitogenic response in wild-type splenocytes, no LPS-induced proliferative response was observed in splenocytes from either TLR4- or MyD88-deficient mice (Fig. 1B). Thus, the responses of both TLR4- and MyD88-deficient mice to *S. minnesota* Re-595 LPS are almost completely abrogated.

We next examined the response of these mice to LPS prepared from *Porphyromonas gingivalis* 381, a Gram-negative bacterium and peridontopathic organisms of major importance (25). *P. gingivalis* LPS displays an interesting property in its ability to activate cells from the otherwise LPS-hyporesponsive C3H/HeJ mice (25). As shown in Fig. 1(C), *P. gingivalis* LPS induced TNF-α in a dose-dependent manner in macrophages from wild-type mice. *P. gingivalis* LPS also induced TNF-α in TLR4-deficient macrophages, similar to C3H/HeJ macrophages, although the level was about one-third that of wild-type macrophages. In contrast, MyD88-deficient macrophages did not produce any detectable TNF-α, even when stimulated with high concentrations of LPS. Splenocytes from TLR4-deficient mice exhibited a significant, albeit lower, proliferative response to *P. gingivalis* LPS. In contrast, proliferation of splenocytes was not observed with MyD88-deficient splenocytes (Fig. 1D). Thus, TLR4-deficient mice were partially defective and MyD88-deficient mice almost completely defective in their response to *P. gingivalis* LPS. These results indicate that MyD88 is essential for, and that TLR4 contributes in part to, the signaling elicited by *P. gingivalis* LPS.

To rule out the possibility that the cells from TLR4- and MyD88-deficient mice are inert to all stimuli, we analyzed their responsiveness to other stimuli. Splenocytes from TLR4- and MyD88-deficient mice proliferated normally in response to IL-4 plus anti-IgM antibody or anti-CD40 antibody (Fig. 2A). IFN-γ-induced augmentation of MHC class II expression on peritoneal macrophages was also enhanced to a similar

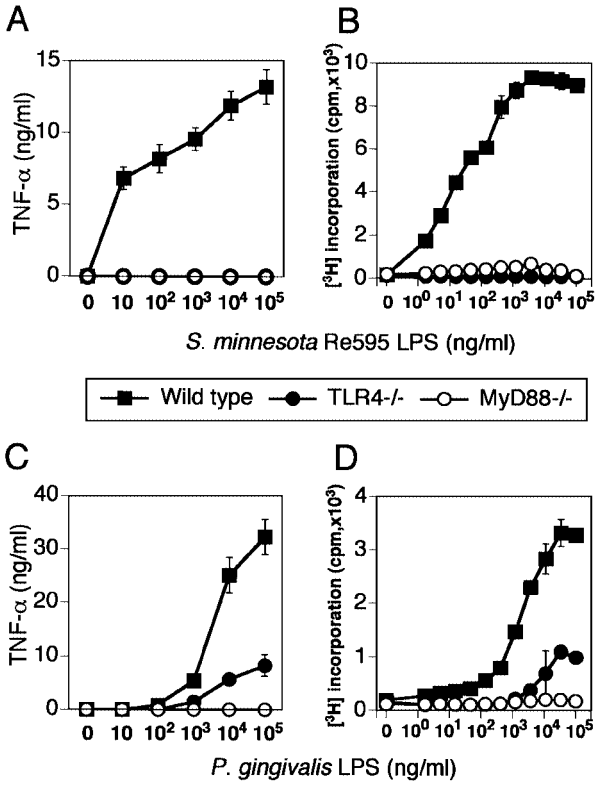

Fig. 1. Responsiveness to *S. minnesota* Re-595 and *P. gingivalis* LPS. (A) Mice were i.p. injected with 2 ml of 4% thioglycollate. Three days later, peritoneal exudate cells were isolated. The cells from wild-type, and TLR4- and MyD88-deficient mice were cultured with the indicated amount of *S. minnesota* Re-595 LPS (Sigma) for 24 h. Concentrations of TNF-α in the culture supernatants were measured by ELISA (Genzyme, Cambridge, MA). (B) Splenocytes (1×10^5) were isolated and cultured with the indicated concentrations of *S. minnesota* Re-595 LPS for 48 h. Then 1 μCi of [^{3}H]thymidine (DuPont, Boston, MA) was pulsed for the last 8 h. ^{3}H incorporation was measured by a β scintillation counter (Packard, Meriden, CT). (C) Peritoneal macrophages from wild-type, and TLR4- and MyD88-deficient mice were cultured with the indicated concentrations of *P. gingivalis* LPS for 24 h. Concentrations of TNF-α in the culture supernatants were measured by ELISA. Preparation of *P. gingivalis* LPS is as described previously (25). (D) Splenocytes were cultured with the indicated concentrations of *P. gingivalis* LPS for 48 h. Then 1 μCi of [^{3}H]thymidine was pulsed for the last 8 h. ^{3}H incorporation was measured by a scintillation counter.

extent in wild-type, and TLR4- and MyD88-deficient mice (Fig. 2B). Peritoneal macrophages from both TLR4- and MyD88-deficient mice phagocytosed latex microspheres normally (Fig. 2C). Thus, the responses of macrophages and splenocytes from either TLR4- or MyD88-deficient mice to these other stimuli were not impaired, indicating that these mutant cells are specifically defective in their response to LPS.

In addition to LPS from Gram-negative bacteria, Gram-positive bacterial cell wall preparations and their components, such as PGN, are known to activate host macrophages (26). Therefore, we investigated the responsiveness of mouse cells to cell wall preparation from *Staphylococcus aureus* and PGN from *S. aureus*. The cell wall preparation from *S. aureus* induced TNF-α production in cells from wild-type mice in a dose-dependent manner. TLR4-deficient macrophages

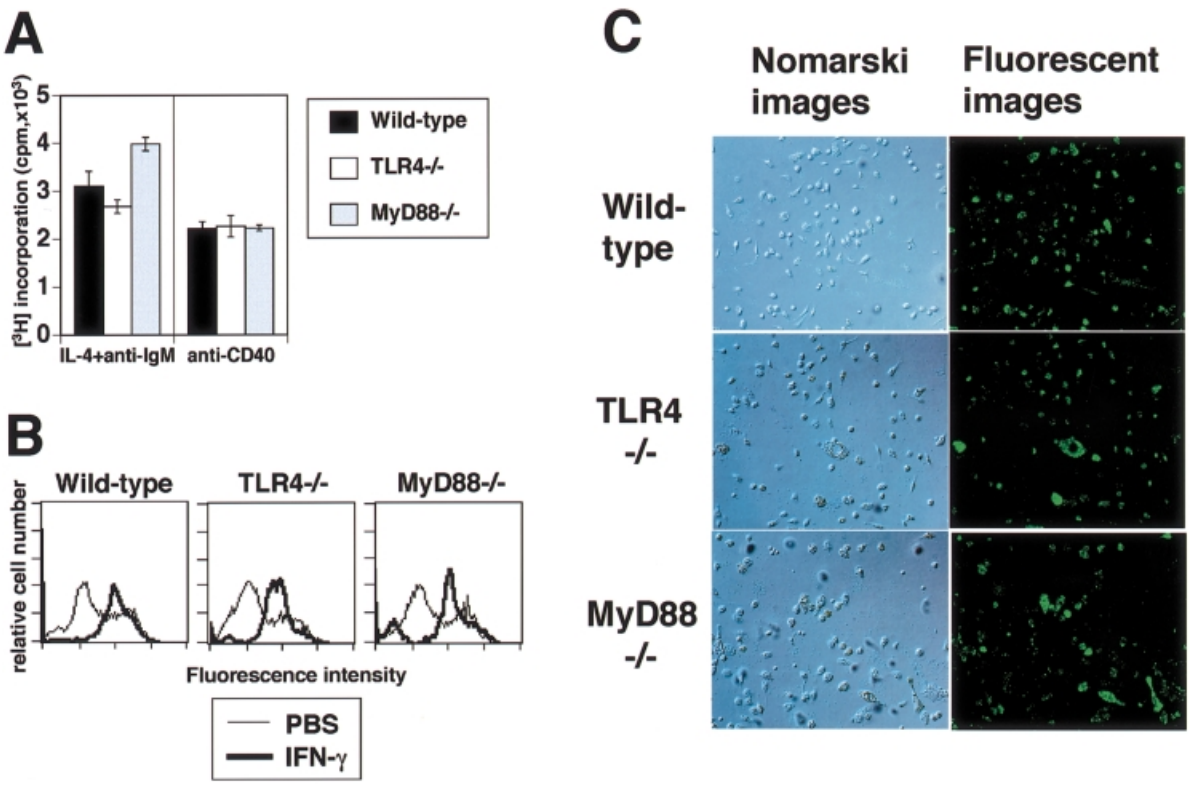

Fig. 2. Normal responses to IL-4 and IFN-γ and normal phagocytic activity in TLR4- and MyD88-deficient mice. (A) Splenocytes were cultured with IL-4 (Genzyme) plus anti-IgM Ab or anti-CD40 Ab (PharMingen, San Diego, CA) for 48 h. Then 1 μCi of [^{3}H]thymidine was pulsed for the last 8 h. ^{3}H incorporation was measured by a scintillation counter. ^{3}H incorporations (c.p.m.) in cells from wild-type, and TLR4- and MyD88-deficient mice without stimulation were 161, 134 and 175 respectively. (B) Mice were injected i.p. with 5000 U of IFN-γ (Genzyme) or PBS. Three days later, peritoneal macrophages were collected and stained with biotin-conjugated anti-I-Ab antibody (PharMingen) followed by streptavidin–FITC (PharMingen). Stained cells were analyzed on FACSCalibur using CellQuest software (Becton Dickinson, Lincoln Pack, NJ). (C) Peritoneal macrophages were isolated 3 days after thioglycollate treatment. These cells were incubated with 0.025% fluorescent latex beads (0.75 mm) (Polyscience, Niles, IL) for 2 h. Then cells were washed vigorously 3 times with PBS to remove non-internalized beads and fixed with 2.5% formaldehyde in PBS for 20 min. Cells were monitored with Axiophot microscope (Carl Zeiss, Thornwood, NY). Left panels show Nomarski images of wild-type, and TLR4- and MyD88-deficient macrophages. Magnification ×20. Fluorescent images of same cells are shown in right panels.

showed significant productions of TNF-α in response to *S. aureus* cell wall, although the production was reduced compared with that of wild-type mice. In contrast, MyD88-deficient macrophages did not produce TNF-α in response to any concentration of *S. aureus* cell wall (Fig. 3A). When stimulated with *S. aureus* PGN, peritoneal macrophages from TLR4-deficient mice produced TNF-α in a dose-dependent manner to almost the same extent as cells from wild-type mice. In contrast, macrophages from MyD88-deficient mice did not produce TNF-α at any concentration added (Fig. 3B). Mycobacterial cell wall components, e.g. lipoarabinomannan, are also known to induce activation of myeloid cells (27). We used crude whole-cell lysates from the *Mycobacterium tuberculosis* Aoyama B strain. Wild-type macrophages produced TNF-α in response to these lysates in a dose-dependent manner. Macrophages from TLR4-deficient mice exhibited a slight defect in TNF-α production compared with wild-type macrophages. In contrast, cells from MyD88-deficient mice did not produce TNF-α in response to the mycobacterial crude cell lysates (Fig. 3C).

We further examined the responses of mutant mice to other bacterial components: LPS from *Escherichia coli* serotype O55:B5, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella flexneri* and *Vibrio cholerae*, and PGN from *Staphylococcus epidermidis*. The responsiveness of wild-type, and TLR4- and MyD88-deficient mice to these bacterial components are summarized in Table 1. TLR4-deficient mice showed reduced, but significant responsiveness to LPS preparations from several bacterial strains. *S. epidermidis* PGN also induced responses in TLR4-deficient cells. In contrast, MyD88-deficient cells did not respond to any LPS or Gram-positive cell wall components. Therefore, it is likely that the recognition and signaling elicited by some LPS or Gram-positive cell wall components is mediated by TLR4 and/or other TLR that use MyD88 as an adaptor molecule.

LPS consists of an *O*-specific chain, a core oligosaccharide and lipid A moieties. In particular, the lipid A portion of LPS is known to be essential for its endotoxic activity (28). Lipid A from different bacterial origins has common structural similarities, but differs in such details as length of their fatty acid carbon chains and degree of phosphorylation (28). The

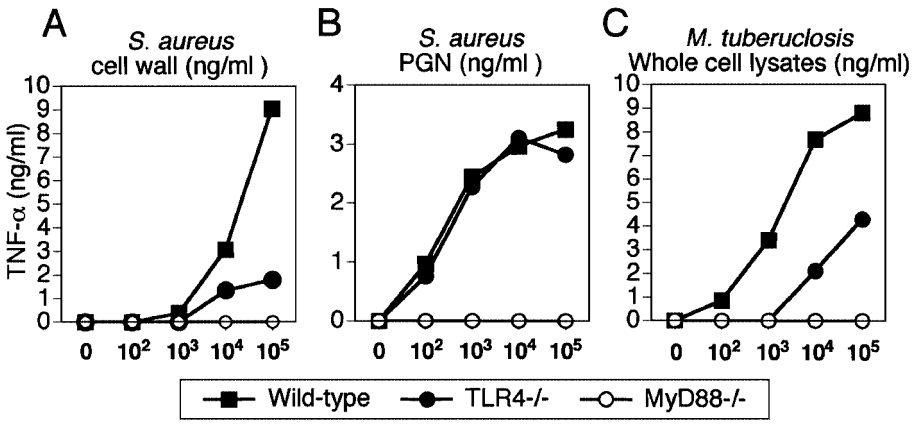

Fig. 3. Responsiveness to Gram-positive bacterial cell wall components and mycobacterial whole-cell lysates. Thioglycollate-elicited peritoneal macrophages from wild-type, and TLR4- and MyD88- deficient mice were cultured with the indicated concentrations of (A) cell wall preparation from *S. aureus*, (B) PGN from *S. aureus*, (Fluka, St Louis, MO) and (C) whole-cell lysates from *M. tuberculosis* Aoyama B strain for 24 h. Then, concentrations of TNF-α in the culture supernatants were measured. Preparation of *S. aureus* cell wall was as described previously (31). Preparation of mycobacterial whole-cell lysates were as described below. *M. tuberculosis* Aoyama B strain (NIHJ 1635) was cultured in Dubos broth (Difco, Detroit, MI) for 1 month. Then cells were collected and resuspended with PBS. Cells were sonicated and used as mycobacterial whole-cell lysates.

Table 1. Responsiveness to bacterial cell wall components

Test specimen[a]	Responsiveness of mice[b]		
	Wild-type	TLR4–/–	MyD88–/–
LPS			
Escherichia coli 055:B5	++	+	–
Klebsiella pneumoniae	++	–	–
Porphyromonas gingivalis	++	+	–
Pseudomonas aeruginosa	++	+	–
Salmonella minnesota Re595	++	–	–
Salmonella typhimurium	++	+	–
Serratia marcescens	++	+	–
Shigella flexneri	++	+	–
Vibrio cholerae	++	+	–
Gram-positive bacterial components			
Staphylococcus aureus cell wall	++	+	–
Staphylococcus aureus PGN	++	++	–
Staphylococcus epidermidis PGN	++	+	–
Mycobacterial whole cell lysates			
Mycobacterium tuberculosis	++	+	–

[a]LPS from *E. coli* serotype O55:B5, *K. pneumoniae*, *P. aeruginosa* serotype 10, *S. typhimurium*, *S. marcescens*, *S. flexneri* serotype 1A and *V. cholerae* serotype Inaba 569B were purchased from Sigma (St Louis, MO). PGN from *Staphylococcus epidermidis* was a gift from Shigeo Kawata (Dainippon Pharmaceutical, Tokyo, Japan) (30).

[b]The responsiveness was assessed by TNF-α production from macrophages and proliferation of splenocytes in response to these stimuli: ++, >50% of wild-type response; +, 10–50% of wild-type response; –, <10% of wild-type response.

lipid A moieties from peridontopathic pathogens such as *P. gingivalis* are especially unique, in that they possess unusually branched and relatively long fatty acids (15–17 carbon atoms) (25). These structural differences among LPS species may have given rise to the diversity of receptors that bind LPS. In contrast, MyD88-deficient mice were not responsive to any of the LPS samples derived from many different bacteria, indicating that all LPS receptors utilize MyD88 as an essential signaling molecule.

MyD88-deficient mice showed no response to the crude cell wall preparation and PGN derived from Gram-positive bacteria or to mycobacterial whole-cell lysates. In contrast, TLR4-deficient mice showed almost the same response to *S. aureus* PGN as wild-type mice, and somewhat reduced, but still significant, response to *S. aureus* cell wall and mycobacterial whole-cell lysates. These indicate that TLR4 may be in part responsible for the recognition of Gram-positive bacterial components and mycobacterial components. Recent studies demonstrate that TLR2 may be a signaling receptor for Gram-positive bacterial components and mycobacterial lipoproteins as well as Gram-negative LPS (21–24). Recognition of Gram-positive bacterial and mycobacterial components might be executed by at least TLR2 and TLR4. Thus, MyD88 may be essential for signaling via TLR family including TLR2 and TLR4.

In interpreting these results we had to consider the possibility of contamination of bacterial cell wall components. The bacterial component that affects the cellular response at the lowest concentration may be LPS from Gram-negative bacteria. Even if a minor component contaminated in the sample may interfere or augment the response of wild-type or TLR4-deficient mice, the fact that MyD88-deficient mice are unresponsive to all bacterial components we tested demonstrates that MyD88 is essential for the cellular response to all bacterial cell wall components.

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Abbreviations

IL-1R	IL-1 receptor
LTA	lipoteichoic acid
LPS	lipopolysaccharide
PGN	peptidoglycan
TLR	Toll-like receptor
TNF	tumor necrosis factor

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