Intracellular TLR4/MD-2 in macrophages senses Gram-negative bacteria and induces a unique set of LPS-dependent genes

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Abstract

Toll-like receptor (TLR)4/MD-2, a sensor for LPS, delivers the MyD88-dependent signal from the cell surface, then traffics to endolysosomes and delivers the TRIF/TICAM-1-dependent signal. Both signals are thought to be dependent on cell surface TLR4/MD-2. Although TLR4/MD-2 is located also in recycling endosomes, the Golgi apparatus or the endoplasmic reticulum, little is known about a role for intracellular TLR4/MD-2 in LPS responses. We here studied intracellular LPS sensing in macrophages. PRAT4A (protein associated with TLR4 A) is a cochaperone for a general chaperone gp96 and required for cell surface expression of TLR4/MD-2. Cell surface TLR4/MD-2 was undetectable on PRAT4A⁻/² thioglycollate-elicited peritoneal macrophages (P-Macs) and bone marrow-derived macrophages (BM-Macs). LPS responses were all abolished in PRAT4A⁻/² P-Macs, whereas a part of LPS responses remained detectable in PRAT4A⁻/² BM-Macs. Of note, LPS responses in PRAT4A⁻/² BM-Macs were not necessarily dependent on TRIF/TICAM-1 signaling. PRAT4A⁻/² BM-Macs showed unimpaired production of both TRIF/TICAM-1-dependent chemokine RANTES (CCL5) and MyD88-dependent chemokine MCP-1 (CCL2). Moreover, up-regulation of co-stimulatory molecules, CD40 and CD86 was not altered. In contrast, TRIF/TICAM-1-dependent production of type I IFN was profoundly impaired. In response to heat-killed bacteria Escherichia coli, BM-Macs also required PRAT4A-independent TLR4/MD-2 for production of MCP-1 (CCL2) and RANTES (CCL5) and for up-regulation of CD40 and CD86, indicating that intracellular TLR4/MD-2 is able to sense phagocytosed bacteria and activate immune responses. These results demonstrate that intracellular TLR4/MD-2 is responsible for unique set of LPS responses.

Keywords: innate immunity, LPS, TLR

Introduction

Toll-like receptors (TLRs) sense a variety of microbial products. Cell surface TLRs including TLR4/MD-2, TLR1/TLR2 and TLR6/TLR2 recognize microbial membrane lipids, whereas TLR3, TLR7, TLR8 and TLR9 reside in intracellular organelles and recognize microbial nucleic acids (1–3). TLRs activate innate immune responses and prime adaptive immune responses by inducing nuclear factor κB-dependent pro-inflammatory cytokines, IFN regulatory factor (IRF)-dependent type I IFNs and co-stimulatory molecules. TLR4/MD-2 senses LPS, a principal membrane component of Gram-negative bacteria. MD-2 is physically associated with TLR4 (4), directly binds to LPS (5) and induces LPS-dependent dimerization of TLR4 (6). TLR4/MD-2 activates two distinct signaling pathways. One is mediated by the adaptor proteins TIRAP (Toll–IL-1 receptor domain-containing adaptor protein) and MyD88 (myeloid differentiation factor 88) and induces production of pro-inflammatory cytokines. The other signaling pathway is activated by a distinct set of signaling adaptors, TRAM (TRIF-related adaptor molecule)/TICAM-2 (TIR domain-containing adaptor molecule-2) and TRIF (Toll/ IL-1 receptor-containing adapter inducing IFN-β)/TICAM-1 (TIR domain-containing adaptor molecule-1), which mediate IRF-3-dependent type I IFN production and up-regulation of co-stimulatory molecules.
These two signaling pathways are sequentially activated by TLR4/MD-2, while TLR4/MD-2 traffics from the cell surface to the endolysosomes after LPS stimulation. TLR4/MD-2 first signals from the cell surface for inducing pro-inflammatory cytokines, whereas type I IFN production is induced only after TLR4/MD-2 is internalized to the endolysosomes (7, 8). TLR4/MD-2, therefore, resides on the cell surface to initiate LPS recognition and signaling (3, 8). Husebye et al. (9) recently reported that TLR4/MD-2 in recycling endosomes is recruited to phagosomes and capable of activating IRF-3-dependent type I IFN production in response to phagocytosed bacteria. Surprisingly, phagosomal TLR4/MD-2 signaling was not influenced by Dynasore, a pharmacological inhibitor of dynamin, which is required for TLR4/MD-2 endocytosis. These results suggest that intracellular TLR4/MD-2 is able to activate IRF-3-dependent type I IFN production without trafficking to the plasma membrane. Cell surface TLR4/MD-2, however, can also be recruited to phagocytic cup during phagocytosis and contributes to cytokine production. Two recent reports showed that LPS on the bacterial surface is able to bind to MD-2 and stimulate TLR4, enhancing both phagocytosis and cytokine production in response to Gram-negative bacteria (10, 11). It remains unresolved whether or how much intracellular TLR4/MD-2 contributes to LPS responses (12).

Three molecules were reported to be required for cell surface expression of TLR4. MD-2 is physically associated with TLR4 and serves as ligand-binding component (4, 5, 13, 14). MD-2 influences cell surface expression of TLR4 (13, 15), although requirement varies, depending on cell types (16, 17). PRAT4A (protein associated with TLR4 A) and a general chaperone gp96 are both required for cell surface expression of TLR4/MD-2 (16, 18, 19). PRAT4A is associated withgp96 and works as a TLR-specific cochaperone for gp96 (20). In C57BL/6 PRAT4A−/− dendritic cells, cell surface TLR4/MD-2 profoundly decreased, but LPS responses, especially CD86 up-regulation, still remained detectable (18), suggesting a possibility that TLR4/MD-2 inside the cells is able to respond to LPS. To further address this possibility, we took an advantage that PRAT4A−/− macrophages on the BALB/c background completely lack cell surface expression of TLR4/MD-2. The present study show macrophage responses to LPS or Gram-negative bacteria in the absence of cell surface TLR4/MD-2.

Methods

Mice

We previously established C57BL/6 background PRAT4A−/− mice (18). C57BL/6 background PRAT4A−/− mice were progressively backcrossed to the BALB/c strain for 10 generations and used as BALB/c background PRAT4A−/− mice. TLR4−/− and MyD88−/− mice on Balb/c background were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All animal experiments were done with the approval of the Animal Research Committee of the Institute of Medical Science, The University of Tokyo.

Reagents and cells

The antibodies for flow cytometry (anti-CD11b, anti-CD40 and anti-CD86 Abs) were purchased from eBioscience. Anti-TLR4/MD-2 (MtsS510), anti-TLR4 (Sa15-21) and anti-CD14 (Sa2-8) were previously established in our laboratory (16, 21).

Lipid A purified from Salmonella minnesota (Re-595) and Poly(I:C) were purchased from Sigma–Aldrich and InvivoGen, respectively.

Bone marrow-derived macrophages (BM-Macs) were prepared as described previously (16). Briefly, bone marrow cells were plated at 0.8 × 10^7 cells ml⁻¹ in 10-cm dishes with 10% FCS–D-MEM supplemented with 10 ng ml⁻¹ recombinant murine M-CSF (PeproTech, Rocky Hill, NJ, USA). At day 6, cells were harvested and used for each analysis. Thioglycollate-elicited peritoneal macrophages (P-Macs) were removed by lavage with 10 ml of HBSS from mice abdominal cavity that had been received intraperitoneal injection of 2 ml thioglycollate 3 days before.

Flow cytometry

Cell surface staining and analyses on the FACSCalibur (BD Biosciences) were described previously (16).

Quantitative real-time PCR

Quantitative real-time PCR was conducted as described previously (16). Briefly, total RNA (1 μg) was reverse transcribed into cDNA using ReverTra Ace qPCR RT Kit (TOYOBO) according to the manufacturer’s instruction. Quantitative real-time PCR analyses were carried out using a 7300 Fast Real Time PCR System (Applied Biosystems) with TaqMan Gene Expression Assays for mouse IFN-β (Mm00439546). Each sample was normalized using TaqMan Gene Expression Assays for mouse β-actin (Mm00607939).

Cytokines and nitrite measurement

Mouse BM-Macs and P-Macs were plated at 1 × 10^5 per well on 96-well plates and treated by indicated stimulants for 24 h. Nitrite concentration was determined by Griess reagent using chemicals from Sigma–Aldrich. In brief, 100 μl Griess reagent was added into 100 μl samples in 96-well plates and sat at room temperature for 10 min. Absorbance at 570 nm was read with a microplate reader (iMark; BioRad).

Macrophages were stimulated with a variety of TLR ligands. After 24 h, supernatant was collected and concentrations of tumor necrosis factor (TNF)-α, IL-6, IL-12, RANTES (CCL5) and MCP-1 (CCL2) were determined by ELISA Kits (R&D systems). Concentrations of MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), G-CSF and KC were measured by Bio-Plex suspension array system (BioRad) according to the manufacturer’s instruction.

Statistical analysis

Data from triplicate samples were used for statistical analysis. Statistical significance was calculated by Student’s t-test. A P value of <0.05 was considered to be significant.

Results

Complete lack of cell surface TLR4/MD-2 on PRAT4A−/− macrophages under the BALB/c background

On the C57BL/6 background, cell surface TLR4/MD-2 on PRAT4A−/− BM-Macs was decreased but still detectable.
whereas on the BALB/c background, cell surface TLR4/MD-2 was completely undetectable on PRAT4A−/− macrophages. Cell surface expression of TLR4/MD-2, TLR4 and CD14 on BM-Macs (A) or P-Macs (B) from Wild-type (wt), PRAT4A−/− and TLR4−/− mice was analyzed by flow cytometry. Cell population highly expressing CD11b was gated as BM-Macs or P-Macs. Open histograms show the staining by each antibody. Shaded histograms show control staining with the second reagent alone. Shown data are representative of more than three independent experiments.

In this regard, these PRAT4A−/− cells were indistinguishable from TLR4−/− cells. As reported previously (18), cell surface expression of CD14 was not altered by the lack of PRAT4A in all the cell types studied. Thus, cell surface expression of TLR4/MD-2 on macrophages is completely dependent on PRAT4A on the BALB/c background.

**PRAT4A-independent TLR4/MD-2 responses in BM-Macs but not P-Macs**

LPS responses in PRAT4A−/− P-Macs were studied to see how much LPS responses are dependent on cell surface
expression of TLR4/MD-2. Lipid A-dependent production of nitric oxide (NO), TNF-α, IL-6 and RANTES (CCL5) was completely abolished in both PRAT4A⁻/⁻ and TLR4⁻/⁻ BM-Macs (Fig. 2A and B). In addition, PRAT4A⁻/⁻ P-Macs failed to induce IFN-β mRNA in response to lipid A (Fig. 2C). PRAT4A⁻/⁻ BM-Macs also showed impaired production of NO in response to lipid A stimulation (Fig. 3A). IFN-β mRNA induction was impaired when compared with wild-type (wt) or MyD88⁻/⁻ BM-Macs but still significantly induced by LPS (Fig. 3B), revealing a difference from PRAT4A⁻/⁻ P-Macs. Endosomal TLR4/MD-2 signaling is reported to be restricted to TRIF/TICAM-1-dependent signaling (7, 8). If TLR4/MD-2 inside the cells is able to sense LPS, LPS responses were expected to be all dependent on TRIF/TICAM-1 but not on MyD88. As expected, MyD88-dependent NO production was not detected, whereas MyD88-independent induction of IFN-β mRNA was also significantly impaired (Fig. 3A and B). To further characterize PRAT4A⁻/⁻-independent LPS responses in BM-Macs, we studied lipid A-dependent production of a variety of cytokines by BM-Macs from PRAT4A⁻/⁻, MyD88⁻/⁻ and TLR4⁻/⁻ mice (Fig. 3C). Unimpaired production by PRAT4A⁻/⁻ BM-Macs was observed not only in TRIF/TICAM-1-dependent chemokine RANTES (CCL5) but also in MyD88-dependent chemokine MCP-1 (CCL2). Further, PRAT4A⁻/⁻ BM-Macs showed significantly higher production of other MyD88-dependent cytokines including TNF-α, IL-6, KC (CXCL-1), MIP-1α (CCL3) and MIP-1β (CCL4) than MyD88⁻/⁻ BM-Macs (Fig. 3C).
Intracellular TLR4/MD-2 up-regulates co-stimulatory molecules

It is well established that LPS up-regulates co-stimulatory molecules like CD40 and CD86 on macrophages in a manner dependent on TRIF/TICAM-1 (22). PRAT4A/−/− BM-Macs or P-Macs were stimulated with various doses of lipid A, and cell surface expression of CD40 and CD86 was studied. PRAT4A/−/− P-Macs showed impaired induction of these co-stimulatory molecules (Fig. 4A and B). BM-Macs showed higher induction of CD40 and CD86 than P-Macs (Fig. 4C and D). PRAT4A/−/− BM-Macs were still lower than wt BM-Macs and similar to MyD88−/− BM-Macs in induction of CD40 (Fig. 4C). CD86 induction in PRAT4A/−/− and MyD88−/− BM-Macs was not impaired at all (Fig. 4D). These data suggest that intracellular LPS sensing is capable of up-regulating co-stimulatory molecules through activation of the TRIF/TICAM-1-dependent signaling pathway.

Bacterial sensing by intracellular TLR4/MD-2

Functional roles for intracellular LPS sensing were further addressed. TLR4/MD-2 were recently shown to be recruited from recycling endosomes to phagosomes in order to sense phagocytosed heat-killed Gram-negative bacteria (9). Contribution of cell surface TLR4/MD-2 in phagosomal signaling remains controversial (12). Heat-killed Escherichia coli were, therefore, next used to stimulate wt or PRAT4A/−/−, MyD88−/− or TLR4−/− macrophages. Heat-killed E. coli induced production of NO, TNF-α, IL-6 and RANTES (CCL5) in P-Macs (Fig. 5A and B). All the production was dependent on TLR4. A low, but significant production of RANTES (CCL5), was only observed in PRAT4A/−/− P-Macs. Heat-killed E. coli was able to up-regulate CD40 and CD86 in a manner dependent on TLR4 (Fig. 5C and D). Up-regulation of CD40 and CD86 in PRAT4A/−/− P-Macs was impaired but significantly detectable (Fig. 5C and D). PRAT4A/−/− BM-Macs also produced NO, TNF-α, IL-6, RANTES (CCL5) and MCP-1 (CCL2) (Fig. 6B). Production of these cytokines was dependent on TLR4 particularly at 3.5 × 10^4 or 3.5 × 10^5 particles ml^-1 E. coli. PRAT4A/−/− BM-Macs showed significant production of not only RANTES (CCL5) but also TNF-α and MCP-1 (CCL2). PRAT4A/−/− BM-Macs showed higher production of TNF-α and MCP-1 (CCL2), but lower RANTES (CCL5) production, than MyD88−/− BM-Macs (Fig. 6B). In response to E. coli, up-regulation of CD40 and CD86 in PRAT4A/−/− BM-Macs was comparable with that in wt or MyD88−/− BM-Macs (Fig. 7A and B).

Discussion

After activating MyD88-dependent signaling pathway on the cell surface, the LPS/MD-2/TLR4 complex is internalized to
the endolysosomes and delivers TRIF/TICAM-1-dependent signaling pathway (7, 8). All the LPS responses in macrophages were, therefore, thought to start from the cell surface. Our results, however, showed that TLR4/MD-2 inside the BM-Macs is a functional LPS receptor and able to activate a unique set of LPS-inducible genes, such as RANTES, MCP-1 (CCL2), CD40 and CD86. Husebye et al. (9) previously suggested that intracellular TLR4/MD-2 in recycling endosomes is recruited to phagosomes and preferentially activates IRF-3-dependent type I IFN production. Dynasore, an inhibitor of dynamin, inhibited TLR4/MD-2 internalization, but not TLR4/MD-2 recruitment to phagosomes, suggesting that cell surface TLR4/MD-2 contributes little to phagosomal signaling. Considering that Dynasore also inhibit phagocytosis itself, a requirement for cell surface TLR4/MD-2 in phagosomal signaling is still controversial (12). The present study employed a different approach to address this question and showed LPS sensing in the absence of cell surface TLR4/MD-2.

PRAT4A is a substrate-specific cochaperon for a general chaperon gp96 (16, 18, 20). PRAT4A is associated with TLR4/MD-2 and required for its cell surface expression. The maturation of TLR4 sugar chains beyond high-mannose-type immature glycosylation require PRAT4A (18). TLR4 in PRAT4A-silenced cells showed immature glycosylation. Such immature form of TLR4 is, however, likely to be able to sense LPS. TLR9, e.g., resides in the endoplasmic reticulum (ER) with immature sugar chains but is able to sense microbial DNA after translocating to the endolysosomes (23). Intracellular TLR4/MD-2 with immature sugar chains was here shown to sense LPS. In contrast to Husebye’s results, intracellular TLR4/MD-2 recognized phagocytosed E. coli and activated a unique set of LPS responses, which were not restricted to TRIF/TICAM-1-dependent responses. Production of TRIF/TICAM-1-dependent IFN-β as well as MyD88-dependent TNF-α was heavily impaired, whereas production of MyD88-dependent MCP-1 (CCL2) and TRIF-dependent RANTES (CCL5) was not altered by the lack of cell surface TLR4/MD-2 (Fig. 3). MyD88 recruitment to TLR4 in the phagosome was reported (9) but has never been shown to mediate LPS signaling inside the cells. The present report, for the first time, suggests that intracellular TLR4/MD-2 activates MyD88-dependent signaling pathway.

It is important to understand a mechanism how LPS is delivered to intracellular TLR4/MD-2. Two different molecules, Scavenger receptors and CD14, are involved in LPS uptake via endocytosis. Type I and II scavenger receptors induce the slow LPS uptake in various cell types, such as endothelial, hepatic cells and macrophages (24, 25). On the other hand, cell surface CD14 induces rapid LPS uptake into the
endolysosomes and the Golgi apparatus (26). Alternatively, intracellular TLR4/MD-2 could recognize LPS on phagocytosed E. coli. In accordance with our data, intracellular TLR4/MD-2 is recruited to phagosome and senses phagocytosed bacteria (9). These two pathways would be differentially used, depending on each cell type.

Intracellular TLR4/MD-2 is similar to nucleic acid-sensing TLR7 and TLR9, which is also recruited to the endolysosomes and senses endocytosed nucleic acids. TLRs are subdivided into cell surface and intravesicular TLRs and TLR4/MD-2 was described to belong to cell surface TLRs. The present study suggested that TLR4/MD-2 inside the cells is not just an immature form but another functional form which can belong to intravesicular TLRs like TLR7 and TLR9. TLR7 and TLR9 sense nucleic acids in the endolysosomes but not on the cell surface. Nucleic acid sensing in the endolysosomes is thought to be a safety mechanism avoiding innate immune responses to self nucleic acids. Whereas self nucleic acids are rapidly degraded by RNase or DNase before reaching the endolysosomes, microbial nucleic acid is protected by microbial membrane and able to reach the endolysosomes (27, 28). TLR4/MD-2 has also been shown to respond to a variety of endogenous ligands (29). Ectopic expression of an endogenous ligand gp96 on the cell surface triggers TLR4/MD-2-dependent autoimmune disease (30). Given that gp96 normally resides in the ER as a chaperon for TLR4/MD-2, interaction of gp96 with TLR4/MD-2 in the ER would not activate TLR4/MD-2. Intracellular LPS sensing may be another safety mechanism restricting innate autoimmune responses. In this context, our previous report is noteworthy. When LPS sensing on the cell surface was reduced in bone marrow-derived cells by transferring PRAT4A/C0/C0 bone marrow cells, recipient mice became resistant to endotoxin shock (18), indicating that PRAT4A-independent intracellular LPS sensing does not mediate LPS toxicity. PRAT4A, controlling inflammatory responses via TLR4, might be a novel target for therapeutic intervention in endotoxin shock.

Cell surface expression of TLR4 was completely dependent on PRAT4A on Balb/c background but not on C57BL/6.
background. C57BL/6 background PRAT4A<sup>−/−</sup> BM-Macs still expressed TLR4/MD-2 on the cell surface (18), suggesting PRAT4A-independent cell surface expression of TLR4/MD-2. We have previously reported that PRAT4B, a molecule similar to PRAT4A, has a role in cell surface TLR4 expression in BaF3 cells (31). PRAT4B may be responsible for PRAT4A-independent cell surface TLR4 expression particularly on the B57BL/6 background. This possibility will be addressed in a future study.

The activity of intracellular TLR4/MD-2 seems to vary, depending on a subset of macrophages. Intracellular LPS sensing in P-Macs is much weaker than that in BM-Macs. An unknown mechanism enabling intracellular LPS sensing may work in BM-Macs but not in P-Macs. Alternatively, intracellular LPS sensing may be actively suppressed in P-Macs. It is also possible that a role for cell surface TLR4/MD-2 is distinct between BM-Macs and P-Macs. Lipid A-dependent production of MCP-1 (CCL2) and RANTES (CCL5) by PRAT4A<sup>−/−</sup> BM-Macs was not impaired at all when compared with wt BM-Macs, suggesting that cell surface TLR4/MD-2 does not contribute to production of these chemokines in BM-Macs. These issues will be addressed in a future study.

The present results propose the second criterion dissecting LPS responses by cell surface versus intracellular LPS sensing, in addition to the first criterion dependent on MyD88 and TRIF/TICAM-1. Future study has to focus on a role for intracellular LPS sensing in immune responses and a mechanism enabling intracellular LPS sensing in macrophages.

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**References**


