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TLR4-Mediated Podosome Loss Discriminates Gram-Negative from Gram-Positive Bacteria in Their Capacity to Induce Dendritic Cell Migration and Maturation

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Chronic infections are caused by microorganisms that display effective immune evasion mechanisms. Dendritic cell (DC)-dependent T cell-mediated adaptive immunity is one of the mechanisms that have evolved to prevent the occurrence of chronic bacterial infections. In turn, bacterial pathogens have developed strategies to evade immune recognition. In this study, we show that Gram-negative and Gram-positive bacteria differ in their ability to activate DCs and that Gram-negative bacteria are far more effective inducers of DC maturation. Moreover, we observed that only Gram-negative bacteria can induce loss of adhesive podosome structures in DCs, a response necessary for the induction of effective DC migration. We demonstrate that the ability of Gram-negative bacteria to trigger podosome turnover and induce DC migration reflects their capacity to selectively activate TLR4. Examining mice defective in TLR4 signaling, we show that this DC maturation and migration are mainly Toll/IL-1 receptor domain-containing adaptor-inducing IFNβ-dependent. Furthermore, we show that these processes depend on the production of PGs by these DCs, suggesting a direct link between TLR4-mediated signaling and arachidonic metabolism. These findings demonstrate that Gram-positive and Gram-negative bacteria profoundly differ in their capacity to activate DCs. We propose that this inability of Gram-positive bacteria to induce DC maturation and migration is part of the armamentarium necessary for avoiding the induction of an effective cellular immune response and may explain the frequent involvement of these pathogens in chronic infections. The Journal of Immunology, 2010, 184: 000–000.

Pathogenic bacteria are an important cause of morbidity and mortality worldwide. Most bacterial infections represent acute episodes that are effectively controlled by innate host defense mechanisms. If infections persist longer than one week, presentation of bacterial Ags by dendritic cells (DCs) to T cells leads to the induction of effective adaptive immune responses that will ultimately eliminate the invading pathogen, and prevent the occurrence of a chronic infection. Chronic bacterial infections are often caused by Gram-positive microorganisms (staphylococci or streptococci), which cause chronic soft tissue infections, or by Mycobacterium and Bartonella spp. Apparently, these pathogens developed effective mechanisms to prevent the induction of cellular immune responses. The signaling pathways responsible for the pattern recognition of these microorganisms likely play an important role in the subsequent activation of adaptive cellular responses. APCs, such as DCs, play an important role in inducing adaptive immunity, but surprisingly little is known about the activation of DC functions by these various classes of pathogens.

On the basis of composition and structure of their cell wall, bacteria can be classified as either Gram-positive or Gram-negative. These differences in outer membrane composition give rise to differential recognition by pattern recognition receptors (PRRs). TLRs constitute a family of PRRs that mediate cellular responses to a large variety of pathogens (viruses, bacteria, and parasites) by specific recognition of so-called “pathogen-associated molecular patterns.” Activation of TLRs, which comprise at least 11 different members that function either as homo- or heterodimers (1), leads to activation of NFκB-dependent and IFN-regulatory factor–dependent signaling pathways (2). LPS, an important cell wall component present on the prototype Gram-negative bacterial pathogens in humans, the enterobacteriaeae and Neisseria spp., is mainly recognized by TLR4. In contrast, peptidoglycans, lipoteichoic acid, and lipoproteins, structural components of Gram-positive bacteria predominantly activate TLR2 (3–5), often in a heterodimeric association with TLR1 or TLR6. In addition, TLRs can modulate each others’ function to affect immune responses (2). In addition, several nonenterobacteriaceal Gram-negative bacteria have been described, such as Bartonella spp., Chlamydia spp., Porphyromonas gingivalis, that contain LPS variants that cannot activate TLR4. As a consequence, these are mainly recognized by TLR2, similar to Gram-positive microorganisms (6, 7). DCs are among the most potent APCs of the immune system (8).
and they express a variety of PRRs, such as C-type lectins, NOD-like receptors, as well as TLRs (9–11). Activation of TLRs present on DCs, leads to the induction of both innate as well as adaptive immune responses. Upon ligation of TLRs, DCs become activated, mature to increase expression of costimulatory molecules and produce cytokines, and migrate to the lymph nodes where T and B cells can be activated. So far, most studies addressing the effects of TLR activation on DCs involve the use of highly purified or synthetic ligands. Much less is known about how DCs respond to intact pathogens, let alone different classes of bacteria.

In the current study, we sought to investigate how entire Gram-negative and Gram-positive bacteria affect DC maturation and especially the adhesive and migratory behavior of DCs. In previous studies, we observed that podosomes, specialized adhesion structures found in immature DCs (iDCs), dissolve in response to the purified TLR4 ligand LPS. Dissolution of podosomes was identified as an essential first step toward a highly migratory DC phenotype (12). In the current study, we show that only Gram-negative bacteria are capable of inducing podosome dissolution. These effects are completely dependent on TLR4 and downstream signals and mediated by production of PGs by the DCs. Moreover, Gram-negative pathogens that fail to stimulate TLR4 resemble Gram-positive bacteria in their inability to induce podosome dissolution. Ineffective DC activation and migration may thus represent one of the conditions necessary to establish a chronic infection by these pathogenic microorganisms.

Materials and Methods

Chemicals and Abs

The following Abs were used: anti-hCD80 and mlgG1 (Becton Dickinson, Franklin Lakes, NJ), anti-hCD83 (Beckman Coulter, Bicetre, France, The Netherlands), mlgG2a and mlgG2b (BD Pharmingen, San Diego, CA), anti-hC7 (R&D Systems, Minneapolis, MN), and antivinculin (Sigma-Aldrich, St. Louis, MO). Alexa Fluor 488-labeled secondary Abs were from Molecular Probes (Leiden, The Netherlands), FITC-labeled secondary Abs from Zymed (San Francisco, CA), and PE-labeled secondary Abs from Beckman Coulter and Becton Dickenson. Texas Red-conjugated phallolidin (Molecular Probes) was used to stain F-actin. Indomethacin and LPS [which was purified before use as described previously (13, 14)] were obtained from Sigma-Aldrich. Indomethacin was used at a concentration of 50 μM, because lower concentrations gave partial effects and concentrations >100 μM were toxic. Pam3Cys (Pam3Cys-SKIKK or Pam3CSK4) and FSL-1 [S-(2,3-bispalmitoyloxypropyl)-CGDPKHSPKSF] were obtained from EMC Microcollections (Tuebingen, Germany). TNF-α and MyD88−/− (21) mice were obtained from S. Akira (Osaka University, Osaka, Japan). The MyD88−/− mice were backcrossed more than eight times on the C57BL6 background. Lps2 mice were obtained from B. Beutler (The Scripps Research Institute, La Jolla, CA).

Preparation of mouse DCs

Bone marrow was isolated from the femurs of C57BL/6 wild type (WT), TLR2−/− or TLR4−/− mice. Bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS, 50 μg 2-ME (Sigma-Aldrich), mouse IL-4 (10 ng/ml) (DNAx, Schering-Plough, Palo Alto, CA), and GM-CSF (10 ng/ml) (PeproTech EC, London, U.K.). Immature bone marrow-derived DCs (BMDCs) were harvested at day 7. Expression of MHC class I and II, costimulatory molecules, and DC-specific markers on DCs were measured by flow cytometry. The immature BMDCs expressed high levels of MHC class I and II, CD11c and CD11b, moderate levels of CD80, CD86, CD40, and GR-1 and no expression of F4/80 could be detected (data not shown).

Flow cytometry

Cells (1 × 10^6) were incubated with 2% (v/v) human serum in PBA (PBS containing 1% [w/v] BSA and 0.05% [w/v] NaN3) for 15 min at 4°C. After washing with cold PBA, the cells were incubated with primary Ab (5 μg/ml) for 30 min at 4°C. Subsequently, the cells were incubated with PE-labeled goat anti-mouse. Cells were washed and resuspended in 100 μl PBA. Fluorescence was measured using a FACScalibur with CellQuest software (Becton Dickinson).

Fluorescence microscopy

Coverslips were coated with fibronectin (20 μg/ml) (Roche, Mannheim, Germany) in PBS at 1 h at 37°C. Cells were seeded on fibronectin-coated coverslips, left to adhere for 1–6 h after stimulation or being left unstimulated, the cells were fixed in 3.7% (v/v) formaldehyde in PBS for 10 min. Cells were permeabilized in 0.1% (v/v) Triton X-100 in PBS for 5 min and blocked with 2% (v/v) BSA in PBS. The cells were incubated with primary Ab for 1 h. Subsequently, the cells were washed with PBS and incubated with Alexa Fluor 488-labeled secondary Abs for 45 min. Finally, cells were incubated with Texas Red-conjugated phallolidin for 30 min prior to embedding in Mowiol (Sigma-Aldrich). Images were collected on a Leica DMRA fluorescence microscope with a 63× PL APO 1.3 NA immersion lens or a 40× PL Fluotar 1.0 NA oil immersion lens and a COHU high-performance integrating CCD camera (COHU, San Diego, CA). Pictures were analyzed with Leica Q-fluor software V1.2.0 (Leica Microsystems, Bannockburn, IL) and Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA) software.

Migration assay

Cells were seeded on fibronectin-coated coverslips, stimulated as indicated, and stained with an antimycilin mAb and phallloidin-Texas Red to detect F-actin. The number of cells displaying podosomes was counted in seven images per condition per experiment, this corresponds to 50–115 cells per experiment. The number of independent experiments is indicated in the legends. An average with SEM is given.

Cytokine and PGE2 measurement

Concentrations of human TNF-α, IL-6, and IL-8 were measured using a human inflammation cytokine bead array (CBA) (Becton Dickinson) according to the manufacturer’s instructions. This CBA has an upper limit of 1 × 10^9 pg/ml. Concentrations of human TNF-α were determined using specific RIAs as described previously (22). Concentrations of human IL-6 were measured by using a commercial ELISA kit (Sanquin, Amsterdam, The Netherlands). Concentrations of PGE2 were measured by using a commercial ELISA kit (Assay Designs, Ann Arbor, MI).
evaporation of the medium. Cell migration was tracked using a previously described migration system (23). Cells were recorded for 1 h, followed by analysis of individual cells. The speed was defined as traversal path during the entire experiment divided by the imaging time.

Transwell migration
Migration of DCs toward CCL21 was assessed by a transwell migration assay. DCs were left unstimulated or stimulated with LPS, *S. enteritidis*, *N. meningitidis*, *S. aureus*, or *S. pneumoniae* for 48 h. The 1 × 10⁵ cells in 200 μl RPMI 1640 with GM-CSF and IL-4 were transferred into the upper chamber of 5-μm pore size polycarbonate filters in 24-well Transwell chambers (Costar, Corning). The lower chamber contained 600 μl RPMI 1640 with GM-CSF and IL-4 and with or without 100 ng/ml CCL21 (R&D Systems). Cells were allowed to migrate for 2 h. The migrated cells from the bottom chamber were harvested and counted by flow cytometry in a FACScan (Becton Dickinson), acquiring events for a period of 60 s using WinMDI software. All conditions were tested in duplicate.

T cell proliferation
T cell proliferation was assessed by [³H]thymidine incorporation. MLR was induced by DCs left unstimulated or stimulated with LPS, *S. enteritidis*, *N. meningitidis*, *S. aureus*, or *S. pneumoniae* for 48 h. PBL (1 × 10⁵) were cocultured with 1 × 10⁵ DCs in 96-well round-bottom plates for 4 d. PBL proliferation was assessed by measuring [³H]thymidine incorporation (1 μCi/well, 8–16 h pulse, MP Biomedicals, Irvine, CA).

Statistical analysis
An ANOVA test or a two-tailed Student t test was used for statistical analysis. The p < 0.05 (and p < 0.01 for the MLR) was considered significant.

Results
Gram-negative bacteria are superior to Gram-positive bacteria in inducing DC maturation
We compared the intact Gram-negative bacteria *N. meningitidis* and *S. enteritidis*, as well as the Gram-positive bacteria *S. aureus* and *S. pneumoniae* for their ability to activate DCs. The effects of these pathogens on DC maturation was determined by measuring the induction of cytokine secretion as well as the expression of the costimulatory molecules CD80 and CD83, and the chemokine receptor CCR7, which is essential for DC migration to the draining lymph nodes. Exposure of DCs to Gram-negative bacteria resulted in high levels of TNF-α, IL-6, and IL-8 and the extent of cytokine secretion observed in response to whole bacteria was comparable to that induced by LPS (Fig. 1A). In the CD80, CD86, and CCR7 expression measurements, the mean fluorescence intensity values with isotype controls were between 3.5 and 4.3 for mIgG1 (CD80), between 3.5 and 12.4 for mIgG2b (CD83), and between 6.2 and 7.8 for mIgG2a (CCR7). In addition to stimulating cytokine production, exposure of DCs to Gram-negative bacteria gave rise to profound upregulation of CD80, CD83, as well as CCR7 compared with iDCs (Fig. 1B). By contrast, secretion of cytokines and expression of maturation markers was lower when DCs were exposed to Gram-positive bacteria instead of Gram-negative bacteria or LPS (Fig. 1B). In addition to promoting cytokine production and DC maturation, DCs stimulated with LPS or Gram-negative bacteria were effective inducers of T cell proliferation in a MLR, whereas DCs stimulated with *S. pneumoniae* were not (Fig. 1C). Unexpectedly, DCs stimulated with the Gram-positive strain *S. aureus* were also potent stimulators of T cell proliferation (Fig. 1C), which could be explained by the presence of *S. aureus* super-Ags. Super-Ags induce T cell proliferation by binding to MHC molecules on the DCs as well as TCRs on the T cells (24). Hence, this type of stimulation bypasses the need for costimulatory molecules and is thus independent of the maturation state of the DCs.

To efficiently activate T cells in vivo, DCs must travel to the draining lymph nodes. Hence, the induction of migratory capacity is an important aspect of DC maturation.

Therefore, random DC migration on fibronectin was investigated. About 20–40% of the unstimulated iDCs or Gram-positive–stimulated DCs were migratory, whereas exposure to Gram-negative bacteria or LPS not only raised migration speed (results not shown), but also increased the total number of migrating cells to ~70% (Fig. 1D). The failure of Gram-positive bacteria to induce CCR7 expression indicates that the latter may also show a reduced ability to stimulate DC migration toward CCL21 and CCL19. To test this assertion, transwell migration toward CCL21 was investigated. Although the observed differences were small and did not reach statistical significance, Gram-negative bacteria or LPS consistently induced more migration toward CCL21 than Gram-positive bacteria (Supplemental Fig. 1). Taken together, these results show that intact Gram-negative bacteria are more efficient inducers of DC maturation and migration than Gram-positive bacteria.

Strains of Gram-negative but not Gram-positive bacteria induce podosome loss in DCs
Next to phenotypic alterations, DC maturation is accompanied by profound cell behavioral changes. Whereas iDCs are strongly adhesive and attach to the substrate through specialized adhesions known as podosomes, mature DCs (mDCs) show much less matrix attachment. This reduced cell-matrix adhesion allows mDCs to migrate at much higher speeds. Previously, we showed that podosome dissolution, in response to maturation stimuli, precedes induction of high-speed migration (12). Because we observed major differences in the migratory properties of DCs exposed to intact Gram-negative and Gram-positive bacteria, we examined if their ability to induce podosome dissolution was also affected. Indeed, although all the Gram-negative bacterial species tested induced podosome dissolution successfully, the Gram-positive bacteria failed to induce podosome dissolution (Fig. 2A, 2B). It is important to note that Gram-positive bacteria do not actively promote podosome dissolution, because the addition of PGE₂, which rapidly induces podosome loss (12), can rescue podosome loss in DCs stimulated with Gram-positive bacteria (Fig. 2C). In addition, we observed that the Gram-negative species *Klebsiella pneumoniae* (ATCC 10031) was also capable of inducing podosome loss (data not shown) and because the LPS used here was derived from *Escherichia coli*, *E. coli* are expected to induce the same responses. Furthermore, Gram-negative strains, such as *Mycobacterium BCG*, and additional isolates of *S. pneumoniae*, either with or without capsules, gave similar results as the other Gram-negative strains tested (data not shown). These results indicate that our findings are likely to apply to a broad range of bacteria. We conclude that Gram-negative bacteria effectively induce many aspects of DC maturation, including podosome dissolution, but that Gram-positive bacteria fail to do so. Moreover, we show that podosome turnover accurately predicts DC maturation in response to exposure to bacterial pathogens.

We next examined whether the observed differences between Gram-negative and Gram-positive bacteria might reflect the ability of the DCs to either bind and/or internalize these pathogens. Therefore, the binding of FITC-labeled bacteria to DCs was analyzed by flow cytometry. We did not observe differences in the extent of binding of Gram-negative and Gram-positive bacteria (Fig. 2D). In addition, DCs incubated with FITC-labeled bacteria were analyzed by making z-stacks with confocal microscopy to confirm phagocytosis. DCs internalized Gram-negative equally effective as Gram-positive bacteria (Fig. 2F). We conclude that the failure of Gram-positive bacteria to induce DC maturation is not due to a lack of binding or internalization.

**Purified and synthetic TLR ligands closely mimic intact bacteria**
Gram-negative bacteria are primarily recognized by TLR4, whereas Gram-positive bacteria activate TLR2. Therefore, we investigated whether activating these receptors with purified/synthetic ligands would mimic the effect of intact bacteria. As we previously
showed (12), E. coli-derived LPS, a TLR4 ligand, was able to induce podosome loss. However, the TLR2/1 ligand Pam3Cys and the TLR2/6 ligand FSL-1 alone or in combination failed to dissolve podosomes (Fig. 3A, 3B). Also prolonged stimulation with TLR2 ligands did not induce podosome loss, suggesting that the difference is not due to delayed responses to Gram-positive bacteria. TLR2 ligands were able to evoke some effect on the DCs, because stimulation with Pam3Cys or FSL-1 induced production of TNF and IL-6, although the levels were significantly lower than those observed in response to LPS (Fig. 3C). A potential synergy between the TLR4 and TLR2 ligands was investigated by combining a suboptimal concentration of LPS (6 ng/ml), derived from LPS serial dilutions (Fig. 3D), with TLR2 ligands. However, no synergy between LPS and TLR2 ligands could be observed (Fig. 3E). We conclude that also purified TLR4 and TLR2 ligands differ in their capacity to induce podosome dissolution.

Loss of podosomes and induction of migration is critically dependent on TLR4 signaling on binding to LPS

To test the role of LPS on intact bacteria we examined two strains of intracellular Gram-negative bacteria, B. quintana and C. pneumoniae, which each carry a structurally distinct form of LPS that is not capable of TLR4 signaling (6, 25, 26). Stimulation of iDCs with B. quintana or C. pneumoniae had no effect on the amount of podosomes (Fig. 4A), suggesting that podosome loss induced by Gram-negative bacteria requires TLR4 activation. Consistent with this notion, addition of the TLR4 antagonist B. quintana LPS (6) completely blocked the LPS- or Gram-negative bacteria-induced podosome loss (Fig. 4B), confirming that the response indeed is TLR4 mediated.
We also tested the effects of differential TLR activation on podosome turnover using mouse BMDCs. Again, podosome dissolution was observed after exposure to LPS and Gram-negative bacteria, but not in response to Pam3Cys, FSL-1, or Gram-positive bacteria (Fig. 4C, 4D), showing that these differential responses are conserved between mice and humans. In addition to BMDCs derived from WT mice, TLR2 and TLR4 knockout BMDCs were also used. We observed that the LPS- and Gram-negative bacteria-induced podosome dissolution was intact in TLR2−/− BMDCs, whereas abrogated in TLR4−/− BMDCs (Fig. 4C, 4D). WT, TLR2−/−, as well as TLR4−/− BMDCs similarly underwent rapid podosome dissolution in response to PGE2 (data not shown).

FIGURE 2. Gram-negative bacteria induce podosome loss, whereas Gram-positive bacteria do not. A and B, iDCs were left untreated or stimulated with LPS, N. meningitidis, S. enteritidis, S. aureus, or S. pneumoniae at MOI 20 for 16 h. A, Podosomes were stained with an antivinculin mAb (green) and phalloidin-Texas Red to detect F-actin (red). Representative images are depicted. Scale bar indicates 20 μm (original magnification ×40). B, The average number of cells displaying podosomes of four experiments (± SEM) is shown. Significant differences (p < 0.05) from control are indicated with an asterisk (*). C, iDCs were left untreated or stimulated with S. aureus or S. pneumoniae at MOI 20 for 16 h with or without 10 μg/ml PGE2 for 5 min or PGE2 alone. The average number of cells displaying podosomes of five experiments (± SEM) is shown. Significant differences (p < 0.05) from control are indicated with an asterisk (*). D and E, iDCs were incubated for 45–60 min with FITC-labeled bacteria. D, The amount of DCs that had bound (or internalized) bacteria was analyzed by flow cytometry. A representative experiment is shown. E, Internalization was confirmed by making z-stacks using a Zeiss LSM 510-meta microscope. Representative images are depicted. Scale bar indicates 10 μm.
showing that extracellular signals other than TLR4 ligands can still induce podosome dissolution in these cells. Together, these results show that podosome dissolution induced by either LPS or Gram-negative bacteria requires TLR4 signaling.

Podosome loss in DCs induced by Gram-negative bacteria requires mainly Toll/IL-1 receptor domain-containing adaptor-inducing IFNβ-dependent signaling

To date, two major signaling routes downstream of TLR4 have been described: one downstream of the adaptor protein MyD88, and the other dependent on the adaptor protein Toll/IL-1 receptor (TIR) domain-containing adaptor-inducing IFNβ (TRIF) (27). To investigate the involvement of these two signaling routes, we used MyD88−/− and Lps2 mice. The latter carry a natural mutation resulting in TRIF deficiency (28). First, we established the capacity of BMDCs from these mice to resolve podosomes similar to WT BMDCs when incubated with PGE2. Both WT and mutated BMDCs underwent rapid podosome dissolution in response to PGE2 (data not shown) indicating that DCs from these mutant mice responded normally. In contrast, responses to LPS or *N. meningitidis* as well as *S. enteritidis* were significantly impaired in Lps2 BMDCs relative to WT BMDCs (Fig. 5A, 5B). For the MyD88−/− BMDCs, the results were less apparent. Although podosome loss seemed impaired in response to LPS and *S. enteritidis*, only with *N. meningitidis* was a significant reduction in podosome dissolution observed (Fig. 5A, 5B). We therefore concluded that podosome loss induced by Gram-negative bacteria was critically dependent on TRIF, but may also involve MyD88-mediated signaling.

Podosome loss induced by Gram-negative bacteria is the result of TLR4-mediated production of PGs

We showed previously that PGE2 can induce rapid podosome loss and that LPS-induced podosome loss is dependent on PG production by the DCs (12). Therefore, we investigated whether the TLR4-mediated podosome loss in response to Gram-negative bacteria was similarly
dependent on the production of PGs. Addition of indomethacin, a potent inhibitor of PG production (29), significantly blocked podosome loss induced by Gram-negative bacteria (Fig. 6A). These findings suggest that this response is, at least in part, dependent on the production of PGs by the DCs. In addition, we tested whether medium conditioned by DCs stimulated with different TLR ligands or bacteria, was capable of inducing podosome turnover. Indeed, medium conditioned by LPS or Gram-negative bacteria, but not Gram-positive bacteria or TLR2 ligands, was capable of inducing podosome disassembly (Fig. 6B, 6C). Again, these responses were partly abrogated when this medium had been conditioned in the presence of indomethacin (Fig. 6C). Because DCs stimulated with LPS or Gram-negative bacteria produce more TNF-α, IL-6, and IL-8, TNF-α is also able to induce podosome dissolution (12), we investigated if the LPS-induced podosome loss could be mediated by signaling of TNF-α or IL-1. Blocking TNF-α or IL-1 signaling by using TNF-binding protein or an IL-1 receptor antagonist, respectively, had no effect on LPS-induced podosome loss (Supplemental Fig. 2). In addition, no effect on LPS-induced podosome loss was observed on treatment with IL-1 converting enzyme-inhibitor (Supplemental Fig. 2), which blocks IL-1β and IL-18 and decreases IL-1α, TNF-α, IL-6, and IFN-γ (30, 31). Together, these findings suggest that podosome dissolution induced by Gram-negative bacteria or LPS is not mediated by the production of these cytokines. Finally, we compared the amount of PGE2 present in the supernatants of DCs stimulated with Gram-negative or Gram-positive bacteria. The levels of PGE2 were consistently higher in supernatants of DCs stimulated with Gram-negative bacteria, in comparison with unstimulated DCs or DCs exposed to Gram-positive bacteria (Fig. 6D). These findings further support the idea that PGs, particularly PGE2,
FIGURE 5. LPS- and Gram-negative bacteria-induced podosome loss mediated by TRIF and MyD88.

A and B. Immature BMDCs from WT, LPS2 (TRIF<sup>−/−</sup>), and MyD88<sup>−/−</sup> mice were left untreated or stimulated with LPS, Pam3Cys, FSL-1, N. meningitidis, S. enteritidis, S. aureus, or S. pneumoniae for 16 h. A. Podosomes were stained with an antivinculin mAb (green) and phalloidin-Texas Red to detect F-actin (red). Representative images are depicted. Scale bar indicates 20 μm (original magnification ×63). B. The average number of cells displaying podosomes of four experiments (± SEM) is shown. Significant differences (p < 0.05) from WT are indicated with an asterisk (*).
produced by DCs in response to TLR4 activation, mediated podosome dissolution and led to a migratory phenotype, as is depicted in Fig. 7.

**Discussion**

In the current study, we demonstrated that intact Gram-negative bacteria, but not Gram-negative bacteria containing aberrant forms of LPS or Gram-positive bacteria, transform adhesive iDCs into a highly migratory cell type through TLR4-mediated signals (Fig. 7). These findings demonstrate that Gram-positive and Gram-negative bacteria profoundly differed in their ability to activate DCs and stimulate DC migration. Based on these findings, we propose that the defective DC migration and maturation by Gram-positive microorganisms is one of their strategies for avoiding the induction of an effective cellular immune response. In contrast, potent TLR4-dependent activation of DCs impairs the capacity of enterobacteria to promote chronic infections.

Chronic infections caused by microorganisms that display effective immune evasion mechanisms still form a major worldwide threat. Specific T cell-mediated immunity, induced by interaction of APCs is one of the main antibacterial mechanisms preventing the occurrence of a chronic infection. The development of an effective adaptive immune response depends on a proper activation and maturation of the APCs, most notably the DCs. This not only involves upregulation of costimulatory molecules and secretion of cytokines such as IL-12, but also transformation from tissue-resident iDCs into highly migratory mDCs (8, 12, 32, 33), allowing efficient migration to draining lymph nodes. In this study, we used both human monocyte-derived DCs as well as mouse BMDCs, whereas we have shown previously that human DCs isolated directly from the blood also develop podosomes which dissolve in response to maturation stimuli (12). Hence, the presence of podosomes and their disappearance in response to maturation stimuli appears to be a universal property of (myeloid) DCs, irrespective of their origin or method of purification.

Instead of testing purified microorganism-derived components, we compared intact Gram-positive and Gram-negative bacteria. Although both types of bacteria are equally well bound and internalized by DCs, we found that only Gram-negative bacteria effectively upregulated the costimulatory molecules CD80 and CD83. In addition, DCs are induced to secrete proinflammatory cytokines TNF-α, IL-6, and IL-8, needed for attracting and stimulating T cells (34, 35), in response to Gram-negative bacteria. However, we and others (36) found that cytokine secretion in response to Gram-positive bacteria is severely impaired. Consistent with these observations, DCs exposed to the Gram-negative bacteria *N. meningitidis* and *S. enteritidis* were effective inducers of T cell proliferation, whereas DCs exposed to Gram-positive *S. pneumoniae* were not, suggesting that Gram-negative bacteria are also more capable of stimulating DC-mediated T cell activation. However, stimulation of DCs with Gram-positive *S. aureus* induced profound T cell proliferation. These seemingly contradictory results most likely reflect the action of *S. aureus* super-Ags. These super-Ags are known to induce proliferation of T cells in the absence of co-stimulation, bypassing the need of DC maturation (24, 37).

On activation, DCs dramatically alter their adhesive properties, which allows them to migrate to and into the draining lymph nodes to become more migratory.
stimulate T cells. By comparison, iDCs are much less migratory and express chemokine receptors (CXCR4, CCR5) that mainly respond to steady-state chemokines, depending on their cellular localization (38). During DC maturation, podosome dissolution represents a first step toward a migratory phenotype. In addition, the activity of beta1 and beta2 integrins, both of which can be found enriched in podosomes, is decreased during maturation (12, 39). Furthermore, these cells gradually become responsive to the chemokines CCL19 and CCL21. These two chemokines were shown to play an important role in directing migration of DCs to the lymph nodes (12, 38). In this study, we find that intact Gram-negative bacteria more effectively induce podosome dissolution and cell migration than Gram-positive bacteria. This increase in cell migration appears to be, at least in part, dependent on the expression of CCR7, the chemokine receptor for CCL19 and CCL21 (40), as migration toward CCL21 is higher in response to stimulation with Gram-negative bacteria.

The regulation of podosome formation and loss during differentiation and in response to inflammatory stimuli differs between cell types. In less differentiated cells of the myeloid lineage, stimulation can lead to increased adhesion and podosome formation, for instance IL-13 or CSF in macrophages, receptor activator of NFκB ligand in osteoclasts and Raw264.7 cells, and phorbol 12-myristate 13-acetate in THP-1 and HL-60 cells (41–44). On activation, most cells of the innate immune system even further increase their adhesive capacity. In contrast, DCs become less adhesive in response to inflammatory stimuli. DCs (as well as phorbol 12-myristate 13-acetate–stimulated Th precursor-1 and HL-60 cells) undergo podosome dissolution in response to PGE2 or TNF-α (12, 42), whereas eosinophils or endothelial cells generate podosomes in response to TNF-α (45, 46).

Because we used intact bacteria in this study, we explored whether the activation of DC by Gram-negative bacteria exclusively depends on LPS and TLR4 activation or if other cell wall components or PRRs could also be involved. By using TLR4 antagonist and BMDCs derived from TLR4−/− mice, we demonstrate that the effects of Gram-negative bacteria are completely dependent on TLR4 signaling. Consistent with these findings, it was shown earlier that a LPS-deficient strain of *N. meningitidis* is incapable of bringing about efficient DC activation (47, 48). In addition, both *B. quintana* and *C. pneumonia*, express a form of LPS that prevents activation of TLR4 and cannot induce podosome loss in DCs, further demonstrating the critical role of TLR4 in DC activation (6, 25, 26). Although both TLR2 and TLR4 can activate MyD88 (49–52), only TLR4 can also induce TRIF-dependent signaling (27, 53). We observed that Gram-negative bacteria-mediated TLR4 signaling mainly involves TRIF. This is in line with previous studies suggesting that the effects of living or heat-killed *E. coli* on DCs are TLR4/TRIF mediated (54). Podosome loss, induced by LPS or Gram-negative bacteria, could be caused by cytokines that are secreted in response to these stimuli or other proinflammatory mediators produced in response to TLR activation. As we failed to observe any effect when interfering with the secretion or signaling of TNF-α or IL-1, we conclude that at least these cytokines are not responsible for the podosome loss induced by LPS or Gram-negative bacteria. Activation of TLR signaling leads to the production of IFN-α or IFN-β. However, addition of IFN-α did not affect podosomes in DCs (data not shown), which makes it also unlikely that IFNs contribute to podosome dissolution. Instead, TLR4-mediated dissolution of podosomes more likely depends on PGE2. This PG, which is widely used to promote migration of clinical DC isolates (55), is produced by DCs in response to LPS but not synthetic TLR2 ligands (data not shown). We previously demonstrated that PGE2 binding to the PG receptors EP2 and EP4 results in an almost immediate dissolution of podosomes (12). Moreover, the levels of PGE2 in supernatants of DCs stimulated with Gram-negative bacteria or LPS are higher than those in supernatants of unstimulated DCs or DCs exposed to Gram-positive bacteria. The ability of these conditioned supernatants to induce podosome loss and the effects of cyclooxygenase (COX) inhibition on podosome dissolution, support a role for PGs, particularly PGE2, in podosome dissolution.

The cellular production of PGs downstream of TLR4 may involve JNK and p38MAPK signaling, which are activated in response to TLR4 triggering (56) and known to induce COX enzymes (57, 58). Our observation that TLR4-mediated podosome loss is mainly TRIF dependent suggests that TRIF-dependent signaling is mainly responsible for activation of COX enzymes and the production of PGs. The observation that Gram-negative bacteria induce strong DC maturation and migration, whereas Gram-positive pathogens are much less effective could explain the inability of classical Gram-negative human pathogens such as enterobacteriaceae or *N. meningitidis* to cause chronic infections. An exception may be severely immunocompromised patients, who are at high risk of having infections of Gram-negative bacteria and sepsis develop (59) and therefore routinely receive antibiotics directed against these bacteria (selective gut decontamination) (60). Gram-positive bacteria (e.g., staphylococci, streptococci,
Mycobacterium spp.) or Gram-negative bacteria containing an LPS variant that cannot stimulate TLR4, are well known to evade immune detection (Bartonella spp., Chlamydia) and a frequent cause of chronic bacterial infections.

Chronic infections of the soft tissues and bones are often caused by staphylococci or streptococci, but rarely by Gram-negative pathogens (61, 62). The occurrence of a chronic infection involves complex interactions between pathogen and host. Based on our findings, induction of differential DC activation by distinct classes of microorganisms may be a critical factor in determining the etiology of chronic infections. We propose that the lack of TLR4-mediated PGE2 production impairs DC maturation and migration and therefore may hamper the development of a proper adaptive immune response.

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Disclosures

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