CellPress

Molecular Cell
Review

A Cross-Disciplinary Perspective on the Innate Immune Responses to Bacterial Lipopolysaccharide

Yunhao Tan[1] and Jonathan C. Kagan[1,*]
[1]Division of Gastroenterology, Boston Children's Hospital and Harvard Medical School, Boston, MA 02115, USA
*Correspondence: jonathan.kagan@childrens.harvard.edu
http://dx.doi.org/10.1016/j.molcel.2014.03.012

The study of innate immunity to bacteria has focused heavily on the mechanisms by which mammalian cells detect lipopolysaccharide (LPS), the conserved surface component of Gram-negative bacteria. While Toll-like receptor 4 (TLR4) is responsible for all the host transcriptional responses to LPS, recent discoveries have revealed the existence of several TLR4-independent responses to LPS. These discoveries not only broaden our view of the means by which mammalian cells interact with bacteria, but they also highlight new selective pressures that may have promoted the evolution of bacterial immune evasion strategies. In this review, we highlight past and recent discoveries on host LPS sensing mechanisms and discuss bacterial countermeasures that promote infection. By looking at both sides of the host-pathogen interaction equation, we hope to provide comprehensive insights into host defense mechanisms and bacterial pathogenesis.

Introduction

Precise spatial and temporal regulation of the innate immune response is essential for the host to defend against pathogen infection. However, when such responses become overamplified or dysregulated, the resulting disruption of homeostasis may lead to the development of immunopathology. In line with this, a deleterious outcome that correlates with bacterial infection is sepsis, which is a clinical syndrome with a high mortality rate (Angus et al., 2001). Specifically, during Gram-negative bacterial infection, lipopolysaccharide (LPS), the major cell wall component of these microbes, plays a dominant role in the induction of sepsis and hence is referred to as endotoxin. On the molecular level, a hallmark of sepsis is the uncontrolled release of proinflammatory cytokines (Cohen, 2002). However, for more than a century, the mechanism of sepsis induction has remained elusive due to the obscure nature of the cellular receptor for LPS. In the past two decades, a series of elegantly designed studies unraveled the host surface molecules responsible for LPS sensing. These molecules include LPS binding protein (LBP), CD14, MD-2, and TLR4 (Gioannini and Weiss, 2007). Among them, TLR4 represents a central mediator of innate and adaptive immune responses to LPS (Medzhitov, 2009; Poltorak et al., 1998a). The observation that TLR4-deficient cells lose all the canonical responses to LPS, such as expression of proinflammatory cytokines and interferons (IFNs), has established the dogma that TLR4 accounts exclusively for all the host responses to LPS (Beutler, 2000; Beutler et al., 2001). Strikingly, recent studies have revealed the existence of host responses to LPS that do not require TLR4 (Hagar et al., 2013; Kayagaki et al., 2013; Zanoni et al., 2009, 2011). These new discoveries allow the field to move away from the TLR4-centric view of LPS signaling and begin focusing on questions of how the diverse LPS response pathways in mammalian cells are integrated. Moreover, these discoveries reveal opportunities to uncover novel immune-evasion strategies used by successful bacterial pathogens. In this review, we adopt a host-pathogen interaction perspective by highlighting both sides of this equation. We first focus on the identification and operation of the LPS response pathways that are induced by bacteria, thus painting a picture that would lead to a more thorough understanding of Gram-negative bacteria-induced sepsis. We then focus on the means by which nature has permitted some bacteria to evade these LPS response pathways. This latter discussion reveals a much more comprehensive view of host-microbe interactions and highlights the fact that during the evolutionary "arms race" with the host, bacterial pathogens have developed sophisticated mechanisms to subvert host defense.

Identification of Proteins that Function as Sensors of Extracellular LPS

Crucial to the innate immune system is its rapid responsiveness to the presence of infectious agents. In the case of Gram-negative bacterial infection, mammalian cells have evolved the capability to sense picomolar concentrations of LPS (Gioannini and Weiss, 2007). Such a level of sensitivity is thought to facilitate microbial detection and the control of infection as rapidly as possible. Tremendous efforts have been devoted to elucidating the host molecules that participate in LPS sensing over the last 25 years.

The isolation of LBP from acute phase rabbit serum represented a landmark in our knowledge of host LPS sensing (Tobias et al., 1986). LBP exhibits high affinity to LPS and hence mediates the extraction and disaggregation of LPS from the cell wall of Gram-negative bacteria. Furthermore, LBP functions as an opsonin to facilitate the binding of bacteria or LPS-coated red blood cells to macrophages, which can subsequently induce tumor necrosis factor alpha (TNFα) secretion (Schumann et al., 1990). Thus, it has been proposed that LBP delivers LPS to a putative receptor expressed on the macrophage surface. The first candidate for this putative receptor was CD14. Nongenetic evidence in support of this idea came from the use of monoclonal antibodies against CD14 that downregulated the adhesion of LBP-LPS complexes to macrophages (Wright et al., 1990). Additionally, a chemically modified LPS variant has been shown to crosslink CD14 on the surface of human THP-1 monocytes (Tobias et al., 1993). Originally described as a marker for myeloid

CellPress

lineage cells, CD14 is anchored on the macrophage surface by its C-terminal glycosylphosphatidylinositol (GPI) lipid modification, without any transmembrane segments (Haziot et al., 1988). Interestingly, a soluble form of CD14 (sCD14) that lacks its GPI membrane anchor exists in plasma and plays a role in sensitizing CD14-negative cells (e.g., epithelial and endothelial cells) toward LPS stimulation (Frey et al., 1992; Pugin et al., 1993). Genetic proof of the importance of CD14 in LPS detection derives from studies using mice lacking this gene. CD14-deficient mice are resistant to doses of LPS that are lethal to wild-type mice (Haziot et al., 1996), and CD14-deficient cells are defective for cytokine expression induced by low concentrations of LPS. However, these mice also suggested that CD14 was not solely responsible for LPS responses, as CD14-deficient cells were still able to respond to high doses of LPS (Haziot et al., 1996; Perera et al., 1997). This observation, coupled to that fact that CD14 does not possess a transmembrane domain, suggested that additional protein(s) might mediate signal transduction from the cell surface to the cytoplasm.

The identification of TLR4 by a genetic approach solved this puzzle. Long before the dawn of molecular biology, it was reported that mouse strains such as C3H/HeJ and C57BL/10ScCr were resistant to the lethal effect of LPS treatment (Coutinho et al., 1977; Heppner and Weiss, 1965). Consequently, the genomic region containing the mutation that renders C3H/HeJ mice hyporesponsive to LPS was named the *Lps* locus (Poltorak et al., 1998b). Shortly after the discovery of the human TLR4 gene (Medzhitov et al., 1997), Craig Gerald noted that the human TLR4 locus was syntenic with the mouse LPS locus (Gerard, 1998), suggesting that TLR4 was involved in LPS detection and signaling. Positional cloning of the functional gene in the LPS locus revealed that TLR4 was indeed required for LPS responses in mice (Poltorak et al., 1998a). Reverse genetics approaches adopted by Shizuo Akira and colleagues to generate TLR4 knockout mice substantiated the central role of TLR4 in LPS detection (Hoshino et al., 1999). Indeed, TLR4-deficient mice are resistant to traditional murine models of LPS-induced sepsis, and TLR4-deficient macrophages are unable to induce the expression of any genes in response to LPS.

While mouse genetics defined the critical role of TLR4 in LPS signaling, attempts to reconstitute the TLR4-LPS signaling pathway in vitro revealed that an additional accessory molecule(s) was required. For example, expression of TLR4 in the human HEK293 cells or mouse Ba/F3 cells did not confer LPS responsiveness to these cell lines (Shimazu et al., 1999), suggesting that another molecule is required to permit TLR4 to signal in response to LPS. Bioinformatic analysis provided clues to the identity of this unknown molecule. In the early 1990s, it was discovered that radioprotective protein 105 (RP105) could convey signals that are important for B cell activation and resistance to radioactive stress (Miyake et al., 1994). Subsequently, a secreted protein called MD-1 was found to interact with the leucine-rich repeat domain of RP105 and increase the expression of this protein. Based on the N-terminal sequence homology between RP105 and TLR4, it was speculated that a protein similar to MD-1 might interact with TLR4, facilitating the function of this receptor. Indeed, computational analyses identified a protein homologous to MD-1, which was therefore named MD-2 (Shimazu et al., 1999). MD-2 expression conferred LPS responsiveness to the Ba/F3 cell line stably expressing TLR4, and MD-2 physically interacted with the extracellular domain of TLR4. Perhaps most interestingly, MD-2 binds to LPS directly (Shimazu et al., 1999; Viriyakosol et al., 2001) and does so with high affinity. Derivation of MD-2 knockout mice solidified its role in LPS signaling, as these mice phenocopy TLR4 deficiency in all assays of LPS-induced gene expression (Meng et al., 2011; Nagai et al., 2002b). Taken together, CD14 and MD-2 were revealed as critical molecules required for LPS sensing, whereas TLR4 was responsible for inducing LPS-induced signal transduction.

Recognition of LPS by the TLR4/MD-2 Complex

Knowledge from charactering the composition of LPS provides a solid basis for our understanding of how it is recognized by the TLR4/MD-2 complex. Serving as the major outer membrane component of Gram-negative bacteria, LPS is not a simple, homogenous compound. Rather, it is a mixture of lipid and carbohydrates with high degree of structural complexity. In general, LPS is comprised of three components: the O antigen, the core oligosaccharide, and lipid A. Among these, lipid A accounts for most of the immunostimulatory effects of LPS (Rietschel et al., 1994). In the case of *Escherichia coli* LPS, the lipid A backbone consists of two phosphorylated glucosamine molecules, to which six acylated lipid chains with a carbon length of 12 to 16 are attached covalently. The phosphorylation status, the number, and the carbon length of acyl chains determine the agonistic activity of lipid A to the TLR4/MD-2 complex (Raetz et al., 2007). As will be discussed in more detail in this review, alteration in lipid A structure is a virulence strategy exploited by bacteria to avoid host detection. Interestingly, the recognition by TLR4/MD-2 of lipid IVa, the precursor of lipid A that only contains four acyl chains, exhibits species-specific activity. For instance, lipid IVa acts as an antagonist on human macrophages, whereas it is a weak agonist on mouse macrophages (Golenbock et al., 1991).

A growing body of structural studies revealed the underlying mechanism of LPS recognition by TLR4/MD-2. In the absence of LPS, MD-2 forms a heterodimer with the horseshoe-like extracellular domain of TLR4 (Kim et al., 2007; Park et al., 2009). Such binding is mediated largely by hydrophilic interactions. The MD-2 binding interface on TLR4 is comprised of the A and B patches. The A patch contains mostly negatively charged residues, whereas the B patch is mainly positively charged. Respectively, the TLR4-binding surface of MD-2 consists of residues that are charge-complimentary to TLR4. MD-2 belongs to a small β cup fold protein family, with the LPS-binding pocket formed by two antiparallel β sheets (Ohto et al., 2007). The high affinity binding between LPS and MD-2 is achieved by the optimized distribution of the hydrophobic residues inside the pocket. In particular, five of the six acyl chains in lipid A fit inside the MD-2 cavity, whereas the sixth acyl chain extends on the surface of MD-2. Such partial exposure of the sixth acyl chain is essential for the hydrophobic interaction with the C-terminal portion of the TLR4 extracellular domain from another TLR4/MD-2 complex, thereby forming the M-shaped TLR4/MD-2/LPS dimer (in a 2:2:2 format) (Park et al., 2009). LPS-induced dimerization of the two TLR4/MD-2 complexes in the extracellular space likely facilitates the dimerization of the TLR4 intracellular TIR domain that subsequently initiates TLR signaling.

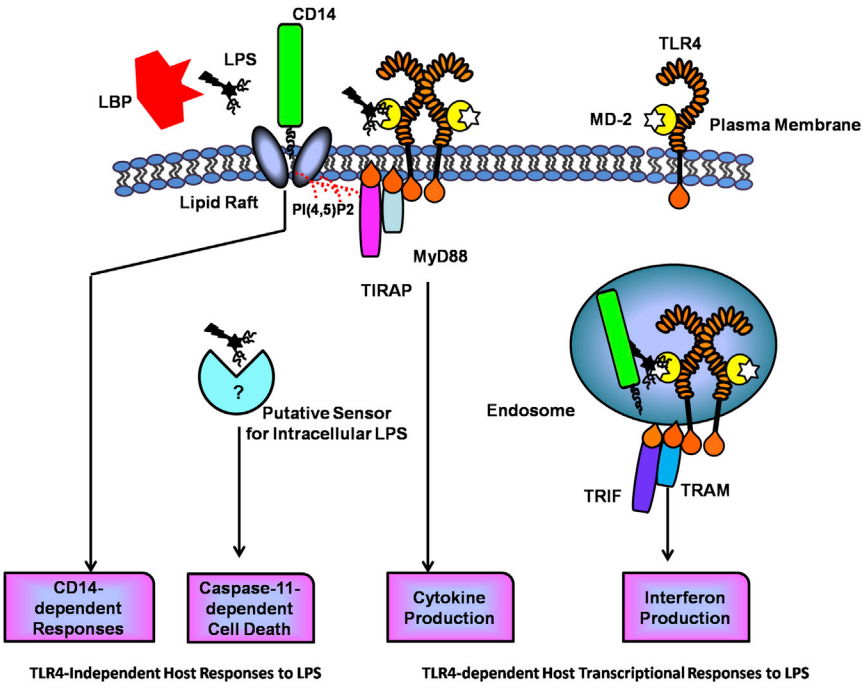

Figure 1. Diverse Host Responses to LPS
Macrophages are capable of sensing LPS in the extracellular milieu and the cytosol. Detection of extracellular LPS is achieved by sequential actions of LBP, CD14, and the TLR4/MD-2 complex. TLR4 induces distinct host transcriptional responses at different cellular locations, which is determined by the sorting adaptors TIRAP and TRAM. The LPS receptor CD14 also acts independently of TLR4 to promote endocytosis, calcium fluxes, and NFAT activation, the latter two activities occurring only in DCs. Finally, an unknown intracellular sensor induces Caspase-11-dependent responses to intracellular LPS.

Orchestrated Action of LBP, CD14, TLR4, and MD-2 in Sensing Extracellular LPS

Following the discovery and functional characterization of LBP, CD14, TLR4, and MD-2, fascinating questions emerged as how these molecules coordinate with each other to elicit host LPS response. To this end, the Weiss group engineered an auxotrophic *Neisseria* strain to produce ^{14}C-labeled LPS via metabolic labeling (Giardina et al., 2001). Due to the absence of the O antigen, the *Neisseria* LPS is also referred to as lipooligosaccharide. The application of radio-labeled LPS ensures the sensitive and quantitative detection of intermolecular LPS transfer. Results from several elegantly designed studies demonstrated that in the presence of albumin, LBP can bind to LPS-rich surfaces (e.g., bacterial outer membranes), which somehow alters the organization in the membrane to permit the binding of LPS by CD14, thus creating monomeric complexes between these two molecules (Gioannini and Weiss, 2007; Prohinar et al., 2007). CD14 then transfers LPS to the TLR4/MD-2 complex, thereby leading to cell activation (Gioannini et al., 2004, 2005) (Figure 1). Remarkably, it has been estimated that this LPS-transfer cascade can convert a single *E. coli* bacterium into enough LPS-CD14 complexes that can activate a thousand macrophages (Gioannini and Weiss, 2007), massively enhancing the host response.

TLR4-Dependent Signaling Responses to LPS

A general working mechanism for TLR-mediated signal transduction is that, upon ligand detection, the cytosolic TIR domain of TLRs recruits one or more cytoplasmic TIR domain-containing proteins through TIR-TIR interactions. MyD88, TIRAP, TRIF, and TRAM are the best characterized TIR domain-containing proteins that serve as adaptors in TLR signaling. Upon TLR activation, these adaptors bridge TLRs to downstream kinases of the IRAK and MAPK families. Together with TRAF family E3 ligases, the TLR signaling cascade activates several transcriptional regulators, such as NF-κB, AP-1, and IFN regulatory factors (IRF), which collectively drive the expression of genes involved in the host immune responses (Akira et al., 2006). Specifically, the adaptor pair MyD88 and TIRAP elicits the production of proinflammatory cytokines by activating the NF-κB- and MAPK-dependent pathways (Horng et al., 2002; Yamamoto et al., 2002), whereas the TRAM and TRIF pair activates the kinases TBK1 and IKKε to promote type I IFN expression (Yamamoto et al., 2003a, 2003b). Thus, recruitment of these adaptors ensures differential signaling outcomes by different TLRs. Among all the TLRs, TLR4 is unique in a sense that it can use all of the four adaptors, leading to the production of both proinflammatory cytokines and type I IFN. These observations raised the question of how TLR4 differentially propagates the MyD88- and TRIF-dependent pathways to mediate two distinct signaling outcomes.

The answer to this question was provided by the studies deciphering the cell biological properties of these aforementioned adaptor proteins (Figure 1). At steady state, TIRAP localizes to the plasma membrane and endosomes via interactions with various acidic phosphoinositides (and phosphatidylserine) that are found on these compartments (Kagan and Medzhitov, 2006; Bonham et al., 2014). These protein-lipid interactions are mediated by an N-terminal lipid binding domain of promiscuous specificity. The promiscuity of TIRAP's lipid binding domain allows it to survey multiple organelles for the presence of activated TLRs (Kagan and Medzhitov, 2006; Bonham et al., 2014). In the case of TLR4, enrichment of phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] in plasma membrane subdomains such as lipid rafts and actin-rich membrane ruffles recruits TIRAP to these locations (Kagan and Medzhitov, 2006). Similarly, the plasma membrane and endosomal localization of TRAM is determined by an N-terminal bipartite localization domain, which is comprised of a myristoylation site and a PI-binding motif (Kagan et al., 2008; Rowe et al., 2006). Upon sensing LPS, TIRAP is thought to interact with TLR4 at PI(4,5)P2-rich plasma membrane subdomains and recruit cytosolic MyD88, which eventually leads to the assembly of myddosome, a protein complex

consisting of TIRAP, MyD88, and IRAK family kinases (Bonham et al., 2014). This complex promotes proinflammatory cytokine production. Subsequently, TLR4 is internalized into endosomes where it engages TRAM, which further recruits TRIF to trigger type I IFN production (Kagan et al., 2008). Importantly, the plasma membrane signaling of TLR4 could be uncoupled from its endosomal signaling, as dynasore (a small molecule that blocks endocytosis) inhibited the TRIF-dependent pathway, whereas it exerted no effect on the MyD88-dependent pathway initiated from the plasma membrane (Kagan et al., 2008). Consequently, TIRAP and TRAM could be categorized as "sorting adaptors" that function to define both the subcellular sites of TLR signaling and the nature of the signaling pathways activated (Figure 1) (Kagan, 2012a).

The cell biological study of TLR4 and its associated adaptors has not only explained the operation of the LPS signaling pathways but has also revealed several emerging concepts in the operation of innate immune signaling in general. For example, TLR4 is not the only receptor that separates its subcellular site of ligand binding from the site of signal transduction. Recent work revealed that TLR2, TLR9, and the cytosolic RIG-I-like receptors (RLRs) also follow this cell biological "rule" (Barbalat et al., 2009; Sasai et al., 2010; Dixit et al., 2010). Second, sorting adaptors such as TIRAP and TRAM are localized at the site of signal transduction before any microbe is detected and thus define the subcellular sites of innate immune signal transduction. The family of sorting adaptors in the innate immune system now extends beyond those used by TLR4 and includes the RLR adaptor MAVS (Kagan, 2012b). Finally, as will be discussed below, cell biological analysis of TLR4 revealed the existence of cellular responses to LPS that do not actually involve this receptor (Figure 1).

TLR4-Independent Responses to Extracellular LPS

The traditional view of CD14 is that it functions to deliver LPS to the TLR4/MD-2 complex, thus acting to sensitize mammalian cells to low concentrations of LPS. Recent studies have revealed a more complex role for this LPS-binding protein. Biochemical analysis indicated that CD14 is required for the recruitment of TLR4 to lipid rafts upon LPS binding (Triantafilou et al., 2002), an observation that suggested CD14 might have more functions than simply delivering LPS to TLR4/MD-2. Further supporting evidence for this notion came from studies demonstrating that CD14 deficiency does not ablate MyD88 and TRIF signaling to similar extents. TRIF signaling appears to be most dependent on CD14, as opposed to MyD88 signaling (Jiang et al., 2005; Perera et al., 1997). An explanation for this finding came from studies that established a crucial role for CD14 in promoting the endocytosis of TLR4, a process that is necessary for TRIF-dependent signal transduction (Zanoni et al., 2011).

Perhaps the most notable feature of CD14-dependent TLR4 endocytosis is that this activity does not require the signaling functions of TLR4. Two pieces of evidence are in support of this finding. First, a TLR4 mutant that lacks all signaling functions retains the ability to be internalized in response to LPS treatment (Zanoni et al., 2011). Second, cells deficient in both of the signaling adaptors MyD88 and TRIF also retain the ability to internalize TLR4 in response to LPS (Zanoni et al., 2011). Thus, CD14 controls a cellular response to LPS that does not depend on TLR4 signal transduction (Figure 1). Characterization of the CD14-dependent endocytosis pathway reveals the involvement of additional regulatory components, such as phospholipase Cγ2 (PLCγ2), intracellular calcium, the tyrosine kinase Syk, and the ITAM containing adaptors DAP12 and FcεRγ (Chiang et al., 2012; Zanoni et al., 2011). That this newly defined cellular response to LPS proceeds independently of TLR4, but is required by TLR4 to induce TRIF-dependent signaling, highlights the interconnections between the signaling pathways activated when mammalian cells encounter Gram-negative bacteria. It remains to be determined whether the CD14-dependent recruitment of TLR4 to lipid rafts is required for receptor internalization, what other proteins are internalized into cells via the CD14-dependent endocytosis pathway, and how cargo for this pathway is determined.

The role of CD14 in promoting endocytosis in response to LPS occurs in all cell types examined that coexpress TLR4 and CD14, including macrophages, dendritic cells (DCs), fibroblasts, and B cells engineered to express both genes (Zanoni et al., 2011). Remarkably, recent studies revealed another TLR4-independent response to LPS that occurs in DCs specifically (Figure 1). In DCs, LPS upregulated several genes that are not expressed in macrophages, such as IL-2, Nur77, and mPGES-1, the latter being an enzyme that promotes the synthesis of prostaglandin E2 (Zanoni et al., 2012). The products of these genes regulate processes that distinguish DC and macrophages functions in the activation of naive T cells. For example, mPGES-1 controls edema formation, which is needed to promote efficient delivery of antigens to the lymph nodes that drain infected tissues (Zanoni et al., 2012). Importantly, Nur77 expression is important for DC homeostasis, as it regulates the lifespan of LPS-activated DCs. The expression of these genes is dependent on NF-κB, as well as the calcium-regulated transcription factors of the NFAT family (Zanoni et al., 2009). Whereas TLR4 can induce NF-κB activation, it cannot activate NFAT or induce calcium influxes. These latter two activities are triggered by CD14 and proceed normally in cells derived from TLR4 knockout mice (Figure 1). Thus, a common theme emerges whereby CD14 activates an interconnected set of TLR4-dependent and -independent responses that are required to promote the expression of genes that control innate and adaptive immunity.

TLR4-Independent Responses to Intracellular LPS

In this section, we continue the exploration of TLR4-independent responses to LPS, but we now move to the intracellular environment of mammalian cells. The study of intracellular LPS responses has been almost nonexistent, as extensive characterization of cellular responses induced by extracellular LPS has dominated the past two decades. Nevertheless, scattered evidence suggested the existence of TLR4-independent host responses to LPS (Vogel et al., 1980; Haziot et al., 2001; Gerth et al., 2005). Two recent studies revealed that intracellular LPS can trigger the Caspase-11-dependent formation of noncanonical inflammasomes by a process that does not require TLR4 (Hagar et al., 2013; Kayagaki et al., 2013). This LPS response pathway differs from the TLR4-independent responses discussed above in that it does not depend on CD14. These reports move us even further from the TLR4-centric view of LPS signaling that has so dominated the last 15 years (Beutler, 2000; Beutler

et al., 2001) and suggests the existence of another sensor of LPS that is yet to be identified (Figure 1).

Inflammasomes are macromolecular protein complexes in the cytosol of macrophages, the formation of which is triggered by various pathogens and stress stimuli (Lamkanfi and Dixit, 2012). The assembly of canonical inflammasomes results in the activation of Caspase-1, which subsequently triggers two distinct signaling outcomes: first, the processing and secretion of proinflammatory cytokines, such as IL-1, and second, the induction of an inflammatory cell death called pyroptosis. In comparison, the activation of noncanonical inflammasomes is triggered by Caspase-11. Caspase-11-deficient mice (not Caspase-1-deficient mice) are resistant to LPS-induced sepsis, implying that Caspase-11 also participates in host response to LPS (Kayagaki et al., 2011; Wang et al., 1996). However, the upstream signal leading to Caspase-11 activation remains elusive (Figure 1).

Based on the cumulative observations that various Gram-negative bacteria activate Caspase-11-dependent inflammasome assembly, it has been speculated that a molecule unique to Gram-negative bacteria might promote Caspase-11 activation (Aachoui et al., 2013). Following the previous finding that the B subunit of cholera toxin (CTB) and LPS could trigger the activation of Caspase-11-dependent inflammasomes (Kayagaki et al., 2011), CTB was found to act as a vehicle for delivering LPS inside the cell. Evidence supporting this idea came from the observation that LPS serotypes that could not bind to CTB were unable to induce Caspase-11-dependent cellular responses (Kayagaki et al., 2013). Such observations were further validated by direct transfection or electroporation of LPS into macrophages, which led to Caspase-11 activation (Kayagaki et al., 2013; Hagar et al., 2013). Alternative means of LPS delivery into cells also promoted Caspase-11 activation, such as coating the Gram-positive bacteria *Listeria monocytogenes* with LPS (Hagar et al., 2013). Altogether, these results suggest that LPS is the molecule that induces Caspase-11 activation. However, how LPS "naturally" enter the cytosol during bacterial infection and triggers Caspase-11 activation is unknown (Aachoui et al., 2013). Furthermore, studies to identify upstream activators of Caspase-11 have so far been unsuccessful, but it is clear that neither CD14, MD-1, MD-2, RP105, nor TLR4 are required. Finally, it is noteworthy that Caspase-11 activation requires transcriptional priming by TLR ligands (Kayagaki et al., 2011; Kayagaki et al., 2013). During the onset of murine sepsis, LPS serves dual roles: it first primes Caspase-11 expression and subsequently activates cellular responses dependent on Caspase-11 activity.

While we do not yet know the identity of this new LPS receptor, studies on the types of LPS that can activate Caspase-11 suggest a similar mechanism of recognition to that of MD-2. For example, tetra-acylated LPS, which is known to activate the TLR4/MD-2 pathways poorly, is a weak activator of Caspase-11 (Hagar et al., 2013; Kayagaki et al., 2013). These results suggest a common feature of LPS signaling induced by MD-2/TLR4 and by the Caspase-11 activator. In human cells, Caspase-4 and Caspase-5 are homologs to mouse Caspase-11. It would be interesting to determine whether human macrophages respond to intracellular LPS in a manner similar to mouse macrophages, and if the answer is positive, which one of these aforementioned Caspases is required for such response.

Available data also raises the question of exactly where within cells this new LPS receptor resides and signals from. Since LPS must be delivered into cells to activate Caspase-11, it is unlikely that the sensor would be surveying the extracellular environment. Rather, this sensor is likely present within the cell. While the simplest interpretation of the current data is that the receptor is present in the cytosol, the experiments using CTB suggest an alternative. CTB is one of the best characterized protein toxins and is well-known to enter cells and deliver its catalytically active A subunit (CTA) to the lumen of the endoplasmic reticulum (ER) (Chinnapen et al., 2007). However, cell biological analysis indicates that CTB cannot enter the cytosol (Chinnapen et al., 2007). These observations therefore raise the question of how CTB can deliver LPS to the cytosol if it cannot access the cytosol itself. It is possible that, like CTA, CTB delivers LPS to the lumen of the ER where it then releases LPS to somehow access the cytosol. An alternative is that the receptor may be an ER-resident protein that can detect LPS in the ER lumen. A final possibility is that the LPS receptor may reside in the cytosol after all and that in macrophages CTB does access this compartment.

Strategies Taken by Pathogenic Bacteria to Specifically Evade TLR Signal Transduction

It is now clear that there are several LPS sensors that operate in mammalian cells to detect Gram-negative bacteria (Figure 1). The collective actions of these receptors promote the cellular responses to LPS that are important for fighting infection and the development of symptoms associated with sepsis. Based on these diverse yet interconnected LPS response pathways, it could be considered surprising that mammals ever suffer from bacterial infections. However, these potent antimicrobial mechanisms likely provided selective pressure for bacteria to evolve immune evasion strategies that evade many of our defenses. In the following sections, we provide examples of strategies used by Gram-negative bacteria to evade our formidable immune defenses, with a focus on the means by which the LPS response pathways are either evaded or disrupted. We highlight bacterial strategies that likely evolved to specifically evade recognition by the host and provide examples of bacterial survival strategies that result in an evasion of TLR signaling "accidentally" (Figure 2). This latter set of examples is intriguing, as it underscores the numerous ways in which studies of the cell biology of infection can be superimposed on studies of cell biology of innate immunity.

Direct Evasion of Innate Immune Detection by Alterations in the Structure of LPS

As LPS is sensed directly by the TLR4/MD-2 complex, altering the structure of LPS, especially the lipid A component, is a common strategy employed by extracellular and intracellular pathogens to avoid host detection (Figure 2A). *Yersinia pestis*, the causative agent of plague, provides an example of how extracellular pathogens evade TLR signaling by modifying the lipid A structure. In the natural environment, *Y. pestis* is a rodent pathogen that can survive in the gut of fleas (Perry and Fetherston, 1997). Human infection is usually caused by flea bites. During its infectious cycle, *Y. pestis* adapts to different temperatures in different host species: 21°C–25°C in fleas and 37°C in humans

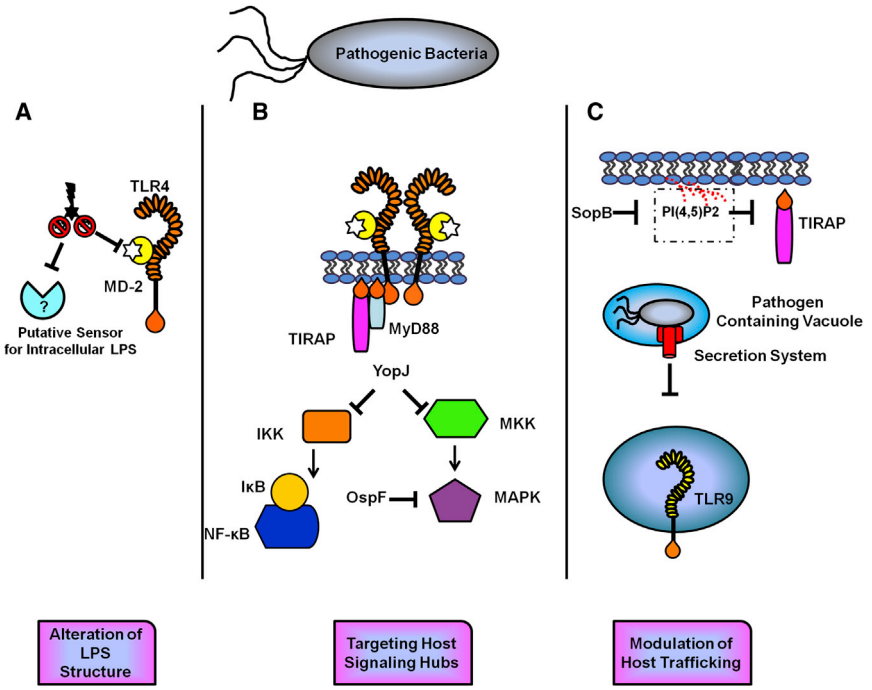

Figure 2. Bacterial Strategies to Avoid Detection by the Host Innate Immune System

(A) Evasion of host LPS sensors. Many Gram-negative bacteria modify their LPS structures to evade detection by surface localized TLR4/MD-2 complex as well as the putative intracellular LPS sensor.

(B) Interference of host TLR signaling by bacterial type III effectors. The *Yersina* effector YopJ blocks activation of NF-κB and MAPK pathways by acetylating members of IKK and MKK families. The *Shigella* effector OspF inhibits MAPK pathway by irreversibly removing the phosphate group from the phosphothreonine residue of members of the MAPK family.

(C) Accidental avoidance of TLR signaling by bacterial effectors that modulate membrane trafficking. The *Salmonella* effector SopB dephosphorylates PI(4,5)P2 to promote invasion, which indirectly disrupts the proper localization of TIRAP, thereby abrogating TLR4 signaling from the plasma membrane. To establish a niche for intracellular replication, almost all vacuolar pathogens deliver effector proteins by various secretion systems to avoid fusion with lysosomes. An indirect outcome of such pathogen-induced detour of host membrane trafficking is the avoidance of detection by endosomal TLRs, such as TLR9.

or rodents. Mass spectrometry demonstrated that *Y. pestis* mainly produces hexa-acylated lipid A at 25°C, whereas it synthesizes tetra-acylated lipid A at 37°C (Kawahara et al., 2002). The production of hexa-acylated lipid A is correlated with the resistance to the cationic antimicrobial peptides produced by the flea innate immune system (Rebeil et al., 2004). On the contrary, the tetra-acylated lipid A is structurally similar to lipid IVa, which is nonstimulatory to the human TLR4/MD-2 complex and is partially stimulatory to the murine counterpart, thus facilitating immune evasion by this bacterium (Montminy et al., 2006). Notably, an engineered *Y. pestis* strain constitutively expressing hexa-acylated LPS at 37°C stimulates potent proinflammatory signals in reporter cell lines and fails to establish systematic infection in wild-type mice (Montminy et al., 2006). Furthermore, the protective effect is provided by the TLR4 pathway, as TLR4 knockout mice succumb when infected with this engineered strain. Besides *Y. pestis*, several other extracellular pathogens modify lipid A structure to avoid host detection, including *Helicobacter pylori* (Moran et al., 1997) and *Porphyromonas gingivalis* (Coats et al., 2009; Rangarajan et al., 2008).

The above-described discovery of an as-yet-unidentified intracellular LPS sensor suggests that intracellular pathogens may also be under pressure to alter the structure of their LPS. Indeed, several intracellular pathogens alter their LPS structure during infections. For example, upon entering host cells, *Salmonella enterica* serovar Typhimurium mediates deacylation and palmitoylation of its lipid A component by a process dependent on the bacterial two-component PhoP-PhoQ system (Guo et al., 1997). These modifications decrease the stimulatory effect of *S. typhimurium* LPS in inducing inflammation as well as increase the bacterial resistance to antimicrobial peptides, thereby contributing to the intracellular survival of *S. typhimurium* (Guo et al., 1997, 1998). *Francisella tularensis,* the etiological agent of tularemia, is well-known for producing LPS with low immunostimulatory effect. When added to mononuclear cells, the amount of TNFα production induced by *F. tularensis* LPS is about 10^3-fold weaker in comparison to that induced by *E. coli* LPS (Sandström et al., 1992). Detailed analyses revealed that *F. tularensis* is capable of synthesizing mono-phosphorylated and tetra-acylated lipid A (Phillips et al., 2004; Vinogradov et al., 2002). Furthermore, three of the acylated lipid side chains contain 18 carbons whereas the remaining one contains 16. These features largely account for the immunologically silent properties of *F. tularensis* LPS, which is pivotal for this bacterium to survive inside the host cell. Recently, it has been reported that *Shigella flexneri* can remodel its lipid A structure specifically during its intracellular growth phase (Paciello et al., 2013). Mass spectrometry analysis demonstrated that the majority of *S. flexneri* LPS purified from infected epithelial cells contained tri-or tetra-acylated lipid A. On the contrary, bacteria grown in broth produced hexa-acylated lipid A. The hypoacylated intracellular LPS possesses weak immunostimulatory potency in that it triggers significantly lower amount of cytokine production via the TLR4 signaling pathway. Further, during *S. flexneri* infection, hypoacylated LPS dampened inflammasome activation in macrophages, as indicated by the reduced release of IL-1β (Paciello et al., 2013). Overall, as could be summarized from numerous studies on the modification of LPS structure by extracellular and intracellular bacteria, an emerging theme is that alteration of LPS structure, in particular the lipid A component, represents a common virulence strategy adopted by bacterial pathogens to evade host innate immune detection. It should be noted however, that alterations in acyl chain length or number have never been found to completely prevent TLR4 signaling. These modifications only

make the bacterial LPS a weak activator. Perhaps for this reason, additional bacterial immune evasion strategies have evolved.

Disruption of TLR4 Signaling by Bacterial Type III Secretion Systems

Bacterial pathogens utilize virulence proteins and secretion systems to disrupt host signal transduction pathways (Figure 2B). Seven secretion systems have been identified in bacteria (Tseng et al., 2009). Among these, the type III secretion system (T3SS) is utilized by various Gram-negative bacteria to deliver virulence proteins into the host cytosol. These virulence proteins are commonly referred to as "effectors" in that they manipulate various host functions to promote bacterial invasion and survival inside the host.

YopJ is one of the first identified *Yersinia* type III effectors that disrupts host innate immune response by inhibiting TNFα production in infected macrophages (Palmer et al., 1998). Biochemical analyses revealed that YopJ interferes with both NF-κB and MAPK pathways by interacting with members of the IKK and MKK families (Figure 2B), which regulate signaling by TLRs and other innate immune receptors (Orth et al., 1999, 2000). Mass spectrometry demonstrated that YopJ is a serine/threonine acetyl transferase that mediates the attachment of an acetyl group to the cognate serine and threonine residues in the activation loop of IKKs and MKKs (Mukherjee et al., 2006). These acetylated substrates could no longer be phosphorylated by their upstream protein kinase, which in turn abrogates the signal transduction cascades induced by upstream receptors. Therefore, YopJ represents a type III effector that is dedicated to paralyzing the host innate signaling pathways. Functional and structural homologs of YopJ exist in other pathogenic bacteria (Orth et al., 2000), suggesting that the means by which this effector disrupts innate immune signaling represents a common immune evasion strategy.

Unlike YopJ, which modifies upstream molecules of the NF-κB and MAPK pathway, the *Shigella* type III effector OspF targets members of MAPK family via a posttranslational modification named eliminylation (Brennan and Barford, 2009). This modification cleaves the C-OP bond (whereas canonical phosphatases attacks the CO-P bond) on the phosphothreonine residue located in the aforementioned activation loop of p38 and ERK1/2, thereby irreversibly inactivating these MAPKs (Li et al., 2007; Figure 2B). Biochemically, the OspF-mediated eliminylation is distinct from dephosphorylation in that the removal of a hydroxyl group from the cognate threonine prevents it from being phosphorylated by the upstream kinases, which explains the irreversible nature of this modification. Thus, OspF is the founding member of a new bacterial phosphothreonine lyase family. The effect of OspF on host gene expression is profound (Arbibe et al., 2007). On one hand, an obvious outcome of OspF disruption of MAPK activity is the selective inactivation of MAPK-dependent genes. On the other hand, although it does not block NF-κB activation, OspF represses the induction of some NF-κB-dependent genes such as IL-8. Detailed analysis revealed the mechanism of this unexpected observation: OspF-mediated inactivation of ERK1/2 prevents the phosphorylation of histone H3, which negatively impinges on the chromatin reorganization events, eventually rendering the promoter binding sites of the NF-κB-dependent genes less accessible to NF-κB (Arbibe et al., 2007). In conclusion, by targeting MAPK kinases, OspF selectively modulates host gene expression to counteract host innate immune responses and promote *S. flexneri* infection.

An apparent advantage of targeting MAPKs and IKKs is that bacterial pathogens could counteract the detection by multiple TLRs (and other receptors), as these proteins are the converging points downstream of various immune receptors and therefore serve as the master regulators of the innate immune response (Figure 2B). Compared to the ample examples of bacterial interference with signaling hub function, our knowledge of bacterial effectors targeting individual TLRs is limited. This could partially be due to the limitation of the assays available to monitor the particular biochemical or cell biological activities of TLRs. In retrospect, our understanding of host innate immune receptors usually precedes that of pathogen immune evasion strategies. For instance, the discovery of the antiviral protein MAVS, which is the critical sorting adaptor in the RLR signaling pathways, led to the identification of an immune evasion mechanism used by hepatitis C virus (HCV). HCV encodes a protease called NS3/4A, which cleaves MAVS from mitochondria and mitochondria-associated membranes (Horner et al., 2011; Li et al., 2005), thus inactivating the ability of this adaptor to induce antiviral responses. Such a discovery could not have been made prior to the identification of MAVS and the importance of its localization to mitochondria and other membranes. Analogously, we anticipate that an increased understanding of TLR function at the receptor proximal level will uncover additional bacterial immune evasion strategies that manipulate specific TLR functions.

Accidental Evasion of TLR-Mediated Signaling Responses by Bacterial Pathogens

The examples listed above highlighted bacterial immune evasion strategies that probably evolved to disrupt TLR-mediated recognition or signaling. In this next section, we examine how some strategies exist that likely evolved to serve some other need in the host-pathogen encounter but resulted in the ability of the pathogen to evade TLR-mediated host defenses. In this regard, these strategies of immune evasion evolved by accident (Figure 2C).

Precise communication between different compartments of the cell depends on various membrane trafficking events. These principles also govern the operation of the innate immune signaling. As previously discussed, the compartmentalization of TLR4 signaling components determines the distinct outcome of signaling events (Kagan et al., 2008). Additionally, compartmentalization of nucleic-acid-sensing TLRs in endolysosomal networks represents an important mechanism for maintaining self-tolerance, as the intracellular localization of these TLRs prevents the recognition of self-nucleic acids by limiting receptor accessibility (Barton et al., 2006). The protease-dependent activation of endosomal TLRs serves as an additional layer of regulation of these receptors (Ewald et al., 2008). For instance, the activation of TLR9, the endosomal sensor for unmethylated CpG DNA motifs, requires the collective action of several endosomal proteases (Ewald et al., 2008; Park et al., 2008). Therefore, membrane trafficking events regulate the activity of the TLR network from several distinct aspects.

Several bacterial pathogens exist that alter membrane trafficking pathways within eukaryotic cells. The ability to alter

membrane trafficking is most widely used to either promote invasion of the bacteria into normally nonphagocytic cells or to prevent delivery of the phagosomes in which they reside to lysosomes. These activities are common in bacterial pathogens that infect cells that do not express endosomal TLRs, such as epithelial cells, or even phagocytic protists. As such, the ability to either promote invasion or to block delivery to lysosomes cannot evolve to prevent delivery to a TLR-containing compartment. Rather, these aforementioned pathogenic tactics are likely evolved to facilitate the establishment of a niche permissive for bacterial intracellular growth. *S. typhimurium* offers examples of means by which it evade TLR signaling from the plasma membrane and from endosomes, but these evasion strategies probably did not evolve to do so.

A hallmark of *S. typhimurium* pathogenicity is its ability to invade nonphagocytic cells. Featured by the drastic rearrangement of host actin cytoskeleton, the invasion process is dependent on the orchestrated actions of *Salmonella* pathogenicity island 1 effectors (Zhou and Galán, 2001). One of these effectors, SopB, is a PI phosphatase that dephosphorylates PI(4,5)P2 during infection (Hernandez et al., 2004; Terebiznik et al., 2002). Furthermore, overexpression of this effector leads to rapid depletion of PI(4,5)P2 from plasma membrane subdomains, which changes the physical properties of the plasma membrane (Terebiznik et al., 2002). Therefore, a direct cell biological consequence mediated by SopB during *S. typhimurium* infection is to promote membrane fission, which facilitates the invasion process (Terebiznik et al., 2002). Because the TLR sorting adaptor TIRAP is localized to the plasma membrane via interactions with PI(4,5)P2, it is expected that injection of SopB into mammalian cells would release TIRAP from the membrane and diminish the ability of TLR4 to promote inflammatory responses during *S. typhimurium* infection. Consistent with this idea, ectopic expression of SopB abrogated the plasma membrane localization of TIRAP and further disrupted the TLR4-induced NF-κB activation by a process dependent on the PI phosphatase activity of SopB (Kagan and Medzhitov, 2006). Thus, by altering the plasma membrane PI composition, an effector protein designed to promote bacterial invasion accidentally interferes with the proper localization of the sorting adaptor TIRAP, which likely renders plasma membrane TLR4 signaling less robust (Figure 2C).

Upon entry into the host cell, *S. typhimurium* employs a different T3SS called SPI-2 that injects into cells effectors to establish the *Salmonella* containing vacuole (SCV) for its intracellular replication (Figueira and Holden, 2012). Although the SCV acidifies during maturation, this compartment is devoid of mannose-6-phosphate receptor (MPR) and its associated proteases, such as cathepsins (Drecktrah et al., 2007; Hang et al., 2006). The SPI-2 effector protein SifA is responsible for interfering with MPR trafficking. SifA associates with a host protein SKIP, which further sequesters the small GTPase Rab9 by forming a "hetero-trimer" (McGourty et al., 2012). Importantly, Rab9 regulates the trafficking of MPR from the Golgi apparatus to the late endosomes under normal conditions (Barbero et al., 2002). Thus, by disrupting Rab9 function, SifA prevents the recruitment of MPR and associated cathepsins to SCV. Since cathepsins are required for the activation of TLR9, the SifA-induced alteration in membrane trafficking likely protects *S. typhimurium* from TLR9 detection (Figure 2C).

Strategically similar to *S. typhimurium*, *Legionella pneumophila*, the causative agent of Legionnaire's disease, manipulates host trafficking pathways to promote its survival inside professional phagocytes. Shortly after being internalized, *L. pneumophila* employs a type IV secretion system to establish the *Legionella*-containing vacuole (LCV), an ER-like compartment that evades lysosome fusion (Isberg et al., 2009). A central characteristic of LCV maturation is the recruitment of small GTPases, such as Arf1 and Rab1, which are regulators of trafficking events between the ER and the Golgi apparatus (Kagan et al., 2004; Nagai et al., 2002a). In line with this, *L. pneumophila* devotes a cohort of effectors to manipulate almost every aspect of Rab1 function with distinct biochemical mechanisms that include the following: nucleotide exchange, nucleotide hydrolysis, reversible AMPylation, and PCylation (Sherwood and Roy, 2013). A direct consequence of manipulating Rab1 function is the recruitment of host ER-derived material to the surface of LCV, which contributes to the evasion of lysosome fusion, whereas an indirect consequence of this event is the avoidance of TLR9 detection (Figure 2C). Consistent with this notion, when compared to heat-killed *L. pneumophila*, live *L. pneumophila* induced significantly less IL-12 p40 production in both macrophages and DCs (Newton et al., 2007). Furthermore, the IL-12 p40 production in response to this bacterium could be suppressed by ODN1826, a TLR9 inhibitor (Newton et al., 2007). These results confirm that *L. pneumophila* is capable of avoiding TLR9 detection during infection. Of note, humans are a terminal host of *L. pneumophila*e; the natural host of this bacterium is various amoeba species that thrive in soil or water environments, which do not possess TLRs (Swanson and Hammer, 2000). Thus, the selective pressure on *L. pneumophila* to avoid lysosomal degradation by its natural hosts inadvertently equipped this bacterium with the ability to avoid host intracellular TLR detection.

Conclusions and Future Perspectives

In the review, we have outlined the historical and recent discoveries of how mammalian cells detect Gram-negative bacteria, with a focus on LPS sensing and microbial strategies of immune evasion. Standing from the host and bacteria interface, we are fascinated by the diverse signaling pathways and the multiangle approaches employed by the host to detect bacteria. We also note that these comprehensive approaches of detecting bacteria likely provide selective pressure for the evolution of immune evasion strategies that promote infection.

From the host perspective, the investigation of host LPS sensing in the past two decades have been fruitful. We have obtained comprehensive knowledge with regards to the signaling networks, transcriptional regulation, and host responses associated with TLR4. Furthermore, by studying the cell biology of TLR4, new regulatory mechanisms of innate immunity have been uncovered. First, the sites of ligand detection is usually separated from the subcellular sites of signal transduction. This observation places protein trafficking pathways at the very center of the regulation of innate immunity. Second, the subcellular sites of signal transduction often determine the nature of the signaling pathways activated. These principles apply not only to

TLRs but also to other host molecules essential for innate immune response to pathogens. Perhaps most excitingly, recent advances have demonstrated that TLR4 is not the only receptor capable of activating cellular responses to LPS. For example, the surface molecule CD14 (not TLR4) is capable of activating endocytosis and the NFAT transcription factors in response to LPS. The latest discovery of the Caspase-11-dependent cell death induced by intracellular LPS further adds to the growing list of such nontranscriptional host responses to LPS. Accordingly, many new questions are emerging from these novel discoveries, some of which were highlighted in this review.

From the pathogen perspective, we have learned that successful bacterial pathogens have developed tactics to dismantle host innate immune defenses during their long coevolution with the host. First, bacteria can modify their LPS structure to avoid detection by the host. With the discovery of the intracellular LPS sensing mechanism, we now know that such driving forces for LPS variation comes not only from the cell surface but also from within the cell. Second, bacterial pathogens utilize secretion systems and effectors to disrupt innate immune signaling. The known targets of these virulence factors are the signaling hubs of the host innate immune signaling, such as NF-κB, or MAPK pathways. By doing so, pathogens could protect themselves from detection by multiple receptors. However, the studies on the *Shigella* effector OspF exemplifies the idea that bacteria do not shut down all cellular responses to LPS but instead block a subset of responses. It is conceivable that limited induction of host inflammatory responses is either beneficial to promote bacterial dissemination in the intestinal environment or required for bacterial pathogens to compete with commensal bacteria at the site of infection. In conclusion, future studies on host and bacterial interactions in the aspect of innate immunity will continue to reveal novel host detection mechanisms and bacterial strategies to modulate the host innate immune response.

ACKNOWLEDGEMENTS

We would like to thank the members of the Kagan lab for helpful discussions. J.C.K. is supported by NIH grants AI093589, AI072955, and P30 DK34854 and an unrestricted gift from Mead Johnson & Company. J.C.K. holds an Investigators in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund.

REFERENCES

Aachoui, Y., Leaf, I.A., Hagar, J.A., Fontana, M.F., Campos, C.G., Zak, D.E., Tan, M.H., Cotter, P.A., Vance, R.E., Aderem, A., and Miao, E.A. (2013). Caspase-11 protects against bacteria that escape the vacuole. Science *339*, 975–978.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell *124*, 783–801.

Angus, D.C., Linde-Zwirble, W.T., Lidicker, J., Clermont, G., Carcillo, J., and Pinsky, M.R. (2001). Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit. Care Med. *29*, 1303–1310.

Arbibe, L., Kim, D.W., Batsche, E., Pedron, T., Mateescu, B., Muchardt, C., Parsot, C., and Sansonetti, P.J. (2007). An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses. Nat. Immunol. *8*, 47–56.

Barbalat, R., Lau, L., Locksley, R.M., and Barton, G.M. (2009). Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat. Immunol. *10*, 1200–1207.

Barbero, P., Bittova, L., and Pfeffer, S.R. (2002). Visualization of Rab9-mediated vesicle transport from endosomes to the trans-Golgi in living cells. J. Cell Biol. *156*, 511–518.

Barton, G.M., Kagan, J.C., and Medzhitov, R. (2006). Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat. Immunol. *7*, 49–56.

Beutler, B. (2000). Tlr4: central component of the sole mammalian LPS sensor. Curr. Opin. Immunol. *12*, 20–26.

Beutler, B., Du, X., and Poltorak, A. (2001). Identification of Toll-like receptor 4 (Tlr4) as the sole conduit for LPS signal transduction: genetic and evolutionary studies. J. Endotoxin Res. *7*, 277–280.

Bonham, K.S., Orzalli, M.H., Hayashi, K., Wolf, A.I., Glanemann, C., Weninger, W., Iwasaki, A., Knipe, D.M., and Kagan, J.C. (2014). A promiscuous lipid-binding protein diversifies the subcellular sites of toll-like receptor signal transduction. Cell *156*, 705–716.

Brennan, D.F., and Barford, D. (2009). Eliminylation: a post-translational modification catalyzed by phosphothreonine lyases. Trends Biochem. Sci. *34*, 108–114.

Chiang, C.Y., Veckman, V., Limmer, K., and David, M. (2012). Phospholipase Cγ-2 and intracellular calcium are required for lipopolysaccharide-induced Toll-like receptor 4 (TLR4) endocytosis and interferon regulatory factor 3 (IRF3) activation. J. Biol. Chem. *287*, 3704–3709.

Chinnapen, D.J., Chinnapen, H., Saslowsky, D., and Lencer, W.I. (2007). Rafting with cholera toxin: endocytosis and trafficking from plasma membrane to ER. FEMS Microbiol. Lett. *266*, 129–137.

Coats, S.R., Jones, J.W., Do, C.T., Braham, P.H., Bainbridge, B.W., To, T.T., Goodlett, D.R., Ernst, R.K., and Darveau, R.P. (2009). Human Toll-like receptor 4 responses to *P. gingivalis* are regulated by lipid A 1- and 4′-phosphatase activities. Cell. Microbiol. *11*, 1587–1599.

Cohen, J. (2002). The immunopathogenesis of sepsis. Nature *420*, 885–891.

Coutinho, A., Forni, L., Melchers, F., and Watanabe, T. (1977). Genetic defect in responsiveness to the B cell mitogen lipopolysaccharide. Eur. J. Immunol. *7*, 325–328.

Dixit, E., Boulant, S., Zhang, Y., Lee, A.S., Odendall, C., Shum, B., Hacohen, N., Chen, Z.J., Whelan, S.P., Fransen, M., et al. (2010). Peroxisomes are signaling platforms for antiviral innate immunity. Cell *141*, 668–681.

Drecktrah, D., Knodler, L.A., Howe, D., and Steele-Mortimer, O. (2007). Salmonella trafficking is defined by continuous dynamic interactions with the endolysosomal system. Traffic *8*, 212–225.

Ewald, S.E., Lee, B.L., Lau, L., Wickliffe, K.E., Shi, G.P., Chapman, H.A., and Barton, G.M. (2008). The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. Nature *456*, 658–662.

Figueira, R., and Holden, D.W. (2012). Functions of the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system effectors. Microbiology *158*, 1147–1161.

Frey, E.A., Miller, D.S., Jahr, T.G., Sundan, A., Bazil, V., Espevik, T., Finlay, B.B., and Wright, S.D. (1992). Soluble CD14 participates in the response of cells to lipopolysaccharide. J. Exp. Med. *176*, 1665–1671.

Gerard, C. (1998). Bacterial infection. For whom the bell tolls. Nature *395*, 217–219.

Gerth, A., Grosche, J., Nieber, K., and Hauschildt, S. (2005). Intracellular LPS inhibits the activity of potassium channels and fails to activate NFkappaB in human macrophages. J. Cell. Physiol. *202*, 442–452.

Giardina, P.C., Gioannini, T., Buscher, B.A., Zaleski, A., Zheng, D.S., Stoll, L., Teghanemt, A., Apicella, M.A., and Weiss, J. (2001). Construction of acetate auxotrophs of *Neisseria meningitidis* to study host-meningococcal endotoxin interactions. J. Biol. Chem. *276*, 5883–5891.

Gioannini, T.L., and Weiss, J.P. (2007). Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. Immunol. Res. *39*, 249–260.

Gioannini, T.L., Teghanemt, A., Zhang, D., Coussens, N.P., Dockstader, W., Ramaswamy, S., and Weiss, J.P. (2004). Isolation of an endotoxin-MD-2

complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. Proc. Natl. Acad. Sci. USA *101*, 4186–4191.

Gioannini, T.L., Teghanemt, A., Zhang, D., Levis, E.N., and Weiss, J.P. (2005). Monomeric endotoxin:protein complexes are essential for TLR4-dependent cell activation. J. Endotoxin Res. *11*, 117–123.

Golenbock, D.T., Hampton, R.Y., Qureshi, N., Takayama, K., and Raetz, C.R. (1991). Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. J. Biol. Chem. *266*, 19490–19498.

Guo, L., Lim, K.B., Gunn, J.S., Bainbridge, B., Darveau, R.P., Hackett, M., and Miller, S.I. (1997). Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes phoP-phoQ. Science *276*, 250–253.

Guo, L., Lim, K.B., Poduje, C.M., Daniel, M., Gunn, J.S., Hackett, M., and Miller, S.I. (1998). Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell *95*, 189–198.

Hagar, J.A., Powell, D.A., Aachoui, Y., Ernst, R.K., and Miao, E.A. (2013). Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science *341*, 1250–1253.

Hang, H.C., Loureiro, J., Spooner, E., van der Velden, A.W., Kim, Y.M., Pollington, A.M., Maehr, R., Starnbach, M.N., and Ploegh, H.L. (2006). Mechanism-based probe for the analysis of cathepsin cysteine proteases in living cells. ACS Chem. Biol. *1*, 713–723.

Haziot, A., Chen, S., Ferrero, E., Low, M.G., Silber, R., and Goyert, S.M. (1988). The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. J. Immunol. *141*, 547–552.

Haziot, A., Ferrero, E., Köntgen, F., Hijiya, N., Yamamoto, S., Silver, J., Stewart, C.L., and Goyert, S.M. (1996). Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. Immunity *4*, 407–414.

Haziot, A., Hijiya, N., Gangloff, S.C., Silver, J., and Goyert, S.M. (2001). Induction of a novel mechanism of accelerated bacterial clearance by lipopolysaccharide in CD14-deficient and Toll-like receptor 4-deficient mice. J. Immunol. *166*, 1075–1078.

Heppner, G., and Weiss, D.W. (1965). High susceptibility of Strain A mice to endotoxin and endotoxin-red blood cell mixtures. J. Bacteriol. *90*, 696–703.

Hernandez, L.D., Hueffer, K., Wenk, M.R., and Galán, J.E. (2004). *Salmonella* modulates vesicular traffic by altering phosphoinositide metabolism. Science *304*, 1805–1807.

Horner, S.M., Liu, H.M., Park, H.S., Briley, J., and Gale, M., Jr. (2011). Mitochondrial-associated endoplasmic reticulum membranes (MAM) form innate immune synapses and are targeted by hepatitis C virus. Proc. Natl. Acad. Sci. USA *108*, 14590–14595.

Horng, T., Barton, G.M., Flavell, R.A., and Medzhitov, R. (2002). The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature *420*, 329–333.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J. Immunol. *162*, 3749–3752.

Isberg, R.R., O'Connor, T.J., and Heidtman, M. (2009). The *Legionella pneumophila* replication vacuole: making a cosy niche inside host cells. Nat. Rev. Microbiol. *7*, 13–24.

Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., Huber, M., Kalis, C., Keck, S., Galanos, C., et al. (2005). CD14 is required for MyD88-independent LPS signaling. Nat. Immunol. *6*, 565–570.

Kagan, J.C. (2012a). Signaling organelles of the innate immune system. Cell *151*, 1168–1178.

Kagan, J.C. (2012b). Defining the subcellular sites of innate immune signal transduction. Trends Immunol. *33*, 442–448.

Kagan, J.C., and Medzhitov, R. (2006). Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell *125*, 943–955.

Kagan, J.C., Stein, M.P., Pypaert, M., and Roy, C.R. (2004). *Legionella* subvert the functions of Rab1 and Sec22b to create a replicative organelle. J. Exp. Med. *199*, 1201–1211.

Kagan, J.C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2008). TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat. Immunol. *9*, 361–368.

Kawahara, K., Tsukano, H., Watanabe, H., Lindner, B., and Matsuura, M. (2002). Modification of the structure and activity of lipid A in *Yersinia pestis* lipopolysaccharide by growth temperature. Infect. Immun. *70*, 4092–4098.

Kayagaki, N., Warming, S., Lamkanfi, M., Vande Walle, L., Louie, S., Dong, J., Newton, K., Qu, Y., Liu, J., Heldens, S., et al. (2011). Non-canonical inflammasome activation targets caspase-11. Nature *479*, 117–121.

Kayagaki, N., Wong, M.T., Stowe, I.B., Ramani, S.R., Gonzalez, L.C., Akashi-Takamura, S., Miyake, K., Zhang, J., Lee, W.P., Muszyński, A., et al. (2013). Noncanonical inflammasome activation by intracellular LPS independent of TLR4. Science *341*, 1246–1249.

Kim, H.M., Park, B.S., Kim, J.I., Kim, S.E., Lee, J., Oh, S.C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O.J., and Lee, J.O. (2007). Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. Cell *130*, 906–917.

Lamkanfi, M., and Dixit, V.M. (2012). Inflammasomes and their roles in health and disease. Annu. Rev. Cell Dev. Biol. *28*, 137–161.

Li, X.D., Sun, L., Seth, R.B., Pineda, G., and Chen, Z.J. (2005). Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc. Natl. Acad. Sci. USA *102*, 17717–17722.

Li, H., Xu, H., Zhou, Y., Zhang, J., Long, C., Li, S., Chen, S., Zhou, J.M., and Shao, F. (2007). The phosphothreonine lyase activity of a bacterial type III effector family. Science *315*, 1000–1003.

McGourty, K., Thurston, T.L., Matthews, S.A., Pinaud, L., Mota, L.J., and Holden, D.W. (2012). *Salmonella* inhibits retrograde trafficking of mannose-6-phosphate receptors and lysosome function. Science *338*, 963–967.

Medzhitov, R. (2009). Approaching the asymptote: 20 years later. Immunity *30*, 766–775.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A., Jr. (1997). A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature *388*, 394–397.

Meng, J., Gong, M., Björkbacka, H., and Golenbock, D.T. (2011). Genome-wide expression profiling and mutagenesis studies reveal that lipopolysaccharide responsiveness appears to be absolutely dependent on TLR4 and MD-2 expression and is dependent upon intermolecular ionic interactions. J. Immunol. *187*, 3683–3693.

Miyake, K., Yamashita, Y., Hitoshi, Y., Takatsu, K., and Kimoto, M. (1994). Murine B cell proliferation and protection from apoptosis with an antibody against a 105-kD molecule: unresponsiveness of X-linked immunodeficient B cells. J. Exp. Med. *180*, 1217–1224.

Montminy, S.W., Khan, N., McGrath, S., Walkowicz, M.J., Sharp, F., Conlon, J.E., Fukase, K., Kusumoto, S., Sweet, C., Miyake, K., et al. (2006). Virulence factors of *Yersinia pestis* are overcome by a strong lipopolysaccharide response. Nat. Immunol. *7*, 1066–1073.

Moran, A.P., Lindner, B., and Walsh, E.J. (1997). Structural characterization of the lipid A component of *Helicobacter pylori* rough- and smooth-form lipopolysaccharides. J. Bacteriol. *179*, 6453–6463.

Mukherjee, S., Keitany, G., Li, Y., Wang, Y., Ball, H.L., Goldsmith, E.J., and Orth, K. (2006). Yersinia YopJ acetylates and inhibits kinase activation by blocking phosphorylation. Science *312*, 1211–1214.

Nagai, H., Kagan, J.C., Zhu, X., Kahn, R.A., and Roy, C.R. (2002a). A bacterial guanine nucleotide exchange factor activates ARF on Legionella phagosomes. Science *295*, 679–682.

Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T., Kosugi, A., Kimoto, M., and Miyake, K. (2002b). Essential role of MD-2 in LPS responsiveness and TLR4 distribution. Nat. Immunol. *3*, 667–672.

Newton, C.A., Perkins, I., Widen, R.H., Friedman, H., and Klein, T.W. (2007). Role of Toll-like receptor 9 in *Legionella pneumophila*-induced interleukin-12 p40 production in bone marrow-derived dendritic cells and macrophages from permissive and nonpermissive mice. Infect. Immun. *75*, 146–151.

Ohto, U., Fukase, K., Miyake, K., and Satow, Y. (2007). Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa. Science *316*, 1632–1634.

Orth, K., Palmer, L.E., Bao, Z.Q., Stewart, S., Rudolph, A.E., Bliska, J.B., and Dixon, J.E. (1999). Inhibition of the mitogen-activated protein kinase kinase superfamily by a *Yersinia* effector. Science *285*, 1920–1923.

Orth, K., Xu, Z., Mudgett, M.B., Bao, Z.Q., Palmer, L.E., Bliska, J.B., Mangel, W.F., Staskawicz, B., and Dixon, J.E. (2000). Disruption of signaling by *Yersinia* effector YopJ, a ubiquitin-like protein protease. Science *290*, 1594–1597.

Paciello, I., Silipo, A., Lembo-Fazio, L., Curcurù, L., Zumsteg, A., Noël, G., Ciancarella, V., Sturiale, L., Molinaro, A., and Bernardini, M.L. (2013). Intracellular *Shigella* remodels its LPS to dampen the innate immune recognition and evade inflammasome activation. Proc. Natl. Acad. Sci. USA *110*, E4345–E4354.

Palmer, L.E., Hobbie, S., Galán, J.E., and Bliska, J.B. (1998). YopJ of *Yersinia pseudotuberculosis* is required for the inhibition of macrophage TNF-alpha production and downregulation of the MAP kinases p38 and JNK. Mol. Microbiol. *27*, 953–965.

Park, B., Brinkmann, M.M., Spooner, E., Lee, C.C., Kim, Y.M., and Ploegh, H.L. (2008). Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. Nat. Immunol. *9*, 1407–1414.

Park, B.S., Song, D.H., Kim, H.M., Choi, B.S., Lee, H., and Lee, J.O. (2009). The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature *458*, 1191–1195.

Perera, P.Y., Vogel, S.N., Detore, G.R., Haziot, A., and Goyert, S.M. (1997). CD14-dependent and CD14-independent signaling pathways in murine macrophages from normal and CD14 knockout mice stimulated with lipopolysaccharide or taxol. J. Immunol. *158*, 4422–4429.

Perry, R.D., and Fetherston, J.D. (1997). *Yersinia pestis*—etiologic agent of plague. Clin. Microbiol. Rev. *10*, 35–66.

Phillips, N.J., Schilling, B., McLendon, M.K., Apicella, M.A., and Gibson, B.W. (2004). Novel modification of lipid A of *Francisella tularensis*. Infect. Immun. *72*, 5340–5348.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., et al. (1998a). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085–2088.

Poltorak, A., Smirnova, I., He, X., Liu, M.Y., Van Huffel, C., McNally, O., Birdwell, D., Alejos, E., Silva, M., Du, X., et al. (1998b). Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region. Blood Cells Mol. Dis. *24*, 340–355.

Prohinar, P., Re, F., Widstrom, R., Zhang, D., Teghanemt, A., Weiss, J.P., and Gioannini, T.L. (2007). Specific high affinity interactions of monomeric endotoxin.protein complexes with Toll-like receptor 4 ectodomain. J. Biol. Chem. *282*, 1010–1017.

Pugin, J., Schürer-Maly, C.C., Leturcq, D., Moriarty, A., Ulevitch, R.J., and Tobias, P.S. (1993). Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc. Natl. Acad. Sci. USA *90*, 2744–2748.

Raetz, C.R., Reynolds, C.M., Trent, M.S., and Bishop, R.E. (2007). Lipid A modification systems in gram-negative bacteria. Annu. Rev. Biochem. *76*, 295–329.

Rangarajan, M., Aduse-Opoku, J., Paramonov, N., Hashim, A., Bostanci, N., Fraser, O.P., Tarelli, E., and Curtis, M.A. (2008). Identification of a second lipopolysaccharide in *Porphyromonas gingivalis* W50. J. Bacteriol. *190*, 2920–2932.

Rebeil, R., Ernst, R.K., Gowen, B.B., Miller, S.I., and Hinnebusch, B.J. (2004). Variation in lipid A structure in the pathogenic yersiniae. Mol. Microbiol. *52*, 1363–1373.

Rietschel, E.T., Kirikae, T., Schade, F.U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A.J., Zähringer, U., Seydel, U., Di Padova, F., et al. (1994). Bacterial endotoxin: molecular relationships of structure to activity and function. FASEB J. *8*, 217–225.

Rowe, D.C., McGettrick, A.F., Latz, E., Monks, B.G., Gay, N.J., Yamamoto, M., Akira, S., O'Neill, L.A., Fitzgerald, K.A., and Golenbock, D.T. (2006). The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. Proc. Natl. Acad. Sci. USA *103*, 6299–6304.

Sandström, G., Sjöstedt, A., Johansson, T., Kuoppa, K., and Williams, J.C. (1992). Immunogenicity and toxicity of lipopolysaccharide from *Francisella tularensis* LVS. FEMS Microbiol. Immunol. *5*, 201–210.

Sasai, M., Linehan, M.M., and Iwasaki, A. (2010). Bifurcation of Toll-like receptor 9 signaling by adaptor protein 3. Science *329*, 1530–1534.

Schumann, R.R., Leong, S.R., Flaggs, G.W., Gray, P.W., Wright, S.D., Mathison, J.C., Tobias, P.S., and Ulevitch, R.J. (1990). Structure and function of lipopolysaccharide binding protein. Science *249*, 1429–1431.

Sherwood, R.K., and Roy, C.R. (2013). A Rab-centric perspective of bacterial pathogen-occupied vacuoles. Cell Host Microbe *14*, 256–268.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J. Exp. Med. *189*, 1777–1782.

Swanson, M.S., and Hammer, B.K. (2000). *Legionella pneumophila* pathogenesis: a fateful journey from amoebae to macrophages. Annu. Rev. Microbiol. *54*, 567–613.

Terebiznik, M.R., Vieira, O.V., Marcus, S.L., Slade, A., Yip, C.M., Trimble, W.S., Meyer, T., Finlay, B.B., and Grinstein, S. (2002). Elimination of host cell PtdIns(4,5)P(2) by bacterial SigD promotes membrane fission during invasion by *Salmonella*. Nat. Cell Biol. *4*, 766–773.

Tobias, P.S., Soldau, K., and Ulevitch, R.J. (1986). Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. J. Exp. Med. *164*, 777–793.

Tobias, P.S., Soldau, K., Kline, L., Lee, J.D., Kato, K., Martin, T.P., and Ulevitch, R.J. (1993). Cross-linking of lipopolysaccharide (LPS) to CD14 on THP-1 cells mediated by LPS-binding protein. J. Immunol. *150*, 3011–3021.

Triantafilou, M., Miyake, K., Golenbock, D.T., and Triantafilou, K. (2002). Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J. Cell Sci. *115*, 2603–2611.

Tseng, T.T., Tyler, B.M., and Setubal, J.C. (2009). Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. BMC Microbiol. *9* (*Suppl 1*), S2.

Vinogradov, E., Perry, M.B., and Conlan, J.W. (2002). Structural analysis of *Francisella tularensis* lipopolysaccharide. Eur. J. Biochem. *269*, 6112–6118.

Viriyakosol, S., Tobias, P.S., Kitchens, R.L., and Kirkland, T.N. (2001). MD-2 binds to bacterial lipopolysaccharide. J. Biol. Chem. *276*, 38044–38051.

Vogel, S.N., Moore, R.N., Sipe, J.D., and Rosenstreich, D.L. (1980). BCG-induced enhancement of endotoxin sensitivity in C3H/HeJ mice. I. In vivo studies. J. Immunol. *124*, 2004–2009.

Wang, S., Miura, M., Jung, Yk., Zhu, H., Gagliardini, V., Shi, L., Greenberg, A.H., and Yuan, J. (1996). Identification and characterization of Ich-3, a member of the interleukin-1beta converting enzyme (ICE)/Ced-3 family and an upstream regulator of ICE. J. Biol. Chem. *271*, 20580–20587.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., and Mathison, J.C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science *249*, 1431–1433.

Yamamoto, M., Sato, S., Hemmi, H., Sanjo, H., Uematsu, S., Kaisho, T., Hoshino, K., Takeuchi, O., Kobayashi, M., Fujita, T., et al. (2002). Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature *420*, 324–329.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003a). Role of

adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science *301*, 640–643.

Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. (2003b). TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat. Immunol. *4*, 1144–1150.

Zanoni, I., Ostuni, R., Capuano, G., Collini, M., Caccia, M., Ronchi, A.E., Rocchetti, M., Mingozzi, F., Foti, M., Chirico, G., et al. (2009). CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation. Nature *460*, 264–268.

Zanoni, I., Ostuni, R., Marek, L.R., Barresi, S., Barbalat, R., Barton, G.M., Granucci, F., and Kagan, J.C. (2011). CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. Cell *147*, 868–880.

Zanoni, I., Ostuni, R., Barresi, S., Di Gioia, M., Broggi, A., Costa, B., Marzi, R., and Granucci, F. (2012). CD14 and NFAT mediate lipopolysaccharide-induced skin edema formation in mice. J. Clin. Invest. *122*, 1747–1757.

Zhou, D., and Galán, J. (2001). *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. Microbes Infect. *3*, 1293–1298.