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Microbe-inducible trafficking pathways that control Toll-like Receptor signaling

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Abstract

The receptors of the mammalian innate immune system are designed for rapid microbial detection, and are located in organelles that are conducive to serve these needs. However, emerging evidence indicates that the sites of microbial detection are not the sites of innate immune signal transduction. Rather, microbial detection triggers the movement of receptors to regions of the cell where factors called sorting adaptors detect active receptors and promote downstream inflammatory responses. These findings highlight the critical role that membrane trafficking pathways play in the initiation of innate immunity to infection. In this review, we describe pathways that promote the microbe-inducible endocytosis of Toll-like Receptors (TLRs), and the microbe-inducible movement of TLRs between intracellular compartments. We highlight a new class of proteins called Transporters Associated with the eXecution of Inflammation (TAXI), which have the unique ability to transport TLRs and their microbial ligands to signaling-competent regions of the cell, and we discuss the means by which the subcellular sites of signal transduction are defined.

Graphical Abstract

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Keywords

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The mammalian innate immune system serves as the frontier of the host to protect against pathogenic microbes and to maintain mutualism with symbiotic microbes ^{1,2}. As originally postulated by Charles Janeway Jr. ³, hallmarks of the innate immune system comprise two fundamental features: 1) the ability to distinguish self-molecules from non-self molecules of potentially infectious agents; and 2) the ability to induce adaptive immunity and long-term protection against infectious agents ³. Central to the composition of the innate immune system is a group of structurally diverse protein families collectively known as the pattern recognition receptors (PRRs). PRRs are categorized into at least five families according to their structural characteristics, including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), Nucleotide-binding domain, Leucine-rich Repeat (LRR)-containing proteins (NLRs), and AIM2-like receptors (ALRs) ⁴. These PRR families are further divided into membrane-bound sensors (TLRs and CLRs) and cytosolic sensors (RLRs, NLRs, and ALRs) based on their primary subcellular positions ¹. PRRs survey the extracellular space, intracellular compartments, and the cytosol for the presence of conserved microbial components (*e.g.*, LPS, RNA, DNA) and specific microbial activities (*e.g.*, toxins, secretion systems, and enzymatic activities of virulence factors) ⁵. Upon sensing their cognate microbial ligands, PRRs induce a plethora of host responses that lead to the immediate control of infection and the subsequent induction of adaptive immune responses ⁶.

Our knowledge of distinct PRR families has expanded greatly since the identification of the first mammalian TLRs in the late 1990s ^{7,8}. The field has benefited from technical advances in genomics, transcriptomics, and proteomics, resulting in the acquisition of considerable insight into the regulation of PRR signaling ⁹. Today, the PRR signaling network provides fertile ground for cell biological studies that attempt to reveal how different components of these networks are connected and operate in space and time ⁹. The importance of understanding these cell biological aspects of innate immunity can be underscored by the fact that mislocalized PRR regulatory proteins render the host susceptible to infection ¹⁰ or prone to autoimmune disease upon the accidental recognition of self-molecules ^{11,12}. In this review, we highlight past and recent advances in the cell biological regulation of PRR signaling, with a focus on the TLR family. Specifically, we explore the emerging view that microbe-inducible trafficking pathways are important to both promote and inactivate TLR signal transduction, with unique signaling pathways emanating from specific organelles. This idea has placed membrane trafficking pathways at the very center of TLR regulation, with significant importance for advancing our understanding of basic biology and human health.

General features of the TLR family

TLRs are type I transmembrane proteins that are composed of three distinct structural elements: a leucine rich repeat containing ectodomain involved in ligand binding, a

transmembrane domain, and a cytosolic tail harboring the Toll/IL-1 receptor homology (TIR) domain that binds downstream adaptor proteins¹³. Structural studies revealed that ligand-induced receptor dimerization is a common means of activating TLR signal transduction. A total of 13 mammalian TLRs have been identified and subsets of TLRs are expressed differentially in humans and mice. Humans express TLRs-1-10; whereas mice express TLRs-1-9 and TLRs-11-13. TLRs are categorized broadly as either cell surface or endosomal receptors¹. Specifically, TLRs-1, -2, -4, -5, and -6 localize at the plasma membrane, and recognize surface molecules derived from microorganisms. In contrast, TLRs-3, 7-9, 11-13 localize within the endosomal network where they sense nucleic acid species and components of intracellular parasites^{1,4} (Table 1). Therefore, the steady-state positions of TLRs correlate with the nature of the ligands they detect, and likely evolved this feature to maximize rapid recognition.

Microbe-inducible endocytosis pathways that regulate TLR signaling

While the primary sites of TLR residence ensure detection of microbial ligands, the site of ligand recognition does not always represent the site of signal transduction. Rather, microbe-induced receptor trafficking is required to promote cellular responses directed by these receptors. In this section, we introduce how microbial ligands induce the movement of TLRs to promote diverse host responses.

Much of our current knowledge of ligand-induced TLR trafficking originated from the characterization of TLR4. As the prototype of the mammalian TLR family, TLR4 senses extracellular lipopolysaccharide (LPS), the major outer membrane component of gram-negative bacteria^{7,8,14}. Upon sensing LPS, TLR4 engages four TIR domain-containing adaptors (MyD88, TIRAP, TRAM, and TRIF) to activate diverse transcription factors that induce pro-inflammatory cytokine production (via NF- κ B and AP-1) and type I interferon (IFN) responses (via IFN regulatory factors (IRFs))^{15–18}. Although TLR4 is required for the induction of transcriptional responses to picomolar concentration of LPS, TLR4 alone is not sufficient to complete the LPS recognition process. Indeed, expression of TLR4 alone did not confer LPS responsiveness to reporter cell lines¹⁹, and biophysical studies demonstrated that TLR4 has a relatively low affinity to LPS (K_d at the order of 10^5) in solution^{20,21}. Indeed, several host accessory proteins operate with TLR4 to facilitate high-affinity detection of LPS in the extracellular milieu. These proteins are LPS-binding protein (LBP), CD14, and MD-2^{19,22,23}. The detailed mechanism of how these proteins operate coordinately to sense extracellular LPS has been reviewed elsewhere²⁴. In brief, the LPS-binding affinity of these proteins increases gradually from LBP, CD14, to the TLR4/MD-2 complex, thereby ensuring the unidirectional delivery of LPS from the extracellular space to TLR4²⁵.

Recent studies have revealed that, in addition to their LPS-binding/transport activities, CD14 and MD-2 function to transport TLR4 to signaling-competent subcellular locations. An example of this principle came from studies of CD14, a glycosylphosphatidylinositol (GPI)-anchored protein that resides on discrete plasma membrane sub-domains known as the lipid rafts²³. Due to the lack of a cytosolic signaling domain, CD14 was once considered to function merely to deliver LPS to TLR4. However, CD14 is now recognized to transport

TLR4 to lipid rafts where the receptor engages the adaptor proteins TIRAP and MyD88 to induce rapid NF- κ B activation and pro-inflammatory cytokine production²⁶. Whereas the molecular mechanism underlying this process is defined poorly, CD14-dependent synthesis of PI(4,5)P2 via PIP5K I α /PIP5K I γ , and cellular processes regulated by the small GTPase Arf6, promote the recruitment of TLR4 to lipid rafts (or the formation of lipid rafts around TLR4) by altering plasma membrane lipid composition^{27,28}.

CD14 also controls the LPS-induced endocytosis of TLR4. Notably, LPS-induced TLR4 endocytosis proceeds normally in cells expressing signaling-incompetent alleles of TLR4 (29, 33), thereby revealing endocytosis as an LPS-inducible response that is not mediated by TLR4 signal transduction²⁹. This finding is significant for two reasons. First, as will be described in detail later in this review, TLR4 induces the expression of type I IFNs and other inflammatory mediators from endosomes specifically. Understanding TLR4 endocytosis is therefore important to understand the inflammatory activities induced by this receptor. Second, prior to this finding, it was widely believed that TLR4 mediated all cellular responses to LPS³⁰. The discovery that TLR4 signaling does not promote its own endocytosis therefore reveals the first LPS-induced host response that is not dependent on TLR4 signal transduction. In subsequent years, other TLR4-independent LPS responses have emerged, with a notable example being the LPS-inducible assembly of non-canonical inflammasomes^{31,32}.

Ample evidence supports the conclusion that LPS-induced endocytosis proceeds independent of TLR4 signaling. For example, LPS promotes the endocytosis of TLR4 and CD14. This process is extremely rapid, with over 80% of CD14 being removed from the cell surface within two minutes of LPS treatment³³. Notably, the kinetics of LPS-induced CD14 endocytosis in TLR4-deficient macrophages tracks with that of wild type (WT) macrophages^{29,33}. In contrast, TLR4 endocytosis does not occur in cells lacking CD14. Thus, TLR4 is merely cargo for an LPS-inducible endocytic process that is mediated by CD14. Key signaling components facilitating this CD14-dependent endocytosis pathway include the immunoreceptor tyrosine-based activation motif (ITAM) adaptors DAP12 and Fc γ R, the tyrosine kinase Syk, phospholipase C γ 2 (PLC γ 2), and the inositol 1,4,5-trisphosphate receptors (IP3Rs)^{29,34}. Furthermore, second messengers such as Ca²⁺ and the reactive oxygen species (ROS) generated by LPS stimulation also promote receptor endocytosis³⁴. Since ITAM adaptor proteins, Syk, and PLC γ 2 are required for the internalization of other innate immune receptors, such as Dectin-1 and Fc γ R1^{35–37}, a common inflammatory endocytosis pathway may exist to promote the transport and possible signaling activities of multiple immune receptors.

Mechanistic analysis of the process by which TLR4 is selected for CD14-dependent endocytosis revealed that the cytosolic tail of TLR4 is dispensable for the initial step of receptor internalization. Indeed, a TLR4 allele that lacks its entire cytosolic domain could be endocytosed in response to LPS³³. Classic models of cargo selection for endocytosis involve direct interactions between the cytosolic tail of the cargo protein and the cognate endocytic adaptors³⁸. Thus, the observation that the TLR4 tail is not necessary for endocytosis highlights the unusual nature of TLR4 endocytosis, in that interactions occurring in the extracellular space select TLR4 as cargo for endocytosis³³. In keeping with

this finding, MD-2, the aforementioned high-affinity LPS-binding protein, functions as the cargo selection agent to promote TLR4 endocytosis. MD-2 is a secreted protein that localizes on the cell surface through an association with the extracellular domain of TLR4¹⁹. Structurally, MD-2 adopts a β -cup fold conformation and in contrast to TLR4, it interacts extensively with LPS by accommodating five out of six acyl chains from the lipid A component of LPS^{20,39,40}. The remaining sixth acyl chain extends out from the LPS-binding pocket of MD-2 and binds the extracellular domain of TLR4 in a distinct TLR4/MD-2 heterodimer, thereby inducing dimerization of two TLR4/MD-2 complexes⁴¹. Thus, MD-2 activates TLR4 signaling by mediating receptor dimerization^{40,41}. The cargo selection role of MD-2 in TLR4 endocytosis was revealed by the findings that MD-2 deficiency abolishes TLR4 endocytosis and that MD-2 alleles unable to bind or dimerize TLR4 fail to rescue the defect in TLR4 endocytosis in MD-2-deficient macrophages³³. In contrast, CD14 endocytosis is unaffected by MD-2 deficiency. Thus, whereas CD14 promotes the process of LPS-induced endocytosis, MD-2 promotes the selection of cargo for this pathway (Figure 1). Taken together, these observations establish that MD-2 controls two essential and interconnected cellular responses to LPS: TLR4 trafficking and TLR4 signaling.

Overall, a common feature of CD14 and MD-2 is that while individually these proteins lack the ability to directly induce inflammatory responses, they transport TLR4 to distinct subcellular sites to induce diverse inflammatory responses. Therefore, CD14 and MD-2 could be categorized as Transporters Associated with the eXecution of Inflammation-“TAXI” proteins³³, which have dual abilities to transport ligands and receptors to precise subcellular locations, thereby triggering distinct host responses. TAXI proteins can be operationally distinguished from classic ligand-binding chaperones, which should transport microbial products to PRRs, but have no receptor transport functions. As will be described later in the manuscript, other proteins that interact with TLR2 ligands also exhibit TAXI functions in transporting ligands and receptors.

Within cells, TLR4 has been observed in multiple endosomal compartments, including Rab5-positive early endosomes, Rab11-positive recycling endosomes, multi-vesicular bodies (MVBs), and phagosomes^{42,43}. Although it has not been definitely demonstrated, TRIF-dependent type I IFN production likely emanates from early endosomes (or phagosomes) as TLR4 is rapidly internalized into the lumen of MVBs as these vesicles mature^{42,43}. The role of Rab11-positive endosomes in TLR4 signaling has attracted attention in recent years. Loss-of-function analysis revealed that Rab11 promotes maximal TLR4 signaling from endosomes⁴². Rab11 also promotes the delivery of MHC-I molecules to phagosomes containing gram-negative bacteria, which enhances cross presentation in dendritic cells (DCs)⁴⁴. Cross presentation is a process by which antigen presenting cells (APCs) employ MHC-I molecules to present antigens of extracellular origin to CD8⁺ T cells⁴⁵; whereas MHC-I molecules in APCs normally present endogenous antigens to CD8⁺ T cells⁴⁵. As such, cross presentation allows APCs to induce protective immune responses against pathogens (or tumors) that may possess immune-evasive strategies⁴⁵. In resting DCs, MHC-class I molecules are present in recycling endosomes marked with vesicle-associated membrane proteins (VAMPs) such as VAMP3/cellubrevin, and VAMP8/endobrevin. TLR4 signaling via the adaptor MyD88 triggers the IKK-2/IKK- β -dependent phosphorylation of

SNAP23 (Synaptosomal-Associated Protein, 23kDa) residing on phagosomes. Phosphorylated SNAP23 then engages the SNARE machineries to mediate fusion between phagosomes and Rab11-positive vesicles⁴⁴. Therefore, Rab11 works in concert with the TLR4-MyD88-IKK β -SNAP23 pathway to promote membrane trafficking events that contribute to cross presentation. Other regulators of vesicle trafficking, such as the hepatocyte growth factor regulated tyrosine kinase substrate (HRS), and the AP-3 complex, are implicated in shuttling TLR4 to the aforementioned destinations^{46,47}. Lastly, a Rab10-mediated process may replenish surface TLR4 expression after LPS stimulation⁴⁸. The precise molecular mechanism of the cargo-selection for intracellular TLR4 trafficking and the complete repertoire of host factors that regulate these trafficking events represent potentially fruitful directions that warrant further investigation.

The idea that microbial detection can promote receptor trafficking also applies to the operation TLR2. Similar to TLR4, TLR2 engages the adaptor pair MyD88 and TIRAP to propagate signal transduction. However, a fundamental difference between these two TLRs is that TLR4 forms a homodimer via MD-2 to trigger signal transduction, whereas TLR2 forms heterodimers with TLR1 or TLR6 to mediate ligand binding and inflammatory signal transduction⁴⁹. The TLR2 heterodimers employs several accessory molecules, such as CD14, CD36, and Mannose-binding lectin (MBL), to facilitate ligand recognition^{50–52}. In particular, CD14 facilitates ligand recognition by the TLR1/2 heterodimer⁵¹, whereas CD36 functions as the co-receptor for the TLR2/6 heterodimer⁵². Altogether, the flexibility in receptor pairing and the diversity in accessory protein utilization enable TLR2 to sense a broad spectrum of microbial surface products^{53,54}.

Akin to the role of CD14 in TLR4 signaling at the plasma membrane, CD36 shuttles the TLR2/6 heterodimer to lipid rafts in a ligand-inducible manner⁵⁵. In addition to its role in mediating uptake of lipotechoic acids (LTA) or the gram-positive bacterium *Staphylococcus aureus*, CD36 triggers the internalization of the TLR2/6 heterodimer^{56,57}. Specifically, this endocytosis process is regulated by the protein kinase Syk with the aid of Fc receptors⁵⁷. A small segment in the cytosolic tail of CD36 coordinates ligand uptake and TLR2 signaling⁵⁶, as point mutation alleles of CD36 (Y463F, C464S) affecting this segment not only impair ligand uptake, but also diminish TLR2-mediated inflammatory cytokine production⁵⁶. In corroboration with a previous report that palmitoylation of CD36 at C464 is important for its association with lipid rafts⁵⁸, these findings demonstrate that proper localization of CD36 is crucial for facilitating ligand recognition and TLR2 trafficking. Furthermore, these studies highlight that lipid rafts are the primary cellular site where TLR2 signaling initiates.

While TLR2 signaling mainly occurs at the plasma membrane, TLR2 can also be internalized upon ligand stimulation^{50,56,57} and colocalize with phagosomes loaded with zymosan particles⁵⁹. Moreover, TLR2 is recruited to phagosomes harboring intracellular bacterial pathogens, such as *Borrelia* and *Chlamydia* species^{60,61}. These observations raise the questions of whether the intracellular pool of TLR2 can mediate signaling from phagosomes or whether intracellular TLR2 signaling activates transcriptional programs different from surface TLR2 signaling. However, care must be taken that the mere presence of TLR2 on phagosomes might not predict that TLR2 actually signals from this subcellular

compartment. TLR2 can be found on phagosomes containing red blood cells instead of microbial ligands, indicating that TLR2 might be simply sampling phagosomal contents^{49,59}. By using bacterial and viral pathogens as tools, two studies provided a direct answer to these questions. For example, MBL, the aforementioned TLR2 accessory molecule, opsonizes *S. aureus* and subsequently promotes bacterial phagocytosis⁵⁰. Notably, MBL mediates the induction of TLR2 signaling from phagosomes by clustering *S. aureus* or the purified ligand LTA and presents these ligands to the TLR2/6 heterodimer, which subsequently activates MyD88-dependent pro-inflammatory responses⁵⁰. In this regard, MBL and CD36 can be considered TAXI proteins of TLR2 heterodimers, in that they promote the transport of ligands and receptors to signaling competent organelles (Figure 1).

A second study also revealed the interconnected nature of phagosomal TLR2 trafficking and intracellular TLR2 signaling. Infection of CD11b⁺Ly6C⁺ inflammatory monocytes with vaccinia virus led to the production of type I IFN that is dependent upon MyD88⁶². Notably, blockade of vaccinia virus endocytosis diminished type I IFN production while leaving virus-induced inflammatory cytokine production unimpaired⁶². Collectively, these studies establish that TLR2 can signal from phagosomes and induce inflammatory cytokine or type I IFN production via MyD88 in a cell type-specific manner. However, it must be noted that intact pathogens, such as *S. aureus* and vaccinia virus, display numerous microbial ligands. Thus, further studies are warranted to delineate the molecular identity of the ligands recognized by TLR2 under infection conditions.

Microbe-inducible trafficking pathways taken by endosomal TLRs

Endocytosis is not the only microbe inducible trafficking pathway that controls TLR signal transduction. Several studies in recent years have highlighted how endosomal TLRs will move in functionally important ways upon detection of their nucleic acid ligands¹. Studies of TLR9, the receptor for unmethylated CpG containing DNA, perhaps best exemplify this paradigm. Upon CpG-DNA stimulation, several host factors, including granulin, HMGB-1, LL-37, and FcγRIIa (CD32) have been implicated in delivering ligands to TLR9 (Figure 2)^{20,63,64}. The molecular mechanisms underlying the operation of these TLR9 accessory molecules have been reviewed elsewhere⁶⁵. In brief, identified by a proteomic screen searching for TLR9-interacting proteins in Raw264.7 cells, granulin is a secreted protein that can be found in the serum at high-concentration⁶⁶. Further analysis reveals that granulin is also able to bind CpG-DNA, thereby forming a granulin-DNA-TLR9 complex that promotes ligand recognition by TLR9⁶⁷. Accordingly, granulin-deficient mice produce less TNFα when stimulated with CpG-DNA. Moreover, the interaction between TLR9 and CpG-DNA in granulin-deficient macrophages is much weaker compared to that of their wild type counterparts, further validating the role of granulin in facilitating ligand recognition by TLR9⁶⁷. HMGB-1 is a versatile host protein that functions at the extracellular space, cytosol, and the nucleus to regulate various cellular processes, such as modulating chromatin structure, regulating transcriptional activities⁶⁸. Additionally, it serves as a marker for necroptosis and a danger signal for tissue damage⁶⁸. In the context of TLR9 signaling, CpG-DNA stimulation enhances the expression and secretion of HMGB-1, which in turn binds to CpG-DNA and delivers it to TLR9^{63,69}. HMGB-1-deficient macrophages and DCs produce less pro-inflammatory cytokines upon CpG-DNA stimulation in comparison to their

wild type counterparts^{63,69}. Furthermore, supplementing exogenous HMGB-1 could rescue the defect in cytokine production caused by HMGB-1 deficiency⁶³. These discoveries establish the role of HMGB-1 in promoting ligand recognition by TLR9. However, the same study also reported that the cytosolic pool of HMGB-1 associates with TLR9 prior to CpG-DNA stimulation⁶³. As such, further studies are required to elucidate how the extracellular and intracellular pools of HMGB-1 coordinate to facilitate TLR9-CpG-DNA interaction. Finally, LL-37 is a well-known broad-spectrum antimicrobial peptide composed of 37 amino acids⁷⁰. By disrupting bacterial membrane integrity, this peptide protects host from bacterial infection at mucosal surfaces⁷⁰. Recent studies demonstrated that besides its antimicrobial effect, LL-37 forms a complex with CpG-DNA and enhances DNA detection in B cells and a subset of DCs known as plasmacytoid DCs (pDCs)^{64,71,72}. Furthermore, LL-37 binds to self-DNA, forming an LL-37-DNA complex resistant to DNase degradation⁶⁴. The LL-37-DNA complex could be internalized by pDCs and transported to endosomes and thereby inducing TLR9 responses⁶⁴. Furthermore, delivery of self-DNA by LL-37 exacerbates progression of the autoimmune skin disease psoriasis, owing to aberrant pDC activation^{64,72}. However, direct interactions between LL-37 and TLR9 have not been reported, indicating that this peptide might only facilitate the ligand delivery process but not the formation of a downstream signaling complex. Besides LL-37, FcγRIIa (CD32) also promotes self-DNA containing immune complex recognition by TLR9 in human pDCs⁷³. Notably, CD32 mediates DNA immune complex uptake and the subsequent delivery of this ligand into TLR9-positive lysosomes, thereby triggering TLR9 signaling. Microscopic observation revealed that CD32, DNA immune complex and TLR9 colocalize with each other in lysosomes⁷³. CD32 blockade by a neutralizing antibody dampens TLR9-dependent responses to DNA immune complex⁷³. Whether CD32 regulates TLR9 trafficking after DNA immune complex sensing requires further investigation. Overall, an emerging theme from these studies is that diverse host factors promote ligand sensing by TLR9, but how these proteins function coordinately with each other during TLR9 signaling activation and whether these factors (granulin, HMGB-1, LL37, and CD32) function as TAXI proteins that couple ligand recognition to the intracellular transport of TLR9 await further investigation.

The outcomes of TLR9 signaling display cell-type specificity and are determined by the subcellular compartments where TLR9 activation occurs. For instance, macrophages and conventional DCs (cDCs) produce inflammatory cytokines (TNFα, IL-6, and IL-12p40) upon CpG-DNA stimulation, whereas plasmacytoid DCs (pDCs) produce type I IFN in addition to pro-inflammatory cytokines^{74–76}. Such differences were originally ascribed to the putative pDC-specific expression pattern of transcription factors, such as a high steady-state level of IRF7 expression^{75,76}. However, cell biological analysis using different types of TLR9 ligands revealed that differential trafficking of TLR9 to specific intracellular compartments determines the observed distinct signaling outcomes in pDCs. Specifically, the aggregation-prone form of CpG-DNA (CpG-A) traffics to early endosomes, where TLR9 promotes the IRF7-dependent induction of type I IFN induction⁷⁶. In contrast, the monomeric form of CpG-DNA (CpG-B) traffics to late endosomes/lysosomes (labeled with lysotracker), where TLR9 signaling triggers NF-κB activation to produce inflammatory cytokines⁷⁶. The mechanisms by which distinct forms of CpG-DNA are targeted to different endosomal compartments remain unclear. However, encapsulation of CpG-B with DOTAP, a

cationic liposomal transfection reagent, results in a shift in the distribution of CpG-B from late endosomes to early endosomes. This shift in locale correlated with a gain in the ability of CpG-B to induce type I IFN responses in pDCs ⁷⁶. Interestingly, DOTAP-mediated delivery of CpG-A into macrophages and cDCs, the cell types which normally do not produce type I IFN upon TLR9 stimulation, also activates type I IFN responses ⁷⁶. It is worth noting here that most studies of TLR9 signaling have used synthetic CpG-DNA that contains a phosphorothioate backbone that is distinct from the natural phosphodiester (PO) backbone. Recent studies have indicated that the mechanisms by which this unnatural DNA are detected by TLR9 are distinct from those elicited by natural DNA ⁷⁷. While further work is needed to better explain the actions of the TLR9 network in response to natural ligands, these findings suggest that intracellular compartments/endosomes are heterogeneous in terms of their abilities to initiate distinct signaling outcomes.

The aforementioned discoveries raise the question of what host factor(s) confers signaling specificity via endosomal TLRs. Two studies revealed the critical role of the adaptor protein complex AP-3 in inducing type I IFN expression downstream of TLR9 ^{78,79}. At the steady state, AP-3 regulates vesicle trafficking between endosomes and lysosome-related organelles (LRO). LROs resemble lysosomes in several ways, but usually contain cell-type specific components for specialized functions ⁸⁰. Iwasaki and colleagues discovered that upon ligand stimulation, AP-3 binds to TLR9 and mediates the movement of this receptor from VAMP3-positive endosomes to LAMP2-positive LROs ⁷⁸. Biochemically, AP-3 bridges the interaction between the E3 ubiquitin ligase TRAF3 and the transcription factor IRF7, thereby forming a signaling complex that promotes type I IFN production ⁷⁸. In keeping with these findings, AP-3 deficiency abolishes type I IFN production from CpG-DNA stimulated pDCs, further substantiating the tight coupling of receptor compartmentalization to the initiation of specific signaling outcomes (Figure 2) ^{78,79}. Of note, whereas AP-3 is absolutely required for type I IFN signaling from pDCs, its role in mediating inflammatory cytokine production in the same cell type is less clear and warrants further investigation. Additional host factors required for LRO biogenesis, including BLOC1 and BLOC2, are crucial for TLR9 signaling in pDCs, as BLOC1 or BLOC2 deficient mice produced less type I IFN in the serum after CpG-DNA stimulation in comparison to their WT counterparts ⁷⁹. While these studies provide clear evidence for CpG-inducible TLR9 trafficking and signaling, much additional work is needed, as the precise nature of the different intracellular compartments that harbor resting and signaling endosomal TLRs is unclear.

In addition to LRO regulatory proteins, a non-canonical form of autophagy, also known as the LC-3 associated phagocytosis (LAP), controls TLR9 signaling upon detection of self-DNA containing immune complexes in pDCs (Figure 2) ^{81,82}. Canonical autophagy is a multi-functional host pathway involved in regulation of development, alleviation of cellular stress, degradation of damaged organelles, and host defense against intracellular pathogens ⁸¹. A key cell biological feature of canonical autophagy is the formation of the autophagosome—a double membrane organelle-surrounding cargo proteins in the cytosol ⁸¹. The molecular mechanism of autophagosome assembly is currently a subject of intense study. This process is orchestrated by a cascade of reactions, including the activation of protein kinases (*e.g.* Ulk1 and Ulk2) and the subsequent protein conjugation processes mediated by various autophagy proteins (ATGs) ^{81,83}. Whereas non-canonical autophagy

utilizes the same ATG protein conjugation system as its canonical counterpart, non-canonical autophagy proceeds normally in the absence of Ulk1/2 activation⁸⁴. Furthermore, induction of non-canonical autophagy leads to the formation of a single membrane LC-3 positive structure distinct from the double membrane structure of the canonical autophagosome⁸⁵. In response to DNA-immune complexes, non-canonical autophagy is initiated with the aid of phosphatidylinositol 3-kinase (PI3K) and subsequently facilitates the delivery of DNA-immune complexes and TLR9 to phagosomes, thereby leading to type I IFN production⁸². This non-canonical autophagy-mediated type I IFN response is independent of AP-3 function, as AP-3-deficient pDCs are still able to produce type I IFN in response to DNA immune complexes⁸². Biochemical characterization of the LAP complex revealed that additional components, including Rubicon, UVRAG, Beclin-1, and VPS34, associate with PI3K to initiate LAP formation^{82,86}. Moreover, mice carrying mutations in several LAP components develop autoimmune disease-like phenotypes resembling systemic lupus erythematosus (SLE)⁸⁷. Further studies are warranted to determine the molecular crosstalk between LAP and CD32-mediated TLR9 signaling activation in the context of DNA immune complex stimulation. These findings demonstrate that multiple host trafficking pathways shuttle TLR9 to distinct intracellular compartments to trigger signaling events in response to ligands that exist in divergent forms.

Operating in concert with the aforementioned host factors, the enzymes controlling phosphoinositide (PI) metabolism exert a fundamental layer of control over the intracellular trafficking and signaling outcomes of endosomal TLRs. In general, as distinct panels of PI kinases and phosphatases reside on different organelles, the PIs generated by these enzymes function as “barcodes” that endow these organelles with unique membrane properties⁸⁸. Consistently, perturbing PI metabolism alters the trafficking and signaling outcomes of endosomal TLRs. For instance, chemical inhibition of the PI3K p110 δ catalytic subunit, which is preferentially expressed in pDCs and is responsible for PI(3,4,5)P₃ generation, abolishes TLR9-induced IFN responses without affecting TNF α and IL-6 production⁸⁹. Further cell biological analysis revealed that p110 δ inhibition blocks IRF7 nuclear translocation, thereby disrupting IFN responses. Whereas the precise biochemical processes and the trafficking events contributing to the abnormal IRF7 nuclear translocation remain elusive, these findings demonstrated the crucial roles of PI3K and PI(3,4,5)P₃ in TLR9 signaling⁸⁹. Similarly, another host PI kinase, PIKfyve, which catalyzes the production of PI(3,5)P₂, crucially determines signaling outcomes upon TLR9 activation in a cell type-specific manner⁹⁰. Using RAW264.7 cells expressing different tagged alleles of TLR9 as a surrogate model to study TLR9 trafficking, the same study showed that PIKfyve inhibitor blocks the trafficking of TLR9 to LAMP1-positive IRF7 endosomes while does not affect the trafficking of this receptor to VAMP3-positive NF- κ B endosomes upon CpG-A stimulation⁹⁰. Mechanistically, inhibition of PIKfyve activity prevents the synthesis of PI(3,5)P₂ on endosomal membranes⁹⁰, thereby diminishing the recruitment of AP-3 to TLR9-positive endosomes. Such perturbation of AP-3 localization impedes the trafficking of TLR9 to IRF7 endosomes and diminishes IFN production. However, the findings obtained from chemical inhibitors need to be interpreted with caution. First, given the delicate nature of pDCs, whether they could tolerate YM201636 treatment is unclear^{90,91}. Second, dependent upon their chemical properties, different inhibitors targeting the same host protein

usually leads to distinct phenotypes. For example, apilimod, a compound that also targets PIKfyve, dampens TLR9-mediated IFN and IL12p40 production without altering CpG-DNA induced TLR9 trafficking^{92,93}. Instead, apilimod-mediated inhibition of PIKfyve activity induces the transcription factor ATF3, which specifically binds to the promoter of IFN gene and therefore represses IFN gene transcription⁹³. Altogether, regardless of the perturbation of diverse cellular processes resulted from PIKfyve inhibition, these findings collectively established the important roles of this enzyme and PI(3,5)P2 in TLR9 signaling.

Apart from its role in TLR9 signaling, PIKfyve also positively regulates TLR7 signaling by promoting the intracellular transport of ssRNA⁹⁴. As PI(3,5)P2, the catalytic product of PIKfyve, enhances the activity of a lysosomal transient receptor potential channel (TRP) called Mucolipin-1. Activation of Mucolipin-1 by PI(3,5)P2 subsequently increases ssRNA delivery into lysosomes⁹⁴. Consistently, both YM201636-mediated inhibition of PIKfyve activity and Mucolipin-1 deficiency impair the trafficking of ssRNA to lysosomes and thereby reduce TLR7 responses⁹⁴. The precise mechanism of Mucolipin-1 induced lysosomal entry of ssRNA is currently unknown, and the additional host factors required for intracellular ssRNA transport warrant further investigation. Overall, these emerging findings illuminate that PIs and the cognate host enzymes involved in their biogenesis regulate the trafficking of TLRs, which further highlights the interconnected nature of innate immune signaling and general cell biological processes.

From a post translational perspective, the host ubiquitinylation machineries also regulate TLR9 (and TLR7) activation and trafficking in response to their cognate ligands. A recent genome-wide siRNA screen reported that ubiquitinylation of TLR7 and TLR9 is essential for HRS-mediated transport of these receptors from endosomes to MVBs and phagosomes. A TLR9 allele devoid of all available residues for ubiquitinylation fails to reach phagosomes and therefore signals poorly in response to CpG-DNA. This observation substantiated that ubiquitinylation is important for sorting TLR9 to the proper intracellular compartments where TLR9 signaling occurs. Whereas the host E3 ligase(s) responsible for ubiquitinating these receptors is unknown, the ability of HRS to govern intracellular transport of both endosomal and surface TLRs suggests that this protein is the nexus of TLR trafficking^{46,95}.

Sorting adaptor proteins: Landmarks that determine the subcellular sites of TLR signaling

As noted above, TLRs themselves do not determine the subcellular sites where signal transduction initiates. Instead, TAXI proteins move ligands and TLRs from subcellular sites of ligand recognition to sites of signal initiation⁹⁶. But what determines the sites of signaling?

Several studies in recent years have identified specific factors that determine the subcellular site(s) of TLR signal transduction. These factors are called sorting adaptors, and are the only known proteins that are found at the sites of signaling prior to microbial detection. In this regard, TLR sorting adaptors can be considered “landmarks” of where within the cell TLR signaling will eventually occur. The known TLR sorting adaptors are TIRAP, TRAM (and dMyD88 in *Drosophila*). Each of these proteins contain a TIR domain that functions to

detect dimerized (ligand-bound) TLRs at the plasma membrane or endosomes, depending on the receptor^{4,97}. Studies to understand the mechanisms by which TLR sorting adaptors are positioned at the sites of signaling have revealed the importance of phosphoinositides in these activities. An example of this principle can be found from the studies of TIRAP, whose N-terminal domain exhibits affinity for a broad spectrum of PI species, such as PI(4,5)P₂ and PI(3)P^{77,98}. The PI(4,5)P₂ binding property of TIRAP allows it to localize to subdomains of the plasma membrane that are rich in membrane ruffles⁹⁸. These structures are likely to be the operational equivalent of lipid rafts, although direct assessment of the localization of TIRAP to rafts has not been performed. Current views suggest that upon LPS encounter, TIRAP senses a dimerized TLR4 complex upon its delivery to lipid rafts by CD14^{26,98}. Subsequently, TIRAP bridges ligand-bound TLR4 to MyD88 which seeds the formation of the myddosome, a supramolecular organizing center (SMOC) composed of TIRAP, MyD88, and IRAK family kinases^{99,100}. The formation of the myddosome triggers a cascade of downstream signaling events, which culminates in the activation of NF- κ B and the production of inflammatory cytokines^{101,102}. The idea of the myddosome was based originally on *in vitro* analysis of the MyD88-IRAK kinases complex¹⁰³, but whether this complex exists in living cells and whether it is stably present or ligand-inducible has been unclear. Studies of TIRAP revealed this adaptor as a regulator of myddosome assembly, and revealed that this SMOC is assembled inducibly upon microbial detection^{77,100}. Of note, several post-translational modifications (*i.e.* phosphorylation, proteolytic cleavage, and ubiquitinylation) target distinct regions of TIRAP, indicating that the activity of TIRAP, as well as the assembly of myddosome, may be subject to multiple layers of regulation^{104–106}.

Due to the promiscuous nature of its PI-binding motif, TIRAP can also be found on endosomes via interactions with PI(3)P⁷⁷. From endosomes, TIRAP can detect ligand-bound TLR7 and TLR9 complexes, and consequently initiate myddosome-mediated inflammatory responses that are similar to those elicited by plasma membrane-localized TLR4⁷⁷. Consistently, blockade of TIRAP-TLR7/9 interactions by a synthetic peptide (2R9) inhibits signal transduction downstream of TLR7/9¹⁰⁷. Of note, restricting the specificity of TIRAP to a single lipid species, such as PI(4,5)P₂ or PI(3)P results in a mutant adaptor that can only survey a single organelle for active TLRs⁷⁷. Thus, in the case of TIRAP, the promiscuous PI-binding activity of this sorting adaptor is important to survey multiple sites in the cell for multiple microbe-detection events. In this regard, it is possible that the affinity of TIRAP for PI(3)P explains how the TLR2/6 heterodimer induces signal transduction from phagosomes⁵⁰.

The identification of TRAM as a sorting adaptor stems from the characterization of endosomal TLR4 signaling⁴³. Concomitant with myddosome formation at the cell surface, TLR4 is internalized in a CD14-dependent manner^{29,43}. The internalization of TLR4 is coupled to the activation of PI3K, which converts PI(4,5)P₂ to PI(3,4,5)P₃¹⁰⁵. This conversion appears important to dissociate TIRAP from membranes and allow TLR4 to engage TRAM on endosomes¹⁰⁵. Similar to TIRAP, TRAM is localized at both the plasma membrane and endosomes^{43,108}. However, the molecular mechanism determining the localization of TRAM differs from that of TIRAP, as TRAM relies on its N-terminal bipartite region modified by myristoylation to maintain its membrane association, whereas the localization of TIRAP depends on the aforementioned PI-binding domain^{43,98,108}.

TRAM senses endosomal TLR4 and recruits the signaling adaptor TRIF, which triggers type I IFN expression and prolongs the duration of NF- κ B activation^{43,102,108}. Several host factors including the kinase TBK1 and the E3 ligase TRAF3, are essential components in the TRIF signaling axis as determined by genetic and *in vitro* biochemical studies^{109–111}. Whether these TRIF-mediated signaling processes are propagated by the formation of the Trifosome, a putative SMOC similar to the myddosome¹⁰¹, warrants further research. Altogether, in response to ligand stimulation, the sorting adaptors TIRAP and TRAM determine the sites of signal transduction by sensing active TLRs and promoting the assembly of SMOCs that elicit inflammatory responses.

Recent studies on TLR2 have revealed intriguing similarities to TLR4, in terms of the location-specific uses of the sorting adaptors TIRAP and TRAM. Whereas early studies suggested that TLR2 only engaged the adaptor proteins MyD88 and TIRAP, a role for TRIF and TRAM in TLR2 signaling is now apparent^{15,16,112–115}. For instance, an inhibitory peptide derived from the vaccinia virus A46 protein that disrupts the binary interactions between TLR4-TIRAP and TLR4-TRAM, respectively, blocks TLR2-dependent type I IFN production¹¹⁴. Furthermore, TRAM interacts with MyD88 and shuttles MyD88 to intracellular structures in response to TLR2 ligands¹¹⁴. Finally, TRAM deficiency reduces host type I IFN production in response to *S. aureus*, further suggesting the involvement of TRAM in TLR2 signaling¹¹⁴. Nevertheless, as MyD88 also activates TLR2 signaling via TIRAP, how TRAM and TIRAP coordinate their interactions with MyD88 during TLR2 signaling requires further clarification. With regards to the role of TRIF in TLR2 signaling, a recent study showed that TRIF is required for the full capacity of TLR2-mediated *Ccl4* and *Ccl5* gene expression, as TRIF deficiency reduces the expression levels of *Ccl4* and *Ccl5* upon TLR2 ligand stimulation¹¹⁵. Consistent with this observation, pharmacological inhibition of endocytosis also impairs *Ccl4* and *Ccl5* expression downstream of TLR2, further substantiating the possibility of endosomal TLR2 signaling¹¹⁵. Others have shown that upon infection by *Borrelia burgdorferi*, the causative agent of Lyme disease, TRIF facilitates the production of IL-6 and type I IFN from intracellular compartments, and the aforementioned adaptor protein AP-3 is required for this intracellular signaling process^{113,116}. However, work on the roles of TRAM and TRIF in TLR2 signaling must continue, as the mechanisms underlying the actions of these adaptor pairs are largely unexplored.

Conclusion and future perspectives

In this review, we have highlighted past and recent discoveries on regulatory mechanisms that govern the trafficking of TLRs. In particular, there is a growing appreciation that microbe-induced receptor trafficking aided by TAXI proteins (Table 2) represents a regulatory means to control TLR signaling. Despite these advances, our knowledge of the cell biology of TLR trafficking, especially at the receptor proximal level, remains rudimentary. Indeed, whereas cell biological analysis of TLR signaling has yielded fruitful discoveries that connect distinct components within the pathway, additional questions persist. We consider the question of how diverse TLR accessory proteins coordinate ligand recognition and trafficking of their cognate TLRs as a fertile territory for future research. Additionally, since the heterogeneity of endosomes dictates the signaling outcomes of TLRs

that reside in them, the precise nature of these distinct intracellular signaling compartments awaits further elucidation. Lastly, cell type-specific regulation of TLR trafficking represents an attractive area for future studies. In conclusion, the knowledge obtained from cell biological characterization of the TLR pathway has been and will likely continue to advance our understanding of general cell biology of the host.

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Synopsis

In the context of lipopolysaccharide (LPS) stimulation, CD14 and MD-2 serve as Transporters Associated with the eXecution of Inflammation (TAXI) proteins for TLR4 to trigger plasma membrane myddosome formation and induce pro-inflammatory cytokine production. Subsequent endocytosis of TLR4 activates the TRAM and TRIF-dependent signaling process leading to type I IFN production. Intracellular TLR4 signaling could occur at distinct compartments including endosomes, MVBs, phagosomes. Ubiquitinylation and HRS-mediated sorting processes have been implicated in the intracellular sorting of TLR4.

In the context of lipotechoic acids (LTA) stimulation, MBL functions as a TAXI protein for the TLR2/6 heterodimer. MBL delivers LTA to the phagosome where a TLR2/6 heterodimer may promote myddosome formation to induce pro-inflammatory cytokine production.

Figure 1. Ligand-induced trafficking of surface TLRs

In the context of LPS stimulation, CD14 and MD-2 serve as TAXI proteins for TLR4 to first trigger plasma membrane myddosome formation and induce pro-inflammatory cytokine production. Subsequent endocytosis of TLR4 activates the TRAM and TRIF-dependent signaling process leading to type I IFN production. Intracellular TLR4 signaling could occur at distinct compartments including endosomes, MVBs, phagosomes. Ubiquitylation and HRS-mediated sorting processes have been implicated in the intracellular sorting of TLR4. In the context of LTA stimulation, MBL functions as a TAXI protein for the TLR2/6 heterodimer. MBL delivers LTA to the phagosome where a TLR2/6 heterodimer may promote myddosome formation to induce pro-inflammatory cytokine production.

Figure 2. Ligand-induced trafficking of endosomal TLRs with a primary focus on TLR9

In macrophages and conventional DCs, host factors such as LL-37, HMGB-1, and granulin facilitate the delivery of CpG-DNA to TLR9, which then induces myddosome formation at endosomes to induce pro-inflammatory cytokine production. In pDCs, LL-37 and CD32 mediate the delivery of DNA-immune complexes to TLR9. CpG-DNA induced TLR9 ubiquitinylation and HRS-dependent intracellular sorting are required for the activation of TLR9 signaling. In addition, proteolytic processing of TLR9 by endosomal cathepsins and AEPs licenses TLR9 signaling.

In pDCs, TLR9 trafficking to distinct intracellular compartments induces different signaling outcomes. A study led by Honda et al., proposes the model by which the forms of CpG determine the outcome of the transcriptional responses. In particular, the aggregation-prone CpG-A induces type I IFN production from early endosomes, whereas the monomeric CpG-B induces pro-inflammatory cytokines from late endosomes/lysosomes. In contrast, studies by Sasai et al., and Blasius et al., propose the model by which TLR9 induces inflammatory cytokine production in endosomes, and that AP-3 mediated TLR9 trafficking to LROs induces type I IFN production. Lastly, in response to the DNA-immune complex, the ligand uptake process is mediated by LAP and the subsequently assembled LAPosome delivers DNA-immune complex to phagosomes where TLR9-dependent signaling occurs to induce type I IFN production. Thus, three intracellular compartments in the pDCs have been linked to type I IFN production: the early endosome, the LRO, and the phagosome.

Table 1

TLRs and their microbial ligands

TLR	Localization	Microbial Ligands*
TLR1/TLR2	Plasma membrane	Triacylated lipopeptides
TLR2	Plasma membrane	Bacterial Lipoproteins, Lipoarabinomannan, zymosan
TLR3	Endosome	dsRNA
TLR4	Plasma membrane	LPS
TLR5	Plasma membrane	Flagellin
TLR2/6	Plasma membrane	Diacylated lipopeptides; <i>Mycoplasma</i> MALP-2, FSL-1
TLR7	Endosome	ssRNA
TLR8	Endosome	ssRNA
TLR9	Endosome	Bacterial and viral CpG DNA, <i>Plasmodium</i> haemozoin
TLR10	Plasma membrane	ND
TLR11	Endosome	<i>Toxoplasma</i> Profilin; uropathogenic <i>E.coli</i> (UPEC)
TLR12	Endosome	<i>Toxoplasma</i> Profilin
TLR13	Endosome	Bacterial 23S ribosomal RNA

* Please note that this table focuses on representative microbial ligands detected by TLRs, which does not represent an exhaustive list of TLR ligands. As such, synthetic ligands and host-derived endogenous ligands are not included.

Table 2

TLRs and TAXI proteins

TLR	TAXI proteins
TLR1/TLR2	ND
TLR3	ND
TLR4	CD14, MD-2
TLR5	ND
TLR2/6	CD36, MBL
TLR7	ND
TLR8	ND
TLR9	Granulin *, HMGB-1 *, LL-37 *, CD32 *
TLR10	ND
TLR11	ND
TLR12	ND
TLR13	ND

* Further studies are required

ND: not determined