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How location governs Toll like receptor signaling

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Abstract

Toll like receptors (TLRs) are a family of innate immune system receptors responsible for recognizing conserved pathogen associated molecular patterns (PAMPs). PAMP binding to TLRs initiates intracellular signaling pathways that lead to the up-regulation of a variety of costimulatory molecules and the synthesis and secretion of various cytokines and interferons by cells of the innate immune system. TLR-induced innate immune responses are a prerequisite for the generation of most adaptive immune responses and in the case of B cells, TLRs directly regulate signaling from the antigen-specific B cell receptor. The outcome of TLR signaling is determined, in part, by the cells in which they are expressed and by the selective use of signaling adaptors. Recent studies suggest that, in addition, both the ligand recognition by TLRs and the functional outcome of ligand binding are governed by the subcellular location of the TLRs and their signaling adaptors. In this review we describe what is known about the intracellular trafficking and compartmentalization of TLRs in innate system's dendritic cells and macrophages and in adaptive system's B cells, highlighting how location regulates TLR function.

Toll like receptors (TLRs) are germline encoded receptors that recognize highly conserved motifs present in microorganisms, including bacteria, viruses, fungi and protozoans referred to as pathogen associated molecular patterns (PAMPs) (1). The TLRs are themselves a highly conserved receptor family that was first described in insects where Toll plays a key role in protection against microbial infections (2). The TLRs are class I transmembrane proteins, the extracellular domains of which contain leucine-rich repeats involved in protein-protein interactions and ligand recognition. TLRs contain a single transmembrane α helix and a conserved cytoplasmic domain containing a Toll/interleukin-1 receptor (TIR) domain. Ten TLRs have been identified in humans thus far, and twelve in mice. Both humans and mice express TLR1-9. In addition humans but not mice express TLR10, mice however also have TLR11, 12 and 13 that humans lack. The TLRs differ from one another in the cell types in which they are expressed, their ligand specificity, the signaling adaptors they utilize, and the cellular responses they induce (3). The crystal structures of three TLR complexes bound to their ligands have been solved recently, namely a TLR1/TLR2 heterodimer bound to tri-acylated lipopeptide (4), a TLR3 dimer complexed to double stranded RNA (5) and a TLR4-MD2-eritoran complex (6). Although there are differences in the details of the mechanisms of ligand binding to these TLRs, in all cases the ligands bridge two TLR molecules forming dimers between the ectodomains that have a similar overall architecture and serve to dimerize the cytoplasmic TIR domains (5). The TIR domains initiate downstream signaling by recruiting other TIR domain containing adaptors (7,8). At least four different TLR adaptors have been identified so far including MyD88, TIRAP, TRIF and TRAM (9). Myd88 functions as a universal adaptor and is shared by all TLRs, except TLR3 that

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exclusively recruits TRIF. TLR4 holds a unique status as it employs all four adaptors. Recently another adaptor, SARM was reported in human cells that functions to negatively regulating TRIF (10). The mechanisms that regulate TLR-adaptor interactions are not well understood. Upon binding to the cytoplasmic dimerized TIR domains, the adaptors recruit downstream kinases and signaling molecules that ultimately lead to the activation of MAP family kinases and NF κ B (3).

We are just beginning to understand the complex cell biology that underlies the function of this important family of receptors. A common theme that is emerging concerning the regulation of TLR signaling is one in which the subcellular location of the TLR dictates its function. Here we focus on four aspects of TLR cell biology in immune cells that touch on this theme, namely, the positioning of the TLRs to allow them to act as sentinels for PAMP-containing pathogens, the intracellular trafficking of the TLRs and their ER connection, compartmentalization of TLRs and their ligands and the role of adaptive immune system's antigen receptor signaling in determining the subcellular location of TLRs.

Positioning the TLRs to function as sentinels of PAMPS

From a simplistic point of view the pathogen world divides itself into microbes that replicate outside of host cells and those that replicate inside cells. Similarly, the TLRs that recognize PAMPs on extracellular microbes are expressed on the cell surface and those that recognize PAMPs on intracellular microbes are inside the cell. The well characterized TLRs, TLRs 1–9 can be broadly divided into two groups based on their PAMP specificity. The first group, expressed on the cell surface, recognizes PAMPs in cell wall components and flagellin from both gram positive and gram negative bacteria, yeast and fungi and includes: TLR2 that forms heterodimers with TLR1 or TLR6 that recognize bacterial lipoproteins and lipopeptides; TLR4 that recognizes lipopolysaccharides (LPS) and TLR5 that recognizes flagellin. Current evidence indicates that these TLRs bind to their ligands that are primarily present in the extracellular space and initiate signaling at the cell surface. The importance of the cell surface expression of the TLRs for their function is underscored by the observation that the inability of TLR1 to traffic to the cell surface is associated with impaired innate immune function. A single nucleotide polymorphism within TLR1 (I602S) at the junction of the transmembrane and the intracellular domains, resulted in aberrant trafficking of the receptor to the cell surface and unresponsiveness to its ligands (11). Importantly, this TLR1 polymorphism is associated with decreased incidence of leprosy, suggesting the possibility that *Mycobacterium leprae* coopts TLR1 signaling to facilitate infection (11).

The second group of TLRs, that reside in intracellular compartments, detects PAMPs in nucleic acids derived from bacterial and viral pathogens and includes: TLR3 that recognizes double stranded RNA that is generally a product of viral replication in host cells; TLR7 and TLR8 that recognize single stranded RNA derived from RNA viruses and small interfering RNA (siRNA) and TLR9 that recognizes unmethylated CpG containing DNA of bacterial and viral origin. While access of the cell surface TLRs to their extracellular ligands appears to be straightforward, the ligands for the intracellular TLRs must be transported into the endo-lysosomal compartments in which these TLRs reside. How the intracellular TLRs gain access to their ligands is at present only poorly understood. The intracellular TLRs can be activated by the addition of their ligands to the extracellular medium, indicating that cells can transport nucleic acids from outside the cell into the TLR-containing compartments. Whether internalization is by phagocytosis or pinocytosis versus by receptor mediated endocytosis is not known. For TLR9, it was shown that responses to exogenously added CpG DNA were dependent on both endocytosis and on endosomal maturation (12). Exogenously added CpG DNA internalizes within a few minutes and colocalized with early endosomal markers, EEA-1, Rab5 and TfR (12,13). Blocking endo-lysosomal compartment

acidification by drugs such as chloroquine and bafilomycin A blocks intracellular TLR signaling suggesting that either the intra-compartment pH or the ability of the TLR-containing compartments to fuse with other vesicles is necessary for TLR function (12). To this point, the binding of TLR3 to its double stranded RNA ligand has been shown to be highly pH dependent (14). CpG DNA has also been shown to be internalized from extracellular medium via scavenger receptors on the cell surface. One such cell surface receptor that has been described to mediate the internalization of CpG DNA and subsequent activation of TLR9 in plasmacytoid dendritic cells (pDC) is termed RAGE (15). RAGE binds to high mobility group protein 1 (HMGB1) that binds directly to class A CpG DNA. The internalization of HMGB1-CpG DNA complexes by RAGE results in the activation of the pDC (15). How general the use of RAGE is in CpG DNA internalization by immune cells remains to be determined. It also remains to be determined if there are additional scavenger receptors for other intracellular TLR ligands.

At present, little is known about the mechanisms by which TLR agonists derived from viruses or bacteria that replicate within the cell reach the intracellular TLRs. For TLR7, recent evidence suggests that autophagy may play a role in providing the viral ssRNA and replication intermediates to TLR7-containing compartments (16). Autophagy involves the sequestration of cytoplasmic material into membrane-enclosed compartments termed autophagosomes (17). The content of the autophagosomes is delivered to the lysosomes for degradation by direct fusion of autophagosome with the lysosomes. It is proposed that during this sequestration process TLR ligands, including viral nucleic acids and their replication intermediates, gain access to autophagosomes for subsequent delivery to TLRs in the endo-lysosomal compartments (16). In addition, recent reports suggest that various TLR ligands stimulate autophagy in macrophages and DCs (18,19). However, it remains unclear if TLR ligand-induced autophagy is a general phenomenon to promote the trafficking of pathogenic nucleic acids to the TLRs. Both TLR3-and TLR7-containing vesicles have also been observed near phagosomes that contain apoptotic particles, suggesting that both TLR3 and TLR7 may access their ligands through fusion with these compartments (20).

The intracellular location of the TLRs within endosomes also suggests a possible regulatory mechanism to modulate ligand binding by controlling the microenvironment within the endosome. A recent study provided evidence that the subcellular localization of TLR9 is important for its specificity and, in particular, for the discrimination of microbial from self DNA (21). It was shown that the intracellular localization of TLR9 is specified by its transmembrane α -helix and a chimeric receptor composed of the TLR9 ectodomain and the TLR4 transmembrane and cytoplasmic domain was trafficked to cell surface (21). These chimeric TLRs responded to self, mammalian DNA, whereas native TLR9 that was present in intracellular compartments responded only to microbial CpG-containing DNA. This study suggested that the ability of TLR9 to discriminate between microbial and self DNA is location dependent (21).

Trafficking of intracellular TLRs - the ER connection

Initial studies mapping the subcellular location of the TLR adaptor protein MyD88 in macrophages following treatment with the TLR9 agonist, CpG DNA provided evidence that TLR9 was expressed intracellularly and signaled from intracellular endo-lysosomal compartments (13). Results of studies localizing chimeric TLRs composed of the extracellular domains of TLR4 and the transmembrane and intracellular domains of either TLR3, 7 or 9 showed the chimeric TLRs to be nearly exclusively located in intracellular compartments and provided evidence that the intracellular location of these TLRs was encoded in their transmembrane and/or intracellular domains (21). Although TLR3, 7 and 9 all reside in intracellular compartments, the mechanisms that dictate their location are

different for each. The intracellular location of TLR3 is determined by a 23- amino acid sequence present in the linker region between transmembrane and TIR domain (20). TLR7 has been shown to colocalized with TLR3-containing compartments, however, intracellular localization of TLR7 is defined by its transmembrane domain (20). The motifs that define the intracellular location of TLR9 differ between human and mouse. The transmembrane α -helix was required for the mouse TLR9 localization and in contrast a tyrosine-based motif in the cytoplasmic domain of human TLR9 dictates its localization (21,22). Recent studies suggest that tyrosine-based sorting signals that direct transmembrane proteins to the endo-lysosomal system may also play a role in TLR localization. Indeed TLR3 contains several tyrosines within its cytoplasmic domain that could mediate its intracellular trafficking (23). Also, TLR3 has been shown to be ubiquitinated, a modification that could also serve as a sorting signal for endo-lysosomal targeting.

Although the evidence is persuasive that TLR3, 7 and 9 signal from endosomes, more detailed analyses, primarily in macrophages and DCs, indicated that in resting cells these TLRs may reside in the ER and traffic to the endosomes upon stimulation by their ligands. TLR9 was shown to be concentrated in the ER of dendritic cells prior to stimulation with CpG DNA (24,25). Following stimulation with CpG DNA, TLR9 rapidly redistributed from the ER to the endosomes that contained internalized CpG DNA and later into tubular lysosomal compartments (24,25). The nature of the ligand induced signal that triggers TLR trafficking from the ER to endosomes is not known nor it is understood how the TLR9 ligand would gain access to TLR9 in the ER. It will be of interest to know if redistribution is triggered by the bulk of the cellular TLR9 in the ER or by a small member of endosomal TLR9. Consistent with earlier observations (13), CpG DNA activated TLR9 signaled from endosomes and tubular lysosomes as shown by the accumulation of MyD88 on the membranes of these compartments (24). In addition to TLR9, TLR3 has also been shown to be localized in the ER of human DCs (26). Upon treatment with the TLR3 agonist, dsRNA, TLR3 moved to endosomes that had internalized dsRNA. Evidence that TLR3 signaled from these endosomes was provided by the observation that TLR3 colocalized with c-Src, kinase that is involved in downstream signaling (26).

The mechanism by which ER residing TLRs, including TLR3 and 9, translocate from the ER to endosome and the purpose or advantages of this ligand induced trafficking are only partially understood. Current evidence indicates that trafficking from the ER to endosomes does not involve the normal secretory pathway that would traffic the TLRs to the endocytic system through Golgi apparatus as both TLR3 and 9 do not acquire endo-H resistance that is achieved by the glycosylation in the Golgi complex (24,25). Rather it was proposed that the translocation of both TLR3 and 9 to endosomes is achieved by direct fusion of the ER with the endosomes in a manner similar to ER-mediated phagocytosis where phagosomes are formed by direct association and fusion of ER to the plasma membrane (27). Additional molecular details of TLR trafficking in the ER were provided by a forward genetic mutagenesis study in mice (28). A single point mutation (H412R) in the gene encoding the 12-membrane spanning protein, Unc93B, a highly conserved ER resident protein, resulted in a complete deficiency in signaling for TLRs 3, 7 and 9 (28). This mutation also resulted in deficiencies in a phenomenon termed antigen cross presentation in which antigen presenting cells load peptides derived from exogenous antigens onto MHC class I molecules (29). Mice carrying the Unc93B mutation are highly susceptible to infection with the intracellular pathogens, mouse cytomegalovirus, listeria monocytogenes, and staphylococcus aureus (28). Notably, cells from individuals with a mutation in the gene encoding Unc93B showed impaired cytokine production upon stimulation with TLR3, 7 and 9 ligands and are highly susceptible to various viral infections including herpes simplex virus (30), further suggesting a link between the ER and endosomal TLR signaling. Brinkman *et al.* showed that within the ER, Unc93B physically interacted with TLR3, 7, 9, and 13 and that for TLR 3 and 9 this

interaction was mediated through their transmembrane domains (31). The Unc93B H412R point mutation abolished this interaction. Unc93B was shown to traffic together with TLR7 and 9 to endo-lysosomal compartments in cells treated with TLR ligands (32). In cells expressing the Unc93B H412R point mutation, TLR7 and 9 were not trafficked to endo-lysosomal compartments and remained in the ER (32). Based on these findings it was suggested that Unc93B interacts with TLR3, 7 and 9 in the ER and when the TLRs are triggered by their ligands, facilitates their trafficking to the LAMP-1 positive, endo-lysosomal compartments where signaling to MAP Kinases and NF κ B occurs (32) (Figure 1).

How might the endo-lysosomal compartments contribute to TLR function? Recent studies provided evidence for an unexpected function of the endo-lysosomal compartments in TLR function (33,34). It was shown that within the endo-lysosomes the ectodomain of TLR9 was cleaved to generate a functional receptor. Although both the full length and the processed forms of TLR9 bind the ligand, only the processed form was signaling active. Conditions that blocked receptor proteolysis resulted in a non-functional TLR9 suggesting that proteolytic cleavage of TLR9 in the endo-lysosomes is a prerequisite for its activation (33,34). In addition these results also suggest an alternative explanation for earlier reported results (21) that chimeric TLR9 directed to the plasma membrane was not equivalent to endosomal TLR9 in its ability to respond to DNA-ligands. In contrast to the earlier studies in which it was suggested that TLR9 was recruited to endo-lysosomal compartments only after ligand internalization (24,25), these new studies suggest that trafficking and cleavage of TLR9 in endo-lysosomes is independent of ligand recognition (33,34). Interestingly, the TLR9 isoform that was cleaved within the endolysosomes was Endo-H sensitive suggesting that it passed through the secretory pathway. Chemical inhibitors of the lysosomal acidification also inhibited cleavage of TLR9 potentially providing a mechanism by which chloroquine and bafilomycin-A block TLR9 responses. A similar finding was also reported for TLR7 (33) although this observation at present appears controversial (34). Cleavage might be expected for TLR7, 8 and 9 as each of these has a similar non-LLR loop at the cleavage site defined in TLR9. In contrast, TLR3 does not contain a similar loop and would not be expected to be sensitive to proteolysis. How cleavage of the TLRs regulates signaling and the function of the cleavage product in TLR signaling, if any, remains to be determined.

A second ER resident protein, protein associated with TLR4A (PRAT4A) has also been shown to be involved in the TLR trafficking (35). PRAT4A is a chaperone like protein that plays a role in the trafficking of both cell surface and intracellular TLRs. PRAT4A was initially identified as a TLR4 interacting protein, later TLR1 and 9 were also shown to be associated with PRAT4A. TLR1,2,4,7 and 9 responses to their ligands are compromised in PRAT-deficient cells (35). Cells lacking PRAT4A showed impaired trafficking of TLR2 and TLR4 to the cell surface and TLR9 to the endo-lysosomal compartments (35). In DCs from PRAT4A-deficient mice, TLR7 and 9 responses were completely abolished, however, TLR3 responses were unaffected suggesting that in these cells not all of the nucleic acid sensing intracellular TLRs are regulated identically (35).

Compartmentalized signaling of TLRs

Current evidence from studies of a variety of signaling receptors indicates that the outcome of receptor stimulation is dictated by the receptors' subcellular location (36,37). This also appears so for TLR4 in which case its internalization induced by ligand binding results in a switch in its signaling pathway. Husebye *et al.* showed that following the binding of its agonist, LPS, TLR4 was rapidly endocytosed by a clathrin- and dynamin-mediated process (38). More recently, Kagan *et al.* (39) showed that compartmentalization of TLR4 was required for inducing two different signaling pathways, namely the production of proinflammatory cytokines and induction of type I interferons (Figure 2). Upon binding to

LPS at the plasma membrane, TLR4 first recruited TIRAP-MyD88 and initiated signaling to p38 phosphorylation and I- κ B degradation. TLR4 was subsequently rapidly internalized into early endosomes where it recruited TRAM-TRIF adaptor complex leading to the induction of type-I interferons (39) (Figure 2).

The subcellular compartmentalization of the TLR ligands has also been shown to influence the activation of TLRs. Only pDCs are able to respond to CpG-A DNA, conventional DCs (cDCs) do not respond. An explanation for such a observation was provided by the compartmentalization CpG-A DNA. In pDCs, the IFN-inducing TLR9 ligand, CpG-A DNA, was retained for long periods in the endosomal vesicles, together with the MyD88 IRF-7 complex (40). However, in cDCs, CpG-A DNA was rapidly transferred to lysosomal vesicles. By manipulating the endosomal retention of CpG-A DNA using a cationic lipid, robust TLR9 responses were achieved in cDCs. Thus, the spatio-temporal regulation of TLR9 ligand appears to be a deciding factor in the responsiveness of pDCs and DCs to CpG-A DNA (40).

The regulation of TLR signaling by adaptive immune system receptors

Although TLRs play a central role in innate immune responses, their expression is not restricted to the innate immune cells. Indeed, TLRs are also expressed in both B and T cells of adaptive immune system and function to regulate their responses to antigen through their antigen-specific receptors. A role for TLRs in regulating adaptive immunity is best established for B cells. B cells express a clonally distributed antigen receptor, the BCR. The BCR serves two functions in B cells, to initiate signaling that leads to the transcription of a variety of genes associated with B cell activation and to deliver the antigen into intracellular compartments for processing and presentation on MHC class II molecules (41). In addition, B cells also express both cell surface and intracellular TLRs, including TLR3, 4, 6, 7, 8 and 9 (42). Treatment of B cells with TLR ligands alone has been shown to result in the polyclonal activation of B cells and secretion of antibodies (42,43). BCR signaling has also been shown to synergize with the signaling originated from various TLRs resulting in hyper B cell activation (44–48). In particular, the synergistic interaction of the BCR with TLR9 and 7 in response to DNA- and RNA-containing antigens has been shown to play a fundamental role in the development of systemic autoimmune disorders in which DNA- and RNA-specific antibodies are hallmarks (45–48). The cellular mechanisms by which TLR ligands are delivered to the intracellular TLRs in B cells are of interest. In contrast to DCs and macrophages, B cells are only poorly pinocytic and are not phagocytic. Consequently, B cells rely on the BCR for the antigen uptake under physiological concentration of antigen, suggesting that the intracellular TLRs in B cells may depend on delivery of their ligand, by the BCR, functioning as an antigen scavenging receptor.

We recently showed that antigen binding to the BCR governs the subcellular localization of TLR9 (49) suggesting a novel mechanism to account for the ability of the BCR to synergize in signaling with TLR9 (Figure 3). Following BCR crosslinking the BCR initiates signaling at the plasma membrane and then is endocytosed into early endosomes. Antigen-BCR complexes then traffic to LAMP-1-positive multilamellar, multivesicular bodies that contain class II molecules. In resting B cells TLR9 was present in punctate-appearing, small compartments that are positive for early endosomal markers EEA-1 and transferrin receptor (49). This constitutive localization of TLR9 in endosomes in resting B cells contrasts with the observations in DCs and macrophages that TLR9 is concentrated in the ER in unstimulated cells. Following stimulation of B cells by CpG DNA alone, TLR9 remained in endosomal compartments and signaled to MAP kinase activation from these compartments as shown by staining for phosphorylated p38 and JNK in confocal microscopy (49). However, the subcellular localization of TLR9 was dramatically altered in cells in which the

BCR is crosslinked by antigen and BCR signaling was initiated. Following BCR crosslinking with the antigen alone or together with CpG DNA, TLR9 was recruited to multilamellar multivesicular autophagosomes where it colocalized with the antigen bound internalized BCR (Figure 3). TLR9 recruitment to the autophagosomes involved the tubulin networks. Recruitment required BCR activation of phospholipase D1, an enzyme known to be involved in intracellular trafficking (50) and was completely independent of MyD88 signaling (49). Moreover, BCR driven recruitment of TLR9 to the autophagosome was required for synergistic signaling between BCR and TLR9 as measured by the hyperactivation of MAP kinases to the DNA-containing antigens (49). It is interesting that BCR bound ligand is not delivered to TLR9 in the endosomes en route to the autophagosomes but rather that TLR9 is induced to traffic to autophagosomes. We speculate that TLR9's recruitment provides an opportunity to regulate TLR9's augmentation of BCR signaling. It is of interest to know if other intracellular nucleic acid sensing TLRs are regulated in the same manner. Indeed, we find that dsRNA sensing TLR3 is also recruited to the BCR-containing autophagosomes, suggesting that by inducing the trafficking of at least TLR3 and 9 to the autophagosome, the BCR allows these TLRs to get access to their ligand (Chaturvedi *et al*, unpublished observation).

Conclusion

Although it is still early days in understanding the cell biology of TLRs, recent results have suggested novel mechanisms that might be at play in regulating the function of this important family of innate immune receptors. An emerging theme is that the subcellular location of TLRs controls the access of the TLRs to their ligands and thus regulates responses. It will be interesting to learn more about the molecular mechanism underlying such controls and if any of these provide targets for new therapeutics to regulate TLR activity in allergy and autoimmunity.

References

1. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol.* 2002; 20:197–216. [PubMed: 11861602]
2. Ferrandon D, Imler JL, Hoffmann JA. Sensing infection in *Drosophila*: Toll and beyond. *Semin Immunol.* 2004; 16(1):43–53. [PubMed: 14751763]
3. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol.* 2004; 5(10):987–995. [PubMed: 15454922]
4. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, Lee H, Lee JO. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell.* 2007; 130(6):1071–1082. [PubMed: 17889651]
5. Liu L, Botos I, Wang Y, Leonard JN, Shiloach J, Segal DM, Davies DR. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. *Science.* 2008; 320(5874):379–381. [PubMed: 18420935]
6. Kim HM, Park BS, Kim JI, Kim SE, Lee J, Oh SC, Enkhbayar P, Matsushima N, Lee H, Yoo OJ, Lee JO. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. *Cell.* 2007; 130(5):906–917. [PubMed: 17803912]
7. Barton GM, Medzhitov R. Toll-like receptors and their ligands. *Curr Top Microbiol Immunol.* 2002; 270:81–92. [PubMed: 12467245]
8. Akira S, Sato S. Toll-like receptors and their signaling mechanisms. *Scand J Infect Dis.* 2003; 35(9):555–562. [PubMed: 14620134]
9. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol.* 2007; 7(5):353–364. [PubMed: 17457343]

10. Carty M, Goodbody R, Schroder M, Stack J, Moynagh PN, Bowie AG. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. *Nat Immunol.* 2006; 7(10):1074–1081. [PubMed: 16964262]
11. Johnson CM, Lyle EA, Omueti KO, Stepensky VA, Yegin O, Alpsoy E, Hamann L, Schumann RR, Tapping RI. Cutting edge: A common polymorphism impairs cell surface trafficking and functional responses of TLR1 but protects against leprosy. *J Immunol.* 2007; 178(12):7520–7524. [PubMed: 17548585]
12. Hacker H, Mischak H, Miethke T, Liptay S, Schmid R, Sparwasser T, Heeg K, Lipford GB, Wagner H. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* 1998; 17(21): 6230–6240. [PubMed: 9799232]
13. Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM, Wagner H. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur J Immunol.* 2002; 32(7):1958–1968. [PubMed: 12115616]
14. Leonard JN, Ghirlando R, Askins J, Bell JK, Margulies DH, Davies DR, Segal DM. The TLR3 signaling complex forms by cooperative receptor dimerization. *Proc Natl Acad Sci U S A.* 2008; 105(1):258–263. [PubMed: 18172197]
15. Tian J, Avalos AM, Mao SY, Chen B, Senthil K, Wu H, Parroche P, Drabic S, Golenbock D, Sirois C, Hua J, An LL, Audoly L, La Rosa G, Bierhaus A, et al. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat Immunol.* 2007; 8(5):487–496. [PubMed: 17417641]
16. Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. *Science.* 2007; 315(5817):1398–1401. [PubMed: 17272685]
17. Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. *Nat Cell Biol.* 2007; 9(10):1102–1109. [PubMed: 17909521]
18. Sanjuan MA, Dillon CP, Tait SW, Moshiah S, Dorsey F, Connell S, Komatsu M, Tanaka K, Cleveland JL, Withoff S, Green DR. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature.* 2007; 450(7173):1253–1257. [PubMed: 18097414]
19. Delgado MA, Elmaoued RA, Davis AS, Kyei G, Deretic V. Toll-like receptors control autophagy. *EMBO J.* 2008; 27(7):1110–1121. [PubMed: 18337753]
20. Nishiya T, Kajita E, Miwa S, Defranco AL. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. *J Biol Chem.* 2005; 280(44):37107–37117. [PubMed: 16105838]
21. Barton GM, Kagan JC, Medzhitov R. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. *Nat Immunol.* 2006; 7(1):49–56. [PubMed: 16341217]
22. Leifer CA, Brooks JC, Hoelzer K, Lopez J, Kennedy MN, Mazzoni A, Segal DM. Cytoplasmic targeting motifs control localization of toll-like receptor 9. *J Biol Chem.* 2006; 281(46):35585–35592. [PubMed: 16990271]
23. Sarkar SN, Peters KL, Elco CP, Sakamoto S, Pal S, Sen GC. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. *Nat Struct Mol Biol.* 2004; 11(11):1060–1067. [PubMed: 15502848]
24. Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, Lien E, Nilsen NJ, Espevik T, Golenbock DT. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol.* 2004; 5(2):190–198. [PubMed: 14716310]
25. Leifer CA, Kennedy MN, Mazzoni A, Lee C, Kruhlak MJ, Segal DM. TLR9 is localized in the endoplasmic reticulum prior to stimulation. *J Immunol.* 2004; 173(2):1179–1183. [PubMed: 15240708]
26. Johnsen IB, Nguyen TT, Ringdal M, Tryggestad AM, Bakke O, Lien E, Espevik T, Anthonsen MW. Toll-like receptor 3 associates with c-Src tyrosine kinase on endosomes to initiate antiviral signaling. *EMBO J.* 2006; 25(14):3335–3346. [PubMed: 16858407]

27. Gagnon E, Duclos S, Rondeau C, Chevet E, Cameron PH, Steele-Mortimer O, Paiement J, Bergeron JJ, Desjardins M. Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell*. 2002; 110(1):119–131. [PubMed: 12151002]
28. Tabeta K, Hoebe K, Janssen EM, Du X, Georgel P, Crozat K, Mudd S, Mann N, Sovath S, Goode J, Shamel L, Herskovits AA, Portnoy DA, Cooke M, Tarantino LM, et al. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. *Nat Immunol*. 2006; 7(2):156–164. [PubMed: 16415873]
29. Rock KL, Shen L. Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol Rev*. 2005; 207:166–183. [PubMed: 16181335]
30. Casrouge A, Zhang SY, Eidenschenk C, Jouanguy E, Puel A, Yang K, Alcais A, Picard C, Mahfoufi N, Nicolas N, Lorenzo L, Plancoulaine S, Senechal B, Geissmann F, Tabeta K, et al. Herpes simplex virus encephalitis in human UNC-93B deficiency. *Science*. 2006; 314(5797):308–312. [PubMed: 16973841]
31. Brinkmann MM, Spooner E, Hoebe K, Beutler B, Ploegh HL, Kim YM. The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. *J Cell Biol*. 2007; 177(2):265–275. [PubMed: 17452530]
32. Kim YM, Brinkmann MM, Paquet ME, Ploegh HL. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. *Nature*. 2008; 452(7184):234–238. [PubMed: 18305481]
33. Ewald SE, Lee BL, Lau L, Wickliffe KE, Shi GP, Chapman HA, Barton GM. The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. *Nature*. 2008
34. Park B, Brinkmann MM, Spooner E, Lee CC, Kim YM, Ploegh HL. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. *Nat Immunol*. 2008
35. Takahashi K, Shibata T, Akashi-Takamura S, Kiyokawa T, Wakabayashi Y, Tanimura N, Kobayashi T, Matsumoto F, Fukui R, Kouro T, Nagai Y, Takatsu K, Saitoh S, Miyake K. A protein associated with Toll-like receptor (TLR) 4 (PRAT4A) is required for TLR-dependent immune responses. *J Exp Med*. 2007; 204(12):2963–2976. [PubMed: 17998391]
36. Vieira AV, Lamaze C, Schmid SL. Control of EGF receptor signaling by clathrin-mediated endocytosis. *Science*. 1996; 274(5295):2086–2089. [PubMed: 8953040]
37. Schutze S, Tchikov V, Schneider-Brachert W. Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. *Nat Rev Mol Cell Biol*. 2008; 9(8):655–662. [PubMed: 18545270]
38. Husebye H, Halaas O, Stenmark H, Tunheim G, Sandanger O, Bogen B, Brech A, Latz E, Espevik T. Endocytic pathways regulate Toll-like receptor 4 signaling and link innate and adaptive immunity. *EMBO J*. 2006; 25(4):683–692. [PubMed: 16467847]
39. Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol*. 2008; 9(4):361–368. [PubMed: 18297073]
40. Honda K, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, Taya C, Taniguchi T. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature*. 2005; 434(7036):1035–1040. [PubMed: 15815647]
41. Lanzavecchia A. Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu Rev Immunol*. 1990; 8:773–793. [PubMed: 2188679]
42. Gururajan M, Jacob J, Pulendran B. Toll-like receptor expression and responsiveness of distinct murine splenic and mucosal B-cell subsets. *PLoS ONE*. 2007; 2(9):e863. [PubMed: 17848994]
43. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*. 1995; 374(6522):546–549. [PubMed: 7700380]
44. Viglianti GA, Lau CM, Hanley TM, Miko BA, Shlomchik MJ, Marshak-Rothstein A. Activation of autoreactive B cells by CpG dsDNA. *Immunity*. 2003; 19(6):837–847. [PubMed: 14670301]
45. Christensen SR, Shlomchik MJ. Regulation of lupus-related autoantibody production and clinical disease by Toll-like receptors. *Semin Immunol*. 2007; 19(1):11–23. [PubMed: 17276080]
46. Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchik MJ. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity*. 2006; 25(3):417–428. [PubMed: 16973389]

47. Lau CM, Broughton C, Tabor AS, Akira S, Flavell RA, Mamula MJ, Christensen SR, Shlomchik MJ, Viglianti GA, Rifkin IR, Marshak-Rothstein A. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. *J Exp Med.* 2005; 202(9): 1171–1177. [PubMed: 16260486]
48. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature.* 2002; 416(6881):603–607. [PubMed: 11948342]
49. Chaturvedi A, Dorward D, Pierce SK. The B cell receptor governs the subcellular location of Toll-like receptor 9 leading to hyperresponses to DNA-containing antigens. *Immunity.* 2008; 28(6): 799–809. [PubMed: 18513998]
50. Snyder MD, Pierce SK. A mutation in Epstein-Barr virus LMP2A reveals a role for phospholipase D in B-Cell antigen receptor trafficking. *Traffic.* 2006; 7(8):993–1006. [PubMed: 16882041]

Figure 1. Trafficking of intracellular TLRs to endolysosomes

UNC93B physically interacts with the transmembrane domain of intracellular nucleotide-sensing TLRs, TLR7 and TLR9 in the endoplasmic reticulum. UNC93B1 regulates the trafficking of TLR7 and TLR9 to the endolysosomes upon stimulation with ssRNA or DNA.

Figure 2. Compartmentalized signaling of TLR4

TLR4 binds to LPS-MD2 complexes and engages MyD88- TIRAP adaptors at the cell surface leading to activation of early MAP kinase pathway. TLR4-LPS complex is endocytosed through a dynamin-dependent pathway terminating the MyD88-TIRAP signaling. TLR4-LPS complex within the endosome initiates a second phase of signaling by engaging TRAM-TRIF adaptors that leads to the production of interferon- β .

Figure 3. Synergistic responses of BCR and TLR9 in B cells to DNA-containing antigens
Crosslinking of BCR through DNA-containing antigens, initiates signaling resulting in the recruitment of TLR9 from endosomes to the autophagosomes in a PLD-dependent manner. TLR9 colocalizes with internalized antigen-BCR complexes within the autophagosomes and initiate synergistic signaling to NF- κ B activation and MAP kinase phosphorylation.