# Emerging Principles Governing Signal Transduction by Pattern-Recognition Receptors

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The problem of recognizing and disposing of non-self-organisms, whether for nutrients or defense, predates the evolution of multicellularity. Accordingly, the function of the innate immune system is often intimately associated with fundamental aspects of cell biology. Here, we review our current understanding of the links between cell biology and pattern-recognition receptors of the innate immune system. We highlight the importance of receptor localization for the detection of microbes and for the initiation of antimicrobial signaling pathways. We discuss examples that illustrate how pattern-recognition receptors influence, and are influenced by, the general membrane trafficking machinery of mammalian cells. In the future, cell biological analysis likely will rival pure genetic analysis as a tool to uncover fundamental principles that govern host–microbe interactions.

The innate immune system uses families of pattern-recognition receptors (PRRs) to recognize diverse microbial ligands (Janeway 1989; Janeway and Medzhitov 2002). During infection, these receptors provide signals that upregulate general antimicrobial features of the innate immune system as well as instruct and initiate adaptive immunity (Iwasaki and Medzhitov 2010). A significant challenge faced by innate immune recognition is the reliable detection of highly diverse, rapidly evolving microbial organisms, many of which possess virulence mechanisms that enable survival within distinct host niches. Moreover, recognition must be linked to induction of contextual signals appropriate for the type of infection. The specificity, signal transduction, and cell biology of PRRs have evolved under these selective pressures to enable broad recognition of microbes within each host niche.

Although the collection of PRRs is decidedly less diverse than antigen receptors of the adaptive immune system, the list of players has grown considerably over the past decade (Kawai and Akira 2010). If one classifies these receptors based on common structure and functional domains, then six families emerge: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), AIM-like receptors (ALRs), Nod-like receptors (NLRs), and OAS-like receptors (OLRs) (Geijtenbeek and Gringhuis 2009; Kawai and Akira 2010; Rathi-

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nam and Fitzgerald 2011; Lamkanfi and Dixit 2012; Kranzusch et al. 2013). Collectively, these receptors bind a diverse array of targets, including lipoproteins, polysaccharides, nucleic acids, carbohydrate structures, and a few highly conserved microbial proteins. These ligands are typically shared across large microbial classes, which facilitate broad recognition with such a limited number of PRRs. Moreover, alteration or masking of these ligands to avoid PRR activation often results in reduced microbial fitness.

The molecular recognition challenge faced by PRRs is all the more complex when one considers the need to detect microbes within distinct subcellular niches. Microbes can be extracellular or intracellular within membrane-bound organelles, within the cytosol, or in the nucleus. In addition, both the innate and adaptive immune mechanisms appropriate for eliminating microbes within these distinct environments are quite distinct, so it is vital that PRR signaling communicate the location of a microbe as well its nature. We now understand that members of the PRR families highlighted above localize to distinct subcellular compartments, and, in some cases, localization can change in a dynamic fashion that regulates or influences recognition and signaling. Moreover, in some cases, signal transduction and resulting gene induction can be dramatically influenced by the organelle from which signaling initiates. Thus, the innate immune system has harnessed the organization inherent to cells as a means of achieving regulation and signaling specificity. Activation of PRRs can also feed back on basic cell biological processes, such as phagocytosis and autophagy, to enhance or accelerate the response to microbial infection.

In the following sections, we discuss these links between cell biology and PRRs of mammalian innate immunity. Our discussions of PRR function and signal transduction will be limited to this theme, as a result, in part, of space constraints but also because in-depth reviews of each PRR family have appeared elsewhere. For discussion purposes, we have grouped the transmembrane PRRs together and the cytosolic PRRs together.

## TRANSMEMBRANE PATTERN-RECOGNITION RECEPTORS

### Basic Features of TLRs and CLRs

The 13 mammalian TLRs (10 in humans, 12 in mice) consist of leucine-rich repeat containing ectodomains and cytosolic Toll-IL-1 receptor (TIR) domains (Kawai and Akira 2010). CLRs are a much larger family, whose members share a carbohydrate recognition domain (CRD) (Sancho and Reis e Sousa 2012). In many proteins, this domain mediates carbohydrate binding but it is now clear that not all CRD-containing proteins bind sugars. Here, we will consider only the CLRs that bind carbohydrates and function clearly as PRRs, such as the dectins. As such, the emphasis in this section will mostly be on TLRs, but we will highlight newer findings especially as they relate to links between CLR function and cell biology.

Both TLRs and CLRs can link the recognition of diverse microbes to signaling pathways that promote both innate and adaptive immunity. TLRs induce a core signaling pathway via recruitment of adaptors with TIR domains: MyD88, Trif, Tram, and Tirap (Kawai and Akira 2010). Distinct signal transduction and gene induction can arise from the differential use of adaptors or selective recruitment of specific signaling components, and examples of such specialization will be discussed later in this article. The ultimate outcome of this signal transduction is the activation of NF-κB and interferon regulatory factor (IRF) transcription factors and induction of genes that promote the subsequent immune response (Kawai and Akira 2010). CLRs are more varied in their signal transduction, but we will focus on the dectin family. Dectin-1, -2, and -3 all activate the tyrosine kinase Syk and, subsequently, the transcription factors NF-κB and nuclear factor of activated T cells (Sancho and Reis e Sousa 2012). Although there are certainly differences in gene induction by CLRs and TLRs, conceptually the outcome of their activation is similar.

TLRs and CLRs are expressed by many types of immune cells (Kawai and Akira 2010; Sancho and Reis e Sousa 2012). The CLRs discussed in this review primarily regulate phagocytic cells,

such as macrophages, dendritic cells (DCs), and neutrophils, whereas TLRs are expressed on these cell types as well as lymphocytes and some nonhematopoietic cell types. Expression of individual TLRs can vary between these cell types, and certain cell types only express a few family members. This restricted expression is one mechanism by which distinct responses can be generated to specific microbes. For example, plasmacytoid DCs selectively express TLR7 and TLR9, which, because of the features of these specialized cells, enables these TLRs to induce substantial production of type I interferons (IFNs) (Kadowaki et al. 2001). For the purposes of this review, it is also important to note that most studies of TLR and CLR cell biology have used macrophages. It is quite possible that other specialized cell types possess distinct cell biological features that may impact the function of these receptors.

Individual TLR family members traffic to distinct subcellular locations and, in some cases, receptor localization changes on activation. In general, the TLRs specific for ligands associated with the exterior surfaces of microbes (Table 1), such as bacterial lipopolysaccharides and lipoproteins, are found in the plasma membrane of unstimulated cells (Barton and Kagan 2009). This subset of TLRs includes TLR4 and TLR5 and the heterodimers TLR1/TLR2, TLR2/TLR6, and TLR2/TLR10 (TLR10 is a pseudogene in mice). A second subset of TLRs resides within endosomes. All of these TLRs (TLR3, TLR7, TLR8, TLR9, and TLR13) share specificity for various forms of nucleic acids (Table 1). As discussed in the next section, endosomal localization is thought to facilitate ligand recognition, as microbes must be degraded before their genetic material can be recognized by a TLR. Endosomal localization has also been reported for TLR11 and TLR12, yet these TLRs recognize the protein ligands flagellin and profilin (Yarovinsky et al. 2005; Pifer et al. 2011; Koblansky et al. 2012; Mathur et al. 2012). Although such localization may facilitate ligand recognition by these TLRs, the evidence supporting their exclusive endosomal localization is fairly limited, and it remains possible that one or both of these receptors also localize to the cell surface. In contrast to the diverse subcellular

**Table 1.** The TLRs specific for ligands associated with the exterior surfaces of microbes

| Receptor | Best defined ligand(s) | Key features |
|---|---|---|
| TLR1 | Triacylated bacterial lipoproteins | Forms heterodimers with TLR2 |
| TLR2 | Di- and triacylated bacterial lipoproteins | Forms heterodimers with TLR1 or TLR6 (TLR10 in humans) |
| TLR3 | Double-stranded RNA | Ectodomain must be cleaved to form an active receptor |
| TLR4 | Bacterial lipopolysaccharide | Recognizes ligands indirectly via MD2 |
| TLR5 | Bacterial flagellin | |
| TLR6 | Diacylated bacterial lipoproteins | Forms heterodimers with TLR2 |
| TLR7 | Single-stranded RNA | Ectodomain must be cleaved to form an active receptor |
| TLR8 | Single-stranded RNA | Ectodomain must be cleaved to form an active receptor |
| TLR9 | Unmethylated CpG-containing DNA | Ectodomain must be cleaved to form an active receptor |
| TLR10 | Unknown | Pseudogene in mice; present in humans; forms heterodimers with TLR2 |
| TLR11 | Toxoplasma profilin/bacterial flagellin | Forms heterodimers with TLR12 |
| TLR12 | Toxoplasma profilin | Forms heterodimers with TLR11 |
| TLR13 | Bacterial 23s ribosomal RNA | |
| Dectin-1 | Fungal β-glucan | |
| Dectin-2 | Fungal α-mannans | Forms heterodimers with Dectin-3 |
| Dectin-3 | Fungal α-mannans | Forms heterodimers with Dectin-2 |
| RIG-I | Short dsRNA with 5′ triphosphate | Detects ligands in the cytosol |
| MDA5 | Long dsRNA | Detects ligands in the cytosol |
| cGAS | DNA | Detects ligands in the cytosol |

sites of TLR residence, it is generally thought that the dectin family of CLRs is located at the cell surface. This localization is likely linked to their function as phagocytic receptors. TLRs also can promote phagocytosis, but this activity is not as robust as that observed for the dectins (see discussion below).

Identification of the trafficking machinery responsible for delivery of TLRs to the correct subcellular location is an area of active research. Correct folding of TLR ectodomains in the endoplasmic reticulum (ER) requires the function of at least two chaperones, gp96 and PRAT4A (Takahashi et al. 2007; Yang et al. 2007b). All TLRs exit the ER and enter the secretory pathway but certain TLRs require a dedicated trafficking chaperone UNC93b1 for this step (Kim et al. 2008). UNC93b1 associates with TLRs in the ER and facilitates their loading into COPII vesicles (Brinkmann et al. 2007; Lee et al. 2013). In cells lacking UNC93b1 function, all of the normally endosomal TLRs fail to exit the ER. UNC93b1 binding to TLRs is at least partially determined by interactions between transmembrane domains and also requires certain TLR juxtamembrane residues (Brinkmann et al. 2007; Kim et al. 2013), but common molecular features that mediate UNC93b1 binding or necessitate UNC93b1 chaperone function remain largely undefined. Why only this subset of TLRs requires UNC93b1 for ER exit is not understood, and it is quite possible that other, as yet unidentified, accessory molecules play a similar role in ER exit for the UNC93b1-independent TLRs. Indeed, the efficiency of ER export can dramatically impact the threshold of receptor activation, so it is likely that this step is subject to complex regulation (Fukui et al. 2009, 2011; Hart and Tapping 2012).

We are only beginning to understand which trafficking factors are necessary for proper post-Golgi sorting of TLRs. Over the past several years, several Rab GTPase family members have been implicated in sorting of TLR4 to subcellular compartments, either at steady state or in response to stimulation (Wang et al. 2007, 2010; Husebye et al. 2010). Similarly, proper trafficking of TLR7 and TLR9 to endosomes requires adaptor protein (AP) sorting complexes (Blasius et al. 2010; Sasai et al. 2010; Lee et al. 2013). These examples will be discussed in greater detail in the following sections.

## Recognition of TLR Ligands from/within Distinct Subcellular Compartments

The finding that different TLRs are found in different subcellular locations has prompted studies to identify the significance of differential localization for receptor function. For example, mutant alleles of endosomal TLR9 have been generated that direct this protein to the plasma membrane (Barton et al. 2006; Mouchess et al. 2011). TLR9 at the cell surface can respond to the synthetic ligand CpG DNA (Barton et al. 2006; Mouchess et al. 2011). This result indicates that endosomal localization of TLR9 is not formally required for its signaling functions, although ligand binding may be enhanced at acidic pH (Rutz et al. 2004). Interestingly, cell surface–localized TLR9 was unable to recognize the DNA virus HSV-2, whereas WT TLR9 efficiently detected this virus (Barton et al. 2006). These findings established that the localization of TLR9 to endosomes was not important for ligand binding per se, rather, localization to endosomes was important to ensure that TLR9 detects pathogen-associated DNA. It is therefore likely that two selective pressures resulted in the localization of nucleic acid–sensing TLRs in endosomes. First was the need for rapid detection of viral nucleic acids, which are not likely to be displayed on the surface of any virulent pathogen. Thus, if nucleic acid–sensing TLRs evolved to be located at the cell surface, those cells could not detect their pathogen-associated ligands that are hidden within the virion. The second selective pressure to direct nucleic acid–sensing TLRs into endosomes probably arose from the need to "ignore" self-nucleic acids to prevent autoimmunity. As stated above, nucleic acid–sensing TLRs do not need to be in endosomes to activate signal transduction; they can be localized to the cell surface (at least in the case of TLR9). The ability of these receptors to function from the cell surface may be useful in some situations, as there are reports of cell types that display nucleic acid–sensing TLRs at

their plasma membrane (Lee et al. 2006b; Lindau et al. 2013). However, the risk of potentially detecting self-nucleic acids may be so great that the vast majority of cells that express these receptors restrict their localization to endosomes. Evidence in direct support of this potential risk comes from in vivo studies of mice whose hematopoietic cells displayed TLR9 at the cell surface (Mouchess et al. 2011). These mice display systemic autoinflammatory symptoms.

Additional means of preventing access of nucleic acid–sensing TLRs to self-DNA or RNA exist. For example, the ectodomain of most nucleic acid–sensing TLRs must be cleaved to generate a signaling-competent receptor (Ewald et al. 2008, 2011; Park et al. 2008; Garcia-Cattaneo et al. 2012). The requirement for TLR cleavage before they become signaling competent is similar to the regulatory processes that control other potentially harmful endosomal proteins, such as the cathepsins. Both cathepsins and endosomal TLRs are created as pro-proteins that must be cleaved by endosomal proteases to become active. This cleavage event is mediated by several endosomal proteases including cathepsin L, cathepsin S, and asparagine endopeptidase, but the relative importance of each of these proteins may vary between cell types (Ewald et al. 2008, 2011; Park et al. 2008; Sepulveda et al. 2009). For example, cathepsin L (not S) is required for TLR9 cleavage in B cells, whereas both proteases are necessary in macrophages (Avalos et al. 2013). Recent work has implicated furin proteases in processing of the human TLR7 ectodomain, indicating that the proteolytic regulation of individual nucleic acid–sensing TLRs may be different and perhaps occur in distinct compartments (Hipp et al. 2013). Interestingly, the amino-terminal fragment of TLR9 that is separated by cleavage appears to be required for TLR9 signaling, although the mechanism underlying this requirement is unclear (Peter et al. 2009; Onji et al. 2013).

Recent studies that examine the transport route taken by newly synthesized nucleic acid–sensing TLRs suggest the importance of these cleavage events. Newly synthesized TLR9 is first delivered to the plasma membrane via interactions with the aforementioned chaperone Unc93b1 (Lee et al. 2013). The strong endocytosis motif within Unc93b1 then delivers TLR9 to endosomes, where it is cleaved and poised for activation. That TLR9 transits to endosomes via a plasma membrane intermediate highlights the risk of detecting self-nucleic acids. TLR3 also associates with UNC93b1 and traffics to the plasma membrane before internalization into endosomes (Matsumoto et al. 2003; Pohar et al. 2013). Not all nucleic acid–sensing TLRs follow this transport pathway. For example, TLR7 is delivered directly to endosomes, yet it is also cleaved to generate a functional receptor (Lee et al. 2013). Additional work must be performed to explain the importance of the transport routes taken by TLRs, and the cleavage events that control their activation.

The section above describes the efforts taken by the innate immune system to prevent nucleic acid–sensing TLRs from responding to all possible ligands, especially extracellular (i.e., self) DNA. In this section, we will highlight how the opposite approach is taken for TLRs located at the plasma membrane, in which several regulatory events are in place to ensure rapid and highly sensitive detection of bacterial cell-surface components. The best-studied example of a bacterial cell-surface component that activates innate immunity is lipopolysaccharide (LPS), which is found in the outer membrane of Gram-negative bacteria. LPS activates TLR4 to induce the expression of numerous immunomodulatory genes. However, TLR4 forms few direct contacts with LPS (Kim et al. 2007; Park et al. 2009). Thus, several proteins facilitate LPS-induced signal transduction by TLR4. These include LPS-binding protein (LBP), albumin, CD14, and MD2 (Gioannini et al. 2004; Prohinar et al. 2007; Teghanemt et al. 2007, 2008; Resman et al. 2009; Esparza et al. 2012). LBP is capable of binding LPS from the outer membrane of Gram-negative bacteria by a process facilitated by albumin (Gioannini et al. 2002). LPB then transfers LPS to CD14, which, in turn, transfers LPS to MD2. The relative affinities of LBP, CD14, and MD2 for LPS differ, with each successive protein in this cascade displaying a higher binding constant for this bacterial product. This sequential increase in LPS affinity

probably facilitates the unidirectional flow of ligand between these LPS-binding proteins. LPS-bound MD2 helps to cross-link TLR4, which is the first step in the initiation of signal transduction. CD14 has also been reported to facilitate ligand binding by TLR2, TLR3, and TLR9 (Henneke et al. 2001; Lee et al. 2006a; Baumann et al. 2010). Interestingly, CD36 and the mannose-binding lectin (MBL) are also important for ligand binding by TLR2 (Hoebe et al. 2005; Ip et al. 2008). The relative roles of CD14, CD36, and MBL in the control of TLR2 signaling remain to be defined. Overall, these observations highlight the differing means by which plasma membrane localized TLRs, and endosomal TLRs are regulated at the level of ligand binding. It appears that numerous regulatory factors are in place to increase the sensitivity of ligand binding by cell-surface TLRs that detect microbial products. In contrast, it appears that the unique regulatory mechanisms of endosomal TLR activation (e.g., the need for ectodomain cleavage) are designed to restrict access to their respective ligands. The benefit of this latter approach is likely to ensure that only microbial nucleic acids are detected, thus limiting the possibility of autoimmunity. In this regard, it is worth noting that, relative to plasma membrane localized TLRs, endosomal TLRs are most commonly implicated in autoimmune syndromes.

## Induction of Distinct TLR-Dependent Signaling Pathways from Distinct Subcellular Locations

Although the TLRs appear to be positioned within mammalian cells as a means of regulating ligand binding, recent studies have indicated that they do not, by themselves, define the subcellular sites of innate immune signal transduction. For example, TLR4 can bind to LPS at the plasma membrane, but needs to be transported into detergent-resistant microdomains of the cell surface called lipid rafts to promote MyD88-dependent signal transduction. The transport of TLR4 into rafts is poorly defined, but is dependent on CD14. After signaling via MyD88 at the plasma membrane, TLR4 is then transported into the cell via an endocytosis pathway that is notable for two reasons. First, it is not activated by TLR4, thus establishing that mammalian cells can respond to LPS via TLR4-independent means. TLR4 endocytosis is instead mediated by CD14. On exposure to LPS, CD14 induces the endocytosis of TLR4 and itself by a process dependent on several factors, including the transmembrane adaptors DAP12 and FcεRγ, the tyrosine kinase Syk, and its downstream effector PLC-γ2 (Zanoni et al. 2011; Lin et al. 2013). In addition, reactive oxygen species have been implicated in the regulation of CD14-dependent endocytosis (Chiang et al. 2012). This CD14-dependent pathway represents the first example of a TLR4-independent response to LPS that operates in numerous mammalian cell types, including macrophages, DCs, and fibroblasts.

The second notable feature of this new LPS response pathway is that TLR4 delivery into endosomes is required for its ability to both inactivate MyD88-dependent signaling and promote a second signaling pathway mediated by the TRIF adaptor (Kagan et al. 2008). This TRIF-dependent pathway results in the expression of type I IFNs and helps stabilize the strong transcriptional activity of NF-κB. Thus, in the case of MyD88- and TRIF-dependent pathways, TLR4 must be transported to a region of the cell after ligand binding to promote signal transduction. Evidence exists that TLR4 is also located on Rab11-positive recycling endosomes in resting cells (Husebye et al. 2010). TLR4 can be delivered from recycling endosomes to phagosomes as a means of further enhancing TRIF-mediated signal transduction. The separation of the sites of ligand binding from signal transduction is not unique to TLR4. In fact, TLR2 signaling on inflammatory monocytes also displays this activity. In these cells, TLR2 must be internalized into endosomes before it is able to activate type I IFN expression (Barbalat et al. 2009). Interestingly, within these cells, TLR2 can only induce type I IFNs in response to viral, not bacterial, ligands.

A final example of the dissociation between the sites of ligand binding and signal transduction can be provided by endosomal TLR9. In

plasmacytoid DCs, TLR9 recognizes unmethylated CpG from viruses and induces the expression of inflammatory cytokines and type I IFNs. Both of these responses are dependent on MyD88. It appears that TLR9 activates inflammatory cytokine expression and IFN expression from distinct populations of endosomes (Honda et al. 2005). TLR9-induced IFN expression occurs from lysosome-related organelles (LROs) rich for phosphatidylinositol 3,5-bisphosphate PI(3,5)P2 (Sasai et al. 2010). Delivery of TLR9 to this IFN-inducing LRO population depends on the trafficking adaptor complex AP3 (Sasai et al. 2010). Consequently, AP3 mutant cells do not permit TLR9 to induce IFN expression in response to CpG DNA or DNA viruses, and at least one group has reported that MyD88-dependent inflammatory cytokine expression (e.g., IL-12) proceeds normally in these cells (Blasius et al. 2010; Sasai et al. 2010).

## Sorting Adaptor Proteins: Factors that Define the Subcellular Sites of TLR Signal Transduction

The section above highlights several examples in the TLR network in which the subcellular site of ligand binding is distinct from the site of signal transduction. These observations raise the question of what defines the subcellular sites of signaling, if not the receptors. This task is accomplished by the actions of a structurally unrelated family of proteins called sorting adaptors (Kagan 2012). Sorting adaptors are defined by several functional criteria. First, these are proteins that act at the receptor proximal level, having the ability to bind directly to ligand-bound PRRs. In the case of the TLR network, the known sorting adaptors are TIRAP and TRAM, both of which act at the receptor proximal level to control signaling (Kagan and Medzhitov 2006; Kagan et al. 2008). Second, sorting adaptors are the only signaling proteins in a given pathway that are present at the site of signaling before any microbial encounter. These proteins are therefore poised to rapidly detect the delivery of a ligand-bound TLR into the region of the cell that is permissive for signal transduction. These proteins can therefore be considered the cytosolic "sensors" of activated TLRs.

TIRAP was the first sorting adaptor defined (Kagan and Medzhitov 2006). This protein contains an amino-terminal lipid-binding domain that interacts with several phosphoinositides (Kagan and Medzhitov 2006). Its ability to bind to PI(4,5)P2 permits localization to the inner leaflet of the plasma membrane ( probably in lipid rafts), the site to which CD14 delivers TLR4 (Triantafilou et al. 2002). Once TLR4 enters this region of the plasma membrane, TIRAP can detect the activated receptor and engage MyD88 to promote inflammatory cytokine expression (Kagan and Medzhitov 2006). Concomitant with the initiation of MyD88-dependent signaling from the plasma membrane, CD14 initiates the endocytosis of TLR4 (Zanoni et al. 2011). This process results in the delivery of TLR4 to the second sorting adaptor in the TLR pathway, TRAM. TRAM is localized to both the plasma membrane and endosomes via an amino-terminal bipartite domain that contains a myristate group adjacent to a phosphoinositide-binding domain (Rowe et al. 2006; Kagan et al. 2008). Delivery of TLR4 to endosomal TRAM triggers the TRIF-dependent signaling pathway leading the expression of type I IFNs (Kagan et al. 2008; Tanimura et al. 2008; Tseng et al. 2010). Mislocalization of TIRAP or TRAM to the cytosol results in a defective cellular response to LPS, yet TLR4 endocytosis proceeds normally, thus underscoring the importance of sorting adaptor localization in defining the subcellular sites of signal transduction (Kagan and Medzhitov 2006; Kagan et al. 2008). The fact that TIRAP and TRAM localization is controlled by interactions with phosphoinositides is notable for two reasons. First, a similar mechanism of sorting adaptor localization in *Drosophila melanogaster* exists, suggesting that the use of sorting adaptors in innate immunity is conserved throughout evolution (Marek and Kagan 2012). Second, several posttranslational modifications have been reported within the critical residues of TIRAP's lipid-binding domain (Mansell et al. 2006). These modifications are induced on TLR signaling, and occur on residues known to influence the interactions be-

tween TIRAP and phosphoinositides (Kagan and Medzhitov 2006), perhaps serving as a novel means of regulation via altering protein localization. Consistent with this idea, PI-3 kinase activity, which converts PI(4,5)P2 to PI(3,4,5)P2, can displace TIRAP from membranes in vitro and within mammalian cells (Aksoy et al. 2012). Of note, all of the sorting adaptors in the Toll pathways (TIRAP, TRAM, and *Drosophila* MyD88) are promiscuous lipid-binding proteins in vitro, yet functional studies within cells indicate the importance of specific lipid targets in the regulation of signal transduction (Kagan and Medzhitov 2006; Kagan et al. 2008; Marek and Kagan 2012). It remains unclear why sorting adaptors show this promiscuity of interactions with multiple lipids. Finally, as will be described later in this article, sorting adaptors have been identified in other innate immune signaling pathways, most notably the RLR pathway that detects cytosolic viruses.

## Links between TLRs, Phagocytosis, and Autophagy

Phagocytosis is one of the most ancient mechanisms of host defense, so it is not surprising that PRR function is intimately linked to this process. Phagocytosis involves a complex series of events, including recognition of cargo, membrane, and cytoskeleton remodeling to facilitate engulfment, and finally phagosome maturation (Flannagan et al. 2012). PRRs would appear ideally suited for the task of cargo recognition and initiation of engulfment, and certain CLRs can promote phagocytosis. In particular, heterologous expression of Dectin-1 is sufficient to facilitate uptake of yeast particles (Herre et al. 2004). This activity is likely because of Syk activation, which is downstream from most CLRs (Osorio and Reis e Sousa 2011); although, at least one report suggests that Syk-independent signals may also be involved (Herre et al. 2004). When compared with CLRs, TLRs do not promote phagocytosis robustly, and macrophages or DCs lacking TLR function engulf microbes with comparable efficiency to wild-type cells (Blander and Medzhitov 2004). However, TLR activation has been linked to an up-regulation in macropinocytosis, and some reports have reported increased bacterial uptake associated with TLR signaling (West et al. 2004; Jain et al. 2008). In addition, multiple groups have reported delayed phagosome maturation in TLR-deficient cells based on a variety of readouts (Blander and Medzhitov 2004; Arpaia et al. 2011). TLR signaling accelerates phagosomal acidification and enhances processing of phagosomal proteins for antigen presentation. Remarkably, the TLR effect is phagosome autonomous, meaning phagosomes that do not contain TLR ligands do not undergo accelerated maturation even if TLR activation occurs in other phagosomes in the same cell (Blander and Medzhitov 2006b). The mechanism by which individual phagosomes are "marked" has not been further elucidated. Notably, a role for TLRs in phagosome maturation has not been observed by every group, and the reason for these disparate results remains unexplained (Blander and Medzhitov 2006a; Russell and Yates 2007). Nevertheless, most of the published reports support a role for TLRs in cargo recognition and enhanced phagosome maturation.

The function of PRRs is also linked to autophagy. Autophagy is the process by which cytosolic contents or damaged organelles are surrounded by a nascent double membrane structure (Levine et al. 2011). These autophagosomes undergo a maturation process similar to phagosome maturation. Although originally described as a response to starvation to reclaim nutrients, it is now clear that autophagy also contributes to host defense (Levine et al. 2011). In some instances, autophagy can directly enhance PRR function. Specifically, during certain viral infections, autophagy is necessary to deliver viral nucleic acids from the cytosol to TLR7 in endosomes (Lee et al. 2007). TLR activation can also regulate autophagic processes. Early work suggested that autophagy can be induced directly by TLR signaling. Indeed, increased association of LC3 with endosomal and phagosomal membranes has been observed in macrophages activated by TLR2 and TLR4 ligands (Sanjuan et al. 2007; Xu et al. 2007). Although originally interpreted as evidence of classic autophagy, it now appears that this LC3

recruitment may be a noncanonical form of autophagy that uses a subset of the autophagic machinery. This process does not require the initiating components of classic autophagy (e.g., Ulk-1), and the LC3-positive compartments lack the classic double membrane autophagosome structure (Martinez et al. 2011). How the autophagic machinery alters the composition of endosomal or phagosomal compartments remains unclear, nor is it clear that all instances of noncanonical autophagy are equivalent. TLR-dependent induction of noncanonical autophagy can enhance phagosome maturation and promote microbial killing (Sanjuan et al. 2007). In plasmacytoid DCs, this machinery is required for trafficking of DNA-immune complexes to a TLR9-containing LRO with type I IFN-signaling capability (Henault et al. 2012). This specialized compartment does not require AP-3, indicating that noncanonical autophagy contributes to further heterogeneity and functional specialization of endosomes. The signaling pathways that induce noncanonical autophagy, downstream from TLRs as well as other receptors, remain poorly characterized.

## CYTOSOLIC RECEPTORS THAT DETECT NUCLEIC ACIDS

In addition to surveying the extracellular and endosomal environments for microbial products, PRRs that survey the cytosol for bacteria and viruses exist. Cytosolic PRRs include the RLRs, which detect viral RNA, the NLRs, which activate various inflammasomes (described below), and the newly defined sensor of viral DNA, cyclic GAMP-AMP synthase (cGAS) (Rathinam and Fitzgerald 2011; Lamkanfi and Dixit 2012; Burdette and Vance 2013). Generally, these cytosolic sensors of microbes can be divided into two groups based on their effector functions. One group will be discussed in this section (RLRs and cGAS), which commonly induces transcriptional responses on microbial detection. The second group consists of the NLRs, most of which do not activate potent transcriptional responses but rather induces immediate responses in the cell. The best characterization of these responses is the activation of inflammasomes, which are cytosolic protein complexes that function to promote the processing and secretion of IL-1 family cytokines and induce cell death (Lamkanfi and Dixit 2012). There is very little known about the cell biology processes that regulate NLR functions, and, as such, we will focus our attention on the cytosolic RNA and DNA sensors described above.

The RLRs differ from the TLRs and dectins in two fundamental ways. First, these receptors are expressed in most (perhaps all) mammalian cells, whereas the aforementioned receptors are expressed on subsets of cells in the mouse and human. As such, RLRs provide a comprehensive means of detecting cytosolic microbes. Second, the fact that RLRs survey the cytosol places them in a position in which they can gauge the virulence of the microbe they encounter. The reason for this is that all pathogens (even extracellular pathogens) must have the ability to manipulate the host cells they encounter. Several examples of host cell manipulation exist, ranging from the use of bacterial toxins, specialized secretion systems, or the expression of immune evasion genes by viral pathogens. Although these strategies differ mechanistically, they share the common attribute of delivering some molecule to the cytosol of the host to disrupt host defenses. Consequently, every pathogen has the need to interact with the cytosolic environment in some way. Because RLRs are present in the cytosol, these receptors are ideally positioned only to be activated during pathogenic encounters. In contrast, TLRs and dectins survey the extracellular environments, a location that both pathogens and nonpathogens may occupy. Thus, TLRs and dectin receptors can be classified as microbe-detection receptors, whereas RLRs (and other cytosolic sensors) can be classified as pathogen-detection receptors.

The RLR family includes RIG-I, MDA5, and LGP2. Of these, RIG-I and MDA5 are the best characterized. Although RIG-I and MDA5 are both RNA-binding proteins, they bind different types of RNA. RIG-I binds to small double-stranded RNA species that contain a 5′ triphosphate group and a 3′ polyuridine-rich region (Pichlmair et al. 2006; Saito et al. 2008; Uzri and Gehrke 2009; Rehwinkel et al.

2010). MDA5, in contrast, binds to long double-stranded RNA species that may contain branched high-order structures (Pichlmair et al. 2009). Structural analysis has indicated that, whereas RIG-I binds the terminus of double-stranded RNA, MDA5 uses protein–protein contacts to oligomerize along the length of double-stranded RNA (Berke and Modis 2012; Peisley et al. 2013; Wu et al. 2013a). In both instances, RLR interactions with RNA result in interactions between the CARD domains present in the receptors with the CARD domain present in the protein mitochondrial antiviral signaling (MAVS). These CARD–CARD interactions result in the formation of a large prion-like aggregate of MAVS, which is thought to promote a signaling pathway important for expression of type I IFNs, interferon-stimulated genes (ISGs), cytokines, and chemokines (Hou et al. 2011). MAVS accomplishes this task by promoting interactions between the kinase TBK1 and its substrate, the transcription factor IRF3 (Belgnaoui et al. 2011). Phosphorylated IRF3 then acts together with other inflammatory transcription factors to induce inflammatory gene expression.

Cell biological analysis of the MAVS protein revealed its function as a sorting adaptor, in that it is localized to the eventual sites of RLR signaling before any microbial encounter (Seth et al. 2005). MAVS contains a transmembrane domain at its carboxyl terminus that directs localization to mitochondria, peroxisomes, and mitochondria-associated membranes (MAM) of the endoplasmic reticulum (Seth et al. 2005; Dixit et al. 2010; Horner et al. 2011). Localization of MAVS to each of these compartments is important for the antiviral activities of the RLRs, as mutant alleles of MAVS that are mislocalized to the cytosol are unable to signal (Seth et al. 2005; Dixit et al. 2010). In addition, several viral proteases have been identified that cleave MAVS from membranes, resulting in a cytosolic species that is signaling deficient (Li et al. 2005; Chen et al. 2007; Yang et al. 2007a).

Studies on the significance of differential MAVS localization found that its function differs depending on the organelle on which it resides (Dixit et al. 2010). For example, mitochondria-localized MAVS is capable of inducing the RLR-dependent expression of type I IFNs, ISGs, cytokines, and chemokines. In contrast, peroxisome-localized MAVS primarily induces the expression of ISGs and chemokines, but cannot induce the expression of type I IFNs. The actions of MAVS on both of these compartments are coordinated by MAM-localized MAVS (Horner et al. 2011), which creates an innate immune synapse that helps synergize compartment-specific signaling events and promote robust antiviral innate immunity. Thus, a common feature of the RLR and TLR networks is the ability of these receptors to induce different and complementary responses in a location-specific manner.

Although the RLR pathway is best known for its ability to detect viral RNA, it can also detect DNA viruses, albeit indirectly. In some instances, the DNA of viruses can be transcribed by the host-encoded RNA polymerase III to create RNA ligands that activate RIG-I dependent antiviral responses (Ablasser et al. 2009; Chiu et al. 2009). In this regard, RLRs may have a role in the detection of both RNA and DNA viruses; however, their role in the detection of DNA viruses is likely limited. As such, numerous studies have attempted to identify a general sensor of cytosolic DNA viruses, and many candidate sensors have been reported, including cGAS, IFI16, DAI, DDX41, MRE11, and DNA-PK (Burdette and Vance 2013). Although all of these proteins remain candidates, the cGAS protein has received the most attention as genetic studies in humans and mice support its role as the major sensor of cytosolic DNA (Sun et al. 2013). cGAS is a broadly expressed cytosolic protein that binds directly to double-stranded DNA, irrespective of sequence (Sun et al. 2013). DNA binding by cGAS activates its intrinsic enzymatic activity that catalyzes the creation of cyclic GMP-AMP (cGAMP) (Wu et al. 2013b). cGAMP contains phosphodiester linkages between the 2′-hydroxyl group of GMP and the 5′-phosphate of AMP, as well as a phosphodiester link between the 3′-hydroxyl of AMP and 5′-phosphate of GMP (Ablasser et al. 2013; Diner et al. 2013; Gao et al. 2013b; Zhang et al. 2013). cGAMP produced by cGAS binds

to an endoplasmic reticulum–localized protein called STING, which is an adaptor that promotes interactions between TBK1 and IRF3 to induce the expression of type I IFNs (Tanaka and Chen 2012). Consequently, cGAS- or STING-deficient mice or cells are highly sensitive to DNA virus infection (Ishikawa and Barber 2008; Gao et al. 2013a; Li et al. 2013). Recent work has extended the role of the STING-dependent pathway to include the detection of *Mycobacterium tuberculosis* (Manzanillo et al. 2012; Watson et al. 2012). Interestingly, detection of Mycobacteria by the STING pathway activates an autophagic response that promotes the maturation of bacteria-containing phagosomes into lysosomes, a process that facilitates killing of the pathogen (Watson et al. 2012).

Cell biological analysis of the cGAS-STING pathway has indicated a complex coordination between signal transduction and protein localization. For example, transfection of mammalian cells (as a mimic of viral infection) triggers the relocalization of STING from the ER to small vesicles that stain positive for TBK1, the exocyst component Sec5, and the autophagy regulators ATG9a and LC-3 (Ishikawa et al. 2009; Saitoh et al. 2009). The identity of these vesicles remains unclear but the movement of STING to this unusual compartment is thought to be associated with the onset of signal transduction (Ishikawa et al. 2009; Saitoh et al. 2009).

## CONCLUDING REMARKS HIGHLIGHTING THEMES

This article was designed to not only provide an overview of the PRR pathways activated in mammalian cells, but to highlight themes that appear to operate in these pathways, regardless of their specific genetic components. The simplest theme that has emerged is that the pathways of the innate immune system are most different at the receptor proximal level. Each pathway is activated by a distinct receptor in response to a distinct ligand, yet most converge on common responses such as gene transcription or autophagy. It is unknown how many other cellular responses are activated by PRRs, and a major challenge faced by the community is to develop novel assays to study more immediate (nontranscriptional) responses that occur on microbial detection. A second theme that has emerged is that regulatory processes are in place to govern the transport and localization of PRRs and their associated sorting adaptors, the latter of which defines the subcellular sites of innate immune signal transduction. A second major challenge faced by the community lies in this area, in which tools are still lacking to monitor the movement of endogenous proteins in primary cells. The use of such tools will be needed to better understand the cell type–specific actions of PRRs, some of which are highlighted in this article. A third theme that emerges is that of the comprehensiveness of immune surveillance by the PRR families. Most subcellular compartments are surveyed by one or more PRRs, including the endolysosomal network, the plasma membrane, and the cytosol. This ubiquity of surveillance poses a formidable challenge to the microbial world, but may have also resulted in the evolution of virulence strategies designed to interfere with PRR functions. Additional means of interrogating the PRR-induced signaling pathways will likely reveal novel means of microbial immunoevasion, and perhaps provide clues for therapeutic intervention.

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