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Defining the subcellular sites of innate immune signal transduction

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Innate immune activation by microbial detection receptors is a complex process involving at least 100 proteins and multiple signaling pathways. Although there continues to be a need to identify additional regulators of host-microbe interactions, a larger conceptual challenge is our lack of understanding of how the known regulators interact in space and time. This review offers a framework to explain the long appreciated (but poorly understood) observation that innate immune signaling pathways are activated from multiple organelles. Using the Toll-like receptors (TLRs) and the retinoic acid-inducible gene 1 protein (RIG-I)-like receptors (RLRs) as examples, I propose that the receptors do not necessarily define the sites of signaling. Rather, a structurally unrelated class of proteins called 'sorting adaptors' functions in this capacity.

Localization of TLRs: ideally positioned for ligand binding, but not signaling

Each TLR family member is a type I transmembrane protein that contains a horseshoe-shaped ectodomain that detects molecules that are common to broad classes of microorganisms [1]. Classic examples of such molecules include bacterial lipopolysaccharide (LPS), lipoproteins, flagellin, and double-stranded RNA [2]. Some TLRs detect microbial ligands directly, whereas others require accessory proteins for high-affinity interactions with their ligands [1]. In their cytosolic tail, TLRs contain a Toll interleukin-1 receptor homology (TIR) domain [3], which functions to recruit downstream TIR-domain-containing adaptors to initiate signal transduction [4]. Despite their common structural features, different TLR family members are transported to different organelles upon translation. The evolutionary pressure to direct a receptor to a given location appears to be linked to the need for rapid responsiveness to a microbial encounter. For this reason, TLRs that detect bacterial cell surface components are often found at the surface of mammalian cells; most notably phagocytes. Examples of such receptors are TLR4 (which detects LPS) [5], TLR2 (bacterial lipoproteins) [6], and TLR5 (flagellin) [7]. Likewise, TLRs that detect nucleic acids are not found at the plasma membrane, but are located in late endosomal compartments [8–11]. In this location, these receptors are poised to detect microbial genomes after they are released by the hydrolytic enzymes present in these organelles. Although most cells that express TLRs display them in the aforementioned locations, there are exceptions, because some cells have been reported

to display nucleic acid sensing TLRs at their plasma membrane [12,13]. In recent years, studies of mislocalized TLRs have revealed the critical importance of proper subcellular localization for the efficient binding of the microbial ligands they detect [14,15]. The means by which the biosynthetic trafficking machinery delivers newly synthesized TLRs to their proper subcellular destination has been reviewed elsewhere [16–18] and will not be discussed further.

Upon ligand binding, TLRs are thought to self-associate to create a scaffold of TIR domains that recruit soluble cytoplasmic adaptor proteins [19]. Myeloid differentiation primary response gene 88 (MyD88), TIR domain containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon- β (TRIF), and TRIF-related adaptor molecule (TRAM) are the known adaptors that initiate TLR signal transduction [11,20–25]. These adaptors are differentially utilized by different TLRs, and serve as the biochemical link between ligand-bound receptors and various serine/threonine kinases that induce inflammatory cytokine expression. MyD88 and TIRAP promote the expression of nuclear factor (NF)- κ B-dependent cytokines, whereas TRAM and TRIF lead to the expression of type I interferons (IFNs). In some instances, most notably plasmacytoid dendritic cells (pDCs), MyD88 can induce the expression of both cytokines and IFNs [26–28]. Although these adaptors are usually considered mere intermediates in a signaling pathway, they can also be considered the cytosolic sensors of activated TLRs. In this regard, the view that adaptors are mere intermediates is altered to view these proteins as critical regulators of the earliest cytosolic events that initiate signal transduction.

For many years, it had been assumed that TLRs are immobile in the membranes where they detect their microbial ligand. As such, receptor localization would be expected to determine where within the cell signal transduction can occur. However, recent work has revealed that, in the case of both plasma membrane localized and endosomal TLRs, these receptors must be transported to new regions of the cell to initiate signal transduction [29–32].

In this review, I focus on where within the cell innate immune signaling first occurs, and how the specificity of signaling locale is achieved. Several examples will be given of proteins that share no primary sequence similarity, but can be grouped based on their ability to define the site where innate immune signaling occurs. I suggest that these proteins be dubbed 'sorting adaptors', the defining features of which are: (i) that they are prepositioned at the location of innate immune signal transduction; and (ii) define the type of signaling pathway activated from that location.

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Adaptor protein localization determines specificity of signaling locale

The LPS receptor TLR4 has served as an excellent model to study how TLRs can be transported to new locations upon microbial detection. LPS recognition by TLR4 is facilitated by interactions with the LPS-binding cofactors CD14 and MD-2 [33–35]. When CD14 detects LPS, it transfers this microbial product to a plasma-membrane-localized heterodimer of MD-2 and TLR4. Upon ligand binding, TLR4 is recruited to phosphatidylinositol 4,5-bisphosphate (PIP₂)-rich regions of the plasma membrane, such as lipid rafts in epithelial cells and sites of phagocytosis in macrophages [31,32]. Interestingly, TLR2 is also thought to be mobilized into lipid rafts after engaging its various ligands [36]. It is within these sites that TLR4 (and perhaps TLR2) is thought to oligomerize and engage TIRAP and MyD88 to induce inflammatory cytokine expression [37]. Subsequent to this event, TLR4 is internalized into endosomes (or phagosomes), where it engages TRAM and TRIF to induce type I IFN expression [29,30,38,39].

Analysis of how TLR4 endocytosis is regulated has revealed that TLR4 does not induce its own internalization. Rather, TLR4 is cargo for an LPS-inducible endocytosis pathway that is mediated by CD14 [32]. After transferring LPS to the MD-2/TLR4 complex, CD14 engages phospholipase C (PLC) γ 2, which induces the calcium-dependent internalization of TLR4 [32,40]. The immunoreceptor tyrosine-based activation motif (ITAM)-containing transmembrane proteins DAP12 and Fc epsilon receptor gamma (Fc ϵ R γ) facilitate this process, probably by activating the tyrosine kinase Syk. Although genetic knockouts of Syk have yet to be used to examine its role in TLR4 endocytosis and TRIF signaling, RNAi- and small molecule-based studies suggest that this kinase is important for these events [32,41,42].

The discovery that TLR4 must be delivered to PIP₂-rich regions of the plasma membrane to activate MyD88 signaling [31,37], and then endocytosed to activate TRIF signaling [30], suggests that the initial site of receptor–ligand interaction does not determine where signal transduction occurs. Cell biological analysis of TIRAP and TRAM has revealed that their localization within cells determines the sites of TLR4 signaling.

TIRAP and TRAM are peripheral membrane proteins whose localization is dictated by interactions with acidic phospholipids [30,37,43]. In the case of TIRAP, this protein is localized to PIP₂-rich regions of the plasma membrane by a process dependent on an N-terminal lipid-binding domain that interacts selectively with this phosphoinositide.

Although the upstream sensory protein TLR4 and the downstream signaling adaptor MyD88 must be recruited to PIP₂-rich regions of the plasma membrane to signal, TIRAP is present in this location prior to microbial encounters [37] (Figure 1a). Altering the localization of TIRAP by ablating the PIP₂-binding domain renders this protein cytoplasmic, and MyD88-dependent signal transduction cannot occur [37]. The localization of TIRAP to PIP₂-rich plasma membrane subdomains therefore defines where within the cell TLR4 can activate MyD88-dependent signaling.

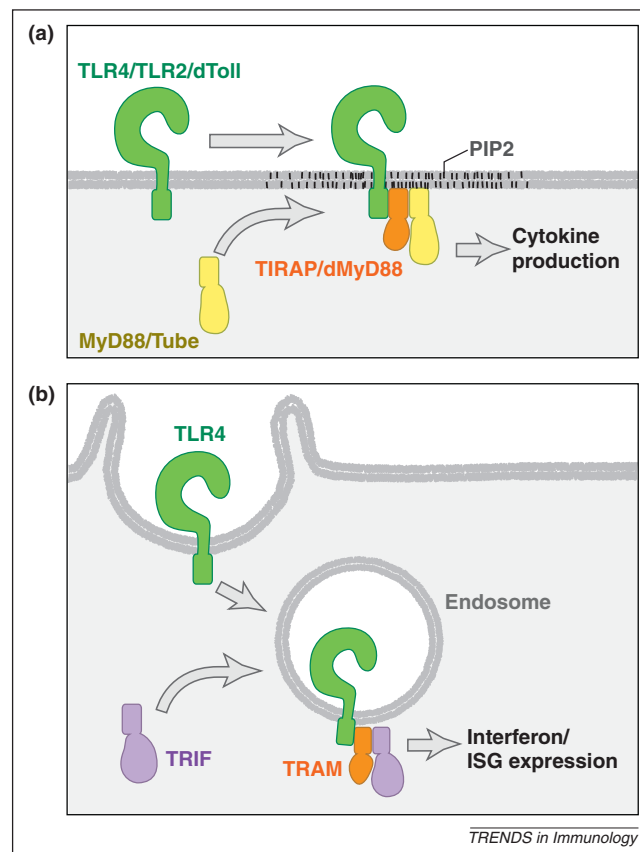


Figure 1. The subcellular site of innate immune signal transduction is defined by the localization of inflexible sorting adaptors. (a) Signaling by plasma-membrane-bound Toll family members involves their recruitment to a new subdomain of the cell surface, where they can engage a sorting adaptor. For the Toll family members, the sorting adaptors TIRAP and dMyD88 are located in phosphatidylinositol 4,5-bisphosphate (PIP₂)-rich regions of the cell surface. (b) TRIF-dependent signaling by Toll-like receptor (TLR)4 involves its delivery to endosomes, where the sorting adaptor TRAM resides. Abbreviations: ISG, interferon stimulated gene; MyD88, myeloid differentiation primary response gene 88; TIRAP, TIR domain containing adaptor protein; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing interferon- β .

In the case of the TRAM, similar cell biological rules apply. TRAM contains a bipartite localization domain consisting of an N-terminal myristoylation motif and a phosphoinositide-binding domain [30,43]. This bipartite domain directs TRAM to both the plasma membrane and endosomes. The upstream receptor TLR4 and the downstream adaptor TRIF must be recruited to endosomes in order to induce type I IFN expression [30,38,39], but it is only TRAM that is resident on endosomes prior to signaling [30,39] (Figure 1b). Mislocalizing TRAM to the cytosol [43], or forcing this adaptor to be mainly located at the cell surface [30], diminishes the ability of TLR4 to induce type I IFN expression. By contrast, forcing TRAM to be located only on early endosomes results in very high type I IFN expression [30]. These data indicate that like TIRAP, TRAM defines the site in the cell where TLR4 and TRIF converge to induce signal transduction.

Interestingly, the TRAM gene encodes a splice variant called TRAM adaptor with GOLD domain (TAG) that displays a distinct subcellular distribution from that of TRAM [44]. Although TRAM utilizes the above-described bipartite motif to localize to early endosomes, this domain has

been replaced by a Golgi dynamics (GOLD) domain in TAG. The GOLD domain directs TAG to late endosomes [44], where it functions to interfere with TRAM-mediated signal transduction through interactions with a newly defined protein called transmembrane emp24 domain-containing protein 7 (TMED7) [45]. Thus, the TRAM gene provides an intriguing example of how localizing an adaptor to different endosomes results in a protein with either proinflammatory (TRAM) or anti-inflammatory (TAG) functions.

The importance of adaptor protein localization for signal transduction appears to be an evolutionarily conserved aspect innate immunity. For example, the fruitfly *Drosophila melanogaster* encodes a Toll signaling pathway that has often been used as a reference for the mammalian network [46]. Although Toll and its downstream serine/threonine kinases are similar to their mammalian orthologs in both structure and function [46], the cytoplasmic adaptors that link the receptor to these kinases bear little similarity to the mammalian orthologs [47,48]. Flies do not encode any genes similar to TIRAP, TRIF, or TRAM, but they do encode a protein similar to MyD88 called dMyD88 (or dmMyD88) [49,50]. Despite displaying strong structural similarities, dMyD88 and mammalian MyD88 exhibit distinct subcellular distributions and functional characteristics [51]. From a cell biological perspective, dMyD88 is more similar to TIRAP and TRAM in that it is prepositioned at the site of signaling via interactions with phosphoinositides; most notably PIP2 [51]. dMyD88 functions to recruit the cytosolic adaptor Tube to PIP2-rich regions of

the plasma membrane to permit Toll signaling (Figure 1a). In response to bacterial encounters, transgenic flies expressing a cytosolic dMyD88 allele in place of wild-type (WT) dMyD88 display low levels of Toll signaling, and cannot survive the infection [51]. Thus, like TIRAP and TRAM, dMyD88 probably defines the subcellular site where Toll signaling can occur.

Sorting adaptors: structurally unrelated signaling proteins that define sites of innate immune signal transduction

Based on our knowledge of where within the cell various signaling proteins are located, some general principles emerge. It is now clear that there are two classes of innate immune signaling factors. One class is flexible in its signaling locale, in that it can participate in signaling events that occur from multiple compartments of the cell. The second class is inflexible in its signaling locale and can only participate in signaling events that occur on a subset of organelles. The mechanism by which these flexible and inflexible factors are categorized relates directly to their means of membrane localization. The inflexible factors are localized to a given organelle by interactions with general components of the organelle itself, such as phosphoinositides in the case of TIRAP, TRAM, and dMyD88 [30,37,51] (Figure 2). Their localization results from interactions with a general membrane component, therefore, these factors cannot participate in signaling events that occur from a different organelle. By contrast, the flexible factors appear

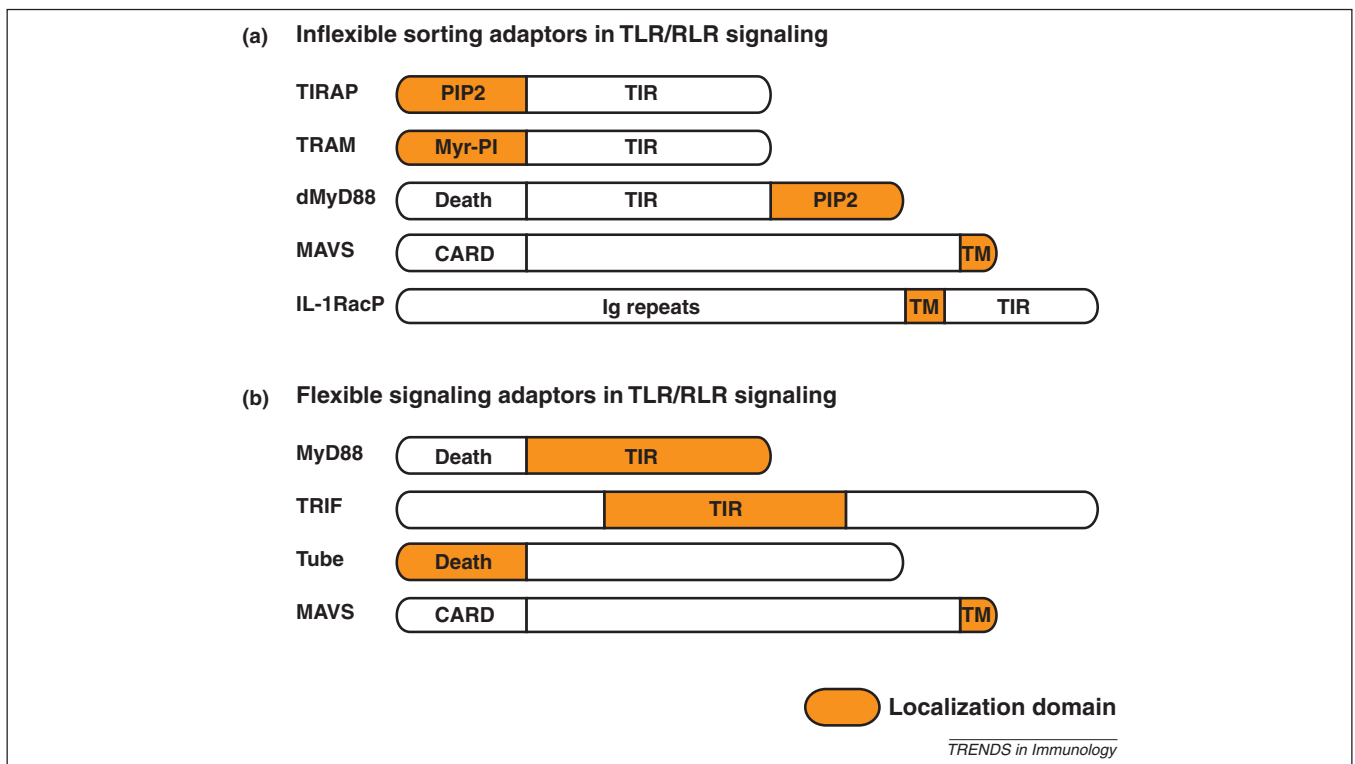


Figure 2. Sorting and signaling adaptors that function in innate immune pathways. **(a)** Proteins implicated as sorting adaptors are shown. All sorting adaptors contain a localization motif that differs from their signaling domains. Thus, all sorting adaptors should be placed at the site of signal transduction prior to the cell encountering any microbial or inflammatory stimulus. **(b)** Proteins implicated as signaling adaptors are shown. Note that for Toll-like receptor (TLR) signaling adaptors, the Toll interleukin-1 receptor homology (TIR) domains are localization domains, whereas for sorting adaptors, the TIR domains are not localization domains. Also, note that although mitochondrial antiviral-signaling protein (MAVS) exhibits many attributes of a sorting adaptor, it also functions as a signaling adaptor. Abbreviations: dMyD88, a protein similar to myeloid differentiation primary response gene 88 (MyD88); IL-1RacP, interleukin-1 receptor accessory protein; RLR, retinoic acid-inducible gene 1 protein (RIG-I)-like receptors; TIRAP, TIR domain containing adaptor protein; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing interferon- β .

to be cytosolic components, whose recruitment to a given membrane occurs upon microbial detection [31,37,39,51] (Figure 2). As such, these flexible factors can be recruited to any organelle harboring an active receptor.

At the receptor proximal level, flexible regulators are *bona fide* signaling adaptors that interact directly with downstream enzymes (Figure 2). Examples of this include MyD88 (which interacts with the interleukin-1 receptor-associated kinase (IRAK) family of kinases) [52,53], TRIF (which interacts with TNF receptor associated factor (TRAF) family of E3 ligases) [54,55], and the *Drosophila* Tube protein (which interacts with Pelle kinases) [48]. Inflexible regulators such as TIRAP, TRAM, and dMyD88 bind downstream signaling enzymes indirectly, but bind directly to the activated receptors. These inflexible regulators define the site of signal transduction by recruiting signaling adaptors to the active receptor. Classically, inflexible regulators such as TIRAP and TRAM are defined as bridging adaptors. However, the very term adaptor implies a bridging function. I therefore suggest that the term sorting adaptor has the spatial connotation that is so important for their function in defining the locale of signal transduction (Figure 2). It is notable that, in the case of TLR and Toll signaling pathways, sorting adaptors and signaling adaptors often act together (e.g., TIRAP and MyD88). Why would this be the case?

There are two major benefits of utilizing sorting and signaling adaptor pairs to promote immune signaling. The first benefit is that sorting adaptor localization facilitates the reliable activation of signal transduction, probably because these adaptors are the first cytosolic proteins to detect activated receptors. In mammals, evidence in support of the need for reliable responsiveness comes from studies showing that cytosolic alleles of TIRAP or TRAM cannot detect active receptors efficiently, and therefore TLR4 signaling occurs with diminished efficacy [37,43]. Studies in *Drosophila* have further underscored the importance of sorting adaptor localization to execute a reliable innate immune response. For example, flies encoding cytosolic alleles of dMyD88 exhibit a highly variable ability to control bacterial replication during infections [51]. This vastly different ability of flies of the same genotype to control bacterial replication is in contrast to the situation with flies expressing WT dMyD88, which nearly always control bacterial growth [49,51]. These examples indicate that the reliability of signaling pathway activation is achieved through the proper subcellular positioning of sorting adaptors prior to any microbial encounter.

In principle, one could achieve a rapid and reliable innate immune response by prepositioning the signaling adaptors in sites of signal transduction. For example, MyD88 could have evolved the ability to bind to PIP2 directly. In this case, MyD88 would be in an ideal position for rapid responsiveness to TLR4 activation. However, the benefit of rapid responsiveness to activated TLR4 would come at the cost of restricting its movement to a single subcellular site. Thus, the second major benefit of utilizing sorting and signaling adaptor pairs is to permit signaling adaptors and downstream enzymes to be recruited to multiple organelles. By placing distinct sorting adaptors in distinct locations, the same downstream enzymes can

function from many locations in the cell. In the case of Toll signaling, the localization of TIRAP to PIP2-rich regions of the plasma membrane facilitates MyD88 recruitment to this location [37], but MyD88 also retains the flexibility to be recruited to other locations in the cell. For example, MyD88 can be recruited to the interleukin 1 receptor (IL-1R) [56], which signals from a region of the plasma membrane that contains low levels of PIP2 [37]. In this regard, the sorting adaptor model would mandate the existence of a sorting adaptor that is prepositioned in PIP2-poor regions of the cell surface to recruit MyD88.

Although never discussed in this context, the IL-1 receptor accessory protein (IL-1RacP) may function as a sorting adaptor for the recruitment of MyD88 to PIP2-poor regions of the plasma membrane. IL-1RacP is a transmembrane protein that is a central component of the signaling complex induced by IL-1 β [57]. IL-1R signaling appears to occur from caveolae, and the receptor is subsequently internalized into caveosomes [58,59]. Relative to lamellipodia, where TIRAP is located [37], caveolae contain low amounts of PIP2 [60]. Similar to the function of TIRAP, the primary function of IL-1RacP is to recruit MyD88 to IL-1R, although it modestly contributes to ligand binding as well [57,61,62].

I predict that although TIRAP serves as a sorting adaptor to recruit MyD88 to plasma membrane subdomains that are enriched in PIP2, IL-1RacP serves as a sorting adaptor to recruit MyD88 to plasma membrane subdomains that are devoid of PIP2 (Figure 2). This prediction may help explain the reliance of some receptors on TIRAP for inducing MyD88-dependent responses (e.g., TLR4, TLR2/1, and TLR2/6) [63,64] and other receptors on IL-1RacP for inducing MyD88-dependent responses (e.g., IL-1R, IL-33R) [61,65]. It is important to note, however, that the ability to implicate IL-1RacP as a *bona fide* sorting adaptor awaits detailed cell biological analysis of the subcellular localization of this protein and its signaling receptor.

Expanding the use of sorting adaptors to intracellular organelles

The aforementioned properties of sorting–signaling adaptor pairs would not only benefit an organelle containing diverse subdomains such as the cell surface, but should also benefit the diverse organelles present in the cytosol of mammalian cells. In this regard, I speculate that sorting adaptors exist in non-plasma membrane compartments. Some obvious organelles to consider in this discussion are endosomal vesicles, peroxisomes and mitochondria. The ability of TRAM to function as an endosomal sorting adaptor for TLR4 suggests that signaling pathways operating from endosomes can indeed benefit from the use of sorting–signaling adaptor pairs. But are there others?

Nucleic acid sensing TLRs such as TLRs 3, 7, 8, and 9 induce innate immune signaling from endosomes [16], and in the case of the latter three receptors, they signal through MyD88. I speculate that a dedicated set of sorting adaptors exists that are uniquely found on endosomes. The localization of these endosomal sorting adaptors would permit the recruitment of MyD88 to a subset of endosomes and endow these organelles with unique signaling capabilities.

It is possible that in the case of TLR9, the ability of MyD88 to be recruited to distinct populations of endosomes (e.g., PI(3,5)P2-rich endosomes and lysosome-related organelles) results from the actions of differentially localized sorting adaptors. The diversification of sorting adaptors on different subsets of endosomes also has the advantage of diversifying the downstream effectors that can be recruited to such compartments, thus explaining the specificity of the signaling response induced by TLR9 in these organelles [66].

Another biological process for which these principles may apply is in the antiviral immune responses induced by RLRs [67]. Despite their distinct structural characteristics, RLRs and TLRs have much in common. Like the TLRs, RLRs recognize microbial infections, utilize downstream adaptors to activate cytokine and IFN expression, and can induce signal transduction from multiple organelles [67].

There are two RLRs that have well-established pro-inflammatory functions: RIG-I and melanoma differentiation-associated protein-5 (MDA5) [26,68,69]. Both RLRs are RNA helicases that survey the cytosol for the presence of viral RNA. The distinction between self and viral RNA is thought to occur through the recognition of specific molecular features that are found within viral nucleic acids. For example, RIG-I signaling occurs when it encounters RNA containing 5' triphosphate groups, short double-stranded regions and/or uridine-rich 3' regions [70–72]. MDA5 signaling, in contrast, occurs when it encounters long stretches of double-stranded RNA [73,74]. The RNA detected by RLRs can be of microbial origin, such as viral genomes, mRNAs or replication intermediates, but can also be produced by the host. For example, the host-encoded RNA polymerase III

can transcribe the DNA of Epstein–Barr virus to produce RNA that activates RIG-I signaling [75,76]. Thus, like the TLRs, RLRs have a widespread role in detecting multiple types of viral infections.

Upon detecting viral RNA, RLRs engage the adaptor protein mitochondrial antiviral-signaling protein [MAVS; also known as interferon-beta promoter stimulator 1 (IPS-1), CARD adaptor inducing interferon-beta (Cardif), or virus-induced signaling adaptor (VISA)] to induce the expression of IFNs and other inflammatory mediators [77–80]. MAVS is located on the limiting membranes of peroxisomes, mitochondria and mitochondria-associated membrane (MAM) [81,82] (Figure 3). These organelles share several homeostatic functions in lipid synthesis and metabolism [83], but have only recently been implicated in the control of antiviral immunity. MAVS signaling from mitochondria induces the expression of type I IFNs and antiviral factors called interferon stimulated genes (ISGs) [80]. By contrast, MAVS signaling from peroxisomes induces ISG expression without inducing the expression of type I IFNs [81]. Recently, it was shown that mitochondria and peroxisomes interact at the MAM during viral infection [82]. This coalescence of organelles on the MAM has been deemed the 'intracellular immune synapse', where the actions of peroxisomes and mitochondrial MAVS are coordinated to ensure effective antiviral immunity (Figure 3).

After detecting a viral infection, RLRs are recruited to an inflexible regulator (MAVS) at sites of signaling (Figure 3). Altering MAVS localization to the cytosol renders this protein unable to induce antiviral signaling [80,81], despite the fact that the signaling domains of this

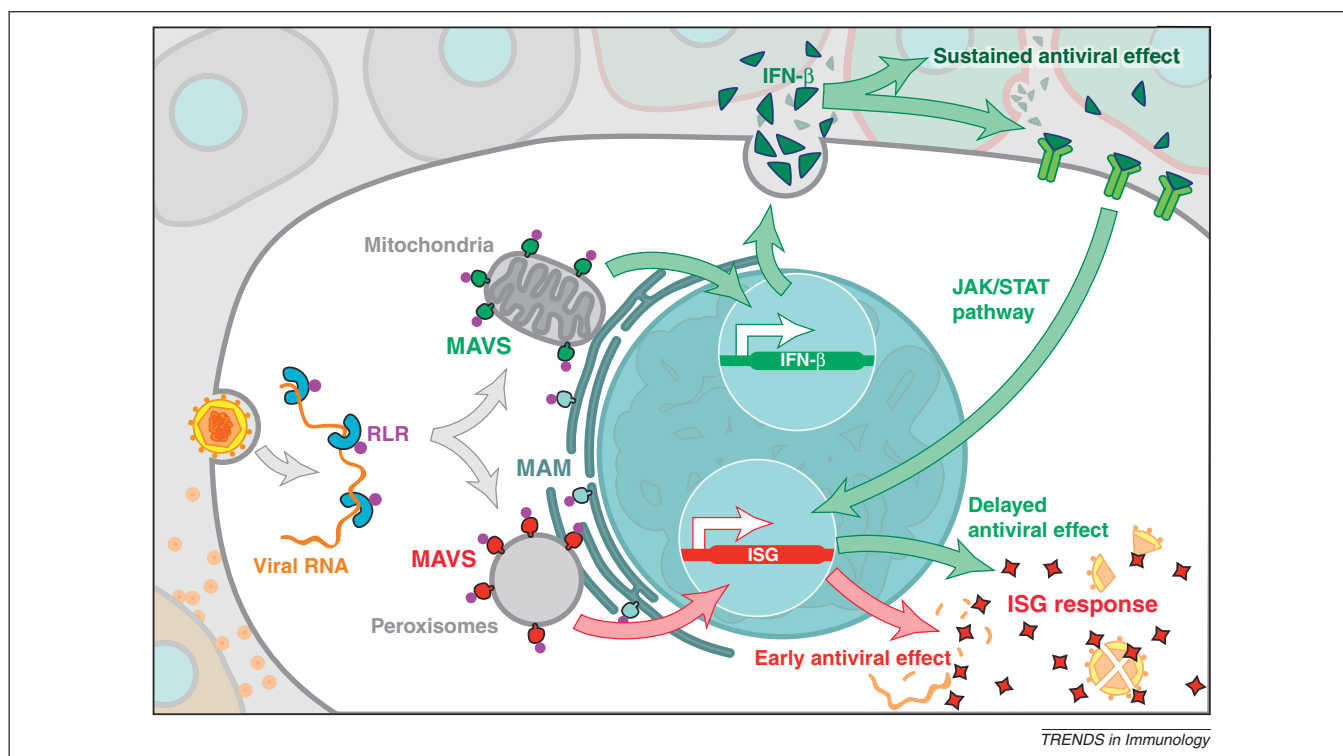


Figure 3. Retinoic acid-inducible gene 1 protein (RIG-I)-like receptor (RLR)-mediated detection of viral RNAs leads to receptor transport to the sorting–signaling adaptor hybrid mitochondrial antiviral-signaling protein (MAVS). MAVS is located on mitochondria, peroxisomes and the mitochondria-associated membrane (MAM). The docking of these organelles at the MAM creates an innate immune synapse that maximizes antiviral innate immunity.

protein remain intact. Thus, similar to the TLR system, the subcellular localization of a downstream adaptor protein, not the site of RLR–virus interaction, determines the initial site of signal transduction.

MAVS therefore has properties that are common to other sorting adaptors, such as defining the site of RLR signaling, and recruiting downstream signaling enzymes to these sites (see examples below). However, this protein also has properties of a signaling adaptor. For example, MAVS can interact with downstream enzymes that facilitate RLR signaling, such as TBK1, TRAF3, TRAF2, and TRAF6 [84]. Thus, MAVS appears to be a hybrid sorting–signaling adaptor (Figure 2), which perhaps explains why RLR signaling only requires this single adaptor to engage downstream enzymes.

It remains to be determined how RLRs are delivered to MAVS to promote antiviral signal transduction. It is possible that the interactions between MAVS and RLRs are facilitated by an intermediate protein, whose function would be to recruit RLRs to the site of MAVS residence. In this regard, this intermediate protein would serve a function analogous to CD14, which delivers TLR4 to endosomes to activate TRAM–TRIF-dependent signaling [32].

Concluding remarks

In this review, I highlighted how cell biological and biochemical analysis of proteins can reveal common biological functions that could not have been predicted by structural and genetic analysis alone. This appears to be the case for TIRAP, TRAM, dMyD88, MAVS, and perhaps IL-1RacP (Figure 2). These proteins share little structural similarity, but all share common biological features of being ‘hard-wired’ to the site in the cell where innate immune signaling occurs. The unifying features of these proteins that define them as sorting adaptors are: (i) they are localized to specific organelles at steady state; (ii) they recruit signaling proteins to their site of residence to initiate signal transduction; and (iii) their mislocalization to the cytosol results in a deficient signaling response. These proteins share no specific structural features, thus, it is difficult to predict additional sorting adaptors bioinformatically. Rather, detailed biochemical and cell biological analysis is necessary to expand the list of sorting adaptors to include regulators of additional signaling pathways.

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