Published in final edited form as:
Cell. 2014 February 13; 156(4): 705–716. doi:10.1016/j.cell.2014.01.019.

A promiscuous lipid-binding protein diversifies the subcellular sites of Toll-like Receptor signal transduction

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Introduction

At some point in their existence, all proteins must move within mammalian cells. Some movement is biosynthetic, in that the protein must be transported from its site of synthesis on the ribosome to its site of action. Another type of movement is signal-dependent, meaning that the protein will move from one location to another in response to some cellular stimulus. Classic cell biological studies identified the cis-acting sequences that direct these types of protein movement within cells; for example (Blobel and Dobberstein, 1975a; Blobel and Dobberstein, 1975b) and (Baeuerle and Baltimore, 1988). In addition to these examples, a more complex type of protein movement exists, where proteins can be found in multiple locations under resting conditions, or can be recruited to multiple locations in response to a stimulus. In this latter instance, it is not obvious how a single localization signal would allow a protein to be targeted to (and function from) multiple organelles. An example of this can be found from the studies of the Toll-like Receptors (TLRs) of the innate immune system.

TLRs are transmembrane receptors that are expressed by a variety of mammalian cell types, but are best studied in professional phagocytes such as macrophages and dendritic cells (DCs) (Akira et al., 2006). TLRs detect a wide range of microbial products, and may be divided into different groups based on their subcellular site of ligand recognition (Barton

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and Kagan, 2009). TLRs 1, 2 and 4–6 reside at the plasma membrane, where they detect molecules displayed on the surface of various pathogens. TLRs 3, 7–9 and 11–13 are localized to various endosomal compartments, where most detect microbial nucleic acids.

Despite residing in distinct subcellular compartments, most TLRs activate a common signal transduction pathway to induce innate and adaptive immunity. TLR signaling usually initiates with the activation of an adaptor protein called MyD88, which is recruited to the conserved TIR domain present in the cytosolic tail of all receptors of this family (Gay et al., 2011; O'Neill and Bowie, 2007). MyD88 forms a protein complex with kinases of the IRAK family called the myddosome (Lin et al., 2010; Motshwene et al., 2009). This complex is thought to induce a cascade of signaling events that activates the NF-κB dependent expression of cytokines, chemokines and other immunomodulatory factors (Gay et al., 2011; Motshwene et al., 2009); (Medzhitov and Horng, 2009). Because TLRs reside on distinct organelles, the myddosome must have the capacity to be assembled in multiple subcellular locations. How myddosome assembly can be promoted from multiple locations is unknown. Answering this question will fill a fundamental gap in our understanding of how immune signaling pathways are integrated into the cellular infrastructure within which they operate.

A simple explanation for how MyD88 can be recruited to diverse organelles would be through interactions with the TIR domains of activated TLRs. However, 2-hybrid analyses performed in yeast and mammalian cells indicated that MyD88 has limited ability to interact with TLRs directly (Brown et al., 2006; Ulrichts et al., 2007). For this reason, an intermediate protein is likely required to link activated TLRs to the recruitment of MyD88. Consistent with this model, the cell surface TLRs utilize "sorting adaptors" to accomplish this task. Sorting adaptors are the only regulators of TLR signaling that are located at the subcellular site of signal transduction, prior to any microbial encounter (Kagan, 2012a). Their placement at the eventual site of signaling allows sorting adaptors to function as sensors of activated TLRs, and recruit downstream signaling adaptors (*e.g.* MyD88) to induce inflammatory cytokine expression. Most plasma membrane-localized TLRs use the sorting adaptor TIRAP (also known as Mal) to recruit MyD88 to the cell surface (Fitzgerald et al., 2001; Horng et al., 2002; Kagan and Medzhitov, 2006; Yamamoto et al., 2002). The ability of TIRAP to function as a sorting adaptor is dependent on its amino terminal localization domain, which interacts with plasma membrane-localized phosphatidylinositol-4,5 bisphosphate (PI(4,5)P2) and other lipids (Kagan and Medzhitov, 2006). The use of sorting adaptors extends beyond the MyD88-dependent pathways, as analogous systems exist in other immune signaling pathways in mammals and *Drosophila melanogaster* (Kagan, 2012a; Kagan et al., 2008; Marek and Kagan, 2012).

Despite the apparent importance of sorting/signaling adaptor pairs for controlling TLR signaling from the cell surface, a sorting adaptor for the exclusively endosomal TLRs has not been described. As such, it is unclear how MyD88-dependent innate immune responses are activated by endosomal TLRs. TIRAP was initially excluded as a sorting adaptor for endosomal TLRs because TIRAP-deficient cells retain the ability to respond to synthetic TLR7 and TLR9 ligands (Horng et al., 2002; Yamamoto et al., 2002). However, these studies also demonstrated that the requirement of TIRAP for signaling from cell surface-localized TLRs can be bypassed when high concentrations of ligand are used. Primary macrophages and DCs are highly endocytic. This property, combined with the common use of phosphorothioate-linked (nuclease-resistant) nucleic acids to activate endosomal TLRs, led us to hypothesize that these ligands could accumulate to high concentrations within endosomes, masking a requirement for TIRAP. In light of this hypothesis, we decided to reassess the role of TIRAP in endosomal TLR signaling.

Herein we report that TIRAP is required for signaling downstream of endosomal TLRs in response to natural ligands, such as viral nucleic acids. We show that TIRAP is present in myddosomes induced by both cell surface and endosomal TLRs, and is required for myddosome formation. Further, we show that the ability of TIRAP to bind to multiple lipid species is critical for its ability to function from multiple subcellular compartments. These findings provide a molecular explanation for the ability of MyD88 to be recruited to more than one organelle, and highlight how a promiscuous-lipid binding domain can be used to diversify the subcellular sites of innate immune signal transduction.

Results

TIRAP is required for endosomal TLR signaling in response to viral infection

To determine if TIRAP would function as a sorting adaptor for endosomal TLRs, we readdressed the genetic requirement for this protein in the signaling pathway activated by TLR9. TLR9 is an excellent model for these studies for two reasons. First, it is the best-characterized endosome-localized TLR (Barbalat et al., 2011) and its ligand (unmethylated CpG-containing DNA) is easy to produce (Hemmi et al., 2000). Second, whereas several viruses activate TLRs at the cell surface and endosomes (Barbalat et al., 2009; Georgel et al., 2007; Kurt-Jones et al., 2004), substrains of herpes simplex virus (HSV) exist that only activate TLR9 (Sato et al., 2006). Thus, TLR9 provides a useful model for the study of both synthetic (CpG DNA) and natural (HSV) activators of endosomal TLRs.

Wild type (WT) and TIRAP-knockout (KO) primary bone-marrow derived macrophages (BMDM) were stimulated with CpG DNA or three substrains of HSV-1 (Sato et al., 2006). The cell populations were then assessed for the expression of the cytokines interleukin-1β (IL-1β) and IL-6. As expected (Horng et al., 2002; Yamamoto et al., 2002), TIRAP KO BMDM had no defect in responding to CpG DNA, while their response to bacterial lipopolysaccharide (LPS), a ligand for the cell-surface TLR4, was impaired (Figure 1A). Interestingly, whereas all three substrains of HSV induced the expression of IL-1β and IL-6 in WT BMDM, TIRAP KO BMDM were defective for these responses (Figure 1B). Among the HSV substrains tested, Kos A and Kos CE only engage TLR9, whereas Kos K engages both cell surface TLR2 and endosomal TLR9 (Sato et al., 2006). Our finding that TIRAP is required for cytokine expression in response to HSV substrains that only engage TLR9 suggests that this adaptor plays a role in signaling from endosomal receptors. Interestingly, as was observed for ligands that activate cell surface-localized TLRs (Horng et al., 2002), the requirement for TIRAP in TLR9 signaling can be bypassed by increasing the dose of a subset of HSV substrains (Figure S1A, MOI=10). The requirement of TIRAP for antiviral responses was unique to the TLR pathway, as WT and TIRAP KO BMDM expressed the interferon (IFN) inducible gene viperin comparably when infected with mammalian reovirus (Figure S1B), a known activator of the cytosolic RIG-I like Receptors (Dixit et al., 2010). Collectively, these data suggest an important role of TIRAP in the response to natural (viral) activators of endosomal TLR9.

To corroborate these findings, we sought an alternative cell type to study the role of TIRAP. Plasmacytoid dendritic cells (pDCs) are an intriguing option, as they exclusively utilize endosomal TLRs to detect infections (Gilliet et al., 2008). As such, all of the plasma membrane-localized TLRs that are established to require TIRAP are non-functional in these cells (Sato et al., 2006). Moreover, in pDCs, endosomal TLRs exhibit an intriguing behavior in that they can induce cytokine and IFN production from different populations of endosomes, with IFN production occurring from endosomes rich in 3′ phosphoinositides (Sasai et al., 2010). We reasoned that if TIRAP was not required for endosomal TLR signaling, as previously believed (Horng et al., 2002; Yamamoto et al., 2002), then pDCs would have no need to express the gene encoding this adaptor. To address this possibility, a

comparison of gene expression was performed between highly purified pDCs and conventional DCs (cDCs). This analysis revealed that pDCs express much lower levels of the cell-surface TLRs 1, 2, 4 and 6 than cDCs (Figure 1C). This observation may (in part) explain the inability of pDCs to respond to ligands for plasma membrane-localized TLRs (Sato et al., 2006). pDCs expressed higher levels of some endosomal TLRs (TLR7 and TLR12) than their cDC counterparts. Interestingly, both cell types expressed comparable levels of TLR9, TIRAP and MyD88 (Figure 1C), suggesting that TIRAP may indeed have a function downstream of endosomal TLRs in pDCs. To address this possibility, pDCs were infected with either HSV or influenza virus, natural activators of endosomal TLR9 and TLR7, respectively (Diebold et al., 2004; Lund et al., 2004). Infected cells were then assessed for their ability to induce the expression of IFNα or the cytokine IL-12p40. Because the expression of these cytokines is initiated from different populations of endosomes (Sasai et al., 2010), this experiment allows us to determine if a population of endosomes exists that selectively requires TIRAP function. In comparison to WT pDCs, TIRAP KO pDCs were impaired in the ability to produce IFNα in response to HSV or influenza virus (Figure 1D). In contrast, the production of IL-12p40 was not impaired in TIRAP KO pDCs, suggesting that TIRAP regulates signaling from endosomes that were previously defined as being rich for 3′ phosphoinositides (Sasai et al., 2010). Another sorting adaptor may exist to control TLR signaling to induce IL-12p40. Overall, these data establish that, in response to natural activators of innate immunity, TIRAP is required for signaling by endosomal TLRs in multiple cell types. Furthermore, the use of HSV strains that only activate TLR9, and the use of pDCs that only permit signaling by endosomal TLRs, eliminates the possibility of contaminating bacterial products (*e.g.* LPS) explaining these observations.

Immortalized macrophages are a model for investigating endosomal TLR signaling

To define the means by which TIRAP regulates TLR signaling from multiple organelles, we sought a cell type that would be easy to propagate and amenable to genetic manipulation. Immortalized BMDM (iBMDM) have emerged as a useful tool in this regard, as they retain the signaling properties of their primary cell counterparts (Dixit et al., 2010; Halle et al., 2008) and TIRAP KO iBMDM have been used to dissect the functions of this adaptor (Nagpal et al., 2009). Similar to our observations made in primary BMDM, TIRAP was required for iBMDM to respond to several substrains of HSV, including two that engage only TLR9 (Kos A and Kos CE) (Figure 2A) (Sato et al., 2006). We found that iBMDM are less phagocytic than primary BMDM (Figure 2B). This was expected, because endocytosis and phagocytosis rates are higher in non-mitotic macrophages (*e.g.* primary BMDM) than in dividing macrophages (Berlin et al., 1978). We hypothesized that this lower phagocytic activity would prevent CpG DNA from building to high concentrations within endosomes, perhaps revealing a requirement for TIRAP in responses to synthetic TLR9 ligands. Indeed, while WT iBMDM responded to CpG DNA comparably to primary WT BMDM, TIRAP KO iBMDM were completely unresponsive (Figure 2C). However, high doses of CpG DNA partially overcame the requirement for TIRAP in these cells (Figure S1C). To determine if TIRAP deficiency was responsible for the unresponsiveness of these cells to TLR9 ligands, this adaptor was stably reintroduced into TIRAP KO iBMDM via retroviral transduction. The retroviral vector used also encodes an internal ribosomal entry site upstream of eGFP, which allowed for the use of fluorescence activated cell sorting (FACS) to isolate a population of cells with uniform levels of expression (data not shown). Rescuing TIRAP expression in TIRAP KO iBMDM restored responsiveness to plasma membrane and endosomal localized TLR ligands (Figure 2D). Furthermore, rescuing TIRAP expression enabled an enhanced response to all substrains of HSV examined (Figures 2A and S1D). Among the substrains examined is HSV 7134 (Figure S1D), which lacks the expression of ICP0 (van Lint et al., 2010). ICP0 is an immune evasion protein that may interfere with TLR

signaling by causing the degradation of TIRAP and/or MyD88 (van Lint et al., 2010). To determine if ICP0 is acting to prevent TLR signaling in our assays, side-by-side experiments were performed with HSV 7134 and an isogenic substrain whose expression of ICP0 was restored (7134R). We observed no difference in the ability of these strains to induce TIRAP-dependent cytokine expression (Figure S1D). These data suggest that ICP0 may act at a later stage of infection or in a different cell type to interfere with TLR signaling. Overall, the observation that the signaling defects of TIRAP KO cells can be rescued by the expression of a TIRAP cDNA provides formal genetic proof of its role in signal transduction activated by endosomal TLRs. iBMDM therefore provide a genetically tractable model to study the role of TIRAP in signaling from endosomal TLRs through the use of natural and synthetic ligands.

TIRAP promotes the assembly of myddosomes at the plasma membrane and endosomes

Based on the experiments described above, it was possible that TIRAP acts to assemble myddosomes after activation of TLRs found at the plasma membrane and endosomes. The crystal structure of the myddosome indicates that the adaptor MyD88 is a core component of this signaling complex (Lin et al., 2010). However, the natural kinetics of myddosome assembly induced by TLR ligands remain undefined. To assess myddosome assembly, iBMDM were stimulated with LPS or CpG DNA, and endogenous MyD88 immunoprecipitates were subjected to western analysis for the presence of the other known components of the myddosome, the kinases IRAK2 and IRAK4 (Lin et al., 2010; Motshwene et al., 2009). Within minutes of stimulating cells with either LPS or CpG DNA, both kinases were recruited to MyD88 (Figures 3A and 3B). Immunoprecipitations with IRAK2 antisera yielded comparable results, with MyD88 and IRAK4 being recruited to IRAK2 within minutes of stimulation (Figure S2A). Consistent with prior work on the activation of MAP kinases and NF-κB (Horng et al., 2002; Schnare et al., 2000), LPS induced maximal assembly of the myddosome more rapidly than CpG DNA (Figures 3A and 3B). Because the myddosome is not detected in resting cells, its assembly can be used to monitor the earliest cytosolic events that occur during TLR signaling.

To determine if TIRAP is a component of LPS or CpG DNA-induced myddosomes, we utilized the transgenic TIRAP KO cells described above. The TIRAP allele expressed in these cells contains a biotinylation sequence and an HA-epitope tag to facilitate biochemical analysis. LPS and CpG DNA-induced myddosomes containing IRAK4 and MyD88 could be detected by direct isolation of biotinylated TIRAP through the use avidin-coated beads (Figure 3C and 3D). Moreover, TIRAP could be recruited to MyD88-immunoprecipitates in LPS treated cells (Figure S2B). These data indicate that TIRAP is a stable component of the myddosomes induced by either TLR4 or TLR9.

To determine if TIRAP was required for myddosome formation, TIRAP KO iBMDM were examined. Myddosome formation in response to both LPS and CpG DNA was impaired in TIRAP KO cells, but rescued in TIRAP KO cells expressing the TIRAP transgene (Figure 3E). These data establish that TIRAP controls the assembly of (and is a component of) myddosomes formed in multiple subcellular compartments.

Promiscuous lipid binding diversifies the subcellular sites of TIRAP residence

TIRAP is a peripheral membrane protein that is enriched at the cell surface (Kagan and Medzhitov, 2006). Our discovery that TIRAP regulates TLR signaling from endosomes therefore raises the question of how a plasma membrane-localized adaptor can function from endosomes. The localization of TIRAP is strictly dependent on an amino terminal lipid-binding domain that interacts with acidic phosphoinositides (PIs) and phosphatidylserine (PS) (Kagan and Medzhitov, 2006). To confirm these observations, the ability of TIRAP to

bind lipids was assessed by PIP-strip analysis, which permits the identification of several possible protein-lipid interactions in a single experiment. GST-TIRAP interacted with all phosphorylated PIs and PS (Figure 4A). As a control for specificity of these interactions, we used a mutant that is defective for lipid-binding *in vitro* and localization *in vivo* (TIRAP 4X) (Kagan and Medzhitov, 2006). This mutant TIRAP allele did not bind to any lipids in this analysis (Figure 4A). Among the lipids TIRAP interacts with, PI(4,5)P2 is considered solely important for its function in controlling signaling by plasma membrane-localized TLRs (Aksoy et al., 2012; Kagan and Medzhitov, 2006). In this regard, TIRAP is similar to many other lipid-binding proteins in that its ability to bind PIs promiscuously is not considered functionally significant (Kavran et al., 1998).

Because some of the lipids that TIRAP interacts with are enriched on endosomes (*e.g.* PI(3)P and PI(3,5)P2) (De Matteis and Godi, 2004), we considered the possibility that a pool of TIRAP would be located on endosomes. This possibility was addressed by examining the localization of the lipid-binding domain of TIRAP in primary BMDM. We transfected these cells with plasmids encoding the GFP-tagged lipid-binding domain of TIRAP (TIRAP-loc) along with a plasmid encoding a cherry-tagged pleckstrin homology (PH) domain from PLCδ1 (PLC), which binds to PI(4,5)P2 uniquely (Botelho et al., 2000). We reasoned that if TIRAP binds only to PI(4,5)P2 within cells, then the subcellular distribution of these two proteins should be identical. However, if TIRAP binds to more lipids than PI(4,5)P2, then the distributions of these proteins should be overlapping but distinct. Confocal microscopy revealed that both proteins colocalized extensively at the plasma membrane, which is an abundant site of PI(4,5)P2 (Botelho et al., 2000). Interestingly, TIRAP's lipid-binding domain also labeled intracellular vesicles that were devoid of the PLCδ1 PH domain (Figure 4B, inset), suggesting that TIRAP binds additional lipid targets inside cells, as it does *in vitro*.

To determine if the intracellular TIRAP-positive compartments were endosomes, live time-lapse microscopy was performed in primary BMDM expressing the GFP-tagged lipid-binding domain of TIRAP. Primary BMDM were highly active and exhibited extensive ruffling of the plasma membrane (Movie S1). Numerous TIRAP positive vesicles were formed at these sites of membrane ruffling, and these vesicles accumulated Alexa-647 labeled dextrans that were added to the culture media shortly after imaging began (Figure 4C and Movie S1). These data indicate that the lipid-binding domain of TIRAP is capable of localizing to the plasma membrane and *bona fide* endosomes. Promiscuous lipid binding by TIRAP may therefore be important for its ability to function as a sorting adaptor for TLRs located at the cell surface and endosomes.

Distinct lipid targets of the TIRAP localization domain permit TLR signaling from the plasma membrane and endosomes

To determine the function of the individual lipids with which TIRAP interacts, the lipid-binding domain of TIRAP was replaced with domains of singular specificity (Figure 5A, top panel). We replaced the endogenous lipid-binding domain of TIRAP with the PLCδ1 PH domain described above, which binds exclusively to PI(4,5)P2 (hereafter referred to as PLC-TIRAP). By a similar strategy, a PI(3)P-specific TIRAP allele was generated called PX-TIRAP. This adaptor has the localization domain of p40-phox, which binds exclusively to PI(3)P on endosomes (Kagan and Medzhitov, 2006; Sato et al., 2001). PX-TIRAP was of particular interest because of our finding that TIRAP preferentially regulates IFNα expression induced by TLR7 and TLR9 (Figure 1D), which occurs from endosomes containing 3′ phosphoinositides in pDCs (Sasai et al., 2010). Finally, SLP2a-TIRAP was generated, which binds PS, a general component of the cell surface and endosomal membranes (Kuroda and Fukuda, 2004). These constructs were fused to GFP, stably

transduced into TIRAP KO iBMDM and sorted by FACS to isolate clones of comparable expression levels. The resulting cell populations were examined by confocal microscopy, which verified their expected localization (Figure 5A, bottom panel). PLC-TIRAP was detected primarily at the cell surface, PX-TIRAP was enriched on intracellular vesicles previously identified as endosomes (Kagan and Medzhitov, 2006), and SLP2a-TIRAP was detected at the cell surface and endosomes.

This set of stable macrophage lines was stimulated with LPS or CpG DNA to determine which lipids were important for TLR signal transduction. As expected, TIRAP KO cells expressing WT TIRAP regained the ability to permit TLR4 signaling from the cell surface and TLR9 signaling from endosomes (Figure 5B). The cell surface-exclusive PLC-TIRAP restored responsiveness to the TLR4 ligand LPS, but interestingly, this allele did not restore TLR9 signaling in response to CpG DNA (Figure 5B). These data indicate that whereas PI(4,5)P2 binding by TIRAP is sufficient for signaling from the plasma membrane, it is not sufficient for signaling from endosomes. Remarkably, examination of the function of PX-TIRAP yielded the opposite results. TIRAP KO cells expressing PX-TIRAP (localized to endosomes via PI(3)P), responded robustly to CpG DNA but did not respond to LPS (Figure 5B). Similar results were obtained with the PS-binding SLP2a-TIRAP allele (Figure 5B).

The localization of TIRAP was also critical for myddosome formation. Consistent with Figure 3B, TIRAP KO cells reconstituted with WT TIRAP formed myddosomes in response to both LPS and CpG DNA (Figure 5C). By contrast, PLC-TIRAP was able to rescue myddosome formation solely in response to LPS, while PX-TIRAP and SLP2a-TIRAP were able to rescue myddosome formation in response to CpG DNA, but not LPS (Figure 5C). Overall, these data establish that distinct targets of the promiscuous TIRAP lipid-binding domain are functional, and allow this adaptor to promote TLR signaling from more than one site in the cell.

Discussion

It has become clear in recent years that TLRs can induce signal transduction from diverse locations in the cell, with the cell surface and endosomal membranes being the best-defined sites (Kagan, 2012b). However, the question remained as to how this diversification of receptor locale can be accommodated with the common need to activate MyD88-dependent signaling. Our finding that TIRAP can function as a sorting adaptor for MyD88 at the cell surface and endosomes provides a molecular explanation to this question and fills an important gap in our knowledge of how diverse subcellular compartments can support TLR signaling.

Several lines of evidence indicate that TIRAP can function from endosomes. First, TIRAP KO primary and immortal BMDM are defective for TLR signaling in response to HSV infection. This finding is in contrast to results obtained with non-degradable nucleic acids containing phosphorothioate linkages, which bypass the need for TIRAP in primary BMDM. These data highlight the utility of natural activators of innate immunity to dissect TLR signal transduction pathways. Second, TIRAP is expressed in cells that do not express plasma membrane-localized TLRs (pDCs), and TIRAP KO pDCs are defective for IFN responses induced by TLR7 or TLR9. Because plasma membrane-localized TLRs are not functional in pDCs, these data eliminate any possibility that contaminating TLR2 or TLR4 ligands can explain our findings. Third, TIRAP is a component of myddosomes induced by TLR4 and TLR9, and is required for their formation. Collectively, using multiple activators, assays and cell types, these data establish that TIRAP has a widespread role in controlling TLR signal transduction in response to bacteria and viruses.

We do note however, that not all MyD88-dependent responses are mediated by TIRAP. In addition to the MyD88-dependent signaling pathways activated by the IL-1 receptor (IL-1R) family (Horng et al., 2002; Yamamoto et al., 2002), we found that TLR7/9-induced IL-12p40 expression in pDCs was TIRAP-independent. This finding is in contrast to TLR7/9-induced IFNα expression in the same cells. Thus, like TLR4 (Kagan, 2012a), at least some endosomal TLRs may engage distinct sorting adaptors to induce compartment-specific cellular responses. The sorting adaptor(s) that mediates MyD88 recruitment to these TIRAP-independent receptors remains unknown. An alternative explanation to these observations is that endosomes which induce IL-12p40 expression may contain higher concentrations of TLR ligands than IFN-inducing endosomes, rending some signaling pathways more dependent on TIRAP than others. While it is formally possible that some TLRs simply recruit MyD88 to their cytosolic TIR domains directly (bypassing the need for a sorting adaptor), the increasing incidence of sorting adaptor dependence for MyD88 recruitment makes this possibility unlikely.

TIRAP is one of numerous phosphoinositide-binding proteins that can bind to multiple lipids (Kavran et al., 1998). This promiscuity of lipid binding has been a point of inquiry, as we and others have suggested that PI(4,5)P2 is mainly responsible for controlling its localization and function (Aksoy et al., 2012; Kagan and Medzhitov, 2006). Our finding that a PI(4,5)P2-specific allele of TIRAP is sufficient to support TLR4 signaling is consistent with this idea. However, PI(4,5)P2-specific alleles of TIRAP cannot support TLR9 signaling. These observations argue strongly that the role of TIRAP in TLR signaling from endosomes involves interactions with lipids other than PI(4,5)P2. Our functional analysis of TIRAP alleles that bind to single lipids revealed that PI(3)P and PS are sufficient to support TLR9 signaling. From these data, it becomes clear that multiple targets of the lipid-binding domain of TIRAP are functionally important.

The analysis of TIRAP alleles that bind single lipids was also informative in considering the precise subcellular sites of TLR signaling. We found that PI(4,5)P2-specific and PS-specific alleles of TIRAP are localized to the plasma membrane, but only PI(4,5)P2 specificity confers LPS responsiveness. Because the PS-specific TIRAP allele is functional when the correct stimulus is applied to the cells (CpG DNA), these data cannot be explained by this protein being misfolded or poorly expressed. Rather, these findings may highlight the precise requirement for TIRAP to be present in a region of the cell surface that is devoid of PS (or at least enriched for PI(4,5)P2). This suggestion is consistent with prior work demonstrating that localization of TIRAP to the plasma membrane is not sufficient for TLR4 signaling (Kagan and Medzhitov, 2006); TIRAP must be localized to PI(4,5)P2-rich regions specifically. Likewise, our analysis of TLR9 signaling highlights the importance of TIRAP localization to endosomes containing PS, the 3′ phosphoinositide PI(3)P, and probably PI(3,5)P2 (Sasai et al., 2010). These observations are of note when considering that the various membrane-bound organelles in mammalian cells have been proposed to consist of a phosphoinositide code (PI code) (Kutateladze, 2010). This PI code is “read” by various lipid-binding proteins to assemble different protein structures on different membranes, effectively allowing the PI code to determine (in part) the activities of each organelle (Kutateladze, 2010). Our analysis of this PI code, through the use of TIRAP alleles that bind to single lipids, provides a functional means to identify the precise subcellular sites of TLR signal transduction. We suggest that a comprehensive map of the sites of innate immune signal transduction can be created by further study of the link between the PI code and sorting adaptor localization.

From a broader perspective, this new function for TIRAP in controlling TLR signaling from multiple organelles is reminiscent of the activities of the protein MAVS, the receptor-proximal adaptor in the antiviral RIG-I like Receptor signaling pathway (Seth et al., 2005).

Both MAVS and TIRAP can be considered sorting adaptors, in that they define the subcellular sites of signal transduction induced by their respective upstream receptors. Like TIRAP, MAVS is located on several organelles in the cell, all of which are important for antiviral signal transduction (Dixit et al., 2010; Horner et al., 2011; Seth et al., 2005). Thus, a common theme appears to be emerging whereby the sites of innate immune signal transduction can be diversified simply by altering the subcellular sites of sorting adaptor residence.

In summary, we have revealed a novel means by which a promiscuous lipid-binding protein can be used in nature. We suggest that the sorting adaptor TIRAP evolved specifically to survey multiple organelles for the presence of activated TLRs. In this context, the promiscuity of lipid binding by TIRAP provides a molecular explanation for how the subcellular sites of TLR signal transduction are so diverse. Our work also demonstrates a novel means by which functional diversity can be achieved at the level of a single protein, as TIRAP can now be considered to function in bacterial detection at the plasma membrane and virus detection in endosomes. These studies therefore highlight how promiscuity, rather than specificity, can be beneficial in biological systems, and provide a mandate to examine the function of other promiscuous lipid-binding proteins in the innate immune system, and beyond.

Materials and Methods

Cell culture, stable transductions, microscopy

WT (C57B/6) and TIRAP KO iBMDM were a gift from D. Golenbock (UMass) and were cultured in complete DMEM (Gibco) containing 10% FBS and 5% L929 conditioned supernatant. Primary BMDM from WT (C57B/6) or TIRAP KO mice (Jax 017629) were prepared as described (Kagan and Medzhitov, 2006). Cells were stimulated LPS (Invivogen) at 100ng/mL or phosphorothioate-linked CpG DNA (TCCATGACGTTCCTGACGTT (MGW Operon) at 1μM, unless otherwise indicated.

TIRAP alleles were introduced into TIRAP KO iBMDM by retroviral transduction and sorted by FACS to normalize GFP expression. When biotin-based myddosome isolations were performed, TIRAP alleles were introduced in iBMDM stably expressing the biotin ligase BirA. Where indicated, cells were fixed with 2% paraformaldehyde and stained with anti-GFP (Clontech) according to manufacturer's instructions. Antibody staining was detected with the secondary antibody labeled with Alexafluor-488 and analyzed by confocal microscopy.

Primary BMDM were transfected by nucleofection using mouse macrophage transfection reagent (Lonza VPA-1009) according to manufacturer's instructions. Cells were imaged 4 hours later by confocal microscopy. Where indicated, dextran-Alexa Fluor 647 (Life Technologies) was used at 10μg/mL.

Flt3 ligand-induced bone marrow derived pDC culture

Bone marrow cells were isolated and cultured for 7 days in RPMI supplemented with 10% FBS, 50 units/mL penicillin, 50μg/mL streptomycin, 10mM HEPES (Invitrogen) and 2-mercaptoethanol (Sigma) and 100ng/mL Flt3-ligand (GEMINI). On day 8, cells in the supernatant were harvested, counted and plated for subsequent experiments.

Viral infections

KOS A, KOS CE, and KOS K (Sato et al., 2006) viruses were propagated and titered on Vero cells as described (Knipe and Spang, 1982). The ICP0-null (7134) and restored

(7134R) virus was grown and titred on U2OS cells (Cai and Schaffer, 1989). Virus was diluted in PBS containing 0.1% (wt/vol) glucose and 1% (vol/vol) heat-inactivated FBS. Cells were infected at the MOI indicated for 1 h at 37 °C, washed twice with PBS, and overlaid with DMEM containing 1% (vol/vol) heat-inactivated FBS. Infected cells were incubated at 37 °C for the indicated length of time. 2×10^6/well pDCs were stimulated with either 2×10^6 PFU (plaque forming unit) of HSV-2 (186 syn+) or 2×10^7 PFU of Influenza A/PR/8 for 24 hours. Supernatant was then harvested and ELISA was performed to measure IFN-α and IL-12p40. BMDM were infected with the Type 3 Dearing strain of reovirus at MOI: 10, 100 or 1000 for 3 hours, and analyzed by qPCR as described below.

Microarray sample generation, data acquisition and processing

pDC and cDC samples for microarrays were generated as described (Iparraguirre et al., 2008). In brief, DPE-GFPxRAG1$^{-/-}$ transgenic mice (Mempel et al., 2006; Mrass et al., 2006), in which pDCs are identified by GFP expression (Iparraguirre et al., 2008) were injected with Flt-3ligand-expressing B16F10 tumor cells for expansion of DC subsets. 12–14 days later, spleens were harvested and stained for CD11b and CD11c. pDCs and cDCs were sorted using a MoFlo cell sorter (DakoCytomation) based on high GFP expression and a CD11c^{hi}CD11b^{hi} phenotype, respectively. Samples were prepared in 2 independent experiments. Total RNA, isolated with the RNeasy kit (Qiagen), was processed, amplified, labeled and hybridized to mouse MOE430v2 GeneChip microarrays (Affymetrix) by the University of Pennsylvania Microarray Core Facility using standard protocols. Data were processed using Affymetrix GeneChip Operating System v1.4 software, and probe intensity files (.cel files) were imported and analyzed in Partek Genomics Suite (v6.6, Partek). Data were $\log_2$-transformed and normalized with GC Robust Multi-Array Averaging (GCRMA). Significance Analysis of Microarrays (SAM, samr v2.0, Stanford University) (Tusher et al., 2001) was applied to identify differentially expressed genes in mDCs and pDCs. The most significant probe set for each gene of interest was used to calculate fold-changes (in Partek) between gene expression levels in mDCs versus pDCs that were graphed using Prism software (v. 5.0c, GraphPad Software). Microarray data sets are available through the Gene Expression Omnibus (GEO); accession number GSE50436.

Phagocytosis assay

5×10^5 cells were kept on ice in 500uL of complete media (described above) and incubated with Fluoresbrite Carboxy YG 2.0 micron beads (PolySciences) at 10 beads/cell. Cells were added to a 37°C water bath for indicated times. Phagocytosis was stopped with ice-cold PBS. Cells were washed 3× with ice-cold PBS, and analyzed by FACS.

Myddosome isolation assay

Cells were plated on 10cm tissue-culture treated dishes and grown to confluency (10^7 cells/plate overnight). Cells were stimulated with ligand as indicated, then lysed in 700μL of buffer containing 1% NP-40, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10% glycerol and protease/phosphatase inhibitors (Roche). 100 μL of cleared lysate was retained for analysis (input) and remaining 600μL was incubated overnight at 4°C with 1μg anti-MyD88 (R&D sciences) or anti-IRAK2 (ProSci). The following day, 50μL of protein G sepharose (GE healthcare) was added for 1 hour. Alternatively, cleared lysates were incubated with Neutravidin agarose beads (Thermo) for 2 hours. Beads were washed 3× with lysis buffer, then proteins were extracted by adding 50μL 2× Laemmli buffer, electrophoresed and immunoblotted with the indicated antibodies using standard conditions. The following antibodies were used: anti-MyD88 (R&D), anti-IRAK2 (Prosci), anti-HA (3F10, Roche), anti-GFP (JL-8, Clontech) and anti-actin (ac-15, Sigma). Anti-IRAK4 was kindly provided by Shizuo Akira.

Real time quantitative PCR

Total RNA was extracted from 2×10^6 cells using RNA Bee (Tel-Test Inc) according to manufacturer's instructions and analyzed with TaqMan one-step qPCR reagents. Expression was plotted relative to GAPDH, shown as mean and standard deviation of 3 technical replicates. Each graph is representative of at least 3 independent experiments.

Plasmids

TIRAP-TAP IRES GFP was produced as follows. Human TIRAP was appended with 3xHA and a BirA target site (de Boer et al., 2003; Mechold et al., 2005) by overlap extension PCR. The biotin ligase BirA (Mechold et al., 2005) was subcloned into pMSCV2.2 by standard procedures. WT TIRAP-GFP, PLC-TIRAP-GFP and PX-TIRAP-GFP were described previously (Kagan and Medzhitov, 2006), and are present in the retroviral vector MSCV202 IRES-hCD2. pEGFP-N1-based TIRAP loc-GFP (amino acids 1–85 of TIRAP fused in frame to GFP) was described (Kagan and Medzhitov, 2006). SLP2a-TIRAP-GFP was cloned similarly to the TIRAP alleles described above. Briefly, the C2A domain of murine SLP2a was cloned from a murine spleen cDNA library and fused in frame to amino acid 86 of TIRAP-GFP. This SLP2a-TIRAP-GFP fusion cDNA was then subcloned into the retroviral vector MSCV2.2 IRES-hCD2. mCherry-PH was purchased from Addgene (plasmid 36075).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the members of the Kagan lab for helpful discussions, and Dr. John W. Tobias (UPenn Bioinformatics Core) for his contribution to the microarray data analysis. K. B. holds a fellowship from the National Science Foundation. The NIH grants AI093589, AI072955, P30DK34854 and an unrestricted gift from Mead Johnson & Company support the work performed in the lab of J. Kagan. J.K. is also supported by the Bill and Melinda Gates Foundation (OPP1066203). A.I. is supported by NIH grant AI064705 and D.K. is supported by NIH grant AI063106. W.W. is supported by a fellowship from the Cancer Institute New South Wales and NHMRC grant APP1010680. J.K. holds an Investigators in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund.

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- The sorting adaptor TIRAP controls TLR signaling from the cell surface and endosomes
- From multiple organelles, TIRAP regulates the assembly of the myddosome
- TIRAP binds to multiple lipids *in vitro* and within macrophages
- Promiscuous lipid binding by TIRAP permits TLR signaling from diverse organelles

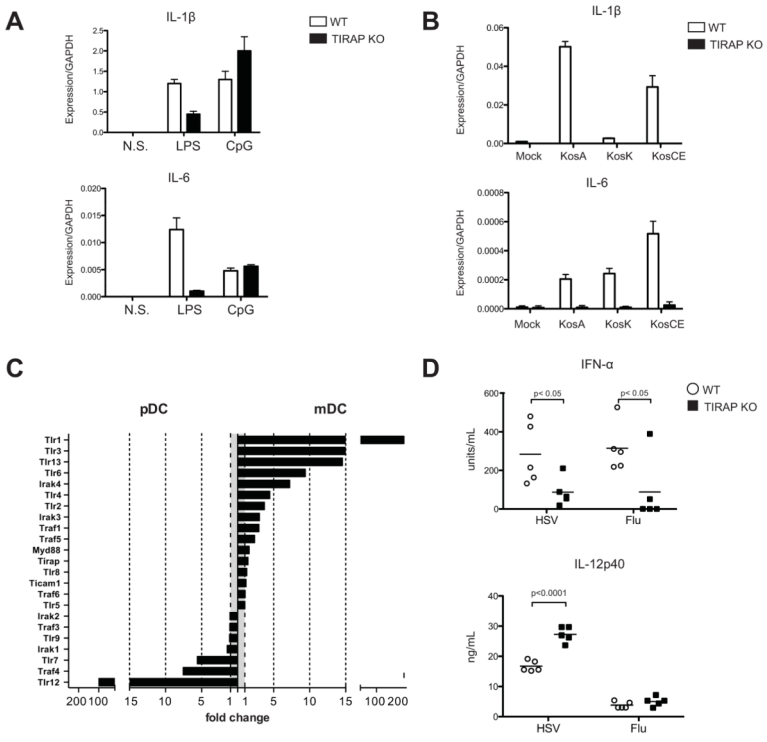

Figure 1. TIRAP is required for endosomal TLR signaling in response to natural ligands

(A, B) Primary WT or TIRAP KO BMDM were treated with 100ng/mL LPS or 1μM CpG DNA (A) or infected with HSV (B) for 3 hours. Total mRNA was extracted and analyzed for expression of IL-1β and IL-6. Note that TIRAP KO BMDM are defective in responding to natural ligands. (C) Microarray gene expression profiles from sorted cDCs and pDCs were analyzed for transcripts associated with TLR-signaling pathways. Values on the x-axis represent the fold difference in gene expression between mDCs and pDCs. Grey area depicts equal gene expression in cDCs and pDCs. Note that TIRAP is expressed in pDCs comparably to cDCs, despite the absence of cell-surface TLRs. (D) pDCs from WT or TIRAP KO mice were infected with HSV-2 (186 syn+) at MOI=1 or influenza (A/PR/8) at MOI=10. Supernatants were collected after 24 hours and analyzed by ELISA. Error bars represent SD. See also Figure S1.

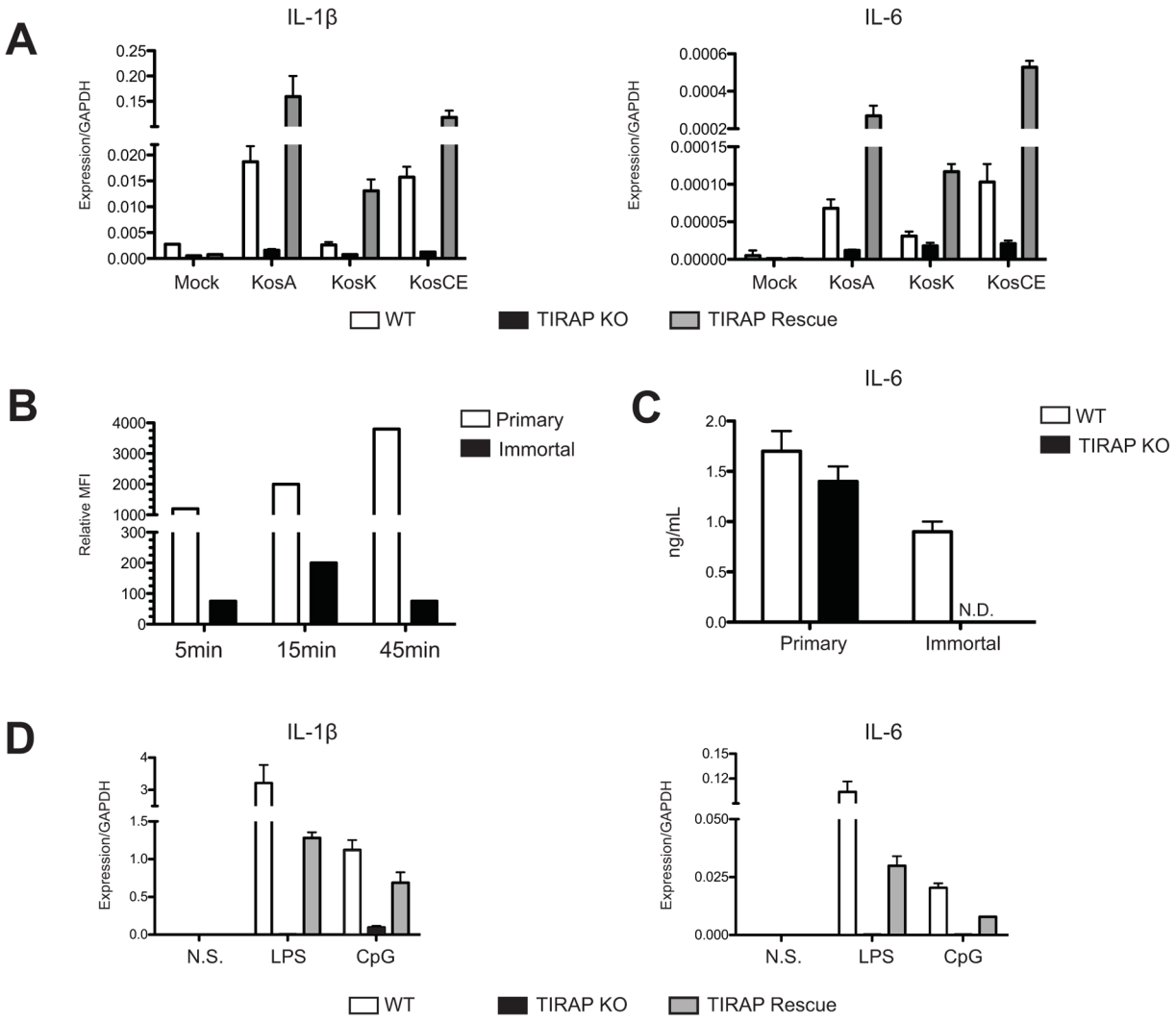

Figure 2. Immortalized BMDM responses to CpG DNA mimic primary cell responses to natural ligands

(A) WT, TIRAP KO or TIRAP-expressing TIRAP KO iBMDM were infected with indicated HSV strains (MOI=1) for 3 hours and analyzed by qPCR. (B) Primary or iBMDM were incubated with fluorescent beads at 37°C for the indicated times and phagocytosis was analyzed by flow cytometry. Mean fluorescence intensity was plotted relative to cells incubated with beads on ice for 45 min. (C) WT or TIRAP KO primary and iBMDM were stimulated with 1μM CpG DNA. After 24 hours, supernatants were collected and analyzed for IL-6 by ELISA. N.D. indicates that no signal was detected. (D) WT, TIRAP KO or TIRAP-expressing TIRAP KO iBMDM were treated with LPS or CpG DNA for 3 hours and analyzed by qPCR. Error bars represent SD.

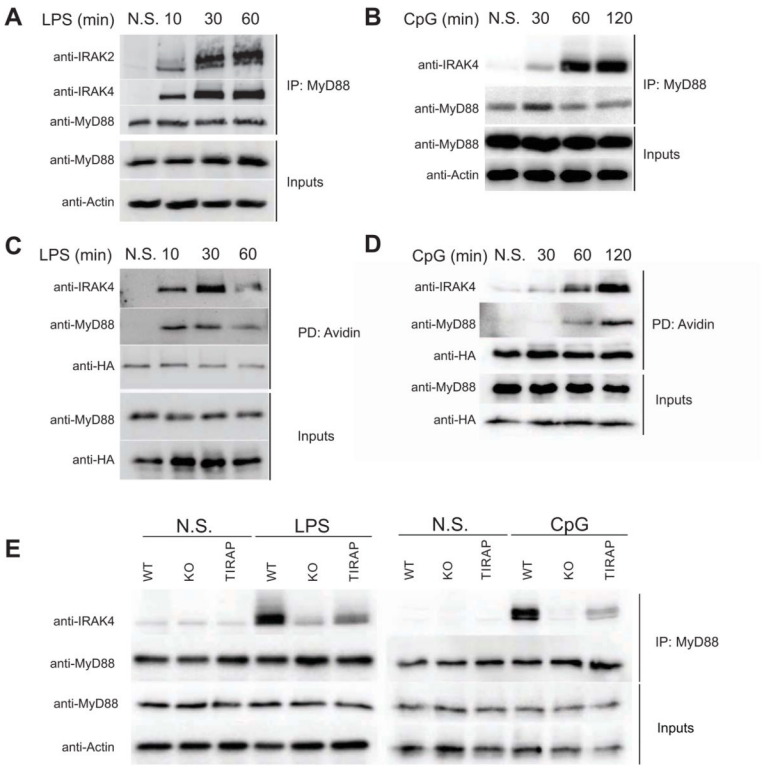

Figure 3. TIRAP is a critical constituent of the myddosome

(A–B) WT iBMDM were stimulated with LPS (A) or CpG DNA (B) for the indicated times. MyD88 was immunoprecipitated from lysates and analyzed by western blot. (C–D) TIRAP-transgenic iBMDM were stimulated with LPS (C) or CpG DNA (D) and biotin-TIRAP was precipitated from cleared lysates with avidin-coated agarose before western analysis. (E) WT, TIRAP KO or TIRAP-expressing TIRAP KO iBMDM were treated with LPS for 1 hour or CpG DNA for two hours and analyzed as in (A). N.S. indicates cells that were not stimulated. See also Figure S2.

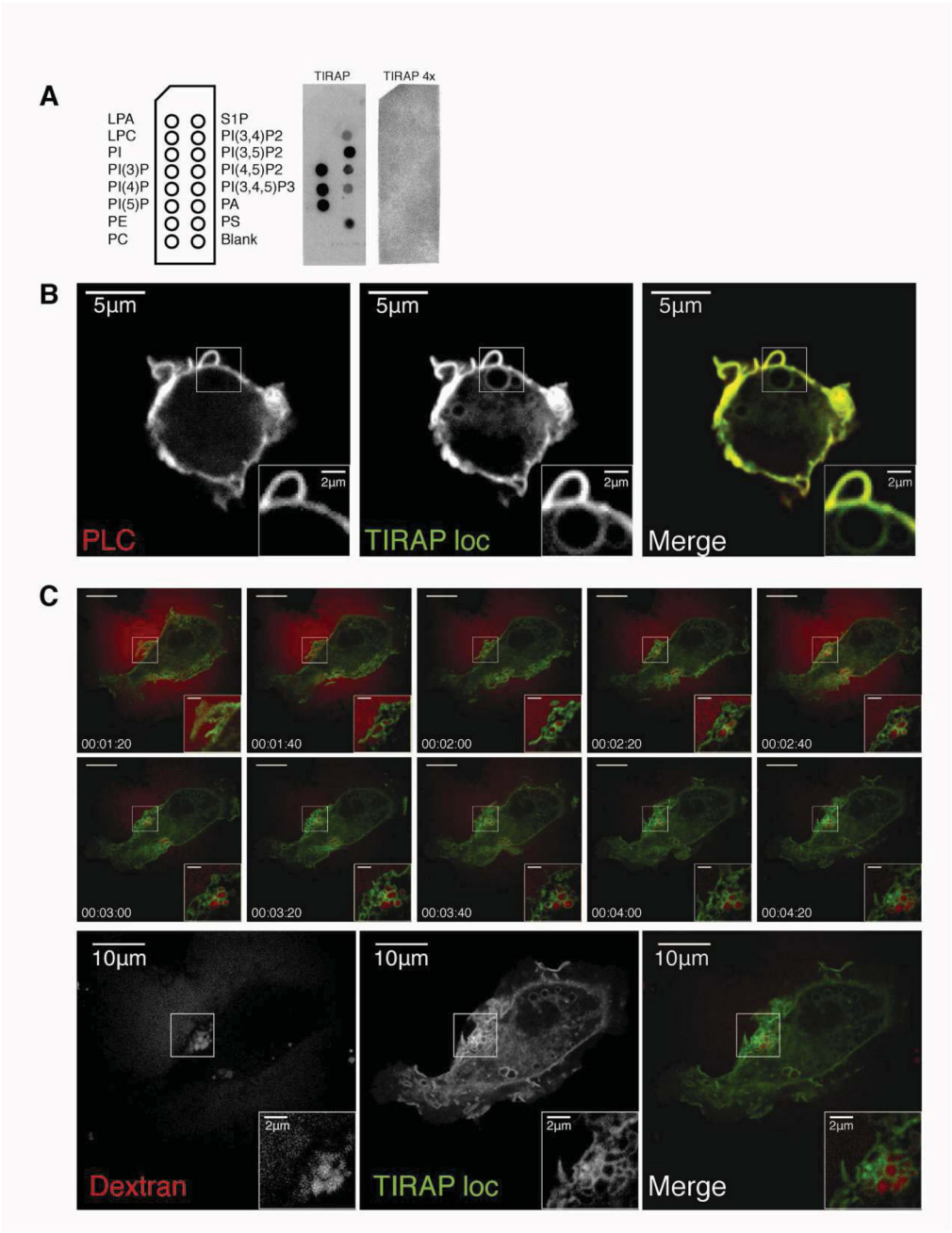

Figure 4. Promiscuous lipid binding diversifies the localization of TIRAP

(A) GST-tagged TIRAP or TIRAP 4X proteins were incubated with PIP strips containing various lipids (shown in left panel) and assessed for protein binding by far western analysis. (B) TIRAP KO primary BMDM expressing TIRAP loc-GFP and PLC-cherry were analyzed by confocal microscopy. All images are representative of at least three independent experiments where over 200 cells were examined per condition and >95% of the cells displayed similar staining. Selective localization of TIRAP to intracellular vesicles compared to the PLCδ1 PH domain demonstrates that TIRAP binds to multiple lipids. (C) TIRAP KO primary BMDM expressing TIRAP-loc GFP were analyzed by confocal

microscopy. One image was captured every 20 seconds for 20 min. (C) Shows representative frames from one such capture (See Movie S1 for full-length movie).

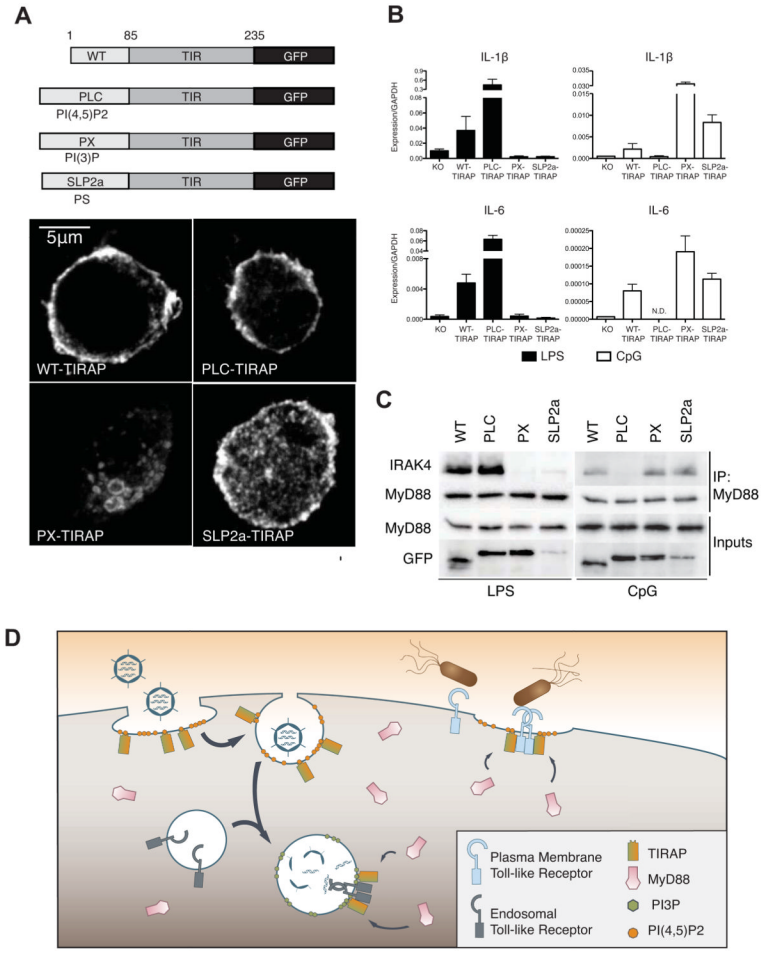

Figure 5. Promiscuous lipid binding by TIRAP diversifies the sites of TLR signaling

(A) Confocal microscopic analysis of TIRAP KO iBMDM stably transduced with GFP-tagged TIRAP alleles that contain different lipid binding domains (depicted in the top panel). The micrographs demonstrate selective localization of TIRAP dependent on its lipid binding domain. All images are representative of at least three independent experiments where over 200 cells were examined per condition and >95% of the cells displayed similar staining. (B) Cells from (A) were stimulated with LPS or CpG DNA and analyzed by qPCR. Note that selective binding of TIRAP to distinct lipids permits signaling from distinct compartments. (C) Cells from (A) were stimulated with LPS or CpG DNA for one hour and myddosome formation was assessed. (D) Model depicting how the promiscuous lipid-binding domain of TIRAP promotes signal transduction from the plasma membrane and endosomes. TLRs found at the cell surface signal from a PI(4,5)P2-rich subdomain, and TIRAP is recruited to that location via interactions with PI(4,5)P2. TLRs found on endosomes signal from a domain rich in 3′ phosphoinositides (depicted is PI(3)P). These lipids direct TIRAP to endosomes to promote TLR signaling. Error bars represent SD.