Recycling Endosomes and TLR Signaling—The Rab11 GTPase Leads the Way

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The ability of Toll-like receptors (TLRs) to activate innate immunity depends on their transport to pathogen-containing organelles. In this issue of *Immunity*, Husebye et al. (2010) report that delivery of TLR4 to phagosomes occurs via a recycling endosome intermediate, which is controlled by the GTPase Rab11a.

The study of TLR signal transduction has experienced somewhat of a rebirth in recent years. As genetic approaches to identify positive regulators of TLR signaling has waned, some of the most exciting research is now geared toward understanding how these signaling networks are integrated into the cellular infrastructure within which they operate (Barton and Kagan, 2009). It is now known that TLRs are found in multiple locations within various immune cells and that their localization and trafficking patterns are important for their signaling functions. In this issue of *Immunity*, Husebye et al. (2010) suggest the existence of a surprising trafficking route taken by TLR4, which is important for the induction of type I interferon (IFN) expression in response to bacterial lipopolysaccharide (LPS).

The classic model of TLR4 signaling dictates that in response to an encounter with a gram-negative bacterium, microbial LPS activates two distinct signaling pathways from the plasma membrane. One signaling pathway is mediated by the adaptor proteins TIRAP and MyD88, which function as a sorting-signaling adaptor pair to activate NF-κB and MAP kinases and induce the expression of inflammatory cytokines (Barton and Kagan, 2009). The second signaling pathway is mediated by a distinct set of sorting-signaling adaptors called TRAM and TRIF, respectively (Barton and Kagan, 2009). When compared to the TIRAP-MyD88 pathway, the TRAM-TRIF pair activates NF-κB and MAP kinases with delayed kinetics, as well as induces interferon regulator factor 3 (IRF3)-dependent IFN expression. Despite the parallels between these two pathways, it remained confusing as to why the TRAM-TRIF signaling pathway was induced with delayed kinetics as compared to the rapidly acting TIRAP-MyD88-dependent pathway.

Detailed cell biological dissection of TLR4 has led to some clarity on this matter. It was first found that the MyD88-dependent activation of NF-κB could be enhanced by disrupting receptor endocytosis (Husebye et al., 2006). This work, along with the finding that TIRAP localization is mediated by interactions with the plasma membrane-localized phosphatidylinositol 4,5-bisphosphate (PIP2), strongly supported the idea that TLR4 induces signal transduction from the plasma membrane (Kagan and Medzhitov, 2006). In contrast, TLR4 endocytosis was necessary to induce the TRIF-TRAM-dependent IFN expression (Kagan et al., 2008). The requirement for TLR4 endocytosis before TRIF signaling can be triggered provided a mechanistic explanation to why MyD88 and TRIF signal with different kinetics, given that endocytosis must occur before TRIF-dependent signaling can be activated.

Husebye et al. have extended the work on TLR4 trafficking and made the interesting discovery that receptor transport was governed by the small GTPase Rab11a. Rab11a is best known for its function in recycling previously internalized endosomal membranes to the cell surface (Bajno et al., 2000; Cox et al., 2000). In addition, Rab11a regulates the fusion of recycling endosomes with the plasma membrane at sites of phagocytosis, a process which is important for the cell to maintain the surface area of the plasma membrane (Cox et al., 2000).

Husebye et al. report that TLR4 localizes to Rab11a positive recycling endosomes in various mammalian cell types, and, most interestingly, they find that TLR4 is recruited from these compartments to bacteria-containing phagosomes. The recruitment to phagosomes only occurs if the enclosed bacterium contains TLR4 ligands (e.g., phagosomes containing gram-positive bacteria or *Yersinia* containing weak TLR4 agonists did not recruit TLR4 from recycling endosomes). This finding was surprising because there was a general belief in the field that TLR4 would behave similarly to TLR2 (Underhill et al., 1999), whose recruitment to phagosomes was thought to occur independently of the cargo.

Husebye et al. found that MyD88, TRAM, and IRF3 were also recruited to bacteria-containing phagosomes. Surprisingly, the microscopy presented suggests that virtually 100% of the total pool of these signaling factors is recruited to phagosomes. Although inconsistent with other signaling pathways, in which only a fraction of the cellular pool participates in a given signaling event (for example, see Ea et al., 2006), these data certainly warrant further inquiry into this matter.

To functionally dissect the site of TLR4 signaling, the authors enlisted the use of a highly specific inhibitor of dynamin GTPases called Dynasore. Dynasore blocks LPS-induced TLR4 endocytosis (Kagan et al., 2008) and the activation of the E3 ubiquitin ligase TRAF3 (Tseng et al., 2010), which is critical for TRIF-mediated signaling. The authors show that Dynasore inhibits TRIF-induced IFN expression (but not MyD88-dependent TNF expression), which confirms that TLR4 signaling through MyD88 and TRIF acts sequentially by a process governed by endocytosis of the receptor complex. In addition, the authors found that

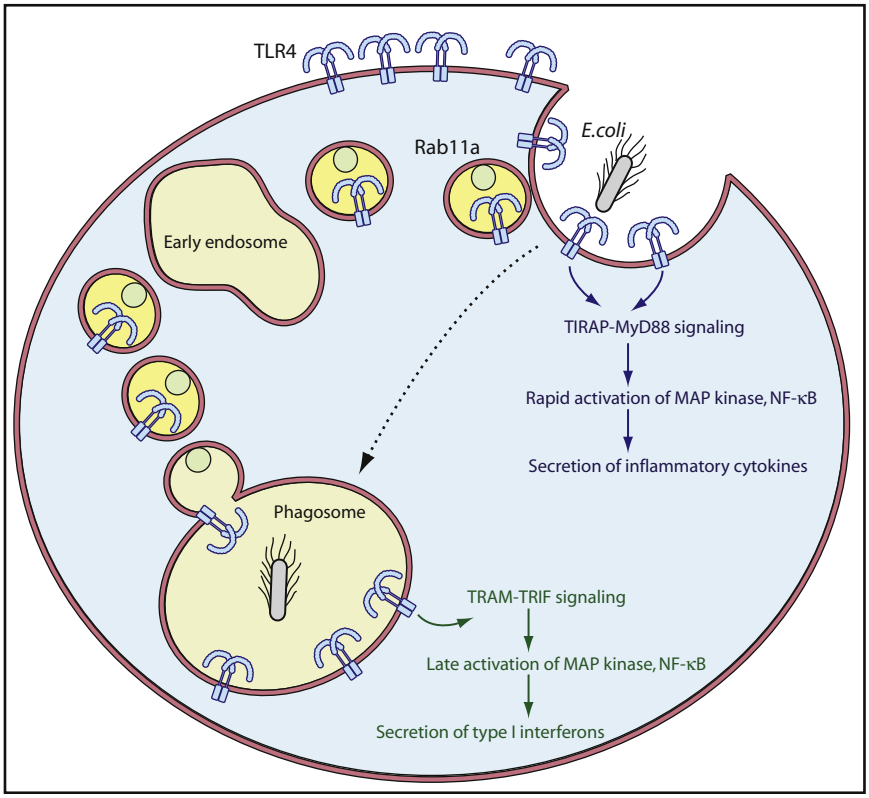

Figure 1. Recycling Endosomes Are Delivered to Bacteria-Containing Phagosomes to Promote TLR Signaling

In macrophages, the process of phagocytosis requires the delivery of recycling endosomes to the site of bacterial contact. This process was thought to function primarily to provide membrane to the forming phagosome, but may also function to deliver TLR signaling proteins to the same location. Recycling endosomes continue to deliver membranes and signaling proteins to the formed phagosome, which promotes the TRIF-dependent expression of type I interferons.

enhancing phagocytosis by using opsonized *E.coli* massively increased TRIF signaling, which further confirms that entry into cells is a rate limiting step in the activation of IFN expression. It is interesting to note, though, that the increase in MyD88 signaling (which occurs by signaling events at the plasma membrane) was only marginally enhanced by opsonizing *E.coli*. This suggests that the total number of TLR4 molecules activated at the cell surface may not change dramatically by opsonization, but that more of the receptors are delivered to endosomes. This finding raises the question of whether a subset of TLR4 molecules that enters the cell to engage TRIF and activate IFN expression exists.

Dynasore treatment 30 min after phagocytosis was used for determining the role of this GTPase in TLR4 trafficking to formed phagosomes. Because dynamins are required for events at the plasma membrane, it was not surprising that Dynasore neither inhibited the transport of TLR4 from recycling endosomes to previously formed phagosomes nor IFN expression. However, in agreement with previous studies (Kagan et al., 2008), Dynamin was necessary for LPS-induced IFN expression. Why would dynamin be necessary for LPS, but not *E.coli*-induced IFN expression? The explanation likely lies in the timing of dynamin inhibition. As stated above, the studies with *E.coli* were performed under conditions in which phagosomes were given 30 min to form and then dynamin function was disrupted. In this instance, the entry of TLR4 to the endosomal system occurs normally, which would therefore permit the initiation of TRIF-mediated signaling. In contrast, the studies with soluble LPS were performed with cells pretreated with Dynasore, and thus, the entry of TLR4 into endosomes is blocked. These results suggest that "decision" to induce IFN expression occurs rapidly upon endocytosis or phagocytosis and that the only way to disrupt this process is to block dynamin function during the entry event.

Turning back to Rab11a, the authors demonstrate that inactivating this GTPase by using RNAi interfered with TRIF-mediated IRF3 activation, but not MyD88-dependents signaling events. This data, along with the observation that Rab11a promotes the continuous delivery of TLR4 to previously formed phagosomes, led the authors to suggest a variant of the sequential model of TLR4 signaling (Barton and Kagan, 2009; Kagan et al., 2008). Both models agree that TLR4 induces TIRAP-MyD88 signaling from the plasma membrane, but this new data suggest that the TRIF-activating pool of TLR4 is actually a different population of TLR4 than the MyD88-activating pool. Husebye et al. propose that the MyD88-activating pool of TLR4 would be found at the plasma membrane, whereas the TRIF-activating pool would originate from recycling endosomes (presumably never "seeing" LPS at the plasma membrane) (Figure 1).

One of the confounding problems with reconciling these two models is the fact that recycling endosomes do not simply deliver TLR4 to phagosomes, as described in this paper. Recycling endosomes play a well established and critical role in the process of phagocytosis by providing endomembranes to the forming phagosome (Bajno et al., 2000; Cox et al., 2000). The general function of this process is to ensure that the surface area of the plasma membrane does not shrink as large particles are internalized. Notably, the same regulator of TLR4 delivery to phagosomes (Rab11a) is necessary for delivery of recycling endosomes to forming phagosomes (Cox et al., 2000). As such, when Rab11a is inhibited, phagocytosis should be diminished, and consequently, endocytosis of TLR4 will also be diminished. Thus, it is possible that the observed defects in TRIF-mediated signaling in cells lacking Rab11a are not solely due to an inability to deliver TLR4 from recycling endosomes to previously formed phagosomes. Rather, Rab11a may either regulate the delivery of TLR4 from recycling endosomes to the forming phagosome or indirectly regulate the internalization of TLR4 from the plasma membrane by regulating the cointernalization of the

bacteria with engaged TLR4 or both. The report by the authors that their cells lacking Rab11a have no defects in phagocytosis supports their model, but it is also counter to much previous literature on the role of recycling endosomes in controlling the very formation of phagosomes (Bajno et al., 2000; Cox et al., 2000). Future studies will need to be performed to definitively implicate this novel route of transport. Despite this potential caveat, it is clear that Rab11a regulates TLR4 transport in several cell types and thus plays an important role in controlling LPS-induced signal transduction.

As with any provocative study, several questions arise. What is the signal that originates from phagosomes that induce the recruitment of recycling endosomes to this bacterial containing compartment? Do all cells that undergo TLR signaling deliver TLR4 to phagosomes by the same process? Is there any function for the delivery of recycling endosomes to phagosomes in terms of the control of antigen presentation?

In summary, the study by Husebye et al. adds to the idea that pattern recognition receptors have been integrated in a well-oiled cell biological machine, which ensures rapid ignition of innate immune signaling, and (we presume), rapid inactivation of signaling when needed. Future work will need to be done to build upon this rapidly burgeoning field of innate immune cell biology.

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A Flt3L Encounter: mTOR Signaling in Dendritic Cells

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The signaling pathway of the cytokine Flt3L in dendritic cells (DCs) is poorly defined. In this issue of *Immunity*, Sathaliyawala et al. (2010) report that the kinase mTOR functions as a mediator of Flt3L signaling in the development and homeostasis of DCs, particularly of the $CD8^+$ and $CD103^+$ DCs.

The fms-like thyrosine kinase 3 receptor (Flt3) and its ligand (Flt3L) are key regulators of dendritic cell (DC) commitment and development. Flt3 is expressed on the surface of early mutlipotent heamatopoietic progenitors, committed DC precursors, and differentiated DC in mouse lymphoid tissues. Flt3 expression and its signaling has been shown to be essential for steady-state DC development and homeostasis as loss of Flt3 or its ligand resulted in a substantial reduction in DC numbers in mouse lymphoid tissues (Schmid et al., 2010). The ligand for Flt3 can promote DC differentiation from both mouse and human hematopoietic progenitors. Administration of Flt3L induces marked expansion in numbers of all DC subsets including the plasmacytoid DC (pDC) and both $CD8^+$ and $CD8^-$ conventional DC (cDC) in mouse spleen, albeit with a strong bias toward the expansion of the $CD8^+$ cDC subset (Schmid et al., 2010). Flt3L-supplemented bone marrow (BM) cultures support the generation of pDCs and the two cDC subsets phenotypically and functionally equivalent to those identified in mouse spleen (Naik et al., 2005). Despite the importance of Flt3 and its ligand in DC development, the signaling pathway downstream of Flt3 in DC differentiation and homeostasis remains poorly characterized.

The mammalian target of rapamycin (mTOR) is a serine-threonine kinase that acts as a central regulator for protein synthesis and cell growth. mTOR activation is induced by PI3K-Akt signaling pathway upon receptor binding to cytokine or growth factors, which leads to phosphorylation of ribosomal protein S6 that regulates ribosomal biogenesis, protein translation, and cell growth (Engelman et al., 2006). The PI3K-mTOR