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CD14 Controls the LPS-Induced Endocytosis of Toll-like Receptor 4

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SUMMARY

The transport of Toll-like Receptors (TLRs) to various organelles has emerged as an essential means by which innate immunity is regulated. While most of our knowledge is restricted to regulators that promote the transport of newly synthesized receptors, the regulators that control TLR transport after microbial detection remain unknown. Here, we report that the plasma membrane localized Pattern Recognition Receptor (PRR) CD14 is required for the microbe-induced endocytosis of TLR4. In dendritic cells, this CD14-dependent endocytosis pathway is upregulated upon exposure to inflammatory mediators. We identify the tyrosine kinase Syk and its downstream effector PLCγ2 as important regulators of TLR4 endocytosis and signaling. These data establish that upon microbial detection, an upstream PRR (CD14) controls the trafficking and signaling functions of a downstream PRR (TLR4). This innate immune trafficking cascade illustrates how pathogen detection systems operate to induce both membrane transport and signal transduction.

INTRODUCTION

The endocytosis of immunity-related receptors has emerged as a critical control step in the signal transduction process. The first receptors activated during any host-pathogen interaction are members of the Toll-like Receptor (TLR) family, which are responsible for detecting microbial products and inducing innate and adaptive immunity (Akira and Takeda, 2004). Different TLR family members are found in different subcellular compartments, ranging from the plasma membrane to early, late, and recycling endosomes (Barton and Kagan, 2009). While it was believed originally that the endocytosis of plasma membrane-localized TLRs downregulates their signaling functions after a microbial encounter (Husebye et al., 2006; Latz et al., 2003), new evidence indicates that receptor delivery to endosomes also activates specific signal transduction pathways (Kagan et al., 2008; Tanimura et al., 2008).

Virtually all of our knowledge of TLR transport is limited to regulators that promote the folding or transport of newly synthesized receptors (e.g., GP96, PRAT4a, and UNC93B1) (Kim et al., 2008; Takahashi et al., 2007; Yang et al., 2007). In the absence of each of these regulators, specific sets of TLRs cannot exit the endoplasmic reticulum after translation and, consequently, TLR ligands present in the extracellular and endosomal spaces are not detected. Unlike the emerging knowledge on the trafficking of newly synthesized receptors, almost nothing is known about the regulators that control TLR endocytosis or transport after microbial detection. Filling this gap in our knowledge is of fundamental importance, as microbe-induced receptor transport is a critical control step in TLR-mediated signal transduction.

The first-described example of microbe-induced TLR transport came from studies of the LPS receptor TLR4, which induces distinct signaling pathways from two different organelles (Kagan et al., 2008; Tanimura et al., 2008). The first signaling pathway is activated from the plasma membrane after TLR4 encounters LPS (Latz et al., 2003). This pathway is mediated by a pair of sorting and signaling adaptor proteins called TIRAP and MyD88, respectively (Kagan and Medzhitov, 2006). These adaptors induce proinflammatory cytokine expression by linking TLR4 to downstream enzymes that activate NF-κB and AP-1 (Akira and Takeda, 2004). TLR4 is then internalized into the endosomal network where the second signaling pathway is triggered through the adaptors TRAM and TRIF (Kagan et al., 2008; Tanimura et al., 2008). These adaptors mediate the activation of the transcription factor Interferon Regulatory Factor-3 (IRF3), which regulates Type I Interferon (IFN) expression (Akira and Takeda, 2004). Thus, in the case of TLR4, the LPS-induced endocytosis of the receptor is essential for its signaling functions. While the general endocytic machinery is undoubtedly involved in internalization of plasma membrane-localized TLRs, there are no known membrane proteins that regulate TLR endocytosis specifically upon microbial recognition.

In considering this problem, we reasoned that since TRIF-mediated IFN expression requires TLR4 endocytosis, cell surface proteins that control endosomal signaling may do so by regulating TLR4 entry into the cell. One such regulator is CD14. CD14 is a GPI-linked protein that is found on the surface of many (but not all) TLR4 expressing cells (Wright et al., 1990). CD14 was the first identified Pattern Recognition Receptor (PRR) that binds

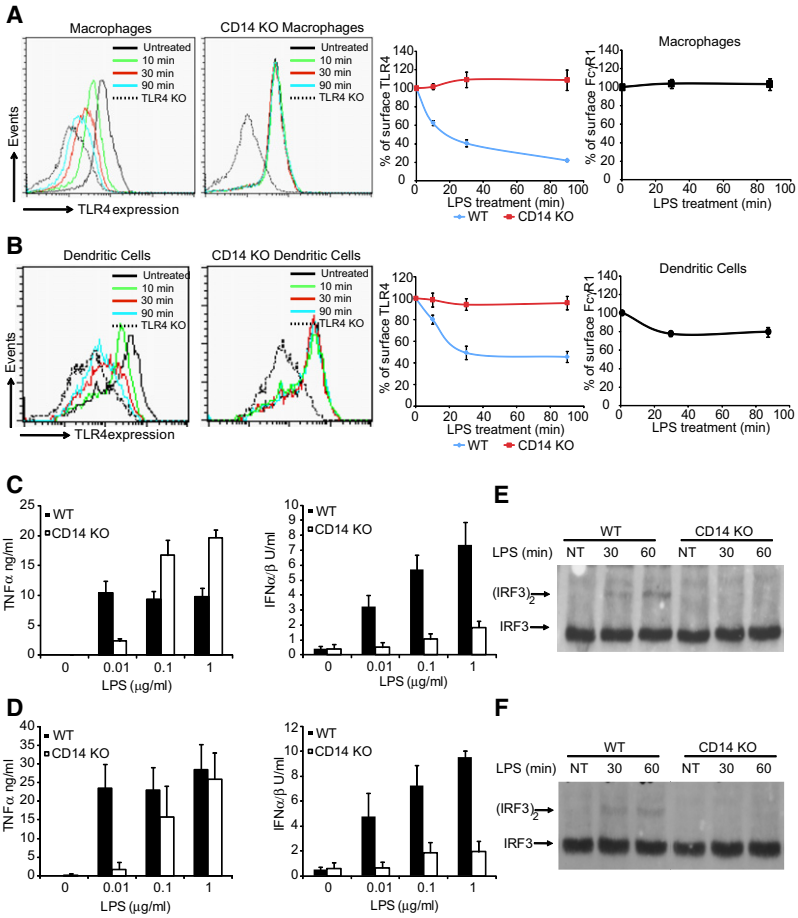

Figure 1. CD14 Is Required for LPS-Induced TLR4 Endocytosis

(A and B) WT or CD14-deficient (KO) mouse BMDM (A) or DCs (B) were untreated or treated with LPS (1 μg/ml) for the times indicated. Flow cytometry was then used to examine receptor endocytosis by determining the surface levels of the endogenous proteins indicated. The third and fourth panels in (A) and (B) represent the mean fluorescence intensity (MFI) of specific receptor staining at each time point.

(C and D) BMDM (C) or DCs (D) were treated with the concentrations of LPS indicated for 18 hr and the amounts of secreted cytokines were determined.

(E and F) BMDM (E) or DCs (F) were treated with LPS (1 μg/ml) for the times indicated and the presence of active (dimerized) IRF3 in cell extracts was determined by native PAGE.

See also Figure S1.

directly to LPS (Wright et al., 1990), and is known to chaperone LPS molecules to the TLR4-MD-2 signaling complex (da Silva Correia et al., 2001; Gioannini et al., 2004; Moore et al., 2000). Notably, while CD14 is marginally important for MyD88-dependent TNFα expression, it is essential for TRIF-mediated IFN expression (Jiang et al., 2005). Thus, we hypothesized that CD14 specifically regulates TRIF-mediated IFN expression because it regulates TLR4 endocytosis. Here, we show that this prediction is correct and that CD14 controls a microbe-specific endocytosis pathway that functions to internalize both TLR4 and promote TRIF-mediated signal transduction. Remarkably, TLR4 is internalized independently of its signaling functions but dependent on regulators of Fc Receptor and Dectin-1-mediated endocytosis. Collectively, these data suggest the existence of an inflammatory endocytosis pathway that can be activated by multiple upstream receptors to promote antimicrobial immunity.

RESULTS

CD14 Is Required for LPS-Induced TLR4 Endocytosis

To identify proteins that specifically regulate the LPS-induced endocytosis of TLR4, we used a highly sensitive assay to detect endogenous TLR4 by flow cytometry. Using the loss of cell surface expression as a readout for TLR4 endocytosis, we showed previously that LPS induces TLR4 internalization in mouse bone marrow derived macrophages (BMDM) (Kagan et al., 2008). The loss of TLR4 surface staining was a bona fide endocytic event as it was inhibited by dynasore (Kagan et al., 2008), an inhibitor of dynamin GTPases that control most endocytic processes in mammalian cells. Using this assay, we determined if CD14 regulates TLR4 transport as a means of controlling IFN production.

LPS-induced TLR4 endocytosis was examined in BMDM and immature dendritic cells (DCs) from wild-type (WT) and CD14-deficient mice. LPS induced the rapid endocytosis of TLR4 in WT BMDM and DCs, but not in cells from CD14-deficient mice (Figures 1A and 1B). The endocytosis of TLR4 was a specific response, as levels of a different endocytic receptor (FcγR1) were largely unaffected by LPS treatment (Figures 1A and 1B). To complement this FACS-based assay, TRIF-mediated signaling events from endosomes were examined (Kagan et al., 2008). CD14-deficient BMDM and DCs were defective for TRIF-mediated IFN production but were not defective for MyD88-mediated TNFα production (Figures 1C and 1D). Of note, at low LPS concentrations, CD14 was needed for TNFα production, which likely reflects its role in delivering LPS to TLR4 (da Silva Correia et al., 2001; Gioannini et al., 2004).

To more specifically address the role of CD14 in signaling from the plasma membrane or endosomes, we examined protein complexes that define each pathway. TLR4 signaling from the plasma membrane induces the formation of the Myddosome, a complex containing MyD88 and IRAK4 that activates NF-κB (Motshwene et al., 2009). TLR4 signaling from endosomes induces the dimerization of the transcription factor IRF3 (Kagan et al., 2008). We monitored the formation of the Myddosome by coimmunoprecipitations of MyD88 and IRAK4 in WT and CD14-deficient immature DCs. LPS treated WT and CD14-deficient DCs induced Myddosome formation (Figure S1A available online), suggesting that TLR4 signaling from the plasma membrane does not absolutely require CD14. In addition,