REVIEW

Cell type-specific role of raftlin in the regulation of endosomal TLR signaling

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Clathrin-dependent endocytic pathway is crucial for cell entry of extracellular pathogens and their components. The innate immune system utilizes this pathway to detect pathogen-associated molecular patterns (PAMPs) within endosomes. In the clathrin-mediated endocytosis, cargo selection depends on AP-2 adaptor and its accessory proteins, but the molecular mechanism of PAMP selection to be internalized is largely unknown. The endosomal Toll-like receptors (TLRs) 3, 7, 8, and 9 recognize viral or bacterial nucleic acids, as well as host-derived nucleic acids incorporated into the endosomal compartments, where type I interferon (IFN)-producing signals are arisen. In addition, lipopolysaccharide (LPS) receptor TLR4 transmits signals to produce IFN-β from endosomes after the clathrin-dependent endocytosis of LPS-TLR4. The cytosolic protein Raftlin that possesses membrane-anchoring motif mediates cellular uptake of TLR3/4 ligands in human cells through association with clathrin-AP-2 complex. Raftlin was first identified as a major raft protein in B cells that modulates B-cell or T-cell receptor-mediated signaling. In this review, we will focus on the Raftlin function in innate immunity and discuss the molecular mechanisms of cellular uptake and delivery of TLR ligands.

Keywords: clathrin-mediated endocytosis; dendritic cells; double-stranded RNA; innate immunity; lipopolysaccharide; Toll-like receptor

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Introduction

Innate immune system is essential for induction of adaptive immunity. A variety of soluble and membrane-bound pattern-recognition receptors (PRRs) have been provided to sense microbial infection, which elicit anti-microbial immune responses [1-3]. Toll-like receptors (TLRs), a type I transmembrane protein family, recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan and viral nucleic acids, and transmit signals linking innate and adaptive immunity [3]. TLRs also detect self-molecules derived from damaged or dying cells, which promotes inflammatory response and development of autoimmune disorders [4, 5]. Among human TLRs 1-10, the nucleic acid-sensing TLRs 3, 7, 8 and 9 localize to the endosomal compartments, where they encounter the incorporated viral- or host-derived nucleic acids and induce type I interferon (IFN) and proinflammatory

cytokine production [6-9]. Although cell surface receptors involving in the uptake and delivery of extracellular nucleic acids (exNAs) have not been fully identified, accumulating evidence demonstrate that clathrin-dependent endocytic pathway plays an important role in cellular uptake of exNAs including polyinocinic:polycytidylic acid (poly(I:C)) and CpG oligodeoxynucleotides (ODNs) [10, 11]. In addition, cell-surface TLR4 is internalized in the clathrin- and dynamin-dependent manner after the binding to LPS [12] to induce IFN-β production through Toll-IL-1 receptor domain-containing adaptor molecule (TICAM)-2 (also called TRAM) and TICAM-1 (also called TRIF) [13-15]. Thus, innate immune system utilizes endocytic pathway to detect extracellular microbial components, but precise molecular mechanisms of PAMP loading remain unknown. Recently, we have reported that the cytosolic protein Raftlin that possesses membrane-anchoring motif mediates cellular uptake of poly(I:C), CpG ODNs and LPS in human dendritic cells (DCs), macrophages and epithelial cells through association with clathrin-AP-2 complex [16,17]. In this review, we will summarize the molecular mechanisms of exNA internalization and focus on the role of Raftlin in the regulation of endosomal TLR signaling.

Raftlin function in adaptive immunity

Raftlin was first identified as a major raft protein in human B cell line, Raji, by proteome analysis [18]. Like Src family protein tyrosine kinase such as Lck and Lyn that localize in lipid rafts, Raftlin possesses the N-terminal fatty acylation sites. The Gly2 N-myristoylation site and the Cys3 S-palmitoylation site are important for localization of Raftlin in lipid rafts. Raftlin colocalized with B-cell receptor (BCR) before and after BCR stimulation in human B cells, but their direct interaction was undetected by immunoprecipitation assay. Using Raftlin-deficient DT40 chicken B cells, Raftlin was shown to act as a positive regulator of BCR signal transduction through the maintenance of lipid raft and recruitment of raft proteins [18].

The physiological function of Raftlin was subsequently analyzed by generating Raftlin-deficient mice, which demonstrated the normal B cell signals. Mouse B cells express a Raftlin-homologue, Raftlin-2 that might compensate Raftlin function in Raftlin-deficient B cells [19]. On the other hand, in Raftlin-deficient T cells that do not express Raftlin-2, T-cell receptor (TCR)-mediated cytokine production, especially IL-17 production was reduced. In addition, T-cell-dependent antibody production was impaired in Raftlin-deficient mice. Raftlin uniformly localized on the plasma membrane of T cells, and is accumulated in the immunological synapse upon TCR stimulation [19]. Based on the biochemical analyses, Raftlin appears to influence the accumulation of tyrosine kinase Lck into lipid rafts, resulting in the modulation of TCR signaling including tyrosine phosphorylation of the signaling molecules and intracellular Ca^{2+} mobilization [19]. Thus, Raftlin localizes to lipid rafts in B- and T-cells and is involved in BCR- and TCR-mediated signaling in adaptive immunity.

Raftlin function in innate immunity

Sensing exNAs by endosomal TLRs

TLR3 recognizes viral double-stranded (ds) RNA [20,21] and incomplete stem structures formed in viral single-stranded (ss) RNA [22], TLR7 and TLR8 sense viral ssRNA and synthetic imidazoquinoline compounds [23-26], and TLR9 recognizes unmethylated CpG-containing DNA [27, 28]. TLR3, 8 reside in the early endosome of myeloid dendritic cells (DCs), whereas TLR7, 9 present in the endolysosome of plasmacytoid DCs and B cells. All of these TLRs require an endoplasmic reticulum (ER)-resident multi-transmembrane protein Unc93B1 and LRRC59 to exit ER and traffic to the distinct endosomal compartments [29-33], where they undergo proteolytic processing to generate functional cleaved/associated form of receptors [9, 34-38]. Internalization and delivery of ligands to the distinct organelles is prerequisite for endosomal TLR-initiated innate immune responses.

Notably, endosomal TLRs are involved in the recognition of self nucleic acids released from damaged or dying cells [4, 5]. TLR7 and TLR9 are closely associated with autoimmune diseases through induction of type I IFNs and antibody production against self-RNA/DNA [4, 5]. Recent study using human TLR8-transgenic mice clearly demonstrated the connection between TLR8 and autoimmune inflammation [39]. In addition, TLR3 recognizes self noncoding RNA derived from stressed or necrotic cells, and induces IFN-β and proinflammatory cytokines [40]. However, molecular structure of endogenous nucleic acids recognized by these TLRs and their recognition mechanisms remain to be elucidated.

Raftlin is essential for clathrin-dependent endocytosis of TLR3 ligands

Human TLR3 localizes both on the cell surface and endosomal membrane in fibroblasts, macrophages and epithelial cells [21], while myeloid DCs express TLR3 intracellularly [6]. Although cell-surface TLR3 appears to recognize poly(I:C) since anti-human TLR3 mAb inhibits poly(I:C)-induced IFN-β production from fibroblasts [21], TLR3-mediated signaling arises from the endosomes in both cell types. Upon ligand recognition in the endosomes, TLR3 transmits signals via an adaptor protein TICAM-1 that

Table 1. Molecules involving in the internalization of poly(I:C)/ODN

Proteins	NAs	Cell	Localization	Function	Ref.
CD14	poly(I:C) small fragment (~200 bp)	bone marrow-derived macrophages, CHO/CD14, CHO/CD14/TLR3	cell surface	direct binding, mediate cellular uptake	44
SR-A	poly(I:C)	human bronchial epithelial cell BEAS-2B, primary human bronchial epithelia	cell surface	binding, mediate cellular uptake	45
SR-As	viral dsRNA (200, 1000bp)	MEFs, human fibroblasts	cell surface	binding	46
CD11b/CD18	poly(I:C) (300-750 bp)	peritoneal macrophages, RAW264.7	cell surface	direct binding, endocytosis	47
Raftlin	poly(I:C), structured RNA derived from poliovirus, B/C-type CpG/GpC ODN	human monocyte-derived DCs, human epithelial cell lines (HeLa, HEK293)	cytoplasm	mediate endocytosis by interacting with clathrin-AP-2 complex	16,22
RAGE	CpG ODN (A/C-type>B-type)	HEK293T/RAGE	cell surface	direct binding, mediate cellular uptake	48
DEC205	B/C-type CpG ODN (for mouse DEC205) B-type CpG ODN (for human DEC205)	CHO-K1/DEC205, mouse $CD8^+$ DC, mouse B cell	cell surface	direct binding, mediate cellular uptake	49
KIR3DL2	C-type CpG ODN	human NK cell	cell surface	direct binding, mediate cellular uptake	60
ARF6	B-type CpG ODN	RAW264.7	cell surface	regulate cellular uptake	61

activates the transcription factors IRF3, NF-κB, and AP-1, leading to induction of type I IFN and inflammatory cytokine production as well as DC maturation [11, 41, 42]. Poly(I:C), a synthetic viral dsRNA analog, is a potent TLR3 agonist that is efficiently internalized into cells and activates endosomal TLR3 [11]. Curiously, in vitro transcribed dsRNAs (100 bp~1000bp) that are functional when directly delivered to endosomes are unable to activate TLR3 when extracellulary added to human myeloid DCs [43], suggesting the failure of dsRNA uptake in contrast to poly(I:C).

Using proteome analyses of poly(I:C)-binding proteins, we identified the cytoplasmic protein Raftlin as a component of clathrin-dependent endocytic machinery of poly(I:C) [16]. When Raftlin was knocked down in human myeloid DCs and epithelial cells, extracellular poly(I:C) failed to enter cells, remaining on the cell surface as speckles. Clustering of the uptake receptor occurs without internalization in Raftlin knockdown cells. Accordingly, TLR3-mediated IFN-β production was never induced. In myeloid DCs and epithelial cells, Raftlin predominantly resides in the cytosol in contrast to the lipid raft localization in B and T cells. Upon poly(I:C) stimulation, Raftlin translocates from the cytosol to the plasma membrane, where it co-localizes with poly(I:C) and then trafficked to the TLR3-positive endosomes along with poly(I:C). Immunoprecipitation analysis demonstrated that Raftlin physically associates with clathrin heavy chain after poly(I:C) stimulation and then dissociated [16]. Hence, Raftlin is recruited to the plasma membrane when poly(I:C) bound to the uptake receptor, where associates with clathrin to modulate cargo sorting and delivery. In steady state, Raftlin interacts with clathrin adaptor AP-2 β-subunit [17], suggesting that Raftlin regulates clathrin-dependent endocytosis of poly(I:C) as an accessory molecule of AP-2 through recognition of poly(I:C)-uptake receptor. Notably, internalization of transferin is independent of Raftlin irrespective of its dependency of clathrin [16].

Besides dsRNA, TLR3 recognizes incomplete stem structures formed in viral ssRNA [22] and self stem-loop RNA derived from damaged or dying cells [40]. Extracellular virus-derived 'structured' RNAs that contain incomplete stems and show nuclease-resistant feature are internalized Raftlin-dependently similar to poly(I:C) uptake [22]. Structured RNA-induced TLR3-mediated IFN-β and cytokine production did not occur when Raftlin was knocked down. Hence, Raftlin cooperates with the uptake receptor to mediate cell entry of ds/structured RNA, which is critical for activation of TLR3 (Figure 1).

Importantly, Raftlin homolog, Raftlin2, is expressed in a species- and cell type-specific manner [16, 17, 19]. Human myeloid DCs and epithelial cells express only Raftlin, while mouse conventional DCs express Raftlin2 in addition to Raftlin. Poly(I:C) is internalized in Raftlin-deficient mouse DCs, but not in $Raftlin^{-/-}$/ $Raftlin2^{-/-}$ DCs, suggesting that

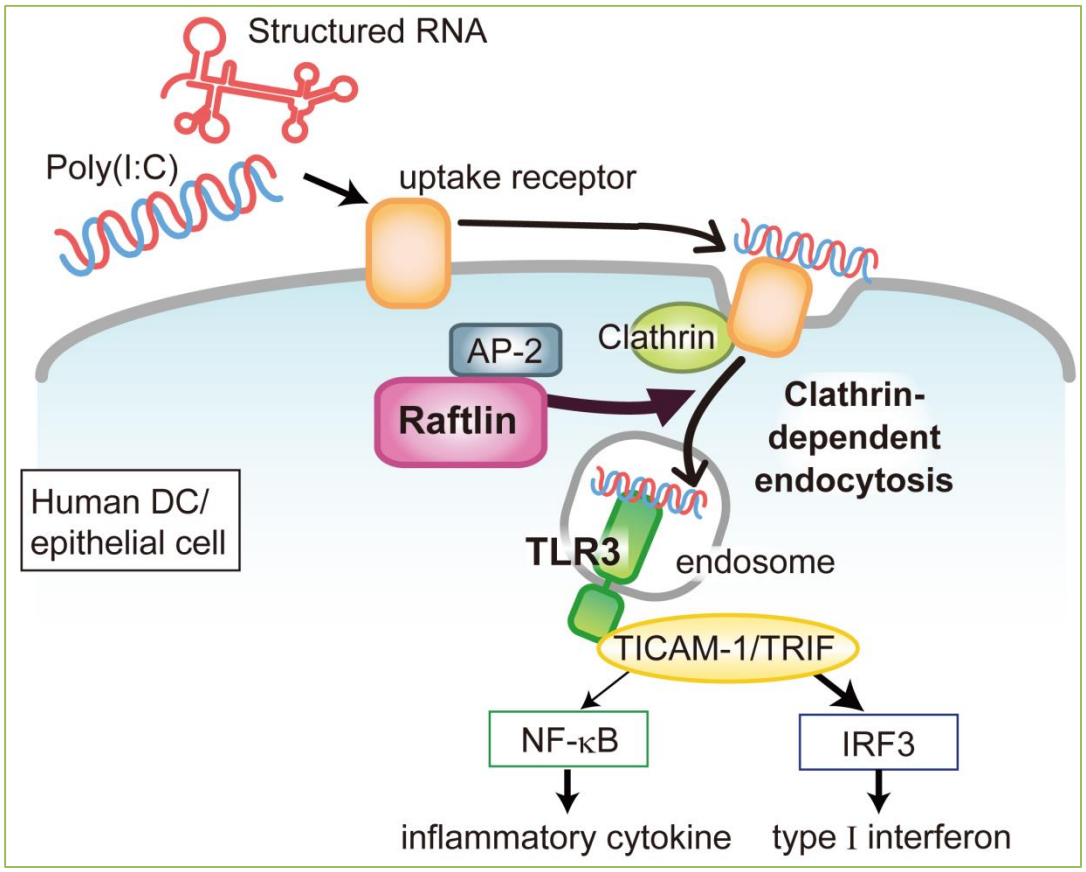

Figure 1. TLR3-mediated signaling. Extracellular poly(I:C) and structured RNA are recognized by cell surface uptake receptor and delivered to the TLR3-resident endosomes through Rarftlin- and clathrin-dependent endocytosis in human myeloid DCs and epithelial cells. Upon ligand recognition, TLR3 activates IRF3 and NF-κB via TICAM-1, leading to production of type I IFN and inflammatory cytokines, respectively.

Raftlin-2 functionally compensates for Raftlin in mouse DCs [16,17].

Raftlin mediates cell entry of phosphorothioated ODNs

In human myeloid DCs and epithelial cells, B/C-type, but not A-type, CpG (TLR9 agonist) or GpC (TLR9 non-agonist) ODNs inhibited cellular binding and uptake of poly(I:C), and vice versa [10]. Raftlin-knocked down HEK293 cells failed to take up B/C-type CpG ODN and abolished TLR9-driven NF-κB activation [16]. It appears that B/C-type ODNs share the uptake receptor with poly(I:C) and their internalization is regulated by Raftlin. B/C-type ODNs are fully phosphorothioated, while A-type ODNs are phosphodiester linkage. Hence, the structural feature of nucleic acids recognized by common uptake receptor for poly(I:C)/ODN is not simple, but is a nuclease-resistant structure.

Uptake receptor for exNAs

The uptake receptors for poly(I:C) or CpG ODNs have been reported by several groups (Table 1). The membrane-bound CD14 enhanced poly(I:C)-mediated TLR3 activation in mouse macrophages by facilitating uptake of poly(I:C) into an intracellular compartment [44]. The scavenger receptor class-A (SR-A) was also identified as a cell surface receptor for poly(I:C) in human bronchial epithelial cells and mouse cells [45, 46]. Limmon *et al*. showed that SR-A antagonists or anti-SR-A antibody inhibited poly(I:C)-induced cytokine production in human bronchial epithelial cell line [46]. Moreover, CD11b/CD18 (CR3) was found to be a surface receptor for poly(I:C) to mediate cellular responses in mouse macrophages [47]. CR3 bound ~200bp-length short poly(I:C) and facilitated the internalization of short poly(I:C). However, knockout of these molecules resulted in only partial abrogation of poly(I:C)-induced TLR3 activation [44-47], suggesting a redundant or cell type-specific function of these receptors in poly(I:C) internalization. Indeed, human myeloid DCs do not express CD14 on the cell surface and an inhibitor for SR-A does not affect poly(I:C) uptake in human myeloid DCs and epithelial cells (Matsumoto *et al*, unpublished data), indicating the presence of another uptake receptor in those cells.

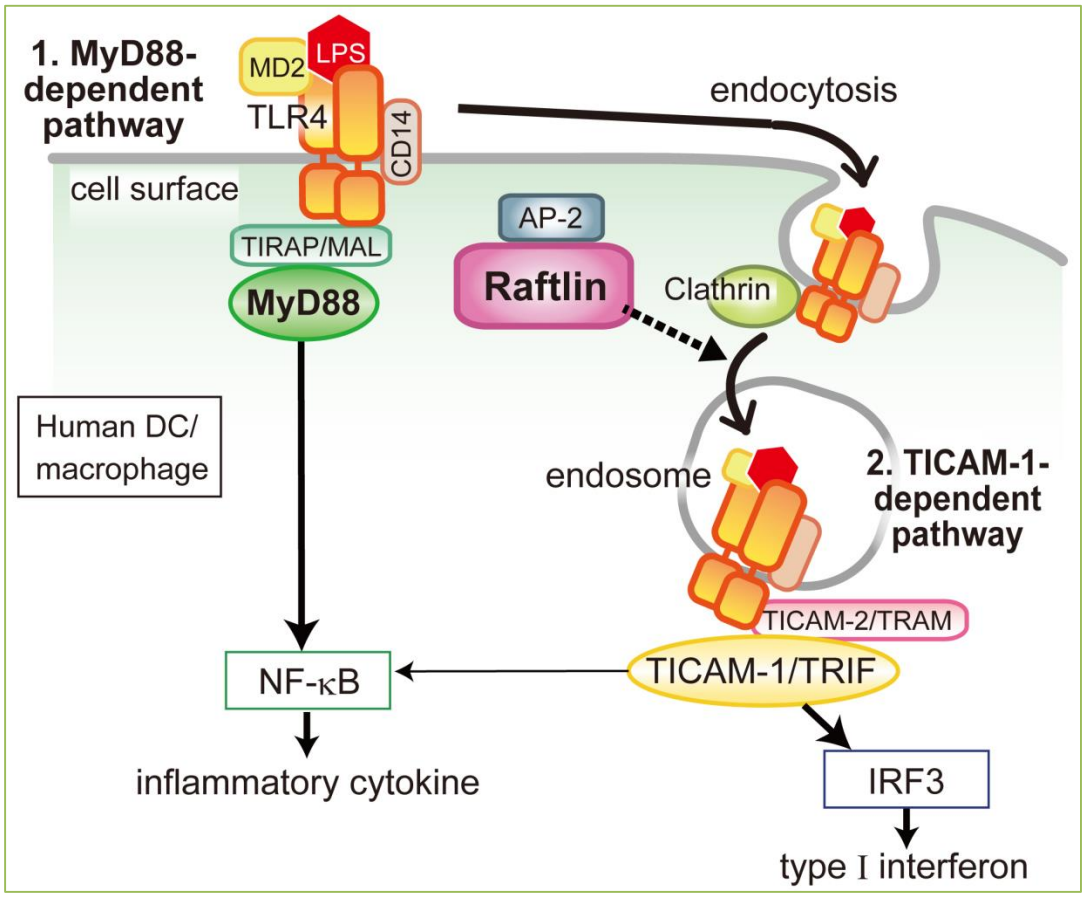

Figure 2. TLR4-mediated signaling. Extracellular LPS is recognized by cell surface TLR4-MD-2 complex cooperatively with CD14, which recruits TIRAP/Mal and MyD88 at the plasma membrane and activates NF-κB to induce inflammatory cytokine production (MyD88-dependent pathway). LPS-TLR4 is subsequently internalized into the endosomes via Raftlin- and clathrin-dependent endocytosis in human macrophages and DCs, where TICAM-2-TICAM-1-mediated IRF3 activation occurs, leading to production of IFN-β (TICAM-1-dependent pathway).

Concerning DNA receptors, the receptor for advanced glycation end-products (RAGE) bound directly to both DNA and RNA oligonucleotide and promoted ODN uptake into endosomes [48]. B-type CpG ODN bound to RAGE more efficiently than A-type CpG ODN in solution, but cell surface RAGE expressed on HEK293T cells was associated with A-type CpG ODN more prevalently than with B-type CpG ODN and enhanced CpG-A ODN uptake [48]. Additionally, DEC-205 has been recently identified as an uptake receptor for B/C-type, but not A-type CpG ODNs in mouse cells [49]. Lahoud *et al*. demonstrated that human DEC-205 preferentially bound B-type, but not C-type, CpG ODN in in vitro binding assay [49]. In our experiments, however, human DEC-205 does not involved in poly(I:C)-induced TLR3 activation (Tatematsu and Matsumoto, unpublished data). Hence, these molecules appear to be independent on the uptake of poly(I:C) and B/C-type ODN in human DCs/epithelial cells. It appears that several uptake machinery participate in the cell entry of extracellular RNAs/DNAs in a cell type- and/or species-specific manner.

Involvement of Raftlin in the TLR4-TICAM-1 signaling

Besides nucleic acid-sensing TLRs, TLR4 transmits signals to produce IFN-β from endosomes via TICAM-2-TICAM-1 signaling pathway in response to extracellular LPS [13-15]. Upon LPS recognition, TLR4-MD-2 complex undergoes dimerization, which activates TIRAP-MyD88 signaling pathway at the plasma membrane resulting in the NF-κB-driven proinflammatory cytokine production [50]. LPS-bound TLR4 is then internalized into the endosomes in a clathrin- and dynamin-dependent manner [12], where activates TICAM-2-TICAM-1 signaling pathway resulting in the activation of IRF3 leading to production of IFN-β. The subcellular shuttling of TLR4 and switching of the signaling complex are regulated by several mechanisms. Kagan and colleagues demonstrated that the membrane bound CD14 controls LPS-induced TLR4 endocytosis in mouse macrophages and DCs [51]. The tyrosine kinase Syk

and PLCγ2 regulate this process dependent on CD14 but independent of TLR4 [51]. Furthermore, the p110δ isoform of PI(3)K acts as a regulator of TLR4 endocytosis in mouse DCs through modulating PtdIns(4,5)P_2 metabolism [52].

We have studied about the role of Raftlin in LPS-induced TLR4-mediated signaling and found that LPS-induced IFN-β promoter activation, but not NF-κB activation, was impaired in Raftlin knockdown HEK293 cells overexpressing human TLR4/MD-2 or TLR4/MD-2/CD14 [17]. The LPS-induced IFN-β production was also decreased in Raftlin knockdown human monocyte-derived macrophages and DCs. Raftlin was resided in the cytoplasm in human macrophages and moved to the plasma membrane upon LPS stimulation, where it colocalized with TLR4 followed by internalization along with TLR4 [17]. Raftlin physically associates with TLR4 and clathrin in response to LPS stimulation, suggesting the involvement in the Raftlin- and clathrin-dependent TLR4 endocytosis (Figure 2). Notably, Raftlin/Raftlin-2 participates in LPS-induced IFN-β production in mouse splenic DCs, but not in bone marrow-derived macrophages (BMDMs) [17], indicating that Raftlin controls LPS-induced TLR4 internalization and TICAM-1 signaling in a cell type-specific manner. Because surface CD14 level of BMDMs is higher than that of splenic DCs and human monocyte-derived DCs and macrophages express CD14 negative and low, respectively, surface CD14 level might affect the dependency of Raftlin in TLR4 endocytosis upon LPS stimulation. Taken together, Raftlin regulates TLR3- and TLR4-mediated endosomal TICAM-1 signaling through the induction of ligand uptake.

Perspectives

The synthetic ligands for endosomal TLRs have been applied to next generating vaccine adjuvants for infectious diseases and tumors [53, 54]. Especially, synthetic TLR3-specific ligand is a pivotal vaccine adjuvant for antitumor immunotherapy [55]. Since adjuvants are mainly targeted to antigen-presenting DCs to activate adaptive immunity [56], efficient delivery of TLR ligands into the endosomes of DCs is important in the efficacy of vaccine treatments. The elucidation of molecular mechanisms of how synthetic TLR ligands are internalized is important for the development of novel adjuvants. Recent studies on the molecular mechanisms of TLR ligand uptake implicate that there are several cell surface receptors mediating endocytosis of TLR ligands, which work species- and cell type-dependent manner. In addition, endocytic pathway is regulated by several molecules/mechanisms including accessory proteins for clathrin adaptor AP-2, Rab GTPases and the phosphatidylinositide conversion [57-59]. In human myeloid DCs and epithelial cells, Raftlin is required for endocytosis of poly(I:C) and poly(I:C)-induced TLR3-mediated signaling, although poly(I:C) uptake receptor recognized with Raftlin remains to be identified. Mass analysis of Raftlin-associating molecules in human epithelial cells has revealed that several molecules related to clathrin-dependent endocytosis are interacted with Raftlin [17]. Further study on the Raftlin-associating proteins provides new insight into the clathrin-mediated endocytic pathway in innate immunity.

Conflicting interests

The authors have declared that no conflict of interests exist.

Author contributions

M.M. conceived and designed the experiments, analyzed the data and wrote the paper. M.T. conceived and performed the experiments and analyzed the data.

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Abbreviations

AP-2: clathrin-associated adaptor protein-2; DC: dendritic cell; exNAs: extracellular nucleic acids; LPS: lipopolysaccharide; ODN: oligodeoxynucleotides; PAMP: pathogen-associated molecular pattern; poly(I:C): polyinosinic: polycytidylic acid: TICAM: Toll-interleukin-1 receptor domain-containing adaptor molecule.

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