Mucolipin 1 positively regulates TLR7 responses in dendritic cells by facilitating RNA transportation to lysosomes

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Abstract

Toll-like receptor 7 (TLR7) and TLR9 sense microbial single-stranded RNA (ssRNA) and ssDNA in endolysosomes. Nucleic acid (NA)-sensing in endolysosomes is thought to be important for avoiding TLR7/9 responses to self-derived NAs. Aberrant self-derived NA transportation to endolysosomes predisposes to autoimmune diseases. To restrict NA-sensing in endolysosomes, TLR7/9 trafficking is tightly controlled by a multiple transmembrane protein Unc93B1. In contrast to TLR7/9 trafficking, little is known about a mechanism underlying NA transportation. We here show that Mucolipin 1 (Mcoln1), a member of the transient receptor potential (TRP) cation channel gene family, has an important role in ssRNA trafficking into lysosomes. Mcoln1−/− dendritic cells (DCs) showed impaired TLR7 responses to ssRNA. A mucolipin agonist specifically enhanced TLR7 responses to ssRNAs. The channel activity of Mcoln1 is activated by a phospholipid phosphatidylinositol (3,5) bisphosphate (PtdIns(3,5)P2), which is generated by a class III lipid kinase PIKfyve. A PIKfyve inhibitor completely inhibited TLR7 responses to ssRNA in DCs. Confocal analyses showed that ssRNA transportation to lysosomes in DCs was impaired by PIKfyve inhibitor as well as by the lack of Mcoln1. Transportation of TLR9 ligands was also impaired by the PIKfyve inhibitor. These results demonstrate that the PtdIns(3,5)P2–Mcoln1 axis has an important role in ssRNA transportation into lysosomes in DCs.

Keywords: endolysosome, RNA, TLR7, trafficking

Introduction

Toll-like receptors (TLRs) sense a variety of microbial products. Cell surface TLRs, including TLR4/MD-2, TLR1/TLR2 and TLR6/TLR2, recognize microbial membrane lipids, whereas TLR3, TLR7, TLR8 and TLR9 localize to intracellular organelles and recognize microbial nucleic acids (NAs) (1–3). TLR7 and TLR9, innate immune sensors of microbial NAs (4), erroneously respond to self-derived NAs, inducing anti-nuclear auto-antibody production in murine models of systemic lupus erythematosus (SLE) (5–7). The discrimination between self and pathogen by TLR7 or TLR9 is error prone and needs to be strengthened by mechanisms restricting RNA/DNA-sensing in endolysosomes rather than on the cell surface. While self-derived RNA/DNA is rapidly degraded by RNase or DNase, microbial RNA/DNA is resistant to degradation because it is encased in bacterial cell walls or viral particles. Microbial RNA/DNA is therefore able to reach endolysomes to stimulate TLR7 or TLR9 (8). Aberrant transportation of self-derived RNA/DNA to endolysosomes has been shown to exacerbate autoimmunity (7, 9, 10).

To limit NA-sensing by TLR7 or TLR9 in endolysosomes, TLR7/9 transportation is tightly controlled. If TLR9 is forced to be expressed on the cell surface, TLR9 signals in response to extracellular self-derived DNA, leading to systemic lethal inflammation (11). Trafficking of TLR7 and TLR9 is dependent on Unc93B1, a multiple transmembrane protein (12, 13). Unc93B1 is associated with TLR7 or TLR9 and essential for their transportation. In mice harboring a mutation in the Unc93B1 gene, TLR7 and TLR9 fail to traffic out of
the endoplasmic reticulum (ER) and to respond to NAs in endolysosomes. Unc93B1 also has a role in balancing TLR7 and TLR9 responses. A point mutation of aspartic acid at position 34 (D34A) alters the balance of TLR7 and TLR9 toward TLR7 (14, 15), leading to TLR7-dependent, systemic lethal inflammation.

When compared with sensor transportation, much less is known about the molecular mechanism underlying NA transportation. PIKfyve, a phosphatidylinositol 3-phosphate 5-kinase, is known to be required for CpG-B translocation from early to late endosomes (16). The PIKfyve inhibitor YM201636 induces accumulation of CpG-B in early endosomes in a macrophage cell line. PIKfyve synthesizes phosphatidylinositol (3,5) bisphosphate (PtdIns(3,5)P$_2$), suggesting a role of PtdIns(3,5)P$_2$ in DNA trafficking. The target of PtdIns(3,5)P$_2$ in CpG-B trafficking is unknown so far. PtdIns(3,5)P$_2$ is reported to directly bind to mucolipin 1 (Mcoln1) and induces Ca$^{2+}$ release (17). Mcoln1 is a member of the transient receptor potential (TRP) cation channel gene family. Mcoln1 primarily localizes on the lysosome membrane and has a role in lysosomal endocytosis and exocytosis (18). Human mutations of Mcoln1 cause mucolipidosis type IV (MLIV), a lysosomal storage disease characterized by abnormally large lysosomes containing electron-dense inclusions and lipid storage bodies (19, 20). Considering that Mcoln1 is a target of PtdIns(3,5)P$_2$ and has a role in endolysosomal trafficking, Mcoln1 may have a role in NA trafficking in innate immune cells. To address this issue, TLR7 and TLR9 responses in Mcoln1$^{-/-}$ mice were assessed in the present study. Mcoln1$^{-/-}$ cells showed impaired TLR7 responses to single-stranded RNAs (ssRNAs), and activation of mucolipin enhanced RNA localization in lysosomes. These results suggest a role of Mcoln1 in RNA transportation to the lysosomes.

**Methods**

**Mice and cells**

The Mcoln1$^{-/-}$ mice used for this research project were B6.129-Mcoln1tm1Asoy/Mmhh, stock number 034789-MU. They were obtained from the Mutant Mouse Regional Resource Center, an NIH funded strain repository (MU MMRRC; U42OD010918; MMRRC ICSC—U42OD010983), and were donated to the MMRRC by Abigail Soyombo-Shoola, PhD, University of Texas Southwestern Medical Center. C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were maintained in the animal facility of the Institute of Medical Science, the University of Tokyo. All animal experiments were approved by the Institutional Animal Care and Use Committee.

The induction of bone marrow conventional dendritic cells (BM-cDCs) and BM plasmacytoid DCs (BM-pDCs) was described previously (15). Briefly, BMs were flushed from mouse tibiae and femurs. After lysis of RBCs, cells were cultured in RPMI1640 medium (Gibco) supplemented with 10% FCS (Thermo), penicillin-streptomycin-glutamine (Gibco) and 50 μM Beta-2-mercaptoethanol (Nacalai Tesque) at 37°C for 1 week. For BM-cDCs, 1 x 10$^7$ BM cells were cultured with 10 ng ml$^{-1}$ GM-CSF. For BM-pDCs, 2 x 10$^7$ BM cells were cultured with 100 ng ml$^{-1}$ Flt3-ligand and stained by anti-CD11c and anti-B220. Stained cells were subjected to sorting with FACS Aria (BD) and the CD11c$^+$B220$^+$ population was collected as BM-pDCs.

**Reagents and antibodies**

Lipid A Re:595 was purchased from Sigma-Aldrich. CpG-A 1585 (5’-G*G*GGTCA ACGTTGAG*G*G*G*G*G-3’; asterisks represent phosphorothioated bases) was synthesized by Hokkaido System Science. CpG-B 1688 (5’-TC CATGA CGT TCCT GATGCT-3’, all phosphorothioated), RNA9.2s-DR (5’-UGUC CUUCA AUGUCC UU CAA-3’, all phosphorothioated) and Poly U (19mer, all phosphorothioated) were synthesized by FASMAC. R848, Imiquimod and Loxoribine were purchased from Invivogen. CpG-A 1586-rhodamine and RNA9.2s-DR-rhodamine were used for flow cytometry. In some experiments, BM-pDCs were stimulated with 10 ng/ml of each TLR ligand for 18 h, and the surface expression of CD11c was determined by flow cytometry.

**Results**

**TLR7 and TLR9 responses are impaired in Mcoln1$^{-/-}$ BM-cDCs.** WT or Mcoln1$^{-/-}$ BM-cDCs were stimulated with TLR ligands for 24h and production of IL-6 (A) or TNF-α (B) was determined by ELISA. The results are represented by the mean value with standard deviation from triplicate wells. *P < 0.05; **P < 0.01. The data are representative of three independent experiments.
were synthesized by Hokkaido System Science. CpG-B 1688-rhodamine and Poly U-rhodamine were synthesized by FASMAC. The mucolipin agonist (ML1-SA1) and PIKfyve inhibitor (YM201636) were purchased from Calbiochem. Recombinant mouse GM-CSF and recombinant mouse Flt3 ligand were purchased from PeproTech. PE-conjugated anti-CD11c and APC-conjugated anti-B220 were purchased from eBioscience.

**Fig. 2.** Impaired TLR7 and TLR9 responses in Mcoln1−/− BM-pDCs. WT or Mcoln1−/− BM-pDCs were stimulated with TLR7 and TLR9 ligands for 24 h. Production of IL-6 (A), TNF-α (B) and IFN-α (C) was determined by ELISA. The results are represented by the average values with standard deviation. *P < 0.05; **P < 0.01. The data are representative of three independent experiments.

**ELISA**

1 × 10^5 per well of BM-cDCs or BM-pDCs were seeded in 96-well plate, followed by treating with TLR ligands. After 24 h culture, supernatant was harvested and subjected to ELISA assay. IL-6 and TNF-α were determined by Ready-SET-Go! ELISA (eBioscience), IFN-α was measured by VeriKine™ Mouse Interferon Alpha ELISA Kit (PBL Assay Science).
Sample plates were analyzed by an iMark Microplate Absorbance Reader (BIO-RAD).

**Flow cytometry**

Cells were stained and analyzed by FACSCalibur (BD Biosciences) as described previously (15).

**Immunoprecipitation and immunoblotting**

2 × 10⁷ BM-cDCs were lysed in lysis buffer [1% Triton X100, 20mM Tris/HCl, 150mM NaCl, 1mM CaCl₂, 1mM MgCl₂, 10% Glycerol, Complete Inhibitor (Roche)] for 30min on ice. Anti-TLR7- or anti-TLR9-coupled agarose beads were supplied to the supernatant and rotated for 2h at 4°C. After incubation, the beads were washed by washing buffer (0.1% Triton X100, 20mM Tris/HCl, 150mM NaCl, 1mM CaCl₂, 1mM MgCl₂, 10% Glycerol) and mixed with 2x sample buffer. The samples were boiled for 5min at 96°C and subjected to SDS-PAGE. After electrophoresis, proteins in the gel were transferred to a polyvinylidene difluoride membrane, and the membrane was blocked with 3% skimmed milk (Difco) in Tris-buffered saline and Tween 20 for 1h at room temperature. Then samples were immunoprobed with anti-TLR7 (polyclonal, eBioscience) or anti-TLR9 (polyclonal, homemade). The signal was visualized by Amersham ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences) and LAS500 (GE Healthcare Life Sciences).

**Fig. 3.** Mucolipin agonist specifically enhances TLR7 responses to ssRNAs. BM-cDCs were pre-treated with DMSO (vehicle) or 10 μM mucolipin agonist (ML1-SA1) for 30min and then stimulated with TLR7 or TLR9 ligands at the indicated concentrations. After 24-h stimulation, supernatants were harvested, and production of IL-6 (A) or TNF-α (B) was determined by ELISA. The results are represented by the average values with standard deviation from triplicate wells. *P < 0.05; **P < 0.01. The data are representative of three independent experiments.
Confocal microscopy

$2 \times 10^6$ BM-cDCs were seeded on a four-compartment cell culture dish (Cellview™ cell culture dish with glass bottom; greiner bio-one). Overnight culture allows the cells to adhere to the bottom. Cells were pre-treated with DMSO or 10 µM ML1-SA1 and incubated with CpG-A-rhodamine, CpG-B-rhodamine, Poly U-rhodamine or RNA9.2s-DR-rhodamine for 90min at 37°C. Supernatant containing ligands were removed and supplemented with LysoTracker® Green DND-26 (Life technologies), incubated for 30min at 37°C. Imaging was performed using an LSM710 confocal microscope with a x63 NA1.4 Plan-Apochromat oil immersion lens (Carl Zeiss Microscopy). Fluorescent images were analyzed by LSM710 ZEN software.

Statistical analysis

Student’s t-test is used for statistical analysis. A P-value of <0.05 was considered to be statistically significant.

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![Graph A](image1.png)

![Graph B](image2.png)

Fig. 4. TLR7 responses to ssRNA in BM-cDCs are sensitive to PIKfyve inhibitor. BM-cDCs were pre-treated with DMSO (vehicle) or PIKfyve inhibitor (YM201636) at the indicated concentrations for 15min and then stimulated with TLR7 or TLR9 ligands. After 24h, supernatants were harvested, and the concentration of IL-6 (A) and TNF-α (B) was determined by ELISA. The results are represented by the average values with standard deviation from triplicate wells. *P < 0.05; **P < 0.01. The data are representative of three independent experiments.
Results

Impaired TLR7 and TLR9 responses in Mcoln1−/− DCs

BM cells were allowed to differentiate into BM-cDCs or BM-pDCs and stained with the maturation markers. No alteration was found in Mcoln1−/− BM cells in terms of cell surface expression of CD11c in BM-cDCs and of CD11c and B220 in BM-pDCs (Supplementary Figure 1A and B, available at International Immunology Online).

To study the role of Mcoln1 in BM-cDC responses, WT and Mcoln1−/− BM-cDCs were stimulated with a variety of TLR ligands and production of IL-6 and TNF-α was determined by ELISA. No alteration was found in responses to a TLR4/MD-2 ligand Lipid A and a TLR9 ligand CpG-B (Fig. 1A and B). Partial, but significant reduction was found in responses to a TLR7 ligand ssRNA (RNA9.2s-DR) and a TLR9 ligand CpG-A. At lower concentrations of TLR7 ligands, loxoribine and R848, Mcoln1−/− BM-cDCs showed significantly lower responses than WT BM-cDCs.

Fig. 5. TLR7 responses to ssRNA in BM-pDCs are sensitive to PIKfyve inhibitor. BM-pDCs were pre-treated with DMSO (vehicle) or PIKfyve inhibitor (YM201636) at the indicated concentrations for 15 min and then stimulated with TLR7 ligands. After 24 h, supernatants were harvested, and the concentration of TNF-α (A) and IFN-α (B) was determined by ELISA. The results are represented by the average values with standard deviation from triplicate wells. *P < 0.05; **P < 0.01. The data are representative of three independent experiments.
A mucolipin agonist specifically enhances TLR7 responses to ssRNAs in BM-cDCs

Impaired TLR7 responses in Mcoln1−/− BM-cDCs and -pDCs suggest that Mcoln1 has a role in TLR7 responses. To address the mechanism underlying a role of Mcoln1, BM-cDCs were stimulated with TLR7 and TLR9 ligands in the presence of a synthetic mucolipin agonist, ML1-SA1. ML1-SA1 synergistically induces a cytosolic Ca2+ increase with an endogenous ligand PtdIns(3,5)P2, (21). ML1-SA1 enhanced IL-6 and TNF-α production by RNA9.2s-DR and IL-6 production by Poly U (Fig. 3). On the contrary, TLR9 responses to CpG-A were partially reduced. TLR7 responses to small chemical ligands R848 and loxoribine and TLR9 responses to CpG-B were not altered at all by ML1-SA1.

TLR7 responses to ssRNA are sensitive to PIKfyve inhibitor

Considering that ML1-SA1 synergistically acts on mucolipin with PtdIns(3,5)P2, PIKfyve, a phosphatidylinositol 3-phosphate 5-kinase, may have a role in RNA transportation (16). The effect of the PIKfyve inhibitor YM201636 on TLR7 and TLR9 responses in BM-cDCs was studied. As reported previously (16), CpG-B responses were significantly but weakly impaired (Fig. 4). Interestingly, TNF-α production was more resistant to YM201636 than IL-6 production (Fig. 4B). Much clearer inhibition was found in stimulation with CpG-A. Further and complete inhibition was observed in responses to ssRNA (Fig. 4A). In ssRNA responses, TNF-α production was inhibited by YM201636 as much as IL-6 production (Fig. 4B). These results suggest that PIKfyve-dependent generation of PtdIns(3,5)P2 is more required for TLR7 responses to ssRNA and TLR9 responses to CpG-A than TLR9 responses to CpG-B.

NAs complexed with a transfection reagent are known to show higher TLR7 and TLR9 responses than NAs alone, probably by enhancing NA delivery to TLR7 and TLR9. Very interestingly, Lipofectamine was able to overcome the inhibition by the PIKfyve inhibitor in ssRNA responses, partially in IL-6 production and completely in TNF-α production (Fig. 4). CpG-A responses were also enhanced by Lipofectamine but CpG-A/Lipofectamine responses remained sensitive to inhibition by the PIKfyve inhibitor (Fig. 4).

The effect of the PIKfyve inhibitor on TLR-dependent production of TNF-α and IFN-α in BM-pDCs was next studied. Production of IFN-α, but not TNF-α, in response to a small chemical ligand R848 was inhibited by the PIKfyve inhibitor. All the TLR7 responses to ssRNA ligands such as RNA9.2s-DR or Poly U were impaired by the PIKfyve inhibitor (Fig. 5). The inhibitory effect of the PIKfyve inhibitor was not seen when ssRNA ligands were complexed with Lipofectamine (Fig. 5).

Production of TNF-α and IFN-α induced by CpG-A was also impaired by the PIKfyve inhibitor. In contrast to TLR7 responses to ssRNA ligands, Lipofectamine failed to rescue CpG-A responses in PIKfyve inhibitor-treated BM-pDCs (Fig. 5). These results demonstrate an important role of PtdIns(3,5)P2 in TLR7 and TLR9 responses.

Expression and processing of TLR7 and TLR9 are not altered in Mcoln1−/− BM-cDCs

Considering that Mcoln1 has a role in lysosomal trafficking, trafficking of TLR7 and TLR9 may be altered in Mcoln1−/− mice. To address this possibility, expression of these TLRs was first...
studied. BM-cDCs were subjected to membrane-permeabilized staining with a TLR7 or TLR9 mAb. Expression of TLR7 and TLR9 was not altered in Mcoln1−/− BM-cDCs (Fig. 6A). Next, TLR7 and TLR9 were immunoprecipitated. Consistent with FACS analyses, no alteration was found in the amount of immunoprecipitated TLR7 and TLR9 (Fig. 6B and C). Moreover, proteolytic cleavage of TLR7 and TLR9 was not altered (Fig. 6B and C). Given that TLR7 and TLR9 are proteolytically cleaved in endolysosomes, trafficking of TLR7 and TLR9 from ER to endolysosomes would not be altered in Mcoln1−/− BM-cDCs.

RNA transportation to lysosomes is impaired in Mcoln1−/− BM-cDCs

Mcoln1 is a gene responsible for MLIV (20), where metabolites accumulate in lysosomes because of altered lysosomal trafficking. NAs might belong to metabolites that require Mcoln1 for proper trafficking to lysosomes. To address this possibility, Mcoln1−/− BM-cDCs were stimulated with rhodamine-labeled ligands such as CpG-A, CpG-B, RNA9.2s-DR or Poly U. Co-localization of these ligands with a lysosome marker lysotracker was studied by confocal microscopy. CpG-B showed co-localization with lysotracker and Mcoln1−/− BM-cDCs did not show any alteration in CpG-B distribution (Fig. 7A). TLR7 ligands, RNA9.2s-DR and Poly U, also showed co-localization with lysotracker in WT BM-cDCs and the co-localization was significantly impaired in Mcoln1−/− BM-cDCs (Fig. 7B and C). These results indicate that an important role of Mcoln1 in RNA trafficking into lysosomes.

The co-localization of CpG-A and lysotracker was significantly decreased in Mcoln1−/− BM-cDCs at 30 min after stimulation (Fig. 8A). The difference was, however,
not seen at 60 and 120 min after stimulation in Mcoln1−/− BM-cDCs (Fig. 8B and C). To confirm this results, CpG-A co-localization with an early endosome marker EEA1 was next studied. CpG-A co-localization with EEA1 was significantly up-regulated in Mcoln1−/− BM-cDCs at 30 min after stimulation (Fig. 9B) but not at 15 or 60 min after stimulation (Fig. 9A and C). CpG-A transportation into lysosomes was delayed in Mcoln1−/− BM-cDCs. These results explain the partial impairment of CpG-A responses in Mcoln1−/− BM-cDCs and indicate the presence of a molecule with redundant function. Mcoln1 is likely to have a role in CpG-A transportation from early endosomes to lysosomes.

**NA transportation to lysosomes is dependent on PtdIns(3,5)P2**

To investigate the role of PtdIns(3,5)P2 in NA transportation to lysosomes, the effect of the PIKfyve inhibitor YM201636 on transportation of CpG-A, CpG-B, RNA9.2s-DR and Poly U was studied. All the NA transportation to lysosomes was significantly inhibited by YM201636 (Fig. 10), demonstrating that NA transportation is dependent on PtdIns(3,5)P2-mediated endosomal trafficking. Lipofectamine rescued RNA9.2s-DR trafficking into lysosomes, but not CpG-A trafficking in YM201636-treated BM-cDCs (Fig. 10A and C). This is consistent with the results that lipofectamine rescued DC responses induced by RNA9.2s-DR but not CpG-A in YM201636-treated DCs (Figs 4 and 5). Although both CpG-A and RNA9.2s-DR are transported by PtdIns(3,5)P2-dependent endosomal trafficking, they are likely to use different transportation pathways in the presence of Lipofectamine.

**Discussion**

Extracellular NAs are sensed by TLRs in endolysosomes, not on the cell surface, to prevent autoimmune responses. Previous reports have revealed that aberrant localization of NAs in endolysosomes predisposes to psoriasis or SLE (7, 9). It is therefore important to clarify the molecular mechanisms behind NA transportation. PIKfyve, which is the mammalian ortholog of yeast Fab1p, has been shown to be required for CpG-B transportation (16). PIKfyve is involved in the generation of PtdIns(3,5)P2. The downstream effector of PtdIns(3,5)P2, however, has not been clarified. Activation of Mcoln1 by PtdIns(3,5)P2 has been reported in a variety of cells (17, 22).
The present study indicates that Mcoln1 is one of the down-stream effectors of PtdIns(3,5)P$_2$ in RNA transportation. TLR7 responses to ssRNA were impaired by the lack of Mcoln1 or a PIKfyve inhibitor and augmented by a mucolipin agonist. ssRNA transportation into lysosomes was inhibited by the lack of Mcoln1 or the PIKfyve inhibitor. Moreover, Lipofectamine rescued ssRNA responses and ssRNA transportation into lysosomes in DCs treated with the PIKfyve inhibitor. Lipofectamine is likely to rescue ssRNA responses by having ssRNA distribute in lysosomes in the presence of the PIKfyve inhibitor. These results strongly suggest an essential role of the PIKfyve–Mcoln1 axis in RNA transportation in DCs. In Mcoln1$^{-/-}$ BM-cDCs, RNA transportation to lysosomes was more severely impaired than DNA transportation. A mechanism regulating RNA transportation in DCs is likely to be distinct from that for DNA transportation.

Although Mcoln1$^{-/-}$ BM-cDCs and -pDCs showed impaired responses to small chemical TLR7 ligands and ssRNA, neither the mucolipin agonist nor the PIKfyve inhibitor altered TLR7 responses to these small chemical ligands. TLR7 responses to small chemical ligands may not require PtdIns(3,5)P$_2$-dependent mucolipin activation, suggesting that Mcoln1 has another role in TLR7 responses in addition to the role in RNA trafficking. Mcoln1 is suggested to have a role in mammalian target of rapamycin (mTOR) activation in Drosophila (23). Given that mTOR influences TLR7 and TLR9 responses (24, 25), mTOR might be involved in the role of Mcoln1 in responses to small chemical TLR7 ligands.

TLR9 responses to CpG-A were also significantly impaired in Mcoln1$^{-/-}$ BM-cDCs and -pDCs. The PIKfyve inhibitor impaired CpG-A responses. These results suggest that Mcoln1 has a role in CpG-A transportation to lysosomes. However, CpG-A transportation to lysosomes was only transiently halted in Mcoln1$^{-/-}$ BM-cDCs at 30 min after CpG-A stimulation. No alteration was found at 120 min after stimulation, demonstrating a redundant role of Mcoln1 in CpG-A transportation into lysosomes. Mcoln2 and Mcoln3 are known to belong to the mucolipin family. These molecules may also have a role in CpG-A transportation.

TLR9 responses to CpG-B were not altered in Mcoln1$^{-/-}$ BM-cDCs and -pDCs. The PIKfyve inhibitor, however, significantly impaired CpG-B responses and CpG-B transportation into lysosomes. These results demonstrate that CpG-B transportation requires PtdIns(3,5)P$_2$-dependent trafficking but not...
Fig. 10. NAs transportation to lysosomes is impaired by a PIKfyve inhibitor in BM-cDCs. BM-cDCs were pre-treated with DMSO (vehicle) or 500 nM PIKfyve inhibitor (YM201636) for 15 min. BM-cDCs were stimulated with 1 μM CpG-A-rhodamine alone or with Lipofectamine (LF2000) (A), 1 μM CpG-B-rhodamine (B), 10 μg ml⁻¹ RNA9.2s-DR-rhodamine alone or with Lipofectamine (LF2000) (C) and 10 μg ml⁻¹ Poly U-rhodamine (D) for 90 min. To visualize lysosomes, cells were incubated with Lysotracker-Green for 30 min before analyses by confocal microscopy. Statistical analyses using correlation coefficient are also shown. ***P < 0.001. The data are representative of three independent experiments. Scale bar, 5 μm.
Mcoln1. Another downstream molecule is likely to be responsible for CpG-B transportation into lysosomes. The present study has revealed that NA transportation into lysosomes is dependent on PtdIns(3,5)P₂. Downstream of PtdIns(3,5)P₂, several molecules including Mcoln1 are likely to function. The selection of downstream effectors seems to be variable, depending on the type of NA.

Human mutations of Mcoln1 cause MLIV, a lysosomal storage disease characterized by abnormally large lysosomes containing electron-dense inclusions and lipid storage bodies (19, 20). RNA is likely to be aberrantly transported and accumulate in lysosomes in MLIV. It is interesting to study TLR7 and TLR9 responses in MLIV. Sphingomyelin is known to inhibit Mcoln1 in lysosomal storage diseases such as Niemann–Pick disease (21). Lipid metabolism may influence innate immunity by modulating transportation of RNA or DNA.

Supplementary data
Supplementary data are available at International Immunology Online.

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