博士論文

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

（樹状細胞における Mucolipin-1 依存的な一本鎖 RNA のリソソームへの輸送を介した TLR7 応答制御機構の解明）

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Abbreviation

Ab: antibody

AP2: adapter-related protein complex-2

ARF6: ADP-ribosylation factor 6

BM-cDCs: bone marrow derived conventional dendritic cells

BM-pDCs: bone marrow derived plasmacytoid dendritic cells

C/EBPβ: CCAAT/enhancer binding protein beta

CD: cluster of differentiation

DAMPs: Damage Associated Molecular Patterns

DMSO: dimethyl sulfoxide

EDTA: etylenediaminetetraacetic acid

ELISA: enzyme linked immunosorbent assay

ER: endoplasmic reticulum

FACS: fluorescence activated cell sorter

FBS: fetal bovine serum

FLT3: fms-related tyrosin kinase 3

GM-CSF: granulocyte-macrophage colony-stimulating factor

HIV-1: human immunodeficiency virus-1

IFN-α: interferon alpha

IgG: immunoglobulin G

IL-6: interleukin-6

IL-12p40: interleukin-12p40

IRFs: interferon regulatory factors

LPS: lipopolysaccharide

LROs: lysosome related organelles

M-CSF: macrophage colony-stimulating factor

mAb: monoclonal antibody

mRNA: messenger RNA

NAs: nucleic acids

NF-κB: nuclear factor kappa B

PAMPs: Pathogen Associated Molecular Patterns

PCR: polymerase chain reaction

PI3K: phosphatidylinositol 3-kinases

PRAT4A: A protein associated with TLR4

PRRs: Pattern Recognition Receptors

PtdIns: phosphatidylinositol

PtdIns(3,5)P_2: Phosphatidylinositol (3,5) bisphosphate

PtdIns(3)P: Phosphatidylinositol (3) bisphosphate

PVDF: polyvinylidene difluoride

RA: rheumatoid arthritis

RASFs: rheumatoid arthritis synovial fibroblasts

SDS-PAGE: sodium dodecyl sulfate polyaclylamidegel electrophoresis

SLE: systemic lupus erythematosus

ssRNA: single stranded RNA

TBST: tris buffered saline tween

TLRs: Toll like receptors

TNF-α: tumor necrosis factor alpha

Unc93b1: Unc-93 homolog B1

1. Introduction

1.1 Innate immunity.

Innate immunity is the first line of defense against infections [1]. The cells belonging to innate immunity are rapidly activated by microbes before the development of adaptive immune responses. In response to microbes, innate immunity stimulates adaptive immune responses and is able to influence the nature of the adaptive responses to make them optimally effective against different types of pathogens. Many cells and tissues are endowed with the ability to contribute to innate immune activity. The specificities of innate immune system have evolved to offense the invaded microbes and are different from those of the adaptive immune system.

The innate immunes system recognizes molecular structures that are conserved within a class of microbes. The small molecular motifs that can stimulate innate immunity are called Pathogen Associated Molecular Patterns (PAMPs). Pathogens including viruses, gram-negative bacteria, gram-positive bacteria and fungi express varied PAMPs such as nucleic acids and proteins that are unique to microbes. The innate immune system also recognizes the endogenous molecules that are released from

damaged tissues or dying cells. These substances are called Damage Associated Molecular Patterns (DAMPs) [2, 3].

1.2 Toll like receptors (TLRs) family.

Several families of cellular receptors distributed in different locations in cells are found to recognize PAMPs and DAMPs. These innate immune receptors are called Pattern Recognition Receptors (PRRs). Among PRR families, Toll like receptors (TLRs) family is one of the best described. TLRs are type I transmembrane proteins with ectodomains containing leucine rich repeats, transmembrane domain and intracellular Toll interleukin 1 (IL-1) receptor (TIR) domain that is involved in the recruitment of signaling adaptor molecules [4].

To date, 10 functional TLRs are found in humans and 13 TLRs are found in mice. TLR1-9 are highly conserved among both species. TLR11, TLR12 and TLR13 have not been functioning in the human genome. The distribution of TLRs has been considered to divide into two groups. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are expressed in intracellular compartments like endoplasmic reticulum (ER), endosome, lysosome and

lysosome related organelles (LROs) [2, 3, 5]. Recently, TLR9 is shown to expressed on the cell surface in many types of cells. The functions of cell surface TLR9 have not been clarified [6].

1.3 Toll like receptors and their ligands.

A variety of molecular patterns of microorganisms and self-components are recognized by different TLRs (Table 1). TLR2/TLR1 and TLR2/TLR6 heterodimers sense various components from bacteria, mycoplasma, fungi and viruses. These components include the lipoproteins of bacteria and mycoplasma. TLR4-MD2 recognizes lipopolysaccharide (LPS) derived from the outer membrane of Gram-negative bacteria. TLR5 recognizes flagellin from flagellated bacteria. TLR11, which has close homology to TLR5, recognizes uropathogenic bacteria and a profilin-like molecule derived from protozoan *Toxoplasma gondii*. TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids (NAs) derived from viruses, bacteria and dead cells [7].

Table 1. Toll like receptors and their ligands.

TLR	Localization	Species	Natural ligands	Synthetic ligands	Recognized pathogens
TLR1	Plasma membrane	Humans and Mice	Triacyl lipopeptides	Pam3CSK4	Bacteria
TLR2	Plasma membrane	Humans and Mice	Lipoproteins, peptidoglycan, LTA, zymosan and mannan	Pam3CSK4	Bacteria, viruses, parasites, self
TLR3	Endolysosome	Humans and Mice	dsRNA	Poly I:C and Poly U	Virus
TLR4	Plasma membrane	Humans and Mice	LPS, RSV, MMTV fusion protein, mannans, and glycoinositolphosphate from *Trypanosoma spp*.	Lipid A derivatives	Gram-negative bacteria, virus and self.
TLR5	Plasma membrane	Humans and Mice	Flagellin	Unknow	Bacteria
TLR6	Plasma membrane	Humans and Mice	Diacylipopetides, LTA and zymosan.	MALP2 and FSL-1	Bacteria and Virus
TLR7	Endolysosome	Humans and Mice	GU-rich ssRNA and short dsRNA	Imidazoquinolines and guanosine analogues	Virus, Bacteria and self
TLR8	Endolysosome	Humans and Mice	GU-rich ssRNA, short dsRNA and bacterial RNA (For human TLR8)	Imidazoquinolines and guanosine analogues	Virus, Bacteria and self
TLR9	Endolysosome	Humans and Mice	CpG DNA and hemozoin from *Plasmodium spp*.	CpG ODNs	Bacteria, Virus, Protozoa and self
TLR10	Endolysosome	Humans	Unknown	Unknow	Unknow
TLR11	Plasma membrane	Mice	Profilin and flagellin	Unknow	Apicomplexan parasites and bacteria (including Salmonella spp. and UPEC)
TLR12	Endolysosome	Mice	Profilin	Unknow	Apicomplexan parasites
TLR13	Endolysosome	Mice	Bacterial 23S rRNA with CGGAAAGACC motif	Unknow	Gram-negative and Gram-positive bacteria

1.4 Localization, trafficking and processing of Toll like receptors.

In resting cells, accessary proteins are required for the trafficking of TLRs. MD2 is essential for the translocation of TLR4 from the Golgi to the plasma membrane [8]. Unc-93 homolog B1 (Unc93b1) mediates TLR3, TLR7, TLR8 and TLR9 trafficking from ER to endosome [9-12]. Recent studies reveal that Unc93b1 regulates cell surface expression of TLR5 and TLR9 [6, 13]. Internalization of cell surface TLR9 is mediated by cooperation of Unc93b1 and adapter-related protein complex-2 (AP2) [14]. A protein associated with TLR4 (PRAT4A) is essential for the proper folding of TLRs (except TLR3) [15], and thereby required for TLR egress from the ER (Fig. 1). PRAT4A (M145K) mutation prevents stimulation-induced TLR9 relocation and weakens the association with TLR4 [16, 17]. Previous reports show that CpG DNA induces TLR9 conformation change [18], and this change is due to the cleavage of TLR9. Moreover, cleavage of TLR7 and TLR3 is proved to be important in NAs sensing [19].

1.5 Localization and trafficking of Toll like receptor ligands.

It has become increasingly evident that the localization and transportation of TLRs is

important to sense their ligands. Latz *et al*. report that CpG DNA moves into early endosomes and is subsequently transported to a tubular lysosomal compartment [18]. Simultaneously TLR9 redistributes from the ER to CpG DNA-containing compartment [18, 20]. It is reported that CD14 contributes to CpG DNA internalization and delivery to TLR9 compartments. In addition, CD14 is able to facilitate the uptake of imiquimod, the chemical ligand of TLR7 [21].

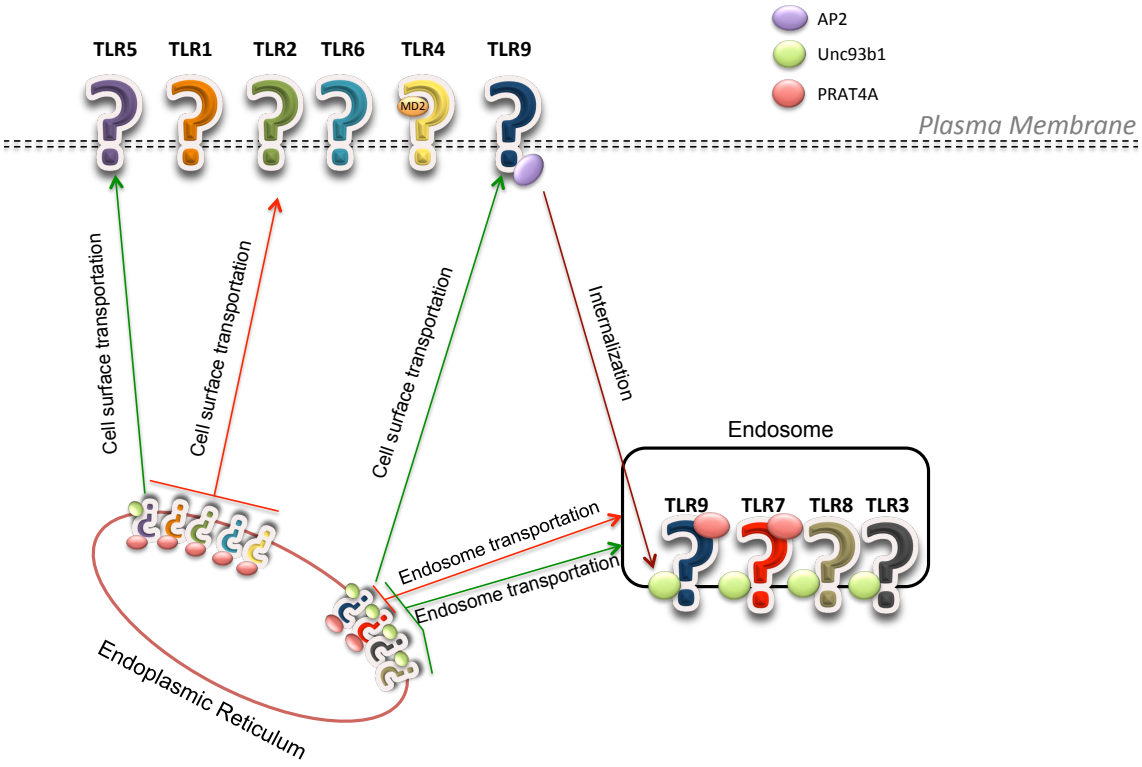


Figure 1. TLRs distribution and accessary proteins for TLRs trafficking. TLRs are divided to two groups depending on their distribution. TLR1, TLR2, TLR4, TLR5, and TLR6 are localized on the cell surface, whereas intracellular TLRs including TLR3, TLR7, TLR8, and TLR9 reside in endolysosomes. TLR9 is also expressed on the cell surface and the internalization is mediated by AP2. Both Unc93b1 and PRAT4A are required for TLRs exit ER and transportation to cell surface or endosome.

1.6 Toll like receptor induced inflammatory mediators.

Activation of TLRs signaling pathways leads to the nuclear translocation of a set of transcription factors, including nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), interferon regulatory factors (IRFs) and CCAAT/enhancer binding protein beta (C/EBPβ). These factors regulate their target genes and remodel chromatin [22]. Thus the protein levels of cytokines, chemokines, and IFNs are elevated after TLRs activation. IL-6 stimulates the inflammatory processes in many autoimmune diseases such as rheumatoid arthritis (RA). Type I IFNs are reported to cause chronic inflammation diseases such as systemic lupus erythematosus (SLE). TNF-α is reported to be involved in atherosclerosis and RA [23-25].

1.7 Aberrant transportation of Nucleic Acids (NAs) predisposes to autoimmune diseases.

TLR7 and TLR9 erroneously respond to self-derived NAs, inducing anti-nuclear autoantibody production in murine models of SLE [26-28]. Self-pathogen discrimination 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. Self-derived RNA/DNA is rapidly degraded by RNase or DNase, whereas 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 endolysosomes to stimulate TLR7 or TLR9 [29]. In the disease state, however, self-derived NAs are shown to become resistant to degradation and reach endolysosomes. Previous reports have revealed that in psoriasis, self-derived DNA and self-derived RNA form complexes with cationic antimicrobial peptide LL37, and traffic to endosomes, where TLR7 and TLR9 are localized [28, 30]. In SLE, self-derived DNA and self-derived RNA form complexes with autoantibodies and traffic to endosomes via Fcγ (CD32) receptor-mediated endocytosis [31, 32]. Rheumatoid arthritis (RA) is characterized by persistent inflammation of the synovial membrane [33]. Emerging evidences reveal that expression of TLR7 is significantly elevated in RA synovial fibroblasts (RASFs) [34, 35]. TLR7 erroneously responds to self-derived ssRNA can exacerbate RA by inducing TNF-α production [35]. Repeated low dose administration of synthetic TLR7 ligands, which results in hyporesponsiveness or tolerance of TLR7, attenuates the joint inflammation in a RA model [36]. A defective TLR9 response, which is caused by the

inhibition of cathepsin K, suppresses autoimmune inflammation of the joints in RA [37]. These findings highlight the important regulatory functions of TLRs in autoimmune disease.

1.8 Molecular mechanism underlying transportation of Toll like receptor 7 and 9.

To limit NA-sensing by TLR7 or TLR9 in endolysosomes, TLR7/9 transportation has to be 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 [38]. Trafficking of TLR7 and TLR9 are dependent on Unc93B1, a multiple transmembrane protein [9, 39]. Unc93B1 is associated with TLR7 or TLR9 and is essential for their transportation. In mice harboring *3d* mutation replacing 412nd histidine with arginine in the Unc93B1 gene, TLR7 and TLR9 fail to traffic out of the ER and to respond to NAs in endolysosomes. Furthermore 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 dominant [40, 41], leading to TLR7-dependent systemic lethal inflammation *in vivo*.

1.9 Roles of Phosphatidylinositides in transportation of RNA/DNA.

When compared to sensor transportation, much less is known about molecular mechanism underlying NAs transportation. Wu. *et al*. show that inhibition of ADP-ribosylation factor 6 (ARF6) impaired cellular uptake of CpG ODN into endosome through the impaired class III phosphatidylinositol 3-kinases (PI3K) activity [42]. Class III PI3K (also known as Vps34) is important in converting phosphatidylinositol (PtdIns) into Phosphatidylinositol (3) bisphosphate (PtdIns(3)P) that is predominant phosphoinositide in early endosomes and autophagosomes [43]. PtdIns(3)P is responsible for recruiting a spectrum of cytosol-localized proteins to these compartments. PtdIns(3)P can be converted into Phosphatidylinositol (3,5) bisphosphate (PtdIns(3,5)P_2) by the phosphatidylinositol 5-kinase (PtdIns(5)K) PIKfyve complex (Fig. 2). Hazeki *et al*. show that PIKfyve inhibitor YM201636 induces accumulation of CpG-B in early endosomes in a macrophage cell line, and thereby impairs TLR9 responses [44].

1.10 Transient receptor potential mucolipin-1, mucolipin-2 and mucolipin-3 (TRPML1, 2 and 3) function as downstream effectors of phosphatidylinositides.

PtdIns(3,5)P_2 is reported to directly bind to amino acid terminus of mucolipin-1 (TRPML1) and induces Ca^{2+} release [45] (Fig. 2). Mucolipin superfamily of ion channels are localized to the endosomal pathways that mediate numerous cellular functions. The mucolipin subfamily consists of three members: mucolipin-1 (TRPML1), mucolipin-2 (TRPML2) and mucolipin-3 (TRPML3) [45]. Mucolipin-1 has been identified as the protein mutated in the lysosomal storage disease (LSD) Mucolipidosis type IV that shows aberrant metabolites transportation and enlarged endolysosome [46, 47]. Likewise, mucolipin-3 is as the one in mice with the varitint-waddler phenotype [48]. Mucolipin-1 localizes in late endosomes and lysosomes [49], mucolipin-2 is found mainly in lysosomes, and mucolipin-3 shuttles between multiple intracellular compartments and the plasma membrane [50].

1.11 The role of mucolipin in the endocytic pathways.

Endosomes are formed via cargo endocytosis from cell surface or reception biosynthetic cargo from the late Golgi complex. The endosomal pathway plays essential

role in endocytic membrane traffic, protein sorting, pathogen uptake, antigen presentation and signaling [51]. Luminal Ca^{2+} is also a key regulator of the endosomal pathway. Release of Ca^{2+} from endosomes and lysosomes is required for several steps of intracellular trafficking, including fusion and fission events [52]. Ca^{2+} is important in inflammatory responses [53-56] (Fig. 2). Mucolipin subfamily mediates Ca^{2+} influx and efflux. The gain-of-function mutation A419P of mucolipin-3 alters ionic selectivity and Ca^{2+} permeability [48]. The homologous mutation V432P of mucolipin-1 confers Ca^{2+} channel constitutive permeability, along with altered trafficking [57].

1.12 The question addressed in this study.

Mucolipin-1 deficient cells are impaired in endosomal trafficking and show enlarged endolysosomes, which are also observed in cells lacking of PtdIns(3,5)P_2 [45]. Previous reports show that PtdIns(3,5)P_2 has an important role in NAs trafficking, in addition, mucolipin-1 is a target of PtdIns(3,5)P_2 [44, 45], PtdIns(3,5)P_2 may regulate NAs trafficking via mucolipin-1. Mucolipidosis type IV cells show impaired transportation of metabolites. NAs belong to metabolites, the transportation of NAs therefore may be regulated by mucolipin-1. To date, the immunological analysis of mucolipin-1 null mice

has not been reported. The present study focused on role of mucolipin-1 in TLR7 and TLR9 responses, particularly in clarifying the mechanisms underlying intracellular NAs transportation. To address this issue, TLR7 and TLR9 responses in *Mcoln1*$^{-/-}$ mice were studied. *Mcoln1*$^{-/-}$ cells showed decreased TLR7 responses to single stranded RNA (ssRNA), and impaired ssRNA localization in lysosomes. PIKfyve inhibitor significantly impaired ssRNA responses and transportation to lysosomes. These results suggest a role of PtdIns(3,5)P_2-mucolipin-1 axis in ssRNA transportation to the lysosomes.

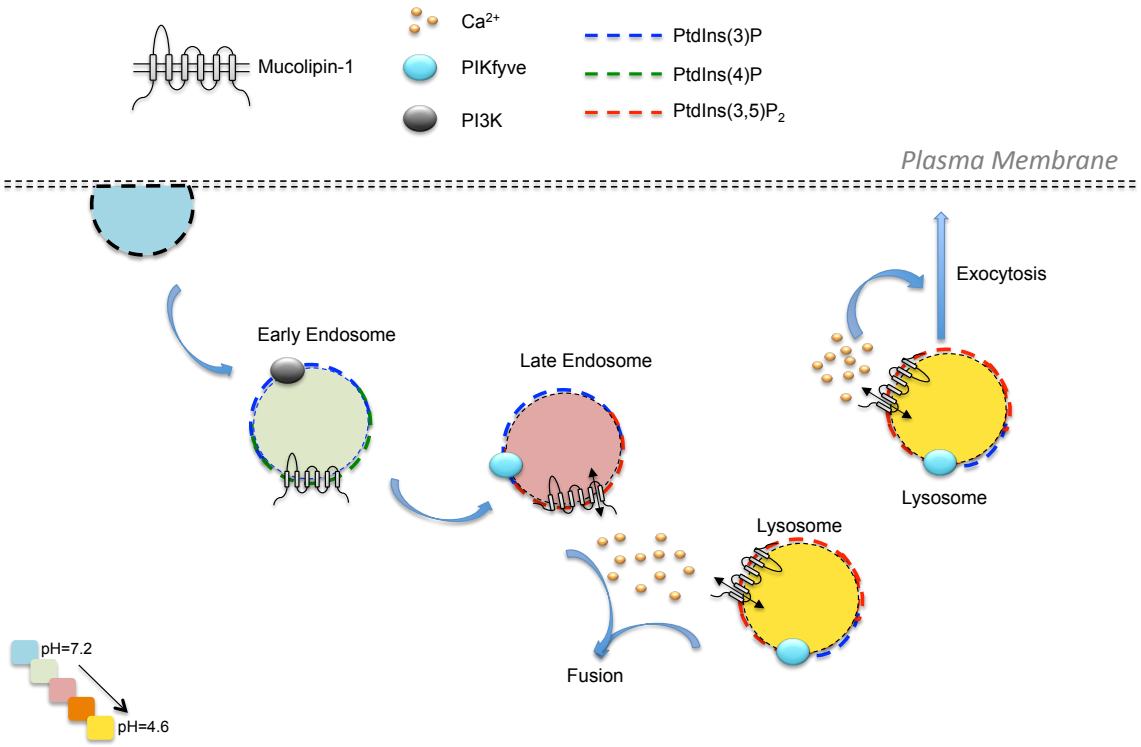


Figure 2. Subcellular distribution of PtdIns and mucolipin-1. A schematic cartoon of endosomal network. PtdIns in different compartments is indicated by different colours. Class III PI3K synthesizes PtdIns(3)P in early endosome. PIKfyve converts PtdIns(3)P to PtdIns(3,5)P_2. PtdIns(3,5)P_2 regulates luminal Ca^{2+} via activation of mucolipin-1. Mucolipin-1 is a Ca^{2+} channel mainly localizes in late endosome and lysosome, and is predicted to have six transmembrane domains with the amino acid terminus binding to PtdIns(3,5)P_2. Luminal Ca^{2+} positively regulates lysosome exocytosis.

2. Materials and Methods

2.1 Mice

Mcoln1$^{-/-}$ mice used for this research project were B6.129-Mcoln1^{tm1Asoy}/Mmmh, stock number 034789-MU. They were obtained from the Mutant Mouse Regional Resource Center, a 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 [58]. C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Bone marrow chimeric mice (BM chimeric) were generated as following protocol. C57BL/6 mice were irradiated at 950 rad at a time. 10 million Bone marrow (from femur and tibia) cells from WT or *Mcoln1*$^{-/-}$ mice were intravenously injected into recipients (C57BL/6 mice, 4 wk of age, female). Water for mice living were supplemented with neomycin. 72 hr later, the neomycin contained water was changed to antibiotics free. BM chimeric mice were used 6 wk after transfer. Chimerisms were confirmed by reverse transcription PCR of mRNA from splenic B cells, and by impaired cell surface expression of CD23 on B cells from *Mcoln1*$^{-/-}$ cells. Mice were maintained in the animal facility of the Institute of Medical Science, the

University of Tokyo (IMSUT). All animal experiments were approved by the Institutional Animal Care and Use Committee.

2.2 Cells

2.2.1 Splenic B cells enrichment.

Most leukocytes express CD43, in addition to granulocytes, macrophages, monocytes, natural killer cells. But resting mature and immature B cells are exception. The purification of splenic B cells is based on this principle. The whole spleen was grinded by slide glass. Debris was filtered off leaving only the cells. Cells were treated with 1ml of room temperature balanced Red Blood Cell lysis buffer (BD PharmLyse BD Science) 1 min, then stopped by 4 ml Fetal Bovine Serum (Thermo). After centrifugation, flicked the cell clump and suspended by RPMI1640 complete culture medium (RPMI1640 (Gibco), 10 % FBS (Thermo), 100 units/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml glutamine (Gibco), 50 μM Beta-2-mercaptoethanol (Nacalai Tesque)). Determined the cells number, spun down the cells then resuspended the cell pellet in 90 μl MACS buffer (2 % FBS, 2 mM EDTA, PBS) per 10^7 cells together with 10 μl CD43 (Ly-48) MicroBeads (Miltenyi Biotec) incubated 15 min at 4°C. Washed cells by

MACS buffer, then proceeded to magnetic separation. Purity of B cell was evaluated by FACSCalibur (BD Biosciences).

2.2.2 Bone marrow derived conventional dendritic cells (BM-cDCs) and plasmacytoid dendritic cells (BM-pDCs).

Mice femur and tibia were cut off in sterile condition. 10 ml syringe and 26 mm needle were used to wash out bone marrow. Bone marrow (BM) cells were treated with Red Blood Cell lysis buffer (BD PharmLyse BD Science). 10×10^6 cells were cultured in RPMI1640 complete culture medium (RPMI1640 (Gibco), 10 % FBS (Thermo), 100 units/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml glutamine (Gibco), 50 μM Beta-2-mercaptoethanol (Nacalai Tesque)) with 10 ng/ml GM-CSF (Peprotech). On the day 7, only floating cells were harvested. 15×10^6 cells were culture in RPMI1640 complete culture medium (RPMI1640 (Gibco), 10 % FBS (Thermo), 100 units/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml glutamine (Gibco), 50 μM Beta-2-mercaptoethanol (Nacalai Tesque)) with 100 ng/ml FLT3 ligand (Peprotech). On the day 7, both floating cells and attached cells were harvested, and sorted by FACSAria Sorting (BD Bioscience).

2.3 ELISA

1×10^5 per well of BM-cDCs or BM-pDCs were seeded in 96 flat bottom wells plate (Falcon), 24 hr stimulation with TLRs ligands. For mucolipin agonist treatment, cells were pre-incubated in 10 μM ML1-SA1 (Calbiochem) for 30 min, followed by stimulating with TLRs ligands. For PIKfyve inhibition assay, cells were pre-incubated in PIKfyve inhibitor (YM201636 (Calbiochem)) for 15 min, followed by stimulating with TLRs ligands. For transfection reagents complex nucleic acids stimulation, ssRNA, CpG-B and CpG-A were packaged with lipofactamine 2000 (Invitrogen). Transfection methods were based on the manual of lipofactamine 2000. Supernatants of samples were harvested to detect inflammatory cytokines such as IL-6, TNF-α and IFN-α.

Serum samples from BM chimeric mice were obtained 4 days before R848 injection as background control (Pre-serum). 2 μg/ 200 μl per mouse of R848 was intravenously injected through vena ophthalmica. After 2 hr, serum samples were collected from facial blood. Sera or supernatants were diluted by Reagent Diluent (1 % BSA (Sigma), 1 % Sucrose (Wako)) relied on cytokine production levels and stimuli. IL-6 and IL-12p40 was detected from serum samples.

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), IL-12p40 was determined by Duo Set ELISA system (R&D systems). Samples plates were analyzed by iMark Microplate Absorbance Reader (BIO-RAD).

2.4 Immunoprecipitation and immunoblotting

Toll like receptors processing were utilized co-immunoprecipitaiton and western blotting. 2×10^7 BM-cDCs were lysed in lysis buffer (1 % Triton X100, 20 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 % Glycerol, Complete Inhibitor (Roche)) for 30 min on ice. Anti-TLR7- or anti-TLR9-coupled agarose beads were supplied to the supernatant and rotated for 2 hr at 4°C. After incubation, the beads were washed by washing buffer (0.1 % Triton X100, 20 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 % Glycerol) and mixed with 2× sample buffer. The samples were boiled for 5 min at 96°C and subjected to SDS-PAGE. After electrophoresis, proteins in the gel were transferred on PVDF membrane, and the membrane was blocked with 3 % skim milk (Difco) in TBST for 1 hr 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).

2.6 FACSCalibur and FACSAria Sorting

During B cell differentiation, maturation and migration, specific markers expressed on cell surface. These processes are measured based on detection of cell surface markers. 3×10^5 whole spleen cells and peripheral blood mononucleated cells were separated into 96 wells plate. Washed by pre-cold FACS buffer (2.5 % FBS, 0.1 % Sodium Azide, 1×PBS) The isotype of antibodies we use were（eBioscience）mouse IgG1-Kappa, thus IgG1 Fc receptor blocking with anti-Fc gamma receptor (CD16/32 eBioscience) was necessary in ahead. Followed by staining with fluorescent dyes labeled antibodies (eBioscience) (Table 2). Samples were evaluated by BD FACSCalibur Flowcytometor (BD Biosciences). Biotin conjugated TLRs antibodies were generated by the Division of Infectious Genetics, IMSUT.

FLT3 ligand induced BM-pDC were sorted by B220 and CD11c double positive population. FACSAria sorting was exerted by FACSAria co-laboratory (IMSUT).

Table 2. Antibodies for FACS analysis.

eBioscience	B220, CD16/32, CD11b, CD11c, CD21, CD23, CD43
Division of Infectious Genetics, IMSUT.	BioA94 (TLR7), BioJ15A7 (TLR9)

2.7 Trypan blue quenching

In order to measure the internalization of ssRNA, trypan blue quenching assay was used. 3×10^5 BM-cDCs were seeded in round bottom 96 wells plates. FITC-conjugated RNA9.2s-DR was incubated with BM-cDCs for 1 hr and 2 hr at 37°C. Cells were briefly washed by 1×PBS one time, and resuspend in 1×PBS or in 2 mg/ml Trypan Blue Stain 0.4 % (Gibco). Fluorescence was evaluated by FACSCalibur (BD Biosciences).

2.8 Confocal Microscopy

2×10^5 BM-cDCs were seeded on 4 compartments 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 ML-SA1, and incubated with CpG-A-rhodamine, CpG-B-rhodamine, Poly U-rhodamine or RNA9.2s-DR-rhodamine for 90 min at 37°C. Supernatant containing ligands were removed and supplemented with LysoTracker® Green DND-26 (Life technologies),

incubated for 30 min at 37°C. Imaging is performed by LSM710 confocal microscope with a ×63 NA1.4 Plan-Apochromat oil immersion lens (Carl Zeiss Microscopy). Fluorescent images were analysed by LSM710 ZEN software.

2.9 Ligands

Toll like receptors specific ligands (Table 3) were used for analyzing the essential role of mucolipin-1 in TLRs function.

Table 3. Toll like receptors ligands.

Sigma-Aldrich	Lipid A (*S.minnesota* <Re-595>)	TLR4
Invivogen	Imiquimod (R837)	TLR7
	Loxoribine	
	R848	
FASMAC	Poly U (uuuuuuuuuuuuuuuuuuu)	
	RNA9.2s-DR (uguccuucaauguccuucaa)	
Hokkaido System Science	CpG-B 1668 (tccatgacgttcctgatgct)	TLR9
	CpG-A 1585 (ggggtcaacgttgaggggggg)	

2.10 Statistical analysis

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

3. Results

3.1 No alteration in maturation of bone marrow derived conventional dendritic cells (BM-cDCs) and bone marrow derived plasmacytoid dendritic cells (BM-pDCs) from *Mcoln1*$^{-/-}$ mice.

BM cells were allowed to differentiate into BM-cDCs or BM-pDCs and stained with the maturation markers. BM-cDCs maturation was not altered by mucolipin-1 in terms of similar cell surface expression of CD11c in *Mcoln1*$^{-/-}$ BM-cDCs (Fig. 3A). Cell surface expression of CD11c and B220 was not altered in *Mcoln1*$^{-/-}$ BM-pDCs indicating that BM-pDCs maturation was not effected by mucolipin-1 (Fig. 3B).

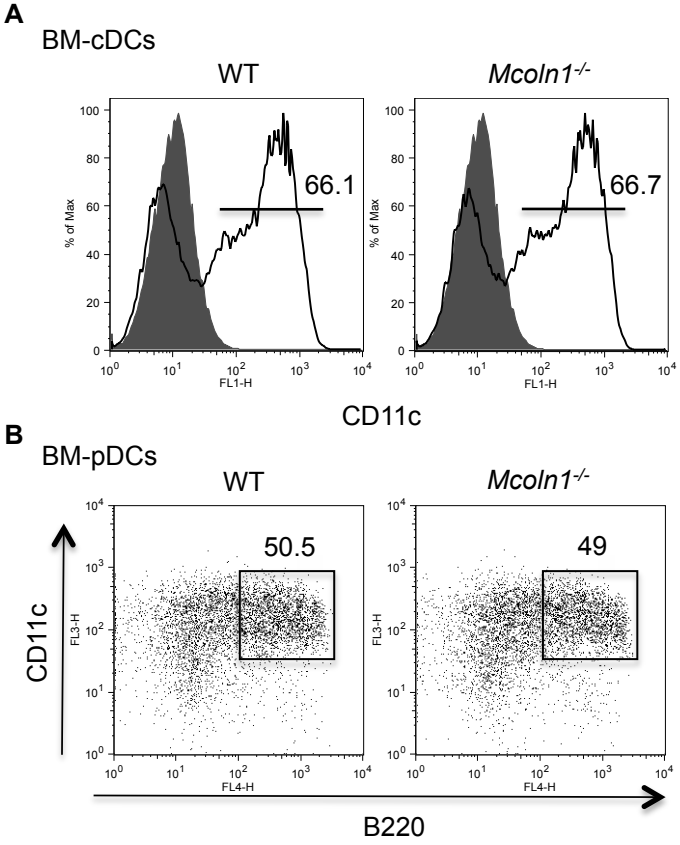


Figure 3. No alteration in maturation of BM-cDCs and BM-pDCs from *Mcoln1*$^{-/-}$ mice. (A) Open histograms showed CD11c expression on WT or *Mcoln1*$^{-/-}$ BM-cDCs. Closed histograms show staining with the second reagent alone. (B) Dot plots showed cell surface expression of CD11c and B220 on WT or *Mcoln1*$^{-/-}$ BM-pDCs. The gates are used for sorting BM-pDCs. The data were representative of three independent experiments.

3.2 Impaired Toll like receptor 7 and 9 responses in *Mcoln1*$^{-/-}$ bone marrow derived conventional dendritic cells (BM-cDCs).

To study roles of Mucolipin-1 in TLR7 and TLR9 responses, GM-CSF induced BM-cDCs from WT and *Mcoln1*$^{-/-}$ 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. 4A and B). On the other hand, partial, but significant reductions were found in responses to a TLR7 ligand ssRNA (RNA9.2s-DR) and a TLR9 ligand CpG-A. At lower concentration of TLR7 ligands R848 and loxoribine, *Mcoln1*$^{-/-}$ BM-cDCs showed significantly lower responses than WT BM-cDCs. The specificity of TLR7 ligands were verified in *Tlr7*$^{-/-}$ BM-cDCs (Fig. 4A and B).

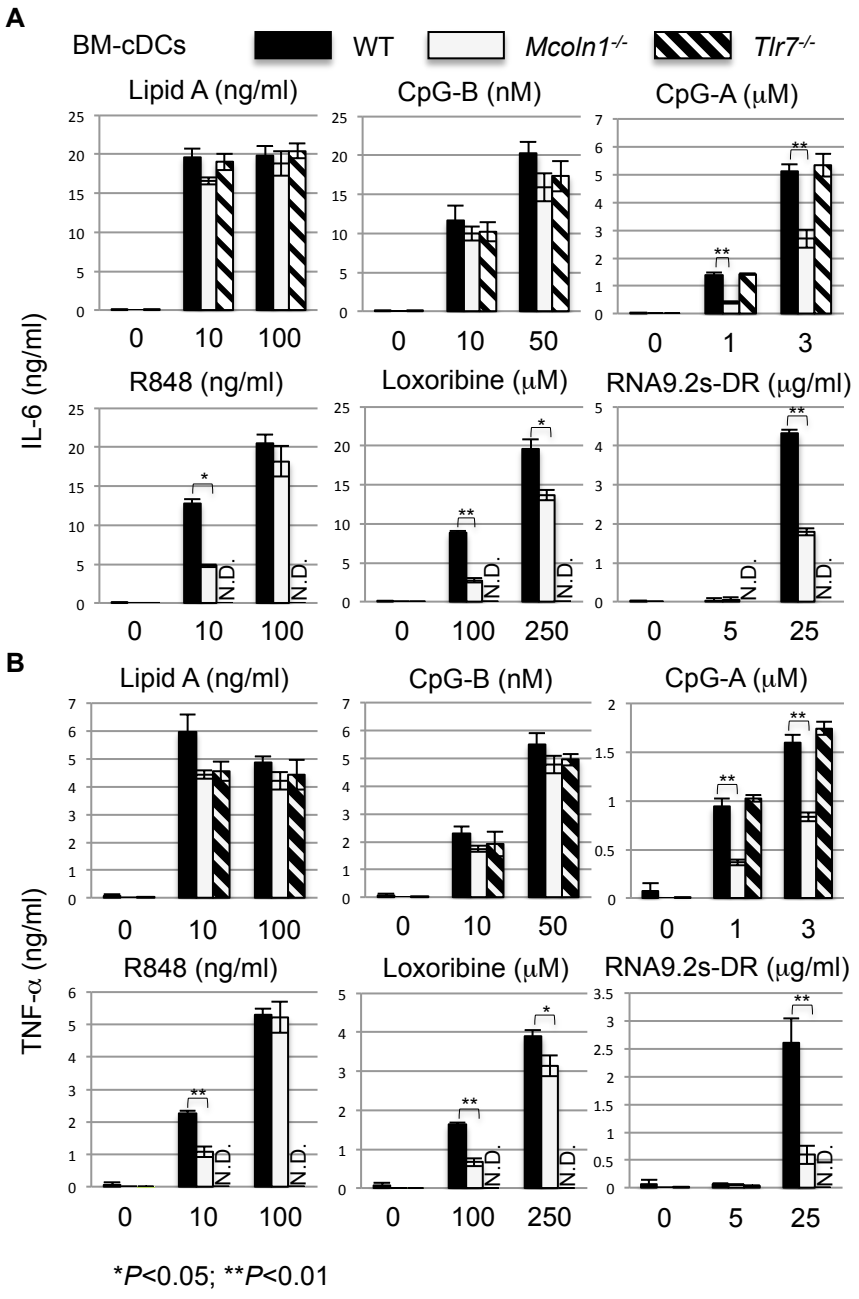


Figure 4. TLR7 and 9 responses are impaired in *Mcoln1*$^{-/-}$ BM-cDCs. WT, *Mcoln1*$^{-/-}$ or *Tlr7*$^{-/-}$ BM-cDCs were stimulated with TLR ligands for 24 hr. Production of IL-6 (A) or TNF-α (B) was determined by ELISA. The results were represented by the mean value with SD from triplicate wells. N.D., Not Detected. **P*<0.05; ***P*<0.01. The data were representative of three independent experiments.

3.3 Impaired Toll like receptor 7 and 9 responses in *Mcoln1*$^{-/-}$ bone marrow derived plasmacytoid dendritic cells (BM-pDCs).

FLT3 induced BM-pDCs were next stimulated with TLR7 and TLR9 ligands. Production of IL-6, TNF-α and IFN-α was evaluated by ELISA. *Mcoln1*$^{-/-}$ BM-pDCs showed impaired TLR7 responses to loxoribine and imiquimod (Fig. 5 and 6). TLR7 ligand Poly U induced TNF-α and IFN-α but not IL-6. *Mcoln1*$^{-/-}$ BM-pDCs showed partial but significant impairment in Poly U-dependent TNF-α and IFN-α production (Fig. 5 and 6). In TLR9 responses to CpG-A, only IFN-α production was partially impaired (Fig. 6). However, TLR9 responses to CpG-B were not altered at all (Fig. 5A and B). The specificity of TLR7 ligands were proved in *Tlr7*$^{-/-}$ BM-cDCs (Fig. 5 and 6).

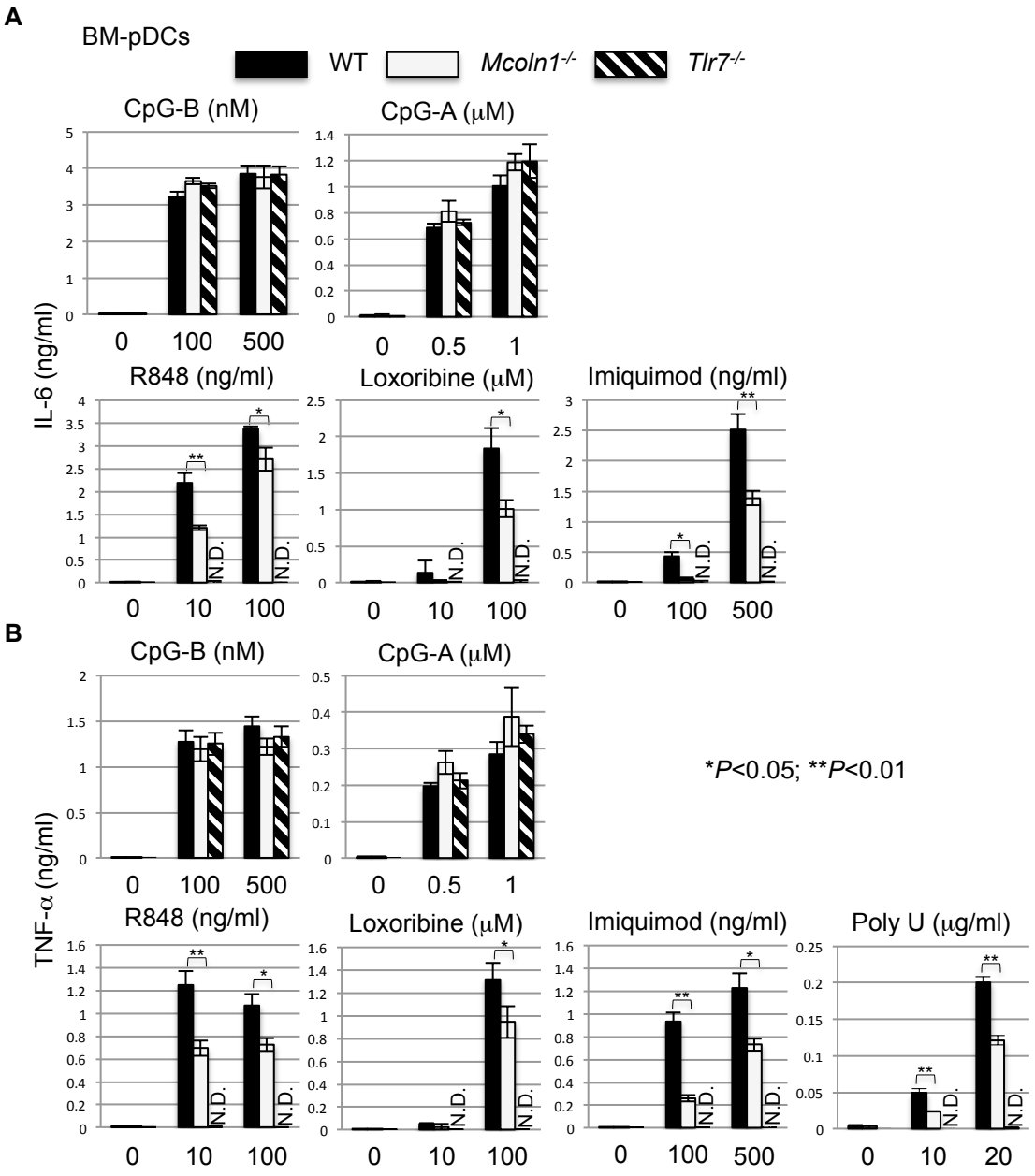


Figure 5. Impaired TLR7 responses in *Mcoln1*$^{-/-}$ BM-pDCs. WT, *Mcoln1*$^{-/-}$ or *Tlr7*$^{-/-}$ BM-pDCs were stimulated with TLR7 and 9 ligands for 24 hr. Production of IL-6 (A), TNF-α (B) was determined by ELISA. The results are represented by the average values with SD. N.D., Not Detected. **P*<0.05; ***P*<0.01. The data were representative of three independent experiments.

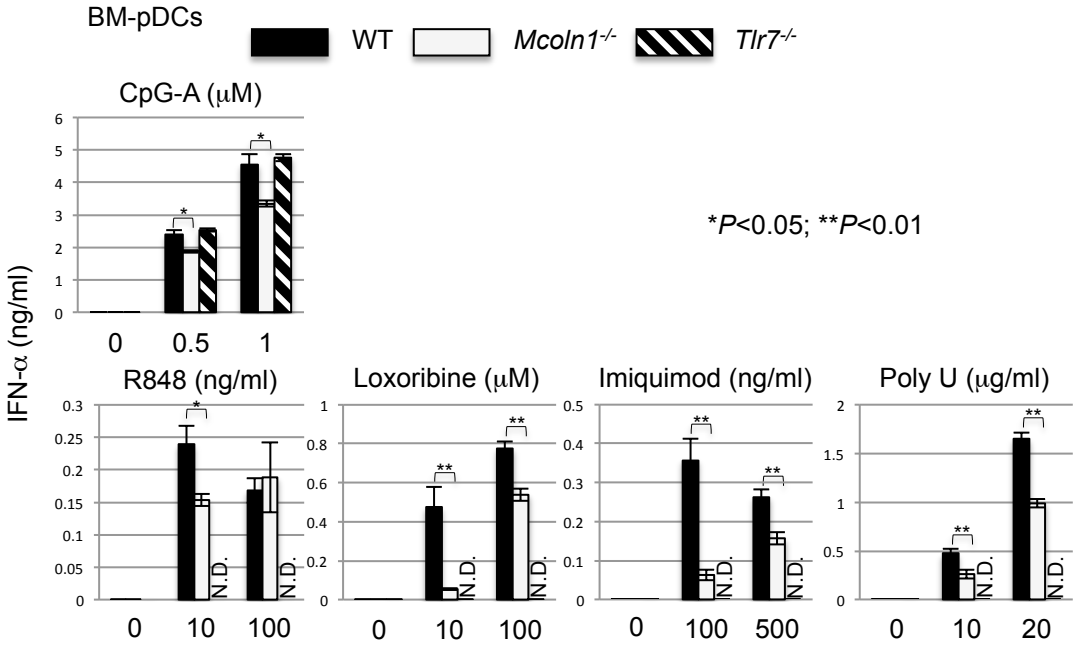


Figure 6. Impaired TLR7 and TLR9 responses in *Mcoln1*$^{-/-}$ BM-pDCs. WT, *Mcoln1*$^{-/-}$ or *Tlr7*$^{-/-}$ BM-pDCs were stimulated with TLR7 and 9 ligands for 24 hr. Production of IFN-α was determined by ELISA. The results are represented by the average values with SD. N.D., Not Detected. **P*<0.05; ***P*<0.01. The data were representative of three independent experiments.

3.4 Mucolipin agonist specifically enhances Toll like receptor 7 responses to single stranded RNA (ssRNA) in bone marrow derived conventional dendritic cells (BM-cDCs).

Results shown in *Mcoln1*$^{-/-}$ BM-cDCs and BM-pDCs (Fig. 4, 5 and 6) suggest that mucolipin-1 has a role in TLR7 and TLR9 responses. To confirm that mucolipin-1 is required for TLR7 and TLR9 responses, BM-cDCs were stimulated with TLR7 and TLR9 ligands in the presence of a synthetic membrane permeable dihydroquinolinyloxo-isoindolinedione compound, which is shown to be mucolipin agonist, ML1-SA1. ML1-SA1 synergistically induces cytosolic Ca^{2+} increase with an endogenous mucolipin ligand PtdIns(3,5)P_2 [45, 59-61]. ML1-SA1 enhanced TLR7 responses to ssRNA (RNA9.2s-DR and Poly U), but not to small chemical ligands (R848 and loxoribine) in BM-cDCs, indicating mucolipin-1 has a role in regulating ssRNA recognition by TLR7 (Fig. 7).

On the contrary, TLR9 responses to CpG-A were partially attenuated by ML1-SA1, indicating that mucolipin-1 may have multiple purposes in regulating CpG-A responses (Fig. 7). TLR9 responses to CpG-B were not altered at all by ML1-SA1, indicating

CpG-B responses are not required mucolipin-1 (Fig. 7).

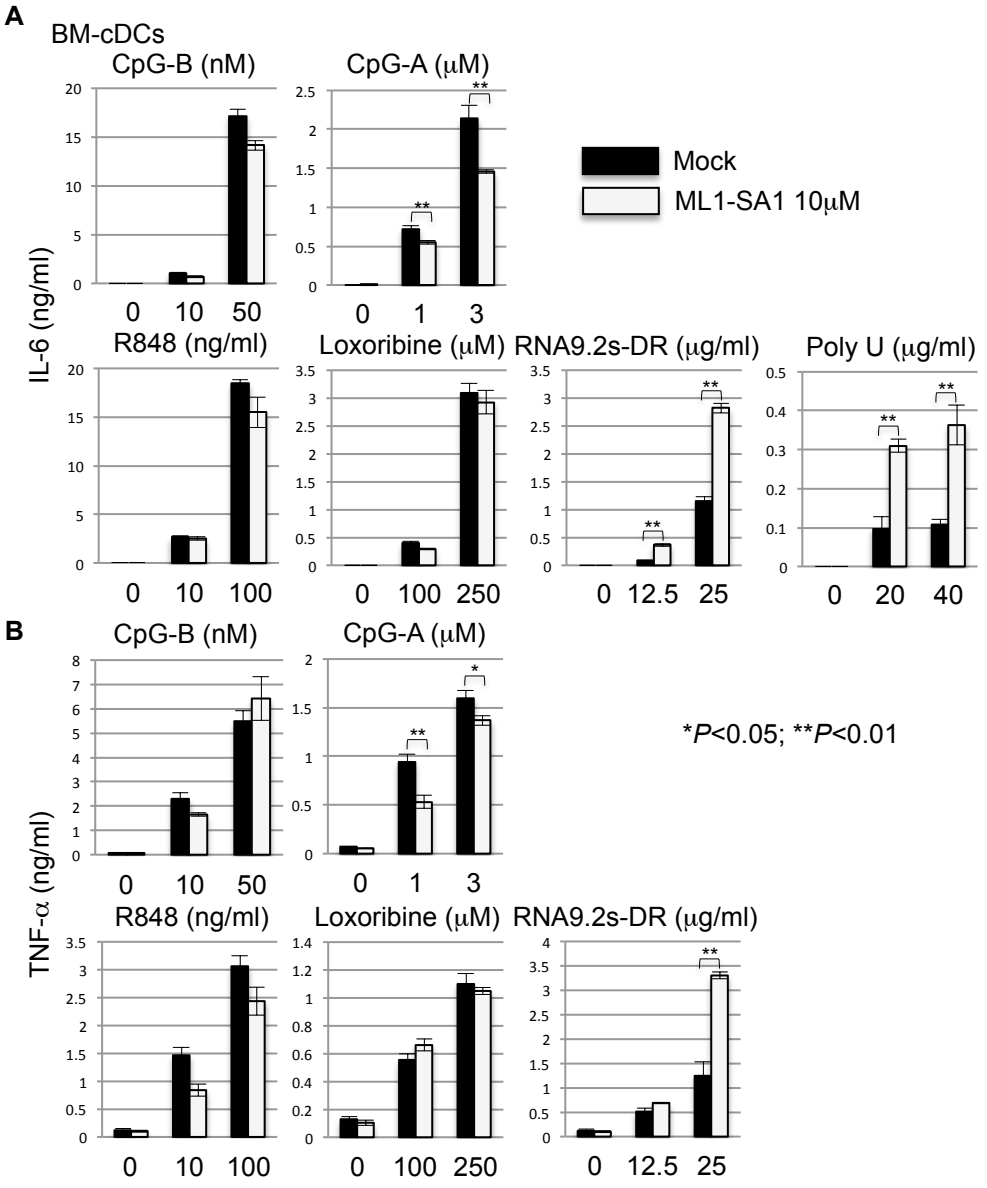


Figure 7. Mucolipin agonist specifically enhances TLR7 responses to ssRNA. BM-cDCs were pre-treated with DMSO (Mock) or 10 μM ML1-SA1 for 30 min, and then stimulated with TLR7 and 9 ligands at indicated concentrations. After 24 hr stimulation, supernatants were harvested, and production of IL-6 (A) or TNF-α (B) was determined by ELISA. The results are represented by the average values with SD. **P*<0.05; ***P*<0.01. The data were representative of three independent experiments.

3.5 Toll like receptor 7 responses to single stranded RNA (ssRNA) is sensitive to PIKfyve inhibitor.

ML1-SA1 synergistically acts on mucolipin-1 with PtdIns(3,5)P_2 [45]. PtdIns(3,5)P_2 is converted by PIKfyve from PtdIns(3)P [62]. PIKfyve complexes with VAC14 and FIG4, which are essential in PIKfyve enzyme activity [63, 64]. The *FIG4* gene, encoding phosphatidylinositol 3,5-bisphosphate 5-phosphatase in human, is important in endosomal trafficking [65]. *FIG4*$^{-/-}$ cells are similar to *Mcoln1*$^{-/-}$ cells in showing enlarged endolysosomes [66]. I reason that PIKfyve may regulate TLR7 and 9 responses. To address this possibility, BM-cDCs were stimulated with TLR7 and TLR9 ligands in the presence of PIKfyve inhibitor YM201636. As reported previously [44], CpG-B responses were significantly but weakly suppressed by PIKfyve inhibitor (Fig. 8). Interestingly, CpG-B induced TNF-α production was more resistant to YM201636 than IL-6 production (Fig. 8B). Much clearer inhibitory effect of PIKfyve inhibitor was found in CpG-A induced cytokine production (Fig. 8). Further and complete inhibition was observed in TLR7 responses to ssRNA, cytokine production induced by ssRNA was dramatically decreased by YM201636 in dose dependent manner (Fig. 8). These

results suggested that in BM-cDCs, PIKfyve-dependent generation of PtdIns(3,5)P_2 was more required for TLR7 responses to ssRNA and TLR9 responses to CpG-A than TLR9 responses to CpG-B.

Interestingly, R848 induced IL-6 and TNF-α production was not effected by PIKfyve inhibitor (Fig. 8), suggesting that PIKfyve kinase may have different roles in regulating TLR7 responses in BM-cDCs.

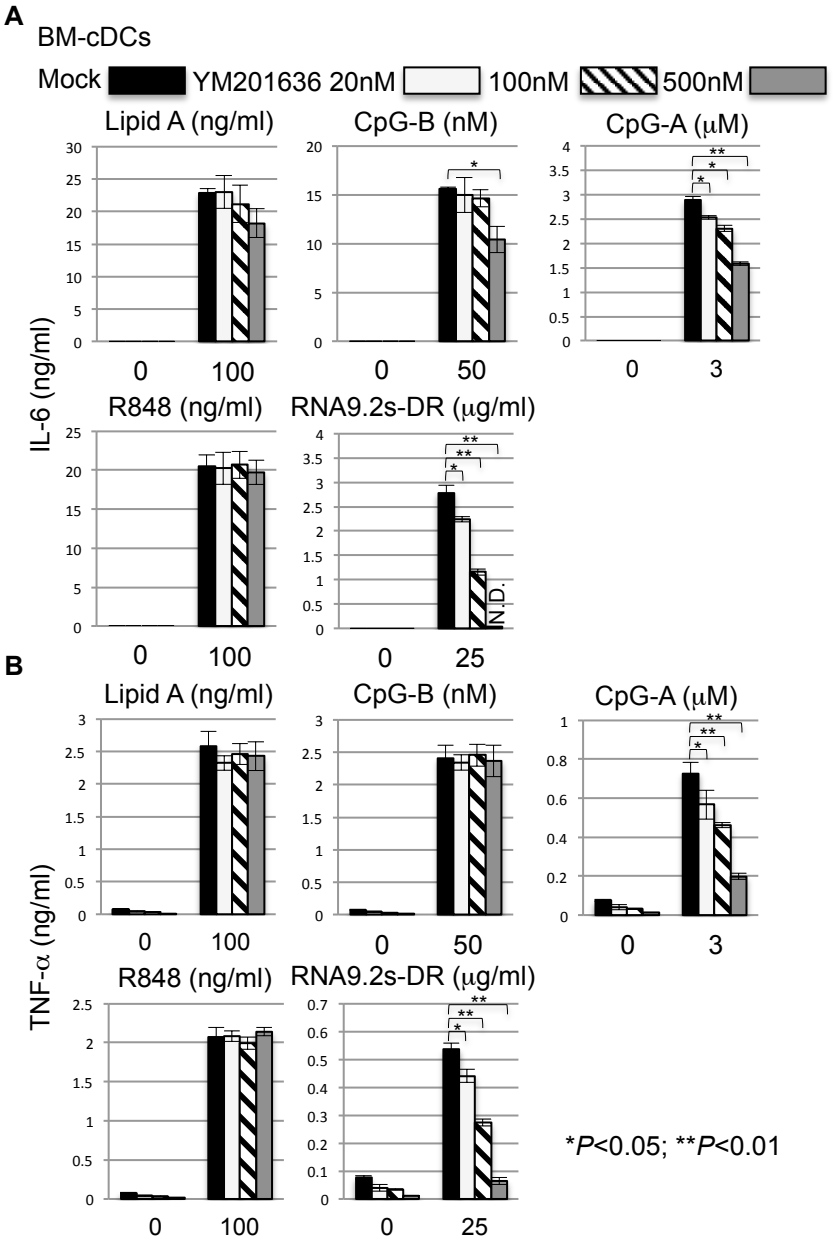


Figure 8. TLR7 responses to ssRNA in BM-cDCs is sensitive to PIKfyve inhibitor. BM-cDCs were pre-treated with DMSO (Mock) or PIKfyve inhibitor YM201636 at indicated concentrations for 15 min, and then stimulated with TLR7 and 9 ligands. After 24 hr, supernatants were harvested, and concentration of IL-6 (A) and TNF-α (B) was determined by ELISA. The results are represented by the average values with SD. N.D., Not Detected. **P*<0.05; ***P*<0.01. The data were representative of three independent experiments.

The inhibitory effect of PIKfyve inhibitor on BM-pDCs was next studied. Consistent with the results in BM-cDCs, cytokine production induced by ssRNAs was impaired by the PIKfyve inhibitor in BM-pDCs (Fig. 9). Interestingly, TLR7 response to a small chemical ligand R848 was impaired significantly in IFN-α production, but not in TNF-α production, by PIKfyve inhibitor (Fig. 9), suggesting that PtdIns(3,5)P_2 generation by PIKfyve is specifically required for R848-dependent IFN-α production.

Collectively, these data indicate that a PtdIns(3,5)P_2-mucolipin-1 axis is required for TLR7 responses to ssRNA.

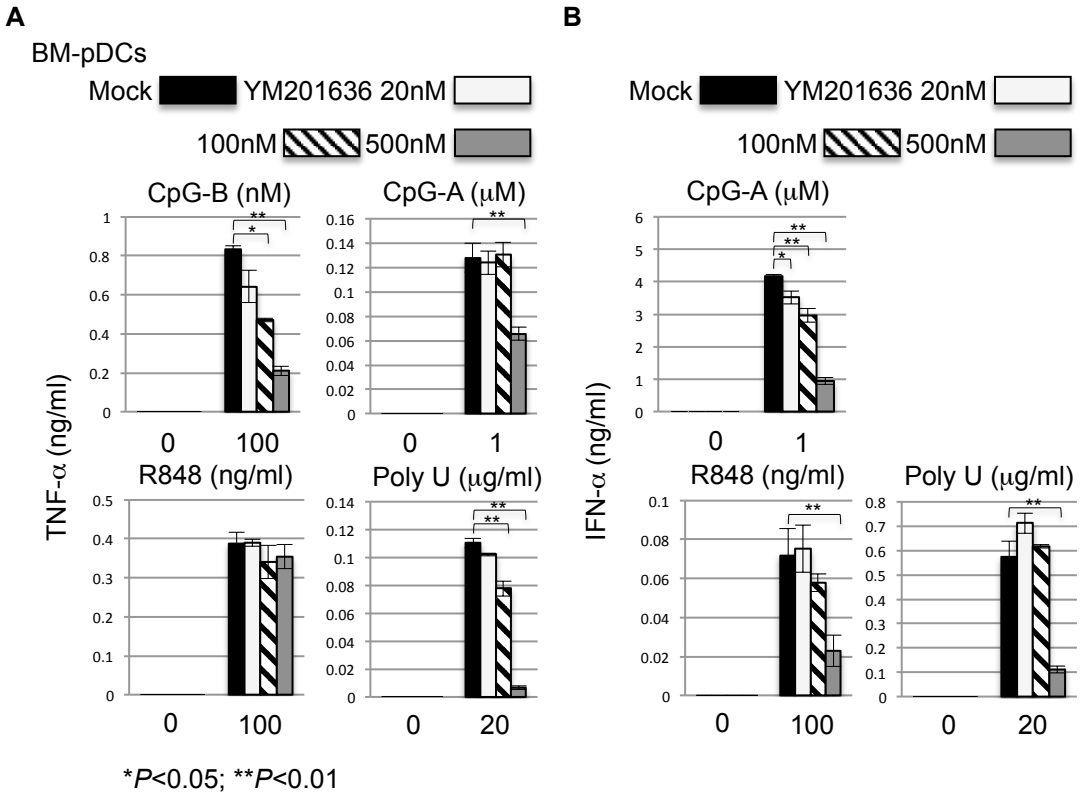


Figure 9. TLR7 responses to ssRNA in BM-pDCs is sensitive to PIKfyve inhibitor. BM-pDCs were pre-treated with DMSO (Mock) or PIKfyve inhibitor YM201636 at indicated concentrations for 15 min, then stimulated with TLR7 ligands. After 24 hr, supernatants were harvested, and concentration of TNF-α (A) and IFN-α (B) was determined by ELISA. The results are represented by the average values with SD. N.D., Not Detected. **P*<0.05; ***P*<0.01. The data were representative of three independent experiments.

3.6 Toll like receptor 7 responses to lipofectamine-complexed single stranded RNA (ssRNA) are not altered by PIKfyve inhibitor in bone marrow derived conventional dendritic cells (BM-cDCs) and bone marrow derived plasmacytoid dendritic cells (BM-pDCs).

Transfection reagents such as lipofectamine promote the uptake of RNA/DNA and facilitate RNA/DNA transportation to TLRs compartments, therefore enhance TLRs responses [67]. Given that the PtdIns(3,5)P_2-mucolipin-1 axis is required for nucleic acids transportation, TLR7 and TLR9 might be able to respond to lipofectamine-complexed nucleic acids even in the absence of the PtdIns(3,5)P_2-mucolipin-1 axis. To examine this possibility, BM-cDCs and BM-pDCs were stimulated with lipofectamine-complexed nucleic acid ligands in the presence of PIKfyve inhibitor. Lipofectamine was able to rescue TLR7 dependent cytokine production in BM-cDCs (Fig. 10A) and BM-pDCs (Fig. 10B). In contrast, lipofectamine failed to rescue CpG-A responses in BM-cDCs (Fig. 10A) and BM-pDCs (Fig. 10B). These results support a possibility that PIKfyve inhibitor impaired ssRNA responses by acting on ssRNA transportation.

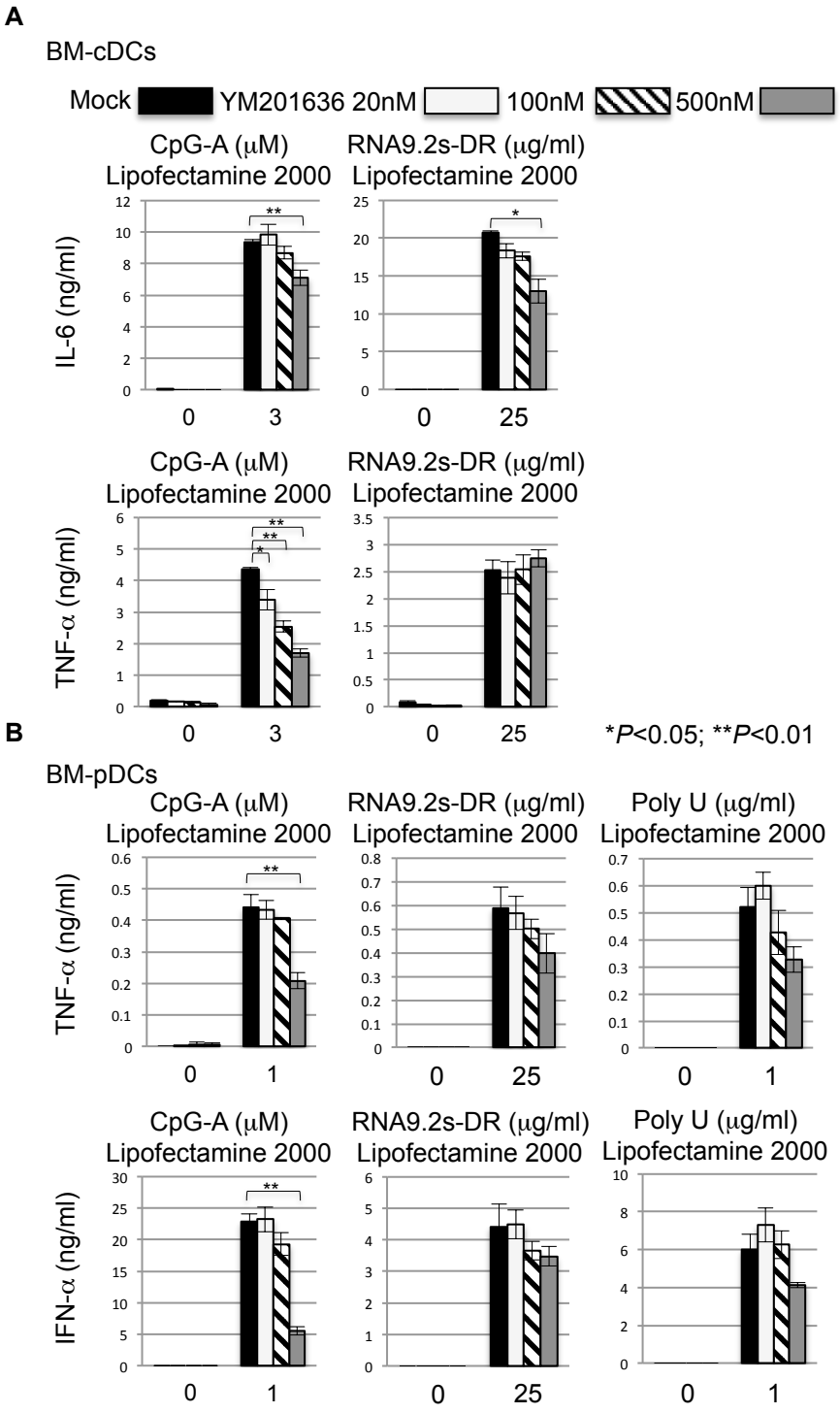


Figure 10. TLR7 responses to lipofectamine-complexed ssRNA are not altered by PIKfyve inhibitor in BM-cDCs and BM-pDCs. BM-cDCs (A) and BM-pDCs (B) were pre-treated with DMSO (Mock) or PIKfyve inhibitor YM201636 at indicated concentrations for 15 min, then stimulated with lipofectamine-complexed nucleic acid ligands. After 24 hr, supernatants were harvested, and concentration of IL-6, TNF-α and

IFN-α was determined by ELISA. The results are represented by the average values with SD. *P<0.05; **P<0.01. The data were representative of three independent experiments.

3.6 Toll like receptor 7 responses to lipofectamine-complexed single stranded RNA (ssRNA) are not altered in *Mcoln1*$^{-/-}$ bone marrow derived conventional dendritic cells (BM-cDCs) and bone marrow derived plasmacytoid dendritic cells (BM-pDCs).

Whether lipofectamine-complexed ssRNA could rescue the TLR7 responses in *Mcoln1*$^{-/-}$ BM-pDCs and BM-cDCs were next studied. Lipofectamine-complexed RNA9.2s-DR or Poly U induced cytokine productions in *Mcoln1*$^{-/-}$ BM-cDCs (Fig. 11A) and BM-pDCs (Fig. 11B) were comparable to those in WT BM-cDCs and BM-pDCs. These results support a possibility that the ligand trafficking rather than sensor trafficking is impaired in *Mcoln1*$^{-/-}$ BM-cDCs.

In contrast, lipofectamine-complexed CpG-A induced IFN-α production was partially reduced in *Mcoln1*$^{-/-}$ BM-pDCs (Fig. 11B), which was similar to naked CpG-A responses (Fig. 6). Production of IL-6 and TNF-a induced by CpG-A with or without lipofectamine was altered in *Mcoln1*$^{-/-}$ BM-pDCs (Fig. 11B). These data indicate that mucolipin-1 may specifically regulate IFN-α production induced by CpG-A.

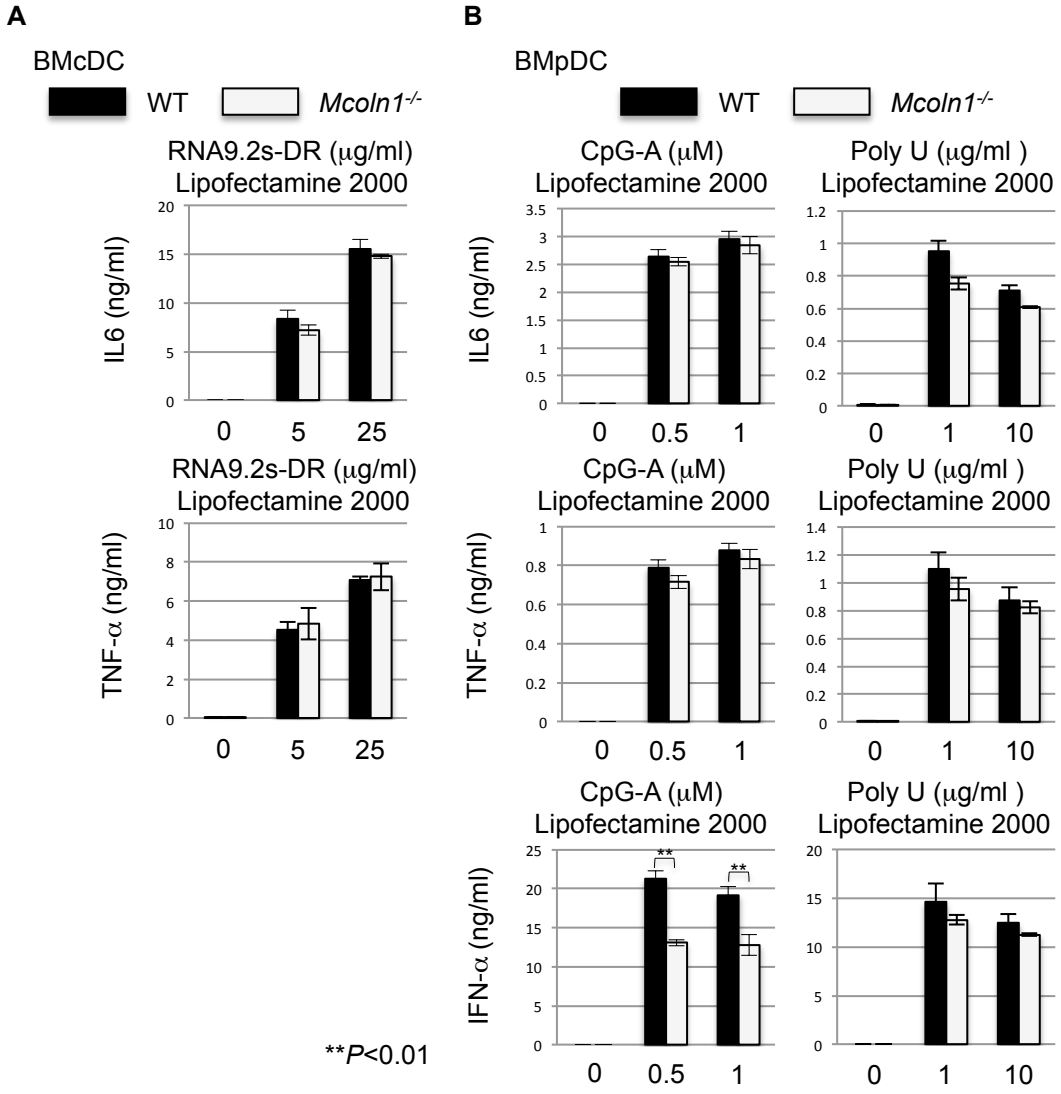


Figure 11. TLR7 responses to lipofectamine-complexed ssRNA are not altered in *Mcoln1*$^{-/-}$ BM-cDCs and BM-pDCs. WT or *Mcoln1*$^{-/-}$ BM-cDCs (A) and BM-pDCs (B) were stimulated with lipofectamine-complexed nucleic acid ligands for 24 hr. Production of IL-6, TNF-α in BM-cDCs and production of IL-6, TNF-α, IFN-α in BM-pDCs was determined by ELISA. The results are represented by the average values with SD. $^{**}P<0.01$. The data were representative of three independent experiments.

3.7 Expression, processing and distribution of Toll like receptor 7 and 9 are not altered in *Mcoln1*$^{-/-}$ bone marrow derived conventional dendritic cells (BM-cDCs).

Considering that mucolipin-1 has roles in lysosomal trafficking, trafficking of TLR7 and 9 may be altered in *Mcoln1*$^{-/-}$ mice. To address this possibility, expression of these TLRs was studied. BM-cDCs were subjected to membrane-permeabilized staining with TLR7 or TLR9 mAb [6, 68]. Expressions of TLR7 and TLR9 were not altered in *Mcoln1*$^{-/-}$ BM-cDCs (Fig. 12A). Next, TLR7 and TLR9 were immunoprecipitated. Consistent with FACS analyses, no alteration was found in the amount of immunoprecipitated TLR7 and TLR9 (Fig. 12B and C). Moreover, proteolytic cleavage of TLR7 and TLR9 was not altered (Fig. 12B and C). Given that TLR7 and TLR9 are proteolytically cleaved in endolysosomes, trafficking of TLR7 and TLR9 from ER to endolysosomes is not likely to be altered in *Mcoln1*$^{-/-}$ BM-cDCs. To examine the distribution of TLR7, I performed a confocal microscopy analysis. Colocalization of TLR7 and LAMP2 was not altered in *Mcoln1*$^{-/-}$ BM-cDCs (Fig. 12D).

Collectively, these data indicate that under steady state, the expression, processing and distribution of TLR7 is not effected in *Mcoln1*$^{-/-}$ BM-cDCs. In addition, the

expression and processing of TLR9 is not altered in *Mcoln1*$^{-/-}$ BM-cDCs.

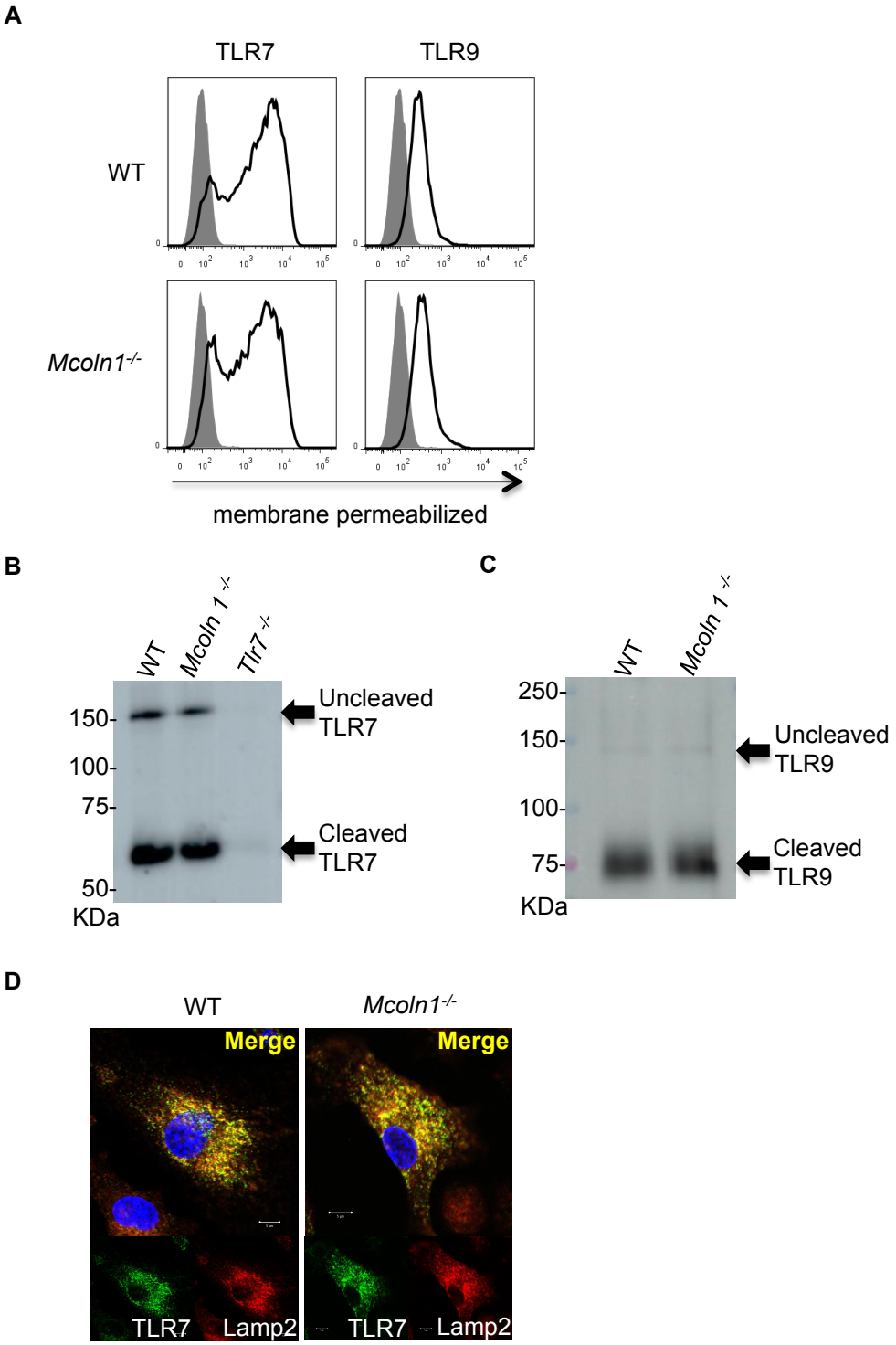


Figure 12. TLR7 or TLR9 processing was not altered in mucolipin-1 deficient BM-cDCs. (A) Open histograms showed membrane-permeabilized staining with anti-TLR7 or TLR9 mAb of WT and *Mcoln1*$^{-/-}$ BM-cDCs. Gray histograms show staining with the second reagent alone. (B, C) WT, *Mcoln1*$^{-/-}$ and *Tlr7*$^{-/-}$ BM-cDCs were subjected to immunoprecipitation with TLR7 mAb (B) or TLR9 mAb (C). Precipitates

were analyzed by immunoprobing with TLR7 or TLR9 polyclonal Ab. Uncleaved and C-terminal cleaved form is indicated with the arrows. The data were representative of three independent experiments. (D) BM-cDCs were counterstained with anti-TLR7 mAb (green) and followed by anti-mouse Alexa-488. Cells were counterstained with anti-LAMP2 (red) to locate the lysosome. Merged images were also shown. The cells were visualized by confocal microscopy. Scale bar, 5 μm.

3.8 Internalization of single stranded RNA (ssRNA) is not altered in *Mcoln1*$^{-/-}$ bone marrow derived conventional dendritic cells (BM-cDCs).

Results shown in Figure 12 led us to explore regulatory function of mucolipin-1 in ssRNA logistics. It is reported that mucolipin-1 has a regulatory function in endocytosis [60], I reason that mucolipin-1 may have roles in ssRNA internalization. WT and *Mcoln1*$^{-/-}$ BM-cDCs were incubated with FITC-labeled RNA9.2s-DR in a time dependent manner at 37°C. Fluorescence on the cell surface was quenched by trypan blue. Internalized ssRNA was analyzed by flow cytometry. No alteration was found between WT and *Mcoln1*$^{-/-}$ BM-cDCs (Fig. 13B). These results suggest that mucolipin-1 is dispensable for ssRNA internalization.

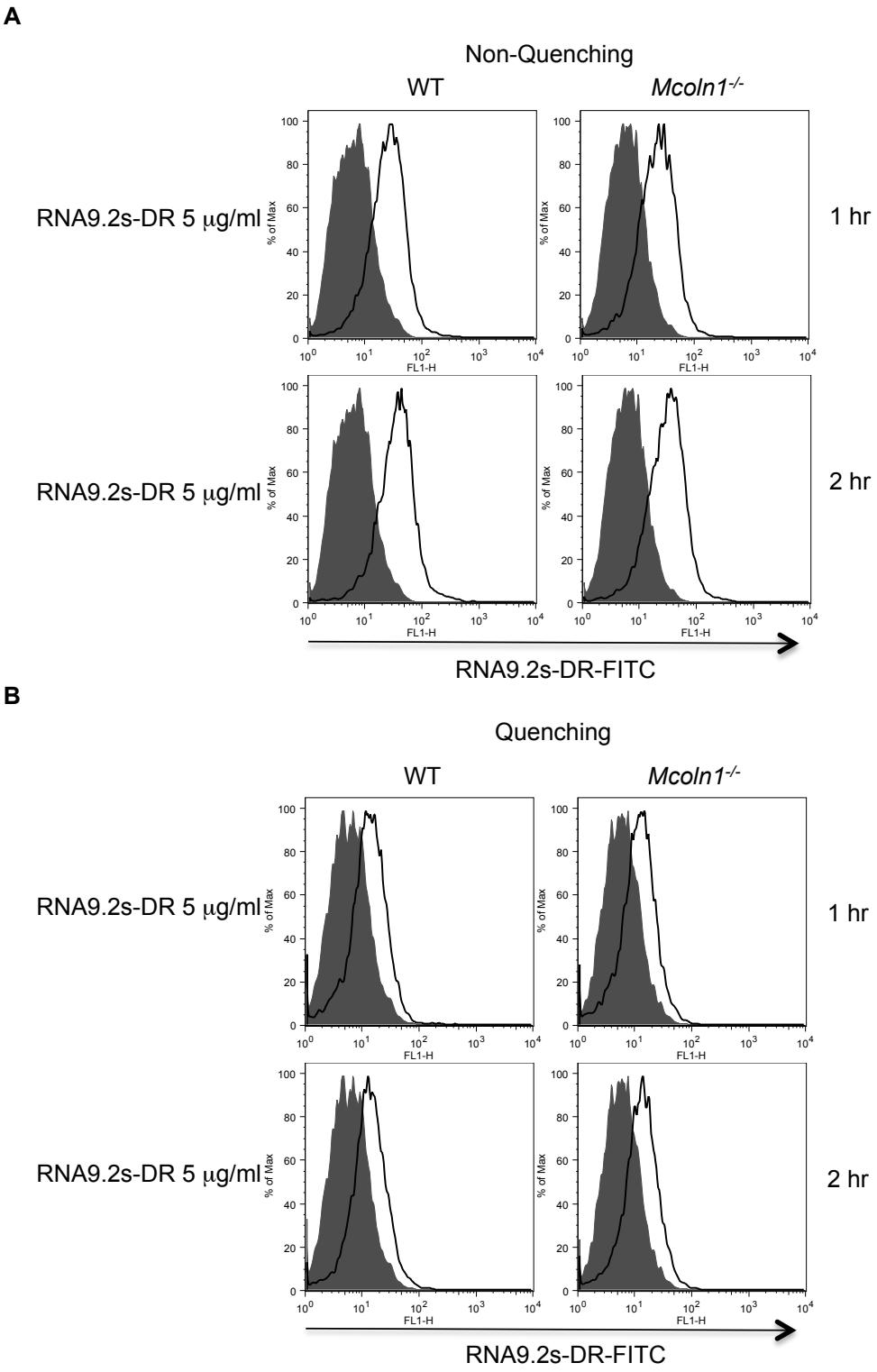


Figure 13. Endocytosis of ssRNA is not altered in *Mcoln1*$^{-/-}$ BM-cDCs. WT and *Mcoln1*$^{-/-}$ BM-cDCs were stimulated with 5 μg/ml RNA9.2s-DR-FITC in indicating time at 37°C. Fluorescence was measured by FACSCalibur without quenching (A) or quenching with 2 mg/ml of trypan blue (B).

3.9 Single stranded RNA (ssRNA) transportation to lysosomes is impaired in *Mcoln1*$^{-/-}$ bone marrow derived conventional dendritic cells (BM-cDCs).

Mucolipin-1 is a gene responsible for mucolipidosis type IV [69], in which metabolites accumulate in lysosomes due to altered lysosomal trafficking. I reason that nucleic acids might belong to metabolites that require mucolipin-1 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. Colocalization of these ligands with a lysosome marker lysotracker was studied by confocal microscopy. CpG-B showed very weak colocalization with lysotracker. *Mcoln1*$^{-/-}$ BM-cDCs did not show any alteration in CpG-B distribution (Fig. 14A). Similarly CpG-A colocalization with lysotracker was not altered in *Mcoln1*$^{-/-}$ BM-cDCs (Fig. 14B). In contrast to CpG-B and CpG-A, TLR7 ligands, RNA9.2s-DR and Poly U, showed high colocalization with lysotracker in WT BM-cDCs and the colocalization was significantly impaired in *Mcoln1*$^{-/-}$ BM-cDCs (Fig. 14C and D). These results indicate that an important role of mucolipin-1 in ssRNA trafficking into lysosomes but not in DNA trafficking into lysosomes. In addition, intracellular transportation of

ssRNA and DNA are likely to be regulated by different mechanisms.

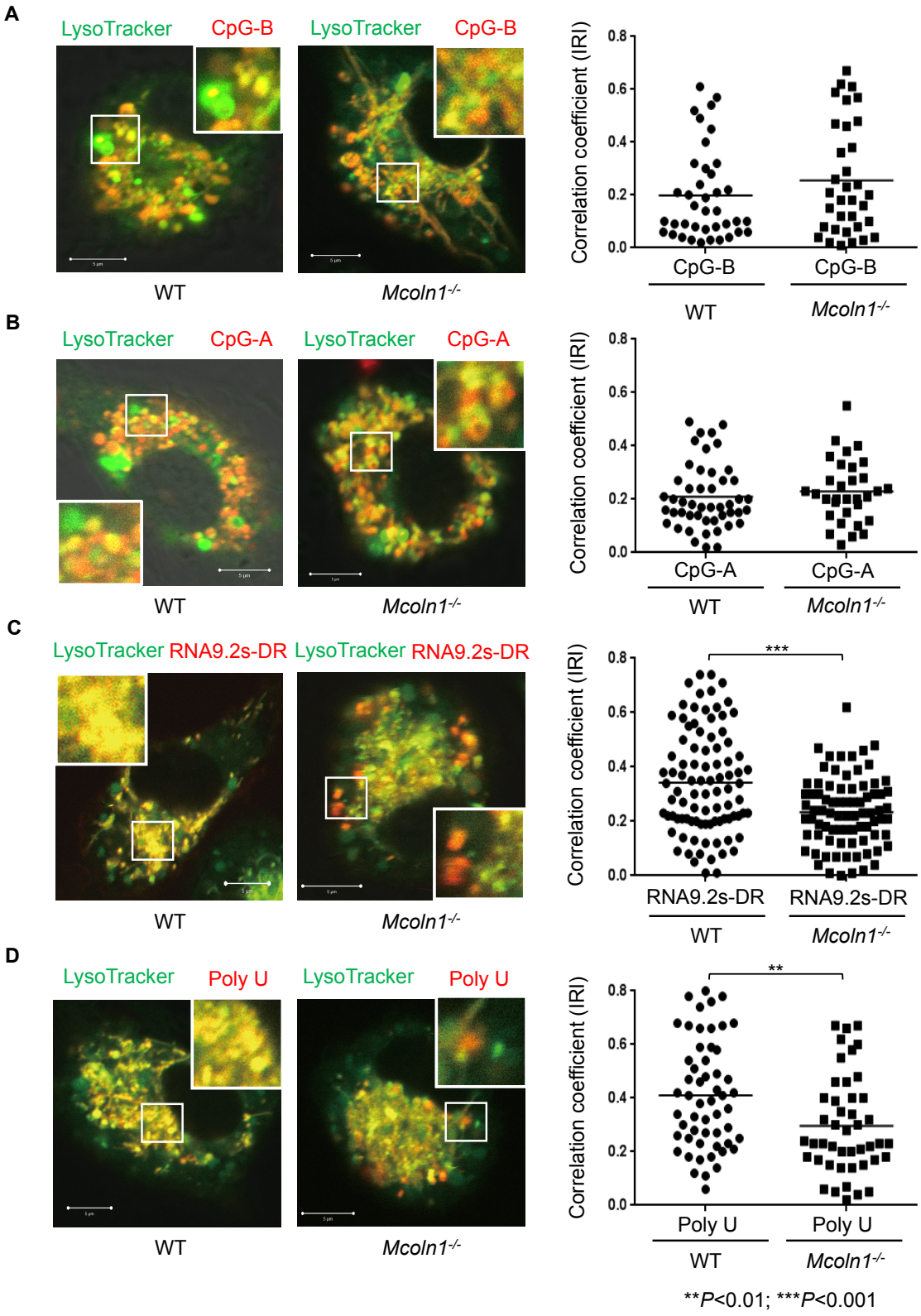

A
LysoTracker
CpG-B
LysoTracker
CpG-B
WT
Mcoln1-/-
Correlation coefficient (IRI)
0.8
0.6
0.4
0.2
0.0
CpG-B
CpG-B
WT
Mcoln1-/-
B
LysoTracker
CpG-A
LysoTracker
CpG-A
WT
Mcoln1-/-
Correlation coefficient (IRI)
CpG-A
CpG-A
WT
Mcoln1-/-
C
LysoTracker
RNA9.2s-DR
LysoTracker
RNA9.2s-DR
WT
Mcoln1-/-
***
Correlation coefficient (IRI)
RNA9.2s-DR
RNA9.2s-DR
WT
Mcoln1-/-
D
LysoTracker
Poly U
LysoTracker
Poly U
WT
Mcoln1-/-
**
Correlation coefficient (IRI)
Poly U
Poly U
WT
Mcoln1-/-
**P<0.01; ***P<0.001

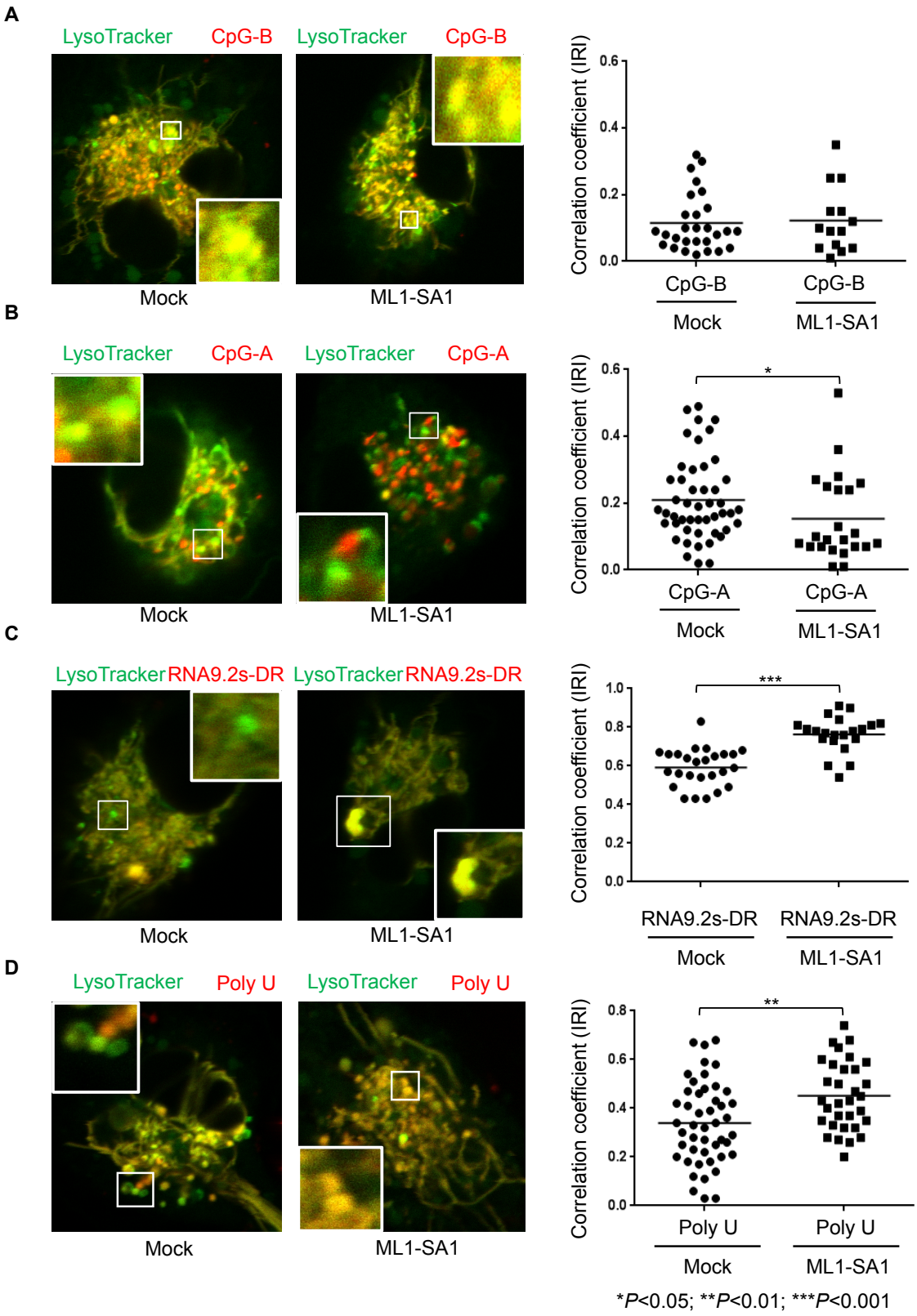
Figure 14. ssRNA transportation to lysosome is impaired in *Mcoln1*$^{-/-}$ BM-cDCs. WT and *Mcoln1*$^{-/-}$ BM-cDCs were stimulated with 1 μM CpG-B-rhodamine (A), 1 μM CpG-A-rhodamine (B), 10 μg/ml RNA9.2s-DR-rhodamine (C) or 10 μg/ml Poly U-rhodamine (D) for 90 min. To visualize lysosomes, cells were incubated with Lysotracker-Green for 30 min before analyses by confocal microscopy. Statistical analyses using correlation coefficient are also shown. ***P*<0.01; ****P*<0.001. Scale bar, 5 μm.

3.10 Single stranded RNA transportation to lysosomes is enhanced by mucolipin agonist ML1-SA1 in bone marrow derived conventional dendritic cells (BM-cDCs).

Mucolipin agonist ML1-SA1 enhances lysosomal exocytosis [60]. Considering that TLR9 responses to CpG-A were impaired by mucolipin agonist ML1-SA1 in BM-cDCs (Fig. 7), the transportation of CpG-A to lysosome may be altered. To address this possibility, ML1-SA1 pre-treated BM-cDCs were stimulated with rhodamine-labeled CpG-B and CpG-A. ML1-SA1 did not alter the colocalization of CpG-B with lysotracker (Fig. 15A). However, ML1-SA1 significantly impaired the colocalization of CpG-A with lysotracker in BM-cDCs were (Fig. 15B). These results suggest that mucolipin-1 negatively regulates CpG-A transportation to lysosome.

ssRNA transportation to lysosomes was reduced in *Mcoln1*$^{-/-}$ BM-cDCs (Fig. 14C and D), and ML1-SA1 specifically enhanced ssRNA responses (Fig. 7), I next examine the transportation of ssRNA in the presence of ML1-SA1. Colocalization of RNA9.2s-DR and Poly U with lysotracker was significantly upregulated by ML1-SA1 (Fig. 15C and D). These results indicate that an important role of mucolipin-1 in ssRNA trafficking into lysosomes. Moreover, the data suggest that DNA and ssRNA employ

different transportation pathways.


A
LysoTracker
CpG-B
LysoTracker
CpG-B
Mock
ML1-SA1
Correlation coefficient (IRI)
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CpG-B
CpG-B
Mock
ML1-SA1
B
LysoTracker
CpG-A
LysoTracker
CpG-A
Mock
ML1-SA1
*
Correlation coefficient (IRI)
0.6
0.4
0.2
0.0
CpG-A
CpG-A
Mock
ML1-SA1
C
LysoTracker
RNA9.2s-DR
LysoTracker
RNA9.2s-DR
Mock
ML1-SA1
***
Correlation coefficient (IRI)
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0.6
0.4
0.2
0.0
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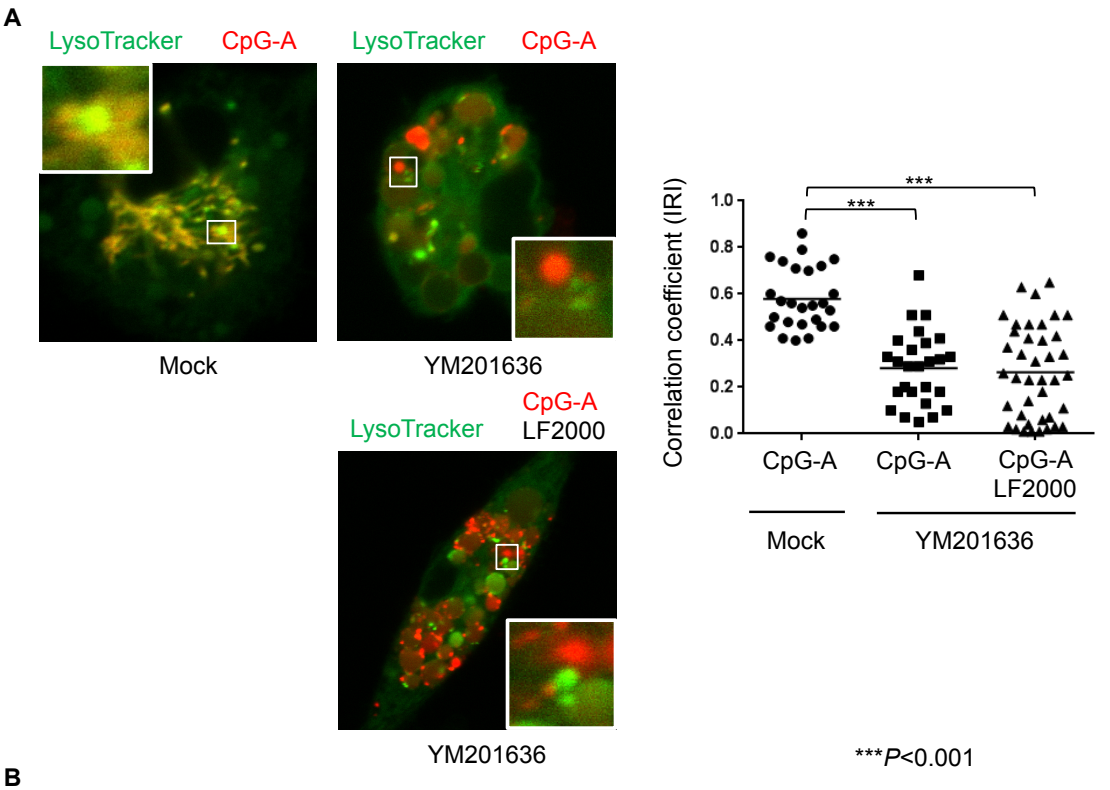
RNA9.2s-DR
Mock
ML1-SA1
D
LysoTracker
Poly U
LysoTracker
Poly U
Mock
ML1-SA1
**
Correlation coefficient (IRI)
0.8
0.6
0.4
0.2
0.0
Poly U
Poly U
Mock
ML1-SA1
*P<0.05; **P<0.01; ***P<0.001

Figure 15. ssRNA transportation to lysosomes is enhanced by mucolipin agonist ML1-SA1 in BM-cDCs. BM-cDCs were pre-treated with DMSO (Mock) or 10 μM ML1-SA1 for 30 min. BM-cDCs were stimulated with 1 μM CpG-B-rhodamine (A), 1 μM CpG-A-rhodamine (B), 10 μg/ml RNA9.2s-DR-rhodamine (C) or 10 μg/ml 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<0.05; **P<0.01; ***P<0.001.

3.11 Single stranded RNA transportation to lysosomes is impaired by PIKfyve inhibitor in bone marrow derived conventional dendritic cells (BM-cDCs).

PIKfyve converts PtdIns(5)P into PtdIns(3,5)P_2, which is an endogenous ligands for mucolipin-1 [44, 63, 64]. Given that mucolipin-1 regulates ssRNA transportation, PIKfyve inhibitor YM201636 may inhibit ssRNA transportation to lyososomes. To address this possibility, YM201636 pre-treated BM-cDCs were stimulated with rhodamine-labeled CpG-A and RNA9.2s-DR. Confocal microscopy images showed that the colocalization of CpG-A and RNA9.2s-DR with lysotracker is drastically impaired by PIKfyve inhibitor (Fig. 16A and B), indicating PIKfyve has a role in ssRNA and DNA transportation to lysosomes. I next examine whether the contrary cytokine production results in lipofectamine-complexed CpG-A and ssRNA in the presence of PIKfyve inhibitor is due to the transportation of nucleic acids regulated by lipofectamine. Lipofectamine failed to rescue CpG-A trafficking to lysosomes in the presence of PIKfyve inhibitor (Fig. 16A). As expected, the suppressive effect of PIKfyve inhibitor in ssRNA transportation to lysosome was abolished by lipofectamine (Fig. 16B).

Collectively, these results indicate that the PtdIns(3,5)P_2-mucolipin-1 axis pathway is required for ssRNA transportation to lysosomes.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Mock
LysoTracker CpG-A
YM201636
LysoTracker CpG-A LF2000
YM201636
Correlation coefficient (IRI)
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0.8
0.6
0.4
0.2
0.0
***
***
CpG-A
CpG-A
CpG-A LF2000
Mock
YM201636
***P<0.001
B
LysoTracker RNA9.2s-DR
Mock
LysoTracker RNA9.2s-DR
YM201636
RNA9.2s-DR
LysoTracker LF2000
YM201636
Correlation coefficient (IRI)
1.0
0.8
0.6
0.4
0.2
0.0
***
***
RNA9.2 s-DR
RNA9.2 s-DR
RNA9.2 s-DR LF2000
Mock
YM201636

Figure. 16 ssRNA transportation to lysosomes is impaired by PIKfyve inhibitor in BM-cDCs. BM-cDCs were pre-treated with DMSO (Mock) or 500 nM YM201636 for 15 min. BM-cDCs were stimulated with 1 μM naked CpG-A-rhodamine and lipofectamine-complexed CpG-A-rhodamine (A), 10 μg/ml naked RNA9.2s-DR-rhodamine and lipofectamine-complexed RNA9.2s-DR-rhodamine (B) for 90 min. To visualize lysosomes, cells were incubated with Lysotracker-Green for 30 min before analyses by confocal microscopy. LF2000: lipofectamine 2000. Statistical analyses using correlation coefficient are also shown. ***P<0.001.

3.12 Toll like receptor 7 responses are altered in *Mcoln1*$^{-/-}$ bone marrow chimeric mice.

To further address the immunological relevance of mucolipin-1 in innate immune responses, BM chimeric mice were used in *in vivo* tests. B cells were purified from spleen of WT, *Mcoln1*$^{-/-}$ and BM chimeric mice reconstituted with WT or *Mcoln1*$^{-/-}$ BM cells. mRNA was isolated from splenic B cells and subjected to reverse transcript PCR. PCR primers were designed according to mucolipin-1 knock out strategy [58]. 480 bp PCR fragment designed for WT allele was undetectable in the splenocyte from BM chimeric mice reconstituted with *Mcoln1*$^{-/-}$ BM cells, confirming that all the B cells were from *Mcoln1*$^{-/-}$ BM cells (Fig. 17A). Because cell surface expression of CD23 was significantly decreased in *Mcoln1*$^{-/-}$ B cells (Fig. 17B), peripheral blood mononuclear cells (PBMCs) were obtained from facial vein of BM chimeric mice and were stained with markers B220, CD21 and CD23. Because of this phenotype, the chimerism of *Mcoln1*$^{-/-}$ BM chimeric mice can be further confirmed by cell surface CD23 expression. All the B cells showed low CD23 expression, confirming that all the B cells were replaced with *Mcoln1*$^{-/-}$ B cells (Fig. 17C). WT and *Mcoln1*$^{-/-}$ BM chimeric mice (10

mice for each group) were administrated with R848 (2 μg/mouse) by vena ophthalmica injection. Sera from each mouse were obtained 4 days before ligands injection and were used as background control in ELISA. Two hours after injection of R848, sera were collected from facial vein. Production of IL-6 and IL-12p40 was detected by ELISA. Both IL-6 and IL-12p40 productions were partially but significantly altered in *Mcoln1*$^{-/-}$ BM chimeric mice (Fig. 17D and E). These data further proved the importance of mucolipin-1 in TLR7 responses *in vivo*.

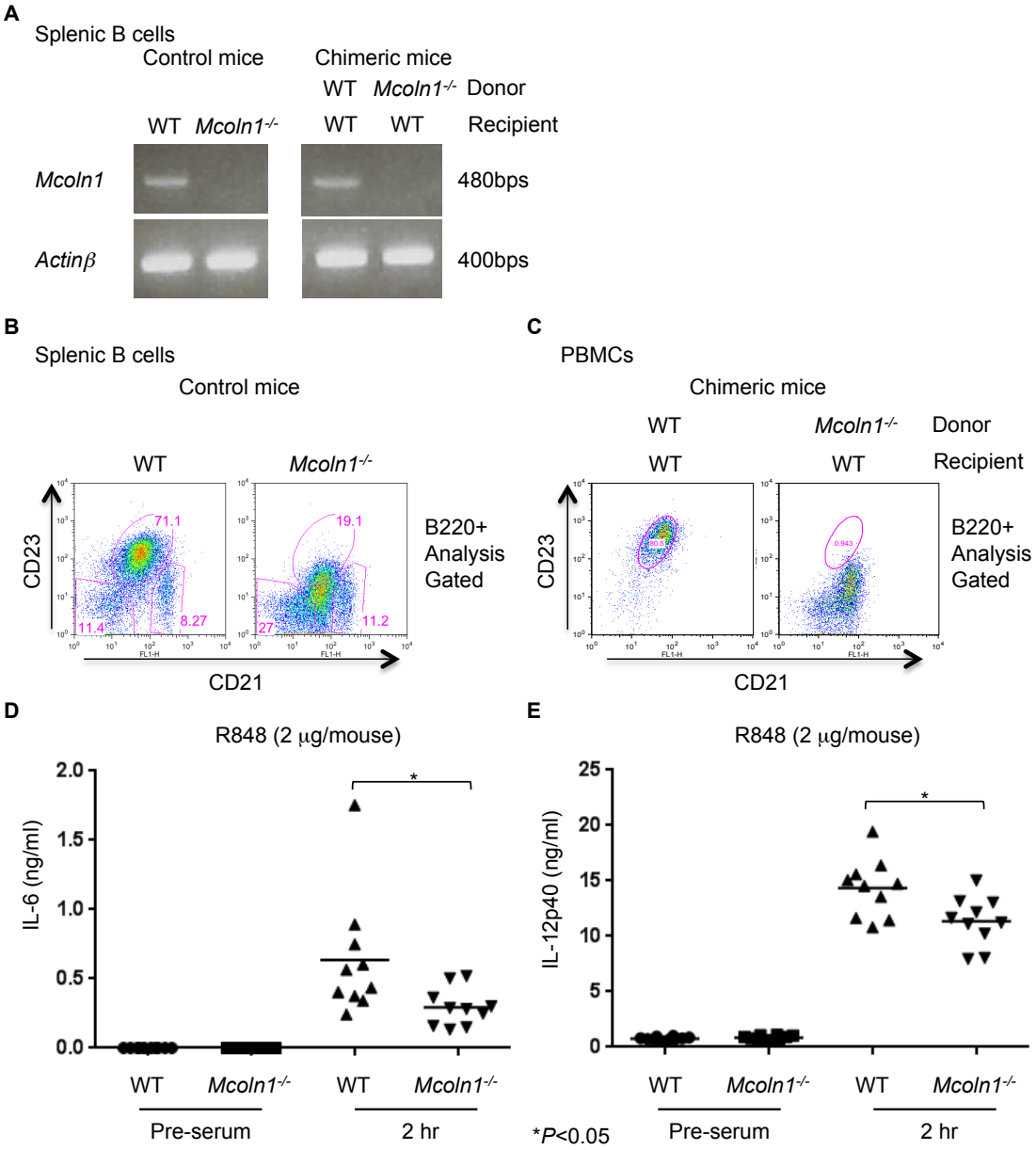

Figure 17. TLR7 responses are altered in *Mcoln1*$^{-/-}$ BM chimeric mice. mRNA was purified from splenic B cells of WT mice, *Mcoln1*$^{-/-}$ mice, WT BM recipient mice and *Mcoln1*$^{-/-}$ BM recipient mice separately. In reverse transcription PCR, primers targeting *Mcoln1*$^{-/-}$ were designed based on previous report [58], *Actinβ* detection was regarded as control (A). B cells, that were purified from WT, *Mcoln1*$^{-/-}$ (B) Chimeric mice (C), stained with markers B220, CD21 and CD23. Cell surface expression of staining markers were detected by FACSCalibur. Serum samples were obtained 4 days before injection as background control (Pre-serum). WT BM chimeric mice (n=10) and *Mcoln1*$^{-/-}$ BM chimeric mice (n=10) were intravenously injected with R848 2 μg/mouse

for 2 hr. Serum samples were obtained, concentration of IL-6 (D) and IL-12p40 (E) were detected by ELISA. Data represent the Mean. *P<0.05.

4. Discussion

The present study shows that a PtdIns(3,5)P_2-muclipin-1 axis has a role in ssRNA transportation to lysosome in bone marrow dendritic cells. *Mcoln1*$^{-/-}$ BM-cDCs and BM-pDCs were significantly impaired in the TLR7 responses to ssRNA. The synthetic mucolipin agonist (ML1-SA1) synergistically activates mucolipin with endogenous ligand PtdIns(3,5)P_2 [60, 61]. BM-cDCs pre-treated with ML1-SA1 showed enhanced responses to ssRNA, demonstrating the importance of mucolipin-1 in TLR7 responses, particularly in ssRNA recognition. BM-cDCs pre-treated with PIKfyve inhibitor demonstrated the important role of a PtdIns(3,5)P_2-muclipin-1 axis in ssRNA recognition (Table 4).

PtdIns(3,5)P_2 is low-abundance endolysosome-specific phosphoinositide. FIG4 deficient cells, which result in 50 % decrease in PtdIns(3,5)P_2 levels, are similar to mucolipin-1 deficient cells in that both cells show enlarged endolysosomes and impaired endocytic trafficking [66]. Recent studies show that PtdIns(3,5)P_2 is able to induce calcium influx/efflux by activating transient receptor potential mucolipin-1, mucolipin-2 and mucolipin-3 [45, 70]. Other phosphoinositides are not able to activate

mucolipin family [45, 70]. PtdIns(3,5)P_2 is produced from PtdIns(3)P by PtdIns(5)K PIKfyve complex that is localized in late endosomes and lysosomes [71]. The present studies showed that TLR7 responses to RNA9.2s-DR and Poly U were diminished by PIKfyve inhibitor YM201636. These data demonstrated that PIKfyve dependent PtdIns(3,5)P_2 generation is important for TLR7 responses to ssRNA. Although BM-cDCs and BM-pDCs lacking mucolipin-1 showed significantly impaired cytokine production in responses to RNA9.2s-DR or Poly U, the cytokine production was still detectable. These results indicate a possibility that PtdIns(3,5)P_2 activates another molecule in addition to mucolipin-1. It is possible that mucolipin-2 and mucolipin-3 have a redundant role in TLR7 responses. Dong *et al*. report that mucolipin-1 is more sensitive than mucolipin-2 and mucolipin-3 to PtdIns(3,5)P_2 [45]. Mucolipin-1, among the target proteins of PtdIns(3,5)P_2, is likely to be predominant mediator in TLR7 responses.

Phosphoinositides are generated in a compartment-specific manner and have important roles in endosomal trafficking [72]. We hypothesized that Mocolipin-1 activation via PtdIns(3,5)P_2 regulates TLR7 logistics. However, TLR7 processing,

which reflected TLR7 trafficking from ER to endosomes [14, 19], was not altered in *Mcoln1*$^{-/-}$ BM-cDCs. Confocal microcopy images showed the colocalization of TLR7 and LAMP2 was not altered in *Mcoln1*$^{-/-}$ BM-cDCs. These data suggest that under steady state, mucolipin-1 does not influence TLR7 trafficking.

Kuo *et al*. show that class III PI3K is essential for CpG-B uptake. Class III PI3K inhibitor (wortmannin) and a loss-of-function mutation in class III PI3K impairs the CpG-B uptake [73]. Class III PI3K converts PtdIns into PtdIns(3)P, which is the substrate of PIKfyve [74]. It has been shown that PIKfyve is required for CpG-B transportation to LAMP1^{+} compartments [44]. Mucolipin-1 has a role in lysosome trafficking, and this process can be inhibited by PIKfyve inhibitor or the lack of FIG4 [60]. PIKfyve is known to form a complex with VAC14 and FIG4, and the complex activity is depended on FIG4 [65, 75]. Considering TLR7 processing was not altered in *Mcoln1*$^{-/-}$ BM-cDCs, I hypothesized that TLR7 responses were impaired due to altered ligand trafficking. Consistent with hypothesis, ssRNAs transportation to lysosome was significantly impaired in *Mcoln1*$^{-/-}$ BM-cDCs. Moreover, mucolipin-1 agonist ML1-SA1 significantly enhanced ssRNA transportation to lysosomes in BM-cDCs. These results

indicated that ssRNA transportation is dependent on the activation of mucolipin-1. Current data showed that the PtdIns(3,5)P_2-mucolipin-1 axis is required for ssRNA responses, I next examined whether PIKfyve regulated ssRNA transportation. As expected, PIKfyve inhibitor drastically impaired ssRNA transportation to lysosomes. Collectively, these results suggest the PtdIns(3,5)P_2-mucolipin-1 axis regulates ssRNA transportation to lysosomes. It is notable that impaired ssRNA transportation in the presence of PIKfyve inhibitor was more remarkable than that in *Mcoln1*$^{-/-}$ BM-cDCs. These data indicate that, in addition to mucolipin-1, as yet unknown effectors downstream of PtdIns(3,5)P_2 contribute to ssRNA transportation.

Transfection reagents such as lipofectamine are able to facilitate the uptake and delivery of NAs, and protect NAs from degradation by nucleases. Therefore, NAs complexed with transfection reagents enhance the TLRs responses. Interestingly, the effect of PIKfyve inhibitor was abolished by complex formation with lipofectamine. Additionally, lipofectamine-complexed ssRNA were able to rescue TLR7 responses in *Mcoln1*$^{-/-}$ BM-cDCs and BM-pDCs. It is reported that synthetic lipofectamine-complexed RNA/DNA potentially mimic viral particles, thus promoting

uptake of RNA [76]. The transportation of RNA via viral particle may be distinct from that of naked RNA. Confocal microscopy images revealed that lipofectamine is able to rescue ssRNA transportation to lysosomes in the presence of PIKfyve inhibitor. These data indicate that lipofectamine might facilitate RNA localization in lysosomes by enabling PIKfyve kinase independent RNA trafficking.

PIKfyve inhibitor significantly impaired CpG-A responses. Of note, PIKfyve inhibitor also impaired lipofectamine-complexed CpG-A responses. Confocal microscopy images showed that PIKfyve inhibitor drastically impaired naked CpG-A and lipofectamine-complexed CpG-A transportation to lysosomes. Nevertheless, CpG-A transportation to lysosomes was not altered in *Mcoln1*$^{-/-}$ BM-cDCs, suggesting that as yet unknown effectors other than mucolipin-1 are responsible for CpG-A transportation. Lipofectamine-mediated CpG-A transportation seems to be PtdIns(3,5)P_2 dependent. Given that lipofectamine-mediated RNA transportation is independent of PtdIns(3,5)P_2, DNA transportation is distinct from RNA transportation even in the presence of lipofectamine.

The mucolipin agonist ML1-SA1 did not enhance, but significantly impaired CpG-A

responses in BM-cDCs. Consistent with this, ML1-SA1 significantly suppressed CpG-A trafficking into lysosomes. However, *Mcoln1*$^{-/-}$ BM-cDCs showed impaired CpG-A responses. It is reported that ML1-SA1 may also activate mucolipin-2 and mucolipin-3 [57, 59], suggesting that mucolipin-2 or mucolipin-3 may negatively regulate CpG-A transportation to lysosomes. If mucolipin-1 does not regulate CpG-A transportation, what is the role of mucolipin-1 in CpG-A responses? TLR9 translocation is important for TLR9 recognition of ligands [18], moreover, Sasai *et al*. show that TLR9 translocation to lysosome related organelles (LROs) is regulated by adapter-related protein complex-3 (AP3) [77]. Vergarajauregui and Puertollano show that AP3 regulates mucolipin-1 trafficking to lysosome by association with di-leucine motifs of mucolipin-1 [78]. Although TLR9 processing demonstrated that under steady state TLR9 trafficking from ER to endolysosomes might not alter, the distribution of TLR9 after stimulation in the endolysosomal systems might be slightly altered in *Mcoln1*$^{-/-}$ cDCs. Further study is required to address this issue.

PIKfyve inhibitor only slightly impaired CpG-B induced responses. These results indicate that mucolipin-1 has a minimal role in CpG-B responses. In contrast to CpG-A

responses, ML1-SA1 did not alter CpG-B responses. Confocal microscopy images revealed ML1-SA1 did not alter the CpG-B transportation to lysosomes, indicating CpG-B transportation is likely to be distinct from CpG-A transportation.

In *Mcoln1*$^{-/-}$ BM-cDCs, CpG-B induced IL-6 and TNF-α production was not altered, whereas CpG-A responses were impaired. Confocal microscopy images showed that neither CpG-B nor CpG-A transportation to lysosomes were altered in *Mcoln1*$^{-/-}$ BM-cDCs, indicating that mucolipin-1 may not regulate their transportation. It is arbitrary to exclude a possibility that TLR9 trafficking in endolysosomal system is slightly altered in *Mcoln1*$^{-/-}$ BM-cDCs.

TLR7 responses to small chemical ligands were impaired in *Mcoln1*$^{-/-}$ BM-cDCs and BM-pDCs (Table 4). However, neither mucolipin agonist nor PIKfyve inhibitor altered TLR7 responses to these small chemical ligands in BM-cDCs. TLR7 responses to small chemical ligands may not require PtdIns(3,5)P_2-dependent mucolipin-1 activation, suggesting that mucolipin-1 has another role in TLR7 responses in addition to RNA-trafficking. Interestingly, Christoph. *et al*. report that in *CD14*$^{-/-}$ BM-cDCs, TLR7 responses to lower concentration of imiquimod is profoundly impaired. Nevertheless,

chemical ligands at higher concentration are able to activate *CD14*$^{-/-}$ BM-cDCs. Moreover, CD14 is essential in ligands uptake and transportation to endolysosome [21]. Chemical ligands at higher doses may be able to activate a mucolipin-1-independent trafficking pathway, and thereby to activate TLR7 in *Mcoln1*$^{-/-}$ DCs. It is important to study all the trafficking pathways employed by TLR7 ligands and TLR9 ligands. It is possible that CD14 and mucolipin-1 contribute to the same trafficking pathway. To note that chemical ligands (R848, loxoribine and imiquimod) induced IFN-α production was partially decreased in *Mcoln1*$^{-/-}$ BM-pDCs. It is reported that TLR7 mediated IFN-α production requires mammalian target of rapamycin (mTOR), rapamycin significantly inhibits loxoribine (TLR7 chemical ligand) induced IFN-α production in BM-pDCs [79]. Wong *et al*. report that a loss-of-function mutant of mucolipin-1 impaired activity of target of rapamycin complex 1 (TORC1) in *Drosophila* [80]. It is possible that decreased IFN-α production induced by chemical ligands is due to impaired mTOR activation in *Mcoln1*$^{-/-}$ BM-pDCs.

Additionally, *in vivo* experiments further showed that mucolipin-1 has regulatory functions in TLR7 responses to chemical ligands. Considering that TLR7 is important

in antiviral responses, *Mcoln1*$^{-/-}$ mice may be sensitive to viral infection. In addition, current data showed that mucolipin-1 has a role in TLR7 responses to Poly U, a uridine (U)-ssRNA which derives from human immunodeficiency virus-1 (HIV-1) [81]. Further study on viral infection using *Mcoln1*$^{-/-}$ mice may shed a light on the importance of mucolipin-1 in antiviral defenses.

Human mutations of mucolipin-1 cause Mucolipidosis type IV, a lysosomal storage disease characterized by abnormally large lysosomes containing electron-dense inclusions and lipid storage bodies [69, 82]. Cells from Mucolipidosis type IV patients present an abnormal endocytosis process of the membrane components to late endosomes and lysosomes, finally leading to abnormal lysosomal storage [83]. Lysosome exocytosis is also affected by Mucolipidosis type IV [84]. RNA is likely to be aberrantly transported and accumulated in lysosomes in Mucolipidosis type IV. It is interesting to study TLR7 and 9 responses in Mucolipidosis type IV. Most LSDs are due to the defective lysosomal enzymes activities, which result in abnormal lysosomal accumulation. In Niemann-Pick (NP) disease sphingomyelins (SMs) accumulate in lysosomes, hence inhibit mucolipin-1 mediated lysosomal trafficking [59]. Lipid

metabolism may influence innate immunity by modulating mucolipin-1 dependent transportation of RNA or DNA.

Table. 4 Summary in functional results

Ligands	TLR	Cytokine	BM-cDCs			BM-pDCs	
			Mcoln1$^{-/-}$	YM201636	ML1-SA1	*Mcoln1*$^{-/-}$	YM201636
CpG-A	TLR9	IL-6	↓	↓	↓	–	
		TNF-α	↓	↓	↓	–	↓
		IFN-α				↓	↓
CpG-A LF2000*		IL-6		↓		–	
		TNF-α		↓		–	↓
		IFN-α				↓	↓
CpG-B		IL-6	–	↓	–	–	
		TNF-α	–	–	–	–	↓
RNA9.2s-DR	TLR7	IL-6	↓	↓	↑	N.D.	N.D.
		TNF-α	↓	↓	↑	N.D.	N.D.
		IFN-α				N.D.	N.D.
RNA9.2s-DR LF2000		IL-6	–	↓			
		TNF-α	–	–			–
		IFN-α					–
Poly U		IL-6	N.D.	N.D	↑	N.D.	N.D.
		TNF-α	N.D.	N.D		↓	↓
		IFN-α				↓	↓
Poly U LF2000		IL-6				–	
		TNF-α				–	–
		IFN-α				–	–
R848	TLR7	IL-6	↓	–	–	↓	
		TNF-α	↓	–	–	↓	–
		IFN-α				↓	↓
Loxoribne		IL-6	↓		–	↓	
		TNF-α	↓		–	↓	
		IFN-α				↓	
Imiquimod		IL-6				↓	
		TNF-α				↓	
		IFN-α				↓	

*: lipofectamine 2000. ↓: decrease. –: not altered. ↑: increase. N.D.: not detected.

Solid Grey: not tested.

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