PtdIns3P phosphatases MTMR3 and MTMR4 negatively regulate innate immune responses to DNA through modulating STING trafficking

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Running title: Regulation of STING function by MTMR3 and MTMR4

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Abstract
The innate immune system plays an essential role in initial recognition of pathogen infection by producing inflammatory cytokines and type I interferons. cGAS is a cytoplasmic sensor for DNA derived from DNA viruses. cGAS binding with DNA induces the production of cGAMP, a second messenger that associates with STING in endoplasmic reticulum (ER). STING changes its cellular distribution from ER to perinuclear Golgi, where it activates the protein kinase TBK1 that catalyzes the phosphorylation of IRF3. Here we found that STING trafficking is regulated by Myotubularin related protein (MTMR) 3 and MTMR4, members of protein tyrosine phosphatases that dephosphorylate 3′ position in phosphatidylinositol (PtdIns) and generate PtdIns3P from PtdIns3,5P2 and PtdIns from PtdIns3P. We established MTMR3 and MTMR4 double knockout (DKO) RAW264.7 macrophage cells, and found that they exhibited increased type I interferon production after interferon-stimulatory DNA (ISD) stimulation and Herpes Simplex Virus 1 infection concomitant with enhanced IRF3 phosphorylation. In DKO cells, STING rapidly trafficked from ER to Golgi after ISD stimulation. Notably, DKO cells exhibited enlarged cytosolic puncta positive for PtdIns3P and STING was aberrantly accumulated in this puncta. Taken together, these results suggest that MTMR3 and MTMR4 regulate the production of PtdIns3P, which plays a critical role in suppressing DNA-mediated innate immune responses via modulating STING-trafficking.

Introduction
Host immune response is a defense system to eliminate to invading pathogens including bacteria, fungi and virus, and largely classified into innate and acquired immune responses. Innate immune response is the first line of host defense system that is triggered upon sensing molecules specific in pathogens, which are called Pathogen-Associated Molecular Patterns (PAMPs), with Pattern-Recognition Receptor (PRRs) that are expressed in various types of host cells. Among them, dendritic cells and macrophages are the major innate immune cells that produce large amounts of pro-inflammatory cytokines and type I interferons (IFNs) and function as antigen presenting cells that potentiate acquired immunity. Releasing cytokines and type I IFNs lead to production of anti-bacterial or anti-viral proteins to prevent pathogen infections. So far, numerous PRRs have been identified, including Toll-Like Receptors (TLRs), Retinoic acid-inducible gene I (RIG-I)-Like Receptor (RLRs), and intracellular sensor for DNA such as cyclic GMP-AMP synthase (cGAS). TLRs localize to the cell surface or intracellular compartments such as the ER, endosome, lysosome or endolysosome, and they recognize their PAMPs such as lipid, lipoprotein, protein and nucleic acid. RLRs recognize cytosolic ssRNA and dsRNA derived from RNA virus, and cGAS recognizes cytosolic double-stranded (ds) DNA derived for DNA virus. Each receptor activation by PAMPs association leads to activate distinct signaling pathways which result in appropriate immune responses specific to given pathogens (1,2).

Various DNA viruses such as human papillomavirus, herpes simplex
virus (HSV), adenovirus, hepatitis B virus release their own genomic DNA to the cytoplasm of host cells upon infection. Released dsDNA binds to a cytoplasmic DNA sensor cGAS (3). cGAS is an enzyme that synthesizes cGAMP from ATP and GTP following binding to viral and host DNA. cGAMP is a cyclic dinucleotide in that 3′-5′ and 2′-5′ ribose on ATP and GTP is connected via phosphodiester linkage, and it acts as the second messenger for adaptor protein, STING(4). STING traffics from the ER to perinuclear region including the Golgi apparatus, then forms cytoplasmic punctate structures along with the protein kinase TBK1. TBK1 phosphorylates the transcription factor IRF3 and phosphorylated IRF3 translocates into nucleus and regulates transcription of type I IFN genes (5,6). Therefore, translocation of STING to Golgi is a hallmark for activation of STING. Various molecules that regulate STING trafficking have been identified. STING trafficking from ER to Golgi apparatus is regulated via the translocon-associated protein (TRAP) complex TRAPβ (SSR2) that is recruited by inactive rhomboid protein 2 (iRhom2) and this complex reaches to Sec-5-containing perinuclear microsome or cytoplasmic punctate structures to assemble with TBK1 and the IKK complex (5). RAB2B-GARIL5 (Golgi-associated RAB2B interactor-like 5) complex is reported as a positive regulator of STING in Golgi apparatus by regulating phosphorylation of IRF3 by TBK1 (7). Moreover, the function of STING is regulated by post-translational mechanism such as E3 ubiquitin ligases, TRIM32 and TRIM56, that conjugate K63-linked polyubiquitination on STING and promote the recruitment of TBK1 (8-10).

Phosphatidylinositol (PtdIns) consists of an inositol ring and acid chains linked by phosphate. Inositol ring in PtdIns contains three phosphorylation sites (3′, 4′ and 5′) and are phosphorylated or dephosphorylated by different combinations. PtdIns are divided to 8 types and each PtdIns has distinct function in terms of signal transduction, organelle trafficking and cytoskeletal regulation (11). These PtdIns are phosphorylated and dephosphorylated at the specific sites in inositol ring by various types of phosphoinositide kinases and phosphatases. Some of these genes such as PIKfyve, PI5P4K and PI3K are participated in innate immune responses (12). The myotubularins (MTMs) are members of the phosphatase superfamily that consists of 1 MTM and 13 MTM-related (MTMR) members which potentially dephosphorylate 3′ site in inositol lipid (13). MTMR3 and MTMR4 are closely related members containing FYVE domain, PH-G domain and the catalytic active site of phosphatase consensus site; catalytic Cys–x5-Arg (Cx5R) motif. Biochemical analysis showed that both MTMR3 and MTMR4 mediate generation of PtdIns from PtdIns3P and PtdIns5P from PtdIns3,5P2 (14,15). MTMR3 and MTMR4 are reported to contribute to the regulation of immune responses. MTMR3 is a risk factor for inflammatory bowel diseases wherein MTMR3 level is increased and PtdIns3P production is reduced (16). Reduction of PtdIns3P results in increased Caspase-1-dependent cytokines production and inflammation by inhibiting autophagy formation.
(16,17), and MTMR4-dependent PtdIns3P production is required for Salmonella-mediated vacuole formation (18).

We previously reported that PtdIns5P that is generated by the lipid kinase PIKfyve plays a crucial role in innate immune responses to RNA virus infection (19). RNA virus infection results in increment of cellular PtdIns5P level in a PIKfyve-dependent manner. PtdIns5P physically binds to IRF3, which facilitates TBK1-mediated phosphorylation and activation of IRF3. In this study, we addressed roles of other phosphatases and kinases that mediate production of PtdIns in innate immune responses. We provided evidence showing that PtdIns3P produced by MTMR3 and MTMR4 plays an important role in the negative regulation of innate immune responses to DNA via modulating STING trafficking.

Results
MTMR3/MTMR4 expression in various types of cells

We first assessed the expression pattern of MTM and MTMR family members in RAW264.7 macrophage cells, murine bone marrow-derived dendritic cells (BMDCs), J774 macrophage-like cells, murine bone marrow-derived macrophages (BMMs) and murine embryonic fibroblast (MEFs) cells by RT-PCR. MTM family genes were widely expressed in all types of cells as tested (Fig. 1A, B). We previously showed that PIKfyve, which contains the FYVE domain that potentially binds to PtdIns, regulates innate immune signaling by generating PtdIns5P production (19). As MTMR3 and MTMR4 also have the FYVE domain, here we focused on MTMR3 and MTMR4, and investigated whether they are involved in the regulation of innate immune signaling.

Dispensable role of MTMR3 in cytokine production after TLR4, RLRs, DNA sensor stimulation

To understand the physiological function of MTMR3 and MTMR4 in innate immune responses, we generated MTMR3 knockout (KO) RAW264.7 cells by using CRISPR/Cas9 system. We designed gRNA in exon 3 regions that contained the PH Gram domain. MTMR3 KO cells showed 16 base pairs (bp) and 4 bp deletion in each allele (Fig. 2A). The expression of Mtmr3 was significantly decreased in MTMR3 KO cells as measured by RT-PCR (Fig. 2B). Our immunoprecipitation-western blot (IP-WB) analyses with anti-MTMR3 antibody demonstrated a loss of MTMR3 protein expression in MTMR3 KO cells (Fig. 2C). Then, we stimulated control and MTMR3 KO RAW264.7 cells with IFN stimulatory DNA (ISD), poly I:C (RLRs ligand) and lipopolysaccharide (LPS) (TLR4 ligand) and measured Ifnb, Il6 and Cxcl10 mRNA by RT-PCR. Expression of these genes was comparable between control and MTMR3 KO cells (Fig. 2D). We also measured production of IL-6 and CXCL10 in the culture supernatant by ELISA, but these cytokines production was also comparable between control and MTMR3 KO cells (Fig. 2E). These results suggest that MTMR3 is dispensable for cytokine expression induced by cGAS, RLRs and TLR4 stimulation.

Dispensable role of MTMR4 in cytokine production after TLR4, RLRs,
**DNA sensor stimulation**

Next, we generated MTMR4 KO RAW264.7 cells by using the same strategy with MTMR3 KO cells. We designed the gRNA at the exon 3 and isolated MTMR4 KO cells that have 4 bp homozygous mutations (Fig. 3A). MTMR4 KO cells showed reduced Mtmr4 mRNA expression and a loss of MTMR4 protein expression (Fig. 3B, C). Ifnb, Il6 and Cxcl10 mRNA expressions after ISD, poly I:C or LPS stimulation were comparable between control and MTMR4 KO cells (Fig. 3D). Moreover, IL-6 and CXCL10 production was also unimpaired in MTMR4 KO cells (Fig. 3E). These results suggest that MTMR4 is also dispensable for the regulation of innate immune responses.

**Enhanced DNA-induced innate immune responses in MTMR3 and MTMR4 double KO and knockdown cells**

MTMR3 and MTMR4 have similar secondary structure and show overlapped expression patterns in various cells as tested, suggesting that they may have complementary functions (Fig. 1). We therefore generated MTMR3 and MTMR4 double knockout (DKO) RAW264.7 cells using CRISPR/CAS9 system. Isolated cells having frameshift mutation in both alleles in both Mtmr3 and Mtmr4 genes were shown in Fig. 4A. Deficiency of these genes was confirmed by RT-PCR and IP-WB against anti-MTMR3 and anti-MTMR4 antibodies (Fig. 4B and C).

We stimulated control and two different lines of MTMR3 and MTMR4 DKO (DKO1 and DKO2) cells with ISD, poly I:C and LPS and measured cytokine mRNA expression. Interestingly, expression of Ifnb, Il6 and Cxcl10 mRNA after ISD stimulation was significantly increased in both DKO1 and DKO2 cells compared to control cells whereas Il10 mRNA expression was unaffected (Fig. 4D). On the other hand, Ifnb, Il6 and Cxcl10 mRNA expression after poly I:C and LPS stimulation was unimpaired. Consistent with these results, IL-6 and CXCL10 production after stimulation with ISD was also significantly increased in DKO1 and DKO2 cells whereas production of these cytokines after poly I:C or LPS was comparable among control, DKO1 and DKO2 cells (Fig. 4E). These results suggest that MTMR3 and MTMR4 negatively regulate DNA-mediated innate immune responses. To further analyze MTMR3 and MTMR4 function in DNA sensing innate immune responses, control and DKO1 cells were infected with DNA virus, HSV-1. Ifnb expression in DKO1 infected with HSV-1 was significantly higher than that in control cell (Fig.4F). Then, we rescued MTMR3 or MTMR4 expression in DKO1 cells by retro virus transfer system (Fig.4G). Ifnb expression after ISD stimulation in DKO cells was higher than that in control cells, and this higher expression of Ifnb was significantly decreased by expression of MTMR3 or MTMR4. These results also suggest that MTMR3 and MTMR4 negatively regulate innate responses against DNA viruses.

A previous report suggested that MTMR3 increases the activation of NLRP3 inflammasome, a protein complex that mediates Caspase-1-dependent IL-1β release in response to various PAMPs or environmental stimuli, via inducing autophagosome formation (16). Therefore, we knocked down MTMR3 and MTMR4 in primary
macrophages and examined IFNβ and IL-1β induction. We electroporated siRNA for Mtmr3 and Mtmr4 into BMMs, and verified knockdown efficacy by qRT-PCR (Fig. 5A). Ifnb expression after ISD stimulation was increased in knockdown cells compared with control cells (Fig. 5B). IL-1β production by cells stimulated with LPS plus Nigericin, a microbial toxin that triggers NLRP3 inflammasome, was significantly reduced by Mtmr3 and Mtmr4 knockdown, suggesting a crucial role of MTMR3 and MTMR4 in the NLRP3 inflammasome activation (Fig. 5C).

MTMR3 and MTMR4 regulate production of PtdIns3P and suppress STING-mediated signaling

MTMR3 and MTMR4 are shown to dephosphorylate PtdIns3,5P2 and PtdIns3P, and generate PtdIns5P and PtdIns, respectively (14,15). It is therefore expected that MTMR3- and MTMR4-deficiencies result in increased PtdIns3P and reduced PtdIns5P production. To visualize cellular distributions of PtdIns3P and PtdIns5P, we expressed YFP-tagged PX domain in p40phox, an NADPH oxidase cytoplasmic component (YFP-PX p40phox) or YFP-tagged PH domain in Docking protein 5 (DOK5) (YFP-PH DOK5), which binds to PtdIns3P or PtdIns5P, respectively, in RAW264.7 cells (20,21). YFP-PX p40phox and YFP-PH DOK5 signals were observed as cytoplasmic puncta structures (Fig. 6A).

To further understand cellular localization of YFP-tagged these markers precisely, cells were costained with a series of organelle maker; EEA1 (early endosome), GM130 (Golgi), Calnexin (ER) or LAMP1 (Lysosome). YFP-PX p40phox localized mainly in the lysosome but partially with early endosome whereas YFP-PH DOK5 localized mainly to unknown dot-like structures but partially localized in endosomal membrane (Fig. S1). We then expressed YFP-PX p40phox and YFP-PH DOK5 in control and MTMR3 and MTMR4 DKO cells. YFP-PX p40phox expression in DKO cells showed increased YFP positive dot-like structures compared to control cells. By contrast, the cellular distribution of YFP-PH DOK5 signals was comparable between control and DKO cells (Fig. 6A, B). These results suggest that DKO cells exhibited accumulation of PtdIns3P.

To understand the contribution of MTMR3 and MTMR4 to innate immune signaling, we performed a reporter assay for the IFNβ promoter. MTMR3 and MTMR4 expression plasmids were transfected together with a luciferase reporter plasmid driven by the IFNβ promoter into HEK293T cells. Overexpression of either or both MTMR3 and MTMR4 did not influence IFNβ promoter activity (Fig 6C, D). Then, we co-overexpressed MTMR3 and MTMR4 along with an adaptor protein; STING (an adaptor for DNA sensor), IPS-1 (an adapter for RLRs), MyD88 (an adapter for TLRs expect for TLR3) or TRIF (an adapter for TLR3 and TLR4) into HEK293T cells. Expression of each adaptor protein alone significantly increased the promoter activity. However, STING-dependent promoter activation was significantly suppressed by co-expression with MTMR3 and MTMR4 whereas promoter activation induced by other adapters was unaffected by MTMR3 and MTMR4 co-overexpression (Fig. 6C). In addition, overexpression of either MTMR3 or
MTMR4 significantly suppressed the IFNβ promoter activity driven by STING overexpression (Fig. 6D). These results suggest that MTMR3 and MTMR4 specifically suppress STING-mediated DNA sensing pathway. Next, we examined phosphorylation of IRF3 and p65 (NF-κB) in control and DKO cells, and found that IRF3 phosphorylation after ISD stimulation was markedly increased in both DKO1 and DKO2 cells compared to control cells whereas IRF3 phosphorylation after poly I:C and LPS stimulation was unimpaired (Fig. 6E). In addition, ISD-induced p65 phosphorylation was not augmented in DKO cells (Fig. 5D). Thus, these results suggest that MTMR3 and MTMR4 repress DNA-induced IRF3 activation.

**Rapid trafficking of STING from ER to Golgi in Mtmr3 and Mtmr4 DKO cells**

It has been shown that STING localizes to ER in steady state and changes its localization to perinuclear Golgi after DNA stimulation (6). To visualize STING trafficking in DKO cells, we established control and DKO cells expressing FLAG-tagged STING using retrovirus transfer system, and co-stained them with anti-FLAG and anti-GM130 antibodies. Co-localization of STING with perinuclear Golgi occurred within 4 hours after ISD stimulation in both control and DKO1 and DKO2 cells (Fig. 7A). Interestingly, STING localization to the perinuclear Golgi was found to occur within 1 hour after ISD stimulation in DKO1 and DKO2 cells whereas control cells did not show STING trafficking to the perinuclear Golgi at this time point, suggesting that STING trafficking from ER to Golgi is more rapid in DKO cells than in control cells, and this rapid translocation may be responsible for increased responses to DNA in DKO cells. Then, we examined activation of STING by utilizing semi-native WB that detects STING dimerization (Fig. 7B). STING dimerization after ISD stimulation was more rapid and increased in MTMR3/4 DKO cells than control cells. These results suggest that accumulation of PtdIns3P by MTMR3 and MTMR4 deficiencies is responsible for STING-dependent responses.

To further address functional relationship between PtdIns3P and STING trafficking, we examined cellular localization of PX p40^phox^ (PtdIns3P) and STING in control and DKO cells (Fig. 7C). In unstimulated condition, STING was not co-localized with PX p40^phox^ positive puncta in both control and DKO1 cells. However, STING was partially incorporated into PX p40^phox^ positive puncta in DKO cells after 4h stimulation with ISD, and this overlap was not observed in control cells. These results also supported by the staining with recombinant fluorescent labeled GST-PX p40^phox^ domain (Fig. S2). These results suggest that STING is accumulated in PtdIns3P positive puncta in DKO cells, which potentiate STING-dependent signaling pathways.

**Reduced PtdIns3P level suppresses ISD-mediated innate immune response**

To understand the roles of PtdIns3P in STING activation, we used Wortmannin, a PI3K inhibitor that is reported to reduce the production of PtdIns3P in cells (22). We visualized cellular PtdIns3P by staining RAW264.7 cells with Alexa647-labeled GST-PX p40^phox^ domain. GST-PX p40^phox^ domain signals showed dot like
structures (Fig. 8A), which were abrogated by Wortmannin treatment (Fig. 8B), suggesting that Wortmannin-treatment reduced PtdIns3P levels. Notably, Wortmannin-treatment reduced ISD-stimulated Ifnb expression and IRF3 phosphorylation in RAW264.7 cells and BMMs (Fig. 7C and D). There results suggest that PtdIns3P is required for ISD-induced IRF3 activation.

Discussion

In this study, we generated single and double KO of MTMR3 and MTMR4 in RAW264.7 cells by Cas/CRISPR system. The Ifnb, Il6 and Cxcl10 mRNA level and IL-6 and CXCL10 production in MTMR3 or MTMR4 single KO cells were comparable with control cells during stimulation with ISD, poly I:C and LPS (Fig. 2 and 3), demonstrating that either MTMR3 or MTMR4 is dispensable for cGAS-, RLRs- and TLR4-mediated signaling. MTMR3 and MTMR4 have similar secondary structures and MTM family genes including Mtmr3 and Mtmr4 are widely expressed in MEF, macrophages and dendritic cells, suggesting the possibility that their function is redundant (Fig. 1). Then, we generated MTMR3 and MTMR4 DKO cells and found that these cells showed significantly increased Ifnb, Il6 and Cxcl10 expression and IL-6 and CXCL10 production following ISD stimulation. Consistent with these results, IRF3 phosphorylation after ISD stimulation was increased in DKO cells (Fig. 4). These results were consistent with the results of the IFNβ promoter assay in which STING-mediated IFNβ promoter activity was repressed by overexpression of MTMR3 and MTMR4 together. Thus, both MTMR3 and MTMR4 play important roles in the negative regulation of DNA sensing innate immune signaling pathways (Fig. 5).

MTMR3 and MTMR4 have abilities to dephosphorylate PtdIns3P and PtdIns3,5P2 to generate PI and PtdIns5P, respectively. MTMR3 and MTMR4 deficiencies may thus induce PtdIns3P increment / PI decrease, and/or PtdIns3,5P2 increment / PtdIns5P decrease. Notably, DKO cells showed increased size and number of YFP-PX p40phox (binding to PtdIns3P) positive dot-like structure compared to control cells, suggesting accumulation of PtdIns3P in DKO cells. In contrast, signals in YFP-PH DOK5 (binding to PtdIns5P)-expressing DKO cells did not show remarkable differences with those in control cells, suggesting that production of PtdIns5P was not abrogated in DKO cells (Fig 5A). Previous reports demonstrated that PtdIns5P production was decreased by inhibition of MTMR3 or MTMR4 activity (23) whereas PtdIns3P production was increased by MTMR4 knockdown, expression of inactive mutant for MTMR4 (MTMR4C407) or MTMR3 knockdown (16,24). Our results supported that PtdIns3P production was increased by MTMR3- and MTMR4-deficiencies, but did not support PtdIns5P reduction. It is unclear why MTMR3- and MTMR4-deficiencies in RAW264.7 cells did not affect PtdIns5P production. It is thought that PtdIns5P production in RAW264.7 cells may be preferentially regulated by other enzymes such as PIKfyve since we previously demonstrated that PtdIns5P production in murine macrophages is decreased by PIKfyve knockdown (19). Thus, PIKfyve may be responsible for PtdIns5P production in...
MTMR3 and MTMR4 DKO cells.

STING localizes in the ER in a steady state and traffics to perinuclear Golgi apparatus after stimulation with ISD (5,6). We hypothesized that accumulation of PtdIns3P by MTMR3- and MTMR4-deficiencies may change the STING localization. Our results showed that STING reached to Golgi after 4 hours stimulation in control and DKO cells, but it translocated more rapidly to Golgi in DKO cells than in control cells (Fig. 6A). It was shown that TBK1 is recruited to STING-positive perinuclear puncta after DNA stimulation, and becomes activated, suggesting that TBK1 translocation to Golgi and subsequent activation is coupled to IRF3 activation. Thus, rapid translocation of STING from ER to Golgi may contribute to enhanced innate immune responses in DKO cells.

It was reported that PtdIns3P is predominantly accumulated in early endosome, but it also widely distributed in other cellular membranes (25). Moreover, PtdIns3P is well-known to regulate autophagosome formation. Autophagy initiates its biogenesis through a pre-autophagosomal double-membrane structure that is a subdomain of ER membrane positive for PtdIns3P and PtdIns3P-binding proteins (26). Consistently, it is reported that knockdown of MTMR3 increased autophagosome formation (17) while increased MTMR3 expression reduced autophagy (16). Autophagy is shown to have an anti-inflammatory role, since disruption of autophagy-related protein in mice results in increased inflammation and type I IFN production during innate immune signaling pathways (27,28). Our results showing that PtdIns3P is increased in DKO cells suggest that the autophagy formation is accumulated in these cells, and inflammation is suppressed. However, MTMR3/4 DKO cells and knockdown cells rather exhibited enhanced type I IFN responses during STING activation whereas MTMR3/4 knockdown in BMMs results in reduced IL-1β production during NLR3 inflammasome activation. This suggests the possibility that autophagosome formation is unimpaired in DKO cells irrespective of increased PtdIns3P, or a rapid translocation of STING to Golgi may negatively regulate PtdIns3P-dependent autophagosome formation via unknown pathways. The relationship between autophagy and MTMR3- and MTMR4-dependent STING regulation should be understood in the future.

Our results demonstrate that STING translocates from ER to Golgi at earlier time point in DKO cells than in control cells. This suggests that accumulation of PtdIns3P by MTMR3/4-deficiencies enhances translocation of STING. ER has a dynamics and complex membrane network that extends to other endosomal organelle. Recent studies suggested that autophagosome biogenesis is initiated at a contact site of ER to other organelle, such as mitochondria and plasma membrane (22,29), suggesting that PtdIns3P is generated at a specific site in ER. Because STING localizes to ER in the steady state, a functional interaction between STING and PtdIns3P in ER may be responsible for the trafficking of STING to Golgi. Indeed, reduced PtdIns3P by Wortmannin treatment reduced ISD-stimulated Ifnb expression and IRF3 phosphorylation. Thus, it is possible that PtdIns3P is required for ISD-mediated innate immune response. However, it is still unclear how
PtdIns3P regulates STING translocation, but several GPCR and ion channel are reported to bind to PtdIns4,5P2 and regulate their functions (30). One possibility is that PtdIns3P may also directly bind to STING and induce the change of STING conformation, which induces STING dimerization and translocation of STING to Golgi. This was also supported by our observation that STING dimer formation is enhanced by MTMR3- and MTMR4-deficiencies (Fig 7B). Association of PtdIns3P with STING is really suggested by our observation in which STING is aberrantly incorporated into PtdIns3P positive puncta in DKO cells (Fig. 6C). Another possibility is that PtdIns3P provides a platform for association of STING and STING-regulatory proteins such as iRhom2, TRIM32 and TRIM56. Although regulatory role of translocation of STING by MTMR3/4 is still unclear, this study showed that MTMR3/4-dependent PtdIns metabolism is important in fine turning innate immune responses to DNA. Self-DNA is aberrantly recognized by cGAS when it is released from the cytoplasm after host cells are died by necrosis or damaged by stresses, drugs, aging and infection. These results in unwanted activation of STING pathway and appear to be highly associated with inflammatory and autoimmune diseases (31). Thus, MTMR3 and MTMR4 may be potential as a target of drug design that can prevent these diseases. Also, it is important to analyze a mutation or SNP in MTMR3 and MTMR4 genes in these diseases.

### Experimental procedures

#### Cells and reagents

Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Nacalai Tesque) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Life Technology) and Plat-E cells were cultured in DMEM containing 100 μM 2-mercaptoethanol (Nacalai Tesque), 1 μg/ml puromycin (Invivogen) and 10 μg/ml Blasticidin (Invivogen) with 10% FBS at 37 °C in humidified 5% CO2. Murine bone marrow cells were treated with 10 ng/ml M-CSF (Proteo Tech) or 10 ng/ml GM-CSF (Proteo Tech), 100 μM 2-mercaptoethanol, and incubated for differentiation 6 to 8 days at 37 °C and 5% CO2 with Roswell Park Memorial Institute (RPMI) 1640 (Nacalai Tesque) containing 100 units / ml penicillin, 100 μg / ml streptomycin (Nacalai Tesque) and 10% FBS, and used as BMM and BM-DC, respectively.

#### Generation of MTMR3 or MTMR4 KO cells

To establish single MTMR3 or MTMR4 KO RAW264.7 cells, guide sequences located in exon 3 of Mtmr3 and exon 3 of Mtmr4 were inserted into pX330-U6-Chimeric_BB-CBh-hSpCas9 (#42230, Addgene) that expresses Cas9 and guide RNA (Mtmr3: sense 5′-GCATAGCCTTGAGTGCATCC-3′; Mtmr4: sense 5′-TGAGGAGGCCCCCCCAGCC-3′). Target DNA sequences for Cas9 were sub-cloned into px330 plasmid that expresses hCas9 and trans-activating CRISPR RNA (trcrRNA). gRNA is located next to PAM sequence (NGG) and 20 bp sequence which potentially shows low off-target effects that do not cross react with other sites in genome. Genomic regions containing guide sequences were inserted into the pCAG
EGxxFP plasmid (32), which is a reporter for genome editing. Then these plasmids were electroporated into RAW264.7 cells by NEON (Invitrogen) at 1680 V and 20 msec. GFP-positive cells were sorted by a BD FACS Aria (BD Bioscience). To obtain MTMR3 and MTMR4 DKO cells, stably expressing Cas/CRISSPR RAW264.7 cells were generated. Synthesized trcrRNA and gRNA (or crRNA) were electroporated into the cells (target gRNA for MTMR3 exon 2: sense 5′-GTTCCCTTCCCTGAACCTCA-3′; MTMR4 exon 2: sense 5′-GGGTGAGGGAGTGGAATTCC-3′). One μl of 1.0 μg/ml crRNA and 3 μl of 0.5 μg/ml tracrRNA were mixed with 6 μl of cell suspension (1x 10^7/ml). RNA and cell mixture were subjected to electroporation using NEON. Electroporated cells were titrated and suspended to 96 well plates. Cells were cultured for around 2 weeks until cellular density reached to 70%. Then, cells were transferred to 24 well plates and DNA were isolated for sequence analysis.

**Knockdown**

Knockdown for targeting sequence is following, Mtmr3; ATGTTACTCGAAGATAAGGTA, Mtmr4; AAGTTGTCTCTTAGTTATAA. Sense and antisense oligo RNA were synthesized with AA overhang (FASMAC). BMMs (4 x 10^5) were mix with 3 μM siRNA and were electroplated with 1400 V, 20 msec and 1 plus by NEON transfection system (Thermo Fisher Scientific).

**Western blot and immunoprecipitation**

Cells were stimulated with the indicated ligand and were lysed with RIPA Buffer (50 mM Tris HCl, 150 mM NaCl, 0.5% Sodium deoxycholate, 1% Nonidet P-40, 0.1% Sodium dodecyl sulfate (SDS)). The supernatant obtained by centrifuging at 800 x g for 10 minutes was used as whole cell lysates. Whole cell lysates were mixed with 2 x SDS sample buffer (1 M Tris-HCl, 10% SDS, 20% Glycerol, 0.01% Bromophenol blue, 0.2 M Dithiothreitol) and heat-treated at 95 °C for 5 minutes. Samples were subjected to SDS-PAGE and proteins were transferred to PVDF membrane (Bio-Rad). Transferred membranes were applied for blocking by TBST buffer (0.5 M Tris, 1.38 M NaCl, 0.027 M KCl, 0.05% tween 20) containing 5% skim milk and were incubated with anti-pIRF3, IRF3, pp65 or p65 antibody (Cell signaling). For IP-WB, control and KO cells cultured in 6 well plates were lysed with IP-buffer (150 mM NaCl, 10 mM Tris-HCl pH8.0, 10 mM EDTA, 2 mM EGTA, 0.2% Tween 20). Lysates were incubated with anti-MTMR3 (Cell signaling) or anti-MTMR4 antibody (ABGENT) with Protein A agarose beads (Thermo Fisher Scientific) and were rotated for o/n at 4 °C. Antibody-bounded Protein A beads were collected by spin down and were washed for three times with IP-buffer. Sample buffer was applied to Protein A beads and samples were performed for WB against anti-MTMR3 antibody (Cell Signaling) or anti-MTMR4 antibody (Proteintech group). As an internal control, cell lysates were blotted with anti-ACTIN antibody (Santa Cruz biotechnology). After incubation with HRP conjugated secondary antibodies (Sigma) for 30 minutes, membranes were reacted with
a luminescent reagent Western Lightning Plus-ECL (PerkinElmer) and proteins were detected by Image Quant LAS-4000 (GE Healthcare).

**Semi-native WB**

STING dimerization was assayed under semi native condition. FLAG-STING expressing cells were stimulated with ISD and were lysed with native sample buffer (1.5 mM Tris HCl, p.H 6.8, 15% glycerol, 1% DOC). Whole cell lysates were run on the standard SDS-PAGE and proteins were transferred to PVDF membrane (Bio-Rad), and blotted with anti-FLAG antibody.

**RNA isolation and RT-PCR**

Total RNA were isolated with Trizol reagent (Invitrogen) and reverse transcribed with ReverTra Ace (Toyobo) according to the manufacturers’ instructions. RT-PCR was performed with the following primers: *Mtm1*, sense 5’-ATCTTAGGAGGATCGCAACG-3’, reverse 5’-TACCGACAAGAGGCTGACTG-3’; *Mtmr1*, sense 5’-TCCATTTATGGGAGCAGTG-3’, reverse 5’-TGCACCAATCTCTCCACTC-3’; *Mtmr2*, sense 5’-AGCAGAAAATGGAGGAAACCAG-3’, reverse 5’-CGCTCCATGCTCTGAAATA-3’; *Mtmr3*, sense 5’-TGGGAATGTATTCTGCTCCA-3’, reverse 5’-AACTGTTTCAAGGTGGCTTCC-3’; *Mtmr4*, sense 5’-TTCCGGTCTATCTCTACTGC-3’, reverse 5’-TGAAGCAAATGCTCATCCTCTC-3’; *Mtmr7*, sense 5’-CCAGTTTGGGAACCTTCTGT-3’, reverse 5’-GGCTCCTGGGTACCATCATC-3’; *Mtmr8*, sense 5’-GGCTCTCTGGAATGCTCAATG-3’, reverse 5’-GAGTGGACAATGCCTCAATG-3’.

Primer for *Ifnb*, *Il6*, *Il10*, *Cxcl10* and 18s were described previously (33,34)

**ELISA**

RAW264.7 cells seeded in 96-well plates were stimulated with 2 μg/mL ISD, 1 μg/mL poly I:C (Invivogen) or 100 ng/mL LPS (Invivogen) for 24 h. Sense and anti-sense ISD sequence were synthesized (Fasmac) and annealed manually. ISD and poly I:C were incubated with Lipofectamine 2000 (Life Technologies) at a ratio of 1:1 in OptiMEM (Life technologies). The IL-6 and CXCL10 concentration in the culture supernatants was measured by a mouse Duoset (R&D Systems) according to the manufacturer’s instructions.

**Luciferase reporter assay**

HEK293T cells were seeded on 24 well plates and were transfected with 100 ng of IFNβ reporter plasmids along with 500 ng of an expression plasmid for MTMR3, MTMR4, STING, IPS-1, MyD88 or TIRF. Ten ng of pRL-TK (Promega) was co-transfected as internal control. MTMR3 and MTMR4 were amplified from mouse cDNA and were subcloned into pFLAG-CMV2 (SIGMA). Other plasmids were described in previously (33). After 24 hours transfection, luciferase activity was measured as described previously.
Fluorescent microscope

Mouse STING gene was subcloned into pMRX-puro vector. pMRX-STING-FLAG-puro was transfected into plat-E cells and the supernatants were transferred to RAW264.7 cells. Then, cells were stained with anti-Flag antibody (Sigma), anti-GM130 (BD bioscience) and DAPI (Dojin). PX domain in p40<sup>pbox</sup> and PH domain in DOK5 were amplified by PCR and subcloned into pYpetC3 vector. pYpetC3-PX p40<sup>pbox</sup> and pYpetC3-PH DOK5 were electroporated into RAW264.7 cells using NEON. Cells were observed with fluorescence microscope LSM 700 (ZEISS) and the image was processed with ZEN software (ZEISS).

Staining with recombinant PX domain

PX P40<sup>pbox</sup> domain was subcloned into pGEX-6p1 (GE healthcare). GST-PX P40<sup>pbox</sup> domain was purified from BL21 E.coli (Takara) after induction with Isopropylβ-D-1-thiogalactopyranoside (IPTG). GST-PX p40<sup>pbox</sup> domain was labeled with Alexa 647 (Thermo Fisher). RAW264.7 cells were fixed with 4 % paraformaldehyde and blocked with 10% FBS/PBS. Fixed cells were permeated by flash frozen using liquid nitrogen. Cells were stained with 20 ng/ml GST-PX p40<sup>pbox</sup> domain and washed with PBS. To further stain with antibody, cells were fixed with 4 % paraformaldehyde.

Statistics

All experiments were independently repeated at least three times. Statistical significance was determined by the Student’s t-test. A p-value of less than 0.05 was considered significant.

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Conflict of interest

The authors declare that they have no competing financial interests.

Author contributions

DDPP and TKawasaki performed the majority of the experiments. MM, TS, TD and DO supported to establish KO cells. SS provided plasmids. DDPP, TKawasaki and TKawai designed the experiment. DDPP, TKawasaki and TKawai wrote the manuscript. TKawai supervised research.

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Figure legends

Figure 1 Expression of Myotubularin family (A and B) Relative expression of the genes encoding myotubularin family members in RAW264.7, BMDC, J774 cells and BMM (A) and MEF cells (B).

Figure 2 Cytokines induction in MTMR3 KO cells (A) Sequence of MTMR3 in KO cells generated by CRISPR/Cas9. Cells that have frame shifted mutation were isolated and defined as MTMR3 KO cells. (B) Gene expression of Mtmr3 in control and MTMR3 KO cells (C) Cell lysates of control and MTMR3 KO cells were subjected to IP/WB analysis with anti-MTMR3 antibody. (D) Control cells and MTMR3 KO cells were stimulated with ISD, poly I:C, or LPS for 24h, and expression of Ifnb, Il6 and Cxcl10 was measured by RT-PCR. (E) Cells were stimulated with ISD, poly I:C or LPS for 24h, and IL-6 and CXCL10 levels in the culture supernatants were measured by ELISA.

Figure 3 Cytokines induction in MTMR4 KO cells (A) Sequence of Mtmr4 in KO cells generated by CRISPR/Cas9. Cells that have frame shifted mutation were isolated and defined as MTMR4 KO cells. (B) Gene expression of Mtmr4 in control and MTMR4 KO cells (C) Cell lysates of control and MTMR4 KO cells were subjected to IP/WB analysis with anti-MTMR4 antibody. (D) Control cells and MTMR4 KO cells were stimulated with ISD, poly I:C, or LPS for 24h, and expression of Ifnb, Il6 and Cxcl10 was measured by RT-PCR. (E) Cells were stimulated with ISD, poly I:C or LPS for 24h, and IL-6 and CXCL10 levels in the culture supernatants were measured by ELISA.

Figure 4 Enhanced innate immune response to DNA in MTMR3 and MTMR4
**DKO cells** (A) Sequence of *Mtmr3* and *Mtmr4* in DKO cells generated by CRISPR/Cas9. Cells that have frame shifted mutation in both genes were isolated and defined as MTMR3/4 DKO cells. (B) Gene expression of *Mtmr3* and *Mtmr4* in control and DKO cells. (C) Cell lysates of control and DKO cells were subjected to IP/WB analysis with the indicated antibodies. (D) Control and DKO cells were stimulated with ISD, poly I:C, or LPS for 24 h and expression of *Ifnb*, *Il6*, *Cxcl10* and *Il10* were measured by RT-PCR. (E) Cells were stimulated with were stimulated with ISD, poly I:C, or LPS for 24 h, and the concentration of IL-6 and CXCL10 in the culture supernatant was measured by ELISA. (F) *Ifnb* expression in control and DKO cells infected with HSV-1 was measured by RT-PCR.

Figure 5  *Mtmr3 and Mtmr4* knockdown in BMMs enhances *Ifnb* expression after ISD stimulation  (A) BMMs were electropolated with siRNA for control or *Mtmr3/Mtmr4*, then mRNA expression for *Mtmr3* and *Mtmr4* mRNA was measured by RT-PCR. (B) *Ifnb* expression was measured by RT-PCR. (C) BMMs primed with LPS were stimulated with Nigericin for 3 h. The concentration of IL-1β in the culture supernatant was measured by ELISA.

Figure 6  *MTMR3 and MTMR4 are required for PtdIns3P production and suppression of STING pathway* (A) Confocal images of PX p40phox (PtdIns3P) and PH DOK5 (PtdIns5P) localization in control and DKO cells. Scale bar = 10 μm. (B) Number of YFP positive puncta in control and DKO cells was counted. (C) HEK293T cells were co-overexpressed with MTMR3 and MTMR4 expression plasmids along with an expression plasmid for STING, IPS-1, MyD88 or TRIF and a reporter plasmid driven by the IFNβ promoter. After 24h, cells were lysed and the luciferase expression was measured. (D) Control and DKO cells were stimulated with ISD, poly I:C or LPS.
for the indicated time points. Cells lysates were subjected to WB with the indicated antibodies.

Figure 7 **MTMR3 and MTMR4 regulate STING translocation.** (A) FLAG-tagged STING was transferred in control and MTMR3/4 DKO1 and DKO2 cells. Cells were stimulated with and without ISD at the indicated time points and were stained with anti-FLAG (Green) and anti-GM130 (Red) antibodies, a Golgi marker. (B) PX p40phox plasmid was transfected in control and MTMR3/4 DKO1 expressing FLAG-STING. Cells were stimulated with or without ISD for 4h and visualized. Scale bar = 10 µm.

Figure 8 **Suppression of PtdIns3P production by PI3K inhibition reduces ISD response.** (A) Confocal images of RAW264.7 cells stained with GST-PX p40phox (PtdIns3P) or GST (control). (B) Confocal image of RAW264.7 cells stained with GST-PX p40phox pre-treated with DMSO or Wortmannin. Scale bar = 10 µm. (C, D) RAW264.7 cells or BMMs pretreated with DMSO or Wortmannin were stimulated with ISD for the indicated periods, and *Ifnb* expression was measured by RT-PCR (C) or IRF3 phosphorylation was evaluated by WB with and anti-total IRF3 or -phosphorylated IRF3 antibodies (D).
Figure 3

A

Mtmr4

ATG

exon 3

TAG

Wild type: GGTGAGGACGCCCTGGAGTACA

Allele #1: GGTGAGGACGCCCTGGAGTACA

Allele #2: GGTGAGGACGCCCTGGAGTACA

B

MTMR4

Input

IP

Control MTMR4 KO

C

Medium

ISD

LPS

poly I:C

Control MTMR4 KO

D

Ilo-I/18s

IL-6

IL-10

IL-12

E

CXCL10

IL-6

IL-10

IL-12

MTMR4 KO

Control MTMR4 KO

Downloaded from http://www.jbc.org/ by guest on April 26, 2019
Figure 7

A

ISD  DAPI  STING  GM130(Golgi)  
Control  MTMR3/4 DKO1  MTMR3/4 DKO2

0 h  

1 h  

4 h  

B

ISD  Control  MTMR3/4 DKO1

MM (kDa)  0 1 3 0 1 3 (h)

72  Dimer

55

43

36  Monomer

C

ISD  

0 h  

PX-p40 (PtdIns3P)  STING  DAPI  Merge

Control  

MTMR3/4 DKO1  

4 h  

Control  

MTMR3/4 DKO1  

Monomer
Figure 8

Panel A: Images of RAW264.7 cells stained with GST-PX and GST.

Panel B: Images showing RAW264.7 cells treated with DMSO and Wortmannin.

Panel C: Bar graphs showing the expression levels of IRF3 and pIRF3 in RAW264.7 cells and BMMs treated with DMSO and Wortmannin, as well as ISD.

Panel D: Western blots showing the expression of IRF3 and pIRF3 in RAW264.7 cells and BMMs treated with DMSO and Wortmannin, at 0, 1, and 3 hours.
PtdIns3P phosphatases MTMR3 and MTMR4 negatively regulate innate immune responses to DNA through modulating STING trafficking
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