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Functional roles of Cot/Tpl2 in mast cell responses to lipopolysaccharide and FcεRI-clustering

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ABSTRACT

Cot/Tpl2, a member of MAP kinase kinase kinase (MAPKKK), is indispensable for the ERK activation, as well as the production of TNF-α, IL-1β, IL-23, and PGE_2 in lipopolysaccharide (LPS)-stimulated macrophages. However, the expression and the functional roles of Cot/Tpl2 in mast cells have not been elucidated. The administration of LPS impairs allergic airway inflammation in a mast cell-dependent manner, and LPS stimulates mast cells to produce not only pro-inflammatory cytokines, such as IL-6 and TNF-α, but also Th2-type cytokines, such as IL-5, IL-10 and IL-13. Here, we examine the role of Cot/Tpl2 by using bone marrow-derived mast cells (BMMCs) from *cot/tpl2* gene-deficient mice. Phosphorylation of ERKs was significantly decreased, whereas that of JNKs and p38 kinase was normal in LPS-stimulated *cot/tpl2*$^{-/-}$ BMMCs compared with wild-type counterparts. LPS-induced mRNA increase was significantly impaired for IL-5, IL-10, IL-13, and TNF-α, but was normal for IL-6, in *cot/tpl2*$^{-/-}$ BMMCs. On the other hand, degranulation by FcεRI-clustering from *cot/tpl2*$^{-/-}$ BMMCs was significantly enhanced compared with the WT control. Although the phosphorylation of ERKs and p38 kinase by FcεRI-clustering was similar in WT and *cot/tpl2*$^{-/-}$ BMMCs, the phosphorylation of Syk was significantly enhanced in *cot/tpl2*$^{-/-}$ BMMCs, which seemed to be due to the increased protein concentration of Syk. These results imply the functional importance of Cot/Tpl2 in mast cells during the course of allergic diseases such as asthma.

1. Introduction

Mast cells are central effector and regulatory cells in Th2-dominated immune responses. The multivalent binding of antigen to receptor-bound IgE and the subsequent aggregation of the high-affinity FcεRI provide triggers for mast cell activation, inducing the release of inflammatory mediators, such as histamine, leukotoriene C4, and prostaglandin D2 [1]. On the other hand, mast cells also play a critical role in host immune defense against gram-negative bacteria through the release of TNF-α [2].

Pathogen-associated molecular patterns (PAMPs) are represented mostly by molecules localized in microbial cell walls. LPS, a prominent PAMP, binds to soluble LPS binding protein (LBP) and then to CD14 [3], which presents the complex to the LPS signaling receptor, Toll-like receptor (TLR) 4 [4]. Signals originating from the LPS-triggered TLR4 receptor activate signaling molecules such as NF-κB [5], AP-1 [6] and MAP kinases including extracellular signal-regulated kinases (ERKs) [7], c-Jun N-terminal kinases (JNKs) [8], and p38 kinases [9], in target cells. The involvement of these three types of MAP kinases in cellular responses such as cytokine production has been documented extensively [8].

Mast cells respond to LPS by producing IL-1β, IL-6, and TNF-α without degranulation [10,11]. Recently, we and others have demonstrated that LPS also induce significant amounts of Th2-associated cytokines such as IL-5, IL-10 and IL-13, but not IL-4, from mast cells through MAP kinase activation [11,12]. The cytokine production was abolished in bone-marrow-derived mast cells (BMMCs) derived from C3H/HeJ mice, in which TLR4 is functionally defective, suggesting it requires functional TLR4 [12]. Various investigators have indicated that exposure to LPS induces airway hyper-responsiveness [13] and worsens allergic airway inflammation [14], indicating LPS as a risk factor for asthma patients. Thus, it is an intriguing possibility that the worsening effect of LPS on asthma is at least partially due to the Th2-type cytokine secretion by mast cells.

A serine/threonine protein kinase, Cot/Tpl2 [15], belongs to the mitogen-activated protein kinase kinase kinase (MAP3K) family, and can activate both the ERK and JNK signaling pathways [16,17]. Cot/Tpl2 has also been implicated in NF-κB activation through IκB-kinase complex or degradation of the inhibitory p105 protein, which

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has recently been reported as a stabilizer/inhibitor of Cot/Tpl2 [18]. The activation of ERK is specifically defective in LPS-stimulated *cot/tpl2*$^{-/-}$ macrophages, which results in the impaired secretion of TNF-α, a key regulator in both inflammation and immunity [19].

We have previously demonstrated that macrophages and DCs from *cot/tpl2*$^{-/-}$ mice produce significantly more IL-12, a heterodimer consisting of p35/p40, in response to TLR ligands, such as CpG DNA and LPS, than their normal counterparts [20]. The increased production of IL-12 was due to the higher induction level of p35 mRNA. Interestingly, *cot/tpl2*$^{-/-}$ macrophages produce significantly less IL-23, a heterodimer (p19/p40) sharing p40 subunit with IL-12, than wild-type macrophages resulting from the decreased induction level of p19 [21]. Thus the Cot/Tpl2 kinase activity in mouse macrophages regulates IL-12/IL-23 expression in opposite directions in response to LPS. However, the functional roles of Cot/Tpl2 in mast cells have not been elucidated.

Here, we examine the functional roles of Cot/Tpl2 in the activation of mast cells using bone marrow-derived mast cells (BMMCs) from *cot/tpl2*$^{-/-}$ mice. Our data suggest that Cot/Tpl2 is an essential regulator of various cytokine expression as well as degranulation in mast cells activated by LPS and FcεRI-clustering, respectively.

2. Materials and methods

2.1. Reagents and materials

LPS from *Escherichia coli* serotype O55:B5, RPMI1640 medium, monoclonal anti-dinitrophenyl (DNP) clone SPE-7, and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide were purchased from Sigma Chemical Co. (St. Louis, MO). DNP-conjugated bovine serum albumin (BSA) was obtained from CALBIOCHEM (Darmstadt, Germany). Polyclonal anti-JNK1 and anti-p38 kinase Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific anti-p38 polyclonal Ab, anti-ERK1/2 polyclonal Ab, phospho-specific anti-ERK monoclonal Ab, and anti-IkBα polyclonal Ab were purchased from Cell Signaling Technology (Beverly, MA).

2.2. Cells

The *cot/tpl2*$^{-/-}$ mouse was generated as described as previously [20]. Animal experiments were conducted in accordance with the institutional guidelines for the care and use of laboratory animals. BMMCs were derived from bone marrow cells of 6–8-week-old female C57BL/6J and *cot/tpl2*$^{-/-}$ mice. These cells were cultured with 50% WEHI-3B-conditioned medium for 2 weeks, and then the cells were harvested for experiments. The cells were >98% mast cells assessed by toluidine blue staining.

2.3. Flowcytometric analysis

Cells were stained with PE or FITC-conjugated antibodies at 4 °C for 20 min. The cell surface expression of proteins was assessed by EPICS® XL-MCL (Beckman Coulter, Brea, CA).

2.4. Cytokine measurement

TNF-α cytokine concentration in the culture supernatants was assessed by ELISA. Briefly, BMMCs (2×10^6 cells/mL) were stimulated with 1 μg/mL LPS or PMA (20 ng/mL)/ionomycin (100 ng/mL) for 8 h. The cytokine concentration was measured by mouse TNF-α ELISA kit (R&D Systems, Minneapolis, MN).

2.5. RT-PCR

Total cellular RNA was isolated as described as previously [12]. Complementary DNA was synthesized from 3 μg total RNA by extension of random primers with 300U ReverTra Ace® reverse transcriptase (TOYOBO Life Science, Japan). The amplification of cDNA was performed with a temperature controller. The primer sequences were described previously [22].

2.6. Northern blot analysis

Northern blot analysis was performed as previously as described [23]. Briefly, 2 μg of the total RNA samples were electro-

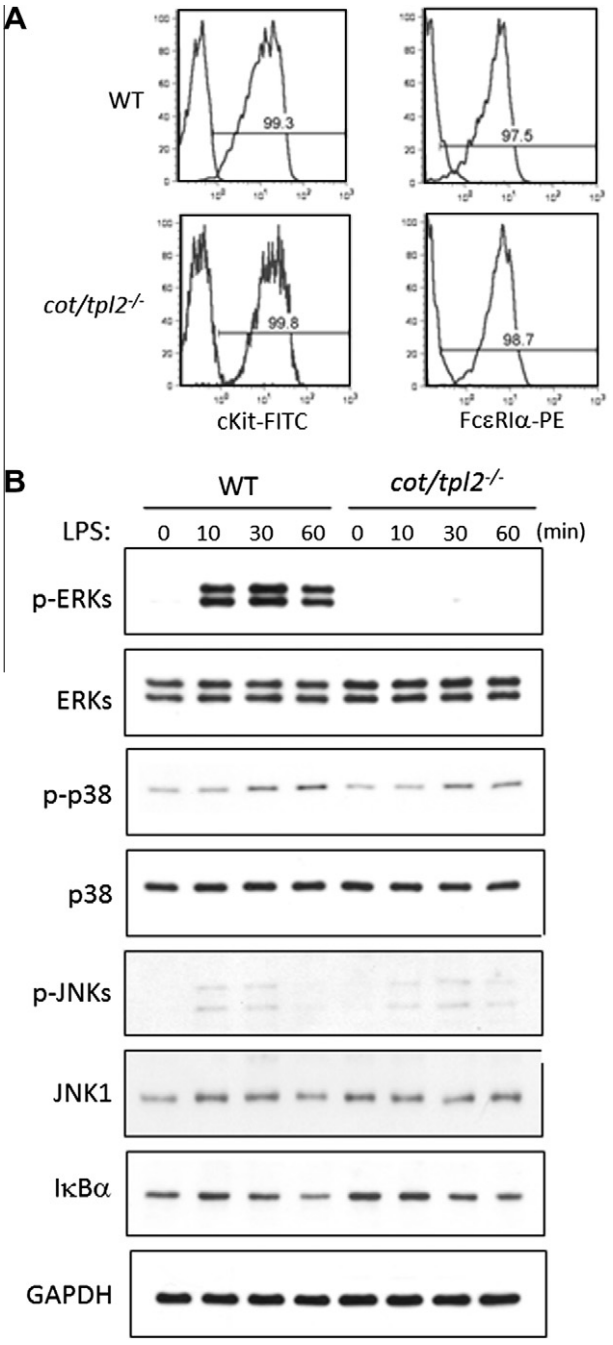

Fig. 1. LPS-induced ERK1/2 phosphorylation was abrogated in *cot/tpl2*$^{-/-}$ BMMCs. (A) Flowcytometric analyses of BMMCs for the cell surface expression of cKit and FcεRIα. BMMCs derived from C57BL/6J and *cot/tpl2*$^{-/-}$ mice were surface stained with FITC-labeled anti-cKit and PE-labeled anti-FcεRIα antibodies. Histograms of each protein expression are shown in combination with the respective isotype-control results. The numbers indicate the percentage of positive cells for the surface expression. (B) BMMCs from WT and *cot/tpl2*$^{-/-}$ mice were stimulated with LPS at 1 μg/ml for indicated time. The cytoplasmic lysates were separated by SDS–PAGE and immunoblotting was performed with indicated antibodies.

phoresed, transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled β-actin or TNF-α cDNA probe.

2.7. Western blot analysis

Cytoplasmic cell lysate was prepared with PLC lysis buffer as previously described [22]. Proteins separated by SDS–polyacrylamide gel electrophoresis were transferred to immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Western blotting analysis was proceeded as described as previously [24], followed by detection using enhanced chemiluminescence system (Perkin–Elmer, Boston, MA) according to the manufacturer's instructions.

2.8. Degranulation assay

The degree of degranulation was determined by measuring the release of β-hexaminidase as described previously [22]. Briefly, BMMCs were bound with 2 μg/mL anti-DNP IgE for 2 h at 37 °C, washed twice, and resuspended in Tyrode's buffer. The cells were stimulated with 100 ng/mL DNP-conjugated BSA or PMA/ionomycin for 40 min. The supernatants and intact-cell lysates were reacted with 1.3 mg/mL *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide in 0.1 M citrate buffer (pH4.5) for 90 min at 37 °C. The reaction was stopped by adding 0.2 M glycine (pH 10.7), and OD_{405} was measured by spectrophotometer.

3. Results

3.1. Cot/Tpl2 is indispensable for LPS-stimulated ERK activation in BMMCs

BMMCs were isolated from bone marrow cells of wild-type (WT) and *cot/tpl2*$^{-/-}$ mice by a standard procedure as described in Section 2. Maturation of mast cells was similarly confirmed for both WT and *cot/tpl2*$^{-/-}$ cells by toluidine blue staining (data not shown) and cell surface expression of c-Kit/FcεRIα (Fig. 1A), indicating that Cot/Tpl2 is basically dispensable for mast cell differentiation.

Signaling activities of MAP kinases and NF-κB were measured by Western blotting for the isolated BMMCs using phospho-specific antibodies and anti-IκB antibody. Stimulation with LPS rapidly induced phosphorylation of ERK1/2, JNKs, and p38 kinase, as well as the degradation of IκBα in WT BMMCs (Fig. 1B), as we previously reported [12]. In *cot/tpl2*$^{-/-}$ BMMCs, however, LPS-stimulated phosphorylation of ERK1/2 was abrogated. On the other hand, the phosphorylation of JNKs and p38 kinase, as well as the degradation of IκBα, was normally induced by LPS in *cot/tpl2*$^{-/-}$ BMMCs.

3.2. Cot/Tpl2 is essential for the gene expression of IL-5, IL-10, IL-13, and TNF-α in LPS-stimulated BMMCs

We and others have previously reported that mast cells produce not only pro-inflammatory cytokines such as TNF-α and IL-6, but also Th2-type cytokines such as IL-5, IL-10 and IL-13, when exposed to LPS [10,12,25,26]. As Cot/Tpl2 has been reported essential for TNF-α production in LPS-stimulated macrophages [19], we measured TNF-α protein in the culture supernatants of WT and *cot/tpl2*$^{-/-}$ BMMCs (Fig. 2A). When stimulated with LPS, *cot/tpl2*$^{-/-}$ BMMCs produced significantly less TNF-α than WT BMMCs. This was in sharp contrast to the TNF-α production induced by PMA/ionomycin stimulation, which was comparable between WT and *cot/tpl2*$^{-/-}$ BMMCs. When TNF-α mRNA levels were examined, *cot/tpl2*$^{-/-}$ BMMCs expressed significantly less TNF-α mRNA in response to LPS than WT BMMCs in both Northern blotting (Fig. 2B) and semi-quantitative RT-PCR (Fig. 2C) analyses.

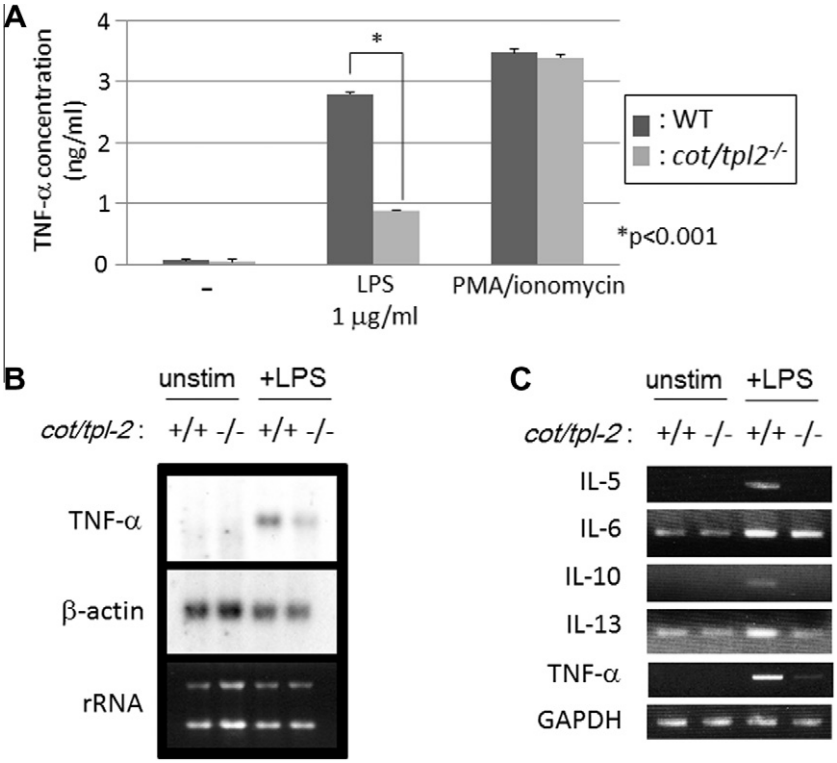

Fig. 2. Cot/Tpl2 is essential for the LPS-stimulated induction of TNF-α and Th2-type cytokines in BMMCs. (A) BMMCs collected from WT and Cot/tpl2$^{-/-}$ mice were stimulated with LPS or PMA/ionomycin for 8 h, and the culture supernatants were collected. The concentrations of TNF-α in the supernatants were assessed by ELISA. The significance of difference was determined by Student's *t*-test ($p < 0.001$). (B, C) Total RNA was collected from LPS-stimulated WT and *cot/tpl2*$^{-/-}$ BMMCs, and the cytokine mRNAs were analyzed by RT-PCR with indicated primers (B) or by Northern blot with the cDNA probe for TNF-α (C).

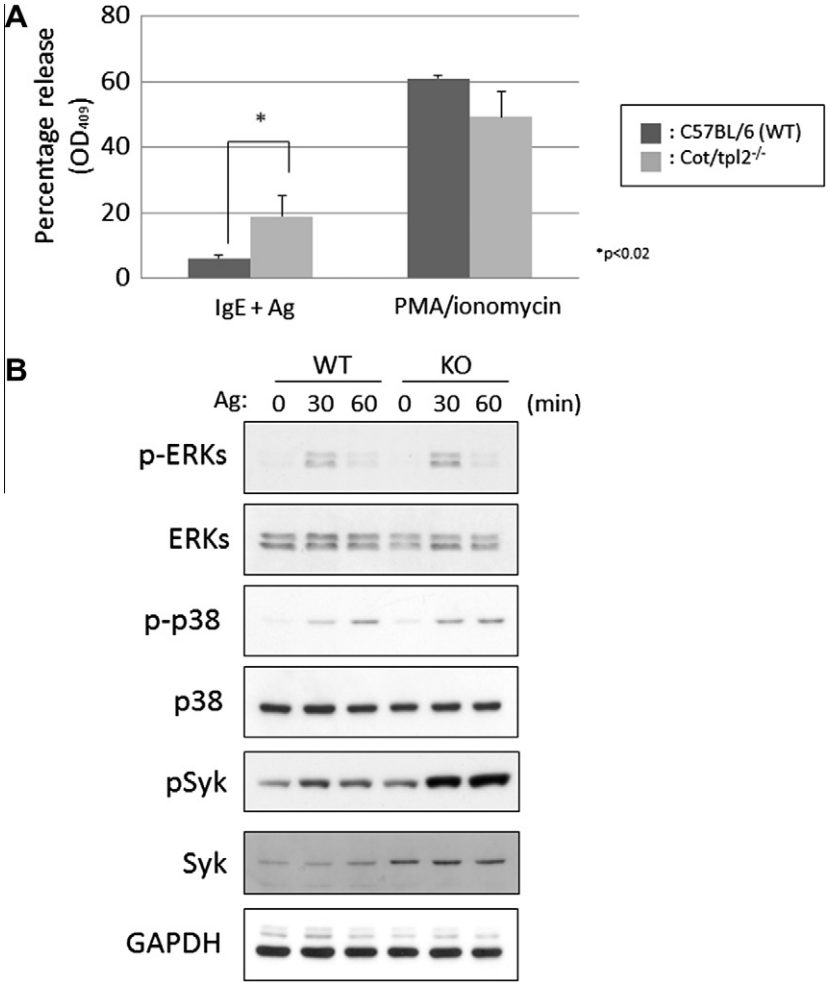

Fig. 3. Antigen-induced degranulation was enhanced in *cot/tpl2*$^{-/-}$ BMMCs. (A) Anti-DNP IgE-bound WT and Cot/tpl2$^{-/-}$ BMMCs were stimulated with DNP-conjugated BSA (100 ng/mL) or PMA/ionomycin for 40 min. Degranulation from BMMCs was assessed by β-hexosaminidase releasing assay. (B) Anti-DNP IgE-bound WT and Cot/tpl2$^{-/-}$ BMMCs were stimulated with DNA-conjugated BSA (100 ng/mL) for indicated time. The cytoplasmic lysates were separated by SDS–PAGE and Immunoblotting was performed with indicated antibodies.

We next examined the effect of *cot/tpl2*-deficiency on the mRNA expression of Th2-type cytokines (IL-5, IL-6, IL-10 and IL-13) in LPS-stimulated BMMCs (Fig. 2C). Similarly to TNF-α, mRNA induction of IL-5, IL-10, and IL-13 by LPS was significantly inhibited in *cot/tpl2*$^{-/-}$ BMMCs. In contrast, the level of IL-6 mRNA induced by LPS was similar between WT and *cot/tpl2*$^{-/-}$ BMMCs.

3.3. FcεRI-mediated degranulation was enhanced cot/tpl2$^{-/-}$ BMMCs

Clustering of FcεRI on mast cells triggers a network of processes that culminates in the secretion of granule-stored mediators [27]. In order to analyze the effect *cot/tpl2*-deficiency on the degranulation processes, clustering of IgE-bound FcεRI on WT and *cot/tpl2*$^{-/-}$ BMMCs were induced by multivalent antigen, followed by measurement of the released β-hexaminidase. The extent of degranulation from *cot/tpl2*$^{-/-}$ BMMCs was significantly enhanced compared with the WT control (Fig. 3A). When the BMMCs were stimulated by PMA/ionomycin, degranulation was similarly observed for WT and *cot/tpl2*$^{-/-}$ BMMCs (Fig. 3A), indicating that the downstream degranulation mechanism is intact and responds normally to the PKC/Ca^{++} signal in *cot/tpl2*$^{-/-}$ BMMCs.

We next analyzed the activation of cell signaling molecules in BMMCs stimulated by FcεRI-clustering. The addition of the multivalent antigen rapidly induced the phosphorylation of ERKs, p38 kinase, and Syk in both WT and *cot/tpl2*$^{-/-}$ BMMCs (Fig. 3B). The induced phosphorylation level of Syk was significantly higher in *cot/tpl2*$^{-/-}$ BMMCs, whereas that of ERKs and p38 kinase was similar between WT and *cot/tpl2*$^{-/-}$ BMMCs. The increased Syk phosphorylation was apparently due to the increased protein concentration of Syk in *cot/tpl2*$^{-/-}$ BMMCs (Fig. 3B). The protein concentration of Syk was consistently higher in *cot/tpl2*$^{-/-}$ BMMCs in several independent experiments.

4. Discussion

In the present study, we sought to examine the role of Cot/Tpl2, a serine/threonine kinase, in the LPS-stimulated cytokine production by mast cells. Mast cells, which are essential inducers of allergic reactions, also play a critical role in host immune defense against gram-negative bacteria through the release of TNF-α [2]. It was previously reported that macrophages derived from *cot/tpl2*$^{-/-}$ mice were severely impaired in the activation of ERKs and the secretion of TNF-α, but normal in the activation of JNKs and p38 kinase, in response to LPS stimulation [19,20], indicating a specific involvement of Cot/Tpl2 in the downstream signal of LPS in macrophages. Here, our present data have added some new information demonstrating that Cot/Tpl2 is also essential as the upstream regulator of ERK activation and TNF-α production in mast cells.

In a previous report, as the TNF-α mRNA was normally induced by LPS, the authors concluded that Cot/Tpl2 is essential for the post-transcriptional regulation of TNF-α expression in macrophages. Using an independently developed line of *cot/tpl2*$^{-/-}$ mice, we previously demonstrated that Cot/Tpl2 is at least partially involved in the TNF-α mRNA increase in LPS-stimulated macrophages [20]. Being similar to our previous report on macrophages, LPS-stimulated increases of TNF-α mRNA and protein secretion were both severely impaired in *cot/tpl2*$^{-/-}$ BMMCs (Fig. 2A and B), indicating that Cot/Tpl2 is essential in mast cells for the TNF-α mRNA expression in response to LPS. The reason for the discrepancy between the previous report and our results is unclear. It is possible that Cot/Tpl2 regulates the LPS-mediated expression of TNF-α at both transcriptional and post-transcriptional levels.

Besides TNF-α, mast cells respond to LPS by also producing significant amounts of Th2-associated cytokines including IL-5, IL-10 and IL-13, but not IL-4 [12]. It is probably of much clinical significance as various investigators have indicated bacterial infection as a risk factor for asthma [13,14]. Our present data have shown that, similarly to TNF-α, Cot/Tpl2 is also essential for the mRNA expression of IL-5, IL-10, and IL-13, in LPS-stimulated mast cells (Fig. 2C). This is especially important, as Cot/Tpl2 may be considered as a candidate for the new therapeutic target of various immune disorders caused by Th2-type responses.

It should be noted, however, that *cot/tpl2*$^{-/-}$ BMMCs were hyper-responsive in degranulation stimulated by FcεRI-clustering (Fig. 3A). Unlike the response to LPS, this is not due to the defective ERK activation, as ERK phosphorylation was normally induced by FcεRI-clustering in *cot/tpl2*$^{-/-}$ BMMCs (Fig. 3B), indicating that Cot/Tpl2 is not essential as the upstream activator of ERK in FcεRI signaling. Instead, *cot/tpl2*$^{-/-}$ BMMCs showed significantly increased phosphorylation of Syk compared with the WT cells, after FcεRI-clustering (Fig. 3B). This appears to be due to the increased protein expression level of Syk (Fig. 3B). Syk is a major player of FcεRI signaling, whose activation, which is caused by its phosphorylation, is essential for the antigen-mediated degranulation [28]. It is postulated that Cot/Tpl2 functions as a positive regulator of Syk protein expression during mast cell differentiation. The elucidation of the detailed mechanism, however, needs further examination.

Unlike TNF-α, the inhibitory effects of *cot/tpl2*-dificiency on LPS-induced Th2-type cytokine production from mast cells do not seem to be mediated through the decreased ERK activity, as pretreatment with PD98059, a specific inhibitor of MEK1/2, failed to inhibit the LPS-mediated production of any of the Th2-type cytokines from BMMCs [12]. As MEK1/2 is a direct upstream kinase of ERKs and Cot/Tpl2 is a further upstream signaling molecule of the MEK1/2-ERK activation pathway, we hypothesize that Cot/Tpl2 controls LPS-stimulated Th2-type cytokine production independently of MEK1–2-ERK activation. In support of our hypothesis, previous reports have indicated the MEK1/2-ERK-independent functions of Cot/Tpl2. For example, macrophages from *cot/tpl2*$^{-/-}$ mice responded to CpG (bacterial) DNA, a ligand of TLR9, with significantly increased expression of IL-12 and IL-10, whereas the CpG DNA-induced phosphorylation of ERKs was intact [20]. Cot/Tpl2 is required in fibroblasts for the transduction of cell migration and gene expression signals originating in the G-protein-coupled receptor proteinase-activated receptor 1 (PAR1) by activating Rac1 and focal adhesion kinase (FAK) in an apparently ERK-independent manner [29].

More importantly, in LPS-stimulated macrophages, Cot/Tpl2 has been reported to mediate the activation of cAMP-responsive element-binding protein (CREB) through its phosphorylation by Msk1 in a manner at least partially independent of ERK activation [30]. CREB is a transcriptional factor involved in the transcriptional up-regulation of both IL-10 [31] and IL-13 [32]. Furthermore, we have found that CREB is more rapidly phosphorylated by LPS in BMMCs than in peritoneal macrophages (data not shown). Thus, it is an intriguing possibility that Cot/Tpl2 mediates LPS-stimulated induction of IL-10 and IL-13 mRNAs through the activation of CREB in an ERK-independent manner.

The mechanism of IL-5 mRNA induction by LPS in mast cells is largely unknown at present. Previous reports showed that IL-5 production is dependent on the protein kinase C (PKC) activity [33], and found functional NFAT binding sites in the proximal promoter of the IL-5 gene [34]. Noticeably, it has been recently reported that Cot/Tpl2 physically associates with protein kinase C (PKC) and nuclear factor of activated T cells (NFAT) c2, and directly regulates transcriptional activity of NFATc2 by its phosphorylation in T cells [35], indicating the possibility that Cot/Tpl2 mediates the induction of IL-5 by LPS through PKC and/or NFAT.

In conclusion, our present findings indicate the unique roles of Cot/Tpl2 in both the LPS-stimulated cytokine expression and the IgE-mediated degranulation of mast cells. As several specific Cot/Tpl2 inhibitors have recently been reported [36], Cot/Tpl2 may be considered as a candidate for a new therapeutic target of allergic diseases such as asthma.

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