Zinc-binding metallothioneins are key modulators of IL-4 production by basophils

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Abbreviations used in this paper: BMBA, bone marrow–derived basophil; Ca, calcium; CaN, calcineurin; CsA, cyclosporin A; FcεRI, Fc epsilon receptor 1; MT, metallothionein; NFAT, nuclear factor of activated T-cells; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; siRNA, short interfering RNA; Th2, T helper 2; TNP, trinitrophenyl; Zip, ZRT/IRT-related protein; Zn, Zinc; ZnT, Zn transporter.

1. Introduction

Zinc (Zn), an essential trace element, forms a structural component of many proteins, including enzymes in cellular signaling pathways and transcription factors, and it is essential for their biological activities (Fukada et al., 2011; Hirano et al., 2008). Defects in Zn homeostasis cause growth retardation, immunodeficiency, and other diseases (Prasad, 1995). Zn homeostasis is tightly controlled by Zip/Slc39 and ZnT/Slc30 family members, Zn transporters that increase or decrease the intracellular Zn, and by Zn-binding proteins such as metallothioneins (MTs) (Andrews, 2001; Palmiter, 2004).

MTs are low molecular weight (~7 kDa) proteins that exhibit a high affinity for divalent heavy metal ions. MTs are grouped into four classes (I-IV) (Thirumoorthy et al., 2011). MT-I and MT-II, which are nearly ubiquitous, are induced by metals and inflammatory stress signals. MT-III is predominantly expressed in neurons and glia. MT-IV is expressed in differentiating stratified squamous epithelial cells (Palmiter, 1998). MTs are reported to assist in the detoxification of heavy metals such as mercury and cadmium, in the homeostasis of essential metals such as copper and zinc, and in protecting cells from oxidative stress caused by hydroxyl free radicals and nitric oxide (Thirumoorthy et al., 2011). MTs also function to buffer Zn, thereby regulating the cytosolic Zn ion concentration. However, it is not known whether MTs regulate intracellular signal transduction.

In mast cells, aggregation of the high-affinity IgE receptor Fc ϵ RI initiates the Zn wave, a phenomenon in which free Zn is released from the endoplasmic reticulum (ER) (Yamasaki et al., 2007). The Zn wave positively modulates NF- κ B's DNA-binding activity, thereby regulating the mast cells' cytokine production downstream of Fc ϵ RI (Yamasaki et al., 2012). In addition, the ZnT-family member ZnT5 is required for the Fc ϵ RI-induced translocation of PKC- β to the plasma membrane, and therefore, for the NF- κ B-dependent cytokine production by mast cells (Nishida et al., 2009). These results indicate that Zn acts as an intracellular signaling molecule in mast cells, and that Zn is essential for the Fc ϵ RI-induced cytokine production by mast cells.

Basophils represent less than 1% of the peripheral blood leukocytes. Like mast cells, they express cell-surface Fc ϵ RI and release cytokines and other chemical mediators after Fc ϵ RI cross-linking (Galli, 2000; Karasuyama and Yamanishi, 2014). Under normal physiological conditions, basophils circulate in the blood while mast cells reside in peripheral tissues. Basophils infiltrate peripheral tissue in response to allergic inflammation, such as in bronchial asthma and atopic dermatitis (Macfarlane et al., 2000; Plager et al., 2006). While basophils have long been neglected in immunological studies because of their small numbers and their morphological similarity to mast cells, they are now recognized as a major source of Th2-promoting cytokines, such as IL-4 and TSLP (Schroeder et al., 2001; Seder et al., 1991; Sokol et al., 2008; Voehringer et al., 2004). However, the molecular mechanisms of basophil IL-4 production have not been fully elucidated.

In the present study, we hypothesized that Zn plays a role in the basophils' IL-4 production. We first examined the expression profiles of Zn-controlling molecules in mouse basophils, and found that FcεRI stimulation significantly induced the *Mt-1* and *Mt-2* genes. We then demonstrated that MTs were required for the FcεRI-induced basophil production of IL-4. We also found that MTs were required to maintain the proper intracellular Zn concentration and to regulate calcineurin (CaN) phosphatase activity, thus impacting the FcεRI-induced nuclear factor of activated T-cell (NFAT)-dependent IL-4 production. Our findings indicate that MT-dependent Zn regulation is essential for basophil IL-4 production.

2. Material and methods

2.1. Mice

C57BL/6 mice (8–10 weeks old) were purchased from CREA (Tokyo, Japan). MT-I/II(-/-) C57BL/6 mice were kindly provided by Dr. Chiharu Tohyama (The University of Tokyo, Japan). All experiments were conducted according to RIKEN's Guidelines for Animal Use and Experimentation.

2.2. Antibodies and reagents

Purified rat anti-mouse CD16/CD32 (2.4G2), biotinylated monoclonal antibodies (mAbs) specific for CD49b (Dx5), purified rat anti-mouse IgE, and purified rat IgG1 kappa (the isotype-matched control) were from BD Pharmingen (San Diego, CA, USA).

FITC-conjugated anti-CD49b (Dx5), PE-anti-mouse CD123, and PE-conjugated anti-Fc ϵ RI α (MAR1) were purchased from eBioscience (San Diego, CA, USA); anti-PKC- β and anti-actin antibodies (Abs) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-AKT and anti-phospho AKT Abs from Cell Signaling Technology (Danvers, MA, USA); and anti-NFATc1 mAb (7A6) from Thermo Scientific (Rockford, IL, USA). [Anti-mouse mast cell protease \(mMCP\)-8 mAb \(TUG8\) was established by the first author \(Ugajin et al., 2009\).](#) Cyclosporin A, Go6976, and wortmannin were from Calbiochem (San Diego, CA, USA).

2.3. Cell preparation and culture

Bone marrow-derived basophils (BMBAs) were prepared by culturing bone marrow cells in the presence of rIL-3 for 10 days, followed by isolating the CD49b⁺ cells using a magnetically activated cell sorter (MACS) system as described previously (Ugajin et al., 2011). To prepare primary basophils, freshly isolated bone marrow cells were MACS-enriched for CD49b⁺ cells, and CD49b⁺CD123⁺ cells were isolated using a FACS Vantage (BD Biosciences, Mountain View, CA, USA). The purity of the sorted cells was >98%.

2.4. Stimulation of basophils

BMBAs (1×10^6 cells/ml) were sensitized with 1 μ g/ml anti-TNP (trinitrophenyl)-IgE (IGEL-b4) in the absence of IL-3 for 6 h at 37 °C, washed, resuspended in Tyrode's buffer, and stimulated with the indicated concentrations of TNP-OVA (BioResearch Technologies, Novato, CA, USA) for the indicated times. *Ex vivo* IgE-mediated stimulation of basophils was performed as described previously (Charles et al., 2009).

2.5. Degranulation assay

The degranulation assay was described previously (Nishida et al., 2009). In brief, BMBAs (1×10^6 cells/ml) were sensitized with 1 μ g/ml anti-TNP IgE in the absence of IL-3 for 6 h, followed by stimulation with 100 ng/ml TNP-OVA in Tyrode's buffer for 30 min. The cells

were centrifuged at 200×g for 5min, and the pellets solubilized with 1% Triton X-100 in Tyrode's buffer. The enzymatic activity of β -hexosaminidase in the supernatants and solubilized cell pellets was then measured with *p*-nitrophenyl *N*-acetyl- β -d-glucosaminide (Sigma-Aldrich) in 0.1 M sodium citrate. The degranulation level was determined by measuring the release of β -hexosaminidase.

2.6. Measurement of IL-4

The IL-4 in cell-culture supernatants was measured with an ELISA kit (R&D, Minneapolis, MN, USA).

2.7. Immunoblotting

Cells were lysed in buffer containing 1% NP-40 and a protease inhibitor cocktail (Active Motif, Carlsbad, CA, USA). Total cell lysates were separated by SDS-PAGE using a 5-20% polyacrylamide gel, transferred to a PVDF membrane, and subjected to immunoblotting with the indicated Abs and HRP-conjugated species-specific secondary Abs. The PVDF membranes were exposed to RX film (Fujifilm, Tokyo, Japan). Densitometric analysis was performed by LAS-1000 (Fujifilm).

2.8. Calcineurin phosphatase assay

Calcineurin (CaN) phosphatase activity was measured using a Calcineurin Phosphatase

Assay Kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) according to the manufacturer's instructions. Briefly, BMBAs pretreated with (background) or without 10 μ M cyclosporin A (CsA, a CaN inhibitor) for 20 min were homogenized with a glass homogenizer in 0.25 M HES buffer and centrifuged at 1000 $\times$ g for 10 min. The supernatants were incubated with 0.75 mM RII phosphopeptide substrate for 10 min. Free phosphate released into the supernatants was determined by a malachite green assay. Phosphate released by CaN was calculated after background subtraction.

2.9. Flow cytometric analysis

Single-cell suspensions were treated with 2.4G2 and normal rat serum, and were stained with the indicated Abs. Cells were analyzed using a FACSCalibur (BD Biosciences, Mountain View, CA, USA), and data were analyzed with CellQuest software (BD Biosciences).

2.10. Zn quantification by flow cytometry

BMBAs were treated with 0.5 μ M Newport green DCF diacetate (Invitrogen, Carlsbad, CA, USA) for 30 min at 37 °C. The fluorescence intensity of the BMBAs was measured using a FACS Caliber (BD Biosciences, Mountain View, CA, USA). The data were analyzed with CellQuest software (BD Biosciences).

2.11. Calcium influx measurement

IgE-sensitized BMBAs were incubated with 10 μ M Indo-1-AM, a cell-permeable Ca-sensor dye (Invitrogen), and Pluronic F-127 (Sigma-Aldrich) for 30 min at 37 °C. The cells were washed and stimulated with antigen. Cells were monitored with a BD LSRII flow cytometer (BD Biosciences). Values were plotted as the ratio of Ca^{2+} -bound Indo-1 (FL5)/ Ca^{2+} to free Indo-1 (FL4).

2.12. Confocal microscopy

For mMCP-8 staining, cytospin preparations were fixed in methanol and treated with blocking solution containing 10% normal goat serum, 0.01% Triton X-100, and 2.4G2. The preparations were then incubated with an Alexa 488-conjugated anti-mMCP-8 mAb. For NFAT2 and PKC- β staining, cells were fixed with 4% paraformaldehyde before spinning. Cytospin preparations were permeabilized with Perm Buffer (BD Biosciences) containing 1% BSA and 2.4G2. The preparations were incubated with anti-NFAT2 mAb or anti-PKC- β mAb, followed by Alexa488-conjugated anti-mouse IgG (Invitrogen) or Alexa488-conjugated anti-rabbit IgG (Invitrogen), respectively. The cells were examined using a TCS SL system (Leica, Bensheim, Germany).

2.13. Real-time PCR analysis

After preparing total cell RNA, first-strand cDNA was synthesized using reverse

transcription with oligo-dT primers. Gene expression was measured using SYBR Green Dye (Applied Biosystems, Foster, CA, USA) with a real-time PCR system, and was normalized to *Gapdh*. The following primers (forward and reverse, respectively) were used for both PCR reactions: for *Gapdh*, 5'-TTCACCACCATGGAGAAGGCCG-3' and 5'-GGCATGGACTGTGGTCATGA-3'; for *Mt1*, 5'-GCCTGCAAGAACTGCAAGTG-3' and 5'-ATAGGAAGACGCTGGGTTGG-3'; for *Mt2*, 5'-TGGAGAACGAGTCAGGGTTG-3' and 5'-TCCTGCAAGAAAAGCTGCTG-3'; for *mll-4*, 5'-ACAGGAGAAGGGACGCCAT-3' and 5'-GAAGCCCTACAGACGAGCTCA-3'.

2.14. Plasmid construction and transfection

The coding region of the mouse *Mt2* gene was isolated from a cDNA library of the C57BL/6 mouse. N-terminally HA-tagged Mt-2 cDNA was inserted into a pCAGI puro expression vector (a gift from Dr. H. Niwa, RIKEN, CDB, Japan). BMBAs were transfected with plasmid vectors or siRNA (Thermo Fisher Scientific, MA, USA) using a two-step electroporator, CUY21ProVibro (Nepa Gene, Chiba, Japan). For electroporation, 1×10^6 BMBAs were re-suspended in 100 μ l of OPTI-MEM, and 12.5 μ g of plasmids or 400 pmol of siRNA were added. Electroporation was carried out as described previously (Yamasaki et al., 2012).

2.15. Luciferase assay

BMBAs were transfected with the luciferase reporter plasmid (pGL4-IL-4) (Kubo et al.,

1994), the renilla reporter plasmid (phRL-TK), and the CAG-puro-HA-MT2 or empty CAG vector. The cells were collected after stimulation and were analyzed using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA).

3. Results

3.1. FcεRI signals induce *Mt-1* and *Mt-2* expression in basophils

To determine how Zn-controlling molecules are involved in basophils' FcεRI-induced cytokine production, we examined the expression profiles of these molecules in primary basophils before and after FcεRI-mediated stimulation by querying the RefDIC (Reference Database of Immune Cells, <http://refdic.rcai.riken.jp/>). This analysis revealed that *Mt-1* and *Mt-2* transcription was significantly elevated after stimulation, while that of *Zips*, *ZnTs*, *Mt-3*, and *Mt-4* was not affected (Supplemental Fig. S1A-C). The *Mt-1* and *Mt-2* mRNA levels also increased in primary basophils and BMBAs after IgE-mediated stimulation (Fig. 1A-B). These results indicated that MT-I and MT-II might be involved in basophil functions.

3.2. MTs are essential for basophils' IgE-mediated IL-4 production

To assess the involvement of MT-I and MT-II in basophil functions, we analyzed BMBAs prepared from MT-I/II(-/-) mice. We first asked if the basophils developed normally in the absence of MT-I and MT-II. The expression levels of FcεRI and CD49b in the MT-I/II(-/-) BMBAs were equivalent to those in wild-type (WT) BMBAs (Fig. 2A). In addition, the expression level of mouse mast cell protease (mMCP-8), a basophil-specific protease (Ugajin et al., 2009), was comparable in MT-I/II(-/-) and WT BMBAs (Fig. 2B). These findings demonstrated that MT-I/II were not required for basophil development. We also

examined the abilities of MT-I/II(-/-) BMBAs to undergo degranulation and to produce IL-4. MT-I/II(-/-) and WT BMBAs released similar amounts of β -hexosaminidase in response to Fc ϵ RI stimulation, indicating that the degranulation in MT-I/II(-/-) BMBAs was normal (Supplemental Fig. S2). However, Fc ϵ RI induced significantly less IL-4 in MT-I/II(-/-) BMBAs than in WT BMBAs (Fig. 2C, left panel). Quantitative PCR analysis of the *Il-4* mRNA levels showed that Fc ϵ RI stimulation increased the *Il-4* transcript levels in WT BMBAs, and that this increase was significantly diminished in MT-I/II(-/-) BMBAs (Fig. 2C, right panel). These findings indicated that MTs contribute to Fc ϵ RI-induced IL-4 production at both the mRNA and protein levels in BMBAs.

We next sought to verify the involvement of MTs in the IL-4 production by primary basophils. Blood basophils prepared from WT and MT-I/II(-/-) mice were passively sensitized with TNP-IgE and then stimulated with anti-IgE using an *ex vivo* basophil stimulation system, as previously described (Charles et al., 2009). Fc ϵ RI induced significantly less IL-4 from MT-I/II(-/-) than from WT blood basophils (Fig. 2D). These results clearly showed that MTs were required for the Fc ϵ RI-induced basophil production of IL-4.

3.3. MTs are selectively required for Fc ϵ RI-induced CaN/NFAT signaling

To determine the molecular mechanisms by which MTs regulate basophils' Fc ϵ RI-induced IL-4 production, we first sought to identify signaling pathways that cause basophils to

produce IL-4. We first examined the effect of the CaN inhibitor cyclosporine A (CsA) on FcεRI-induced IL-4 production. CsA pretreatment significantly diminished the FcεRI-stimulated increase in *Il-4* mRNA (Fig. 3A). The transcription factor NFAT is dephosphorylated by CaN, after which it is translocated to the nucleus to activate target-gene expression (Muller and Rao, 2010). The NFAT family members NFAT1 and NFAT2 were highly expressed in basophils (data not shown). To clarify their roles in basophils' FcεRI-mediated IL-4 production, we transfected BMBAs with siRNAs targeting *Nfatc2* and *Nfatc1* to knock down NFAT1 and NFAT2. We confirmed that the siRNA treatment reduced the *Nfatc2* and *Nfatc1* mRNA expression (Fig. 3D, left and middle panels). The induction of *Il-4* mRNA by FcεRI was significantly inhibited in cells expressing *Nfatc2* or *Nfatc1* siRNA (Fig. 3D, right panel). These results indicated that the CaN/NFAT pathway was responsible for the basophils' FcεRI-mediated IL-4 production. We also examined whether other signaling molecules, such as PKC and PI3K, were involved in producing IL-4. Go6976 and wortmannin, which inhibit PKC-α/β and PI3K, respectively, significantly decreased the amount of *Il-4* mRNA induced by FcεRI stimulation (Fig. 3B-C). Together, these results showed that the PKC, PI3K, and CaN/NFAT signaling pathways were responsible for basophils' FcεRI-induced IL-4 production.

To examine whether MTs were involved in any of these signaling pathways responsible for FcεRI-induced IL-4 production, we first investigated the involvement of MTs in PKC or PI3K activation. We found that the PKC family members PKC-α, PKC-β, and

PKC- δ were expressed in basophils (data not shown). The Fc ϵ RI-induced translocation of PKC- β to the plasma membrane, which is essential for PKC activation, was intact in MT-I/II(-/-) BMBAs (Fig. 4B). The Fc ϵ RI-induced phosphorylation of Akt, a downstream PI3K target, was also equivalent in WT and MT-I/II(-/-) BMBAs (Fig. 4C). However, the Fc ϵ RI-induced nuclear translocation of NFAT2 was decreased in MT-I/II(-/-) BMBAs; the frequency of nuclear NFAT2 localization was lower in the MT-I/II(-/-) than in the WT BMBAs (Fig. 4A). These data indicated that MTs are selectively required for Fc ϵ RI-induced CaN/NFAT signaling.

3.4. Elevated intracellular free zinc inhibits CaN activity in MT-I/II(-/-) basophils

We next sought to identify the upstream targets of MTs involved in NFAT activation, which is known to be regulated by Ca²⁺ mobilization and the Ca²⁺-dependent serine/threonine phosphatase CaN (Muller and Rao, 2010). We found that the CaN phosphatase activity was significantly diminished in MT-I/II(-/-) BMBAs (Supplemental Fig. S3B), although the Fc ϵ RI-induced Ca²⁺ influx was normal (Supplemental Fig. S3A). Thus, MTs contribute to the nuclear translocation of NFAT in basophils by regulating the CaN activity.

To further define the molecular mechanism by which MTs regulate Fc ϵ RI-induced CaN/NFAT signaling, we investigated whether Zn homeostasis was disturbed in MT-I/II(-/-) basophils. For this, we used Newport green DCF diacetate, a cell-permeable Zn-specific fluorescent probe that predominantly localizes to the cytoplasm. We found that the

intracellular Zn levels were higher in MT-I/II(-/-) than in WT BMBAs (Supplemental Fig. S3C). Intracellular Zn levels in 100 nM ZnSO₄-treated WT BMBAs mimicked those in MT-I/II(-/-) BMBAs (Supplemental Fig. S3D). The phosphatase activity of CaN was diminished in ZnSO₄-treated compared to untreated WT BMBAs (Supplemental Fig. S3E). These results supported the idea that MTs impact CaN activity by regulating the intracellular free Zn concentration.

3.5. MT knockdown and reconstitution

To determine whether the impaired IL-4 production observed in MT-I/II(-/-) basophils was directly caused by the loss of MT function, we knocked down MT-II or both MT-I and MT-II in WT BMBAs by transient siRNA transfection. We confirmed that the siRNA treatment reduced the *Mt-1* and *Mt-2* mRNA expression (Fig. 5A, left and middle panels). We found that the FcεRI-induced IL-4 levels in MT-II single-knockdown and MT-I/II double-knockdown BMBAs were significantly lower than in control BMBAs (Fig. 5A, right panel and 5B). We further confirmed that transfecting MT-I/II(-/-) basophils with MT-II cDNA rescued the defective FcεRI-induced IL-4 production. In this experiment, the BMBAs were transiently co-transfected with a murine IL-4 promoter luciferase construct, a renilla luciferase control reporter, and either MT-II cDNA or an empty vector. The FcεRI-induced increase in IL-4 promoter-driven luciferase expression, which was observed in mock-transfected WT BMBAs, was significantly diminished in the mock-transfected

MT-I/II(-/-) BMBAs (Fig. 5C), consistent with the impaired IL-4 production observed in MT-I/II(-/-) BMBAs (Fig. 2C). MT-II overexpression in the MT-I/II(-/-) BMBAs restored the FcεRI-induced IL-4-promoter-driven luciferase activity to normal levels (Fig. 5C). Taken together, these findings showed that MTs are required for regulating the FcεRI-induced IL-4 production in basophils.

4. Discussion

Our previous studies revealed that Zn acts as an essential signaling molecule in FcεRI-stimulated mast cell activation and cytokine production in mouse (Kabu et al., 2006; Nishida et al., 2009; Yamasaki et al., 2012). However, Zn's role in basophil activation has been unclear. Here, we provide evidence that both Zn and the Zn-binding MT proteins are involved in the FcεRI-induced IL-4 production in basophils.

Zn homeostasis is maintained by its transport through fourteen ZIP-family and eight ZnT-family members and by its buffering by four MT-family members. All of these protein families play a role in the intricate regulation of free Zn, and the members involved may depend on the cell type. In mast cells, the ZnT5 expression is up-regulated by FcεRI stimulation, and ZnT5 is required for the FcεRI-induced mast cell cytokine production that occurs through NF-κB activation (Nishida et al., 2009). In the present study, we showed that FcεRI stimulation increased the expression levels of MT-I and MT-II among the Zn-regulating molecules in basophils (Supplemental Fig. S1A-C), suggesting that MT-dependent Zn regulation is involved in basophil function downstream of FcεRI signaling. In fact, our results showed that MTs were involved in the FcεRI-induced IL-4 production but not in degranulation in basophils (Fig. 2C-D and Supplemental Fig. S2).

Basophils are known to produce large amounts of Th2-promoting cytokines, including IL-4 and TSLP (Schroeder et al., 2001; Seder et al., 1991; Sokol et al., 2008;

Voehringer et al., 2004). However, the molecular mechanisms of basophil IL-4 production have not been fully elucidated. Here, using pharmacological inhibitors and RNA-interference experiments, we identified three regulatory pathways responsible for FcεRI-induced IL-4 production: CaN/NFAT signaling, PKC signaling, and PI3K/Akt signaling.

There are four or five NFAT-binding sites in the mouse and human IL-4 promoters (Serfling et al., 2000). Of the five NFAT-family members (NFAT1-5), NFAT2 is the most predominantly expressed in human basophils (Schroeder et al., 2002). FcεRI signals induce NFAT2 accumulation in the nucleus and increase NFAT's binding to the IL-4 promoter (Qi et al., 2011; Schroeder et al., 2002), suggesting that NFAT2 may be required for IgE-mediated basophil IL-4 production. In the present study, treatment with either *Nfatc2* (NFAT1) or *Nfatc1* (NFAT2) siRNA inhibited the FcεRI-induced increase in *Il-4* mRNA in BMBAs (Fig. 3D).

In addition, the CaN inhibitor CsA abolished the FcεRI-mediated induction of *Il-4* mRNA in BMBAs (Fig. 3A). Our findings demonstrated that CaN/NFAT signaling is required for FcεRI-mediated IL-4 production by mouse basophils. In mast cells, PKC is responsible for FcεRI-induced cytokine production through NF-κB activation (Kabu et al., 2006; Nechushtan et al., 2000). We found that the PKCα/β inhibitor Go6976 attenuated the FcεRI-stimulated *Il-4* induction in BMBAs (Fig. 3B), suggesting that PKC positively regulates basophils' FcεRI-induced IL-4 production.

PI3K is reported to be required for IgE-induced mediator release from human basophils (Gibbs and Grabbe, 1999; Miura and MacGlashan, 2000). We found that the PI3K inhibitor wortmannin also attenuated the BMBAs' FcεRI-induced IL-4 production (Fig. 3C), indicating that PI3K activation is required for this IL-4 production in both mouse and human basophils. Thus, the signaling pathways responsible for FcεRI-induced IL-4 production are conserved, and are mediated by the activation of PKC and PI3K and by CaN/NFAT signaling.

Since our results showed that MTs are required for basophils' IL-4 production, we next asked which of the above pathways is regulated by MTs. The FcεRI-mediated nuclear translocation of NFAT was impaired in MT-I/II(-/-) BMBAs (Fig. 4A), while both the FcεRI-induced translocation of PKCβ to the membrane and the phosphorylation of PI3K's downstream target Akt were intact in these cells (Fig. 4B-C). Therefore, MTs are selectively required for FcεRI-induced CaN/NFAT signaling. Ca mobilization activates calmodulin and calmodulin-dependent CaN, thereby inducing NFAT's nuclear translocation (Muller and Rao, 2010). Ca mobilization was intact in MT-I/II(-/-) BMBAs (Supplemental Fig. S3A), [consistent with our finding that the degranulation of MT-I/II\(-/-\) BMBAs was normal \(Supplemental Fig. S2\)](#). However, CaN's phosphatase activity was significantly impaired in MT-I/II(-/-) BMBAs, despite being expressed at normal levels (Supplemental Fig. S3B and data not shown). These results indicated that MTs regulate CaN's phosphatase activity independently of Ca signaling.

CaN is a heterodimer consisting of a catalytic subunit, CaN A, and a regulatory subunit, CaN B. The catalytic domain contains an Fe^{3+} - Zn^{2+} dinuclear center, and Zn is thought to act as a catalytic cofactor (King and Huang, 1984; Rusnak and Mertz, 2000). On the other hand, Zn is reported to inhibit CaN's activity *in vitro* (Aydemir et al., 2009; Huang et al., 2008; Takahashi et al., 2003). In the current study, the intracellular free Zn concentration was higher in MT-I/II(-/-) than in WT BMBAs (Supplemental Fig. S3C) due to the absence of MTs, which are important for storing cytosolic Zn. Experimentally increased Zn levels attenuated the CaN activity in WT BMBAs (Supplemental Fig. S3E). Together, these findings suggest that the MT-mediated regulation of intracellular Zn levels plays an important role in modulating the CaN/NFAT signaling in basophils.

In summary, the present study revealed a novel molecular mechanism that regulates the basophil production of IL-4, involving Zn's sequestration by MTs. This finding suggests that the MTs could be key therapeutic targets for basophil-mediated immune responses and allergic inflammation.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Figure legends

Figure 1. FcεRI-mediated stimulation up-regulates MT expression. BMBAs (A) and primary basophils (CD49b⁺CD123⁺ cells) (B) isolated from the bone marrow cells of C57BL6/J mice were sensitized with 1 μg/ml anti-TNP-IgE and stimulated with 100 ng/ml TNP-OVA for the indicated times. Gene expression levels were measured using SYBR Green Dye with a real-time PCR system, and were normalized to *Gapdh*. The data show the mean±SD. **P*<0.05; ***P*<0.01, Student's *t*-test. Datasets are representative of three (A, B) experiments.

Figure 2. MT-I/II(-/-) basophils have impaired IL-4 production. (A) BMBAs from WT and MT-I/II(-/-) mice were identified as CD49b⁺FcεRI⁺ cells by flow cytometric analysis. (B) Cytospin slides prepared from WT and MT-I/II(-/-) BMBAs were stained with an anti-mMCP-8 mAb (green) and DAPI (blue). Scale bar, 20 μm. The percentage of mMCP-8-positive cells is shown below the photomicrograph. Data show the mean+SD. N.S., not significant, Student's *t*-test. (C) BMBAs from WT (open bars) or MT-I/II(-/-) (closed bars) mice were sensitized with 1 μg/ml anti-TNP-IgE and stimulated with the indicated TNP-OVA concentrations for the indicated times. IL-4 concentrations in the supernatants were determined by ELISA (left panel). *Il-4* transcript levels were measured by real-time PCR and normalized to *Gapdh* (right panel). (D) Blood basophils from WT (open bars) and

MT-I/II(-/-) (closed bars) mice (n=3) were passively sensitized with TNP-IgE and stimulated with anti-IgE using a previously described *ex vivo* basophil-stimulation system (Charles et al., 2009). Plasma IL-4 levels were determined by ELISA. Data show the mean $\pm$ SD.

* P <0.05 or ** P <0.01 by Student's *t*-test, compared with WT basophils. Datasets are representative of three experiments.

Figure 3. Fc ϵ RI-induced IL-4 production is dependent on Calcineurin/NFAT, PKC, and PI3K-mediated pathways.

(A) BMBAs sensitized with 1 μ g/ml anti-TNP-IgE were treated with 10 μ M cyclosporin A (CsA) (closed bars) or vehicle (open bars) for 20 min, then stimulated with 100 ng/ml TNP-OVA for 1 h. (B and C) BMBAs sensitized with 1 μ g/ml anti-TNP-IgE were treated with 5 μ M Go6976 (B, closed bars), 100 nM Wortmannin (C, closed bars), or vehicle (open bars) for 2 h, then stimulated with 100 ng/ml TNP-OVA (Ag) or nothing (Non) for 1 h. IL-4 expression at the mRNA level was assessed by real-time quantitative RT-PCR. (D) NFAT1-knockdown, NFAT2-knockdown, and control BMBAs were sensitized with 1 μ g/ml TNP-IgE, followed by stimulation with 100 ng/ml TNP-OVA (Ag) or nothing (Non) for 1 h. Gene expression levels were assessed by real-time quantitative RT-PCR. Data show the mean $\pm$ SD. * p <0.05, ** p <0.01, compared with vehicle-treated or control BMBAs, Student's *t*-test. A representative dataset of three independent experiments, each which gave similar results, is shown.

Figure 4. MT-I/II(-/-) BMBAs have defective FcεRI-mediated CaN/NFAT signaling.

WT and MT-I/II(-/-) BMBAs were stimulated with 100 ng/ml TNP-OVA (Ag) or nothing (Non) for 30 min (A) or 10 min (B,C). (A) Cells were stained with an anti-NFAT2 Ab (green) and DAPI (4,6 diamidino-2-phenylindole) (blue). Scale bar, 10 μm. The frequency of cells with nuclear NFAT2 staining is shown at right. For each experiment, 100 cells were randomly counted, and the percentage of cells with translocated nuclear NFAT2 was calculated. (B) Cells were stained with an anti-PKC-β Ab (green) and DAPI (blue). Scale bar, 10 μm. The percentage of cells showing PKC-β translocation to the plasma membrane is shown at right. For each experiment, 100 randomly counted cells were analyzed. (C) Cell lysates were immunoblotted with anti-phospho Akt, -Akt, and -actin mAbs. Densitometric values are shown at right. Data show the mean±SD. **P*<0.05, compared with WT BMBA, Student's *t*-test. Datasets shown are representative of three experiments.

Figure 5. Knockdown and reconstitution of MTs.

(A and B) MT-II knockdown (gray bars), MT-I/II double knockdown (closed bars), and control (open bars) BMBAs were sensitized with 1 μg/ml TNP-IgE, followed by stimulation with 100 ng/ml TNP-OVA (Ag) or nothing (Non) for 1 h (A, right panel) or 3 h (B). Gene expression levels were assessed by real-time quantitative RT-PCR (A). The IL-4 concentration in the supernatants was determined by ELISA (B). Data show the mean+SD.

* $p < 0.05$, ** $p < 0.01$, compared with control BMBAs. (C) WT and MT-I/II(-/-) BMBAs were transfected with pGL4-IL-4-Luc and pRL-TK together with vectors expressing MT-II or Mock. The cells were then sensitized with 1 $\mu\text{g/ml}$ TNP-IgE, followed by stimulation with 100 ng/ml TNP-OVA (Ag) or nothing (Non) for 2 h. The normalized luciferase activity obtained from non-stimulated cells was set as 1. Data show the mean + SD. * $p < 0.05$, ** $p < 0.01$, Student's *t*-test. Datasets shown are representative of three experiments.

Figure

A

B

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5