

also involved in responses against *C. rodentium*. The bacterial burdens in the colon after infection with *C. rodentium* showed similar increases in *Il17f*^{-/-}, *Il17a*^{-/-}, and *Il17a*^{-/-}*Il17f*^{-/-} mice, indicating that deficiency of just one of the IL-17 proteins results in full susceptibility to *C. rodentium* infection. Notably, splenomegaly and colon hypertrophy, which are associated with severe colonic inflammation, were more pronounced in *Il17f*^{-/-} mice than in *Il17a*^{-/-} mice, suggesting that IL-17F is more important than IL-17A in protecting colonic epithelial cells from the pathogenic effects of this bacterium. The observation that both IL-17A and IL-17F are required for the protection against *C. rodentium* makes clear contrast to the case of *S. aureus*, infection in which either IL-17A or IL-17F is enough for the protection, suggesting that the defense mechanisms against *S. aureus* and *C. rodentium* infection are different.

We found that β -defensin production was impaired in the infected colons of *Il17a*^{-/-} and *Il17f*^{-/-} mice, suggesting both IL-17A and IL-17F are required for the induction of these molecules in vivo, although either IL-17A or IL-17F alone can promote β -defensin production from ECs (Kao et al., 2004; Liang et al., 2006). Because β -defensins play an important role in immune responses against these pathogens (LeBlanc et al., 2008; Simmons et al., 2002), it seems likely that the defect in β -defensin production contributes to the increased susceptibility of *Il17a*^{-/-} and *Il17f*^{-/-} mice to *C. rodentium*. Although these in vivo data may suggest possible synergistic action between IL-17A and IL-17F in the defense against *C. rodentium*, we could not observe any synergism between these molecules in vitro with a colon epithelial cell line as the target, suggesting that the response of this cell line may be different from colon defensin producer cells in vivo. We also could not observe any synergism between IL-17A and IL-17F in vivo in the protection against *S. aureus*. IL-17A and IL-17F are not required for adaptive immune responses against *C. rodentium*, because *C. rodentium*-specific Ab production in *Il17f*^{-/-}, *Il17a*^{-/-}, and *Il17a*^{-/-}*Il17f*^{-/-} mice was normal.

We found that IL-17A and IL-17F producer cells in the colon are different; IL-17F is primarily produced by colonic ECs and innate immune cells, whereas the major part of IL-17A is produced by Rag2-dependent cells that are likely to be Th17 cells. Furthermore, our data show that IL-17A production is markedly induced after bacterial infection, whereas the induction of IL-17F was not so prominent in infected colons. These observations suggest that colonic EC- and/or innate immune cell-derived IL-17F induce antimicrobial peptides in the ECs, providing protection against initial bacterial invasion and dissemination. This differential action of IL-17A and IL-17F and also the apparent synergy between these two molecules in inducing defensins may explain why these two cytokines are not complementary in the colonic *C. rodentium* infection.

It was reported that *Il17ra*^{-/-} mice show ulcerative syndrome around mucous membranes of the mouth and eyes as a result of colonization of staphylococcus species (Schwarzenberger and Kolls, 2002). These phenotypes closely resemble those observed in *Il17a*^{-/-}*Il17f*^{-/-} mice, suggesting that IL-17RA is involved in both IL-17A and IL-17F signaling. However, we found that IL-17RC is highly expressed in colonic epithelial cells, whereas IL-17RA is preferentially expressed in immune cells such as macrophages and T cells. Because the binding affinity

of IL-17F to IL-17RA is much lower than that of IL-17A (Hymowitz et al., 2001; Wright et al., 2008), and only IL-17F binds to IL-17RC in the mouse (Kuestner et al., 2007), it seemed likely that IL-17A and IL-17F differentially use these receptors. In support of this notion, we showed that the effects of IL-17A and IL-17F are different among colonic epithelial cells, macrophages, and T cells; both IL-17A and IL-17F can induce neutrophil chemoattractants and β -defensins in colonic epithelial cells, whereas only IL-17A can efficiently induce cytokines in macrophages and T cells. These observations suggest involvement of other forms of receptors than IL-17RA–IL-17RC heterodimer complex in the colon. Indeed, IL-17RA and IL-17RC may also form homodimers (Kramer et al., 2006), and IL-17RA interacts with IL-17RB for IL-25 signaling (Rickel et al., 2008). Thus, in addition to the difference of producer cells, cell-type-specific IL-17 receptor distribution and the different binding affinities of IL-17A and IL-17F for these receptors may explain why IL-17A is important in both allergic and host defense responses and IL-17F only contributes innate immune responses in epithelial cells.

A recent study of *Il22*^{-/-} and *Il17rc*^{-/-} mice, which do not respond to IL-17A or IL-17F, demonstrated that IL-22, but not IL-17A and IL-17F, expressed in response to IL-23 is essential for the early host response against *C. rodentium* (Zheng et al., 2008). IL-22 is produced by innate immune cells, such as dendritic cells, during the *C. rodentium* infection and induces the expression of Reg-family antimicrobial proteins in colonic ECs (Zheng et al., 2008). These observations with *Il17rc*^{-/-} mice apparently contradict our results that both IL-17A and IL-17F are involved in the host defense against *C. rodentium*, although our data are compatible with the involvement of IL-22. Further studies to elucidate the relationship between ligand and receptor in the IL-17 system and relative contributions of the bacterial and mouse strains are needed to address these issues. Furthermore, it remains to be investigated whether other IL-17A- and IL-17F-producing cells than ECs and Th17 cells, such as $\gamma\delta$ T cells (Ivanov et al., 2006; Yang et al., 2008), granulocytes (Hue et al., 2006), monocytes (Hue et al., 2006), or mast cells (Kawaguchi et al., 2004), are also involved in host defense against *C. rodentium*.

In conclusion, we have demonstrated the different contributions of IL-17F and IL-17A in allergic responses and protection against bacterial infection. Our findings provide insights into the molecular mechanisms of IL-17A- and IL-17F-mediated responses and should be useful to the development of new therapeutics for allergic diseases and bacterial infections.

EXPERIMENTAL PROCEDURES

Mice

Il17f^{-/-} and *Il17a*^{-/-}*Il17f*^{-/-} mice were generated as shown in Figure S1. *Il17a*^{-/-} (Nakae et al., 2002), *Il17f*^{-/-}, and *Il17a*^{-/-}*Il17f*^{-/-} mice on a 129/Ola \times C57BL/6J background, or mice backcrossed to C57BL/6J (Nihon SLC) or BALB/cA mice (CLEA Japan) for eight or four generations, respectively, were used as described. The sexes and ages (2–3 months old) of the mice were matched in all experimental groups. *Il17f*^{-/-}*Il1rn*^{-/-} and *Il17a*^{-/-}*Il17f*^{-/-}*Il1m*^{-/-} mice were produced by crossing *Il17f*^{-/-} and *Il17a*^{-/-}*Il17f*^{-/-} mice with *Il1m*^{-/-} mice, which were backcrossed for eight generations to BALB/cA mice (Horai et al., 2004). *Rag2*^{-/-} mice were obtained from the Central Institute for Experimental Animals. C3H/HeJ and C3H/HeN or C.B.-17 SCID mice were purchased from Nihon SLC or CLEA Japan, respectively. All mice were kept under specific pathogen-free conditions in an



environmentally controlled clean room at the Center for Experimental Medicine (The Institute of Medical Science, University of Tokyo). The experiments were conducted according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments.

Cell Isolation

Thy1.2⁺, CD4⁺, B220⁺, CD11c⁺, and CD11b⁺ cells were isolated from spleens with an autoMACS (Miltenyi Biotec) after being stained with microbead-conjugated anti-mouse Thy1.2, CD4, B220, CD11c, and CD11b mAbs (Miltenyi Biotec), respectively, according to the manufacturer's instructions. For isolation of thioglycollate-elicited peritoneal macrophages, mice were injected intraperitoneally with 2 ml of 4% thioglycollate (Nissui), and peritoneal cells were collected by washing with PBS 4 days after injection.

Cell Culture

The mouse T cell line (BW5147), B cell line (X5563), macrophage cell line (RAW264), and colonic epithelial cell line (CMT93 or Colon26) were cultured with RPMI 1640 (Sigma) containing 10% FBS. The mouse dendritic cell line (DC2.4) was cultured with RPMI 1640 containing 10% FBS, HEPES, and nonessential amino acids (GIBCO). For the measurement of cytokines, chemokines, and antimicrobial peptide amounts, CMT93 cells, peritoneal macrophages, or CD4⁺ T cells were treated with 5–250 ng/ml recombinant mouse IL-17A or IL-17F (R&D systems) for 6–48 hr.

Measurement of Cytokines, Chemokines, and Antigen-Specific Igs

Concentrations of IFN- γ , IL-6 (OptEIA kit, BD Pharmingen), IL-17A, and IL-17F (DuoSet ELISA kit, R&D systems) in culture supernatants were determined with ELISAs according to the manufacturer's instructions. IL-1 α , IL-1 β , IL-9, IL-10, IL-12/23 p40, IL-12 p70, IL-13, G-CSF, GM-CSF, IFN- γ , CXCL1, CCL2, CCL3, CCL4, and CCL5 amounts in culture supernatants were measured by the Bio-Plex system (Bio-Rad) following the manufacturer's instructions. *C. rodentium*-specific Ig amounts in sera were measured as described previously (Bry and Brenner, 2004).

Flow Cytometry

Cells were stimulated with 50 ng/ml PMA (Sigma), 500 ng/ml ionomycin (Sigma), and 1 μ M monensin (Sigma) for 5 hr. Intracellular cytokine staining was performed as described previously (Komiya et al., 2006). Cells were treated with anti-mouse CD16 and CD32 mAbs (2.4G2) in staining buffer (HBSS containing 2% FCS and 0.1% sodium azide) to block FcR binding and then stained with APC-anti-CD4 mAbs (Gk1.5; BioLegend). After washing, the cells were fixed with 4% paraformaldehyde. After washing with permeabilization buffer (0.1% saponin [Sigma] in staining buffer), cells were incubated with PE-anti-mouse IFN- γ mAbs (XMG1.2; BD Pharmingen), PE-anti-mouse IL-17A mAbs (TC11-18H10; BD Pharmingen), or goat anti-mouse IL-17F polyclonal Abs (AF2057 or BAF2057; R&D systems). For secondary staining, we used Alexa Fluor 488-anti-goat IgG (A-11055; Invitrogen), PE-anti-goat IgG (Santa Cruz), or FITC-streptavidin (BD Pharmingen). The cells were analyzed on a FACSCalibur system (Becton Dickinson) and data were analyzed with FlowJo software (Tree Star).

Real-Time RT-PCR

Total RNA was extracted with Sepasol reagent (Nacalai Tesque) according to the manufacturer's instructions. RNA was denatured in the presence of an oligo dT primer and then reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time RT-PCRs were performed with a SYBR Green qPCR kit (Invitrogen) and an iCycler system (Bio-Rad) with the sets of primers described in Table S2.

Clinical Assessment of Arthritis

Arthritis development in *Il17r^{-/-}* mice was monitored by macroscopic evaluation as described previously (Horai et al., 2004). Arthritis development in each paw was graded by macroscopic evaluation as follows: 0, no change; 1, mild swelling; 2, obvious joint swelling; and 3, severe joint swelling and ankylotic changes.

S. aureus Infection

Bacteria *S. aureus* 834 was prepared as described (Nakane et al., 1995). Bacteria were cultured on tryptic soy agar (Difco) for 12 hr at 37°C, inoculated into tryptic soy broth (Difco), and incubated for another 12 hr. The organisms were collected by centrifugation and resuspended in PBS. The concentration of resuspended cells was adjusted spectrophotometrically at 550 nm. Mice were infected intravenously with 200 μ l of a solution containing 1×10^7 CFU of viable *S. aureus* cells in PBS. For determination of the bacterial burden in the infected tissues, kidneys were homogenized, and diluted in 10-fold steps in sterile PBS. Bacterial CFU was determined by plating each dilution on tryptic soy agar after culture for 12 hr at 37°C.

C. rodentium Infection

C. rodentium infections were performed as described previously (Nagai et al., 2005). In brief, 129/Ola \times C57BL/6J mice were inoculated with 200 μ l of a bacterial suspension (2×10^8 CFU/head) via an oral gavage. For the colony formation assays, colons were harvested and homogenized, and serially diluted homogenates were plated on MacConkey agar (Difco). For histological analysis, colons were fixed with 4% paraformaldehyde in PBS at 4°C overnight and frozen in tissue-freezing medium (Leica Jung). Frozen sections were prepared and stained with anti-*C. rodentium* sera as described previously (Nagai et al., 2005).

Statistics

Unless otherwise specified, all results are shown as mean and the standard error of the mean (SEM). Unpaired Student's *t* tests, Mann-Whitney's *U* tests, or χ^2 tests were used to statistically analyze the results. Differences were considered significant at $p < 0.05$.

SUPPLEMENTAL DATA

Supplemental Data include ten figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at [http://www.immunity.com/supplemental/S1074-7613\(08\)00554-2](http://www.immunity.com/supplemental/S1074-7613(08)00554-2).

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Zinc transporter Znt5/Slc30a5 is required for the mast cell–mediated delayed-type allergic reaction but not the immediate-type reaction

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Zinc (Zn) is an essential nutrient and its deficiency causes immunodeficiency. However, it remains unknown how Zn homeostasis is regulated in mast cells and if Zn transporters are involved in allergic reactions. We show that Znt5/Slc30a5 is required for contact hypersensitivity and mast cell–mediated delayed-type allergic response but not for immediate passive cutaneous anaphylaxis. In mast cells from Znt5^{-/-} mice, Fcε receptor 1 (FcεRI)–induced cytokine production was diminished, but degranulation was intact. Znt5 was involved in FcεRI–induced translocation of protein kinase C (PKC) to the plasma membrane and the nuclear translocation of nuclear factor κB. In addition, the Zn finger–like motif of PKC was required for its plasma membrane translocation and binding to diacylglycerol. Thus, Znt5 is selectively required for the mast cell–mediated delayed-type allergic response, and it is a novel player in mast cell activation.

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Abbreviations used: BMDC, BM-derived mast cell; cDNA, complementary DNA; CHS, contact hypersensitivity; DAG, diacylglycerol; ERK, extracellular signal–regulated kinase; FcεRI, Fcε receptor 1; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; PCA, passive cutaneous anaphylaxis; PKC, protein kinase C; PVDF, polyvinylidene fluoride; Slc, solute-linked carrier; Zip, ZRT/IRT-related protein; Zn, zinc; Znt, Zn transporter.

Zinc (Zn) is one of the essential trace elements. It is a structural component of a great number of proteins, including enzymes and transcription factors, and it is essential for their biological activity (1, 2). Zn has a variety of effects in the immune system (3). Zn-deficient mice have defects in natural killer cell–mediated cytotoxic activity, antibody–mediated responses, host defense against pathogens, and tumors (4–6). In contrast, Zn itself is cytotoxic, and it induces apoptosis in T and B cells (7). Therefore, cells have evolved a complex system to maintain a balance of Zn uptake, intracellular storage, and efflux (8–11). Two solute-linked carrier (Slc) protein families have been identified in Zn transport: the Zn transporter (Znt)/Slc30 and ZRT/IRT-related protein (Zip)/Slc39 (8, 11–13). Znt transporters reduce cytoplasmic Zn availability by promoting Zn efflux from the cytoplasm into the extracellular space or intracellular compartments. The Zip transporters increase

cytoplasmic Zn availability by promoting extracellular Zn uptake and Zn release from compartments into the cytoplasm.

Zn has also been implicated as an intracellular signaling molecule (14–17). A nematode Znt1 orthologue, CDF1, positively affects Ras–extracellular signal–regulated kinase (ERK) signal transduction (18). Slc39a7/Zip7 was found to affect epidermal growth factor/insulin–like growth factor signaling and tamoxifen resistance of breast cancer cells (19). We showed that Slc39a6/Zip6/Liv1 controls the nuclear localization of the Zn finger transcription factor Snail (20). In addition, Toll-like receptor 4–mediated DC maturation is, at least in part, dependent on a Toll-like receptor 4–induced decrease in intracellular free Zn effected by a change in the gene expression profile of Zip

K. Nishida and A. Hasegawa contributed equally to this paper.

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and Znt family members (21). Furthermore, in mast cells, Fcε receptor 1 (FcεRI) stimulation induces an increase in intracellular free Zn, a phenomenon called the "Zn wave" (22). These results support the idea that Zn is involved in intracellular signaling and lead to the prediction that Znts have roles not only in maintaining Zn homeostasis but also in intracellular signaling events (17). Along these lines, we previously showed that Zn might be required for mast cell activation in allergic responses; the Zn chelator TPEN (*N,N,N,N*-tetrakis (2-pyridylmethyl) ethylenediamine) inhibits FcεRI-mediated NF-κB activation and degranulation in mast cells (23). All of these results suggest that Zn has pivotal roles in mast cell function. However, it remains completely unknown how Zn homeostasis is regulated in mast cells and if Znts are involved in mast cell function.

Mast cells are major players in allergy responses such as immediate- and delayed-type hypersensitivity reactions (24–26). Passive cutaneous anaphylaxis (PCA) and contact hypersensitivity (CHS) are mouse models for *in vivo* immediate-type and delayed-type reactions, respectively (27–29). FcεRI stimulation activates several downstream pathways that initiate the allergic inflammatory process by eliciting mast cell degranulation accompanied by the rapid release of preformed chemical mediators such as histamine and serotonin. This process plays critical roles in the immediate-type allergic response. In contrast, the mast cell-mediated delayed responses and IgE-induced chronic allergic inflammatory processes are mainly dependent on cytokine production (30, 31). One important question in mast cell biology is how the early signaling events after FcεRI stimulation are integrated and how specific mast cell responses, such as immediate degranulation versus a delayed-type allergic reaction, are selected. It would be helpful to understand these mechanisms because the identification of molecules that selectively regulate specific mast cell effector functions could provide targets for therapies for mast cell-mediated diseases.

FcεRI stimulation can induce the *de novo* synthesis of proinflammatory cytokines such as IL-6 and TNF-α (32, 33). In particular, both calcium and diacylglycerol (DAG) activate conventional protein kinase C (PKC) isoforms that mediate a critical positive signal necessary for cytokine production in mast cells (34, 35). In addition, conventional PKCs have two regions in their regulatory domain, C1 and C2, in common. The C1 region regulates the activation or translocation of PKC and has two cysteine-rich loops (Zn finger-like motifs) that interact with DAG or phorbol esters (36–40). The C2 region mediates calcium binding. Recently, in T, B, and mast cells, PKCs were shown to be important for NF-κB activation and the upstream function of an adaptor protein complex composed of Bcl-10 and MALT1 (41–43). Collectively, these studies indicate that the PKC/Bcl-10/MALT1/NF-κB cascade plays important roles in cytokine production. Although this linear cascade is well established, it still seems oversimplified. Additional molecules are likely to be involved in the regulation of the PKC–NF-κB signaling pathway.

Inoue et al. (44) reported that mice deficient in Znt5 show severe osteopenia and male-specific sudden death from

bradyarrhythmia during their reproductive period. We demonstrate in this paper that a Znt, Znt5, is required for the mast cell-mediated delayed-type allergic response but not the immediate response. Znt5 is required for the FcεRI-induced translocation of PKC-β to the plasma membrane and, therefore, for NF-κB-dependent cytokine production. These observations provide the first genetic evidence that a Znt, Znt5, is required for immunological responses and has a selective role in allergic reactions.

RESULTS

Znt5 is highly expressed in BM-derived mast cells (BMMCs) but not required for mast cell development

There are eight known members of the Znt family (45). To examine the role of the Znts in mast cells, we checked the expression level of each family member in BMMCs by examining the ReFDIC (Reference Database of Immune Cells) (46). The database showed that Znt5 was among the Znts that were highly expressed in BMMCs (Fig. S1). The messenger RNA (mRNA) level of Znt5 was also enhanced by FcεRI stimulation (Fig. 1 A and Fig. S2). Because Znt5^{-/-} mice are available (44), we focused on Znt5 to clarify its role in mast cell function. First, we determined that Znt5 protein was expressed in BMMCs from WT but not Znt5^{-/-} mice (Fig. 1 B). We also determined that in the BMMCs, Znt5 was mainly located in the Golgi (Fig. 1 C), as reported previously for other cell types (47, 48). Next, we investigated whether Zn homeostasis was disordered in Znt5^{-/-} mast cells using a cell-permeable Zn-specific fluorescent probe, Newport green DCF diacetate, which predominantly localizes to the cytoplasm. The Znt5^{-/-} BMMCs showed an increased level of free Zn in the cytoplasm (Znt5^{+/+} = 0.0723 ± 0.00225 μM; Znt5^{-/-} = 0.106 ± 0.0132 μM; P < 0.05; Fig. 1 D), indicating that the intracellular distribution of free Zn was impaired in the Znt5^{-/-} BMMCs.

We next asked if Znt5 was required for mast cell development. Culturing BM cells from WT and Znt5^{-/-} mice in the presence of IL-3 yielded highly purified mast cell populations. These BMMCs, whether derived from WT or Znt5^{-/-} mice, were indistinguishable in morphology when stained with Alcian blue (Fig. S3 A). The growth rate and total cell numbers in these cultures, as well as the frequency of BMMCs as revealed by flow cytometric analysis of the surface expression of c-kit and FcεRI, were equivalent (Fig. S3 B). In addition to the normal *in vitro* development of the mast cells, the number of mast cells, their morphology, and their anatomical distribution in the ear, skin, and stomach of WT and Znt5^{-/-} mice were comparable (Fig. S4, A and B). Collectively, these findings demonstrated that Znt5 is not required for mast cell development.

Znt5 is selectively required for the CHS but not the PCA reaction

We examined the roles played by Znt5 *in vivo* in PCA and CHS using the Znt5^{-/-} mice. We first tested the importance of Znt5 in the PCA model by evaluating the extravasation of

Evans blue dye in the ears of mice that had been sensitized and challenged with an antigen. However, there was no notable difference in the extravasation kinetics or total amount of Evans blue dye in the ear in the WT and *Znt5*^{-/-} mice (Fig. 2 A), indicating that the mast cell-mediated PCA reaction occurs normally in the absence of *Znt5*.

We next examined the CHS response, which is promoted by mast cell-derived proinflammatory cytokines. WT mice developed robust CHS responses to experimental hapten such as FITC, as assessed by tissue swelling at the site of hapten challenge, which reached a maximum 24 h after antigen stimulation (Fig. 2 B). Consistent with a previous study (31), mast cell-deficient *Kit*^{W-sh/W-sh} mice developed a low-level FITC-induced CHS reaction (Fig. 2 B). The *Znt5*^{-/-} mice exhibited a similar defective CHS reaction (Fig. 2 B). Even 24 h after the antigen challenge, the increase in ear thickness of the *Znt5*^{-/-} mice was <45% of that of WT mice. Consistent with this result, the affected ear of the *Znt5*^{-/-} mice showed significantly reduced FITC-induced cytokine production and

transcription of the genes for IL-6, IL-1 β , MCP, and TNF- α (Fig. 2, C and D). The *Znt5*^{-/-} ears also showed a decrease in the transcription level of the genes for chemokines such as MIP-2 (CXCL2; Fig. 2 C). From these results, we concluded that *Znt5* was selectively required for CHS, but not for PCA, in vivo. To determine whether defectiveness in CHS response in *Znt5*^{-/-} mice is caused by the lack of *Znt5* in mast cell but not in other environmental cells and tissues, we checked *Kit*^{W-sh/W-sh} mice that had been engrafted with mast cell from either WT or *Znt5*^{-/-} mice. We found that engraftment with BMMCs from WT mice, but not *Znt5*^{-/-} mice, restored CHS response in *Kit*^{W-sh/W-sh} mice (Fig. 2 E). Consistent with these results, *Kit*^{W-sh/W-sh} mice reconstituted with *Znt5*^{-/-} BMMCs had significantly reduced FITC-induced cytokine expression level such as TNF- α and IL-6 in the ear (Fig. 2 F). The number of mast cells per mm² of dermis did not change between *Znt5*^{+/+} and *Znt5*^{-/-} BMMC-engrafted *Kit*^{W-sh/W-sh} mice (Fig. S5). Collectively, these findings showed that *Znt5* in mast cells is required for FITC-induced CHS responses.

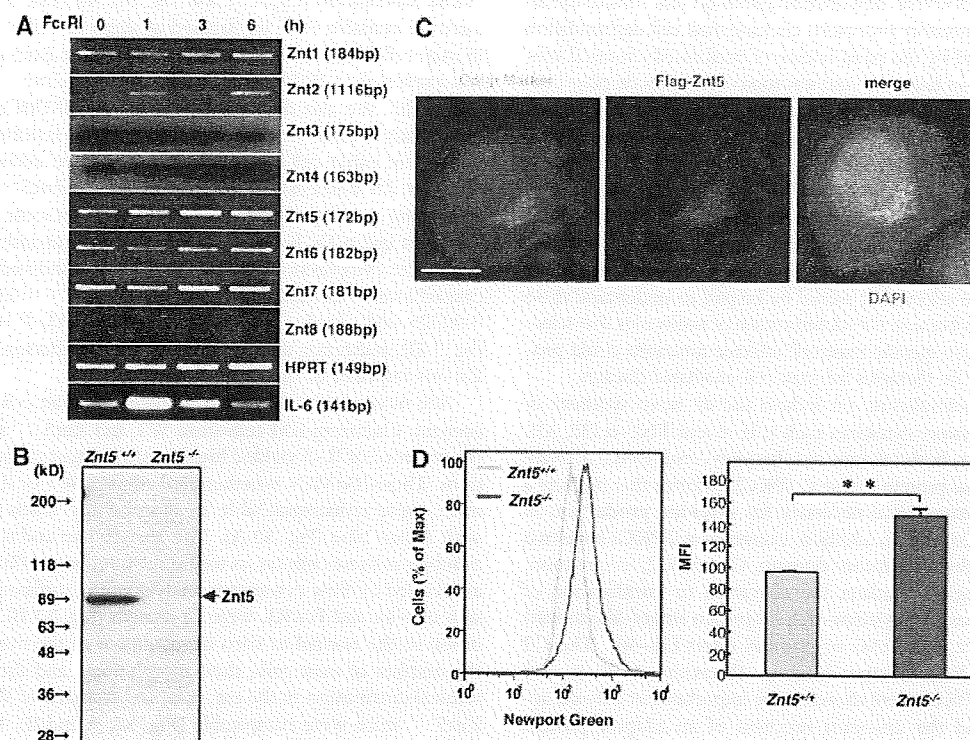


Figure 1. Localization of Znt5 in BMMCs. (A) RT-PCR of mRNAs encoding Znt family members (1–8), IL-6, and hypoxanthine-guanine phosphoribosyl transferase in BMMCs. BMMCs were untreated or treated with DNP-human serum albumin (HSA) for the indicated times. (B) Immunoblotting analysis of Znt5 in BMMCs. The BMMCs from *Znt5*^{+/+} and *Znt5*^{-/-} mice were analyzed by immunoblotting with an anti-Znt5 antibody. (C) Subcellular localization of ectopically expressed Flag-hZnt5 in BMMCs. Cells were retrovirally transfected with Flag-hZnt5 (described in Materials and methods), and then cells were double stained with FITC-conjugated anti-GM130 antibody (green) and Zenon-conjugated anti-flag antibody (red). Nuclei were labeled with DAPI (blue). Bar, 5 μ m. (D) FACS analysis of intracellular free Zn using a Zn indicator, Newport green (left). Blue line, BMMCs from *Znt5*^{+/+} mice; orange line, BMMCs from *Znt5*^{-/-} mice. Histograms show mean fluorescent intensity (right). Data show mean \pm SD. **, $P < 0.01$ (two-tailed Student's t test). A representative dataset of the three (A, C, and D) or two (B) experiments performed, each of which gave similar results, is shown.

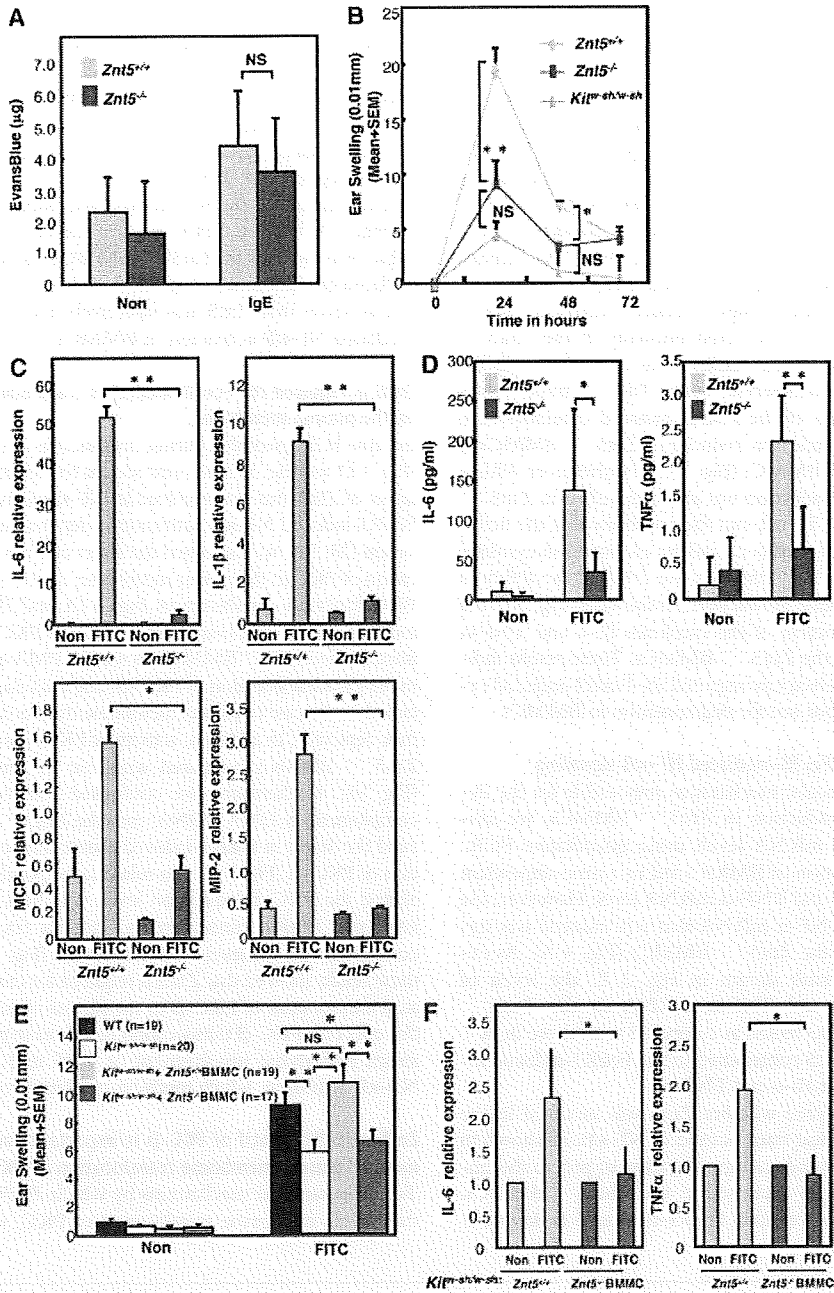


Figure 2. Normal PCA but defective CHS response in Znt5^{-/-} mice. (A) Analysis of PCA in Znt5^{+/+} mice (n = 12) and Znt5^{-/-} mice (n = 11) that received intradermal injections of IgE anti-DNP into the right ear and of saline into the left ear (control, non). After sensitization, mice were intravenously challenged with DNP-BSA. Data show mean + SD of the extravasation of Evans blue into the ears. (B) Analysis of the CHS response in Znt5^{+/+} (n = 14), Znt5^{-/-} (n = 14), and Kitl^{shwshw} (n = 11) mice. Znt5^{+/+}, Znt5^{-/-}, and Kitl^{shwshw} mice were sensitized with FITC. Ear swelling was measured at the indicated times after hapten challenge. Data show mean + SEM. (C) Real-time quantitative RT-PCR of mRNAs for cytokines, chemokines, and GAPDH (loading control) expressed in the ears of Znt5^{+/+} and Znt5^{-/-} mice challenged with FITC after 8 h. Data show the mean + SD (n = 3 per group). (D) Concentrations of IL-6 and TNF-α in Znt5^{+/+} (IL-6, n = 10; TNF-α, n = 5) and Znt5^{-/-} (IL-6, n = 10; TNF-α, n = 5) mice challenged with FITC and measured by enzyme-

Znt5 is required for FcεRI-mediated cytokine production but not for degranulation in BMMCs

FcεRI-induced degranulation and the production of lipid mediators play crucial roles in the PCA reaction, whereas cytokine production is essential for the CHS response (49). To learn the basis for the selective defect in the CHS reaction of *Znt5*^{-/-} mice, we examined the capacities of *Znt5*^{-/-} BMMCs to degranulate, synthesize, and secrete lipid mediators and to produce cytokines. BMMCs derived from *Znt5*^{-/-} mice released similar amounts of β-hexosaminidase to WT BMMCs in response to FcεRI stimulation (Fig. 3 A), indicating that their ability to degranulate was intact. Furthermore, FcεRI stimulation-induced lipid mediator secretion, such as that of leukotrienes C₄, occurred normally in the *Znt5*^{-/-} BMMCs (Fig. 3 B). These results were consistent with the normal PCA reaction observed in the *Znt5*^{-/-} mice. However, the production of the FcεRI-induced cytokines IL-6 and TNF-α was significantly reduced in *Znt5*^{-/-} BMMCs as compared with WT BMMCs (Fig. 3 C). Furthermore, PMA-induced cytokine production was also diminished in *Znt5*^{-/-} BMMCs (Fig. 3 D). To rule out the possibility that the defect in cytokine production was caused by an impaired cytokine transport step in *Znt5*^{-/-} BMMCs, we checked the ability of LPS to induce cytokine production. As shown in Fig. 3 E, the LPS-induced production of the cytokines IL-6 and TNF-α did not decrease in the *Znt5*^{-/-} BMMCs. These results indicated that Znt5 is selectively required for FcεRI-induced cytokine production but not for degranulation in BMMCs.

Znt5 is involved in FcεRI-mediated NF-κB signaling

To define the molecular mechanisms responsible for the defective cytokine production in *Znt5*^{-/-} BMMCs, we analyzed the cytokine mRNA levels using quantitative PCR. The FcεRI stimulation of BMMCs led to a time-dependent increase in the IL-6 and TNF-α mRNA level. However, the level of IL-6 and TNF-α mRNA that was induced was significantly lower in the *Znt5*^{-/-} BMMCs (Fig. 4 A). As expected from the result shown in Fig. 3 E, the levels of LPS-induced IL-6 and TNF-α mRNA in the *Znt5*^{-/-} BMMCs were not decreased as compared with those in WT (Fig. 4 B). Thus, Znt5 contributed to the FcεRI-induced increase of mRNA for IL-6 and TNF-α.

We further analyzed whether Znt5 was involved in the FcεRI-induced nuclear translocation of NF-κB. As shown in Fig. 4 C, the FcεRI-induced translocation of p65 to the nucleus was significantly decreased in the *Znt5*^{-/-} BMMCs. Consistent with these results, FcεRI-mediated IκBα phos-

phorylation and its subsequent degradation were impaired in the *Znt5*^{-/-} BMMCs (Fig. 4 D and Fig. S6). In contrast, the FcεRI-induced activation of the mitogen-activated protein kinases (MAPKs) ERK1/2, JNK1/2, and p38 was not diminished in the *Znt5*^{-/-} BMMCs (Fig. 4 E). We also investigated whether Znt5 was involved in FcεRI-induced calcium-elicited signaling events. We monitored the tyrosine phosphorylation of LAT and PLC-γ1, which contributed calcium signaling (26, 50). As expected from the result shown in Fig. 3 A, LAT and PLC-γ1 tyrosine phosphorylation was apparently normal in *Znt5*^{-/-} BMMCs, as was the FcεRI-induced calcium influx (Fig. S7, A and B). Collectively, these results show that Znt5 was selectively required for FcεRI-mediated NF-κB activation in BMMCs.

Znt5 is required for FcεRI-mediated translocation of PKC-β to the plasma membrane

Because IκBα phosphorylation decreased in *Znt5*^{-/-} BMMCs (Fig. 4 D and Fig. S6), we next sought to identify the upstream target of Znt5 that was involved in NF-κB activation. Because FcεRI-induced NF-κB activation is dependent on PKC activation (23, 51), we examined the effect of Znt5 on the translocation of PKC to the plasma membrane, a process important for PKC function. As shown in Fig. 5 (A and B), the FcεRI-induced plasma membrane translocation of PKC-β was diminished in the *Znt5*^{-/-} BMMCs, as was the PMA-induced plasma membrane translocation of PKC-β, and a similar result was obtained in the case of plasma membrane translocation of PKC-α (not depicted). In addition, activity of PKC was reduced in the *Znt5*^{-/-} BMMCs compared with that in *Znt5*^{+/+} BMMCs (Fig. S8). Furthermore, we confirmed that transfected Znt5 complementary DNA (cDNA) into the *Znt5*^{-/-} BMMCs rescued the defect in FcεRI-induced plasma membrane translocation of PKC-β (Fig. 5 C). To rule out the possibility that Znt5 altered the PKC-β expression level, we checked the protein level after FcεRI stimulation and did not find any decrease compared with the level in WT BMMCs (Fig. S9). Together, these results showed that Znt5 is required for the FcεRI-mediated translocation of PKC-β to the plasma membrane and that this effect on PKC-β translocation is, at least in part, the mechanism underlying involvement of Znt5 in FcεRI-induced NF-κB activation, which results in cytokine production.

Zn finger-like motif of PKC-β is important for the PMA-induced plasma membrane translocation of PKC-β

To understand the mechanisms by which Znt5 is involved in the plasma membrane translocation of PKC, we focused on

linked immunosorbent assay. Data show mean ± SD. (E) Tissue swelling response to vehicle or FITC in FITC-sensitized C57BL/6 WT mice (*n* = 19), *Kit*^{W^{50W}/W⁵⁰ mice (*n* = 20), and *Kit*^{W^{50W}/W⁵⁰ mice that had been selectively repaired of their mast cell deficiency by the intradermal injection of *Znt5*^{+/+} (*n* = 19) or *Znt5*^{-/-} (*n* = 17) BMMCs. Data show mean ± SEM. (F) Cytokine response to vehicle or FITC in *Kit*^{W^{50W}/W⁵⁰ mice that had been selectively repaired of their mast cell deficiency by the intradermal injection of *Znt5*^{+/+} or *Znt5*^{-/-} BMMCs. TNF-α and IL-6 expression level were measured by real-time quantitative RT-PCR. The expression level of nonstimulation was set as 1. Data show the mean ± SD (*n* = 4 per group). *, *P* < 0.05; **, *P* < 0.01. (two-tailed Student's *t* test). Data are from at least two independent experiments with similar results, with two to six (A and B), three (C), two to five (D), seven to ten (E), or four (F) mice per group per experiment.}}}

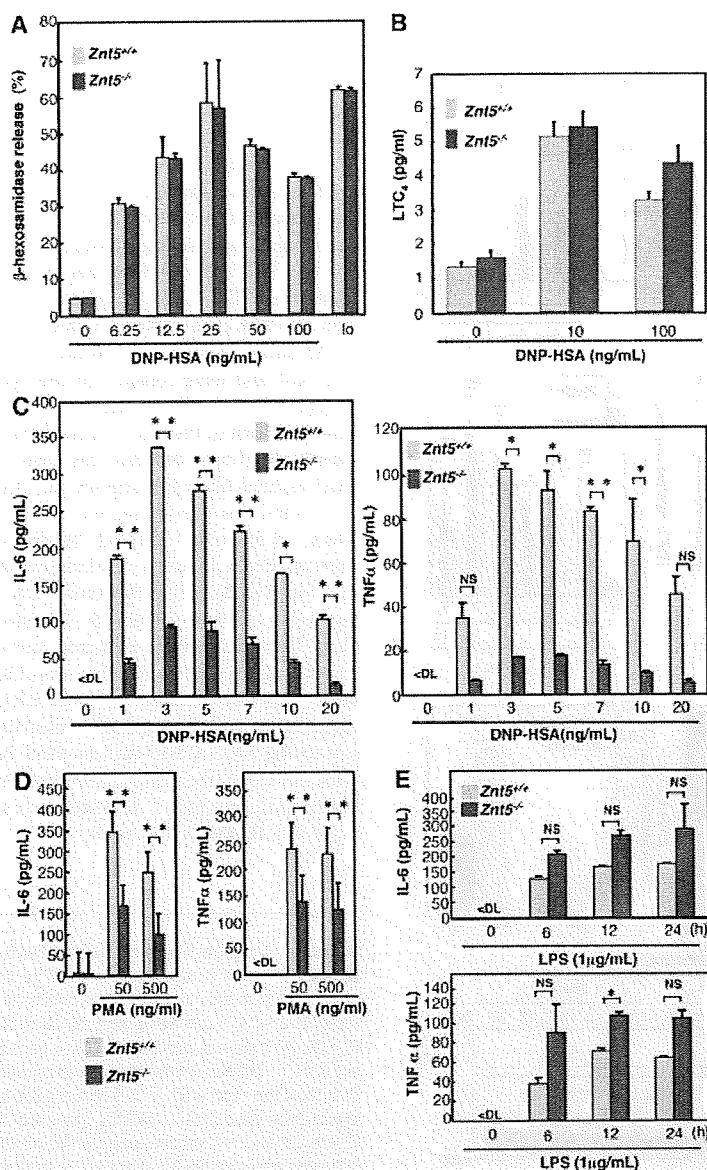
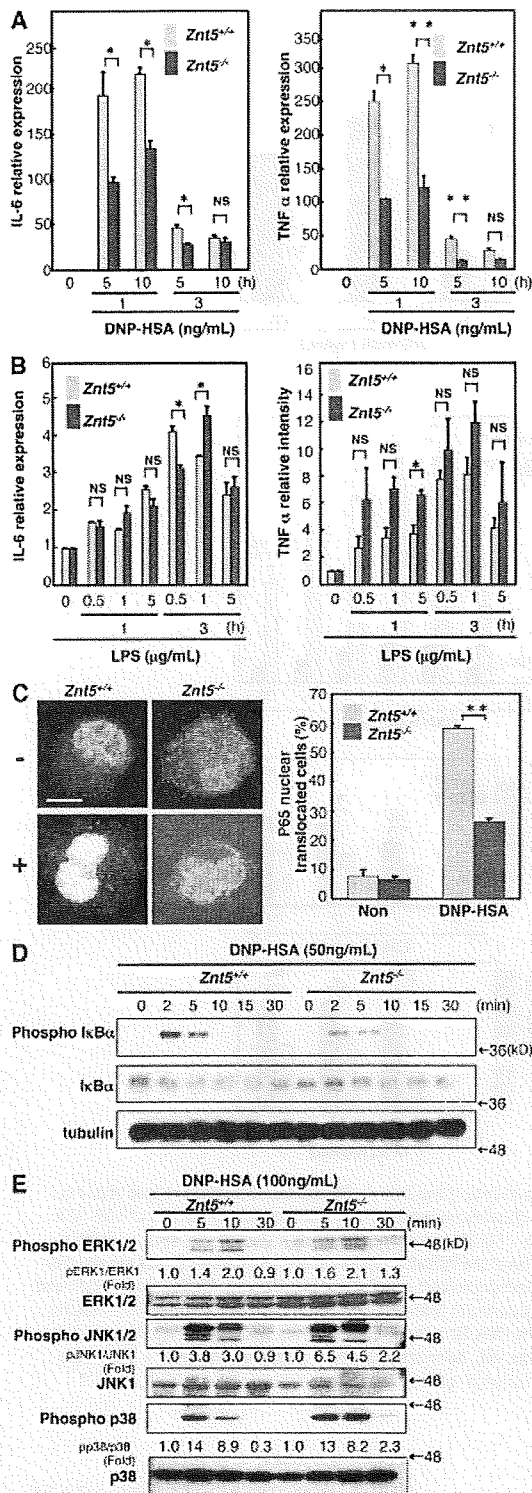


Figure 3. Defective cytokine production in Znt5^{-/-} BMMCs. (A) Degranulation of Znt5^{+/+} and Znt5^{-/-} BMMCs assessed as the release of β -hexosaminidase. BMMCs from Znt5^{+/+} and Znt5^{-/-} mice were sensitized with anti-DNP IgE and stimulated with the indicated concentrations of DNP-HSA. As a control, BMMCs were stimulated with 1 μ M ionomycin (10). Data are the mean \pm SD. (B) Leukotriene synthesis in Znt5^{+/+} and Znt5^{-/-} BMMCs. Anti-DNP IgE-sensitized BMMCs from the two genotypes were stimulated with DNP-HSA at the indicated concentrations. Leukotrienes C₄ was measured by enzyme-linked immunosorbent assay. Data show the mean \pm SD. (C) Enzyme-linked immunosorbent assay of IL-6 and TNF- α in BMMCs from Znt5^{+/+} and Znt5^{-/-} mice sensitized with anti-DNP IgE and stimulated with the indicated concentrations of DNP-HSA. Data show the mean \pm SD. (D) PMA-induced cytokine production in Znt5^{+/+} and Znt5^{-/-} BMMCs stimulated with 50 and 500 ng/ml PMA. Data show the mean \pm SD. (E) LPS-induced cytokine production in Znt5^{+/+} and Znt5^{-/-} BMMCs stimulated with 1 μ g/ml LPS for the indicated time. <DL, below detection limit. Data show the mean \pm SD. A representative dataset of the five (A), four (B), or three (C-E) experiments performed, each of which gave similar results, is shown. *, P < 0.05; **, P < 0.01 (two-tailed Student's t test).



the C1 domain of PKC, the DAG-binding site. The C1 domain of PKC has two Zn finger-like motifs (Fig. 6 A) (39, 40). Based on the known function of the Zn finger, we hypothesized that the Zn finger-like motifs of PKC are required for translocation of PKC- β to the plasma membrane. To demonstrate this hypothesis, we designed WT-PKC- β -GFP and three mutant PKC- β -GFP constructs (Fig. 6 A) and examined their ability to undergo PMA-induced plasma membrane translocation in HeLa cells.

The WT and C1BS mutant form of PKC- β -GFP translocated equally well to the plasma membrane upon stimulation by PMA (Fig. 6 B and Videos 1 and 3). In contrast, the C1AS and C1ABS double mutant constructs did not translocate well and were retained in the cytoplasm (Fig. 6 B and Videos 2 and 4). These data suggested that the C1A Zn finger-like motif of PKC is important for its translocation to the plasma membrane and that this motif is a possible target for Znt5 and the likely binding site for DAG.

To test this hypothesis, we designed a biotin-conjugated PMA, an analogue of DAG (Biotinyl-PMA) (Fig. S10 A). Biotinyl-PMA induced cytokine production, just as PMA did (Fig. S10 B). Using Biotinyl-PMA, we assessed the binding ability of PMA to PKC- β . Transfected PKC- β WT and the C1AS mutant were extracted, run on a gel, and electrotransferred to a polyvinylidene fluoride (PVDF) membrane under native conditions. As shown in Fig. 6 C, Biotinyl-PMA expected to bind the PKC WT construct, but its signal intensity to the PKC C1AS mutant form was lower. Consistent with this result, the signal intensity of Biotinyl-PMA to PKC obtained from Znt5^{-/-} BMMCs was less than that to PKC from WT BMMCs (Fig. 6 D).

Figure 4. Defective Fc ϵ RI-mediated NF- κ B signaling in Znt5^{-/-} BMMCs. Cells were sensitized with anti-DNP IgE and subsequently stimulated for 1 or 3 h with the indicated concentrations of DNP-HSA (A) or LPS (B). The mRNAs for IL-6 and TNF- α were measured by real-time quantitative RT-PCR. (A) Fc ϵ RI-induced IL-6 and TNF- α gene expression in Znt5^{+/+} and Znt5^{-/-} BMMCs. The expression level of nonstimulation was set as 1. Data show the mean \pm SD. (B) LPS-induced IL-6 and TNF- α gene expression in Znt5^{+/+} and Znt5^{-/-} BMMCs. Data show the mean \pm SD. (C, left) Confocal microscopy images of NF- κ B nuclear translocation in cells stimulated with Fc ϵ RI (+) or not (-). Anti-p65 antibody staining (p65, green) and DAPI (nuclei, blue). Bar, 5 μ m. (C, right) Frequency of cells with nuclear NF- κ B. A total of 50 cells were counted randomly in each experiment, and the frequency of NF- κ B nuclear-translocated cells was calculated. Data show mean \pm SD. (D) I κ B α phosphorylation and degradation were determined in Znt5^{+/+} and Znt5^{-/-} BMMCs stimulated with DNP-HSA for the indicated times. Cells were stimulated with Fc ϵ RI and immunoblotted with anti-phospho-I κ B α , anti-I κ B α , and tubulin. (E) Fc ϵ RI-induced MAPK activation in Znt5^{+/+} and Znt5^{-/-} BMMCs. The cell lysates were blotted with anti-phospho-ERK1/2, anti-phospho-JNK1/2, and anti-phospho-p38. The protein loading controls, which were used to normalize densitometric values, were anti-ERK1/2, anti-JNK1, and anti-p38, respectively. A representative dataset of the three (A, B, C, D [I κ B α], and E) or four (D [phospho-I κ B α]) experiments performed, each of which gave similar results, is shown. *, $P < 0.05$; **, $P < 0.01$ (two-tailed Student's t test).

DISCUSSION

Znt5 selectively regulates the mast cell-mediated delayed-type allergic response but not the immediate-type one
 In this paper, we demonstrate that a Znt, Znt5, plays a crucial role in mast cell activation and mast cell-mediated allergic re-

actions. The mast cell is widely recognized as the most important effector cell in IgE-associated allergic disorders such as anaphylaxis. Previous experiments with genetically mast cell-deficient Kit^W/Kit^{W-V} or Kit^{W-sh}/Kit^{W-sh} mice have shown that mast cells are required for the IgE-mediated immediate-type

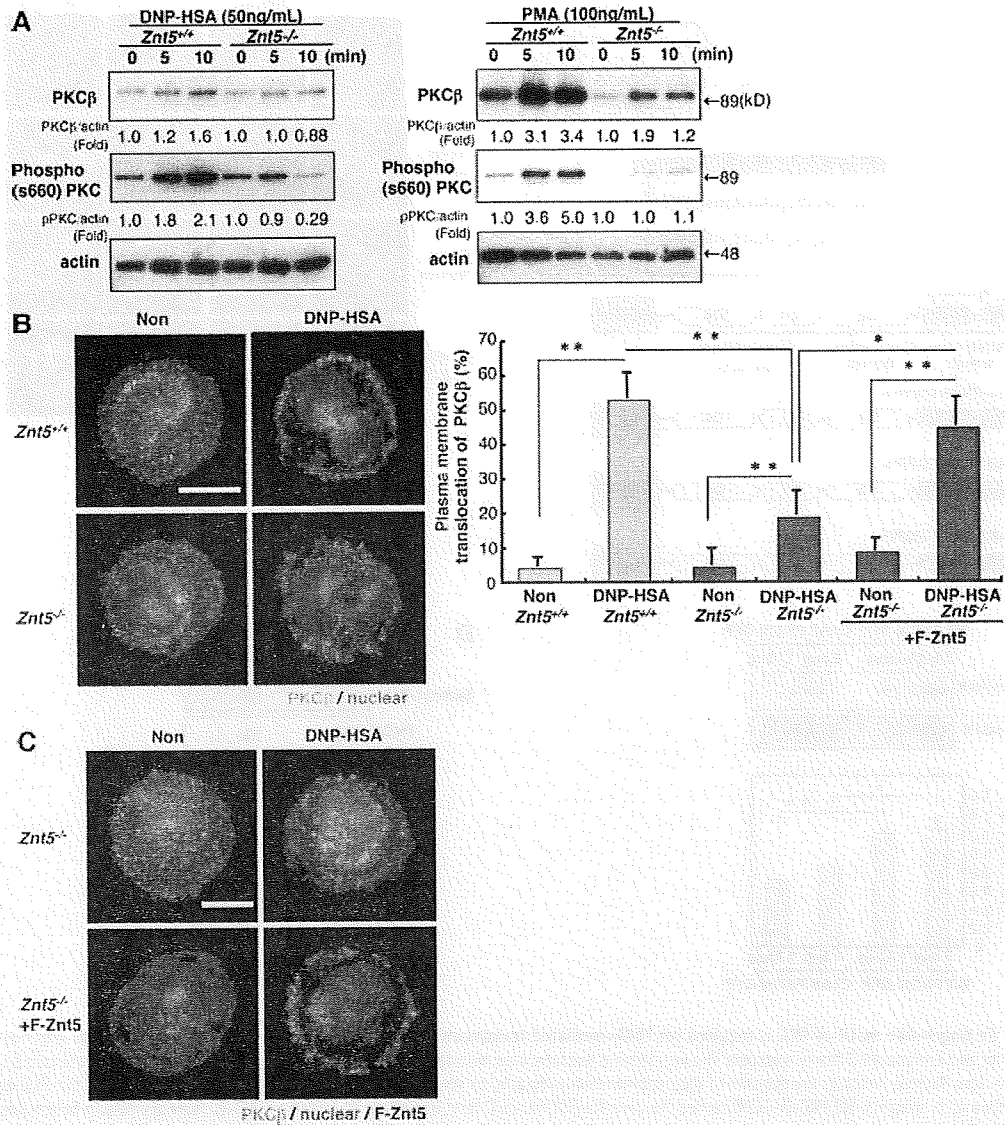


Figure 5. FcεRI or PMA-mediated plasma membrane translocation of PKC-β was impaired in Znt5^{-/-} BMMCs. (A) FcεRI (left)- or PMA (right)-induced plasma membrane translocation of PKC in Znt5^{+/+} and Znt5^{-/-} BMMCs stimulated with DNP-HSA for the indicated times. The plasma membrane fraction was immunoblotted. The protein loading control, used to normalize densitometric values, was anti-actin. (B) PKC-β plasma membrane translocation observed by confocal microscopy. Cells were stimulated with DNP-HSA for 15 min or not (Non). Polyclonal anti-PKC-β antibody staining (green) and DAPI (blue) are shown. Bar, 5 μm. (C) Cells were double stained with anti-PKC-β antibody (green) and Zenon-conjugated anti-flag antibody (red). Nuclei were labeled with DAPI. Bar, 5 μm. The percentage of cells in the plasma membrane translocation of PKC-β is indicated on the right. 30 independent flag-Znt5-positive cells in Znt5^{-/-} BMMCs were counted for each experiment. The values are the mean ± SD. *, P < 0.05; **, P < 0.01, (two-tailed Student's t test). A representative dataset of the three (A and B) or five (C) experiments performed, each of which gave similar results, is shown.

allergic response (24, 27). Thus, the participation of mast cells in the immediate allergic reaction is well established. In addition to this classical role, much evidence has accumulated showing that mast cells play crucial roles in delayed-type aller-

gic reactions and chronic inflammatory diseases, including arthritis, experimental allergic encephalomyelitis, colitis, and CHS (28, 52–54). A variety of mast cell-derived cytokines and chemokines are likely to be essential for delayed-type

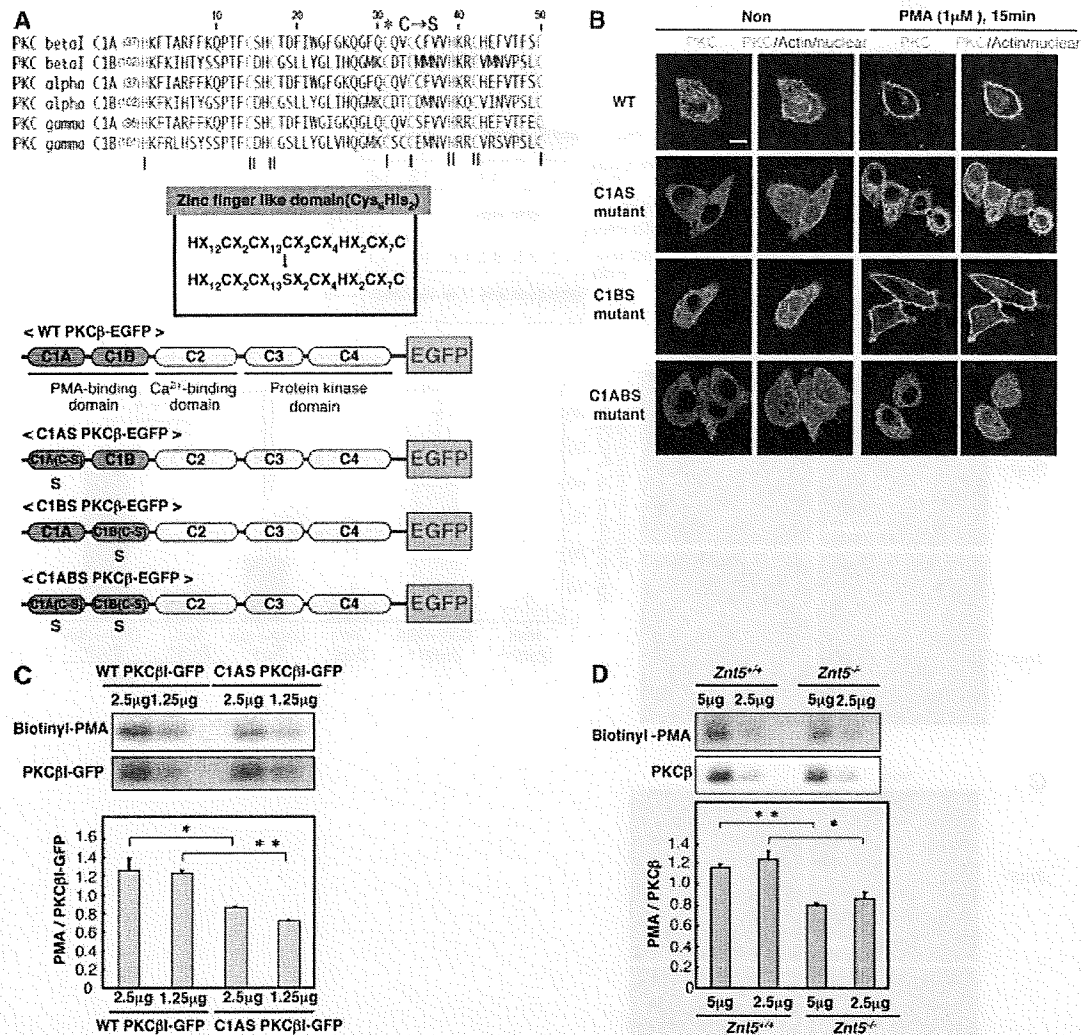


Figure 6. Zn finger-like motif of PKC is required for PMA-mediated translocation of PKC-β to the plasma membrane. (A, top) Alignment of C1 domains of conventional PKC family members. Brown, cysteine residues; blue, histidine residues. I and II indicate residues involved in the two Zn ion coordination sites. Asterisk indicates the position of the cysteine residue replaced by serine in this experiment. (A, bottom) Constructs for the PKC-β-GFP fusion protein and its mutants. WT PKC-β-EGFP carried the gene for EGFP at its C terminus. In the C1 region of PKC-β, there are two Zn finger-like sequences (top). To produce a Zn finger-like motif mutant form of PKC-β, one cysteine residue in each of the two cysteine-rich regions was replaced by serine by site-directed mutagenesis. (B) PMA-induced plasma membrane translocation of PKC-β observed by confocal microscopy. Localization of constructs in transfected HeLa cells stimulated with PMA or not (Non). F-actin and nuclei were labeled with phalloidin-rhodamine (red) and DAPI (blue), respectively. Bar, 10 μm. (C) PMA binding to PKC-β determined using biotinyl-PMA. 293T cells expressing WT-PKC-β-GFP and C1AS-PKC-β-GFP immunoblotted under native conditions (details in Materials and Methods). The probes for PKC-β and biotinyl-PMA were anti-GFP antibody and streptavidin, respectively. (D) Cell lysates from Znt5^{+/+} and Znt5^{-/-} BMDCs resolved by native PAGE and blotted. (C and D) Densitometric values are shown below the blots. Data show mean ± SD. *, P < 0.05; **, P < 0.01 (two-tailed Student's *t* test). A representative dataset of three (B–D) experiments performed, each of which gave similar results, is shown.

allergic reactions and chronic inflammatory diseases. In fact, the mast cell-defective *Kit^W/Kit^{W-V}* mouse does not develop experimental allergic encephalomyelitis, and this defect is rescued by transferred WT BMMCs but not by IL-4-deficient BMMCs (55). These studies indicate that mast cell-derived cytokines are important in inflammatory diseases.

In this paper, we showed that *Znt5^{-/-}* mast cells had an intact degranulation process but impaired cytokine and chemokine production. Most importantly, *Znt5^{-/-}* mice had a defective mast cell-dependent delayed-type allergic reaction, i.e., CHS, but their immediate-type reaction, i.e., PCA, was unimpaired. Therefore, the *Znt5^{-/-}* mouse will be valuable for further dissection of the *in vivo* roles of mast cells in immune and allergic responses and for analyzing the pathological status of a variety of immune-related diseases.

Znt5 is required for FcεRI-mediated NF-κB activation and the translocation of PKC to the plasma membrane

Our results showed that *Znt5* is important for FcεRI-mediated cytokine or chemokine production. As *Znt5* was mainly located in the Golgi, we first examined whether *Znt5^{-/-}* mast cells were defective in cytokine processing/secretion. However, LPS-induced cytokine production was normal, indicating that the transport step was unaffected by the *Znt5* deficiency in mast cells. We also observed that FcεRI induced normal activation of LAT and PLC-γ and calcium mobilization. This observation is consistent with our result showing normal degranulation in *Znt5^{-/-}* BMMCs. From this, we deduced that the degranulation process can be induced in *Znt5^{-/-}* mast cells as a result of intact calcium influx. Our results do not dispute the reported involvement of PKC in the degranulation process (35, 56), but this requirement for PKC may be compensated by other signaling pathways in *Znt5^{-/-}* mast cells. Alternatively, the threshold of the degree of PKC activation required for degranulation and that for NF-κB activation might be different. To support this idea, we performed PKC family inhibitor experiment and showed that PKC inhibitor-treated *Znt5^{-/-}* mast cells were significantly reduced by FcεRI-mediated degranulation (Fig. S11).

We found that FcεRI-mediated NF-κB activation was defective in the *Znt5^{-/-}* BMMCs; FcεRI-induced nuclear translocation of p65 was reduced, and FcεRI-mediated IRBα phosphorylation and its subsequent degradation were impaired. From these results, we concluded that *Znt5* is selectively required for FcεRI-mediated NF-κB activation but not for MAPK activation or calcium mobilization. Because the expression of IL-6 and TNF-α depend on the activation of NF-κB, which is a master transcription factor that regulates the expression of proinflammatory cytokine and chemokine genes (57), our results indicated that *Znt5* is a novel player involved in FcεRI-induced cytokine production through NF-κB activation.

PKC-θ and PKC-β play important roles in NF-κB activation and function upstream of the Bcl10-MALT1 complex in TCR and BCR signaling (41, 42). In mast cells, PKC is

responsible for the activation of NF-κB; for example, a PKC inhibitor suppresses NF-κB activation (23, 51). Furthermore, PKC-β-deficient mast cells are defective in IL-6 production (58). In this paper, we showed that *Znt5* was required for the FcεRI- or PMA-induced translocation of PKC-β to the plasma membrane of BMMCs, and this requirement explains why *Znt5* is needed for NF-κB activation.

A related question is how *Znt5* regulates the plasma membrane translocation of PKC-β, and several of our findings relate to this issue: the C1A Zn finger-like motif of PKC-β was required for translocation of PKC to the plasma membrane; mutating the C1A Zn finger-like motif of PKC-β diminished its ability to bind PMA; PKC obtained from *Znt5^{-/-}* BMMCs showed decreased binding to PMA; *Znt5* was located in the Golgi; and *Znt5^{-/-}* BMMCs showed an increase in cytoplasmic free Zn. Because *Znt5* was located in the Golgi, it probably promotes a Zn efflux from the cytoplasm into the Golgi. Because *Znt5^{-/-}* BMMCs had increased cytoplasmic free Zn, it seems likely that the available Zn in the Golgi was decreased concomitantly.

Together, these observations suggested the hypothesis that in the *Znt5^{-/-}* BMMCs, the Zn available to the C1A Zn finger-like motif of PKC as it transits through the Golgi is decreased, which results in an increase in Zn-free PKC. The mutation of the Zn-binding site of C1A interfered with the plasma membrane translocation of PKC, and this mutation also diminished PMA binding to PKC. Collectively, our findings suggest that binding of Zn to the C1A of PKC induces a conformational change required for DAG binding, which is itself required for the translocation of PKC to the plasma membrane. This hypothesis is supported by our data as well as by findings from other groups that Zn is involved in the translocation of PKC to the plasma membrane (59–61). Furthermore, in experiments using *Znt5^{-/-}* DT40 cells, Suzuki et al. (48, 62) showed that *Znt5* expressed on the ER-Golgi membrane is required for the enzymatic activity of the Zn-dependent alkaline phosphatases (ALPs), which are processed from apoALPs to holoALPs in the ER-Golgi.

In summary, the results in this paper show that a *Znt5*, *Znt5*, is selectively required for the mast cell-mediated delayed-type allergic response but not for the immediate-type allergic reaction. We further showed that *Znt5* is required in the PKC-mediated NF-κB signaling pathway. The identification of *Znt5* as a novel component of FcεRI-mediated PKC activation sheds light on the molecular mechanisms of the processing and activation of PKC and of the Zn-mediated regulation of mast cell-dependent allergic responses, specifically of the delayed type.

MATERIALS AND METHODS

Mice. C57BL/6j mice were obtained from CREA Japan. *Znt5^{-/-}* mice were provided by Y. Nakamura and T. Tanaka (University of Tokyo, Tokyo, Japan), and RIKEN, Center for Genomic Medicine, Kanagawa, Japan). The generation of *Znt5^{-/-}* mouse was reported in detail by Inoue et al. (44). Mast cell-deficient *Kit^{W-sh/W-sh}* mice were provided by the RIKEN BioResource Center. We obtained approval from the Animal Research Committee at RIKEN for all animal experiments performed.

Antibodies and reagents. Anti-Zn5 antibodies were raised by immunizing rabbits with KLH-conjugated peptide (TIQVEKEAYFQHMSG). For the immunization, rabbits were first injected with 200 µg of KLH-conjugated Zn5 peptide in complete Freund's adjuvant and then boosted every week with 50 µg of the antigen in incomplete Freund's adjuvant. Anti-GFP and antiphosphotyrosine (4G10) antibodies were purchased from MBL and Millipore, respectively. Anti-Flag (M2), anti-GM130, anti-PKC-β (C-16), anti-phospho-PKC (S660), and anti-LAT antibodies were obtained from Sigma-Aldrich, BD, Santa Cruz Biotechnology, Inc., Cell Signaling Technology, and Millipore, respectively. G86976 was purchased from EMD.

FITC-induced CHS. The FITC-induced CHS procedure was performed as described previously (31). In brief, mice were sensitized with a total of 200 µl of 2% FITC isomer-I (FITC; Sigma-Aldrich) in a vehicle consisting of acetone-dibutylphthalate (1:1) applied to the skin of the back. 5 d after the sensitization with FITC, the mice were challenged with 40 µl of vehicle alone on the right ear (20 µl on each side of the ear) and 0.5% FITC on the left ear (20 µl on each side). Ear thickness was measured before and at multiple intervals after FITC challenge with an engineer's microcaliper (Ozaki). Some mice from each group were killed 24 h after the FITC or vehicle challenge for cytokine analysis.

For mast cell reconstitution studies, BMMCs were injected intradermally (1.0×10^6 cells in 20 µl/ear for CHS) in 6-wk-old *Kit^{flx/flx}* mice. The mice were used for CHS experiments at 6 wk after transfer of BMMCs.

PCA and histology of the ears. A total of 2 µg IgE was injected subcutaneously into ears for 12 h. After this sensitization, the mice were then challenged with an intravenous injection of 250 µg of polyvalent DNP-BSA (Cosmo Bio Co., LTD.) in 250 µl of saline and 5 mg/ml of Evans blue dye (Sigma-Aldrich). The extravasation of Evans blue into the ear was monitored for 30 min. The mice were then sacrificed, both ears were dissected, and the Evans blue dye was extracted in 700 µl of formamide at 63°C overnight. The absorbance of the Evans blue-containing formamide was then measured at 620 nm. To observe the ear mast cells, 3-µm paraffin sections were fixed and stained with nuclear fast red and Alcian blue, and the Alcian blue-stained cells in each sample were counted. All data are expressed as the number of mast cells per mm² of dermis.

Primary mast cell cultures and cell lines. BMMCs were prepared as described previously (63). In brief, BM cells were flushed from the marrow cavity of mouse femurs, and the mast cells (BMMCs) were selectively grown in RPMI1640 medium supplemented with IL-3 (conditioned medium from an mL-3-producing cell line, CHOmIL-3-3-12M [gift from T. Sudo, Toray Industries, Inc., Kamakura, Japan]), 10% FBS, 10 mU/ml penicillin, and 0.1 mg/ml streptomycin for 4–8 wk. During culture, the medium was changed every 3–4 d, and the cells were transferred to new dishes to eliminate adherent cells. After 5 wk in culture, the BMMCs were ready for *in vitro* experiments and showed the cell surface expression of FcεRI and c-Kit. HeLa cells were maintained in RPMI1640 supplemented with 10% FBS, penicillin, and streptomycin.

Degranulation assay. The degranulation assay was described previously (64). In brief, the degree of degranulation was determined by measuring the release of β-hexosaminidase. 1×10^6 cells/ml were preloaded with 1 µg/ml anti-DNP IgE (SPE-7; Sigma-Aldrich) by a 6-h incubation in medium (without IL-3). To measure β-hexosaminidase release, the sensitized cells were stimulated with 100 ng/ml DNP-HSA for 30 min in Tyrode's buffer. Samples were placed on ice and then spun at 4°C for 5 min. The enzymatic activities of β-hexosaminidase in the supernatants and cell pellets solubilized with 1% Triton X-100 in Tyrode's buffer were measured with p-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma-Aldrich) in 0.1 M sodium citrate, pH 4.5, for 60 min at 37°C. The reaction was stopped by the addition of 0.2 M glycine, pH 10.7. The release of the product 4-p-nitrophenol was detected by absorbance at 405 nm. The extent of degranulation was calculated

by dividing the 4-p-nitrophenol absorbance in the supernatant by the sum of the absorbance in the supernatant and detergent-solubilized cell pellet.

Measurement of cytokines and chemical mediators. Cells were activated as described in the previous sections, and TNF-α and IL-6 in the cell culture supernatants were measured with an ELISA kit (eBioscience). The leukotrienes were quantified using an ELISA kit (Cayman Chemical), according to the manufacturer's instructions.

Cell lysates and immunoblotting. 1×10^6 /ml BMMCs were sensitized with 1 µg/ml IgE for 6 h at 37°C. Cells were stimulated with 100 ng/ml DNP-HSA. After the stimulation, the cells were harvested and lysed with 100 µl of lysis buffer (20 mM Tris-HCl, pH 7.4, at 4°C, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.1% deoxycholate, 1 mM NaVO₄, 3 mM EDTA, and proteinase inhibitors [0.5 mM PMSF, 10 µg/ml aprotinin, 5 µg/ml pepstatin, and 10 µg/ml leupeptin]) for 30 min at 4°C and spun at 15,000 g at 4°C for 15 min. The eluted and reduced samples were resolved by SDS-PAGE using a 5–20% gradient polyacrylamide gel (Wako Chemicals USA, Inc.) and transferred to a PVDF membrane (Immobilon-P; Millipore). For immunoblotting, the membranes were incubated with anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38, anti-ERK, anti-JNK, anti-p38, anti-phospho-IκBα, anti-IκBα, anti-β-tubulin, anti-actin, and anti-PKC-β, respectively. For immunoprecipitation, the cell lysates were incubated for 4 h at 4°C with 1 µg of antibodies bound to protein A Sepharose. The eluted and reduced samples were separated on a 5–20% gradient SDS-polyacrylamide gel and transferred to a PVDF membrane. The membranes were incubated with antiphosphotyrosine. After blotting with the first antibody, the membranes were blotted with HRP-conjugated species-specific anti-IgG (Invitrogen) for 1 h at room temperature. After extensive washing of the membranes, the immunoreactive proteins were visualized using the Renaissance chemiluminescence system (Dupont), according to the manufacturer's recommendations. The PVDF membranes were exposed to RX film (Fujifilm). Densitometric analysis for tubulin and PKC-β was performed using a fluorescence image analyzer (LAS-1000; Fujifilm).

Preparation of plasma membrane protein. Plasma membrane proteins were extracted using a Plasma Membrane Protein Extraction kit (K268-50; BioVision, Inc.), according to the manufacturer's instructions. The plasma membrane fractions were used for immunoblotting and probed with anti-actin, anti-PKC-β, and anti-phospho-PKC (S660). Densitometric analysis for PKC-β, phospho-PKC (S660), and actin was performed using an LAS-1000 fluorescence image analyzer.

Confocal microscopy. Separate aliquots of 5×10^5 cells were sensitized with 1 µg/ml IgE for 6 h. The cells were then stimulated with 100 ng/ml DNP-HSA for 10 min at 37°C and fixed with 4% paraformaldehyde for 30 min. The cells were spun and permeabilized in Perm Buffer (BD) containing 1% BSA for 15 min at room temperature. The cells were washed with 1 ml PBS⁻ (PBS without calcium and magnesium) twice, resuspended in 500 µl of PBS⁻, and attached to glass slides by cytospin (Thermo Fisher Scientific) for 5 min. Primary and secondary staining were performed on the slides, with anti-p65 at a dilution of 1:50, anti-PKC-β at 1:100, Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen) at 1:100, and phalloidin-rhodamine (Invitrogen) at 1:100. Confocal microscopy was performed using the TCS SL system (Leica).

RT-PCR and real-time quantitative RT-PCR analysis. Cells were homogenized with Sepasol RNAI (Nacal Tesque, Inc.), and the total RNA was isolated according to the manufacturer's instructions. For standard RT-PCR, cDNA was synthesized from 500 ng of total RNA by RT (ReverTra Ace; TOYOBO) and 500 ng of oligo (dT) primer (Invitrogen) for 30 min at 42°C. A portion of the cDNA (typically 1/20 vol) was used for standard PCR to detect IL-6, TNF-α, and GAPDH. 25 cycles of PCR were performed with 0.5 U rTaq DNA polymerase and 10 pmol of gene-specific sense and antisense primers. For real-time quantitative RT-PCR, IL-6 and TNF-α gene expression was measured relative to

GAPDH using the SYBR Green system (Applied Biosystems). Primers used in these experiments were purchased from Invitrogen, and the sequences were as follows: IL-6 forward, 5'-GAGGATACCACTCCCAACAG-ACC-3' and reverse, 5'-AAGTGCATCATCGTTGTTTCATACA-3'; TNF- α forward, 5'-CATCTTCTCAAATTCGAGTGACAA-3' and reverse, 5'-TGGGAGTAGACAAGGTACAACCC-3'; GAPDH forward, 5'-TTCACCACCATGGAGAAGCCG-3' and reverse, 5'-GGCATG-GACTGTGGTCATGA-3'; and hypoxanthine-guanine phosphoribosyl transferase forward, 5'-CCCTCCCACTCCCTTCATGACA-3' and reverse, 5'-GATTAGCGATGATGAACCAGGTT-3'. In addition, primers used for amplifying cDNAs for Znt family members and other primer sets are listed in Table S1. PCR products were separated on an agarose gel, stained with 500 ng/ml ethidium bromide, and photographed.

Intracellular Zn measurement. 2×10^6 BMMCs were treated with 2.5 μ M of Newport green for 30 min at 37°C. The cells were washed two times with PBS⁻ before the fluorescence intensity was measured by FACS. The concentration of intracellular free Zn was calculated from the mean fluorescence with the formula $[Zn] = K_D \times [(F - F_{min}) / (F_{max} - F)]$. The dissociation constant of the Newport Green/Zn complex is 1 μ M. F_{max} was determined by the addition of the Zn-specific membrane-permeant chelator TPEN, and F_{min} was determined by the addition of ZnSO₄ and the ionophore pyrithione (65).

Calcium influx measurement. 1×10^6 BMMCs/ml were sensitized with 1 μ g/ml IgE (anti-DNP IgE clone SPE-7; Sigma-Aldrich) for 6 h at 37°C. IgE-sensitized mast cells were washed three times, resuspended in Tyrode's buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, and 5.6 mM glucose), allowed to adhere to a poly-L-lysine-coated glass-bottom dish, and incubated with 5 μ M Fluo-4, a cell-permeant dye, for 30 min at 37°C. Surplus fluorescence indicator and floating cells were removed by at least three washes with Tyrode's buffer. Cells were stimulated with 100 ng/ml DNP-HSA (Sigma-Aldrich) at 37°C. Images of the fluorescent signals were captured every 10 or 30 s from an inverted microscope (Axiovert 200 MOT; Carl Zeiss, Inc.) equipped with an oil Plan Neofluar 100 \times 1.3 NA objective, a charge-coupled device camera (Cool Snap HQ; Roper Scientific), and the system control application SlideBook (Intelligent Imaging Innovation) at 25°C. The captured images were processed with Photoshop software (Adobe) to adjust their size and contrast.

cDNA cloning and plasmid construction. PKC- β was cloned by PCR using cDNA from BMMCs and sequenced on a 3130xl sequencer (ABI-PRISM; Applied Biosystems). The GFP-tagged expression vectors for PKC- β were constructed and subcloned into pEGFP-N1 (Invitrogen). The construct encoding mutant PKC- β -GFP, in which the Zn finger-like motif in the C1 region was mutated, was made using a PCR-based method. The human Znt5 cDNA in pA-Zeoicin was reported in detail by Suzuki et al. (62). HeLa cells were transfected with a total of 1 μ g PKC- β cDNA in pEGFP or with empty vector using Lipofectamine 2000 (Invitrogen).

Retroviral infection. Retroviral infection was performed as previously described (64). The Flag-tagged human Znt5 plasmid (gift from T. Kambe and T. Suzuki, Kyoto University, Kyoto, Japan) was inserted into the BamHI and NotI sites of the retroviral vector pMX (gift from T. Kitamura, University of Tokyo, Tokyo, Japan). This construct was then used to transfect the 293T-based packaging cell line phoenix (gift from G. Nolan, Stanford University, Stanford, CA), with Lipofectamine 2000 (Invitrogen) to generate recombinant retroviruses. BM cells were infected with the retrovirus in the presence of 10 μ g/ml polybrene (Sigma-Aldrich) and IL-3.

Biotinyl-PMA and native PAGE. PMA (Nacal Tesque, Inc.) was conjugated with biotin (Wako Chemicals USA, Inc.). The predicted biotinyl-PMA is shown in Fig. S10. Native PAGE was performed with the NativePAGE Novex Bis-Tris Gel system (Invitrogen). In detail, the cells were lysed with NativePAGE Sample buffer for 10 min at 4°C and spun at 15,000 g at 4°C for 10 min. The samples were separated by native PAGE on 4–16% Bis-Tris Gels

and transferred to a PVDF membrane (Immobilion-P; Millipore). For immunoblotting, the membrane was incubated with Biotinyl-PMA or anti-PKC- β antibody. After the membrane was washed in TBST, the coloring reaction was performed with streptavidin-AP (Roche) or anti-rabbit IgG HRP antibody, respectively. Densitometric analysis for PKC- β and PMA was performed using an LAS-1000 fluorescence image analyzer (Fujifilm).

Statistical analysis. All data were analyzed with Statcell (Microsoft). Data were considered statistically significant for p-values < 0.05 , obtained with a two-tailed Student's *t* test.

Online supplemental material. Fig. S1 shows expression level of Znt family member in BMMCs. Fig. S2 shows expression level of Znt5 after antigen stimulation. Fig. S3 shows normal development of BMMC from Znt5^{-/-} mice. Fig. S4 shows normal development of mast cell in Znt5^{-/-} mice. Fig. S5 shows numbers of mast cells in dermis from vehicle-treated or FITC-challenged ears. Fig. S6 shows quantitative analysis of I κ B α phosphorylation and degradation. Fig. S7 shows normal Fc ϵ R1-induced calcium signaling and influx in Znt5^{-/-} BMMCs. Fig. S8 shows impairment of PKC activity in Znt5^{-/-} BMMCs. Fig. S9 shows normal expression levels of PKC- β in Znt5^{-/-} BMMCs. Fig. S10 shows design of biotinyl-PMA and that biotinyl-PMA can induce cytokine production. Fig. S11 shows that the PKC inhibitor G66976 inhibits Fc ϵ R1-induced degranulation in Znt5^{-/-} BMMCs. Video 1 shows time-lapse images of WT PKC- β -EGFP in HeLa cells after PMA stimulation. Video 2 shows time-lapse images of C1AS PKC- β -EGFP in HeLa cells after PMA stimulation. Video 3 shows time-lapse images of C1BS PKC- β -EGFP in HeLa cells after PMA stimulation. Video 4 shows time-lapse images of C1ABS PKC- β -EGFP in HeLa cells after PMA stimulation. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20082533/DC1>.

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A protective function for interleukin 17A in T cell-mediated intestinal inflammation

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Interleukin 23 (IL-23) and IL-17 have been linked to the pathogenesis of several chronic inflammatory disorders, including inflammatory bowel disease. Yet as an important function for IL-23 is emerging, the function of IL-17 in inflammatory bowel disease remains unclear. Here we demonstrate IL-17A-mediated protection in the CD45RB^{hi} transfer model of colitis. An accelerated wasting disease elicited by T cells deficient in IL-17A correlated with higher expression of genes encoding T helper type 1-type cytokines in colon tissue. IL-17A also modulated T helper type 1 polarization *in vitro*. Furthermore, T cells deficient in the IL-17 receptor elicited an accelerated, aggressive wasting disease relative to that elicited by wild-type T cells in recipient mice. Our data demonstrate a protective function for IL-17 and identify T cells as not only the source but also a target of IL-17 *in vivo*.

The interleukin 23 (IL-23)–IL-17 axis is emerging as a critical regulatory system that bridges the innate and adaptive arms of the immune system. The IL-12 cytokine family member IL-23 is secreted by 'professional' antigen-presenting cells during inflammatory responses to pathogenic as well as nonpathogenic stimuli¹. IL-23 elicits myriad effector molecules, most notably IL-17A, IL-17F and tumor necrosis factor, as well as others, depending on the responder cell^{2,3}. Among the cell types known to respond to IL-23 are antigen-experienced CD4⁺ T lymphocytes, which express surface IL-23 receptor (IL-23R) complexes and rapidly produce proinflammatory cytokines, including interferon- γ (IFN- γ) and IL-17, within hours of IL-23 stimulation^{2,4} (W.O., unpublished observations). Of particular interest is IL-23-mediated production of IL-17, which has been reported to be important in the pathogenesis of several inflammatory disorders, including the initiation and/or progression of experimental autoimmune encephalomyelitis, a mouse model of human multiple sclerosis^{5,6}. IL-17 is thought to exert proinflammatory effects mainly by eliciting the production of CXC chemokines and other chemoattractants from both endothelial and epithelial cells, which nearly ubiquitously express the IL-17 receptor (IL-17R)^{7,8}. Neutrophils and other cells of the immune system are subsequently recruited, thereby amplifying the inflammatory episode^{9,10}.

The IL-23–IL-17 system has also been linked to inflammatory bowel disease (IBD). Genome-wide analyses have identified several uncommon *Il23r* variants inversely correlated with susceptibility to IBD^{11,12}. In addition, there is higher expression of IFN- γ and IL-17 in the intestinal mucosa of patients with Crohn's disease^{13,14}. In mouse colitis

studies using either adoptive transfer of CD45RB^{hi} T cells or infection with helicobacter to induce IBD, investigators have documented expression of IL-23, IL-17 and IFN- γ in the inflamed colon tissue^{15–17}. In these studies, the expression of IFN- γ and IL-17 is dependent on recipient mouse-derived IL-23, and disease development in recipient mice is nearly completely dependent on the presence of IL-23, which indicates IL-23 is a critical factor in the initiation of mouse IBD. This has led to speculation that IFN- γ and IL-17 may act in synergy to initiate colon inflammation. However, as other studies have shown that neutralization of IFN- γ nearly completely abrogates T cell-mediated intestinal damage and wasting disease¹⁸, and the adoptive transfer of IFN- γ deficient cells also fails to induce disease in recipient mice¹⁹, IFN- γ has been shown to be critical for IBD in these models, whereas the function of IL-17 in these experimental systems has remained unclear. Indeed, the function of IL-17A in intestinal inflammation has thus far remained controversial. An inflammatory function for IL-17 in trinitrobenzenesulfonic acid-induced colitis has been described²⁰, yet another report has suggested IL-17 might offer protection in the dextran sodium sulfate-induced colitis model²¹. Notably, chemically induced intestinal damage in those models is T cell independent²² and more accurately reflects the chronic stage of disease after compromise of the epithelial barrier, rather than early disease-initiating events.

Here we show that IL-17A mediates a protective effect on T cell-driven intestinal inflammation *in vivo*. Using a well-established mouse transfer model of colitis, we found that relative to wild-type populations, *Il17a*^{-/-} CD45RB^{hi} cells induced a more aggressive wasting disease when transferred into recipient mice deficient in

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recombination-activating gene 1 (*Rag1*^{-/-}). Cohorts that developed accelerated IBD had higher expression of IFN- γ and osteopontin mRNA in resected colon tissue at the onset of intestinal tissue damage, as well as weight loss, which provides evidence that classical T helper type 1 (T_H1)-associated cytokines, not IL-17, probably drive disease initiation. Consistent with those observations, *Il17a*^{-/-} CD45RB^{hi} cells polarized to T_H1 effectors *in vitro* had higher expression of IFN- γ and the T_H1 'master regulator' T-bet, and recombinant IL-17 inhibited the expression of these factors in developing wild-type T_H1 cells. Furthermore, CD45RB^{hi} cells deficient in IL-17R (*Il17ra*^{-/-}) also elicited an accelerated aggressive wasting disease relative to that in recipients of wild-type T cells in the transfer model. Our findings demonstrate that IL-17A provides protection in this model of T cell-mediated wasting disease and that T cells are responsive to IL-17A *in vivo*, and they identify T cells as both the source and the relevant target of IL-17 in this experimental system. Our observations collectively have implications for strategies that aim to modulate the IL-23-IL-17 immune axis for clinical benefit.

RESULTS

IL-17A modulates T cell-driven colitis

To directly assess the function of IL-17A in T cell-mediated colitis, we adoptively transferred purified CD45RB^{hi} CD25⁻CD4⁺ T cells from *Il17a*^{-/-} mice or wild-type mice into *Rag1*^{-/-} recipients and then monitored wasting disease in the recipients. Wild-type CD4⁺ CD45RB^{hi} T cells predictably induced wasting disease 5-7 weeks after transfer (Fig. 1a). Cohorts that received *Il17a*^{-/-} T cells however, developed an overly aggressive inflammatory disease relative to that of recipients of wild-type cells, as shown by their accelerated decrease in body mass (Fig. 1a,b). There was divergence in body-mass curves by 4 weeks after adoptive transfer, and repeated-measures analysis of variance (ANOVA) showed significant main effects of time ($P < 0.001$) and experimental group ($P < 0.05$) and a time-group interaction ($P < 0.05$) in the composite data analysis of three independent experiments (Fig. 1b). There was a significant difference at 4.0-4.5 weeks after adoptive transfer of CD4⁺ CD45RB^{hi} T cells (post-hoc *t*-test). Recipients of *Il17a*^{-/-} CD4⁺ CD45RB^{hi} T cells reached the

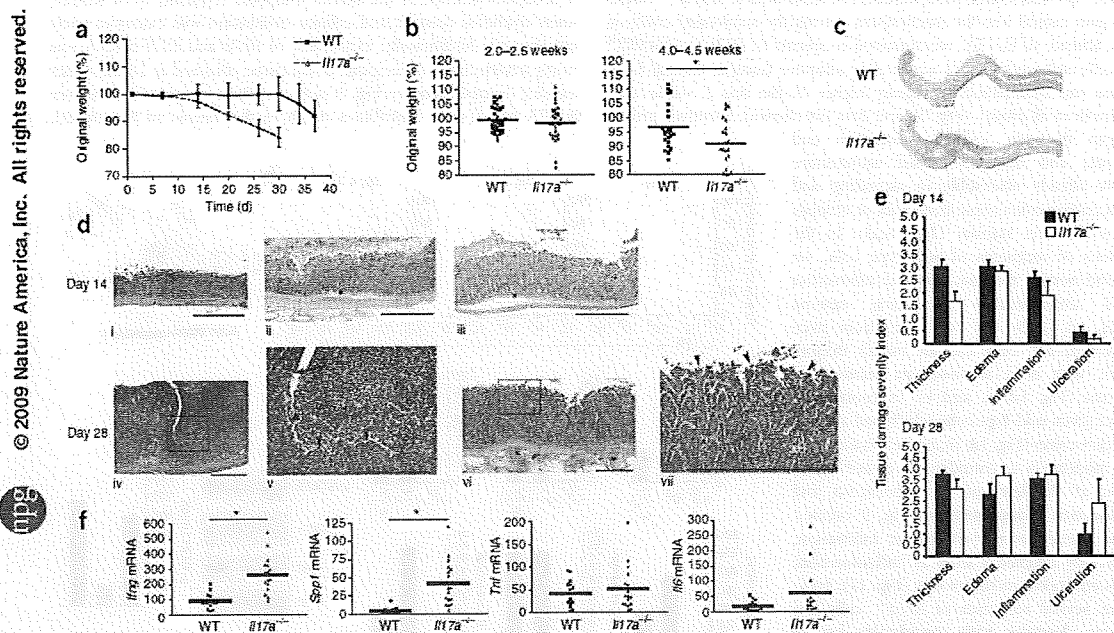


Figure 1 IL-17A-deficient CD45RB^{hi} T cells induce an aggressive wasting disease in *Rag1*^{-/-} recipient mice. (a) Body weights of *Rag1*^{-/-} recipients of intraperitoneally injected purified CD45RB^{hi} CD4⁺ T cells from *Il17a*^{-/-} mice or unmanipulated wild-type (WT) C57BL/6 mice on day 0, presented as percent of original weight. Data are group averages from one experiment (error bars, s.e.m.). (b) Weight loss distributions of each individual mouse (single symbols) and composite statistics of all recipient mice. Means (small horizontal bars) at day 14 ($P = 0.4155$): recipients of wild-type cells ($n = 35$), 99.26 (s.d., 4.168); recipients of *Il17a*^{-/-} cells ($n = 32$), 98.22 (s.d., 6.057). Means at day 28: recipients of wild-type cells ($n = 24$), 96.71 (s.d., 7.493); recipients of *Il17a*^{-/-} cells ($n = 21$), 90.87 (s.d., 8.155). *, $P < 0.01$. Data are representative of three independent experiments. (c) Gross organ morphology of the cecum and ascending colon from the recipient mice in b at day 28. Results are representative of three experiments. (d) Histology of colon tissues from the mice in a. Top, colon tissues at day 14 from an unmanipulated wild-type mouse (i), a recipient of *Il17a*^{-/-} cells (ii) or a recipient of wild-type cells (iii). *, edema. Bottom, colon tissues at day 28 from a recipient of *Il17a*^{-/-} cells (iv and v) (higher magnification of box in iv), or wild-type cells (vi and vii) (higher magnification of box in vi). Arrowheads indicate the presence (iv,v) or absence (vi,vii) of ulcerated epithelium. Scale bars, 500 μ m. Results are representative of two experiments. (e) Quantification of pathological changes in the mice in a, assessed as 'severity scores'. Data are representative of two experiments (error bars, s.e.m.). (f) Quantitative RT-PCR analysis of mRNA transcripts encoding cytokines (vertical axes), measured in ascending colon tissue from the mice in a on day 28. Each dot represents an individual mouse; scores are presented as 'fold increase' relative to baseline expression in colon tissue from unmanipulated wild-type littermates after normalization to expression of *Hprt1* (encoding hypoxanthine guanine phosphonobosyl transferase; change in cycling threshold method). Means for *Ifng* ($P \leq 0.0001$): recipients of wild-type cells ($n = 14$), 89.79; recipients of *Il17a*^{-/-} cells ($n = 17$), 263.8. Means for *Spp1* ($P \leq 0.001$): recipients of wild-type cells ($n = 13$), 4.56; recipients of *Il17a*^{-/-} cells ($n = 17$), 41.25. Means for *Il6*: recipients of wild-type cells ($n = 14$), 60.48; recipients of *Il17a*^{-/-} T cells ($n = 13$), 18.62. *, $P \leq 0.001$ (unpaired, two-tailed Student's *t*-test). Data are representative of three experiments.