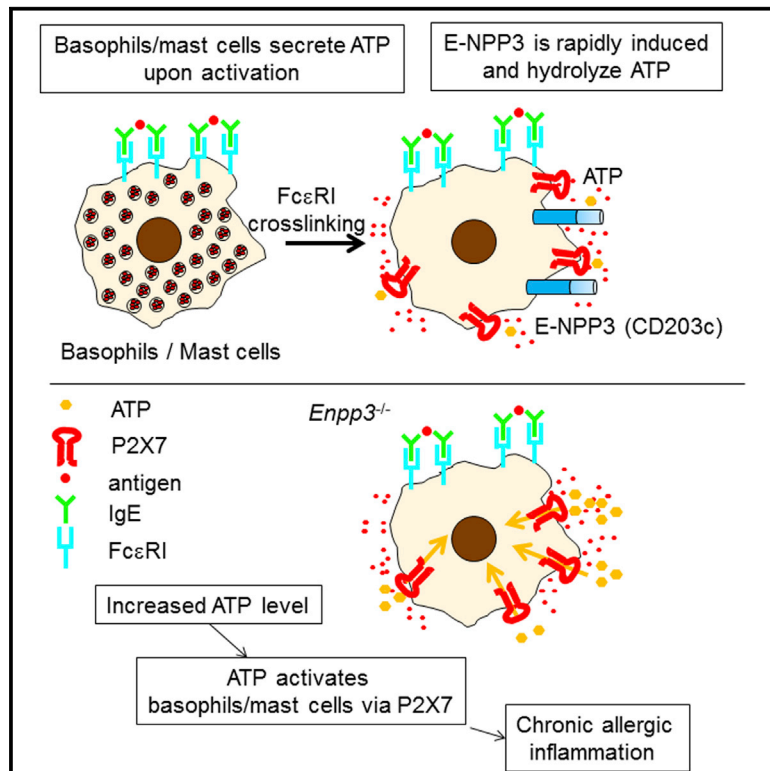


Immunity

The Ectoenzyme E-NPP3 Negatively Regulates ATP-Dependent Chronic Allergic Responses by Basophils and Mast Cells

Graphical Abstract



Authors

Shih Han Tsai, Makoto Kinoshita, ..., Hajime Karasuyama, Kiyoshi Takeda

Correspondence

ktakeda@ongene.med.osaka-u.ac.jp

In Brief

The mechanisms for regulation of chronic allergic responses remain unknown. Takeda and colleagues show that ATP secreted by basophils and mast cells acts on the cells in an autocrine manner. E-NPP3 is rapidly expressed on basophils and mast cells and hydrolyzes ATP to prevent the ATP-dependent chronic allergic inflammation.

Highlights

- E-NPP3 (CD203c) is rapidly induced on basophils and mast cells to hydrolyze ATP
- Activated basophils and mast cells secrete ATP
- ATP acts on basophils and mast cells to induce chronic allergic responses
- E-NPP3 is responsible for suppression of ATP-dependent allergic inflammation



The Ectoenzyme E-NPP3 Negatively Regulates ATP-Dependent Chronic Allergic Responses by Basophils and Mast Cells

Shih Han Tsai,^{1,2} Makoto Kinoshita,^{1,2} Takashi Kusu,^{1,2} Hisako Kayama,^{1,2} Ryu Okumura,^{1,2} Kayo Ikeda,^{1,2} Yosuke Shimada,^{1,2} Akira Takeda,¹ Soichiro Yoshikawa,³ Kazushige Obata-Ninomiya,³ Yosuke Kurashima,^{2,4} Shintaro Sato,^{2,4} Eiji Umemoto,^{1,2} Hiroshi Kiyono,^{2,4} Hajime Karasuyama,^{2,3} and Kiyoshi Takeda^{1,2,*}

¹Laboratory of Immune Regulation, Department of Microbiology and Immunology, Graduate School of Medicine, WPI Immunology Frontier Research Center, Osaka University, Suita, Osaka 565-0871, Japan

²Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan

³Department of Immune Regulation, Tokyo Medical and Dental University Graduate School, Tokyo 113-8519, Japan

⁴Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

*Correspondence: ktakeda@ongene.med.osaka-u.ac.jp

<http://dx.doi.org/10.1016/j.immuni.2015.01.015>

SUMMARY

Crosslinking of the immunoglobulin receptor FcεRI activates basophils and mast cells to induce immediate and chronic allergic inflammation. However, it remains unclear how the chronic allergic inflammation is regulated. Here, we showed that ecto-nucleotide pyrophosphatase-phosphodiesterase 3 (E-NPP3), also known as CD203c, rapidly induced by FcεRI crosslinking, negatively regulated chronic allergic inflammation. Basophil and mast cell numbers increased in *Enpp3*^{-/-} mice with augmented serum ATP concentrations. *Enpp3*^{-/-} mice were highly sensitive to chronic allergic pathologies, which was reduced by ATP blockade. FcεRI crosslinking induced ATP secretion from basophils and mast cells, and ATP activated both cells. ATP clearance was impaired in *Enpp3*^{-/-} cells. *Enpp3*^{-/-}*P2rx7*^{-/-} mice showed decreased responses to FcεRI crosslinking. Thus, ATP released by FcεRI crosslinking stimulates basophils and mast cells for further activation causing allergic inflammation. E-NPP3 decreases ATP concentration and suppresses basophil and mast cell activity.

INTRODUCTION

Basophils and mast cells are distinct cell lineages, but they share some phenotypic and functional properties (Karasuyama et al., 2011; Voehringer, 2013). For example, both basophils and mast cells are activated upon crosslinking of FcεRI with antigen-bound IgE to mediate type 2 immune responses against pathogens such as helminths and ticks (Abraham and St John, 2010; Karasuyama et al., 2011). In addition, these two cell types induce pathologic responses to allergens, composed of immediate and chronic responses.

CD203c was identified as a highly expressed molecule on activated basophils (Bühning et al., 2001) and is now considered a good biomarker candidate for the diagnosis of allergic diseases (Bühning et al., 2004). CD203c is a member of the ecto-nucleotide pyrophosphatase-phosphodiesterase (E-NPP) family consisting of seven structurally related molecules that hydrolyze pyrophosphate and phosphodiester bonds in various substrates (Schetinger et al., 2007; Stefan et al., 2005; Yegutkin, 2008). Among seven family members, E-NPP1, E-NPP2 (also called autotaxin), and E-NPP3 (CD203c) are closely related in structure and hydrolyze nucleotides (Bollen et al., 2000). E-NPP1 was shown to generate pyrophosphate through NTP hydrolysis and thereby inhibit bone mineralization (Hessle et al., 2002; Okawa et al., 1998). E-NPP2 acts as lysophospholipase-D, which hydrolyzes lysophosphatidylcholine into lysophosphatidic acid, thereby mediating several biologic responses such as lymphocyte transmigration and vascular development (Kanda et al., 2008; Umemoto et al., 2011; van Meeteren et al., 2006). In contrast to E-NPP1 and E-NPP2, whose functions are well characterized, the function of E-NPP3 (CD203c) remains undetermined despite its high expression in activated basophils.

Extracellular adenosine 5'-triphosphate (ATP) is released during inflammation and activates immune cells (Idzko et al., 2014; Junger, 2011). Several lines of evidence demonstrated that extracellular ATP activates mast cells via the purinoreceptor P2X7 (Kurashima et al., 2012, 2014; Sudo et al., 1996). In addition, several types of cells including mast cells release ATP upon stimulation (Junger, 2011; Wang et al., 2013). Thus, extracellular ATP modulates mast cell activity in an autocrine manner. However, it remains unclear how ATP-dependent mast cell responses are regulated. In addition, it should also be determined whether extracellular ATP acts on basophils, which share several immunological functions with mast cells.

In this study, we analyzed the physiological function of E-NPP3 (CD203c), which was highly expressed in activated basophils and mast cells. In *Enpp3*^{-/-} mice, the activity of basophils and mast cells was markedly enhanced with increased ATP concentrations. Accordingly, *Enpp3*^{-/-} mice were susceptible

to chronic allergic pathologies mediated by basophils and mast cells. The crosslinking of FcεRI by IgE induced the secretion of ATP from basophils and mast cells, and ATP clearance activity was impaired in *Enpp3*^{-/-} basophils and mast cells. Extracellular ATP and FcεRI crosslinking strongly activated *Enpp3*^{-/-} basophils and mast cells, which was reduced by the addition of P2X7 antagonists or introducing *P2rx7* deficiency. Non-hydrolyzable ATP activated basophils and mast cells from wild-type mice to similar levels observed in *Enpp3*^{-/-} cells. These findings demonstrate that extracellular ATP induced by FcεRI crosslinking is a potent activator of basophils and mast cells that mediate the chronic phases of allergic responses and that E-NPP3 modulates ATP-mediated allergic responses.

RESULTS

Increased Numbers of Basophils and Mast Cells in *Enpp3*^{-/-} Mice

Activated human basophil were reported to express high amounts of E-NPP3 (CD203c), encoded by *Enpp3* (Bühning et al., 2001, 2004). Surface expression of E-NPP3 was rapidly induced on mouse bone-marrow-derived basophils sensitized with anti-dinitrophenyl (DNP) IgE and then stimulated with DNP-human serum albumin (HSA) for 30 min (Figure 1A). Similarly, bone-marrow-derived mast cells showed enhanced expression of surface E-NPP3 after 30 min of crosslinking of FcεRI by DNP-specific IgE (Figure 1B).

To analyze the in vivo function of E-NPP3, we generated mice lacking *Enpp3* by gene targeting (Figures S1A and S1B). The targeted disruption of *Enpp3* was confirmed by mRNA and protein assay (Figures S1C and S1D). *Enpp3*^{-/-} mice were born and grew healthily with no alteration in the numbers of peripheral lymphocytes, eosinophils, and neutrophils (Figures S1E and S1F) or CD49b⁺FcεRI⁺ basophils in bone marrow (Figure 1C). However, frequencies and numbers of CD49b⁺FcεRI⁺ basophils were markedly increased in the peripheral blood and spleen of *Enpp3*^{-/-} mice compared with wild-type mice (Figure 1D). In addition, increased frequencies and numbers of c-kit⁺FcεRI⁺ mast cells were observed in the small and large intestines of *Enpp3*^{-/-} mice (Figure 1E). In parallel with the increased number of basophils and mast cells, serum IgE concentration was increased in *Enpp3*^{-/-} mice (Figure 1F). To determine whether the increased number of basophils and mast cells was caused by abnormalities in hematopoietic or non-hematopoietic cells, we generated bone marrow chimeric mice. In wild-type mice transferred with *Enpp3*^{-/-} bone marrow cells, both basophil and mast cell populations increased, but not in *Enpp3*^{-/-} mice transferred with wild-type bone marrow cells, indicating that ENPP-3 deficiency affected the hematopoietic cell compartment leading to the increased number of basophils and mast cells (Figures S1G and S1H). Thus, *Enpp3*^{-/-} mice showed the increased number of basophils and mast cells in the periphery.

Enhanced Chronic Allergic Inflammation in *Enpp3*^{-/-} Mice

Mast cells are critical mediators of immediate hypersensitivity reactions. Accordingly, we first examined the sensitivity of *Enpp3*^{-/-} mice to passive cutaneous anaphylaxis (Obata et al.,

2007). Mice were sensitized with trinitrophenyl (TNP)-specific IgE intradermally followed by intravenous challenge with TNP-ovalbumin (OVA) and Evans blue dye (Figure 2A). Both wild-type and *Enpp3*^{-/-} mice showed similar extravasation of the dye, indicating that immediate allergic response remains unaltered in the absence of *Enpp3*.

Basophils mediate chronic allergic inflammation in ear skin (Mukai et al., 2005). Therefore, we analyzed the sensitivity of *Enpp3*^{-/-} mice to basophil-dependent allergic inflammation. Mice were sensitized with TNP-specific IgE and then intradermally challenged with TNP-OVA (Figure S2). BALB/c background wild-type mice exhibited ear swelling (Figures 2B and 2C) accompanied by the increased infiltration of basophils, eosinophils, and neutrophils at 4 days after antigen challenge (Figures 2D and 2E). *Enpp3*^{-/-} mice showed more severe ear swelling with increased numbers of infiltrating basophils, eosinophils, and neutrophils compared with wild-type mice. Thus, the basophil-dependent chronic allergic inflammation was exacerbated in *Enpp3*^{-/-} mice.

We next analyzed the sensitivity of *Enpp3*^{-/-} mice to allergen-induced diarrhea (Brandt et al., 2003). Mice were sensitized with OVA+alum twice then orally challenged with OVA every other day, and their weight was monitored (Figure S3A). Compared with wild-type mice, *Enpp3*^{-/-} mice suffered from greater weight loss (Figure 3A). After ten times of OVA challenge, we analyzed the colon by histopathology (Figures 3B and 3C). In *Enpp3*^{-/-} mice, the colon length was shortened and more severe intestinal pathology was observed. Infiltration of mast cells into the intestine was increased in *Enpp3*^{-/-} mice (Figure 3D). Flow cytometric analysis also indicated that mast cell populations in the small and large intestines were increased in OVA-sensitized *Enpp3*^{-/-} mice compared to controls (Figures 3E and S3B). We measured serum concentrations of ATP in mice challenged with OVA (Figure 3F). Serum ATP concentration was significantly elevated in *Enpp3*^{-/-} mice compared with wild-type mice. In OVA-challenged *Enpp3*^{-/-} mice, IL-4 production from CD4⁺ T cells in the small intestine, large intestine, and mesenteric lymph nodes was increased (Figures 3G and S3C). In addition, serum IgE concentrations were augmented in OVA-challenged *Enpp3*^{-/-} mice (Figure 3H), indicating enhanced Th2-cell-type responses in OVA-sensitized *Enpp3*^{-/-} mice. Thus, *Enpp3*^{-/-} mice developed severe allergic intestinal inflammation with a dramatic increase in mast cell populations. Because serum ATP concentrations were elevated in *Enpp3*^{-/-} mice, we analyzed whether severe allergic inflammation in *Enpp3*^{-/-} mice was caused by elevated ATP levels by treating mice with oxidized ATP (oATP), which blocks ATP actions by antagonizing the ATP sensor P2X7 (Figures 3E, 3G, and 3H). Treatment with oATP substantially reduced the number of mast cells and IL-4-producing CD4⁺ T cells as well as serum IgE concentrations in OVA-sensitized *Enpp3*^{-/-} mice. These findings indicate that the severe allergic inflammation observed in *Enpp3*^{-/-} mice was attributed to increased ATP concentrations.

We also investigated a chronic asthma model. Mice were sensitized with OVA+alum twice and then intranasally challenged with OVA six times (Figure S4). *Enpp3*^{-/-} mice were highly sensitive to OVA-induced inflammation and almost all mutant mice died after OVA challenge (Figure 4A). Histopathological

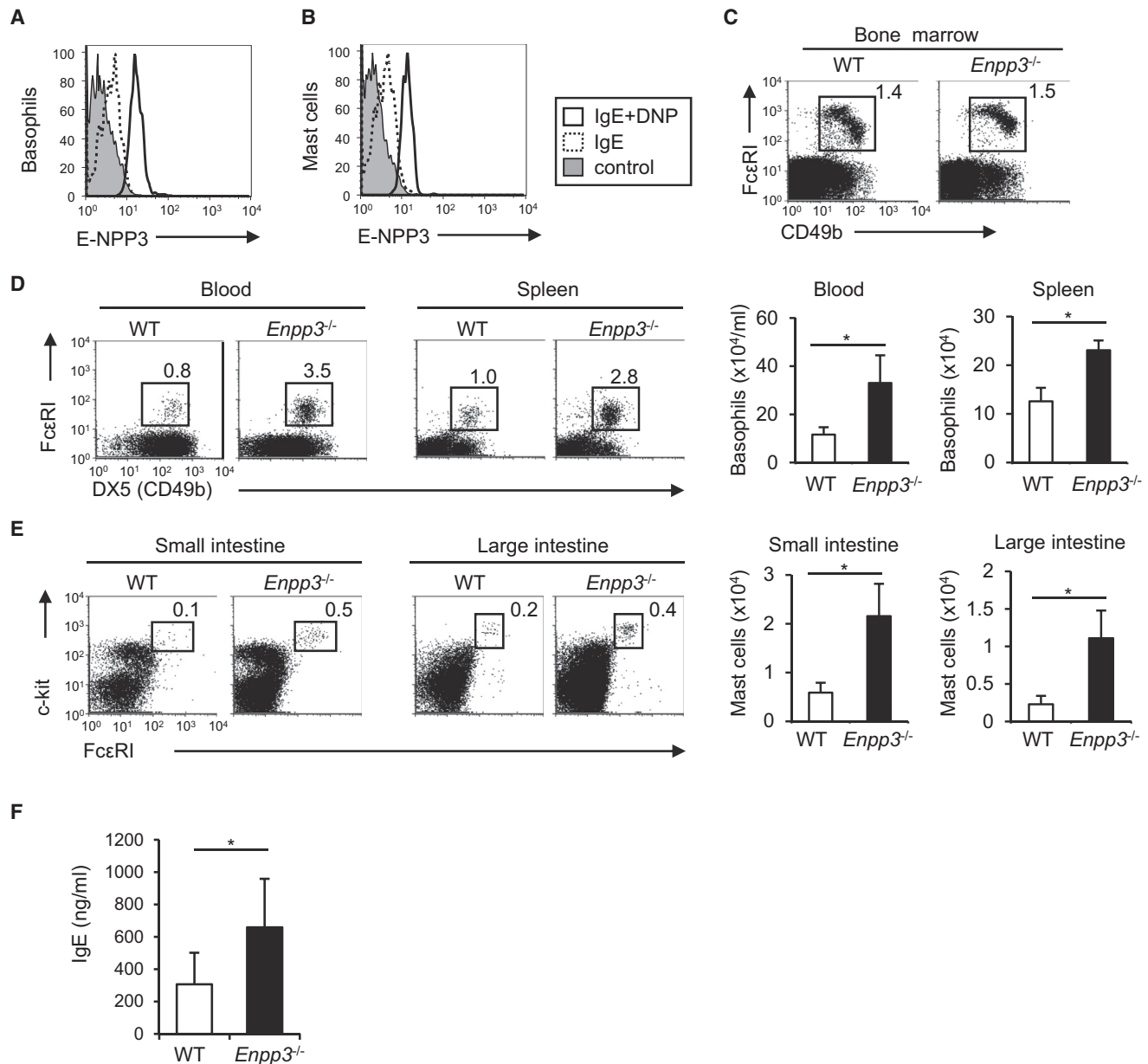


Figure 1. Increased Numbers of Peripheral Basophils and Mast Cells in *Enpp3*^{-/-} Mice

(A and B) Bone-marrow-derived basophils (A) and mast cells (B) were stimulated by FcεRI crosslinking using anti-DNP-IgE and DNP-HSA for 30 min and analyzed for surface E-NPP3 expression by flow cytometry. Solid line, IgE+DNP; dashed line, IgE; gray histogram, non-stimulated control. Data are representative of five independent experiments.

(C and D) Frequencies of basophils in bone marrow (C) and the peripheral blood and spleen (D) from wild-type (WT) and *Enpp3*^{-/-} mice were determined by flow cytometry. Live CD3⁺CD4⁺CD8⁺B220⁺ cells were gated and analyzed for expression of FcεRI and CD49b. Representative dot plots (left) and the means ± SD of total numbers of FcεRI⁺CD49b⁺ cells from five mice (right) are shown. *p < 0.05.

(E) Frequencies of mast cells in the small and large intestines were determined by flow cytometry. Live CD3⁺CD4⁺CD8⁺B220⁺ cells were gated and analyzed for expression of FcεRI and c-kit. Representative dot plots (left) and the means ± SD of total numbers of FcεRI⁺c-kit⁺ cells from five mice (right) are shown. *p < 0.05.

(F) Concentration of serum IgE was measured. Data are means ± SD (n = 5). *p < 0.05.

See also Figure S1.

analysis of the lung revealed infiltration of increased number of inflammatory cells and enhanced mucus production in OVA-challenged *Enpp3*^{-/-} mice (Figures 4B and 4C). Basophil populations in the peripheral blood and the lung increased and serum IgE concentrations were augmented in *Enpp3*^{-/-} mice (Figures

4D and 4E). Serum ATP concentrations were also elevated in *Enpp3*^{-/-} mice challenged with OVA (Figure 4F). oATP treatment ameliorated OVA-induced inflammation and reduced basophil populations and serum IgE levels in *Enpp3*^{-/-} mice (Figures 4A, 4D, and 4E). Taken together, these findings indicate that

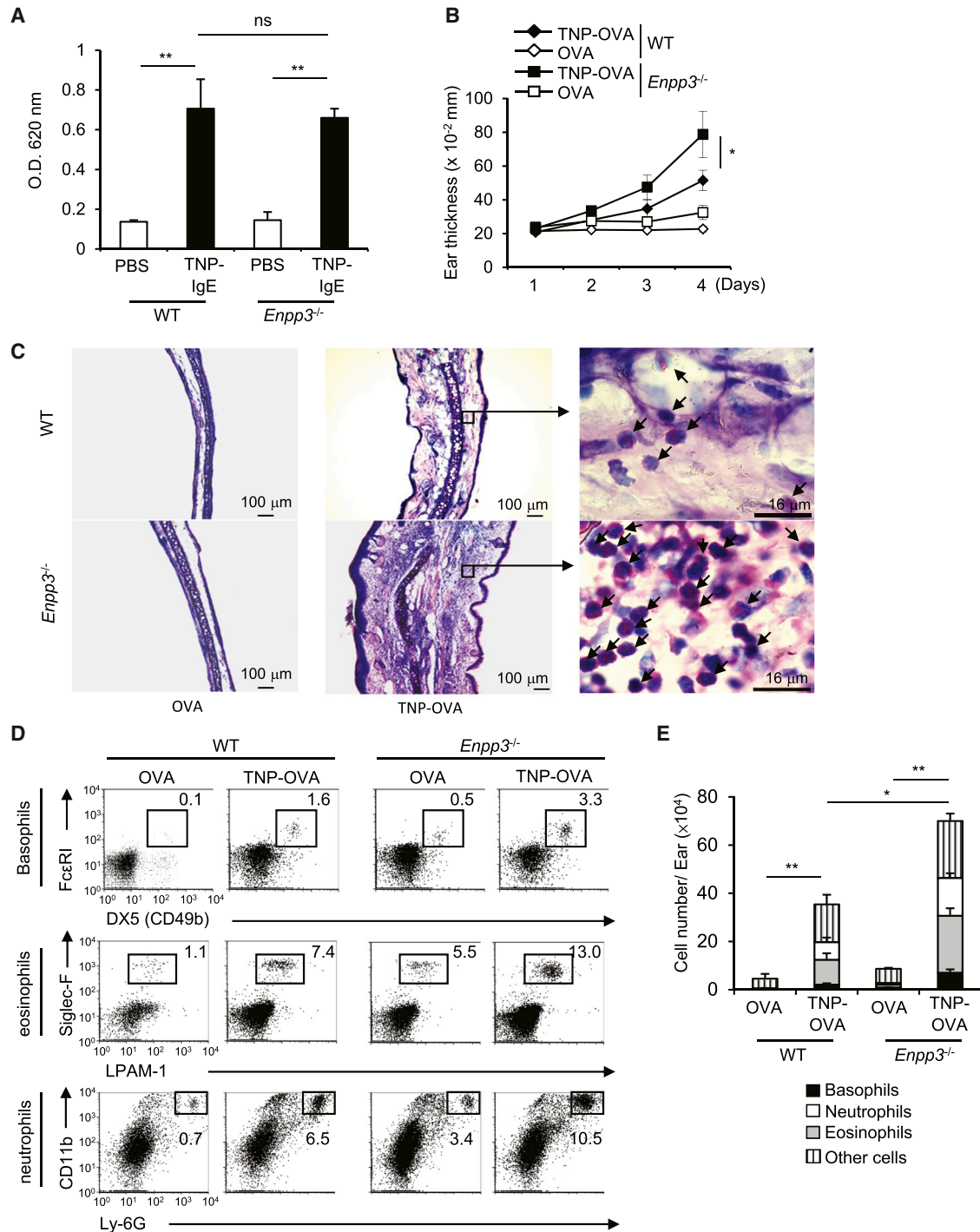


Figure 2. The *Enpp3* Deficiency Exacerbates Basophil-Dependent Allergic Inflammation

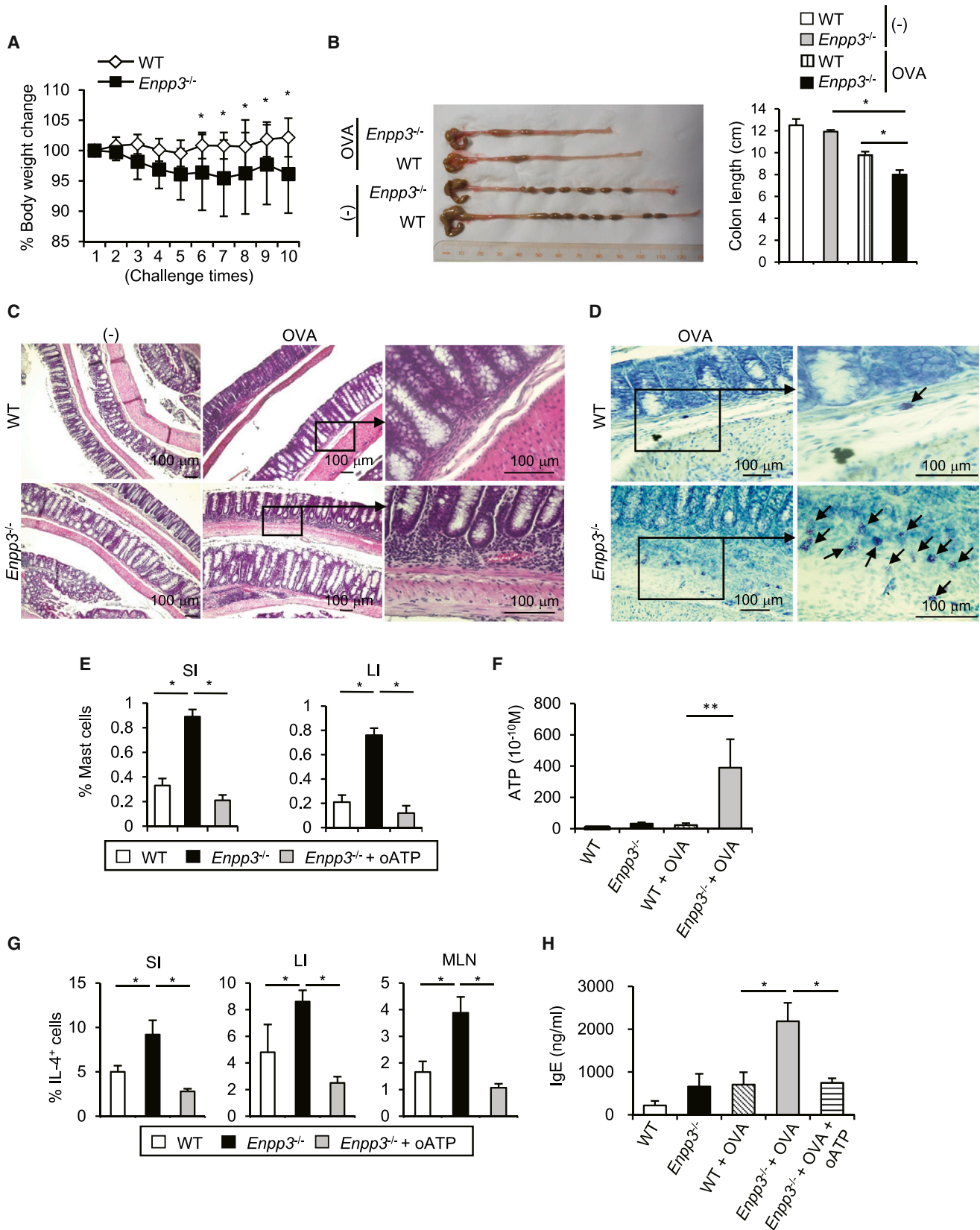
(A) Anti-TNP IgE-sensitized WT and *Enpp3*^{-/-} mice were intravenously challenged with TNP-OVA and Evans blue. At 30 min after the challenge, extravasated dyes in the ear skin were measured by spectrophotometry. Data are shown as means $\pm$ SD (n = 5). **p < 0.01, ns: not significant.

(B) Thickness of ears from anti-TNP IgE-sensitized WT and *Enpp3*^{-/-} mice were measured daily after subcutaneous challenge with TNP-OVA or OVA. Data are shown as means $\pm$ SD (n = 7). *p < 0.05.

(C) Ear specimens from WT and *Enpp3*^{-/-} mice were histologically analyzed with Giemsa staining 4 days after antigen challenge. Black arrows indicate infiltrated eosinophils containing red-colored granules. Data are representative of five mice examined.

(D and E) Frequencies (D) and numbers (E) of basophils (CD3⁺CD4⁺CD8⁺B220⁺FcεRI⁺CD49b⁺), eosinophils (CD3⁺CD4⁺CD8⁺B220⁺Siglec-F⁺LPAM-1⁺), and neutrophils (MHC class II⁺Ly6G⁺CD11b⁺) were determined by flow cytometry and cell counting with trypan blue. Data in (E) are means $\pm$ SD (n = 5). *p < 0.05, **p < 0.01.

See also Figure S2.



(legend on next page)

E-NPP3 is required for the suppression of chronic allergic inflammation by the downregulation of ATP concentration.

Defective ATP Clearance of *Enpp3*^{-/-} Basophils and Mast Cells

Crosslinking of FcεRI by DNP-specific IgE induced the production of cytokines such as IL-4 and IL-6 from basophils and mast cells, respectively (Figure 5A). IgE-dependent cytokine production was enhanced in *Enpp3*^{-/-} cells. Activated mast cells show increased Ki-67 expression (Abraham and St John, 2010; Bischoff et al., 1999). Therefore, we analyzed Ki-67 expression in basophils and mast cells stimulated with FcεRI crosslinking (Figure 5B). The FcεRI crosslinking-dependent increase in Ki-67 staining was enhanced in *Enpp3*^{-/-} mast cells and basophils. Thus, *Enpp3*^{-/-} basophils and mast cells were highly activated.

Several cell types secrete ATP upon stimulation (Corriden and Insel, 2010; Junger, 2011). Therefore, basophils and mast cells were stimulated with DNP-specific IgE for 1 hr and analyzed for ATP release (Figure 5C). FcεRI crosslinking of wild-type basophils and mast cells induced the release of substantial amounts of ATP, and ATP concentrations in supernatants of stimulated *Enpp3*^{-/-} cells were markedly increased compared with that in wild-type cells. We then analyzed whether increased ATP concentrations in stimulated *Enpp3*^{-/-} cells was due to defective ATP clearance. Basophils and mast cells were stimulated by FcεRI crosslinking for the indicated periods and ATP concentrations in supernatants were measured (Figure S5A). At 15 min after stimulation, ATP concentrations were comparable between wild-type and *Enpp3*^{-/-} cells, indicating that FcεRI-dependent ATP release at the early phase of stimulation was not affected in *Enpp3*^{-/-} cells. However, at 30 and 60 min after stimulation, ATP concentrations were increased in *Enpp3*^{-/-} cells compared with wild-type cells. We next added exogenous ATP into the basophil and mast cell culture and measured inorganic phosphate (Pi) and inorganic pyrophosphate (PPi), produced by hydrolysis of ATP to ADP and ATP to AMP, respectively (Figures 5D and S5B). In the supernatants of wild-type basophils and mast cells, free Pi and PPi increased after the addition of exogenous ATP, indicating that wild-type cells have potent ATP hydrolyzing activity. However, increase in Pi and PPi concentrations was substantially dampened in the supernatants of *Enpp3*^{-/-} cells. These findings indicate that ATP clearance ac-

tivity was impaired in basophils and mast cells derived from *Enpp3*^{-/-} mice.

We also assessed the ATP clearance activity in vivo. Wild-type and *Enpp3*^{-/-} mice were intravenously injected with ATP, and at the indicated time points serum was taken to analyze the levels of ATP and PPi. Serum ATP concentration was higher at any time points in *Enpp3*^{-/-} mice (Figure S5C). In addition, at 2 and 4 hr after the administration, fold-increase of ATP concentration compared to the basal concentration was markedly elevated in *Enpp3*^{-/-} mice. However, the fold change at 6 hr time point showed similar pattern between wild-type and *Enpp3*^{-/-} mice (Figure S5D). The concentration of PPi was lower at 1 hr in *Enpp3*^{-/-} mice, but later elevated to the similar concentration as that of wild-type mice (Figure S5E). These findings indicate that kinetics of the ATP clearance in vivo was delayed in *Enpp3*^{-/-} mice, resulting in the increased serum ATP concentration.

Enhanced ATP Responses in *Enpp3*^{-/-} Mice

Because extracellular ATP activates mast cells (Kurashima et al., 2012, 2014; Sudo et al., 1996), we analyzed ATP responses of basophils and mast cells. Culture of wild-type basophils and mast cells in the presence of exogenous ATP led to the secretion of IL-4 and IL-6, respectively (Figure 5E). ATP-induced cytokine production was markedly augmented in *Enpp3*^{-/-} cells. ATP also increased the number of Ki-67-positive basophils and mast cells from wild-type mice, and this was substantially enhanced in *Enpp3*^{-/-} cells (Figure 5F). Thus, *Enpp3*^{-/-} basophils and mast cells showed enhanced ATP-dependent responses.

Next, we assessed whether the enhanced ATP responses were due to the defective clearance of extracellular ATP in *Enpp3*^{-/-} basophils and mast cells by stimulating cells with a non-hydrolyzable ATP, ATPγS (Figure 5F). In wild-type basophils and mast cells, ATPγS increased Ki-67 expression compared with ATP, and their levels were comparable to those of ATPγS-stimulated *Enpp3*^{-/-} cells. We also analyzed in vivo ATP responses. ATP or ATPγS was intravenously injected to mice over three consecutive days and the Ki-67 expression in basophils was assessed (Figure 5G). Almost all blood basophils from *Enpp3*^{-/-} mice were Ki-67 positive, indicating that *Enpp3*^{-/-} basophils were highly activated. In contrast, about 50% of basophils in the peripheral blood of wild-type mice

Figure 3. The *Enpp3* Deficiency Aggravates Allergic Intestinal Inflammation

- (A) Body weights of OVA-sensitized WT (n = 7) and *Enpp3*^{-/-} (n = 7) mice with intragastric challenge with OVA. Data are baseline weights and means ± SD. *p < 0.05.
- (B) Colon length of WT (n = 7) and *Enpp3*^{-/-} (n = 7) mice were measured after ten times OVA administration. Representative pictures are shown (left). Data (right) are means ± SD. *p < 0.05.
- (C and D) Colons were sectioned and stained with H&E (C) or Toluidine blue (D). Black arrows indicate mast cells. Data are representative of three mice examined.
- (E) Mast cells frequencies in small (SI) and large (LI) intestines of OVA-challenged WT mice (n = 5) and *Enpp3*^{-/-} mice with (n = 5) or without (n = 5) oATP treatment were determined by flow cytometry. Means ± SD are shown. *p < 0.05.
- (F) Serum ATP concentrations from non-treated WT (n = 8), *Enpp3*^{-/-} (n = 8), OVA-challenged WT (n = 7), and OVA-challenged *Enpp3*^{-/-} (n = 7) mice were measured. Data are means ± SD. **p < 0.01.
- (G) Cells isolated from small intestine (SI), large intestine (LI), and mesenteric lymph nodes (MLNs) of WT mice (n = 5), *Enpp3*^{-/-} mice (n = 5), and oATP-treated *Enpp3*^{-/-} mice (n = 5) after 10 times OVA administration were stained for intracellular IL-4 in CD4⁺ cells. Means ± SD of the percentage of IL-4⁺ cell in CD4⁺ cells are shown. *p < 0.05.
- (H) Serum concentrations of IgE in non-treated WT (n = 8), *Enpp3*^{-/-} (n = 8), OVA-challenged WT (n = 7), and OVA-challenged *Enpp3*^{-/-} mice with (n = 5) or without (n = 7) oATP treatment were measured. Data are means ± SD. *p < 0.05.
- See also Figure S3.

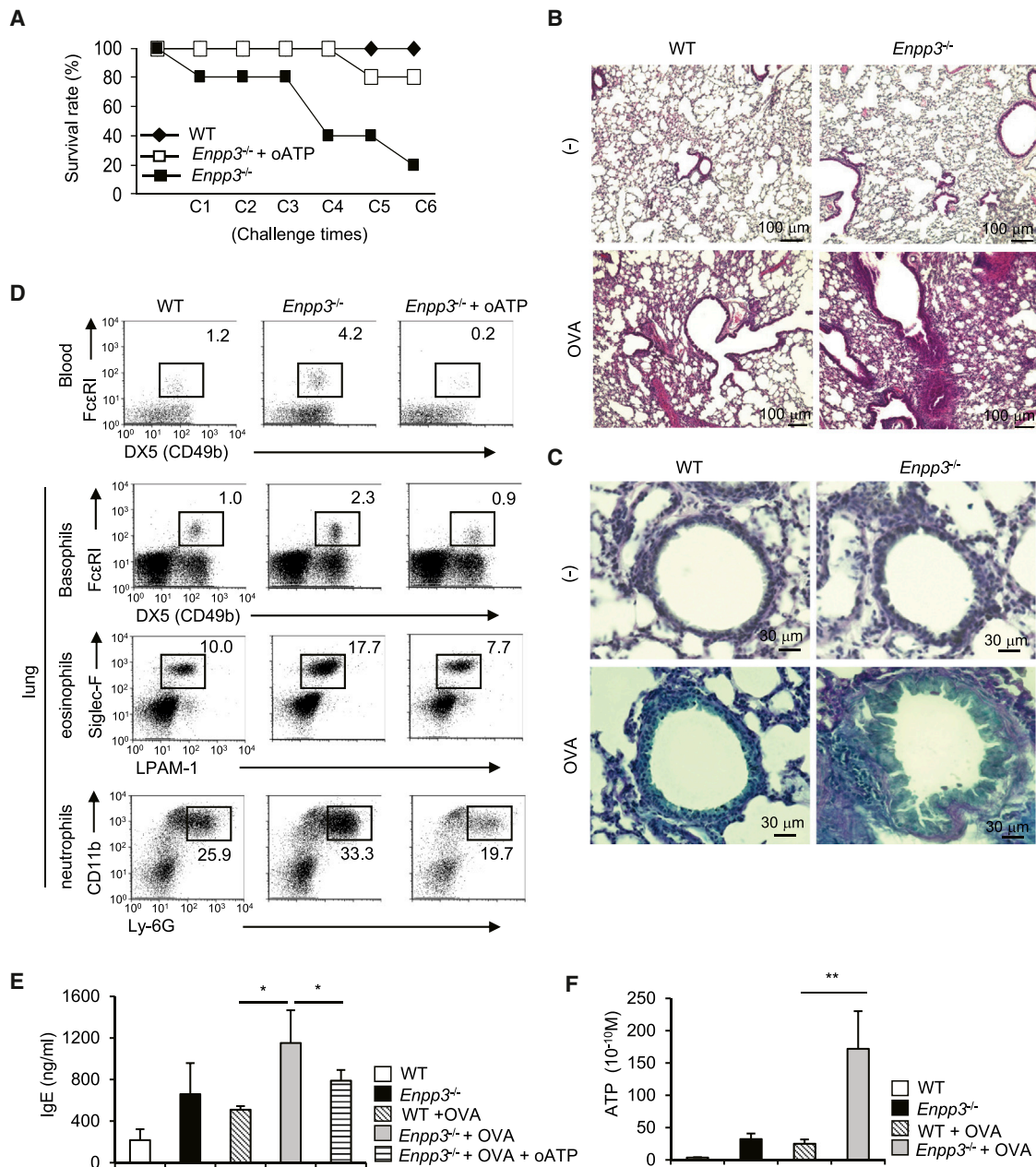


Figure 4. High Sensitivity to Chronic Airway Inflammation in *Enpp3*^{-/-} Mice

(A) Survival rates of WT (n = 7), *Enpp3*^{-/-} (n = 10), and *Enpp3*^{-/-} mice with oATP treatment (n = 5) after OVA challenge are shown.

(B and C) Lungs were sectioned and stained with H&E (B) or Alcian blue (C). Data are representative of three mice examined.

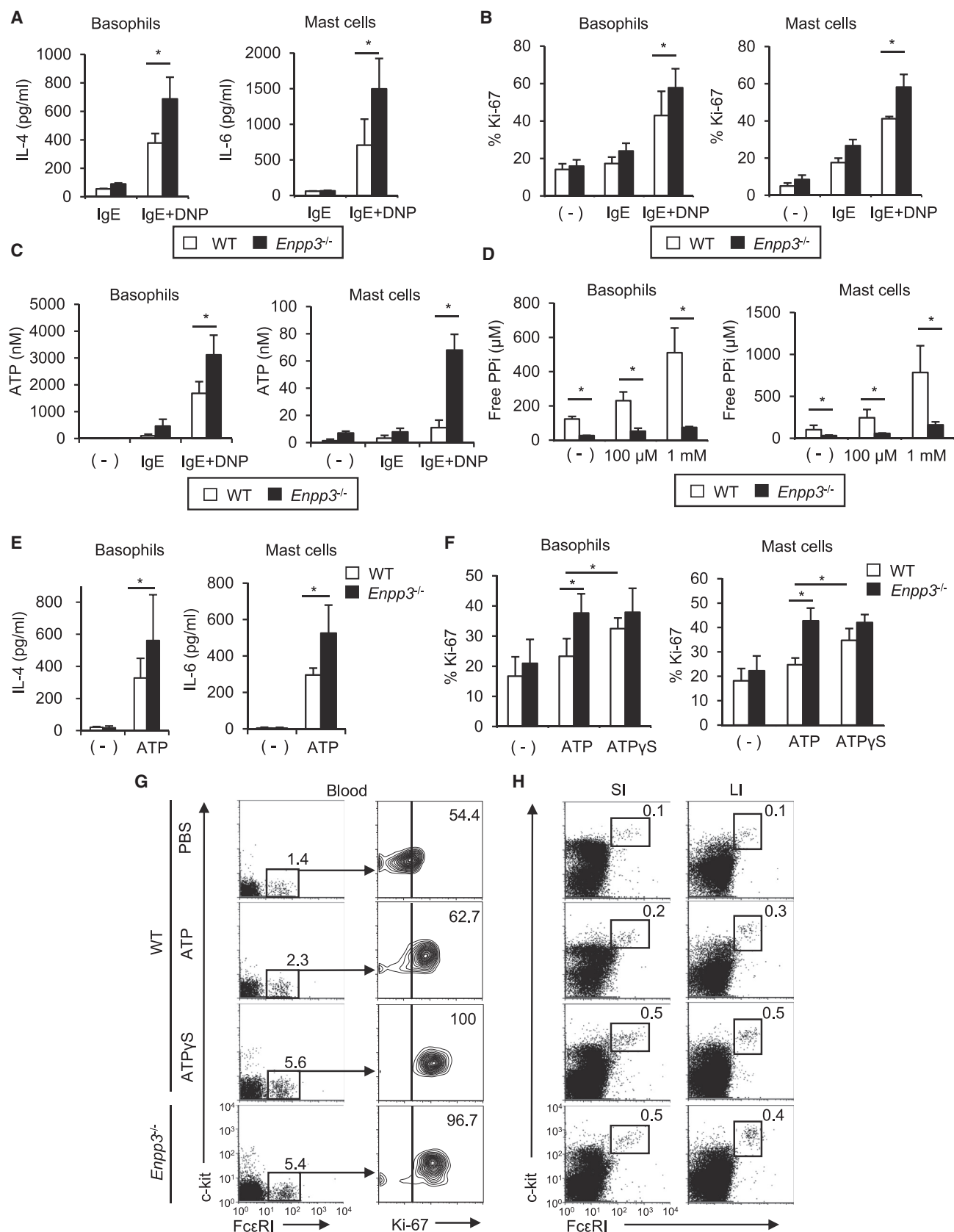
(D) Frequencies of basophils, eosinophils, and neutrophils in the peripheral blood and lung were measured by flow cytometry. Representative dot plots of WT (n = 5), *Enpp3*^{-/-} (n = 3), and *Enpp3*^{-/-} mice with oATP treatment (n = 3) are shown.

(E and F) Concentrations of serum IgE (E) and ATP (F) at 32 days after OVA challenge were determined by ELISA and a luciferin-luciferase assay, respectively. The means $\pm$ SD are shown. Non-treated WT (n = 7), *Enpp3*^{-/-} (n = 7), OVA-challenged WT (n = 7), and OVA-challenged *Enpp3*^{-/-} mice with (n = 5) or without (n = 7) oATP treatment. *p < 0.05, **p < 0.01.

See also Figure S4.

were Ki-67 positive. ATP injection only moderately increased the number of Ki-67-positive basophils in the peripheral blood of wild-type mice, but almost all wild-type basophils were Ki-67 positive after ATP γ S injection. We also analyzed mast cell populations in the small and large intestines after the intravenous in-

jection of ATP or ATP γ S (Figure 5H). ATP only slightly increased mast cell frequencies in wild-type mice, but ATP γ S substantially increased the frequency of wild-type mast cells to similar levels of mast cells in *Enpp3*^{-/-} mice. Taken together, these findings indicate that extracellular ATP activates basophils and mast cells



(legend on next page)

and that E-NPP3 modulates ATP-induced responses through ATP degradation.

We next investigated whether the ATP-dependent increase of basophils and mast cells was attributable to increased proliferation or decreased apoptosis. Basophils and mast cells were treated with ATP, and proliferative activity and apoptotic cell number in vitro were analyzed by measuring ^3H -thymidine uptake and annexin-V-positive cells, respectively (Figures S5F and S5G). ATP induced growth of wild-type basophils and mast cells only marginally. However, *Enpp3*^{-/-} basophils and mast cells showed vigorous proliferation in response to ATP. ATP inhibited apoptosis marginally in wild-type basophils and moderately in wild-type mast cells. ATP-mediated suppression of apoptosis was not dramatically altered in *Enpp3*^{-/-} mice. Thus, in the absence of *Enpp3*, proliferative activity of basophils and mast cells was enhanced, which might result in increased number of peripheral basophils and mast cells.

Regulation of Autocrine ATP-Dependent Activation by E-NPP3

Extracellular ATP activates ERK1 and/or ERK2 (ERK1/2) to induce cellular responses (Shiratori et al., 2010; Swanson et al., 1998). Therefore, we analyzed ATP-induced phosphorylation of ERK1/2 in basophils and mast cells (Figure 6A). Phosphorylation of ERK1/2 was induced by 15 min of ATP stimulation in wild-type basophils and mast cells and ATP-induced phosphorylation of ERK1/2 was enhanced in *Enpp3*^{-/-} cells compared with wild-type cells. ATP-induced ERK1/2 phosphorylation was inhibited by oATP pretreatment. FcεRI crosslinking also induced ERK1/2 phosphorylation. At 15 min after FcεRI crosslinking, phosphorylation of ERK1/2 was similarly induced in wild-type and *Enpp3*^{-/-} basophils and mast cells (Figure 6B). Pretreatment with oATP did not decrease the ATP-induced ERK1/2 phosphorylation, indicating that FcεRI crosslinking directly induced ERK activation. At 60 min after crosslinking, the amount of ERK1/2 phosphorylation was reduced in wild-type basophils and mast cells compared with those observed at 15 min. However, ERK1/2 phosphorylation remained at a high level in *Enpp3*^{-/-} cells even at 60 min after crosslinking (Figure 6C). Because FcεRI crosslinking induces the secretion of ATP and ATP activates ERK1/2, we analyzed the involvement of ATP in sustained ERK1/2 phosphorylation in *Enpp3*^{-/-} cells at

the later time point of FcεRI crosslinking, by pretreating cells with oATP. oATP pretreatment did not induce changes in ERK1/2 phosphorylation of wild-type cells. In contrast, oATP pretreatment substantially reduced ERK1/2 activation. This was in accordance with the observations that ATP activated ERK and that ATP- and FcεRI crosslinking-dependent cellular activation of *Enpp3*^{-/-} basophils and mast cells was reduced by treatment with the ERK inhibitor U0126 (Figures S6A–S6D). These findings indicate that ATP released by FcεRI crosslinking acts on basophils and mast cells in an autocrine manner to further activate the cells and that E-NPP3 negatively regulates ATP-dependent cellular activation.

In order to confirm the autocrine effect of ATP on basophils and mast cells, we co-cultured CD45.2 *Enpp3*^{-/-} basophils and mast cells with CD45.1 wild-type cells. ATP- or FcεRI crosslinking-induced increase of Ki-67 expression was considerably enhanced in *Enpp3*^{-/-} basophils and mast cells compared to wild-type cells (Figure 6D). Furthermore, in basophils and mast cells with siRNA-mediated reduced *Enpp3* expression, ATP- and FcεRI crosslinking-induced cytokine production was increased (Figures 6E and S6E). Thus, the enhanced ATP response of basophils and mast cells in the absence of *Enpp3* is intrinsic to these cells. Taken together, these findings demonstrate that E-NPP3 suppresses the autocrine effect of ATP of basophils and mast cells.

P2X7-Dependent Activation of Basophils and Mast Cells

ATP activates mast cells via the purinoreceptor P2X7 (Kura-shima et al., 2012, 2014; Sudo et al., 1996). Because basophil- and mast cell-dependent chronic inflammation in *Enpp3*^{-/-} mice was ameliorated by oATP treatment, which antagonizes P2X7 actions (Figures 3 and 4), we analyzed whether overactivation of *Enpp3*^{-/-} basophils and mast cells was mediated by P2X7 by using another P2X7 selective antagonist, A839977 (Figure 7A). FcεRI crosslinking markedly augmented the number of Ki-67-positive basophils and mast cells in *Enpp3*^{-/-} mice, which was reduced by pretreatment with oATP. The selective P2X7 antagonist A839977 also reduced the number of Ki-67-positive cells in *Enpp3*^{-/-} mice. The enhanced production of IL-4 and IL-6 from FcεRI-stimulated basophils and mast cells was also decreased by pretreatment with A839977 and oATP (Figure 7B). Thus, experiments with P2X7 receptor

Figure 5. Defective ATP Clearance of *Enpp3*^{-/-} Basophils and Mast Cells

- (A) Bone-marrow-derived basophils (left) and mast cells (right) from WT mice and *Enpp3*^{-/-} mice were stimulated with FcεRI crosslinking for 24 hr, and concentrations of IL-4 and IL-6 in culture supernatants were measured.
- (B) Basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were stimulated with FcεRI crosslinking for 24 hr. The percentage of Ki-67-positive cells was determined by flow cytometry.
- (C) Basophils (left) and mast cells (right) of WT and *Enpp3*^{-/-} mice were stimulated with FcεRI crosslinking for 1 hr, and concentrations of ATP in culture supernatants were measured.
- (D) Basophils and mast cells were incubated for 60 min in the presence of ATP (100 μM or 1 mM final concentration). Concentrations of pyrophosphate (free PPI) in the supernatants were measured.
- (E) Basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were cultured in the presence of 100 μM ATP for 24 hr, and concentrations of IL-4 and IL-6 in culture supernatants were measured.
- (F) Basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were stimulated with 1 μM ATP or ATPγS for 24 hr. The percentage of Ki-67-positive cells was determined by flow cytometry.
- (G and H) WT mice were administered PBS, ATP, or ATPγS. *Enpp3*^{-/-} mice were administered PBS. Expression of c-kit, FcεRI, and Ki-67 in blood CD3⁺CD4⁺CD8⁺B220⁺ cells was analyzed (G). Frequencies of c-kit⁺FcεRI⁺ mast cells in small (SI) and large intestines (LI) of mice were determined by flow cytometry (H).

Data are means ± SD of five independent experiments; *p < 0.05 (A–F). Data are representative of three independent experiments (G and H). See also Figure S5.

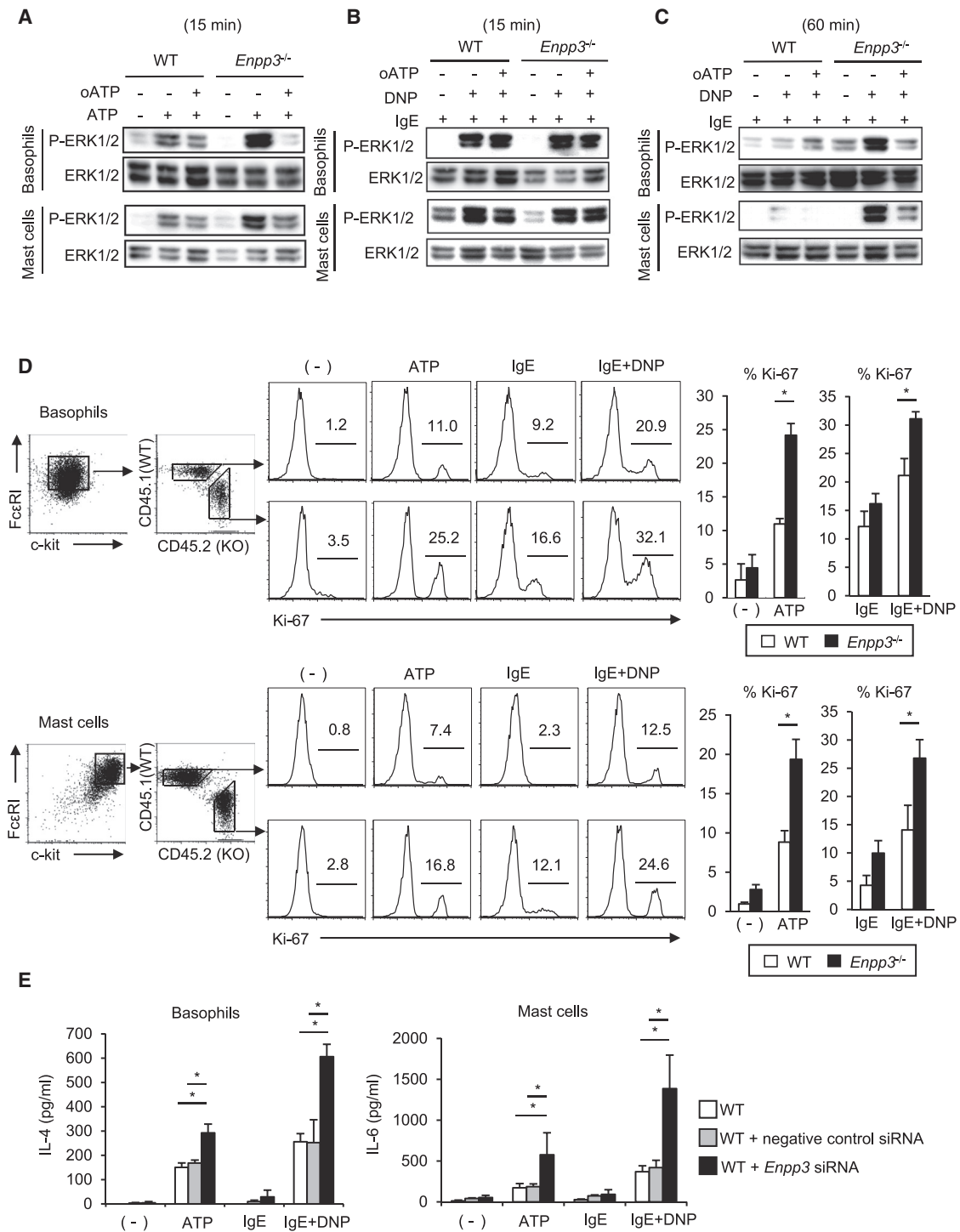


Figure 6. ATP-Dependent Overactivation of ERK1/2 in *Enpp3*^{-/-} Basophils and Mast Cells

(A) Cell lysates were prepared from bone-marrow-derived basophils (upper) and mast cells (lower) from WT and *Enpp3*^{-/-} mice pretreated with 300 μ M oATP for 3 hr and then stimulated by 100 μ M ATP for 15 min. Expression of phospho-ERK1/2 and ERK1/2 were determined by immunoblotting. Data are representative of five independent experiments.

(B and C) Cell lysates were prepared from bone-marrow-derived basophils (upper) and mast cells (lower) of WT and *Enpp3*^{-/-} mice pretreated with 300 μ M oATP for 3 hr and then stimulated by anti-DNP-IgE and DNP-HSA for 15 min (B) or 60 min (C). The levels of phospho-ERK1/2 and ERK1/2 were determined by immunoblotting. Data are representative of three independent experiments.

(legend continued on next page)

antagonists suggested that P2X7 mediates the overactivation of *Enpp3*^{-/-} basophils and mast cells. We next introduced the *P2rx7* deficiency into *Enpp3*^{-/-} mice. In *P2rx7*^{-/-} and *Enpp3*^{-/-}*P2rx7*^{-/-} basophils and mast cells, ATP-dependent cytokine production was severely reduced (Figure 7C). Furthermore, FcεRI crosslinking-induced increase of cytokine production was decreased in *Enpp3*^{-/-}*P2rx7*^{-/-} basophils and mast cells compared with *Enpp3*^{-/-} cells (Figure 7D). In addition, the number of basophils in the peripheral blood and spleen as well as mast cells in the small and large intestines was also reduced in *Enpp3*^{-/-}*P2rx7*^{-/-} mice compared with *Enpp3*^{-/-} mice (Figure 7E). Thus, introduction of the *P2rx7* deficiency into *Enpp3*^{-/-} mice resulted in the decreased activity of basophils and mast cells, demonstrating that P2X7 mediates the ATP-dependent late-phase activation of basophils and mast cells.

DISCUSSION

E-NPP3 (also known as CD203c) is an activation marker of basophils and mast cells (Bühning et al., 2001; Ghannadan et al., 2002). In the present study, we demonstrated that E-NPP3 is a critical negative regulator of basophils and mast cells through the hydrolysis of extracellular ATP, an activator of mast cells (Kurashima et al., 2012, 2014). Basophils were activated by ATP and the overproduction of ATP enhanced chronic allergic responses mediated by basophils and mast cells. Thus, extracellular ATP was demonstrated to be a potent inducer of the chronic phase of allergic responses, and E-NPP3, which is rapidly expressed in activated basophils and mast cells by FcεRI crosslinking, suppresses the ATP-dependent activation by hydrolyzing extracellular ATP.

Several lines of evidence indicated that ATP modulates the functions of neuronal and immune cells (Junger, 2011). ATP is released as a danger-associated molecular pattern from damaged and stressed cells for the clearance of these cells by phagocytes (Elliott et al., 2009; Mariathasan et al., 2006). In addition, ATP is released from non-damaged cells by physiologic stimuli and acts on cells in an autocrine or paracrine manner to modulate cellular functions (Corriden and Insel, 2010). The present study demonstrates that basophils and mast cells are activated by autocrine ATP.

An increase in extracellular ATP was reported in several inflammatory conditions, such as skin inflammation and asthma (Idzko et al., 2007; Kawamura et al., 2012). In addition, the importance of ATP hydrolysis in the regulation of several types of inflammations was demonstrated in mice deficient for CD39 (ecto-nucleoside triphosphate diphosphohydrolase) or CD73 (ecto 5'-nucleotidase) (Deaglio et al., 2007; Friedman et al., 2009; Mizumoto et al., 2002). In those studies, cells responsible for ATP hydrolysis were T cells or dendritic or Langerhans cells.

The present study demonstrated that another type of ATP-hydrolyzing enzyme, E-NPP3, expressed on basophils and mast cells was responsible for the modulation of chronic allergic inflammation via the suppression of ATP-dependent autocrine cell activation.

Basophils and mast cells expressing FcεRI are well known to induce immediate allergic responses through IgE-mediated crosslinking (Gould and Sutton, 2008). In addition, recent evidence indicated that chronic allergic responses are especially induced by basophils through the FcεRI (Karasuyama et al., 2009; Mukai et al., 2005). The present study indicates that ATP is responsible for the pathogenesis of chronic allergic responses. Based on the findings of the present study, we propose the following model during immediate and chronic phases of allergic responses mediated by basophils and mast cells. FcεRI crosslinking induces secretion of several mediators including ATP from basophils and mast cells, which induces the immediate phase of allergic responses. Then, ATP acts on basophils and mast cells in an autocrine manner to further activate basophils and mast cells, leading to the development of chronic-phase allergic inflammation. E-NPP3 (CD203c), which is rapidly expressed on activated basophils and mast cells, negatively regulates ATP-dependent chronic responses.

In *Enpp3*^{-/-} mice, the number of peripheral basophils, but not bone marrow basophils, was increased. In this regard, basophils in the bone marrow, where basophils are generated de novo, might be premature or many factors might contribute to the maintenance or proliferation of basophil precursors. Indeed, anti-basophil mAb treatment drastically reduced the number of peripheral basophils but not bone marrow basophils (Obata et al., 2007). Alternatively, ATP concentration in the bone marrow environment was not altered in *Enpp3*^{-/-} mice (our unpublished results), which might be one of causes for the similar number of bone marrow basophils in both genotypes.

It is interesting to note that the inflammatory mediator ATP was released from basophils and mast cells by FcεRI crosslinking and that the negative regulator of the ATP response, E-NPP3, was simultaneously expressed in these cells. In this regard, ATP-dependent continuous activation might be physiologically responsible for the efficient expulsion of helminths by basophils and mast cells. E-NPP3, in turn, modulates excessive ATP-dependent responses to prevent chronic allergic disorders.

The ATP-induced activation of basophils and mast cells is mediated by P2X7 and ERK MAP kinases. A previous study demonstrated that P2X7-mediated sensing of ATP induced the activation of several signal transduction pathways leading to the activation of microglia (Shiratori et al., 2010). In the present study, the involvement of ERK MAP kinases in the activation of basophils and mast cells was demonstrated. However, other signaling pathways such as p38 and JNK MAP kinases as well

(D) Mixtures of basophils (top) and mast cells (bottom) of WT (CD45.1) and *Enpp3*^{-/-} (KO: CD45.2) mice (1:1 ratio in cell number) were stimulated with ATP or FcεRI crosslinking for 24 hr. The percentage of Ki-67-positive cells was determined by flow cytometry. Data shown in right panel are means ± SD of three independent experiments. *p < 0.05.

(E) Bone-marrow-derived basophils (left) and mast cells (right) from wild-type mice were introduced with *Enpp3*-specific siRNA or control siRNA and then stimulated with ATP or FcεRI crosslinking for 24 hr. The concentrations of IL-4 and IL-6 in culture supernatants were measured. Data are means ± SD of five independent experiments. *p < 0.05.

See also Figure S6.

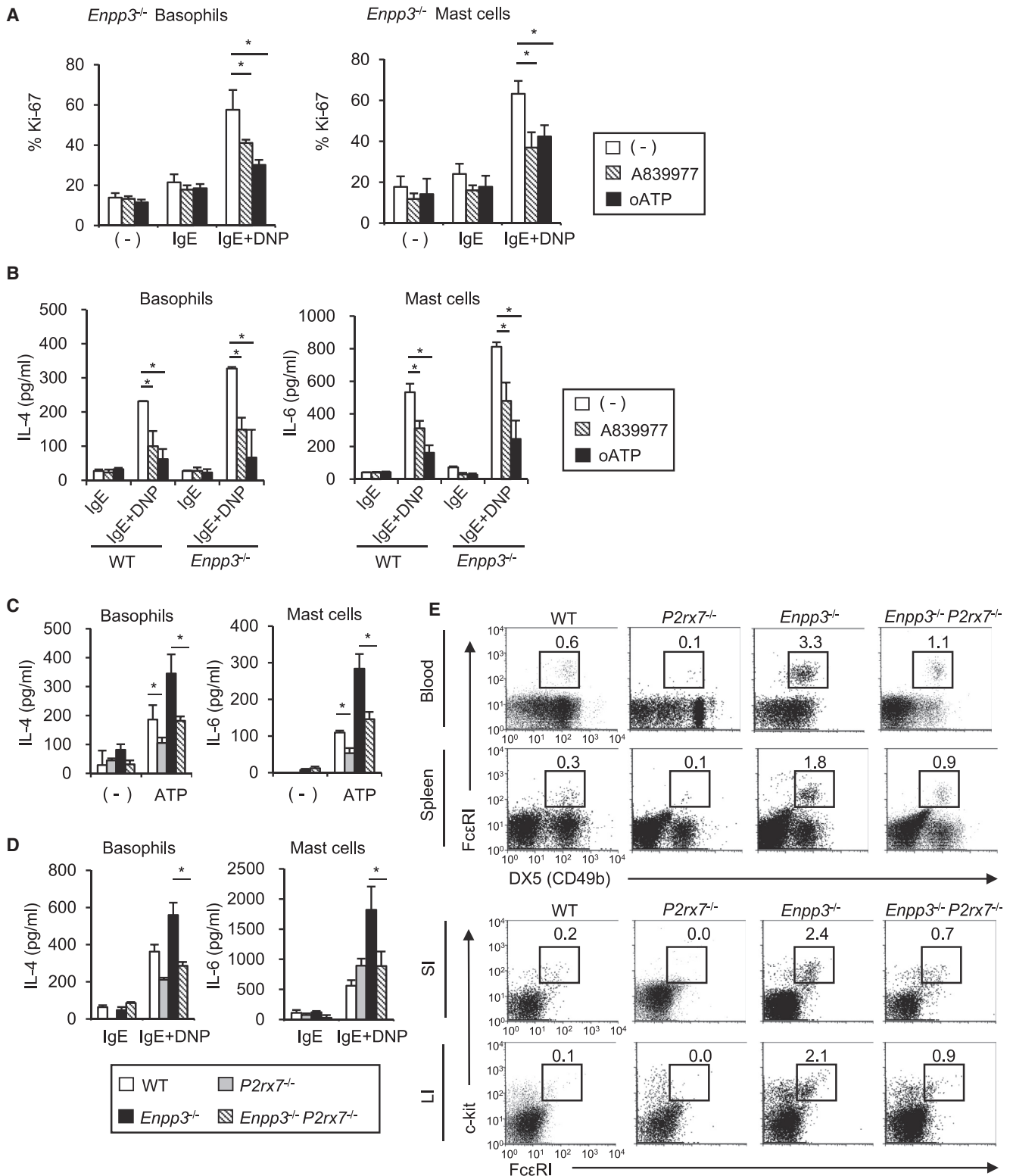


Figure 7. P2X7-Dependent Activation of Basophils and Mast Cells

(A and B) Bone-marrow-derived basophils (left) and mast cells (right) from *Enpp3*^{-/-} mice were pretreated with 300 μ M oATP or 100 nM A839977 for 3 hr and then stimulated by anti-DNP-IgE and DNP-HSA for 24 hr. The percentage of Ki-67⁺ cells was determined by flow cytometry (A). Concentrations of IL-4 and IL-6 in culture supernatants were determined by ELISA (B). Data are means $\pm$ SD of three independent experiments. **p* < 0.05.

(legend continued on next page)

as PKC might also participate in the ATP/P2X7-induced activation of basophils and mast cells.

P2X7 was shown to be responsible for the ATP-dependent overactivation of basophils and mast cells in the absence of E-NPP3. Indeed, several previous studies indicated that P2X7 is implicated in the pathogenesis of airway allergic inflammation in mice and humans (Denlinger et al., 2013; Idzko et al., 2007; Manthei et al., 2012; Müller et al., 2011) as well as contact hypersensitivity in a mouse model (Weber et al., 2010). However, all of these studies focused on dendritic cells as a target of ATP. It is generally considered that late-phase allergic inflammation is mediated by Th2 cells. However, basophils and mast cells are closely associated to Th2-cell-type responses (Karasuyama et al., 2011; Voehringer, 2013). In addition, it was shown that the chronic phase of IgE-dependent allergic inflammation can be induced independently of T cells (Mukai et al., 2005). In the present study, P2X7 on basophils and mast cells was responsible for the late phase of IgE-dependent activation of basophils and mast cells. Thus, P2X7 on basophils and mast cells could be a possible target for the treatment of allergic diseases. However, both P2X3 and P2X7 were highly expressed on basophils (our unpublished results), and therefore P2X3 might also participate in the ATP-dependent activation of basophils and mast cells. Indeed, although ATP-dependent responses were severely reduced, they were not completely blocked in the absence of *P2rx7*. The possible involvement of P2Y receptors should also be considered.

In the present study, we revealed that E-NPP3 is a negative regulator of chronic allergic responses via ATP hydrolysis. Because E-NPP3 is selectively expressed in activated basophils and mast cells, the main cell types involved in allergic responses, the development of a novel drug to activate or induce E-NPP3 might be beneficial for the establishment of an effective therapy for allergic diseases, which recently have increased in number.

EXPERIMENTAL PROCEDURES

Passive Cutaneous Anaphylaxis

Wild-type and *Enpp3*^{-/-} mice on the BALB/c background were sensitized with 100 ng anti-TNP IgE derived from IGLb4 B cell hybridoma in 10 μ l PBS by intradermal injection into the left ear. As a control, PBS was injected into the right ear. At 24 hr after the sensitization, mice were intravenously challenged with 300 μ g TNP14-conjugated ovalbumin (TNP-OVA; Biosearch Technologies) plus 2% Evans blue (Sigma-Aldrich) in 300 μ l PBS. Mice were euthanized 30 min after the antigen challenge, followed by extraction of Evans blue dye from each ear in 700 μ l acetone/water (7:3) at room temperature overnight. The absorbance at 620 nm in the extract was measured with a spectrophotometer. All animal experiments were conducted with approval of the Animal Research Committee of the Graduate School of Medicine at Osaka University and the Animal Care and Use Committee, The University of Tokyo.

IgE-Mediated Chronic Allergic Skin Inflammation

IgE-mediated chronic allergic skin inflammation was induced as previously described (Mukai et al., 2005). In brief, wild-type and *Enpp3*^{-/-} mice on the BALB/c background were sensitized with 200 μ g anti-TNP IgE derived from

IGELb4 B cell hybridoma by intravenous injection. The next day, left ears of IgE-sensitized mice were intradermally challenged with 10 μ g TNP-OVA (Biosearch Technologies) in 10 μ l PBS and right ears were inoculated an equal amount of OVA (grade V; Sigma-Aldrich) by microsyringe. The thickness of ears were measured by a dial thickness gauge (G-1A; Ozaki), and numbers and frequencies of basophils (CD3⁺CD4⁺CD8⁺B220⁺Fc ϵ RI⁺CD49b⁺), eosinophils (CD3⁺CD4⁺CD8⁺B220⁺Siglec-F⁺LPAM-1⁺), and neutrophils (MHC class II⁺Ly6G⁺CD11b⁺) in ears treated with 1 mg/ml collagenase D (Roche) were determined by trypan blue cell counting and flow cytometry. For histological analysis, ear specimens were fixed with 4% paraformaldehyde phosphate buffer solution (Wako) and embedded in paraffin. Sections were stained with Giemsa and analyzed with a BZ-9000 microscope system (Keyence).

Experimental Oral Allergen-Induced Diarrhea

Experimental oral allergen-induced diarrhea was elicited as previously described (Brandt et al., 2003). In brief, wild-type and *Enpp3*^{-/-} mice were sensitized with a mixture of 50 μ g of OVA (grade V; Sigma-Aldrich) and 1 mg of aluminum hydroxide adjuvant (Imject Alum; Thermo Scientific) twice with a 2-week interval. At 2 weeks after the last sensitization, mice were intragastrically challenged with 10 mg of OVA in 200 μ l PBS every 2 days for 10 times after 3 hr of food deprivation. Body weights were recorded before intragastric challenge. In some experiments, OVA-sensitized *Enpp3*^{-/-} mice were intravenously administered with 100 μ l of 5 mM oATP (Sigma-Aldrich) 5 times every 3 days during the period of intragastric challenge. After 10 times of OVA challenge, the mice were sacrificed and used for analyses of cell population and histology. Peripheral blood was harvested for serum ATP measurement 1 hr after the last intragastric challenge. For histological analysis, colon specimens were prepared by a Swiss roll method and fixed with 4% paraformaldehyde phosphate buffer solution (Wako) and embedded in paraffin. Sections were stained with hematoxylin and eosin or toluidine blue and analyzed with a BZ-9000 microscope system (Keyence).

Experimental Chronic Asthma

Chronic asthma was induced as previously described with some modifications (Idzko et al., 2007; Wakahara et al., 2013). Wild-type and *Enpp3*^{-/-} mice were sensitized with a mixture of 50 μ g of OVA (grade V; Sigma-Aldrich) and 1 mg of aluminum hydroxide adjuvant (Imject Alum; Thermo Scientific) twice with a 10-day interval. Then, the OVA-sensitized mice were intranasally challenged with 50 μ g of OVA in 20 μ l PBS on day 17, 19, 21, 28, 30, and 32. In some experiments, OVA-sensitized *Enpp3*^{-/-} mice were administered 100 μ l of 5 mM oATP intravenously on day 11, 14, and 17, as well as 30 min before OVA intranasal challenges. At 16 hr after the last intranasal challenge, peripheral blood was harvested for serum ATP measurement. For histological analysis, lung specimens were fixed with 4% paraformaldehyde phosphate buffer solution (Wako) and embedded in paraffin. Sections were stained with hematoxylin and eosin or Alcian blue and hematoxylin.

In Vitro Stimulation Assay

For IgE crosslinking, bone-marrow-derived basophils and mast cells were pretreated with 1 μ g/ml anti-DNP IgE (SPE-7; Sigma-Aldrich) for 6 hr, and then stimulated with 50 ng/ml DNP-HSA (Sigma-Aldrich) for the indicated periods. In some experiments, bone-marrow-derived basophils and mast cells were pretreated with 300 μ M oATP (Sigma-Aldrich) or 100 nM A839977 P2X7 antagonist (Tocris Bioscience) for 3 hr or with 20 μ M U0126 (Sigma-Aldrich) for 1 hr.

Measurement of ATP Hydrolyzing Activity

ATP hydrolyzing activity was determined by measuring levels of free inorganic phosphate and free inorganic pyrophosphate in the supernatants of cells cultured for 1 hr after the addition of ATP (100 μ M or 1 mM). Levels of free inorganic phosphate and free inorganic pyrophosphate were determined by

(C and D) Bone-marrow-derived basophils (left) and mast cells (right) from WT, *P2rx7*^{-/-}, *Enpp3*^{-/-}, and *Enpp3*^{-/-}*P2rx7*^{-/-} mice were cultured in the presence of 100 μ M ATP (C) or anti-DNP-IgE and DNP-HSA (D) for 24 hr, and concentrations of IL-4 and IL-6 in culture supernatants were measured. Data are means $\pm$ SD of three independent experiments. **p* < 0.05.

(E) Frequencies of basophils in peripheral blood and spleen and frequencies of mast cells in small (SI) and large intestines (LI) from WT, *P2rx7*^{-/-}, *Enpp3*^{-/-}, and *Enpp3*^{-/-}*P2rx7*^{-/-} mice were determined by flow cytometry. Representative dot plots from three independent experiments are shown.

Malachite Green Phosphate Assay Kits (BioAssay Systems) and Pyrophosphate Assay Kit (Fluorometric; Abcam), respectively.

Treatment with ATP and ATP γ S

Mice were administrated 1 mM ATP or ATP γ S in 100 μ l PBS intravenously (i.v.) daily for 3 days before flow cytometric analysis.

Co-culture of Wild-Type and *Enpp3*^{-/-} Basophils and Mast Cells

Bone-marrow-derived basophils and mast cells from CD45.1 wild-type and CD45.2 *Enpp3*^{-/-} mice on the C57BL/6 background were mixed in 1:1 ratio and stimulated with 100 μ M ATP or treated with 1 μ g/ml anti-DNP IgE (SPE-7; Sigma-Aldrich) for 6 hr followed by stimulation with 50 ng/ml DNP-HSA (Sigma-Aldrich). After 24 hr of the stimulation, cells were stained with anti-CD45.1, anti-CD45.2, and anti-Ki-67 Abs.

siRNA-Mediated Knockdown of *Enpp3* in Basophils and Mast Cells

Bone-marrow-derived basophils and mast cells from wild-type mice prepared as above were introduced with *Enpp3*-specific siRNA (sc-142185; Santa Cruz) or control siRNA (silencer negative control; Life Technologies) by using a Lonza transfection system (Amaxa cell line nucleofector kit V; Lonza). At 12 hr after the siRNA introduction, cells were analyzed for expression *Enpp3* mRNA and used for experiments.

Statistical Analysis

Differences between control and experimental groups were evaluated by the Student's *t* test. A *p* value < 0.05 was considered significant. Data are presented as mean $\pm$ SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2015.01.015>.

AUTHOR CONTRIBUTIONS

S.H.T. performed experiments. T.K. generated mutant mice. M.K., H. Kayama, R.O., K.I., Y.S., and E.U. performed animal experiments. S.S., Y.K., and H. Kiyono performed experiments with double mutant mice. A.T., S.Y., K.O.-N., and H. Karasuyama performed experiments with anti-TNP-IgE. M.K. and S.H.T. contributed to the experimental design and data analysis. K.T. planned and directed the research and wrote the paper.

ACKNOWLEDGMENTS

We thank Y. Uno and M. Tajima for generation of *Enpp3*^{-/-} mice, T. Kondo and Y. Magota for technical assistance, S. Pareek for critical reading of the manuscript, and C. Hidaka for secretarial assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, the Japan Science and Technology Agency, and by the Ministry of Health, Labour and Welfare.

Received: July 16, 2014

Revised: August 23, 2014

Accepted: December 9, 2014

Published: February 17, 2015

REFERENCES

- Abraham, S.N., and St John, A.L. (2010). Mast cell-orchestrated immunity to pathogens. *Nat. Rev. Immunol.* 10, 440–452.
- Bischoff, S.C., Sellge, G., Lorentz, A., Sebald, W., Raab, R., and Manns, M.P. (1999). IL-4 enhances proliferation and mediator release in mature human mast cells. *Proc. Natl. Acad. Sci. USA* 96, 8080–8085.
- Bollen, M., Gijssels, R., Ceulemans, H., Stalmans, W., and Stefan, C. (2000). Nucleotide pyrophosphatases/phosphodiesterases on the move. *Crit. Rev. Biochem. Mol. Biol.* 35, 393–432.
- Brandt, E.B., Strait, R.T., Hershko, D., Wang, Q., Muntel, E.E., Scribner, T.A., Zimmermann, N., Finkelman, F.D., and Rothenberg, M.E. (2003). Mast cells are required for experimental oral allergen-induced diarrhea. *J. Clin. Invest.* 112, 1666–1677.
- Bühning, H.J., Seiffert, M., Giesert, C., Marxer, A., Kanz, L., Valent, P., and Sano, K. (2001). The basophil activation marker defined by antibody 97A6 is identical to the ectonucleotide pyrophosphatase/phosphodiesterase 3. *Blood* 97, 3303–3305.
- Bühning, H.J., Streble, A., and Valent, P. (2004). The basophil-specific ectoenzyme E-NPP3 (CD203c) as a marker for cell activation and allergy diagnosis. *Int. Arch. Allergy Immunol.* 133, 317–329.
- Corriden, R., and Insel, P.A. (2010). Basal release of ATP: an autocrine-paracrine mechanism for cell regulation. *Sci. Signal.* 3, re1.
- Deaglio, S., Dwyer, K.M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J.F., Enjyoji, K., Linden, J., Oukka, M., et al. (2007). Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* 204, 1257–1265.
- Denlinger, L.C., Manthei, D.M., Seibold, M.A., Ahn, K., Bleecker, E., Boushey, H.A., Calhoun, W.J., Castro, M., Chinchili, V.M., Fahy, J.V., et al.; NHLBI Asthma Clinical Research Network Investigators (2013). P2X7-regulated protection from exacerbations and loss of control is independent of asthma maintenance therapy. *Am. J. Respir. Crit. Care Med.* 187, 28–33.
- Elliott, M.R., Chekeni, F.B., Trampont, P.C., Lazarowski, E.R., Kadl, A., Walk, S.F., Park, D., Woodson, R.I., Ostankovich, M., Sharma, P., et al. (2009). Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* 461, 282–286.
- Friedman, D.J., Künzli, B.M., A-Rahim, Y.I., Seigny, J., Berberat, P.O., Enjyoji, K., Csizmadia, E., Friess, H., and Robson, S.C. (2009). From the Cover: CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to inflammatory bowel disease. *Proc. Natl. Acad. Sci. USA* 106, 16788–16793.
- Ghannadan, M., Hauswirth, A.W., Scherthaner, G.H., Müller, M.R., Klepetko, W., Schatzl, G., Sperr, W.R., Bühning, H.J., and Valent, P. (2002). Detection of novel CD antigens on the surface of human mast cells and basophils. *Int. Arch. Allergy Immunol.* 127, 299–307.
- Gould, H.J., and Sutton, B.J. (2008). IgE in allergy and asthma today. *Nat. Rev. Immunol.* 8, 205–217.
- Hessle, L., Johnson, K.A., Anderson, H.C., Narisawa, S., Sali, A., Goding, J.W., Terkeltaub, R., and Millan, J.L. (2002). Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *Proc. Natl. Acad. Sci. USA* 99, 9445–9449.
- Idzko, M., Hammad, H., van Nimwegen, M., Kool, M., Willart, M.A., Muskens, F., Hoogsteden, H.C., Luttmann, W., Ferrari, D., Di Virgilio, F., et al. (2007). Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat. Med.* 13, 913–919.
- Idzko, M., Ferrari, D., and Eltzschig, H.K. (2014). Nucleotide signalling during inflammation. *Nature* 509, 310–317.
- Junger, W.G. (2011). Immune cell regulation by autocrine purinergic signalling. *Nat. Rev. Immunol.* 11, 201–212.
- Kanda, H., Newton, R., Klein, R., Morita, Y., Gunn, M.D., and Rosen, S.D. (2008). Autotaxin, an ectoenzyme that produces lysophosphatidic acid, promotes the entry of lymphocytes into secondary lymphoid organs. *Nat. Immunol.* 9, 415–423.
- Karasuyama, H., Mukai, K., Tsujimura, Y., and Obata, K. (2009). Newly discovered roles for basophils: a neglected minority gains new respect. *Nat. Rev. Immunol.* 9, 9–13.
- Karasuyama, H., Mukai, K., Obata, K., Tsujimura, Y., and Wada, T. (2011). Nonredundant roles of basophils in immunity. *Annu. Rev. Immunol.* 29, 45–69.
- Kawamura, T., Ogawa, Y., Nakamura, Y., Nakamizo, S., Ohta, Y., Nakano, H., Kabashima, K., Katayama, I., Koizumi, S., Kodama, T., et al. (2012). Severe dermatitis with loss of epidermal Langerhans cells in human and mouse zinc deficiency. *J. Clin. Invest.* 122, 722–732.
- Kurashima, Y., Amiya, T., Nochi, T., Fujisawa, K., Haraguchi, T., Iba, H., Tsutsui, H., Sato, S., Nakajima, S., Iijima, H., et al. (2012). Extracellular ATP

mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors. *Nat. Commun.* 3, 1034.

Kurashima, Y., Amiya, T., Fujisawa, K., Shibata, N., Suzuki, Y., Kogure, Y., Hashimoto, E., Otsuka, A., Kabashima, K., Sato, S., et al. (2014). The enzyme Cyp26b1 mediates inhibition of mast cell activation by fibroblasts to maintain skin-barrier homeostasis. *Immunity* 40, 530–541.

Manthei, D.M., Jackson, D.J., Evans, M.D., Gangnon, R.E., Tisler, C.J., Gern, J.E., Lemanske, R.F., Jr., and Denlinger, L.C. (2012). Protection from asthma in a high-risk birth cohort by attenuated P2X(7) function. *J. Allergy Clin. Immunol.* 130, 496–502.

Mariathasan, S., Weiss, D.S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W.P., Weinrauch, Y., Monack, D.M., and Dixit, V.M. (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440, 228–232.

Mizumoto, N., Kumamoto, T., Robson, S.C., Sévigny, J., Matsue, H., Enjyoji, K., and Takashima, A. (2002). CD39 is the dominant Langerhans cell-associated ecto-NTPDase: modulatory roles in inflammation and immune responsiveness. *Nat. Med.* 8, 358–365.

Mukai, K., Matsuoka, K., Taya, C., Suzuki, H., Yokozeki, H., Nishioka, K., Hirokawa, K., Etori, M., Yamashita, M., Kubota, T., et al. (2005). Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity* 23, 191–202.

Müller, T., Vieira, R.P., Grimm, M., Dürk, T., Cicko, S., Zeiser, R., Jakob, T., Martin, S.F., Blumenthal, B., Sorichter, S., et al. (2011). A potential role for P2X7R in allergic airway inflammation in mice and humans. *Am. J. Respir. Cell Mol. Biol.* 44, 456–464.

Obata, K., Mukai, K., Tsujimura, Y., Ishiwata, K., Kawano, Y., Minegishi, Y., Watanabe, N., and Karasuyama, H. (2007). Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood* 110, 913–920.

Okawa, A., Nakamura, I., Goto, S., Moriya, H., Nakamura, Y., and Ikegawa, S. (1998). Mutation in Npps in a mouse model of ossification of the posterior longitudinal ligament of the spine. *Nat. Genet.* 19, 271–273.

Schetinger, M.R., Morsch, V.M., Bonan, C.D., and Wyse, A.T. (2007). NTPDase and 5'-nucleotidase activities in physiological and disease conditions: new perspectives for human health. *Biofactors* 31, 77–98.

Shiratori, M., Tozaki-Saitoh, H., Yoshitake, M., Tsuda, M., and Inoue, K. (2010). P2X7 receptor activation induces CXCL2 production in microglia through NFAT and PKC/MAPK pathways. *J. Neurochem.* 114, 810–819.

Stefan, C., Jansen, S., and Bollen, M. (2005). NPP-type ectophosphodiesterases: unity in diversity. *Trends Biochem. Sci.* 30, 542–550.

Sudo, N., Tanaka, K., Koga, Y., Okumura, Y., Kubo, C., and Nomoto, K. (1996). Extracellular ATP activates mast cells via a mechanism that is different from the activation induced by the cross-linking of Fc receptors. *J. Immunol.* 156, 3970–3979.

Swanson, K.D., Reigh, C., and Landreth, G.E. (1998). ATP-stimulated activation of the mitogen-activated protein kinases through ionotropic P2X2 purinoreceptors in PC12 cells. Difference in purinoreceptor sensitivity in two PC12 cell lines. *J. Biol. Chem.* 273, 19965–19971.

Umamoto, E., Hayasaka, H., Bai, Z., Cai, L., Yonekura, S., Peng, X., Takeda, A., Tohya, K., and Miyasaka, M. (2011). Novel regulators of lymphocyte trafficking across high endothelial venules. *Crit. Rev. Immunol.* 31, 147–169.

van Meeteren, L.A., Ruurs, P., Stortelers, C., Bouwman, P., van Rooijen, M.A., Pradère, J.P., Pettit, T.R., Wakelam, M.J., Saulnier-Blache, J.S., Mummery, C.L., et al. (2006). Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development. *Mol. Cell. Biol.* 26, 5015–5022.

Voehringer, D. (2013). Protective and pathological roles of mast cells and basophils. *Nat. Rev. Immunol.* 13, 362–375.

Wakahara, K., Van, V.Q., Baba, N., Bégin, P., Rubio, M., Delespesse, G., and Sarfati, M. (2013). Basophils are recruited to inflamed lungs and exacerbate memory Th2 responses in mice and humans. *Allergy* 68, 180–189.

Wang, L., Sikora, J., Hu, L., Shen, X., Grygorczyk, R., and Schwarz, W. (2013). ATP release from mast cells by physical stimulation: a putative early step in activation of acupuncture points. *Evid. Based Complement. Alternat. Med.* 2013, 350949.

Weber, F.C., Esser, P.R., Müller, T., Ganesan, J., Pellegatti, P., Simon, M.M., Zeiser, R., Idzko, M., Jakob, T., and Martin, S.F. (2010). Lack of the purinergic receptor P2X(7) results in resistance to contact hypersensitivity. *J. Exp. Med.* 207, 2609–2619.

Yegutkin, G.G. (2008). Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim. Biophys. Acta* 1783, 673–694.

Immunity

Supplemental Information

The Ectoenzyme E-NPP3 Negatively Regulates ATP-Dependent Chronic Allergic Responses by Basophils and Mast Cells

Shih Han Tsai, Makoto Kinoshita, Takashi Kusu, Hisako Kayama, Ryu Okumura, Kayo Ikeda, Yosuke Shimada, Akira Takeda, Soichiro Yoshikawa, Kazushige Obata-Ninomiya, Yosuke Kurashima, Shintaro Sato, Eiji Umemoto, Hiroshi Kiyono, Hajime Karasuyama, and Kiyoshi Takeda

Figure S1

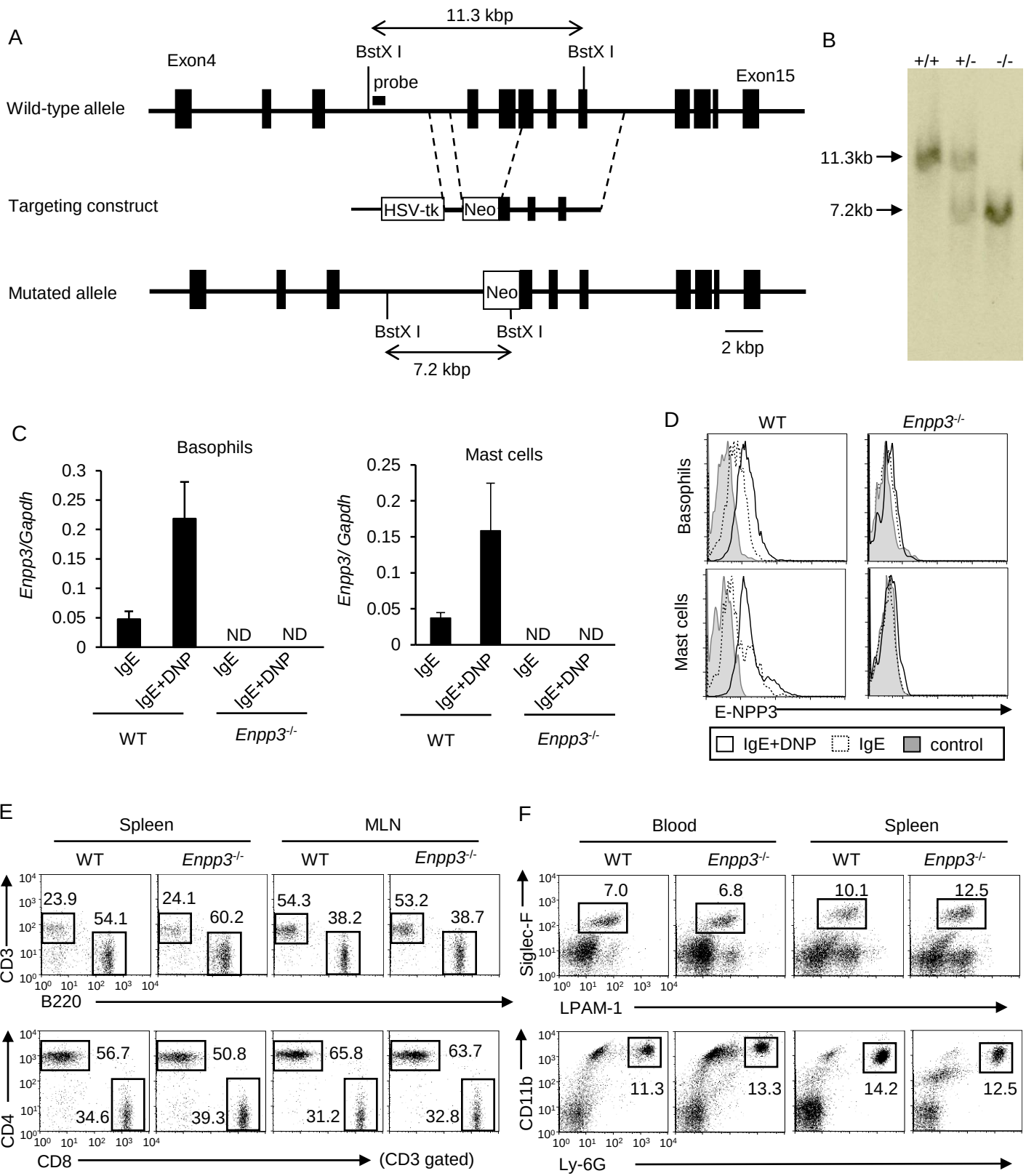


Figure S1

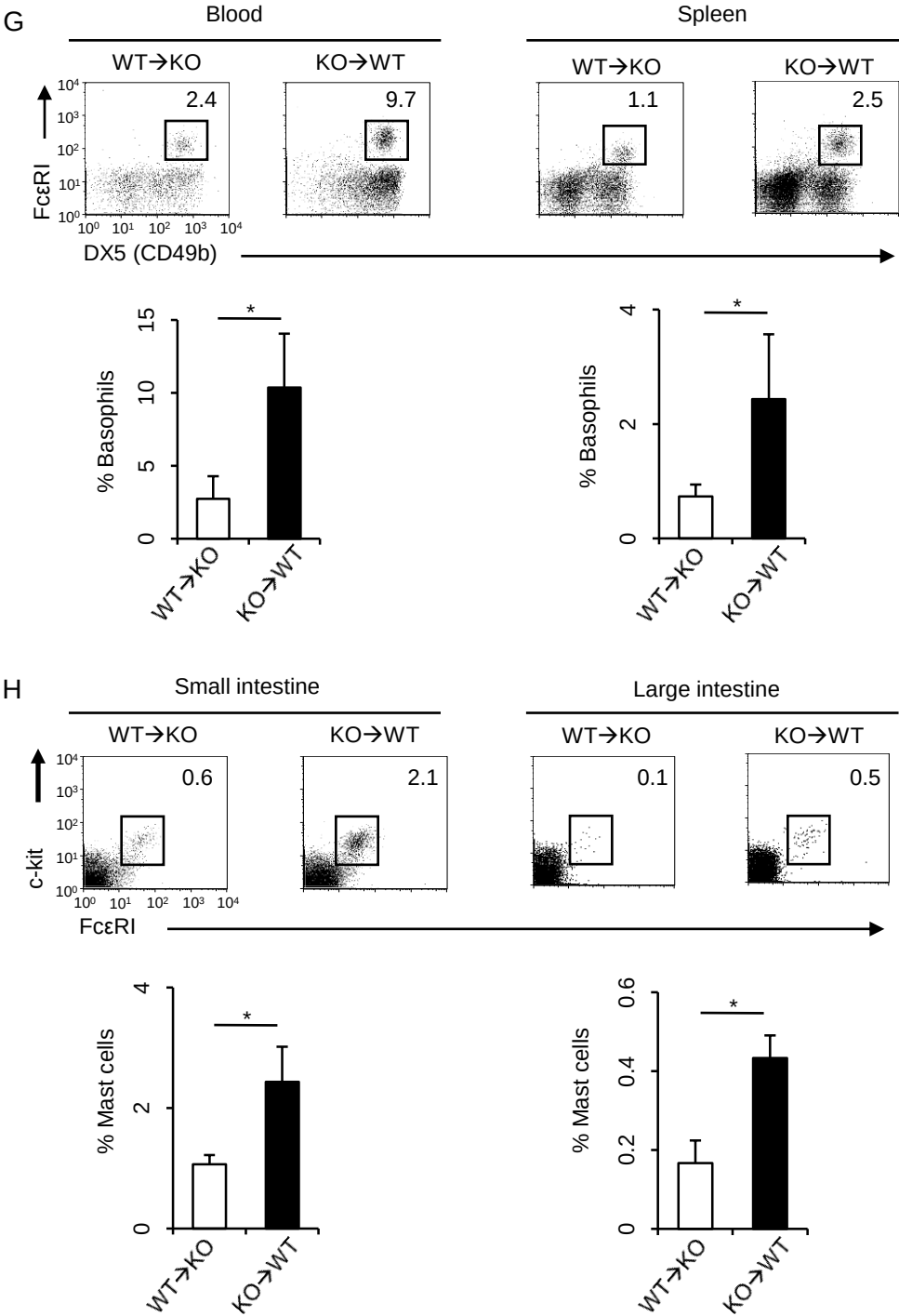


Figure S1, related to Figure 1. Generation of *Enpp3*-deficient mice.

(A) Schematic representation of the *Enpp3* gene targeting strategies. The solid boxes indicate coding regions of the *Enpp3* gene. Neo and HSV-tk indicate a neomycin-resistance gene and a herpes simplex virus thymidine kinase gene, respectively. The positions of the probe and restriction enzyme site for Southern blotting are shown. **(B)** Genomic DNA was isolated from the tails of wild-type (+/+), heterozygous (+/-) and homozygous (-/-) *Enpp3* mutant mice. Southern blot analysis, which was performed after digestion of the genomic DNA with BstXI, shows the correct targeting of the locus. **(C)** Bone marrow-derived basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were stimulated by anti-DNP-IgE and DNP-HSA for 1 h, and analyzed for *Enpp3* mRNA expression by real-time RT-PCR. **(D)** Bone marrow-derived basophils (upper) and mast cells (lower) were stimulated by anti-DNP-IgE and DNP-HSA for 30 min, and analyzed for surface E-NPP3 expression by flow cytometry. Data panels are representative of three independent experiments. **(E)** Frequencies of CD4⁺ and CD8⁺ lymphocytes as well as CD3⁺ and B220⁺ lymphocytes in the spleen (left) and mesenteric lymph node (right) from WT and *Enpp3*^{-/-} mice were examined by flow cytometry. Data are representative of three independent experiments. **(F)** Frequencies of eosinophils (CD3⁻ CD4⁻ CD8⁻ B220⁻ Siglec-F⁺ LPAM-1⁺) and neutrophils (MHC class II⁻ Ly6G⁺ CD11b⁺) in the peripheral blood (left) and spleen (right) from WT and *Enpp3*^{-/-} mice were examined by flow cytometry. Data are representative of three independent experiments. **(G, H)** WT and *Enpp3*^{-/-} (KO) mice (6–7 weeks old) received 8.5 Gy irradiation, and were then intravenously transferred with 5×10^6 bone marrow cells from WT or KO mice in 100 μ l PBS. Eight weeks after bone marrow cell transfer, frequencies of basophils in the peripheral blood and the spleen (G) as well as mast cells in the small and large intestines (H) were determined by flow cytometry. The mean percentages of basophils and mast cells are shown in lower panels (n=5). CD3⁻ CD4⁻ CD8⁻ B220⁻ cells were gated and analyzed for expression of Fc ϵ RI and CD49b (basophils), as well as for Fc ϵ RI and c-kit (mast cells). Data are means $\pm$ SD. *: p<0.05.

Figure S2

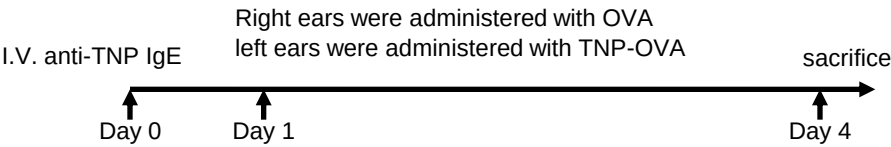
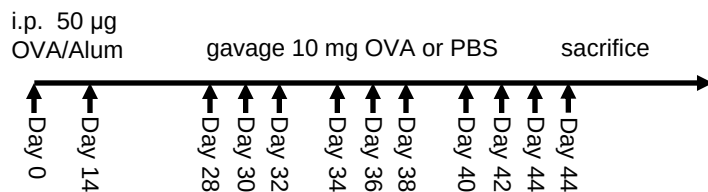


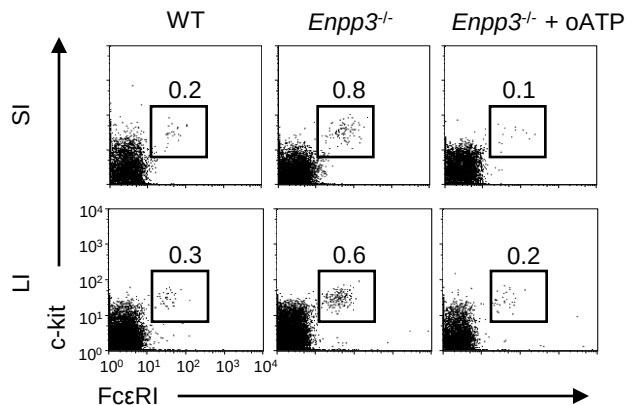
Figure S2, related to Figures 2. Protocol for induction of chronic allergic skin inflammation
Schematic of the protocol to induce IgE-mediated chronic allergic skin inflammation in mice.

Figure S3

A



B



C

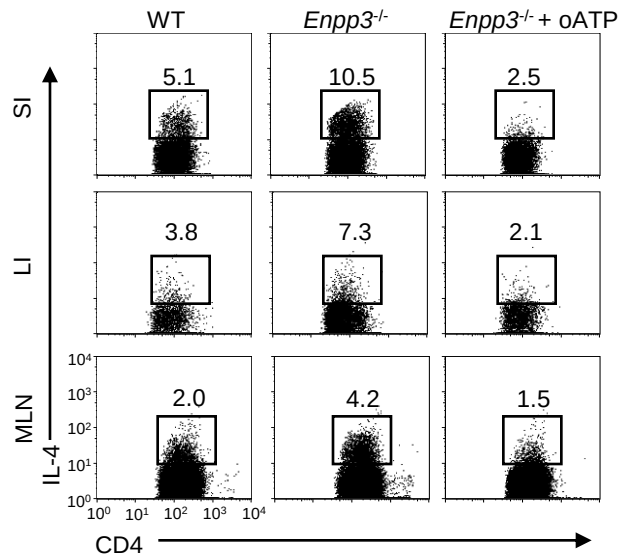


Figure S3, related to Figure 3. Exacerbation of oral allergen-induced intestinal inflammation

(A) Schematic of the protocol to induce experimental oral allergen-induced diarrhea in mice. **(B)** Mast cells frequencies in small (SI) and large intestines (LI) of OVA-challenged WT mice (n=5), *Enpp3*^{-/-} mice with (n=5) or without oATP treatment (n=5) were determined by flow cytometry. Representative dot plots are shown. **(C)** Cells isolated from small intestine (SI), large intestine (LI) and mesenteric lymph nodes (MLN) of WT mice (n=5), *Enpp3*^{-/-} mice (n=5) and oATP-treated *Enpp3*^{-/-} mice (n=5) after 10 times OVA administration, were stained for intracellular IL-4 in CD4⁺ cells. Representative dot plots are shown.

Figure S4

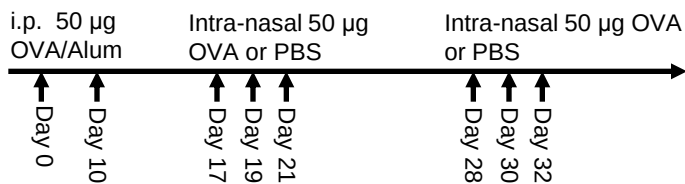


Figure S4 related to Figures 4. Protocol for induction of chronic airway inflammation
Schematic of the protocol to induce experimental chronic asthma in mice.

Figure S5

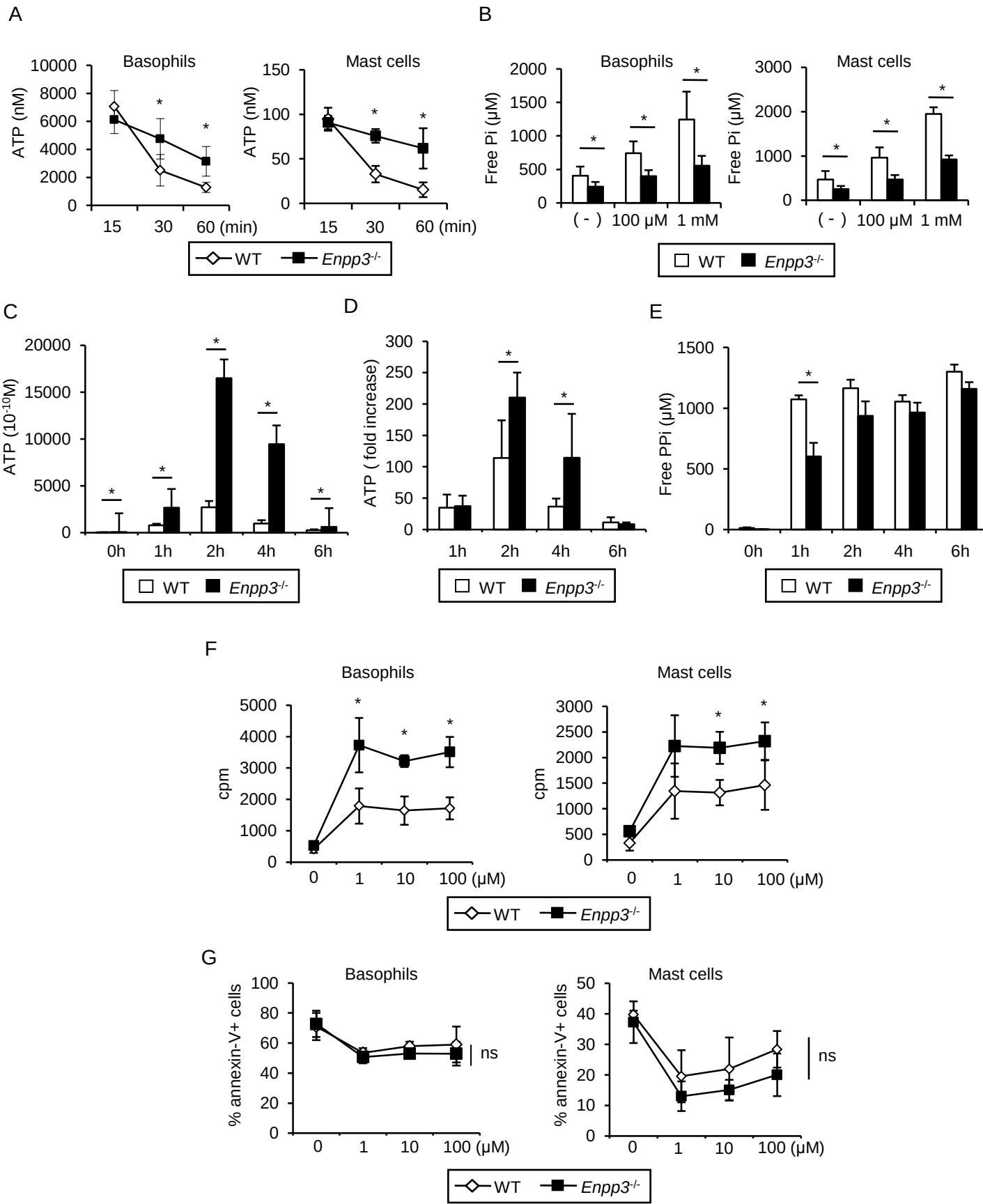


Figure S5, related to Figure 5. Impaired ATP clearance of *Enpp3*^{-/-} mice

(A) Basophils (left) and mast cells (right) of WT and *Enpp3*^{-/-} mice were stimulated with FcεRI crosslinking for the indicated periods, and concentrations of ATP in culture supernatants were measured. Data are means ± SD of five independent experiments *: p<0.05. **(B)** Basophils and mast cells were incubated for 60 min in the presence of ATP (100 μM or 1 mM final concentration). Concentrations, inorganic free phosphate (free Pi) in the supernatants were measured. Data are means ± SD of five independent experiments *: p<0.05. **(C, D, E)** WT and *Enpp3*^{-/-} mice were intravenously injected with 1mM ATP, and sera were collected at the indicated time points. Concentrations of serum ATP were determined by luciferin-luciferase assay (C, D). Concentrations of pyrophosphate (free PPi) in the sera were measured (E). Data are means ± SD of three independent experiments. *: p<0.05. **(F)** Bone marrow-derived basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were cultured in the presence of ATP (1μM, 10μM and 100μM) for 40 h. Thymidine uptake was measured. Data are means ± SD of four independent experiments *: p<0.05. **(G)** Bone marrow-derived basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were cultured in the presence of ATP (1μM, 10μM and 100μM) for 24 h, and percentage of annexin-V-positive cells were determined by flow cytometry. Data are means ± SD of three independent experiments. ns: not significant.

Figure S6

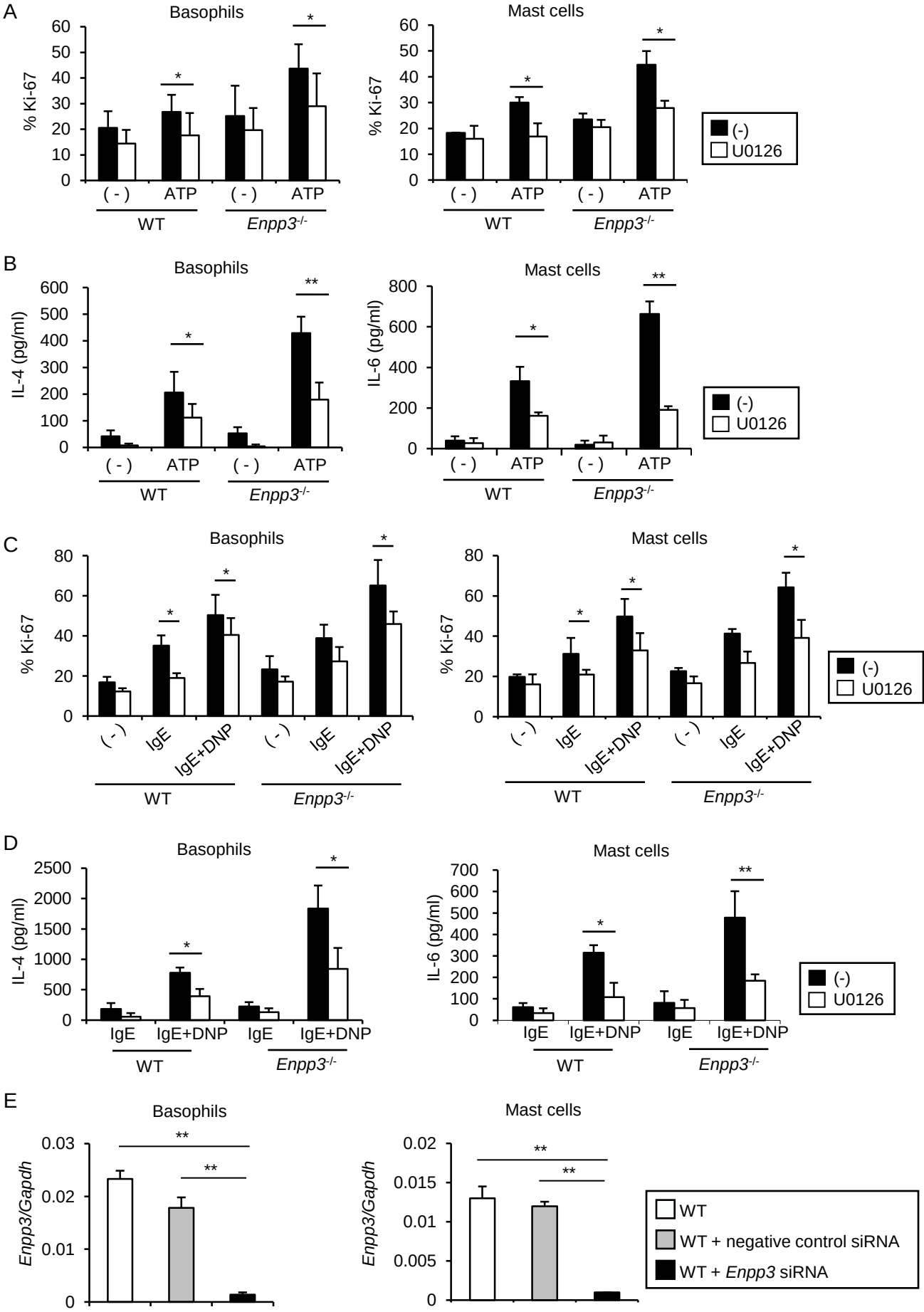


Figure S6, related to Figure 6. ATP-dependent autocrine activation of *Enpp3*^{-/-} basophils and mast cells.

(A, B) Bone marrow-derived basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were pretreated with 20 μ M U0126 for 1 h, and then incubated with 100 μ M ATP for 24 h. The percentage of Ki-67-positive cells was determined by flow cytometry (A), and concentration of IL-4 and IL-6 in culture supernatants was determined by ELISA (B). Data are means $\pm$ SD of three independent experiments *: $p < 0.05$. **(C, D)** Bone marrow-derived basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were pretreated with 20 μ M U0126 for 1 h, and then stimulated by anti-DNP-IgE and DNP-HSA for 24 h. The percentage of Ki-67-positive cells was determined by flow cytometry (C), and concentration of IL-4 and IL-6 in culture supernatants was determined by ELISA (D). Data are means $\pm$ SD of three independent experiments *: $p < 0.05$, **: $p < 0.01$. **(E)** Bone marrow-derived basophils and mast cells from wild-type mice were introduced with *Enpp3*-specific siRNA, control siRNA or vehicle. At 12 h after the transfection, *Enpp3* mRNA expression in each cells was analyzed by real-time RT-PCR. Data are means $\pm$ SD of three independent experiments. **: $p < 0.01$.

Supplemental Experimental Procedures

Mice

P2rx7^{-/-} mice on a BALB/c background were previously described (Kurashima et al., 2012), and were crossed with *Enpp3^{-/-}* mice to generate *P2rx7^{-/-} Enpp3^{-/-}* mice. BALB/c and C57BL/6 mice were purchased from CLEA. All animal experiments were conducted with approval of the Animal Research Committee of the Graduate School of Medicine at Osaka University and the Animal Care and Use Committee, The University of Tokyo.

Generation of Enpp3-deficient mice

The targeting vector was constructed by replacing of a 2.8-kb fragment encoding the seventh and eighth exons of *Enpp3* with a neomycin resistance gene cassette, and HSV thymidine kinase gene driven by a phosphoglycerate kinase promoter for negative selection was inserted into the genomic fragment. Before the double-resistant colonies of G418 and ganciclovir were selected and screened by PCR and Southern blot analysis, V6.5 embryonic stem cells were transfected with a targeting vector. Blastocysts of C57BL/6 females were used for microinjection of homologous recombinants and for the generation of *Enpp3*-deficient mice, heterozygous F1 mice were intercrossed. After intercrossing, *Enpp3*-deficient mice and their wild-type littermates were confirmed by Southern blot analysis. *Enpp3*-deficient mice were backcrossed onto BALB/c mice or C57BL/6 for at least four generations, and *Enpp3*-deficient mice and their wild-type littermates from intercrosses of heterozygous mice were used for experiments. All animal experiments were conducted with approval from the Animal Research Committee of the Graduate School of Medicine at Osaka University.

Reagents

The following antibodies were purchased from BD Biosciences: anti-CD16/32 (2.4G2),

anti-CD11b-PE/Cy7 (M1/70), anti-Siglec-F-PE (E50-2440), anti-IL-4-PE (11B11), anti-CD3 ϵ -FITC (145-2C11), anti-CD3 ϵ -PE/Cy7(145-2C11), anti-Ki-67-PE (B56), and anti-Ly6G-PE (1A8). The following antibodies were purchased from Biolegend: anti-CD49b-PE (DX5), anti-Fc ϵ RI α -APC (MAR-1), anti-c-kit-PE/Cy7 (2B8), anti-LPAM-1(α 4 β 7)-APC (DATK32), anti-CD8a-FITC (53-6.7), anti-CD8a-APC (53-6.7), anti-B220-FITC (RA3-6B2), anti-IL-17A-APC (TC11-18H10.1), anti-CD45.2-Pacific blue (104), anti-CD4-PerCP/Cy5.5 (GK1.5), anti-CD4-FITC (GK1.5), anti-CD4-PE (RM4-5). Anti-MHC class II-FITC (M5/114.15.2) was purchased from eBioscience. Anti-CD45.1-PerCP/Cy5.5 (A20) was purchased from Tonbo biosciences and anti-CD203c-FITC (orb15186) was from Biorbyt. RPMI 1640 medium (Nacalai Tesque) supplemented with 10% fetal bovine serum (Nichirei Bioscience), 100 U/ml penicillin (Gibco), 100 μ g/ml streptomycin (Gibco) and 50 μ M 2-mercaptoethanol (Gibco) was used for experiments as complete medium. Phosphate buffered saline (PBS) and Hank's Balanced Salt Solution (HBSS) were purchased from Nacalai Tesque. Adenosine 5'-triphosphate (ATP) disodium salt hydrate (A1852), adenosine 5'-(γ -thio)triphosphate tetralithium salt (ATP γ S; A1388), and oxidized ATP (oATP; ATP periodate oxidized sodium salt) were purchased from Sigma-Aldrich. IGELb4 B cell hybridoma was previously described (Mukai et al., 2005).

Generation of bone marrow-derived basophils and mast cells

Bone marrow cells from wild-type and *Enpp3*^{-/-} mice were cultured for 14 days with 5 ng/ml recombinant murine IL-3 (PeproTech). After 14 days of culture, bone marrow-derived basophils (Fc ϵ RI⁺ c-kit⁻ cells) and mast cells (Fc ϵ RI⁺ c-kit⁺ cells) were enriched by sorting with FACSaria (BD Biosciences) as previously described (Yoshimoto and Nakanishi, 2012). After FACS sorting, bone marrow-derived basophils and mast cells were cultured with complete medium in the absence of IL-3 for 16 h, and used for experiments.

Real-time RT-PCR

RNA samples were isolated from bone marrow-derived basophils and mast cells using TRIzol reagent (Invitrogen). cDNA was generated from total RNA by treatment with RQ1 DNase I (Promega) before performing reverse transcription with random primers (Toyobo) and Moloney murine leukemia virus reverse transcriptase (Promega). Real-time RT-PCR was performed by cDNA PCR in an ABI 7300 Real-time PCR system (Applied Biosystems) with GoTaq qPCR Master Mix (Promega). The following primer sets were used: *Enpp3*: 5'-CTCATGCCCTGCACTACAGA-3' and 3'-TAGCCTTTGGTTTGCTTGCT-5', *Gapdh*: 5'-CCTCGTCCCGTAGACAAAATG-3' and 5'-TCTCCACTTTGCCACTGCAA-3'. Final values shown are relative expressions after normalizing to the expression of *Gapdh*.

Cell isolation

The spleen and mesenteric lymph nodes were ground with glass slides, and the cells were passed through a 40- μ m mesh filter to obtain single-cell suspensions. Cells from the spleen and peripheral blood were treated with RBC lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA) to deplete red blood cells. For isolation of lamina propria cells, intestines were opened, washed to remove fecal content, shaken in HBSS containing 5 mM EDTA for 20 min at 37°C to remove epithelial cells and fat tissue, cut into small pieces and incubated with RPMI 1640 containing 4% FBS, 1 mg/ml collagenase D (Roche), 0.5 mg/ml dispase (Invitrogen) and 40 μ g/ml DNase I (Roche) for 1 h at 37°C in a shaking water bath. The digested tissues were washed with HBSS containing 5 mM EDTA and resuspended with 5 ml 40% Percoll (GE Healthcare), and then 80% Percoll was added to the bottom of tube to form the 80% (lower)/40% (upper) Percoll layers. After Percoll density gradient centrifugation, the lamina propria lymphocytes and granulocytes were collected at the Percoll gradient interface.

Flow cytometry

Cells were pre-incubated with 2.5 µg/ml anti-CD16/32 (BD Biosciences) for 5 min followed by staining with the indicated antibodies. All data were acquired using FACSCanto II (BD Biosciences). To investigate expression of intracellular IL-4 in CD4⁺ T cells, cells were incubated with 50 ng/ml phorbol myristate acetate (Sigma-Aldrich), 5 mM calcium ionophore (A23187; Sigma-Aldrich), and GolgiStop (BD Biosciences) at 37°C for 4 h. Then, cells were stained with anti-CD4-PerCP/Cy5.5 (BioLegend) at 4°C for 20 min, washed, fixed and permeabilized with the Cytotfix/Cytoperm Kit plus (BD Bioscience), and then stained with anti-IL-4-PE (BD Biosciences) at 4°C for 20 min. For nuclear Ki-67 staining, cells were fixed and permeabilized using diluted Fixation/Permeabilization Concentrate (eBiosciences), and then stained with anti-Ki-67-PE (BD Biosciences) for 30 min at 4°C.

Bone marrow chimera mice

Wild-type and *Enpp3*^{-/-} mice (6–7 weeks old) received 8.5 Gy irradiation and then irradiated mice were administrated 5×10^6 bone marrow cells from normal wild-type and *Enpp3*^{-/-} mice in 100 µl PBS. At 8 weeks after bone marrow cell transfer, frequencies of basophils and mast cells were determined by flow cytometry.

Measurement of IgE, IL-4, IL-6 and extracellular ATP

Concentration of IgE, IL-4 and IL-6 was measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience). To measure the levels of extracellular ATP *in vivo*, serum was harvested from peripheral blood after centrifugation, and ATP levels were measured by a luciferin-luciferase ATP assay (Toyo Ink). For *in vitro* experiments, culture supernatants were harvested after incubation of cells with Tyrode's buffer (140 mM NaCl, 4 mM KCl, 0.2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM

D-glucose) alone or with ATP (100 μ M and 1 mM) for 1 h, and ATP levels were measured by a luciferin-luciferase assay (Toyo Ink).

In vivo ATP hydrolyzing activity

Wild-type and *Enpp3*^{-/-} mice were intravenously injected with 1 mM ATP in 100 μ l PBS. Sera were collected at the indicated periods. Levels of ATP and free inorganic pyrophosphate were measured by a luciferin-luciferase ATP assay (Toyo Ink) and Pyrophosphate Assay Kit (Fluorometric; Abcam), respectively.

Western blot analysis

Bone marrow-derived basophils and mast cells were lysed with RIPA lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 μ g/ml leupeptin) with protease inhibitor cocktail (Roche). The cell lysates were separated by SDS-PAGE and then transferred to polyvinyl difluoride (PVDF; Millipore) membranes. The transferred membranes were blocked with 5% skim milk (BD Biosciences) in 0.1% Tween-20 containing Tris-buffered saline (TBST) for 1 h, and then hybridized with anti-Phospho-p44/42 MAPK (9101; Cell Signaling Technology) or anti-ERK1 (K-23; Santa Cruz Biotechnology) for 2 h at room temperature. After three times washing with TBST, membranes were incubated with hydroperoxidase conjugated donkey anti-rabbit IgG (GE healthcare) for 1 h at room temperature. The enhanced chemiluminescence (SuperSignal; Thermo scientific) method was used for immunodetection and images were captured by a Luminescent Image Analyzer LAS-1000 (Fuji Film) image system.

Apoptosis assay

Bone marrow-derived basophils and mast cells from wild-type and *Enpp3*^{-/-} mice were

incubated with ATP (1 μ M, 10 μ M and 100 μ M) for 24h, and then stained with annexin-V (Annexin-V-fluos staining kit; Roche). The percentages of annexin-V-positive cells were determined by flow cytometry.

Proliferation assay

Bone marrow-derived basophils and mast cells from wild-type and *Enpp3*^{-/-} mice were cultured with ATP (1 μ M, 10 μ M and 100 μ M) for 40h. [³H]-thymidine (1 μ Ci) was added to the culture in the last 16 h. The radioactivity was measured by a 1450 microbeta scintillation counter (Wallac). Data are shown in counts per min (CPM).

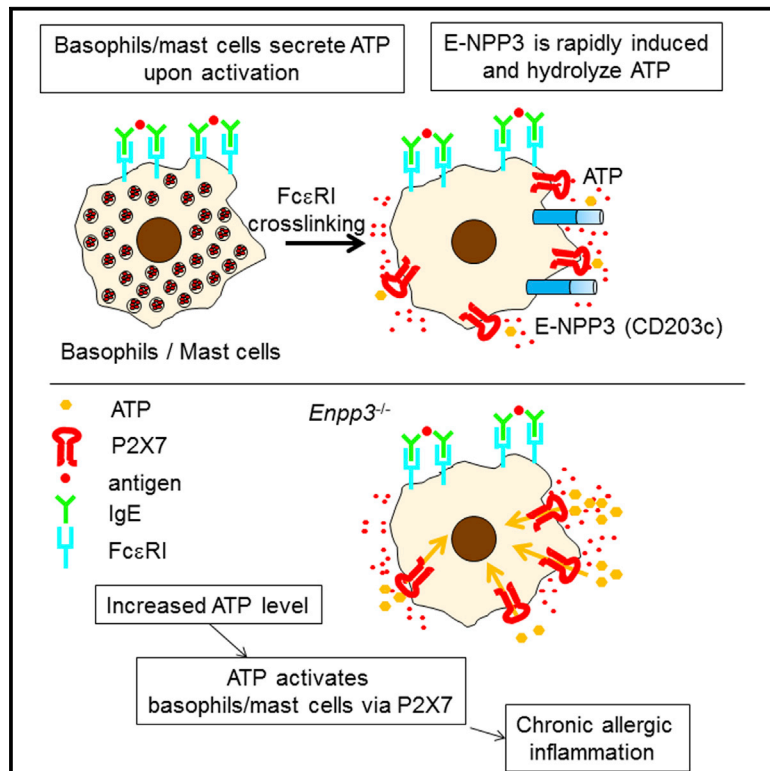
Supplemental References

Yoshimoto, T., and Nakanishi, K. (2012). Generation and characterization of mouse basophils from bone marrow and purification of basophils from spleen. Current protocols in immunology / edited by John E. Coligan ... [et al.] *Chapter 3*, Unit 3 24.

Immunity

The Ectoenzyme E-NPP3 Negatively Regulates ATP-Dependent Chronic Allergic Responses by Basophils and Mast Cells

Graphical Abstract



Authors

Shih Han Tsai, Makoto Kinoshita, ..., Hajime Karasuyama, Kiyoshi Takeda

Correspondence

ktakeda@ongene.med.osaka-u.ac.jp

In Brief

The mechanisms for regulation of chronic allergic responses remain unknown. Takeda and colleagues show that ATP secreted by basophils and mast cells acts on the cells in an autocrine manner. E-NPP3 is rapidly expressed on basophils and mast cells and hydrolyzes ATP to prevent the ATP-dependent chronic allergic inflammation.

Highlights

- E-NPP3 (CD203c) is rapidly induced on basophils and mast cells to hydrolyze ATP
- Activated basophils and mast cells secrete ATP
- ATP acts on basophils and mast cells to induce chronic allergic responses
- E-NPP3 is responsible for suppression of ATP-dependent allergic inflammation



The Ectoenzyme E-NPP3 Negatively Regulates ATP-Dependent Chronic Allergic Responses by Basophils and Mast Cells

Shih Han Tsai,^{1,2} Makoto Kinoshita,^{1,2} Takashi Kusu,^{1,2} Hisako Kayama,^{1,2} Ryu Okumura,^{1,2} Kayo Ikeda,^{1,2} Yosuke Shimada,^{1,2} Akira Takeda,¹ Soichiro Yoshikawa,³ Kazushige Obata-Ninomiya,³ Yosuke Kurashima,^{2,4} Shintaro Sato,^{2,4} Eiji Umemoto,^{1,2} Hiroshi Kiyono,^{2,4} Hajime Karasuyama,^{2,3} and Kiyoshi Takeda^{1,2,*}

¹Laboratory of Immune Regulation, Department of Microbiology and Immunology, Graduate School of Medicine, WPI Immunology Frontier Research Center, Osaka University, Suita, Osaka 565-0871, Japan

²Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan

³Department of Immune Regulation, Tokyo Medical and Dental University Graduate School, Tokyo 113-8519, Japan

⁴Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

*Correspondence: ktakeda@ongene.med.osaka-u.ac.jp

<http://dx.doi.org/10.1016/j.immuni.2015.01.015>

SUMMARY

Crosslinking of the immunoglobulin receptor FcεRI activates basophils and mast cells to induce immediate and chronic allergic inflammation. However, it remains unclear how the chronic allergic inflammation is regulated. Here, we showed that ecto-nucleotide pyrophosphatase-phosphodiesterase 3 (E-NPP3), also known as CD203c, rapidly induced by FcεRI crosslinking, negatively regulated chronic allergic inflammation. Basophil and mast cell numbers increased in *Enpp3*^{−/−} mice with augmented serum ATP concentrations. *Enpp3*^{−/−} mice were highly sensitive to chronic allergic pathologies, which was reduced by ATP blockade. FcεRI crosslinking induced ATP secretion from basophils and mast cells, and ATP activated both cells. ATP clearance was impaired in *Enpp3*^{−/−} cells. *Enpp3*^{−/−}*P2rx7*^{−/−} mice showed decreased responses to FcεRI crosslinking. Thus, ATP released by FcεRI crosslinking stimulates basophils and mast cells for further activation causing allergic inflammation. E-NPP3 decreases ATP concentration and suppresses basophil and mast cell activity.

INTRODUCTION

Basophils and mast cells are distinct cell lineages, but they share some phenotypic and functional properties (Karasuyama et al., 2011; Voehringer, 2013). For example, both basophils and mast cells are activated upon crosslinking of FcεRI with antigen-bound IgE to mediate type 2 immune responses against pathogens such as helminths and ticks (Abraham and St John, 2010; Karasuyama et al., 2011). In addition, these two cell types induce pathologic responses to allergens, composed of immediate and chronic responses.

CD203c was identified as a highly expressed molecule on activated basophils (Bühning et al., 2001) and is now considered a good biomarker candidate for the diagnosis of allergic diseases (Bühning et al., 2004). CD203c is a member of the ecto-nucleotide pyrophosphatase-phosphodiesterase (E-NPP) family consisting of seven structurally related molecules that hydrolyze pyrophosphate and phosphodiester bonds in various substrates (Schetinger et al., 2007; Stefan et al., 2005; Yegutkin, 2008). Among seven family members, E-NPP1, E-NPP2 (also called autotaxin), and E-NPP3 (CD203c) are closely related in structure and hydrolyze nucleotides (Bollen et al., 2000). E-NPP1 was shown to generate pyrophosphate through NTP hydrolysis and thereby inhibit bone mineralization (Hessle et al., 2002; Okawa et al., 1998). E-NPP2 acts as lysophospholipase-D, which hydrolyzes lysophosphatidylcholine into lysophosphatidic acid, thereby mediating several biologic responses such as lymphocyte transmigration and vascular development (Kanda et al., 2008; Umemoto et al., 2011; van Meeteren et al., 2006). In contrast to E-NPP1 and E-NPP2, whose functions are well characterized, the function of E-NPP3 (CD203c) remains undetermined despite its high expression in activated basophils.

Extracellular adenosine 5'-triphosphate (ATP) is released during inflammation and activates immune cells (Idzko et al., 2014; Junger, 2011). Several lines of evidence demonstrated that extracellular ATP activates mast cells via the purinoreceptor P2X7 (Kurashima et al., 2012, 2014; Sudo et al., 1996). In addition, several types of cells including mast cells release ATP upon stimulation (Junger, 2011; Wang et al., 2013). Thus, extracellular ATP modulates mast cell activity in an autocrine manner. However, it remains unclear how ATP-dependent mast cell responses are regulated. In addition, it should also be determined whether extracellular ATP acts on basophils, which share several immunological functions with mast cells.

In this study, we analyzed the physiological function of E-NPP3 (CD203c), which was highly expressed in activated basophils and mast cells. In *Enpp3*^{−/−} mice, the activity of basophils and mast cells was markedly enhanced with increased ATP concentrations. Accordingly, *Enpp3*^{−/−} mice were susceptible

to chronic allergic pathologies mediated by basophils and mast cells. The crosslinking of FcεRI by IgE induced the secretion of ATP from basophils and mast cells, and ATP clearance activity was impaired in *Enpp3*^{-/-} basophils and mast cells. Extracellular ATP and FcεRI crosslinking strongly activated *Enpp3*^{-/-} basophils and mast cells, which was reduced by the addition of P2X7 antagonists or introducing *P2rx7* deficiency. Non-hydrolyzable ATP activated basophils and mast cells from wild-type mice to similar levels observed in *Enpp3*^{-/-} cells. These findings demonstrate that extracellular ATP induced by FcεRI crosslinking is a potent activator of basophils and mast cells that mediate the chronic phases of allergic responses and that E-NPP3 modulates ATP-mediated allergic responses.

RESULTS

Increased Numbers of Basophils and Mast Cells in *Enpp3*^{-/-} Mice

Activated human basophil were reported to express high amounts of E-NPP3 (CD203c), encoded by *Enpp3* (Bühning et al., 2001, 2004). Surface expression of E-NPP3 was rapidly induced on mouse bone-marrow-derived basophils sensitized with anti-dinitrophenyl (DNP) IgE and then stimulated with DNP-human serum albumin (HSA) for 30 min (Figure 1A). Similarly, bone-marrow-derived mast cells showed enhanced expression of surface E-NPP3 after 30 min of crosslinking of FcεRI by DNP-specific IgE (Figure 1B).

To analyze the in vivo function of E-NPP3, we generated mice lacking *Enpp3* by gene targeting (Figures S1A and S1B). The targeted disruption of *Enpp3* was confirmed by mRNA and protein assay (Figures S1C and S1D). *Enpp3*^{-/-} mice were born and grew healthily with no alteration in the numbers of peripheral lymphocytes, eosinophils, and neutrophils (Figures S1E and S1F) or CD49b⁺FcεRI⁺ basophils in bone marrow (Figure 1C). However, frequencies and numbers of CD49b⁺FcεRI⁺ basophils were markedly increased in the peripheral blood and spleen of *Enpp3*^{-/-} mice compared with wild-type mice (Figure 1D). In addition, increased frequencies and numbers of c-kit⁺FcεRI⁺ mast cells were observed in the small and large intestines of *Enpp3*^{-/-} mice (Figure 1E). In parallel with the increased number of basophils and mast cells, serum IgE concentration was increased in *Enpp3*^{-/-} mice (Figure 1F). To determine whether the increased number of basophils and mast cells was caused by abnormalities in hematopoietic or non-hematopoietic cells, we generated bone marrow chimeric mice. In wild-type mice transferred with *Enpp3*^{-/-} bone marrow cells, both basophil and mast cell populations increased, but not in *Enpp3*^{-/-} mice transferred with wild-type bone marrow cells, indicating that ENPP-3 deficiency affected the hematopoietic cell compartment leading to the increased number of basophils and mast cells (Figures S1G and S1H). Thus, *Enpp3*^{-/-} mice showed the increased number of basophils and mast cells in the periphery.

Enhanced Chronic Allergic Inflammation in *Enpp3*^{-/-} Mice

Mast cells are critical mediators of immediate hypersensitivity reactions. Accordingly, we first examined the sensitivity of *Enpp3*^{-/-} mice to passive cutaneous anaphylaxis (Obata et al.,

2007). Mice were sensitized with trinitrophenyl (TNP)-specific IgE intradermally followed by intravenous challenge with TNP-ovalbumin (OVA) and Evans blue dye (Figure 2A). Both wild-type and *Enpp3*^{-/-} mice showed similar extravasation of the dye, indicating that immediate allergic response remains unaltered in the absence of *Enpp3*.

Basophils mediate chronic allergic inflammation in ear skin (Mukai et al., 2005). Therefore, we analyzed the sensitivity of *Enpp3*^{-/-} mice to basophil-dependent allergic inflammation. Mice were sensitized with TNP-specific IgE and then intradermally challenged with TNP-OVA (Figure S2). BALB/c background wild-type mice exhibited ear swelling (Figures 2B and 2C) accompanied by the increased infiltration of basophils, eosinophils, and neutrophils at 4 days after antigen challenge (Figures 2D and 2E). *Enpp3*^{-/-} mice showed more severe ear swelling with increased numbers of infiltrating basophils, eosinophils, and neutrophils compared with wild-type mice. Thus, the basophil-dependent chronic allergic inflammation was exacerbated in *Enpp3*^{-/-} mice.

We next analyzed the sensitivity of *Enpp3*^{-/-} mice to allergen-induced diarrhea (Brandt et al., 2003). Mice were sensitized with OVA+alum twice then orally challenged with OVA every other day, and their weight was monitored (Figure S3A). Compared with wild-type mice, *Enpp3*^{-/-} mice suffered from greater weight loss (Figure 3A). After ten times of OVA challenge, we analyzed the colon by histopathology (Figures 3B and 3C). In *Enpp3*^{-/-} mice, the colon length was shortened and more severe intestinal pathology was observed. Infiltration of mast cells into the intestine was increased in *Enpp3*^{-/-} mice (Figure 3D). Flow cytometric analysis also indicated that mast cell populations in the small and large intestines were increased in OVA-sensitized *Enpp3*^{-/-} mice compared to controls (Figures 3E and S3B). We measured serum concentrations of ATP in mice challenged with OVA (Figure 3F). Serum ATP concentration was significantly elevated in *Enpp3*^{-/-} mice compared with wild-type mice. In OVA-challenged *Enpp3*^{-/-} mice, IL-4 production from CD4⁺ T cells in the small intestine, large intestine, and mesenteric lymph nodes was increased (Figures 3G and S3C). In addition, serum IgE concentrations were augmented in OVA-challenged *Enpp3*^{-/-} mice (Figure 3H), indicating enhanced Th2-cell-type responses in OVA-sensitized *Enpp3*^{-/-} mice. Thus, *Enpp3*^{-/-} mice developed severe allergic intestinal inflammation with a dramatic increase in mast cell populations. Because serum ATP concentrations were elevated in *Enpp3*^{-/-} mice, we analyzed whether severe allergic inflammation in *Enpp3*^{-/-} mice was caused by elevated ATP levels by treating mice with oxidized ATP (oATP), which blocks ATP actions by antagonizing the ATP sensor P2X7 (Figures 3E, 3G, and 3H). Treatment with oATP substantially reduced the number of mast cells and IL-4-producing CD4⁺ T cells as well as serum IgE concentrations in OVA-sensitized *Enpp3*^{-/-} mice. These findings indicate that the severe allergic inflammation observed in *Enpp3*^{-/-} mice was attributed to increased ATP concentrations.

We also investigated a chronic asthma model. Mice were sensitized with OVA+alum twice and then intranasally challenged with OVA six times (Figure S4). *Enpp3*^{-/-} mice were highly sensitive to OVA-induced inflammation and almost all mutant mice died after OVA challenge (Figure 4A). Histopathological

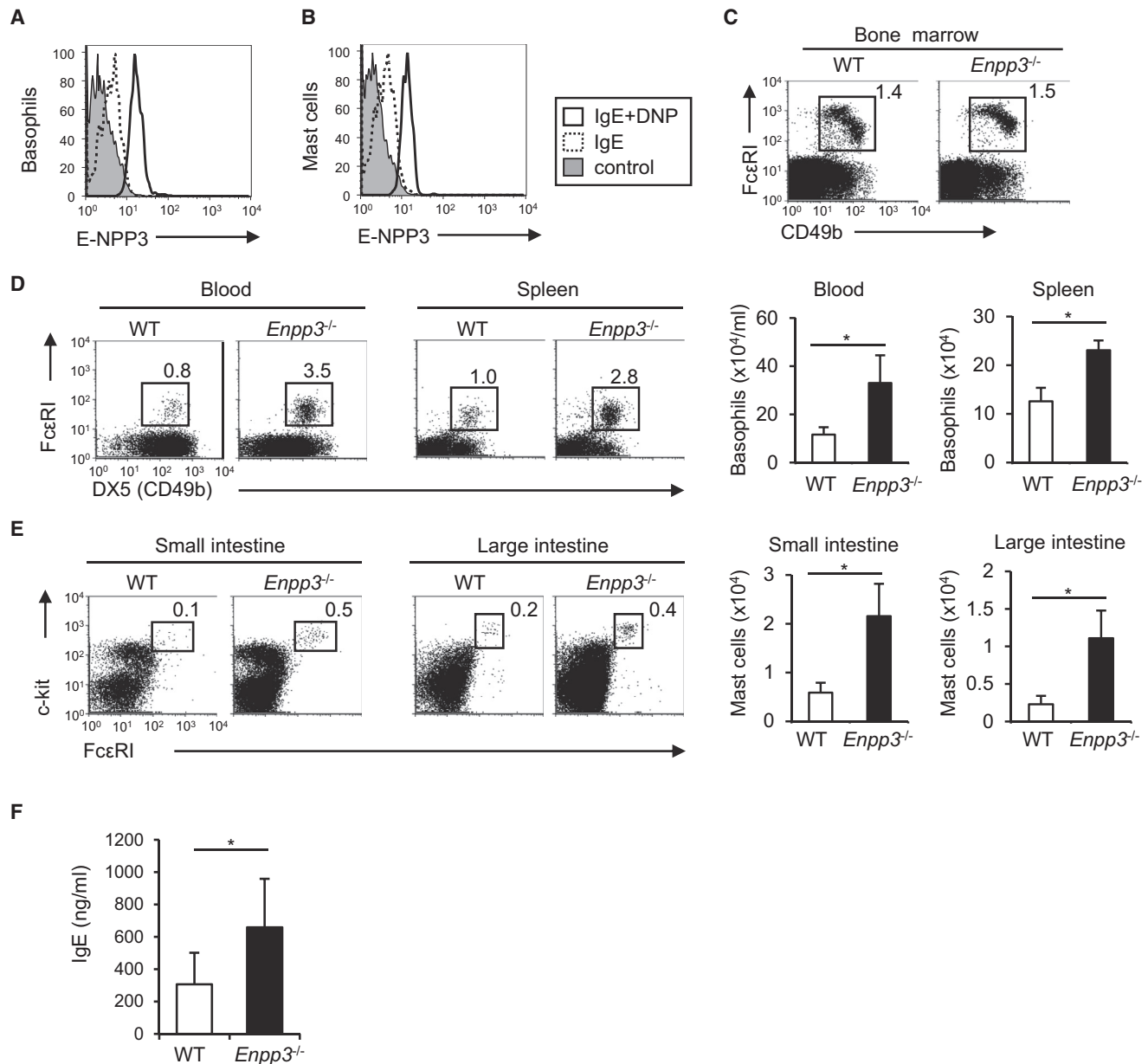


Figure 1. Increased Numbers of Peripheral Basophils and Mast Cells in *Enpp3*^{-/-} Mice

(A and B) Bone-marrow-derived basophils (A) and mast cells (B) were stimulated by FcεRI crosslinking using anti-DNP-IgE and DNP-HSA for 30 min and analyzed for surface E-NPP3 expression by flow cytometry. Solid line, IgE+DNP; dashed line, IgE; gray histogram, non-stimulated control. Data are representative of five independent experiments.

(C and D) Frequencies of basophils in bone marrow (C) and the peripheral blood and spleen (D) from wild-type (WT) and *Enpp3*^{-/-} mice were determined by flow cytometry. Live CD3⁺CD4⁺CD8⁺B220⁺ cells were gated and analyzed for expression of FcεRI and CD49b. Representative dot plots (left) and the means ± SD of total numbers of FcεRI⁺CD49b⁺ cells from five mice (right) are shown. *p < 0.05.

(E) Frequencies of mast cells in the small and large intestines were determined by flow cytometry. Live CD3⁺CD4⁺CD8⁺B220⁺ cells were gated and analyzed for expression of FcεRI and c-kit. Representative dot plots (left) and the means ± SD of total numbers of FcεRI⁺c-kit⁺ cells from five mice (right) are shown. *p < 0.05.

(F) Concentration of serum IgE was measured. Data are means ± SD (n = 5). *p < 0.05.

See also Figure S1.

analysis of the lung revealed infiltration of increased number of inflammatory cells and enhanced mucus production in OVA-challenged *Enpp3*^{-/-} mice (Figures 4B and 4C). Basophil populations in the peripheral blood and the lung increased and serum IgE concentrations were augmented in *Enpp3*^{-/-} mice (Figures

4D and 4E). Serum ATP concentrations were also elevated in *Enpp3*^{-/-} mice challenged with OVA (Figure 4F). oATP treatment ameliorated OVA-induced inflammation and reduced basophil populations and serum IgE levels in *Enpp3*^{-/-} mice (Figures 4A, 4D, and 4E). Taken together, these findings indicate that

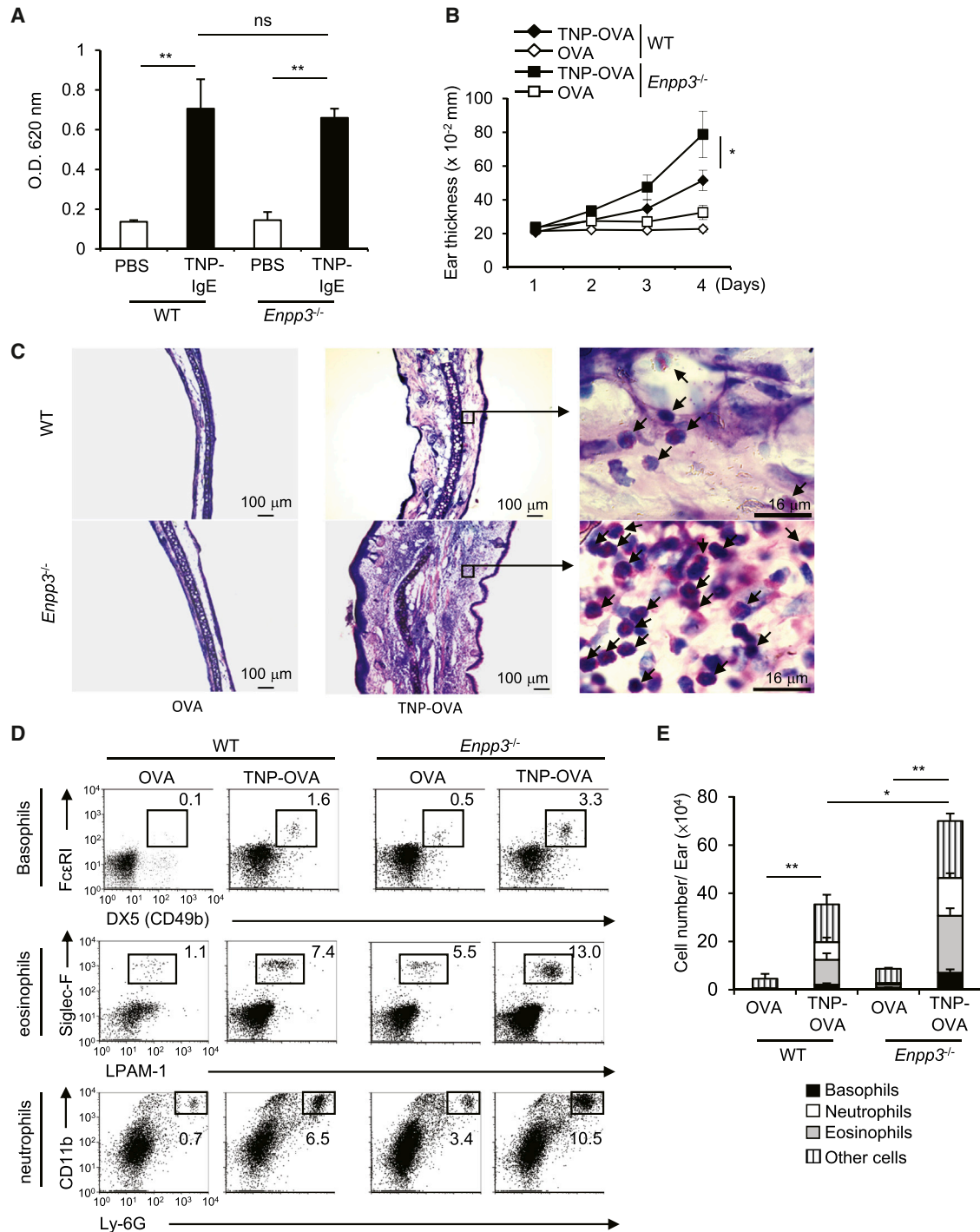


Figure 2. The *Enpp3* Deficiency Exacerbates Basophil-Dependent Allergic Inflammation

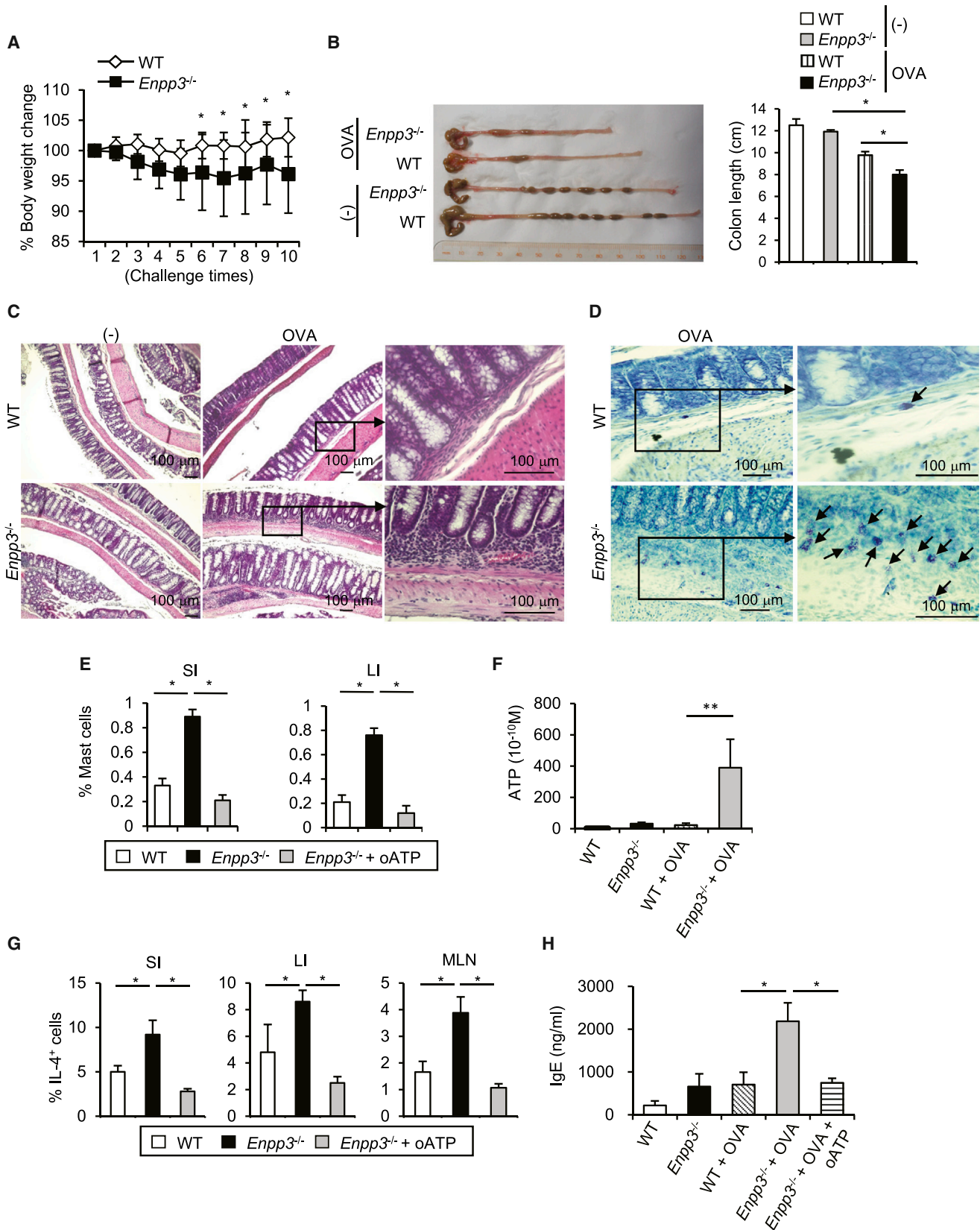
(A) Anti-TNP IgE-sensitized WT and *Enpp3*^{-/-} mice were intravenously challenged with TNP-OVA and Evans blue. At 30 min after the challenge, extravasated dyes in the ear skin were measured by spectrophotometry. Data are shown as means $\pm$ SD (n = 5). **p < 0.01, ns: not significant.

(B) Thickness of ears from anti-TNP IgE-sensitized WT and *Enpp3*^{-/-} mice were measured daily after subcutaneous challenge with TNP-OVA or OVA. Data are shown as means $\pm$ SD (n = 7). *p < 0.05.

(C) Ear specimens from WT and *Enpp3*^{-/-} mice were histologically analyzed with Giemsa staining 4 days after antigen challenge. Black arrows indicate infiltrated eosinophils containing red-colored granules. Data are representative of five mice examined.

(D and E) Frequencies (D) and numbers (E) of basophils (CD3⁺CD4⁺CD8⁺B220⁺FcεRI⁺CD49b⁺), eosinophils (CD3⁺CD4⁺CD8⁺B220⁺Siglec-F⁺LPAM-1⁺), and neutrophils (MHC class II⁺Ly6G⁺CD11b⁺) were determined by flow cytometry and cell counting with trypan blue. Data in (E) are means $\pm$ SD (n = 5). *p < 0.05, **p < 0.01.

See also Figure S2.



(legend on next page)

E-NPP3 is required for the suppression of chronic allergic inflammation by the downregulation of ATP concentration.

Defective ATP Clearance of *Enpp3*^{-/-} Basophils and Mast Cells

Crosslinking of FcεRI by DNP-specific IgE induced the production of cytokines such as IL-4 and IL-6 from basophils and mast cells, respectively (Figure 5A). IgE-dependent cytokine production was enhanced in *Enpp3*^{-/-} cells. Activated mast cells show increased Ki-67 expression (Abraham and St John, 2010; Bischoff et al., 1999). Therefore, we analyzed Ki-67 expression in basophils and mast cells stimulated with FcεRI crosslinking (Figure 5B). The FcεRI crosslinking-dependent increase in Ki-67 staining was enhanced in *Enpp3*^{-/-} mast cells and basophils. Thus, *Enpp3*^{-/-} basophils and mast cells were highly activated.

Several cell types secrete ATP upon stimulation (Corriden and Insel, 2010; Junger, 2011). Therefore, basophils and mast cells were stimulated with DNP-specific IgE for 1 hr and analyzed for ATP release (Figure 5C). FcεRI crosslinking of wild-type basophils and mast cells induced the release of substantial amounts of ATP, and ATP concentrations in supernatants of stimulated *Enpp3*^{-/-} cells were markedly increased compared with that in wild-type cells. We then analyzed whether increased ATP concentrations in stimulated *Enpp3*^{-/-} cells was due to defective ATP clearance. Basophils and mast cells were stimulated by FcεRI crosslinking for the indicated periods and ATP concentrations in supernatants were measured (Figure S5A). At 15 min after stimulation, ATP concentrations were comparable between wild-type and *Enpp3*^{-/-} cells, indicating that FcεRI-dependent ATP release at the early phase of stimulation was not affected in *Enpp3*^{-/-} cells. However, at 30 and 60 min after stimulation, ATP concentrations were increased in *Enpp3*^{-/-} cells compared with wild-type cells. We next added exogenous ATP into the basophil and mast cell culture and measured inorganic phosphate (Pi) and inorganic pyrophosphate (PPi), produced by hydrolysis of ATP to ADP and ATP to AMP, respectively (Figures 5D and S5B). In the supernatants of wild-type basophils and mast cells, free Pi and PPi increased after the addition of exogenous ATP, indicating that wild-type cells have potent ATP hydrolyzing activity. However, increase in Pi and PPi concentrations was substantially dampened in the supernatants of *Enpp3*^{-/-} cells. These findings indicate that ATP clearance ac-

tivity was impaired in basophils and mast cells derived from *Enpp3*^{-/-} mice.

We also assessed the ATP clearance activity in vivo. Wild-type and *Enpp3*^{-/-} mice were intravenously injected with ATP, and at the indicated time points serum was taken to analyze the levels of ATP and PPi. Serum ATP concentration was higher at any time points in *Enpp3*^{-/-} mice (Figure S5C). In addition, at 2 and 4 hr after the administration, fold-increase of ATP concentration compared to the basal concentration was markedly elevated in *Enpp3*^{-/-} mice. However, the fold change at 6 hr time point showed similar pattern between wild-type and *Enpp3*^{-/-} mice (Figure S5D). The concentration of PPi was lower at 1 hr in *Enpp3*^{-/-} mice, but later elevated to the similar concentration as that of wild-type mice (Figure S5E). These findings indicate that kinetics of the ATP clearance in vivo was delayed in *Enpp3*^{-/-} mice, resulting in the increased serum ATP concentration.

Enhanced ATP Responses in *Enpp3*^{-/-} Mice

Because extracellular ATP activates mast cells (Kurashima et al., 2012, 2014; Sudo et al., 1996), we analyzed ATP responses of basophils and mast cells. Culture of wild-type basophils and mast cells in the presence of exogenous ATP led to the secretion of IL-4 and IL-6, respectively (Figure 5E). ATP-induced cytokine production was markedly augmented in *Enpp3*^{-/-} cells. ATP also increased the number of Ki-67-positive basophils and mast cells from wild-type mice, and this was substantially enhanced in *Enpp3*^{-/-} cells (Figure 5F). Thus, *Enpp3*^{-/-} basophils and mast cells showed enhanced ATP-dependent responses.

Next, we assessed whether the enhanced ATP responses were due to the defective clearance of extracellular ATP in *Enpp3*^{-/-} basophils and mast cells by stimulating cells with a non-hydrolyzable ATP, ATPγS (Figure 5F). In wild-type basophils and mast cells, ATPγS increased Ki-67 expression compared with ATP, and their levels were comparable to those of ATPγS-stimulated *Enpp3*^{-/-} cells. We also analyzed in vivo ATP responses. ATP or ATPγS was intravenously injected to mice over three consecutive days and the Ki-67 expression in basophils was assessed (Figure 5G). Almost all blood basophils from *Enpp3*^{-/-} mice were Ki-67 positive, indicating that *Enpp3*^{-/-} basophils were highly activated. In contrast, about 50% of basophils in the peripheral blood of wild-type mice

Figure 3. The *Enpp3* Deficiency Aggravates Allergic Intestinal Inflammation

- (A) Body weights of OVA-sensitized WT (n = 7) and *Enpp3*^{-/-} (n = 7) mice with intragastric challenge with OVA. Data are baseline weights and means ± SD. *p < 0.05.
- (B) Colon length of WT (n = 7) and *Enpp3*^{-/-} (n = 7) mice were measured after ten times OVA administration. Representative pictures are shown (left). Data (right) are means ± SD. *p < 0.05.
- (C and D) Colons were sectioned and stained with H&E (C) or Toluidine blue (D). Black arrows indicate mast cells. Data are representative of three mice examined.
- (E) Mast cells frequencies in small (SI) and large (LI) intestines of OVA-challenged WT mice (n = 5) and *Enpp3*^{-/-} mice with (n = 5) or without (n = 5) oATP treatment were determined by flow cytometry. Means ± SD are shown. *p < 0.05.
- (F) Serum ATP concentrations from non-treated WT (n = 8), *Enpp3*^{-/-} (n = 8), OVA-challenged WT (n = 7), and OVA-challenged *Enpp3*^{-/-} (n = 7) mice were measured. Data are means ± SD. **p < 0.01.
- (G) Cells isolated from small intestine (SI), large intestine (LI), and mesenteric lymph nodes (MLNs) of WT mice (n = 5), *Enpp3*^{-/-} mice (n = 5), and oATP-treated *Enpp3*^{-/-} mice (n = 5) after 10 times OVA administration were stained for intracellular IL-4 in CD4⁺ cells. Means ± SD of the percentage of IL-4⁺ cell in CD4⁺ cells are shown. *p < 0.05.
- (H) Serum concentrations of IgE in non-treated WT (n = 8), *Enpp3*^{-/-} (n = 8), OVA-challenged WT (n = 7), and OVA-challenged *Enpp3*^{-/-} mice with (n = 5) or without (n = 7) oATP treatment were measured. Data are means ± SD. *p < 0.05.
- See also Figure S3.

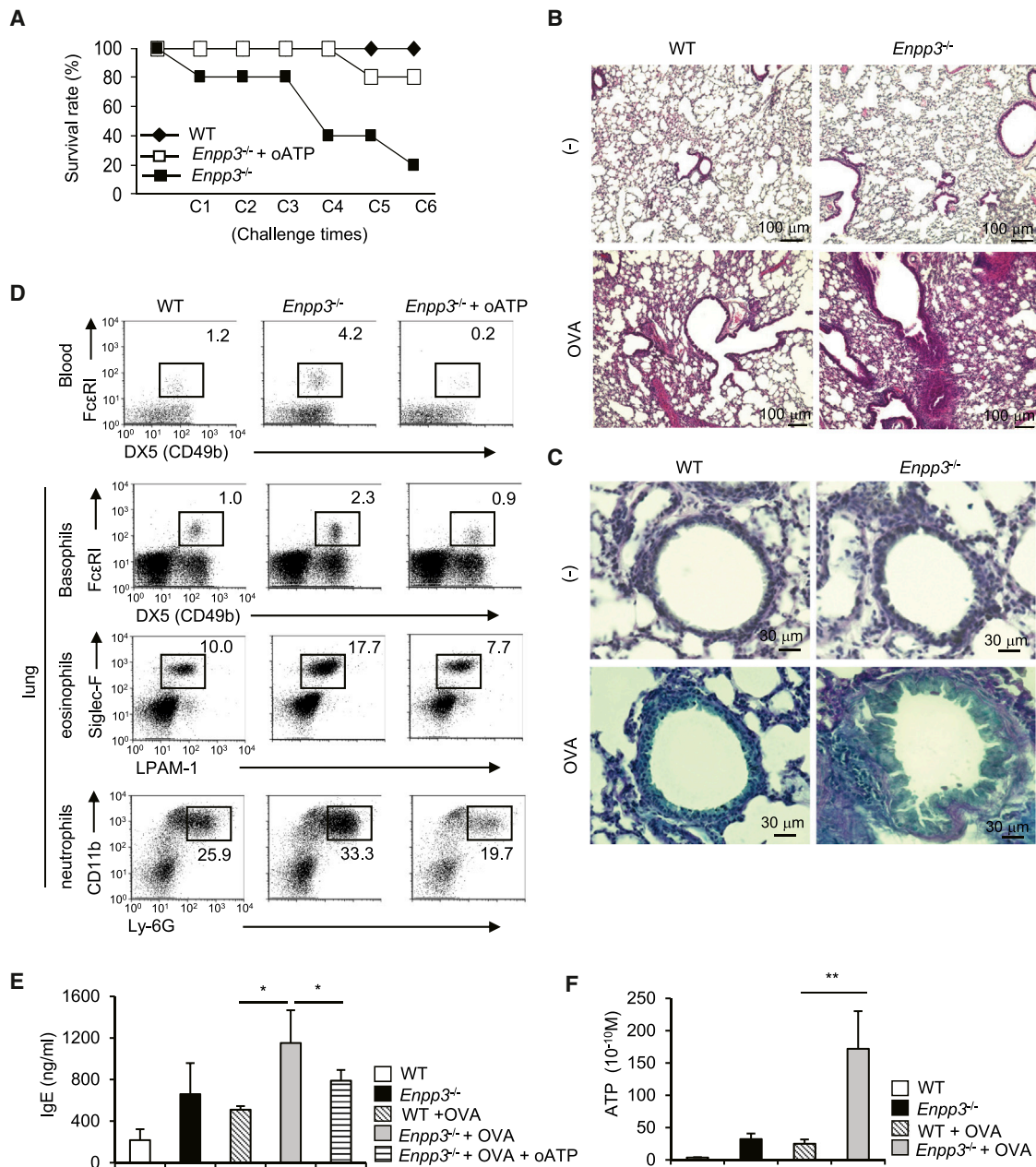


Figure 4. High Sensitivity to Chronic Airway Inflammation in *Enpp3*^{-/-} Mice

(A) Survival rates of WT (n = 7), *Enpp3*^{-/-} (n = 10), and *Enpp3*^{-/-} mice with oATP treatment (n = 5) after OVA challenge are shown.

(B and C) Lungs were sectioned and stained with H&E (B) or Alcian blue (C). Data are representative of three mice examined.

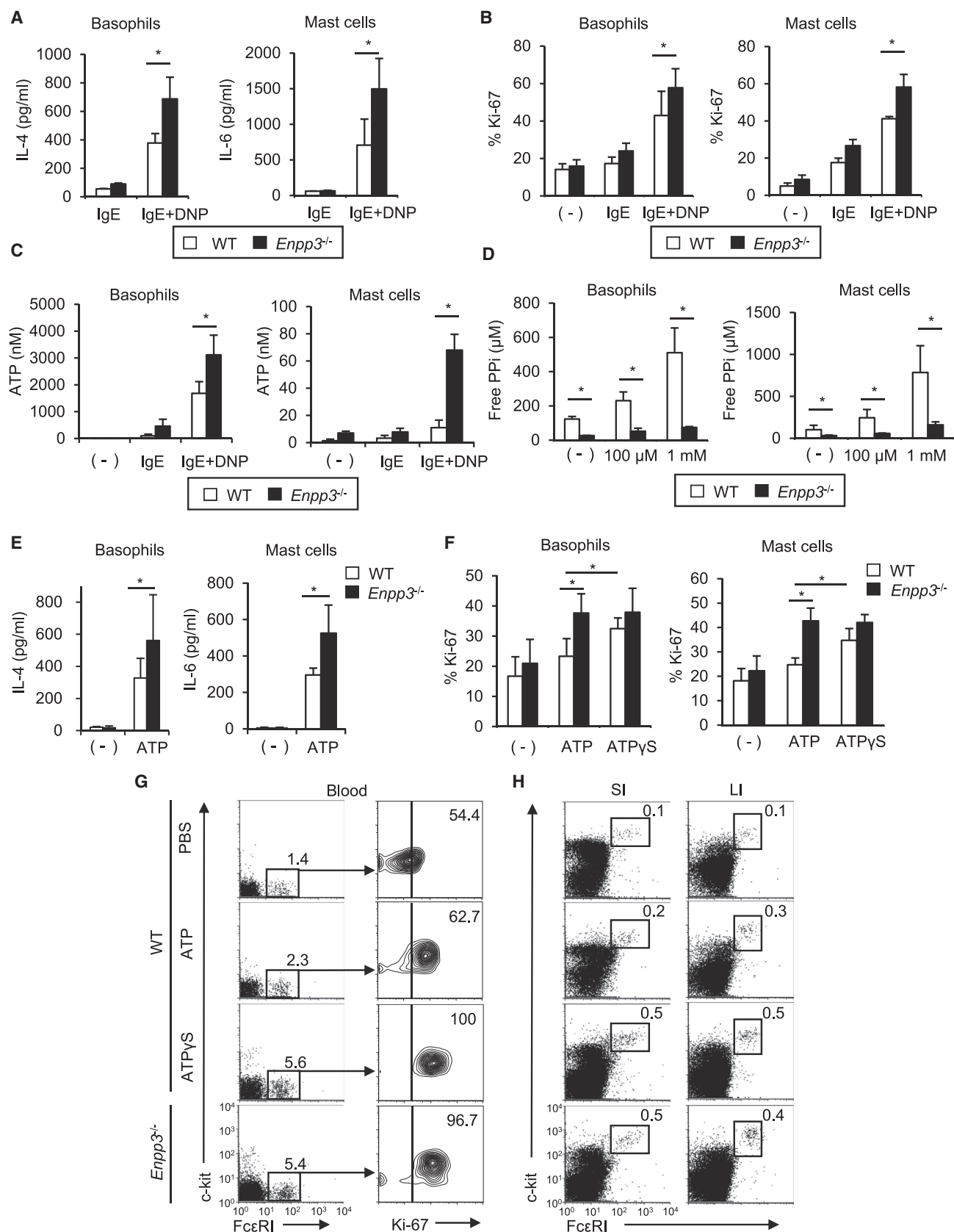
(D) Frequencies of basophils, eosinophils, and neutrophils in the peripheral blood and lung were measured by flow cytometry. Representative dot plots of WT (n = 5), *Enpp3*^{-/-} (n = 3), and *Enpp3*^{-/-} mice with oATP treatment (n = 3) are shown.

(E and F) Concentrations of serum IgE (E) and ATP (F) at 32 days after OVA challenge were determined by ELISA and a luciferin-luciferase assay, respectively. The means $\pm$ SD are shown. Non-treated WT (n = 7), *Enpp3*^{-/-} (n = 7), OVA-challenged WT (n = 7), and OVA-challenged *Enpp3*^{-/-} mice with (n = 5) or without (n = 7) oATP treatment. *p < 0.05, **p < 0.01.

See also Figure S4.

were Ki-67 positive. ATP injection only moderately increased the number of Ki-67-positive basophils in the peripheral blood of wild-type mice, but almost all wild-type basophils were Ki-67 positive after ATP γ S injection. We also analyzed mast cell populations in the small and large intestines after the intravenous in-

jection of ATP or ATP γ S (Figure 5H). ATP only slightly increased mast cell frequencies in wild-type mice, but ATP γ S substantially increased the frequency of wild-type mast cells to similar levels of mast cells in *Enpp3*^{-/-} mice. Taken together, these findings indicate that extracellular ATP activates basophils and mast cells



(legend on next page)

and that E-NPP3 modulates ATP-induced responses through ATP degradation.

We next investigated whether the ATP-dependent increase of basophils and mast cells was attributable to increased proliferation or decreased apoptosis. Basophils and mast cells were treated with ATP, and proliferative activity and apoptotic cell number in vitro were analyzed by measuring ^3H -thymidine uptake and annexin-V-positive cells, respectively (Figures S5F and S5G). ATP induced growth of wild-type basophils and mast cells only marginally. However, *Enpp3*^{-/-} basophils and mast cells showed vigorous proliferation in response to ATP. ATP inhibited apoptosis marginally in wild-type basophils and moderately in wild-type mast cells. ATP-mediated suppression of apoptosis was not dramatically altered in *Enpp3*^{-/-} mice. Thus, in the absence of *Enpp3*, proliferative activity of basophils and mast cells was enhanced, which might result in increased number of peripheral basophils and mast cells.

Regulation of Autocrine ATP-Dependent Activation by E-NPP3

Extracellular ATP activates ERK1 and/or ERK2 (ERK1/2) to induce cellular responses (Shiratori et al., 2010; Swanson et al., 1998). Therefore, we analyzed ATP-induced phosphorylation of ERK1/2 in basophils and mast cells (Figure 6A). Phosphorylation of ERK1/2 was induced by 15 min of ATP stimulation in wild-type basophils and mast cells and ATP-induced phosphorylation of ERK1/2 was enhanced in *Enpp3*^{-/-} cells compared with wild-type cells. ATP-induced ERK1/2 phosphorylation was inhibited by oATP pretreatment. FcεRI crosslinking also induced ERK1/2 phosphorylation. At 15 min after FcεRI crosslinking, phosphorylation of ERK1/2 was similarly induced in wild-type and *Enpp3*^{-/-} basophils and mast cells (Figure 6B). Pretreatment with oATP did not decrease the ATP-induced ERK1/2 phosphorylation, indicating that FcεRI crosslinking directly induced ERK activation. At 60 min after crosslinking, the amount of ERK1/2 phosphorylation was reduced in wild-type basophils and mast cells compared with those observed at 15 min. However, ERK1/2 phosphorylation remained at a high level in *Enpp3*^{-/-} cells even at 60 min after crosslinking (Figure 6C). Because FcεRI crosslinking induces the secretion of ATP and ATP activates ERK1/2, we analyzed the involvement of ATP in sustained ERK1/2 phosphorylation in *Enpp3*^{-/-} cells at

the later time point of FcεRI crosslinking, by pretreating cells with oATP. oATP pretreatment did not induce changes in ERK1/2 phosphorylation of wild-type cells. In contrast, oATP pretreatment substantially reduced ERK1/2 activation. This was in accordance with the observations that ATP activated ERK and that ATP- and FcεRI crosslinking-dependent cellular activation of *Enpp3*^{-/-} basophils and mast cells was reduced by treatment with the ERK inhibitor U0126 (Figures S6A–S6D). These findings indicate that ATP released by FcεRI crosslinking acts on basophils and mast cells in an autocrine manner to further activate the cells and that E-NPP3 negatively regulates ATP-dependent cellular activation.

In order to confirm the autocrine effect of ATP on basophils and mast cells, we co-cultured CD45.2 *Enpp3*^{-/-} basophils and mast cells with CD45.1 wild-type cells. ATP- or FcεRI crosslinking-induced increase of Ki-67 expression was considerably enhanced in *Enpp3*^{-/-} basophils and mast cells compared to wild-type cells (Figure 6D). Furthermore, in basophils and mast cells with siRNA-mediated reduced *Enpp3* expression, ATP- and FcεRI crosslinking-induced cytokine production was increased (Figures 6E and S6E). Thus, the enhanced ATP response of basophils and mast cells in the absence of *Enpp3* is intrinsic to these cells. Taken together, these findings demonstrate that E-NPP3 suppresses the autocrine effect of ATP of basophils and mast cells.

P2X7-Dependent Activation of Basophils and Mast Cells

ATP activates mast cells via the purinoreceptor P2X7 (Kura-shima et al., 2012, 2014; Sudo et al., 1996). Because basophil- and mast cell-dependent chronic inflammation in *Enpp3*^{-/-} mice was ameliorated by oATP treatment, which antagonizes P2X7 actions (Figures 3 and 4), we analyzed whether overactivation of *Enpp3*^{-/-} basophils and mast cells was mediated by P2X7 by using another P2X7 selective antagonist, A839977 (Figure 7A). FcεRI crosslinking markedly augmented the number of Ki-67-positive basophils and mast cells in *Enpp3*^{-/-} mice, which was reduced by pretreatment with oATP. The selective P2X7 antagonist A839977 also reduced the number of Ki-67-positive cells in *Enpp3*^{-/-} mice. The enhanced production of IL-4 and IL-6 from FcεRI-stimulated basophils and mast cells was also decreased by pretreatment with A839977 and oATP (Figure 7B). Thus, experiments with P2X7 receptor

Figure 5. Defective ATP Clearance of *Enpp3*^{-/-} Basophils and Mast Cells

- (A) Bone-marrow-derived basophils (left) and mast cells (right) from WT mice and *Enpp3*^{-/-} mice were stimulated with FcεRI crosslinking for 24 hr, and concentrations of IL-4 and IL-6 in culture supernatants were measured.
- (B) Basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were stimulated with FcεRI crosslinking for 24 hr. The percentage of Ki-67-positive cells was determined by flow cytometry.
- (C) Basophils (left) and mast cells (right) of WT and *Enpp3*^{-/-} mice were stimulated with FcεRI crosslinking for 1 hr, and concentrations of ATP in culture supernatants were measured.
- (D) Basophils and mast cells were incubated for 60 min in the presence of ATP (100 μM or 1 mM final concentration). Concentrations of pyrophosphate (free PPI) in the supernatants were measured.
- (E) Basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were cultured in the presence of 100 μM ATP for 24 hr, and concentrations of IL-4 and IL-6 in culture supernatants were measured.
- (F) Basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were stimulated with 1 μM ATP or ATPγS for 24 hr. The percentage of Ki-67-positive cells was determined by flow cytometry.
- (G and H) WT mice were administered PBS, ATP, or ATPγS. *Enpp3*^{-/-} mice were administered PBS. Expression of c-kit, FcεRI, and Ki-67 in blood CD3⁺CD4⁺CD8⁺B220⁺ cells was analyzed (G). Frequencies of c-kit⁺FcεRI⁺ mast cells in small (SI) and large intestines (LI) of mice were determined by flow cytometry (H).

Data are means ± SD of five independent experiments; *p < 0.05 (A–F). Data are representative of three independent experiments (G and H). See also Figure S5.

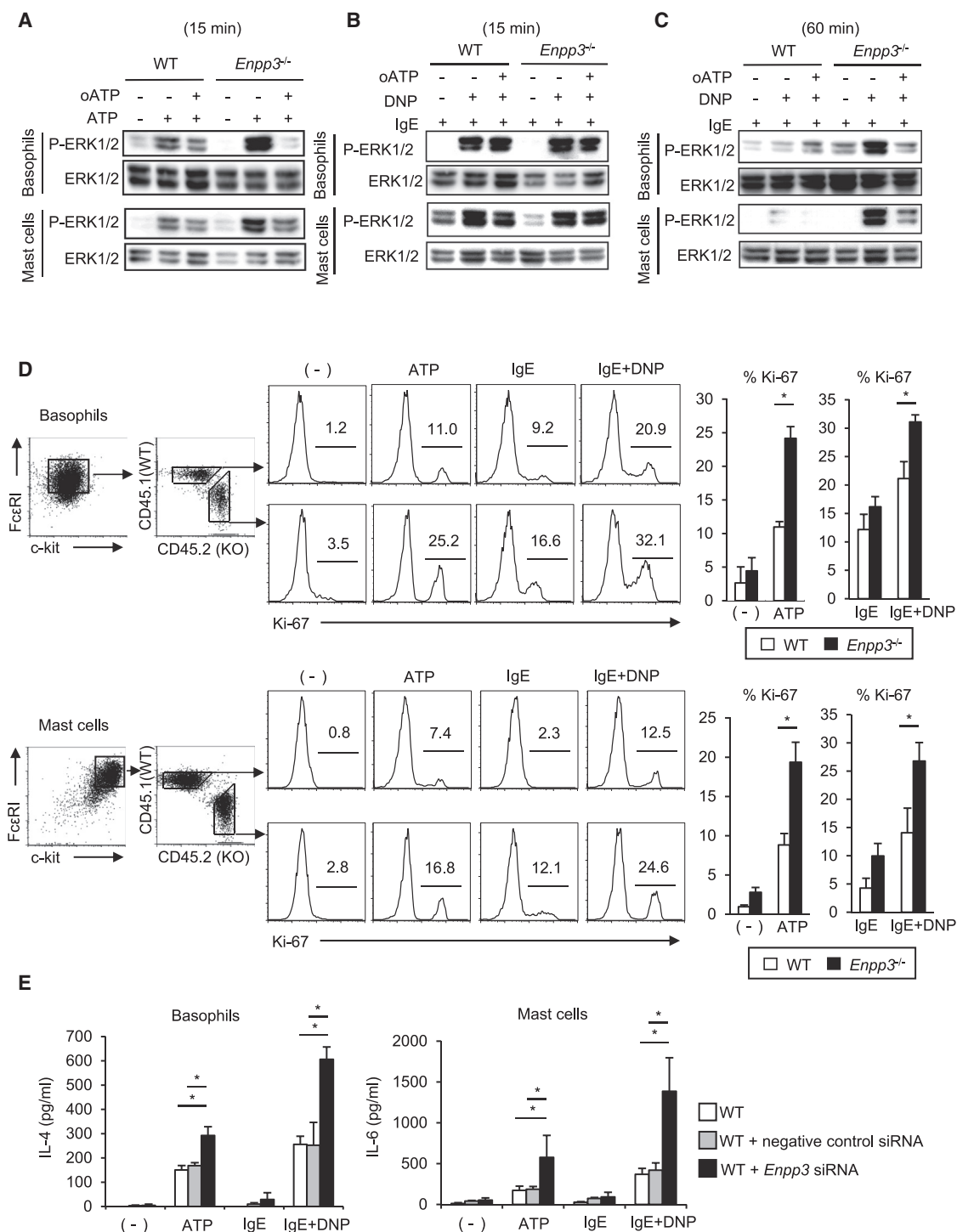


Figure 6. ATP-Dependent Overactivation of ERK1/2 in *Enpp3*^{-/-} Basophils and Mast Cells

(A) Cell lysates were prepared from bone-marrow-derived basophils (upper) and mast cells (lower) from WT and *Enpp3*^{-/-} mice pretreated with 300 μ M oATP for 3 hr and then stimulated by 100 μ M ATP for 15 min. Expression of phospho-ERK1/2 and ERK1/2 were determined by immunoblotting. Data are representative of five independent experiments.

(B and C) Cell lysates were prepared from bone-marrow-derived basophils (upper) and mast cells (lower) of WT and *Enpp3*^{-/-} mice pretreated with 300 μ M oATP for 3 hr and then stimulated by anti-DNP-IgE and DNP-HSA for 15 min (B) or 60 min (C). The levels of phospho-ERK1/2 and ERK1/2 were determined by immunoblotting. Data are representative of three independent experiments.

(legend continued on next page)

antagonists suggested that P2X7 mediates the overactivation of *Enpp3*^{-/-} basophils and mast cells. We next introduced the *P2rx7* deficiency into *Enpp3*^{-/-} mice. In *P2rx7*^{-/-} and *Enpp3*^{-/-}*P2rx7*^{-/-} basophils and mast cells, ATP-dependent cytokine production was severely reduced (Figure 7C). Furthermore, FcεRI crosslinking-induced increase of cytokine production was decreased in *Enpp3*^{-/-}*P2rx7*^{-/-} basophils and mast cells compared with *Enpp3*^{-/-} cells (Figure 7D). In addition, the number of basophils in the peripheral blood and spleen as well as mast cells in the small and large intestines was also reduced in *Enpp3*^{-/-}*P2rx7*^{-/-} mice compared with *Enpp3*^{-/-} mice (Figure 7E). Thus, introduction of the *P2rx7* deficiency into *Enpp3*^{-/-} mice resulted in the decreased activity of basophils and mast cells, demonstrating that P2X7 mediates the ATP-dependent late-phase activation of basophils and mast cells.

DISCUSSION

E-NPP3 (also known as CD203c) is an activation marker of basophils and mast cells (Bühning et al., 2001; Ghannadan et al., 2002). In the present study, we demonstrated that E-NPP3 is a critical negative regulator of basophils and mast cells through the hydrolysis of extracellular ATP, an activator of mast cells (Kurashima et al., 2012, 2014). Basophils were activated by ATP and the overproduction of ATP enhanced chronic allergic responses mediated by basophils and mast cells. Thus, extracellular ATP was demonstrated to be a potent inducer of the chronic phase of allergic responses, and E-NPP3, which is rapidly expressed in activated basophils and mast cells by FcεRI crosslinking, suppresses the ATP-dependent activation by hydrolyzing extracellular ATP.

Several lines of evidence indicated that ATP modulates the functions of neuronal and immune cells (Junger, 2011). ATP is released as a danger-associated molecular pattern from damaged and stressed cells for the clearance of these cells by phagocytes (Elliott et al., 2009; Mariathasan et al., 2006). In addition, ATP is released from non-damaged cells by physiologic stimuli and acts on cells in an autocrine or paracrine manner to modulate cellular functions (Corriden and Insel, 2010). The present study demonstrates that basophils and mast cells are activated by autocrine ATP.

An increase in extracellular ATP was reported in several inflammatory conditions, such as skin inflammation and asthma (Idzko et al., 2007; Kawamura et al., 2012). In addition, the importance of ATP hydrolysis in the regulation of several types of inflammations was demonstrated in mice deficient for CD39 (ecto-nucleoside triphosphate diphosphohydrolase) or CD73 (ecto 5'-nucleotidase) (Deaglio et al., 2007; Friedman et al., 2009; Mizumoto et al., 2002). In those studies, cells responsible for ATP hydrolysis were T cells or dendritic or Langerhans cells.

The present study demonstrated that another type of ATP-hydrolyzing enzyme, E-NPP3, expressed on basophils and mast cells was responsible for the modulation of chronic allergic inflammation via the suppression of ATP-dependent autocrine cell activation.

Basophils and mast cells expressing FcεRI are well known to induce immediate allergic responses through IgE-mediated crosslinking (Gould and Sutton, 2008). In addition, recent evidence indicated that chronic allergic responses are especially induced by basophils through the FcεRI (Karasuyama et al., 2009; Mukai et al., 2005). The present study indicates that ATP is responsible for the pathogenesis of chronic allergic responses. Based on the findings of the present study, we propose the following model during immediate and chronic phases of allergic responses mediated by basophils and mast cells. FcεRI crosslinking induces secretion of several mediators including ATP from basophils and mast cells, which induces the immediate phase of allergic responses. Then, ATP acts on basophils and mast cells in an autocrine manner to further activate basophils and mast cells, leading to the development of chronic-phase allergic inflammation. E-NPP3 (CD203c), which is rapidly expressed on activated basophils and mast cells, negatively regulates ATP-dependent chronic responses.

In *Enpp3*^{-/-} mice, the number of peripheral basophils, but not bone marrow basophils, was increased. In this regard, basophils in the bone marrow, where basophils are generated de novo, might be premature or many factors might contribute to the maintenance or proliferation of basophil precursors. Indeed, anti-basophil mAb treatment drastically reduced the number of peripheral basophils but not bone marrow basophils (Obata et al., 2007). Alternatively, ATP concentration in the bone marrow environment was not altered in *Enpp3*^{-/-} mice (our unpublished results), which might be one of causes for the similar number of bone marrow basophils in both genotypes.

It is interesting to note that the inflammatory mediator ATP was released from basophils and mast cells by FcεRI crosslinking and that the negative regulator of the ATP response, E-NPP3, was simultaneously expressed in these cells. In this regard, ATP-dependent continuous activation might be physiologically responsible for the efficient expulsion of helminths by basophils and mast cells. E-NPP3, in turn, modulates excessive ATP-dependent responses to prevent chronic allergic disorders.

The ATP-induced activation of basophils and mast cells is mediated by P2X7 and ERK MAP kinases. A previous study demonstrated that P2X7-mediated sensing of ATP induced the activation of several signal transduction pathways leading to the activation of microglia (Shiratori et al., 2010). In the present study, the involvement of ERK MAP kinases in the activation of basophils and mast cells was demonstrated. However, other signaling pathways such as p38 and JNK MAP kinases as well

(D) Mixtures of basophils (top) and mast cells (bottom) of WT (CD45.1) and *Enpp3*^{-/-} (KO: CD45.2) mice (1:1 ratio in cell number) were stimulated with ATP or FcεRI crosslinking for 24 hr. The percentage of Ki-67-positive cells was determined by flow cytometry. Data shown in right panel are means ± SD of three independent experiments. *p < 0.05.

(E) Bone-marrow-derived basophils (left) and mast cells (right) from wild-type mice were introduced with *Enpp3*-specific siRNA or control siRNA and then stimulated with ATP or FcεRI crosslinking for 24 hr. The concentrations of IL-4 and IL-6 in culture supernatants were measured. Data are means ± SD of five independent experiments. *p < 0.05.

See also Figure S6.

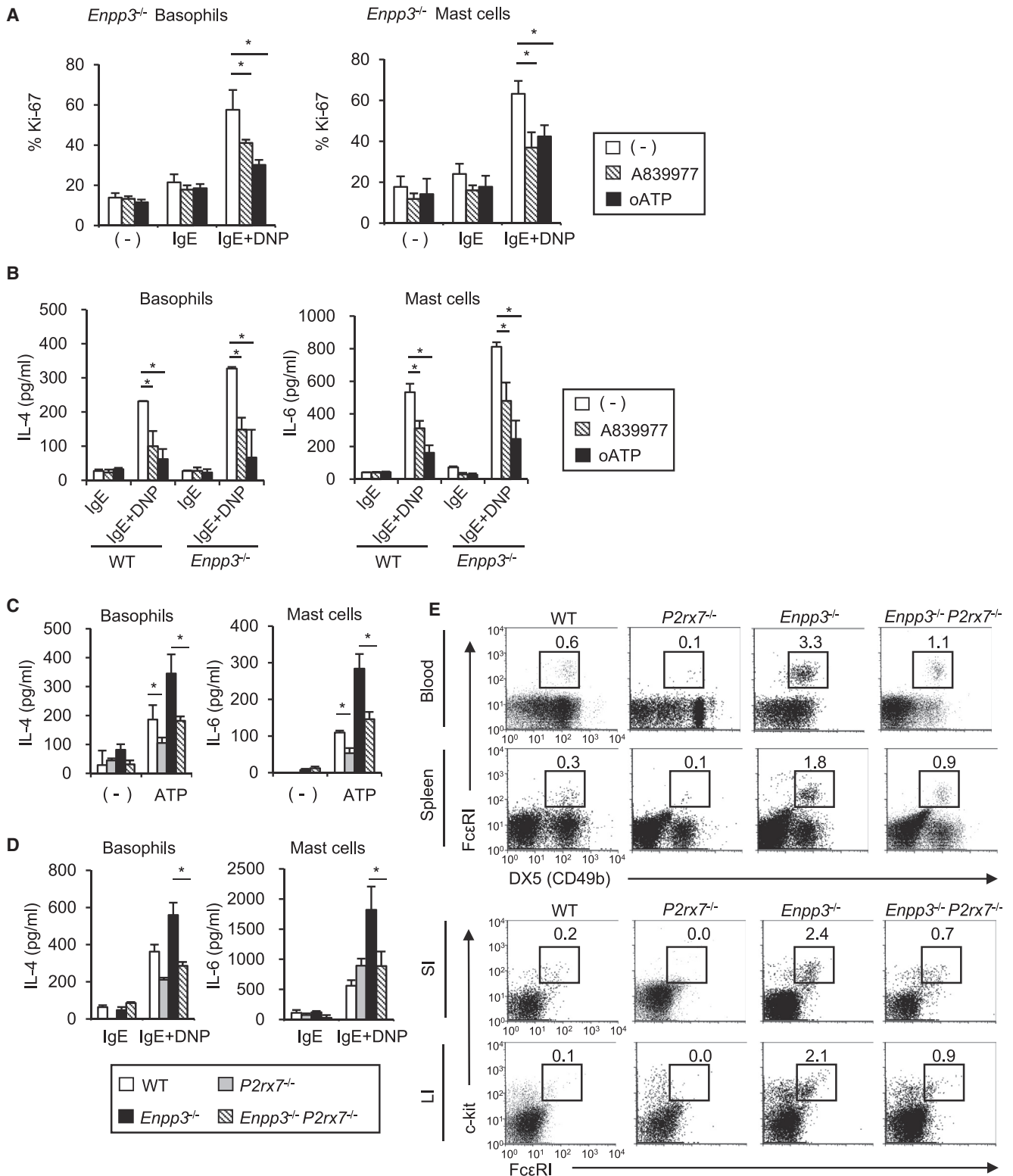


Figure 7. P2X7-Dependent Activation of Basophils and Mast Cells

(A and B) Bone-marrow-derived basophils (left) and mast cells (right) from *Enpp3*^{-/-} mice were pretreated with 300 μ M oATP or 100 nM A839977 for 3 hr and then stimulated by anti-DNP-IgE and DNP-HSA for 24 hr. The percentage of Ki-67⁺ cells was determined by flow cytometry (A). Concentrations of IL-4 and IL-6 in culture supernatants were determined by ELISA (B). Data are means $\pm$ SD of three independent experiments. **p* < 0.05.

(legend continued on next page)

as PKC might also participate in the ATP/P2X7-induced activation of basophils and mast cells.

P2X7 was shown to be responsible for the ATP-dependent overactivation of basophils and mast cells in the absence of E-NPP3. Indeed, several previous studies indicated that P2X7 is implicated in the pathogenesis of airway allergic inflammation in mice and humans (Denlinger et al., 2013; Idzko et al., 2007; Manthei et al., 2012; Müller et al., 2011) as well as contact hypersensitivity in a mouse model (Weber et al., 2010). However, all of these studies focused on dendritic cells as a target of ATP. It is generally considered that late-phase allergic inflammation is mediated by Th2 cells. However, basophils and mast cells are closely associated to Th2-cell-type responses (Karasuyama et al., 2011; Voehringer, 2013). In addition, it was shown that the chronic phase of IgE-dependent allergic inflammation can be induced independently of T cells (Mukai et al., 2005). In the present study, P2X7 on basophils and mast cells was responsible for the late phase of IgE-dependent activation of basophils and mast cells. Thus, P2X7 on basophils and mast cells could be a possible target for the treatment of allergic diseases. However, both P2X3 and P2X7 were highly expressed on basophils (our unpublished results), and therefore P2X3 might also participate in the ATP-dependent activation of basophils and mast cells. Indeed, although ATP-dependent responses were severely reduced, they were not completely blocked in the absence of *P2rx7*. The possible involvement of P2Y receptors should also be considered.

In the present study, we revealed that E-NPP3 is a negative regulator of chronic allergic responses via ATP hydrolysis. Because E-NPP3 is selectively expressed in activated basophils and mast cells, the main cell types involved in allergic responses, the development of a novel drug to activate or induce E-NPP3 might be beneficial for the establishment of an effective therapy for allergic diseases, which recently have increased in number.

EXPERIMENTAL PROCEDURES

Passive Cutaneous Anaphylaxis

Wild-type and *Enpp3*^{-/-} mice on the BALB/c background were sensitized with 100 ng anti-TNP IgE derived from IGLb4 B cell hybridoma in 10 μ l PBS by intradermal injection into the left ear. As a control, PBS was injected into the right ear. At 24 hr after the sensitization, mice were intravenously challenged with 300 μ g TNP14-conjugated ovalbumin (TNP-OVA; Biosearch Technologies) plus 2% Evans blue (Sigma-Aldrich) in 300 μ l PBS. Mice were euthanized 30 min after the antigen challenge, followed by extraction of Evans blue dye from each ear in 700 μ l acetone/water (7:3) at room temperature overnight. The absorbance at 620 nm in the extract was measured with a spectrophotometer. All animal experiments were conducted with approval of the Animal Research Committee of the Graduate School of Medicine at Osaka University and the Animal Care and Use Committee, The University of Tokyo.

IgE-Mediated Chronic Allergic Skin Inflammation

IgE-mediated chronic allergic skin inflammation was induced as previously described (Mukai et al., 2005). In brief, wild-type and *Enpp3*^{-/-} mice on the BALB/c background were sensitized with 200 μ g anti-TNP IgE derived from

IGELb4 B cell hybridoma by intravenous injection. The next day, left ears of IgE-sensitized mice were intradermally challenged with 10 μ g TNP-OVA (Biosearch Technologies) in 10 μ l PBS and right ears were inoculated an equal amount of OVA (grade V; Sigma-Aldrich) by microsyringe. The thickness of ears were measured by a dial thickness gauge (G-1A; Ozaki), and numbers and frequencies of basophils (CD3⁺CD4⁺CD8⁺B220⁺Fc ϵ RI⁺CD49b⁺), eosinophils (CD3⁺CD4⁺CD8⁺B220⁺Siglec-F⁺LPAM-1⁺), and neutrophils (MHC class II⁺Ly6G⁺CD11b⁺) in ears treated with 1 mg/ml collagenase D (Roche) were determined by trypan blue cell counting and flow cytometry. For histological analysis, ear specimens were fixed with 4% paraformaldehyde phosphate buffer solution (Wako) and embedded in paraffin. Sections were stained with Giemsa and analyzed with a BZ-9000 microscope system (Keyence).

Experimental Oral Allergen-Induced Diarrhea

Experimental oral allergen-induced diarrhea was elicited as previously described (Brandt et al., 2003). In brief, wild-type and *Enpp3*^{-/-} mice were sensitized with a mixture of 50 μ g of OVA (grade V; Sigma-Aldrich) and 1 mg of aluminum hydroxide adjuvant (Imject Alum; Thermo Scientific) twice with a 2-week interval. At 2 weeks after the last sensitization, mice were intragastrically challenged with 10 mg of OVA in 200 μ l PBS every 2 days for 10 times after 3 hr of food deprivation. Body weights were recorded before intragastric challenge. In some experiments, OVA-sensitized *Enpp3*^{-/-} mice were intravenously administrated with 100 μ l of 5 mM oATP (Sigma-Aldrich) 5 times every 3 days during the period of intragastric challenge. After 10 times of OVA challenge, the mice were sacrificed and used for analyses of cell population and histology. Peripheral blood was harvested for serum ATP measurement 1 hr after the last intragastric challenge. For histological analysis, colon specimens were prepared by a Swiss roll method and fixed with 4% paraformaldehyde phosphate buffer solution (Wako) and embedded in paraffin. Sections were stained with hematoxylin and eosin or toluidine blue and analyzed with a BZ-9000 microscope system (Keyence).

Experimental Chronic Asthma

Chronic asthma was induced as previously described with some modifications (Idzko et al., 2007; Wakahara et al., 2013). Wild-type and *Enpp3*^{-/-} mice were sensitized with a mixture of 50 μ g of OVA (grade V; Sigma-Aldrich) and 1 mg of aluminum hydroxide adjuvant (Imject Alum; Thermo Scientific) twice with a 10-day interval. Then, the OVA-sensitized mice were intranasally challenged with 50 μ g of OVA in 20 μ l PBS on day 17, 19, 21, 28, 30, and 32. In some experiments, OVA-sensitized *Enpp3*^{-/-} mice were administrated 100 μ l of 5 mM oATP intravenously on day 11, 14, and 17, as well as 30 min before OVA intranasal challenges. At 16 hr after the last intranasal challenge, peripheral blood was harvested for serum ATP measurement. For histological analysis, lung specimens were fixed with 4% paraformaldehyde phosphate buffer solution (Wako) and embedded in paraffin. Sections were stained with hematoxylin and eosin or Alcian blue and hematoxylin.

In Vitro Stimulation Assay

For IgE crosslinking, bone-marrow-derived basophils and mast cells were pretreated with 1 μ g/ml anti-DNP IgE (SPE-7; Sigma-Aldrich) for 6 hr, and then stimulated with 50 ng/ml DNP-HSA (Sigma-Aldrich) for the indicated periods. In some experiments, bone-marrow-derived basophils and mast cells were pretreated with 300 μ M oATP (Sigma-Aldrich) or 100 nM A839977 P2X7 antagonist (Tocris Bioscience) for 3 hr or with 20 μ M U0126 (Sigma-Aldrich) for 1 hr.

Measurement of ATP Hydrolyzing Activity

ATP hydrolyzing activity was determined by measuring levels of free inorganic phosphate and free inorganic pyrophosphate in the supernatants of cells cultured for 1 hr after the addition of ATP (100 μ M or 1 mM). Levels of free inorganic phosphate and free inorganic pyrophosphate were determined by

(C and D) Bone-marrow-derived basophils (left) and mast cells (right) from WT, *P2rx7*^{-/-}, *Enpp3*^{-/-}, and *Enpp3*^{-/-}*P2rx7*^{-/-} mice were cultured in the presence of 100 μ M ATP (C) or anti-DNP-IgE and DNP-HSA (D) for 24 hr, and concentrations of IL-4 and IL-6 in culture supernatants were measured. Data are means $\pm$ SD of three independent experiments. *p < 0.05.

(E) Frequencies of basophils in peripheral blood and spleen and frequencies of mast cells in small (SI) and large intestines (LI) from WT, *P2rx7*^{-/-}, *Enpp3*^{-/-}, and *Enpp3*^{-/-}*P2rx7*^{-/-} mice were determined by flow cytometry. Representative dot plots from three independent experiments are shown.

Malachite Green Phosphate Assay Kits (BioAssay Systems) and Pyrophosphate Assay Kit (Fluorometric; Abcam), respectively.

Treatment with ATP and ATP γ S

Mice were administrated 1 mM ATP or ATP γ S in 100 μ l PBS intravenously (i.v.) daily for 3 days before flow cytometric analysis.

Co-culture of Wild-Type and *Enpp3*^{-/-} Basophils and Mast Cells

Bone-marrow-derived basophils and mast cells from CD45.1 wild-type and CD45.2 *Enpp3*^{-/-} mice on the C57BL/6 background were mixed in 1:1 ratio and stimulated with 100 μ M ATP or treated with 1 μ g/ml anti-DNP IgE (SPE-7; Sigma-Aldrich) for 6 hr followed by stimulation with 50 ng/ml DNP-HSA (Sigma-Aldrich). After 24 hr of the stimulation, cells were stained with anti-CD45.1, anti-CD45.2, and anti-Ki-67 Abs.

siRNA-Mediated Knockdown of *Enpp3* in Basophils and Mast Cells

Bone-marrow-derived basophils and mast cells from wild-type mice prepared as above were introduced with *Enpp3*-specific siRNA (sc-142185; Santa Cruz) or control siRNA (silencer negative control; Life Technologies) by using a Lonza transfection system (Amaxa cell line nucleofector kit V; Lonza). At 12 hr after the siRNA introduction, cells were analyzed for expression *Enpp3* mRNA and used for experiments.

Statistical Analysis

Differences between control and experimental groups were evaluated by the Student's *t* test. A *p* value < 0.05 was considered significant. Data are presented as mean $\pm$ SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2015.01.015>.

AUTHOR CONTRIBUTIONS

S.H.T. performed experiments. T.K. generated mutant mice. M.K., H. Kayama, R.O., K.I., Y.S., and E.U. performed animal experiments. S.S., Y.K., and H. Kiyono performed experiments with double mutant mice. A.T., S.Y., K.O.-N., and H. Karasuyama performed experiments with anti-TNP-IgE. M.K. and S.H.T. contributed to the experimental design and data analysis. K.T. planned and directed the research and wrote the paper.

ACKNOWLEDGMENTS

We thank Y. Uno and M. Tajima for generation of *Enpp3*^{-/-} mice, T. Kondo and Y. Magota for technical assistance, S. Pareek for critical reading of the manuscript, and C. Hidaka for secretarial assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, the Japan Science and Technology Agency, and by the Ministry of Health, Labour and Welfare.

Received: July 16, 2014

Revised: August 23, 2014

Accepted: December 9, 2014

Published: February 17, 2015

REFERENCES

- Abraham, S.N., and St John, A.L. (2010). Mast cell-orchestrated immunity to pathogens. *Nat. Rev. Immunol.* 10, 440–452.
- Bischoff, S.C., Sellge, G., Lorentz, A., Sebald, W., Raab, R., and Manns, M.P. (1999). IL-4 enhances proliferation and mediator release in mature human mast cells. *Proc. Natl. Acad. Sci. USA* 96, 8080–8085.
- Bollen, M., Gijbbers, R., Ceulemans, H., Stalmans, W., and Stefan, C. (2000). Nucleotide pyrophosphatases/phosphodiesterases on the move. *Crit. Rev. Biochem. Mol. Biol.* 35, 393–432.
- Brandt, E.B., Strait, R.T., Hershko, D., Wang, Q., Muntel, E.E., Scribner, T.A., Zimmermann, N., Finkelman, F.D., and Rothenberg, M.E. (2003). Mast cells are required for experimental oral allergen-induced diarrhea. *J. Clin. Invest.* 112, 1666–1677.
- Bühning, H.J., Seiffert, M., Giesert, C., Marxer, A., Kanz, L., Valent, P., and Sano, K. (2001). The basophil activation marker defined by antibody 97A6 is identical to the ectonucleotide pyrophosphatase/phosphodiesterase 3. *Blood* 97, 3303–3305.
- Bühning, H.J., Streble, A., and Valent, P. (2004). The basophil-specific ectoenzyme E-NPP3 (CD203c) as a marker for cell activation and allergy diagnosis. *Int. Arch. Allergy Immunol.* 133, 317–329.
- Corriden, R., and Insel, P.A. (2010). Basal release of ATP: an autocrine-paracrine mechanism for cell regulation. *Sci. Signal.* 3, re1.
- Deaglio, S., Dwyer, K.M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J.F., Enjyoji, K., Linden, J., Oukka, M., et al. (2007). Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* 204, 1257–1265.
- Denlinger, L.C., Manthei, D.M., Seibold, M.A., Ahn, K., Bleecker, E., Boushey, H.A., Calhoun, W.J., Castro, M., Chinchili, V.M., Fahy, J.V., et al.; NHLBI Asthma Clinical Research Network Investigators (2013). P2X7-regulated protection from exacerbations and loss of control is independent of asthma maintenance therapy. *Am. J. Respir. Crit. Care Med.* 187, 28–33.
- Elliott, M.R., Chekeni, F.B., Trampont, P.C., Lazarowski, E.R., Kadl, A., Walk, S.F., Park, D., Woodson, R.I., Ostankovich, M., Sharma, P., et al. (2009). Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* 461, 282–286.
- Friedman, D.J., Künzli, B.M., A-Rahim, Y.I., Seigny, J., Berberat, P.O., Enjyoji, K., Csizmadia, E., Friess, H., and Robson, S.C. (2009). From the Cover: CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to inflammatory bowel disease. *Proc. Natl. Acad. Sci. USA* 106, 16788–16793.
- Ghannadan, M., Hauswirth, A.W., Scherthaner, G.H., Müller, M.R., Klepetko, W., Schatzl, G., Sperr, W.R., Bühning, H.J., and Valent, P. (2002). Detection of novel CD antigens on the surface of human mast cells and basophils. *Int. Arch. Allergy Immunol.* 127, 299–307.
- Gould, H.J., and Sutton, B.J. (2008). IgE in allergy and asthma today. *Nat. Rev. Immunol.* 8, 205–217.
- Hessle, L., Johnson, K.A., Anderson, H.C., Narisawa, S., Sali, A., Goding, J.W., Terkeltaub, R., and Millan, J.L. (2002). Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *Proc. Natl. Acad. Sci. USA* 99, 9445–9449.
- Idzko, M., Hammad, H., van Nimwegen, M., Kool, M., Willart, M.A., Muskens, F., Hoogsteden, H.C., Luttmann, W., Ferrari, D., Di Virgilio, F., et al. (2007). Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat. Med.* 13, 913–919.
- Idzko, M., Ferrari, D., and Eltzschig, H.K. (2014). Nucleotide signalling during inflammation. *Nature* 509, 310–317.
- Junger, W.G. (2011). Immune cell regulation by autocrine purinergic signalling. *Nat. Rev. Immunol.* 11, 201–212.
- Kanda, H., Newton, R., Klein, R., Morita, Y., Gunn, M.D., and Rosen, S.D. (2008). Autotaxin, an ectoenzyme that produces lysophosphatidic acid, promotes the entry of lymphocytes into secondary lymphoid organs. *Nat. Immunol.* 9, 415–423.
- Karasuyama, H., Mukai, K., Tsujimura, Y., and Obata, K. (2009). Newly discovered roles for basophils: a neglected minority gains new respect. *Nat. Rev. Immunol.* 9, 9–13.
- Karasuyama, H., Mukai, K., Obata, K., Tsujimura, Y., and Wada, T. (2011). Nonredundant roles of basophils in immunity. *Annu. Rev. Immunol.* 29, 45–69.
- Kawamura, T., Ogawa, Y., Nakamura, Y., Nakamizo, S., Ohta, Y., Nakano, H., Kabashima, K., Katayama, I., Koizumi, S., Kodama, T., et al. (2012). Severe dermatitis with loss of epidermal Langerhans cells in human and mouse zinc deficiency. *J. Clin. Invest.* 122, 722–732.
- Kurashima, Y., Amiya, T., Nochi, T., Fujisawa, K., Haraguchi, T., Iba, H., Tsutsui, H., Sato, S., Nakajima, S., Iijima, H., et al. (2012). Extracellular ATP

mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors. *Nat. Commun.* 3, 1034.

Kurashima, Y., Amiya, T., Fujisawa, K., Shibata, N., Suzuki, Y., Kogure, Y., Hashimoto, E., Otsuka, A., Kabashima, K., Sato, S., et al. (2014). The enzyme Cyp26b1 mediates inhibition of mast cell activation by fibroblasts to maintain skin-barrier homeostasis. *Immunity* 40, 530–541.

Manthei, D.M., Jackson, D.J., Evans, M.D., Gangnon, R.E., Tisler, C.J., Gern, J.E., Lemanske, R.F., Jr., and Denlinger, L.C. (2012). Protection from asthma in a high-risk birth cohort by attenuated P2X(7) function. *J. Allergy Clin. Immunol.* 130, 496–502.

Mariathasan, S., Weiss, D.S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W.P., Weinrauch, Y., Monack, D.M., and Dixit, V.M. (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440, 228–232.

Mizumoto, N., Kumamoto, T., Robson, S.C., Sévigny, J., Matsue, H., Enjyoji, K., and Takashima, A. (2002). CD39 is the dominant Langerhans cell-associated ecto-NTPDase: modulatory roles in inflammation and immune responsiveness. *Nat. Med.* 8, 358–365.

Mukai, K., Matsuoka, K., Taya, C., Suzuki, H., Yokozeki, H., Nishioka, K., Hirokawa, K., Etori, M., Yamashita, M., Kubota, T., et al. (2005). Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity* 23, 191–202.

Müller, T., Vieira, R.P., Grimm, M., Dürk, T., Cicko, S., Zeiser, R., Jakob, T., Martin, S.F., Blumenthal, B., Sorichter, S., et al. (2011). A potential role for P2X7R in allergic airway inflammation in mice and humans. *Am. J. Respir. Cell Mol. Biol.* 44, 456–464.

Obata, K., Mukai, K., Tsujimura, Y., Ishiwata, K., Kawano, Y., Minegishi, Y., Watanabe, N., and Karasuyama, H. (2007). Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood* 110, 913–920.

Okawa, A., Nakamura, I., Goto, S., Moriya, H., Nakamura, Y., and Ikegawa, S. (1998). Mutation in Npps in a mouse model of ossification of the posterior longitudinal ligament of the spine. *Nat. Genet.* 19, 271–273.

Schetinger, M.R., Morsch, V.M., Bonan, C.D., and Wyse, A.T. (2007). NTPDase and 5'-nucleotidase activities in physiological and disease conditions: new perspectives for human health. *Biofactors* 31, 77–98.

Shiratori, M., Tozaki-Saitoh, H., Yoshitake, M., Tsuda, M., and Inoue, K. (2010). P2X7 receptor activation induces CXCL2 production in microglia through NFAT and PKC/MAPK pathways. *J. Neurochem.* 114, 810–819.

Stefan, C., Jansen, S., and Bollen, M. (2005). NPP-type ectophosphodiesterases: unity in diversity. *Trends Biochem. Sci.* 30, 542–550.

Sudo, N., Tanaka, K., Koga, Y., Okumura, Y., Kubo, C., and Nomoto, K. (1996). Extracellular ATP activates mast cells via a mechanism that is different from the activation induced by the cross-linking of Fc receptors. *J. Immunol.* 156, 3970–3979.

Swanson, K.D., Reigh, C., and Landreth, G.E. (1998). ATP-stimulated activation of the mitogen-activated protein kinases through ionotropic P2X2 purinoreceptors in PC12 cells. Difference in purinoreceptor sensitivity in two PC12 cell lines. *J. Biol. Chem.* 273, 19965–19971.

Umamoto, E., Hayasaka, H., Bai, Z., Cai, L., Yonekura, S., Peng, X., Takeda, A., Tohya, K., and Miyasaka, M. (2011). Novel regulators of lymphocyte trafficking across high endothelial venules. *Crit. Rev. Immunol.* 31, 147–169.

van Meeteren, L.A., Ruurs, P., Stortelers, C., Bouwman, P., van Rooijen, M.A., Pradère, J.P., Pettit, T.R., Wakelam, M.J., Saulnier-Blache, J.S., Mummery, C.L., et al. (2006). Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development. *Mol. Cell. Biol.* 26, 5015–5022.

Voehringer, D. (2013). Protective and pathological roles of mast cells and basophils. *Nat. Rev. Immunol.* 13, 362–375.

Wakahara, K., Van, V.Q., Baba, N., Bégin, P., Rubio, M., Delespesse, G., and Sarfati, M. (2013). Basophils are recruited to inflamed lungs and exacerbate memory Th2 responses in mice and humans. *Allergy* 68, 180–189.

Wang, L., Sikora, J., Hu, L., Shen, X., Grygorczyk, R., and Schwarz, W. (2013). ATP release from mast cells by physical stimulation: a putative early step in activation of acupuncture points. *Evid. Based Complement. Alternat. Med.* 2013, 350949.

Weber, F.C., Esser, P.R., Müller, T., Ganesan, J., Pellegatti, P., Simon, M.M., Zeiser, R., Idzko, M., Jakob, T., and Martin, S.F. (2010). Lack of the purinergic receptor P2X(7) results in resistance to contact hypersensitivity. *J. Exp. Med.* 207, 2609–2619.

Yegutkin, G.G. (2008). Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim. Biophys. Acta* 1783, 673–694.

Immunity

Supplemental Information

The Ectoenzyme E-NPP3 Negatively Regulates ATP-Dependent Chronic Allergic Responses by Basophils and Mast Cells

Shih Han Tsai, Makoto Kinoshita, Takashi Kusu, Hisako Kayama, Ryu Okumura, Kayo Ikeda, Yosuke Shimada, Akira Takeda, Soichiro Yoshikawa, Kazushige Obata-Ninomiya, Yosuke Kurashima, Shintaro Sato, Eiji Umemoto, Hiroshi Kiyono, Hajime Karasuyama, and Kiyoshi Takeda

Figure S1

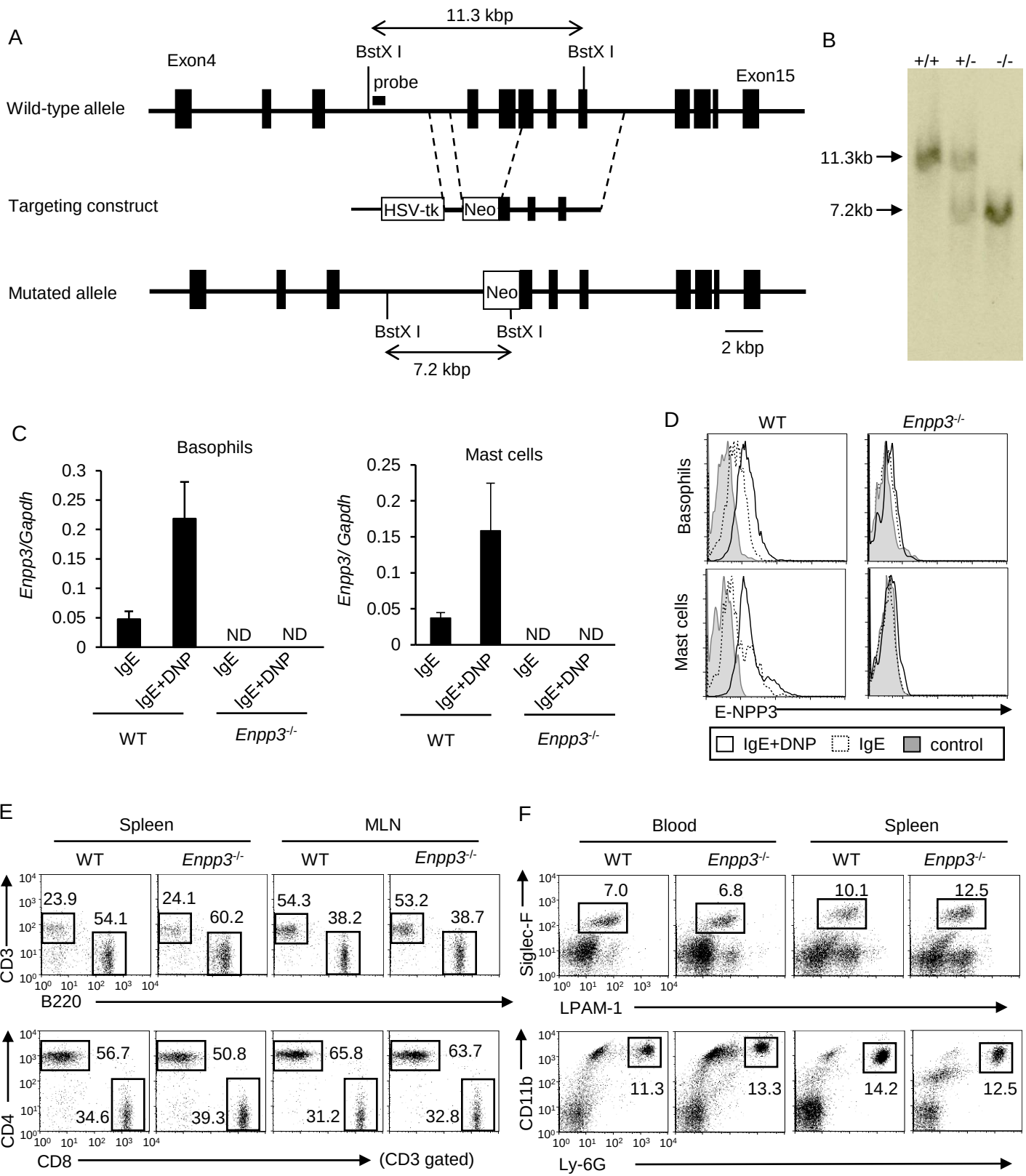


Figure S1

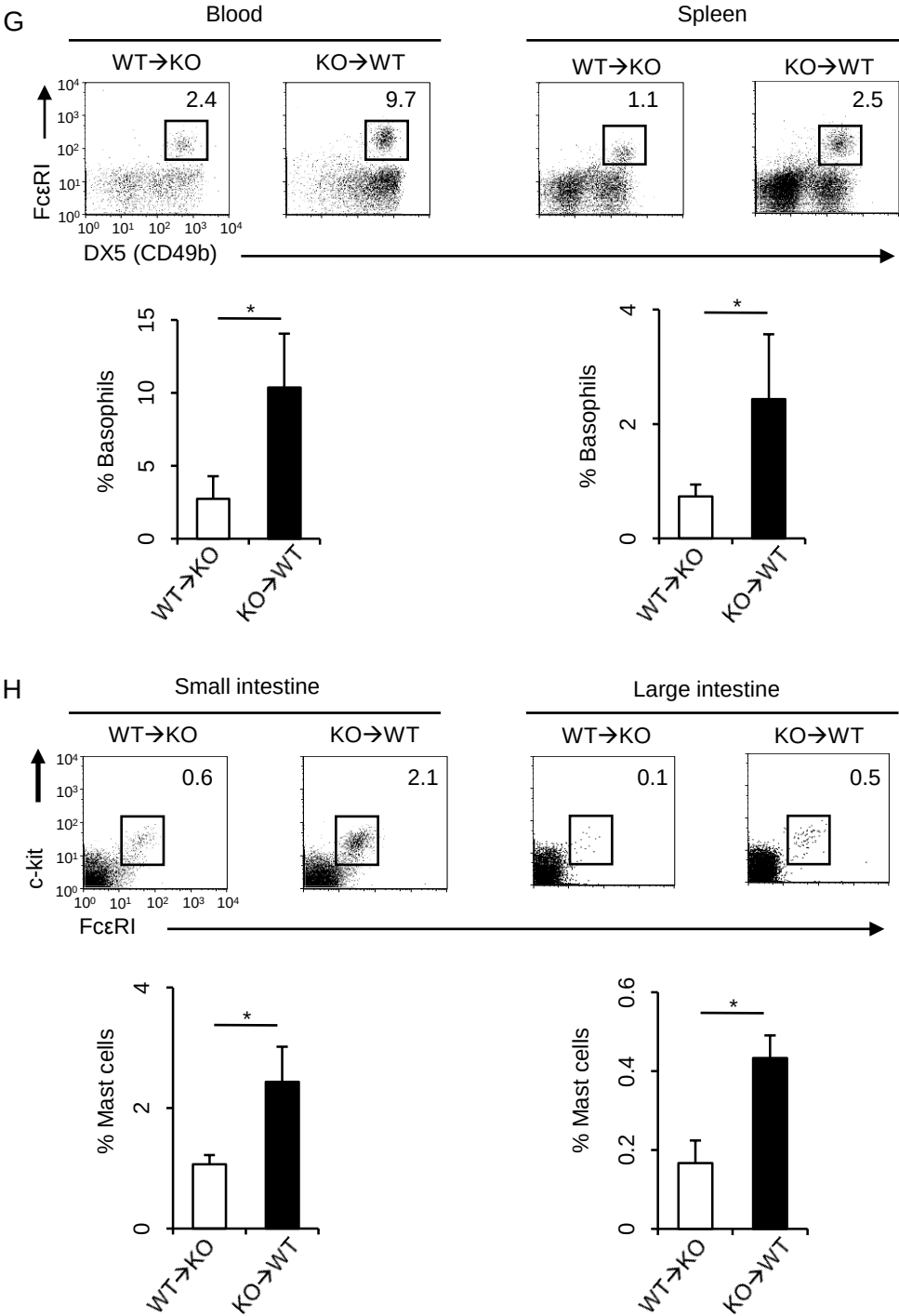


Figure S1, related to Figure 1. Generation of *Enpp3*-deficient mice.

(A) Schematic representation of the *Enpp3* gene targeting strategies. The solid boxes indicate coding regions of the *Enpp3* gene. Neo and HSV-tk indicate a neomycin-resistance gene and a herpes simplex virus thymidine kinase gene, respectively. The positions of the probe and restriction enzyme site for Southern blotting are shown. **(B)** Genomic DNA was isolated from the tails of wild-type (+/+), heterozygous (+/-) and homozygous (-/-) *Enpp3* mutant mice. Southern blot analysis, which was performed after digestion of the genomic DNA with BstXI, shows the correct targeting of the locus. **(C)** Bone marrow-derived basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were stimulated by anti-DNP-IgE and DNP-HSA for 1 h, and analyzed for *Enpp3* mRNA expression by real-time RT-PCR. **(D)** Bone marrow-derived basophils (upper) and mast cells (lower) were stimulated by anti-DNP-IgE and DNP-HSA for 30 min, and analyzed for surface E-NPP3 expression by flow cytometry. Data panels are representative of three independent experiments. **(E)** Frequencies of CD4⁺ and CD8⁺ lymphocytes as well as CD3⁺ and B220⁺ lymphocytes in the spleen (left) and mesenteric lymph node (right) from WT and *Enpp3*^{-/-} mice were examined by flow cytometry. Data are representative of three independent experiments. **(F)** Frequencies of eosinophils (CD3⁻ CD4⁻ CD8⁻ B220⁻ Siglec-F⁺ LPAM-1⁺) and neutrophils (MHC class II⁻ Ly6G⁺ CD11b⁺) in the peripheral blood (left) and spleen (right) from WT and *Enpp3*^{-/-} mice were examined by flow cytometry. Data are representative of three independent experiments. **(G, H)** WT and *Enpp3*^{-/-} (KO) mice (6–7 weeks old) received 8.5 Gy irradiation, and were then intravenously transferred with 5×10^6 bone marrow cells from WT or KO mice in 100 μ l PBS. Eight weeks after bone marrow cell transfer, frequencies of basophils in the peripheral blood and the spleen (G) as well as mast cells in the small and large intestines (H) were determined by flow cytometry. The mean percentages of basophils and mast cells are shown in lower panels (n=5). CD3⁻ CD4⁻ CD8⁻ B220⁻ cells were gated and analyzed for expression of Fc ϵ RI and CD49b (basophils), as well as for Fc ϵ RI and c-kit (mast cells). Data are means $\pm$ SD. *: p<0.05.

Figure S2

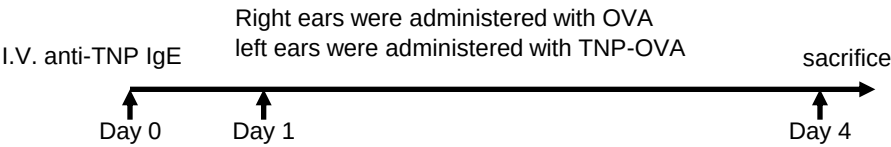
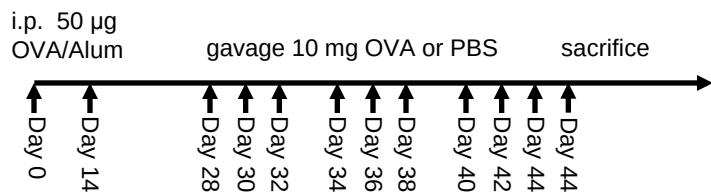


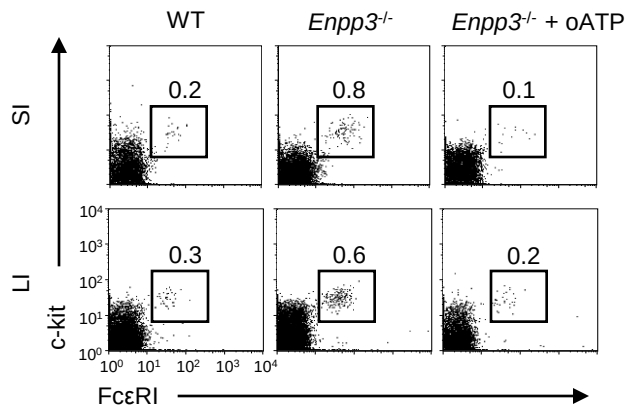
Figure S2, related to Figures 2. Protocol for induction of chronic allergic skin inflammation
Schematic of the protocol to induce IgE-mediated chronic allergic skin inflammation in mice.

Figure S3

A



B



C

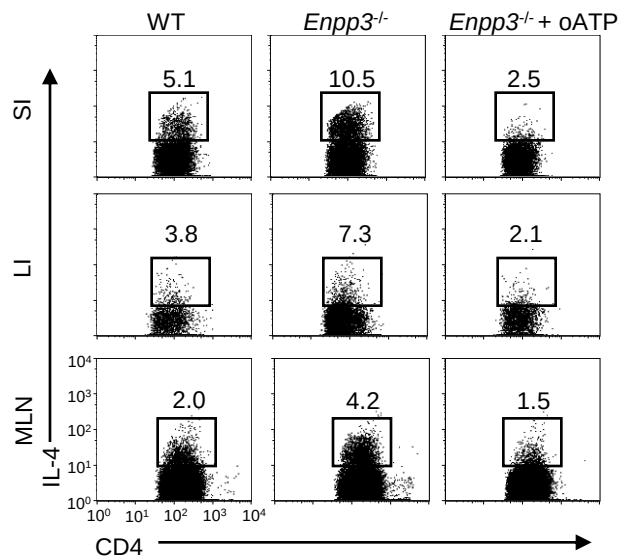


Figure S3, related to Figure 3. Exacerbation of oral allergen-induced intestinal inflammation

(A) Schematic of the protocol to induce experimental oral allergen-induced diarrhea in mice. **(B)** Mast cells frequencies in small (SI) and large intestines (LI) of OVA-challenged WT mice (n=5), *Enpp3*^{-/-} mice with (n=5) or without oATP treatment (n=5) were determined by flow cytometry. Representative dot plots are shown. **(C)** Cells isolated from small intestine (SI), large intestine (LI) and mesenteric lymph nodes (MLN) of WT mice (n=5), *Enpp3*^{-/-} mice (n=5) and oATP-treated *Enpp3*^{-/-} mice (n=5) after 10 times OVA administration, were stained for intracellular IL-4 in CD4⁺ cells. Representative dot plots are shown.

Figure S4

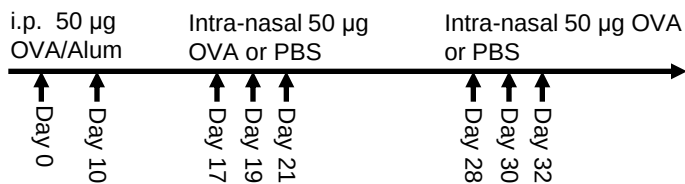


Figure S4 related to Figures 4. Protocol for induction of chronic airway inflammation
Schematic of the protocol to induce experimental chronic asthma in mice.

Figure S5

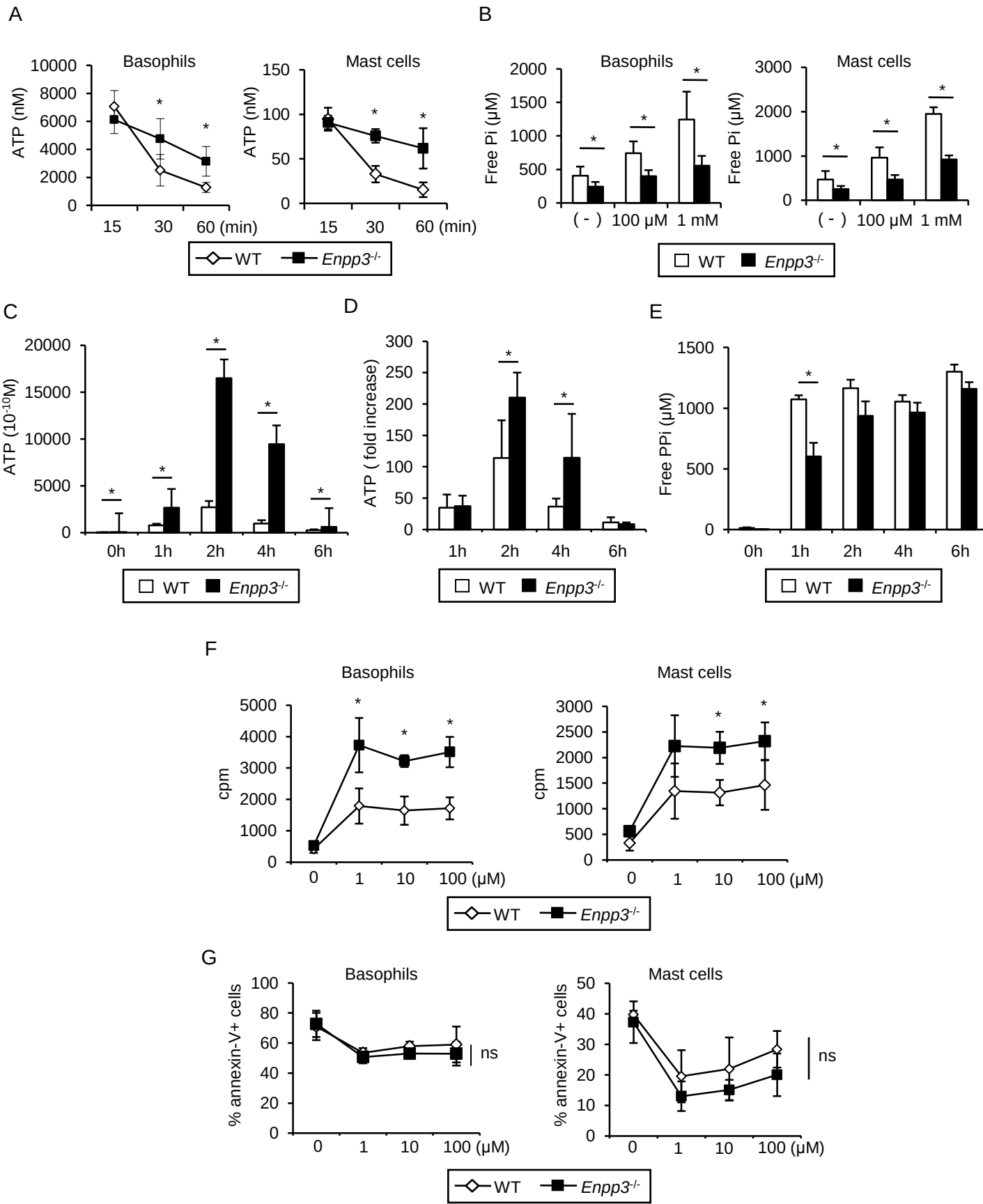


Figure S5, related to Figure 5. Impaired ATP clearance of *Enpp3*^{-/-} mice

(A) Basophils (left) and mast cells (right) of WT and *Enpp3*^{-/-} mice were stimulated with FcεRI crosslinking for the indicated periods, and concentrations of ATP in culture supernatants were measured. Data are means ± SD of five independent experiments *: p<0.05. **(B)** Basophils and mast cells were incubated for 60 min in the presence of ATP (100 μM or 1 mM final concentration). Concentrations, inorganic free phosphate (free Pi) in the supernatants were measured. Data are means ± SD of five independent experiments *: p<0.05. **(C, D, E)** WT and *Enpp3*^{-/-} mice were intravenously injected with 1mM ATP, and sera were collected at the indicated time points. Concentrations of serum ATP were determined by luciferin-luciferase assay (C, D). Concentrations of pyrophosphate (free PPi) in the sera were measured (E). Data are means ± SD of three independent experiments. *: p<0.05. **(F)** Bone marrow-derived basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were cultured in the presence of ATP (1μM, 10μM and 100μM) for 40 h. Thymidine uptake was measured. Data are means ± SD of four independent experiments *: p<0.05. **(G)** Bone marrow-derived basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were cultured in the presence of ATP (1μM, 10μM and 100μM) for 24 h, and percentage of annexin-V-positive cells were determined by flow cytometry. Data are means ± SD of three independent experiments. ns: not significant.

Figure S6

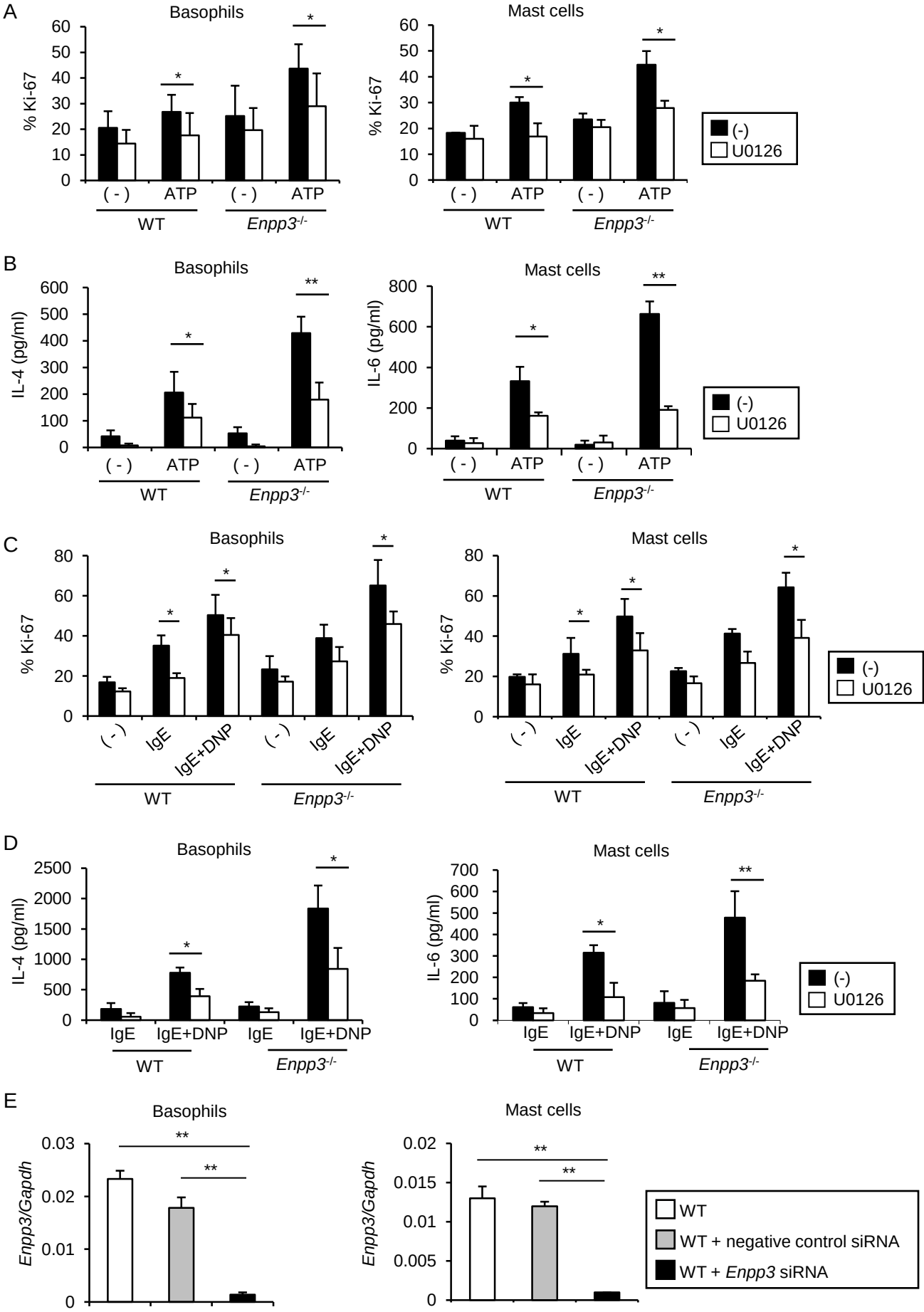


Figure S6, related to Figure 6. ATP-dependent autocrine activation of *Enpp3*^{-/-} basophils and mast cells.

(A, B) Bone marrow-derived basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were pretreated with 20 μ M U0126 for 1 h, and then incubated with 100 μ M ATP for 24 h. The percentage of Ki-67-positive cells was determined by flow cytometry (A), and concentration of IL-4 and IL-6 in culture supernatants was determined by ELISA (B). Data are means $\pm$ SD of three independent experiments *: $p < 0.05$. **(C, D)** Bone marrow-derived basophils (left) and mast cells (right) from WT and *Enpp3*^{-/-} mice were pretreated with 20 μ M U0126 for 1 h, and then stimulated by anti-DNP-IgE and DNP-HSA for 24 h. The percentage of Ki-67-positive cells was determined by flow cytometry (C), and concentration of IL-4 and IL-6 in culture supernatants was determined by ELISA (D). Data are means $\pm$ SD of three independent experiments *: $p < 0.05$, **: $p < 0.01$. **(E)** Bone marrow-derived basophils and mast cells from wild-type mice were introduced with *Enpp3*-specific siRNA, control siRNA or vehicle. At 12 h after the transfection, *Enpp3* mRNA expression in each cells was analyzed by real-time RT-PCR. Data are means $\pm$ SD of three independent experiments. **: $p < 0.01$.

Supplemental Experimental Procedures

Mice

P2rx7^{-/-} mice on a BALB/c background were previously described (Kurashima et al., 2012), and were crossed with *Enpp3^{-/-}* mice to generate *P2rx7^{-/-} Enpp3^{-/-}* mice. BALB/c and C57BL/6 mice were purchased from CLEA. All animal experiments were conducted with approval of the Animal Research Committee of the Graduate School of Medicine at Osaka University and the Animal Care and Use Committee, The University of Tokyo.

Generation of Enpp3-deficient mice

The targeting vector was constructed by replacing of a 2.8-kb fragment encoding the seventh and eighth exons of *Enpp3* with a neomycin resistance gene cassette, and HSV thymidine kinase gene driven by a phosphoglycerate kinase promoter for negative selection was inserted into the genomic fragment. Before the double-resistant colonies of G418 and ganciclovir were selected and screened by PCR and Southern blot analysis, V6.5 embryonic stem cells were transfected with a targeting vector. Blastocysts of C57BL/6 females were used for microinjection of homologous recombinants and for the generation of *Enpp3*-deficient mice, heterozygous F1 mice were intercrossed. After intercrossing, *Enpp3*-deficient mice and their wild-type littermates were confirmed by Southern blot analysis. *Enpp3*-deficient mice were backcrossed onto BALB/c mice or C57BL/6 for at least four generations, and *Enpp3*-deficient mice and their wild-type littermates from intercrosses of heterozygous mice were used for experiments. All animal experiments were conducted with approval from the Animal Research Committee of the Graduate School of Medicine at Osaka University.

Reagents

The following antibodies were purchased from BD Biosciences: anti-CD16/32 (2.4G2),

anti-CD11b-PE/Cy7 (M1/70), anti-Siglec-F-PE (E50-2440), anti-IL-4-PE (11B11), anti-CD3 ϵ -FITC (145-2C11), anti-CD3 ϵ -PE/Cy7(145-2C11), anti-Ki-67-PE (B56), and anti-Ly6G-PE (1A8). The following antibodies were purchased from Biolegend: anti-CD49b-PE (DX5), anti-Fc ϵ RI α -APC (MAR-1), anti-c-kit-PE/Cy7 (2B8), anti-LPAM-1(α 4 β 7)-APC (DATK32), anti-CD8a-FITC (53-6.7), anti-CD8a-APC (53-6.7), anti-B220-FITC (RA3-6B2), anti-IL-17A-APC (TC11-18H10.1), anti-CD45.2-Pacific blue (104), anti-CD4-PerCP/Cy5.5 (GK1.5), anti-CD4-FITC (GK1.5), anti-CD4-PE (RM4-5). Anti-MHC class II-FITC (M5/114.15.2) was purchased from eBioscience. Anti-CD45.1-PerCP/Cy5.5 (A20) was purchased from Tonbo biosciences and anti-CD203c-FITC (orb15186) was from Biorbyt. RPMI 1640 medium (Nacalai Tesque) supplemented with 10% fetal bovine serum (Nichirei Bioscience), 100 U/ml penicillin (Gibco), 100 μ g/ml streptomycin (Gibco) and 50 μ M 2-mercaptoethanol (Gibco) was used for experiments as complete medium. Phosphate buffered saline (PBS) and Hank's Balanced Salt Solution (HBSS) were purchased from Nacalai Tesque. Adenosine 5'-triphosphate (ATP) disodium salt hydrate (A1852), adenosine 5'-(γ -thio)triphosphate tetralithium salt (ATP γ S; A1388), and oxidized ATP (oATP; ATP periodate oxidized sodium salt) were purchased from Sigma-Aldrich. IGELb4 B cell hybridoma was previously described (Mukai et al., 2005).

Generation of bone marrow-derived basophils and mast cells

Bone marrow cells from wild-type and *Enpp3*^{-/-} mice were cultured for 14 days with 5 ng/ml recombinant murine IL-3 (PeproTech). After 14 days of culture, bone marrow-derived basophils (Fc ϵ RI⁺ c-kit⁻ cells) and mast cells (Fc ϵ RI⁺ c-kit⁺ cells) were enriched by sorting with FACSaria (BD Biosciences) as previously described (Yoshimoto and Nakanishi, 2012). After FACS sorting, bone marrow-derived basophils and mast cells were cultured with complete medium in the absence of IL-3 for 16 h, and used for experiments.

Real-time RT-PCR

RNA samples were isolated from bone marrow-derived basophils and mast cells using TRIzol reagent (Invitrogen). cDNA was generated from total RNA by treatment with RQ1 DNase I (Promega) before performing reverse transcription with random primers (Toyobo) and Moloney murine leukemia virus reverse transcriptase (Promega). Real-time RT-PCR was performed by cDNA PCR in an ABI 7300 Real-time PCR system (Applied Biosystems) with GoTaq qPCR Master Mix (Promega). The following primer sets were used: *Enpp3*: 5'-CTCATGCCCTGCACTACAGA-3' and 3'-TAGCCTTTGGTTTGCTTGCT-5', *Gapdh*: 5'-CCTCGTCCCGTAGACAAAATG-3' and 5'-TCTCCACTTTGCCACTGCAA-3'. Final values shown are relative expressions after normalizing to the expression of *Gapdh*.

Cell isolation

The spleen and mesenteric lymph nodes were ground with glass slides, and the cells were passed through a 40- μ m mesh filter to obtain single-cell suspensions. Cells from the spleen and peripheral blood were treated with RBC lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA) to deplete red blood cells. For isolation of lamina propria cells, intestines were opened, washed to remove fecal content, shaken in HBSS containing 5 mM EDTA for 20 min at 37°C to remove epithelial cells and fat tissue, cut into small pieces and incubated with RPMI 1640 containing 4% FBS, 1 mg/ml collagenase D (Roche), 0.5 mg/ml dispase (Invitrogen) and 40 μ g/ml DNase I (Roche) for 1 h at 37°C in a shaking water bath. The digested tissues were washed with HBSS containing 5 mM EDTA and resuspended with 5 ml 40% Percoll (GE Healthcare), and then 80% Percoll was added to the bottom of tube to form the 80% (lower)/40% (upper) Percoll layers. After Percoll density gradient centrifugation, the lamina propria lymphocytes and granulocytes were collected at the Percoll gradient interface.

Flow cytometry

Cells were pre-incubated with 2.5 µg/ml anti-CD16/32 (BD Biosciences) for 5 min followed by staining with the indicated antibodies. All data were acquired using FACSCanto II (BD Biosciences). To investigate expression of intracellular IL-4 in CD4⁺ T cells, cells were incubated with 50 ng/ml phorbol myristate acetate (Sigma-Aldrich), 5 mM calcium ionophore (A23187; Sigma-Aldrich), and GolgiStop (BD Biosciences) at 37°C for 4 h. Then, cells were stained with anti-CD4-PerCP/Cy5.5 (BioLegend) at 4°C for 20 min, washed, fixed and permeabilized with the Cytotfix/Cytoperm Kit plus (BD Bioscience), and then stained with anti-IL-4-PE (BD Biosciences) at 4°C for 20 min. For nuclear Ki-67 staining, cells were fixed and permeabilized using diluted Fixation/Permeabilization Concentrate (eBiosciences), and then stained with anti-Ki-67-PE (BD Biosciences) for 30 min at 4°C.

Bone marrow chimera mice

Wild-type and *Enpp3*^{-/-} mice (6–7 weeks old) received 8.5 Gy irradiation and then irradiated mice were administrated 5×10^6 bone marrow cells from normal wild-type and *Enpp3*^{-/-} mice in 100 µl PBS. At 8 weeks after bone marrow cell transfer, frequencies of basophils and mast cells were determined by flow cytometry.

Measurement of IgE, IL-4, IL-6 and extracellular ATP

Concentration of IgE, IL-4 and IL-6 was measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience). To measure the levels of extracellular ATP *in vivo*, serum was harvested from peripheral blood after centrifugation, and ATP levels were measured by a luciferin-luciferase ATP assay (Toyo Ink). For *in vitro* experiments, culture supernatants were harvested after incubation of cells with Tyrode's buffer (140 mM NaCl, 4 mM KCl, 0.2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM

D-glucose) alone or with ATP (100 μ M and 1 mM) for 1 h, and ATP levels were measured by a luciferin-luciferase assay (Toyo Ink).

In vivo ATP hydrolyzing activity

Wild-type and *Enpp3*^{-/-} mice were intravenously injected with 1 mM ATP in 100 μ l PBS. Sera were collected at the indicated periods. Levels of ATP and free inorganic pyrophosphate were measured by a luciferin-luciferase ATP assay (Toyo Ink) and Pyrophosphate Assay Kit (Fluorometric; Abcam), respectively.

Western blot analysis

Bone marrow-derived basophils and mast cells were lysed with RIPA lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 μ g/ml leupeptin) with protease inhibitor cocktail (Roche). The cell lysates were separated by SDS-PAGE and then transferred to polyvinyl difluoride (PVDF; Millipore) membranes. The transferred membranes were blocked with 5% skim milk (BD Biosciences) in 0.1% Tween-20 containing Tris-buffered saline (TBST) for 1 h, and then hybridized with anti-Phospho-p44/42 MAPK (9101; Cell Signaling Technology) or anti-ERK1 (K-23; Santa Cruz Biotechnology) for 2 h at room temperature. After three times washing with TBST, membranes were incubated with hydroperoxidase conjugated donkey anti-rabbit IgG (GE healthcare) for 1 h at room temperature. The enhanced chemiluminescence (SuperSignal; Thermo scientific) method was used for immunodetection and images were captured by a Luminescent Image Analyzer LAS-1000 (Fuji Film) image system.

Apoptosis assay

Bone marrow-derived basophils and mast cells from wild-type and *Enpp3*^{-/-} mice were

incubated with ATP (1 μ M, 10 μ M and 100 μ M) for 24h, and then stained with annexin-V (Annexin-V-fluos staining kit; Roche). The percentages of annexin-V-positive cells were determined by flow cytometry.

Proliferation assay

Bone marrow-derived basophils and mast cells from wild-type and *Enpp3*^{-/-} mice were cultured with ATP (1 μ M, 10 μ M and 100 μ M) for 40h. [³H]-thymidine (1 μ Ci) was added to the culture in the last 16 h. The radioactivity was measured by a 1450 microbeta scintillation counter (Wallac). Data are shown in counts per min (CPM).

Supplemental References

Yoshimoto, T., and Nakanishi, K. (2012). Generation and characterization of mouse basophils from bone marrow and purification of basophils from spleen. Current protocols in immunology / edited by John E. Coligan ... [et al.] *Chapter 3*, Unit 3 24.