

An immunoglobulin-like receptor, Allergin-1, inhibits immunoglobulin E-mediated immediate hypersensitivity reactions

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Anaphylaxis is a life-threatening immediate hypersensitivity reaction triggered by antigen capture by immunoglobulin E (IgE) bound to the high-affinity IgE receptor (FcεRI) on mast cells. However, the regulatory mechanism of mast cell activation is not completely understood. Here we identify an immunoglobulin-like receptor, Allergin-1, that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM)-like domain, and show it was preferentially expressed on mast cells. Mouse Allergin-1 recruited the tyrosine phosphatases SHP-1 and SHP-2 and the inositol phosphatase SHIP. Coligation of Allergin-1 and FcεRI suppressed IgE-mediated degranulation of bone marrow-derived cultured mast cells. Moreover, mice deficient in Allergin-1 developed enhanced passive systemic and cutaneous anaphylaxis. Thus, Allergin-1 suppresses IgE-mediated, mast cell-dependent anaphylaxis in mice.

Anaphylaxis is a life-threatening allergic reaction that is rapid in onset^{1,2}. The frequency of anaphylaxis is estimated to be 50–2,000 episodes per 100,000 people³. Anaphylaxis can occur after exposure to certain allergens, such as foods, drugs, insect venoms, latex and immunotherapy injection². Mast cells and basophils express FcεRI, the high-affinity receptor for immunoglobulin E (IgE), on the cell surface, and have a central role in IgE-associated anaphylaxis^{4,5}. Crosslinking of FcεRI-bound IgE with multivalent antigen initiates the activation of mast cells and basophils by promoting aggregation of FcεRI. This process results in the degranulation of mast cells and basophils, with the concomitant secretion of chemical mediators, such as histamine, tryptase, carboxypeptidase A and proteoglycans, that are stored in the cytoplasmic granules of these cells; the *de novo* synthesis of proinflammatory lipid mediators, such as prostaglandins and leukotrienes, as well as platelet-activating factor, in the early phase (5–30 min after exposure to antigen); and the synthesis and secretion of cytokines and chemokines in the late phase (2–6 h after exposure to antigen)⁶. This anaphylactic reaction is called type I immediate hypersensitivity.

Studies of mice lacking mast cells (c-Kit-deficient *W/W^v* mice), FcεRI or IgE have provided evidence of the existence of an alternative pathway for anaphylaxis^{7–10}. IgG-allergen immune complexes mediate their activation signal via the low-affinity IgG receptor FcγRIII (CD16; A000537) on basophils, which induces the release of platelet-activating factor rather than histamine. The release of platelet-activating factor is the chief cause of the development of IgG-dependent systemic anaphylaxis in mice¹¹, although the mechanism underlying this type of anaphylaxis in humans remains unclear.

The signaling cascades mediated by FcεRI in mast cells have been extensively investigated¹². FcεRI is a heterotetrameric receptor composed of an IgE-binding α-subunit, a four-transmembrane-spanning β-subunit and two disulfide-bonded γ-subunits (αβγ₂). Aggregation of FcεRI by crosslinking of FcεRI-bound IgE induces activation of the Src family protein tyrosine kinase Lyn that is bound to the FcεRI β-subunit and activation of the nonreceptor protein tyrosine kinase Syk. The activated Syk then phosphorylates tyrosine residues in the immunoreceptor tyrosine-based activation motif of the β- and γ-subunits of FcεRI (ref. 13). This early step in the activation signal leads to the phosphorylation of adaptor proteins, including Lat, SLP76 and Vav, and to the activation of downstream signaling pathways⁶.

FcεRI-mediated mast cell activation is modulated by several cell-surface inhibitory receptors¹⁴, including FcγRIIB (CD32; A000556)¹⁵, PIR-B^{16,17}, gp49B1 (ref. 18), MAIR-I (also called CLM-8, LMIR1 or CD300a)^{19–21}, MAFA^{22,23} and SIRP-α²⁴. The cytoplasmic portion of these inhibitory receptors commonly contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs). When these inhibitory receptors are coligated with FcεRI, the tyrosine residue in the ITIM is phosphorylated and recruits the tyrosine phosphatases SHP-1 (A002156) and SHP-2 (A002157) and/or the inositol phosphatase SHIP (A002154), thereby blocking the early step in the activation signal mediated by FcεRI. The regulatory mechanisms of FcεRI-mediated mast cell activation have not been completely elucidated, and a molecular target that controls allergic responses for the prevention or treatment of anaphylaxis has not been identified. Here we identify a previously unknown inhibitory immunoglobulin-like receptor, allergy inhibitory receptor 1 (Allergin-1), that was

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preferentially expressed on mast cells, and we explore its role in mast cell-mediated allergic responses *in vivo* and *in vitro*.

RESULTS

Identification of the gene encoding Allergen-1

To study the role of cell surface receptors in regulating the differentiation and activation of hematopoietic cells, we cloned several cDNA fragments encoding putative transmembrane proteins from a cDNA library derived from the human bone marrow stromal cell line HAS303 (ref. 25) by using the signal sequence-trap method²⁶. We isolated full-length cDNAs from a random-primed cDNA library derived from the HAS303 cell line. We identified cDNA encoding Allergen-1L (Allergen-1 long form), which included a 19-amino acid leader sequence, a 208-amino acid extracellular region composed of two immunoglobulin-like domains, a 21-amino acid transmembrane domain and a 95-amino acid cytoplasmic domain (Fig. 1a,b). We also identified two cDNAs encoding Allergen-1 isoforms that lacked the first or second immunoglobulin-like domain in the extracellular portion; we called these Allergen-1S1 (Allergen-1 short form 1) and Allergen-1S2 (Allergen-1 short form 2), respectively (Fig. 1b). The extracellular portions of mouse Allergen-1 and human Allergen-1L and those of human Allergen-1S1 and Allergen-1S2 contained six and three potential N-linked glycosylation sites, respectively (Fig. 1a,b).

Genomic DNA database analysis demonstrated that the human gene encoding Allergen-1 consisted of ten exons, but those encoding Allergen-1S1 and Allergen-1S2 lacked exons 4 and 3, respectively (Supplementary Fig. 1). A database search identified mouse and rat homologs of Allergen-1 that contained a single immunoglobulin-like domain with amino acid identities of 50% and 52% with that of human Allergen-1S1 (Fig. 1a,b). Although the rat Allergen-1 cDNA has been reported to be MCA-32 (ref. 27), the molecular and functional characteristics of Allergen-1 have not been reported. The gene encoding Allergen-1 is located on chromosome 17q23.3 in humans (*ALLERGIN-1*) and chromosome 11E1 in mice (*Allergen-1*), near genes of the *Cd300* family that encode immunoglobulin-like receptors that mediate positive or negative signaling in myeloid cells^{28,29} (Fig. 1c). *Allergen-1* was also close to the gene encoding PECAM-1 (CD31), an ITIM-bearing immunoglobulin-like receptor expressed on myeloid cells and platelets. Genomic DNA database analysis demonstrated that like PECAM-1, but unlike CD300, Allergen-1 is encoded by a single gene in both human and mouse (data not shown).

Biochemical characteristics of Allergen-1

To study the biochemical characteristics of Allergen-1, we established transfectants of the RBL-2H3 rat basophil leukemia cell line and Ba/F3 pro-B cell line stably expressing mouse Allergen-1 tagged with Flag

protein at the amino terminus (Supplementary Fig. 2). Immunoprecipitation of Allergen-1 protein from lysates of RBL-2H3 transfectants, followed by immunoblot analysis with antibody to Flag (anti-Flag), showed that mouse Allergen-1 had a molecular mass of ~60 kilodaltons (kDa) under both reducing and nonreducing conditions (Fig. 1d), which suggested that mouse Allergen-1 is a monomeric protein. However, the molecular masses of human Allergen-1L, Allergen-1S1 and Allergen-1S2 seemed to be ~70 kDa, ~40 kDa and ~40 kDa, respectively, under reducing conditions (Fig. 1e), although we also observed bands of greater molecular mass for human Allergen-1S1 and Allergen-1S2 as well as mouse Allergen-1 (Fig. 1d,e). As both mouse and human Allergen-1 contained several potential N-linked glycosylation sites, these 'plural' bands might be dependent on different mobilities of the heavily glycosylated proteins. Indeed, treatment with N-glycosidase F substantially decreased the mass of mouse and human Allergen-1, consistent with that of the polypeptide we predicted from the Allergen-1 cDNA and the presence of potential N-linked glycosylation sites in the extracellular portion (Fig. 1d,e).

Expression of Allergen-1

To investigate the cellular distribution of Allergen-1 expression, we generated monoclonal antibodies to mouse Allergen-1 (Fig. 2a). Flow cytometry of spleen cells demonstrated that Allergen-1 was expressed on myeloid lineage cells, including dendritic cells, macrophages and neutrophils, but not on T cells, B cells or

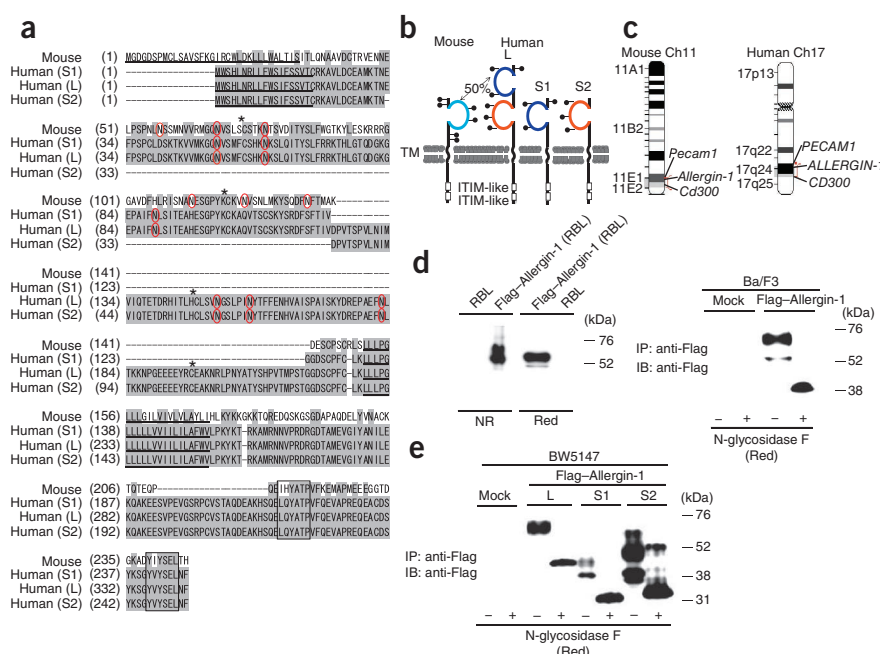


Figure 1 Molecular characteristics of Allergen-1. (a) Amino acid sequences of mouse Allergen-1 and human Allergen-1L (L), Allergen-1S1 (S1) and Allergen-1S2 (S2). Numbers in parentheses (left margin) indicate amino acid positions; shading indicates identical amino acid residues; underlining indicates putative leader and transmembrane sequences; red circles outline potential N-linked glycosylation sites in the extracellular domain; * indicates cysteine residues potentially involved in disulfide bonding of the immunoglobulin-like domains; boxes outline ITIM-like motifs. (b) Mouse and human Allergen-1 proteins. TM, transmembrane domain; black circles indicate potential N-linked glycosylation. The amino acid sequences of the first (blue) and second (orange) immunoglobulin-like domains in human Allergen-1L are identical to those of Allergen-1S1 and Allergen-1S2. (c) Chromosomal locations of *Allergen-1* and *ALLERGIN-1*. Ch, chromosome. (d,e) Immunoprecipitation (IP) of lysates of RBL-2H3 cells (RBL) and Ba/F3 cells and their transfectants expressing Flag-tagged mouse Allergen-1 (d) and BW5147 transfectants (e), as demonstrated in Figure 3a, with Flag-specific mAb (anti-Flag), followed by no treatment (–) or treatment with N-glycosidase F (+) and immunoblot analysis (IB) with polyclonal anti-Flag in reducing (Red) or nonreducing (NR) conditions. Data are representative of two (d) or three (e) independent experiments.

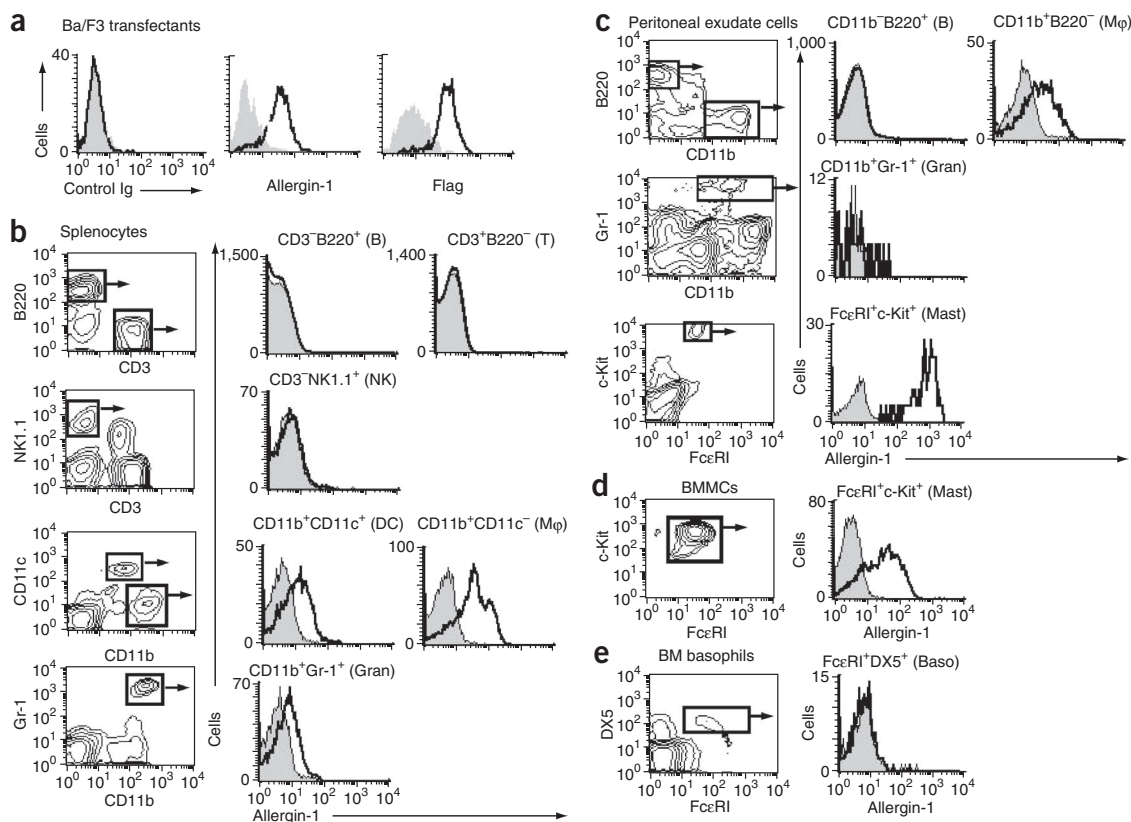


Figure 2 Expression of mouse Allergen-1 protein. (a) Flow cytometry of Ba/F3 cells (shaded) or Ba/F3 transfectants (5×10^5 cells per experiment) expressing Flag-tagged mouse Allergen-1 (thick lines), stained with biotinylated control mouse immunoglobulin, anti-mouse Allergen-1 (TX83) or mAb to Flag, followed by allophycocyanin (APC)-conjugated streptavidin. (b–e) Flow cytometry of splenocytes (1×10^7 cells per experiment; b), peritoneal exudate cells (1×10^6 to 5×10^6 cells per experiment; c), BMMCs (5×10^5 cells per experiment; d) and bone marrow cells (1×10^7 cells per experiment; e) from C57BL/6N mice ($n = 3$), stained with fluorescein isothiocyanate-conjugated (horizontal axes; left) and phycoerythrin-conjugated (vertical axes; left) monoclonal antibodies, or with biotinylated F(ab')₂ fragment of anti-Allergen-1 (open histograms; middle and right) or isotype-matched control antibody (shaded histograms; middle and right), followed by allophycocyanin-conjugated streptavidin. Cells in outlined areas (left) were gated and analyzed for Allergen-1 expression (middle and right). B, B cell; T, T cell; NK, natural killer cell; DC, dendritic cell; Mφ, macrophage; Gran, granulocyte; Mast, mast cell; Baso, basophil. Data are representative of three independent experiments.

natural killer cells (Fig. 2b). However, mast cells from the peritoneal cavity had the highest expression of Allergen-1 (Fig. 2c). Bone marrow-derived cultured mast cells (BMMCs) also expressed Allergen-1 (Fig. 2d). In contrast, we did not detect Allergen-1 on basophils from the bone marrow (Fig. 2e). These results suggest that Allergen-1 may be involved in immune responses mediated by myeloid cells.

We also generated a monoclonal antibody (mAb) that specifically recognized human Allergen-1L and Allergen-1S1 but not Allergen-1S2 (Fig. 3a). In agreement with results obtained for mouse Allergen-1, expressed on myeloid cell lineages, including monocytes, neutrophils and dendritic cells, from the peripheral blood also expressed human Allergen-1L and/or Allergen-1S1 (Fig. 3b). Although T cells and natural killer cells did not express Allergen-1L or Allergen-1S1, we detected weak expression of Allergen-1L and/or Allergen-1S1 on B cells. We noted that unlike mouse Allergen-1, human Allergen-1L and/or Allergen-1S1 had considerable expression on peripheral blood basophils (Fig. 3b). To determine whether Allergen-1 was expressed on human mast cells, we generated cultured mast cells derived from the CD34⁺ cells among the peripheral blood mononuclear cells. These cultured human mast cells also expressed Allergen-1L and/or Allergen-1S1 (Fig. 3c), consistent with results obtained for mouse Allergen-1.

Mouse Allergen-1 recruits phosphatases

The cytoplasmic regions of human and mouse Allergen-1 contain six and five tyrosine residues, respectively. The tyrosine residues at positions 313 and 338 in humans and those at positions 216 and 241 in mice may form part of ITIM-like sequences (Figs. 1a and 4a), which suggests that Allergen-1 may be tyrosine phosphorylated and could recruit phosphatases such as SHP-1 or SHP-2, or SHIP. To test that hypothesis, we treated mouse Allergen-1-expressing RBL-2H3 transfectants with pervanadate, which stimulates protein tyrosine phosphorylation. Immunoprecipitation of Allergen-1 with Flag-specific mAb demonstrated that Allergen-1 was tyrosine phosphorylated and recruited SHP-1, SHP-2 and SHIP (Fig. 4b). To determine which tyrosine residues were phosphorylated and responsible for recruitment of the phosphatases, we established RBL-2H3 transfectants that stably expressed Flag-tagged mutant Allergen-1 in which the tyrosine residue at position 216 or 241, or those at both positions 216 and 241, were replaced with phenylalanine (Supplementary Fig. 2). All the mutant Allergen-1 proteins, as well as wild-type Allergen-1, were tyrosine phosphorylated (Fig. 4b), which suggested that tyrosine residues at positions other than 216 and 241 were also phosphorylated. Allergen-1 with substitution at residues 216 and 241 did not recruit SHP-1 or SHP-2 (Fig. 4b), which indicated that the presence of tyrosine residues at these positions was required for the recruitment

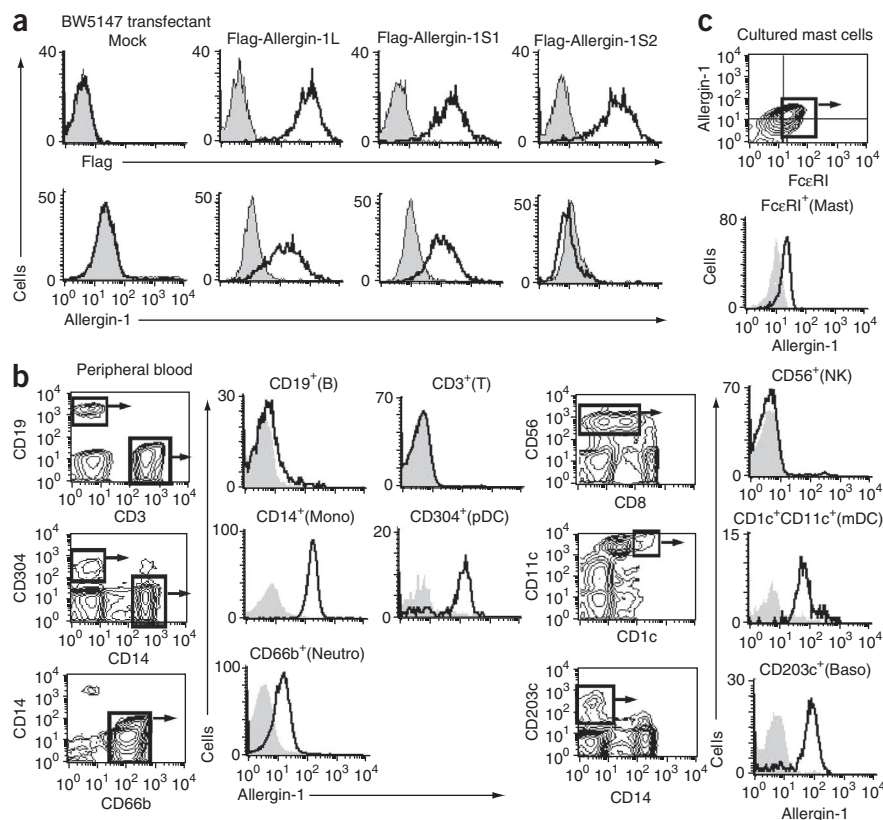


Figure 3 Expression of human Allergen-1 protein. (a) Flow cytometry of BW5147 cells transfected with mock vector or cDNA encoding Flag-tagged human Allergen-1, stained with biotinylated control mouse immunoglobulin (shaded), anti-human Allergen-1 (EX33; thick line, bottom) or mAb to Flag (thick line, top), followed by allophycocyanin-conjugated streptavidin. (b,c) Flow cytometry of peripheral blood mononuclear cells (1×10^6 cells per experiment; b) or cultured mast cells (1×10^5 cells per experiment; c) simultaneously stained with fluorescein isothiocyanate-conjugated (horizontal axes; left) and phycoerythrin-conjugated (vertical axes; left) monoclonal antibodies, or with unlabeled anti-human Allergen-1 (shaded histograms; middle and right) or control immunoglobulin (open histograms; middle and right), followed by Alexa Fluor 647-conjugated anti-human Allergen-1. Cells in outlined areas (left) were gated and analyzed for Allergen-1 expression (middle and right). Mono, monocyte; pDC, plasmacytoid dendritic cell; Neutro, neutrophil; mDC, monocytic dendritic cell. Data are representative of three independent experiments.

of SHP-1 and SHP-2. In contrast, SHIP was recruited by each of the mutant allergen-1 proteins (Fig. 4b), which suggested that the tyrosine residues at position(s) 175, 200 and/or 239 might be involved in the recruitment of SHIP.

Mouse Allergen-1 inhibits FcεRI-mediated degranulation

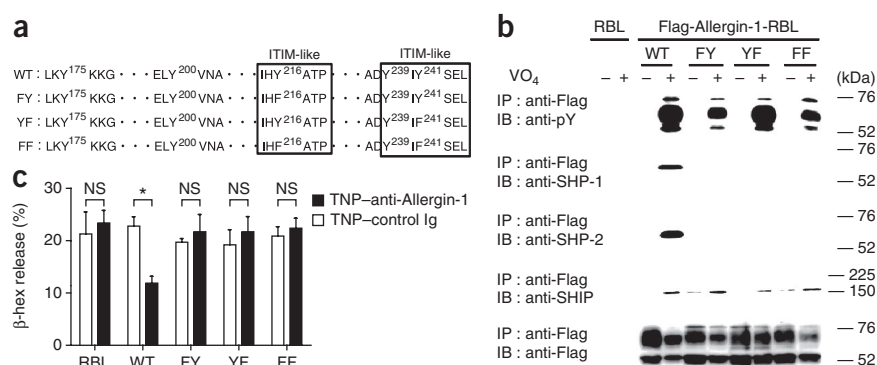
Because mast cells have high surface expression of Allergen-1, we next examined whether mouse Allergen-1 mediates an inhibitory signal for the FcεRI-mediated degranulation of RBL-2H3 cells. We stimulated wild-type Allergen-1-expressing RBL-2H3 transfectants with IgE antibody to 2,4,6-trinitrophenyl (TNP) together with TNP-conjugated control antibody or Allergen-1-specific mAb. Coligation of Allergen-1 with FcεRI resulted in significantly less degranulation than

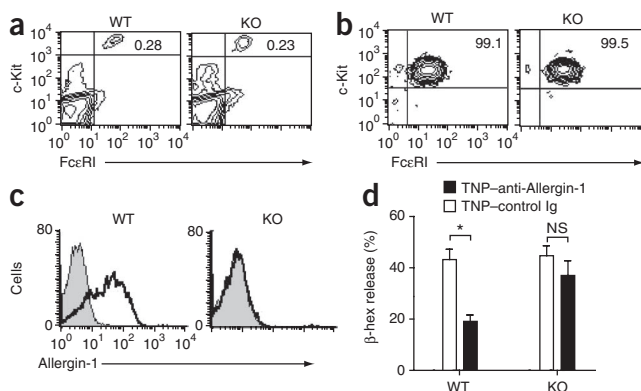
did FcεRI stimulation alone, as determined by the release of β-hexosaminidase from the transfectants (Fig. 4c); this suggested that mouse Allergen-1 inhibits IgE-mediated degranulation of RBL-2H3 cells. However, degranulation was not affected in RBL-2H3 transfectants expressing mutant Allergen-1 with replacement of tyrosine at position(s) 216 and/or 241 with phenylalanine. This finding indicates that both tyrosine residues at positions 216 and 241 were involved in the inhibitory effect on degranulation. Together these results suggest that mouse Allergen-1 recruits SHP-1 and SHP-2 through the phosphorylation of tyrosine residues at positions 216 and 241 and mediates an inhibitory signal for FcεRI-mediated degranulation of RBL-2H3 cells.

Generation of Allergen-1-deficient mice

To study the function of Allergen-1 in immune responses, we generated mice lacking the gene encoding Allergen-1 (*Allergen-1*;

Figure 4 Signal transduction via ITIM-like motifs of mouse Allergen-1. (a) Wild-type (WT) Allergen-1 (top), with tyrosine residues in the cytoplasmic domain at positions 175, 200, 216, 239 and 241; below, site-specific mutant Allergen-1 constructs with replacement of tyrosine (Y) with phenylalanine (F) at position 216 (FY) or 241 (YF) or both (FF). Boxes outline tyrosine residues in ITIM-like motifs. (b) Tyrosine phosphorylation (pY) of Allergen-1 and recruitment of phosphatases in RBL-2H3 transfectants expressing wild-type or mutant Allergen-1, left unstimulated (–) or stimulated (+) with pervanadate (VO_4) and lysed with 1% digitonin buffer, followed by immunoprecipitation of Allergen-1 with mAb to Flag and immunoblot analysis. Data are representative of five independent experiments. (c) Release of β-hexosaminidase (β-hex) from RBL-2H3 cells and their transfectants expressing wild-type or mutant Allergen-1, sensitized with mouse IgE anti-TNP and then stimulated TX83 mAb to Allergen-1 (TNP-anti-Allergen-1), presented relative to release from cells sensitized with mouse IgE anti-TNP and stimulated with TNP-conjugated F(ab')₂ fragments of control immunoglobulin (TNP-control Ig). NS, not significant; * $P = 0.001$ (unpaired Student's *t*-test). Data are representative of five independent experiments (mean and s.d. of triplicates).





Supplementary Fig. 3). Naive *Allergin-1*^{-/-} mice had normal composition of lymphocyte and myeloid cell populations in the spleen, bone marrow, thymus and peritoneal cavity (Fig. 5a, Supplementary Fig. 4 and Supplementary Table 1). Moreover, we did not observe any difference between wild-type and *Allergin-1*^{-/-} mice in the development of mast cells cultured from bone marrow cells in the presence of the cytokines interleukin 3 and stem cell factor (Fig. 5b). These results suggest that disruption of *Allergin-1* did not affect differentiation of hematopoietic cells, including mast cells.

To determine whether Allergin-1 inhibits FcεRI-mediated degranulation of mast cells, as well as of RBL-2H3 cells, we generated BMMCs from wild-type and *Allergin-1*^{-/-} mice (Fig. 5c) and stimulated the cells with IgE anti-TNP together with either TNP-conjugated control antibody or mAb to Allergin-1. Stimulation with mAb to Allergin-1 resulted in significantly less FcεRI-mediated release of β-hexosaminidase from wild-type BMMCs ($P = 0.0008$) but not from *Allergin-1*^{-/-} BMMCs ($P = 0.074$; Fig. 5d). These results indicate that Allergin-1 inhibits FcεRI-mediated degranulation of BMMCs as well as RBL-2H3 cells.

Mouse Allergin-1 suppresses passive systemic anaphylaxis

As Allergin-1 inhibited FcεRI-mediated degranulation of mast cells *in vitro*, we next determined whether Allergin-1 was involved in passive systemic anaphylaxis, an IgE-mediated type I immediate hypersensitivity reaction. We passively sensitized wild-type and *Allergin-1*^{-/-} mice with IgE mAb to TNP and then injected them intravenously with ovalbumin (OVA) or TNP-conjugated OVA (TNP-OVA). We measured rectal temperature, an indicator of passive systemic anaphylaxis, every 6 min after injection. Neither wild-type nor *Allergin-1*^{-/-} mice injected with OVA showed any change in rectal temperature. In contrast, wild-type mice challenged with TNP-OVA showed a progressive decrease in rectal temperature to 4 °C below the basal temperature by 18 min after injection, followed by a gradual recovery to basal temperature (Fig. 6a). *Allergin-1*^{-/-} mice challenged with TNP-OVA showed significantly lower rectal temperatures than the corresponding *Allergin-1*^{+/+} mice during the entire 180-minute observation period (Fig. 6a).

Figure 5 Normal development of mast cells in Allergin-1-deficient mice. (a,b) Flow cytometry of peritoneal exudate cells (5×10^6 cells per experiment; a) or BMMCs (5×10^5 cells per experiment; b) derived from wild-type (WT) or *Allergin-1*^{-/-} (KO) mice and stained with mAb to c-Kit and FcεRI. Numbers in top right quadrants indicate percent mast cells. (c) Flow cytometry of BMMCs derived from wild-type and *Allergin-1*^{-/-} mice and stained with TX83 mAb to Allergin-1 (thick lines) or isotype-matched control antibody (shaded histograms). (d) Release of β-hexosaminidase from BMMCs derived from wild-type and *Allergin-1*^{-/-} mice, stimulated and presented as described in Figure 4c. * $P < 0.001$ (unpaired Student's *t*-test). Data are representative of five independent experiments (error bars (d), s.d. of triplicates).

Moreover, although mast cell-deficient (*W^{sh}/W^{sh}*) mice did not show the decrease in rectal temperature, mice transplanted with BMMCs derived from *Allergin-1*^{-/-} mice showed significantly more passive systemic anaphylaxis than did those given transplantation of BMMCs derived from *Allergin-1*^{+/+} mice (Fig. 6b). These results suggest that Allergin-1 negatively regulates IgE-mediated mast cell activation *in vivo* and suppresses the type I immediate hypersensitivity reaction.

Mouse Allergin-1 suppresses passive cutaneous anaphylaxis

We next determined whether Allergin-1 was also involved in IgE-mediated passive cutaneous anaphylaxis reaction in the ear, which typically develops in the late phase (2–6 h) after allergen exposure. We passively sensitized wild-type and *Allergin-1*^{-/-} mice by intravenous injection of dinitrophenol-specific IgE mAb and then challenged them by epicutaneous application of acetone and olive oil alone in the right ear and dinitrofluorobenzene (DNFB) in acetone and olive oil in the left ear. The net swelling in the left ear, as determined by the difference in the thickness of the left and right ears, of both *Allergin-1*^{+/+} or *Allergin-1*^{-/-} mice was greatly increased by 4 h after the antigen challenge (Fig. 7a). The ear swelling in *Allergin-1*^{-/-} mice was significantly greater than that in wild-type mice during the entire 50-hour observation period after the antigen challenge. In support of those results, histological analysis demonstrated that the ear swelling in *Allergin-1*^{-/-} mice 24 h after application of DNFB was considerably greater than that in wild-type mice (Fig. 7b).

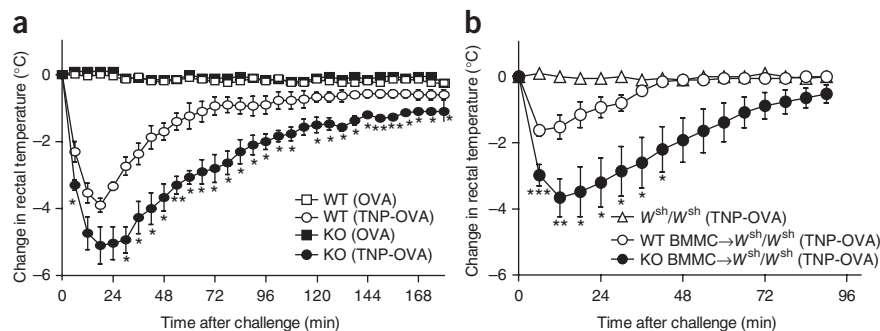


Figure 6 Enhanced systemic anaphylaxis in Allergin-1-deficient mice. (a) Rectal temperatures of wild-type (*Allergin-1*^{+/+}) mice and *Allergin-1*^{-/-} mice ($n = 3$ per genotype) injected intravenously with 20 μg mouse IgE mAb to TNP, then challenged 24 h later with 1 mg OVA or TNP-OVA, assessed every 6 min. (b) Rectal temperatures of mast cell-deficient mice given no BMMCs (*W^{sh}/W^{sh}*; $n = 2$) or given adoptive transfer of BMMCs derived from wild-type mice (WT BMMC → *W^{sh}/W^{sh}*; $n = 5$) or *Allergin-1*^{-/-} mice (KO BMMC → *W^{sh}/W^{sh}*; $n = 5$), then sensitized 3 months later with 20 μg mouse IgE anti-TNP, followed by challenge with 1 mg TNP-OVA. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$, wild-type versus *Allergin-1*^{-/-} mice challenged with TNP-OVA (unpaired Student's *t*-test). Data are representative of five (a) or two (b) independent experiments (mean ± s.e.m.).

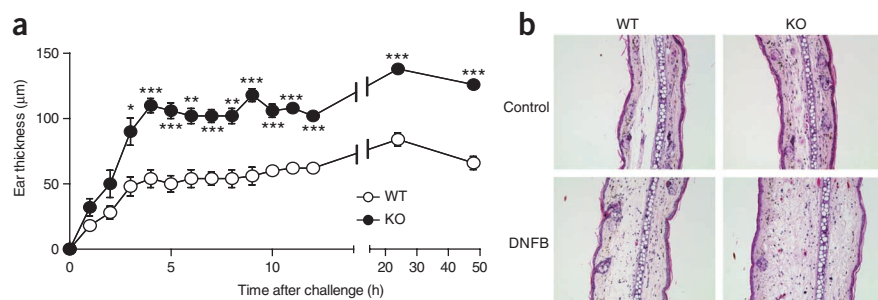


Figure 7 Enhanced passive cutaneous anaphylaxis in Allergin-1-deficient mice. **(a)** Ear swelling of wild-type (*Allergin-1*^{+/+}) mice (*n* = 5) and *Allergin-1*^{-/-} mice (*n* = 5) injected intravenously with 2 μg mouse IgE mAb to dinitrophenol, then challenged 24 h later by epicutaneous application of acetone and olive oil alone (right ear) or DNFB in acetone and olive oil (left ear), calculated as the difference between the thickness of the right and left ear. **P* < 0.05, ***P* < 0.001 and ****P* < 0.0005, wild-type versus *Allergin-1*^{-/-} (unpaired Student's *t*-test). Data are representative of three independent experiments (error bars, s.e.m.). **(b)** Histological sections of the right ears (Control) and left ears (DNFB) of wild-type and *Allergin-1*^{-/-} mice treated as in **a**, stained with hematoxylin and eosin. Scale bar, 0.2 mm. Data are representative of three independent experiments.

DISCUSSION

Progress in mast cell biology has demonstrated that mast cell activation is negatively regulated by several inhibitory mechanisms, including cell surface inhibitory receptors, anti-inflammatory molecules such as transforming growth factor-β³⁰, interleukin 10 (ref. 31), retinol^{32,33} and β₂-adrenoreceptor agonists³⁴. In the present study we have identified the inhibitory immunoglobulin-like receptor Allergin-1, which contains an ITIM-like motif in the cytoplasmic portion and inhibited IgE-mediated degranulation of BMMCs. IgE-mediated mouse mast cell degranulation can be inhibited by crosslinking of FcεRI to the ITIM-containing cell surface receptors, including FcγRIIB³⁵, PIR-B³⁶, gp49B1³⁷, MAIR-I (CLM-8, LMIR or Cd300a)^{19,21}, MAFA^{22,23} and SIRP-α²⁴. Although CD200 does not bear an ITIM, it is still able to inhibit FcεRI-mediated mouse mast cell activation by binding SHIP³⁸. Among the inhibitory receptors expressed on mast cells, FcγRIIB, SIRP-α, CD300a (CMRF35) and LILR-B2 (a human ortholog of PIR-B³⁹) have also been shown to inhibit IgE-mediated degranulation of human mast cells¹⁴. *In vivo* studies have shown that IgE-mediated systemic or cutaneous anaphylaxis is enhanced in mice deficient in FcγRIIB⁴⁰, PIR-B³⁹ or gp49B1 (ref. 41), which demonstrates that these receptors suppress FcεRI-mediated mast cell activation *in vivo* as well as *in vitro*. FcγRIIB and gp49B1 recruit SHIP and SHP-1, respectively, after tyrosine phosphorylation, and these phosphatases are essential for the inhibition of FcεRI-mediated mast cell activation⁴². In contrast, although PIR-B constitutively associates with SHP-1, it is dispensable for PIR-B-mediated inhibition of FcεRI-mediated mast cell activation³⁶. An ITIM-recruiting molecule responsible for the suppression of mast cell activation by PIR-B has not yet been identified.

We demonstrated that mouse Allergin-1 recruited SHP-1, SHP-2 and SHIP in RBL-2H3 cells stimulated with pervanadate. Allergin-1 with substitution of either Tyr216 or Tyr241 recruited neither SHP-1 nor SHP-2, which indicates that SHP-1 and SHP-2 require both tyrosine residues to bind to Allergin-1. Those results are in agreement with the functional analyses showing that the inhibitory effect of Allergin-1 on degranulation of mast cells required both Tyr216 and Tyr241. The KIR inhibitory natural killer cell receptors are also reported to require two ITIMs for the recruitment of tyrosine phosphatases and inhibitory signaling⁴³. Allergin-1 with substitution at residues 216 and 241 was still tyrosine phosphorylated and recruited SHIP when stimulated with pervanadate, which suggests that tyrosine

residues other than those at positions 216 and 241 (that is, position(s) 175, 200 and/or 239) are also phosphorylated and involved in the recruitment of SHIP. Nonetheless, our results suggest that SHP-1 and SHP-2, but not SHIP, are responsible for Allergin-1-based negative regulation of FcεRI-mediated degranulation of mast cells.

At present, a ligand for Allergin-1 has not yet been identified. However, Allergin-1-deficient mice showed enhanced IgE-mediated, mast cell-dependent passive systemic anaphylaxis as early as 6 min after antigen challenge, which suggests that Allergin-1 on tissue mast cells might be tyrosine phosphorylated as a result of *cis* or *trans* interaction with a putative ligand expressed on mast cells themselves or surrounding cells in tissues before or just after antigen capture by FcεRI. PIR-B, which binds to major histo-

compatibility complex class I molecules³⁹, is constitutively tyrosine phosphorylated³⁶; thus, it might be possible that PIR-B modulates basal activation of mast cells. However, FcγRIIB might downmodulate FcεRI-mediated signaling after binding to the IgG immune complex⁴⁴. We observed neither tyrosine phosphorylation of Allergin-1 expressed on BMMCs or RBL-2H3 transfectants nor recruitment of phosphatases by Allergin-1 before stimulation of Allergin-1 with mAb *in vitro*. However, it remains undetermined whether Allergin-1 is constitutively tyrosine phosphorylated *in vivo*. To further understand the molecular mechanism of Allergin-1-mediated regulation of mast cell activation, it will be essential to identify a ligand for Allergin-1.

As Allergin-1 is expressed on mast cells but not on basophils in mice, the enhanced IgE-mediated allergic inflammation in Allergin-1-deficient mice was dependent on mast cells. However, we demonstrated that in humans, basophils as well as mast cells had abundant surface expression of Allergin-1. Studies have demonstrated that basophils are important in IgG-mediated systemic anaphylaxis and IgE-mediated chronic allergic inflammation in mice^{11,45,46}. Although the role of basophils in human allergic inflammation remains undetermined, Allergin-1 might be involved not only in mast cell-dependent allergic responses but also in basophil-dependent allergic responses in humans.

We have demonstrated that Allergin-1 was expressed on macrophages, neutrophils and dendritic cells as well as mast cells and/or basophils in both humans and mice. Human Allergin-1 was also expressed on B cells. This broad expression pattern on various cell types is similar to that of other immunoglobulin-like inhibitory receptors, such as FcγRIIB, PIR-B, gp49B1, MAIR-I and SIRP-α. These receptors are involved in a variety of immune responses other than the activation of mast cells or basophils. For example, FcγRIIB interferes with B cell antigen receptor-mediated signals and inhibits B cell activation. Mice deficient in FcγRIIB are susceptible to induced autoimmune diseases, such as collagen-induced arthritis, and also spontaneously develop lupus in a strain-specific manner¹⁴. SIRP-α binds CD47, which is expressed on many cell populations, including red blood cells. Interaction with SIRP-α and CD47 constitutively inhibits FcγR-dependent and FcγR-independent phagocytosis by macrophages⁴⁷. At present, the function of Allergin-1 in immune responses that involve myeloid cells other than mast cells remains to be defined. Further studies are needed to determine the role of Allergin-1 in allergic and nonallergic immune responses.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Accession codes. UCSD-Nature Signaling Gateway (<http://www.signaling-gateway.org>): A000537, A000556, A002156, A002157 and A002154; GenBank: human *ALLERGIN-1L*, AB542950; human *ALLERGIN-1S1*, AB542951; human *ALLERGIN-1S2*, AB542952; mouse *Allergin-1*, AB542953.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

K.H., S.S., A.F., H.T. and T.S. did experiments and analyzed data; S.S. and K.S. contributed to experimental design and data interpretation; S.T.-H. designed and did experiments, analyzed data and wrote the paper; and A.S. supervised the overall project and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/natureimmunology/>.

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1. Sampson, H.A. *et al.* Second symposium on the definition and management of anaphylaxis: summary report—Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. *J. Allergy Clin. Immunol.* **117**, 391–397 (2006).
2. Sampson, H.A. *et al.* Symposium on the definition and management of anaphylaxis: summary report. *J. Allergy Clin. Immunol.* **115**, 584–591 (2005).
3. Clark, S. & Camargo, C.A. Jr. Epidemiology of anaphylaxis. *Immunol. Allergy Clin. North Am.* **27**, 145–163 (2007).
4. Galli, S.J. Mast cells and basophils. *Curr. Opin. Hematol.* **7**, 32–39 (2000).
5. Schwartz, L.B. Mast cells: function and contents. *Curr. Opin. Immunol.* **6**, 91–97 (1994).
6. Kawakami, T. & Galli, S.J. Regulation of mast-cell and basophil function and survival by IgE. *Nat. Rev. Immunol.* **2**, 773–786 (2002).
7. Dombrowicz, D. *et al.* Absence of FcεRI α chain results in upregulation of FcγRIII-dependent mast cell degranulation and anaphylaxis. Evidence of competition between FcεRI and FcγRIII for limiting amounts of FcR β and γ chains. *J. Clin. Invest.* **99**, 915–925 (1997).
8. Jacoby, W., Cammarata, P.V., Findlay, S. & Pincus, S.H. Anaphylaxis in mast cell-deficient mice. *J. Invest. Dermatol.* **83**, 302–304 (1984).
9. Miyajima, I. *et al.* Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and FcγRIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. *J. Clin. Invest.* **99**, 901–914 (1997).
10. Oettgen, H.C. *et al.* Active anaphylaxis in IgE-deficient mice. *Nature* **370**, 367–370 (1994).
11. Tsujimura, Y. *et al.* Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. *Immunity* **28**, 581–589 (2008).
12. Kraft, S. & Kinet, J.P. New developments in FcεRI regulation, function and inhibition. *Nat. Rev. Immunol.* **7**, 365–378 (2007).
13. Jouvin, M.H. *et al.* Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. *J. Biol. Chem.* **269**, 5918–5925 (1994).
14. Li, L. & Yao, Z. Mast cell and immune inhibitory receptors. *Cell. Mol. Immunol.* **1**, 408–415 (2004).
15. Malbec, O. *et al.* Fc epsilon receptor I-associated Lyn-dependent phosphorylation of Fcγ receptor IIB during negative regulation of mast cell activation. *J. Immunol.* **160**, 1647–1658 (1998).
16. Maeda, A., Kurosaki, M., Ono, M., Takai, T. & Kurosaki, T. Requirement of SH2-containing protein tyrosine phosphatases SHP-1 and SHP-2 for paired immunoglobulin-like receptor B (PIR-B)-mediated inhibitory signal. *J. Exp. Med.* **187**, 1355–1360 (1998).
17. Yamashita, Y., Ono, M. & Takai, T. Inhibitory and stimulatory functions of paired Ig-like receptor (PIR) family in RBL-2H3 cells. *J. Immunol.* **161**, 4042–4047 (1998).
18. Katz, H.R. *et al.* Mouse mast cell gp49B1 contains two immunoreceptor tyrosine-based inhibition motifs and suppresses mast cell activation when coligated with the high-affinity Fc receptor for IgE. *Proc. Natl. Acad. Sci. USA* **93**, 10809–10814 (1996).
19. Yotsumoto, K. *et al.* Paired activating and inhibitory immunoglobulin-like receptors, MAIR-I and MAIR-II, regulate mast cell and macrophage activation. *J. Exp. Med.* **198**, 223–233 (2003).
20. Kumagai, H. *et al.* Identification and characterization of a new pair of immunoglobulin-like receptors LMIR1 and 2 derived from murine bone marrow-derived mast cells. *Biochem. Biophys. Res. Commun.* **307**, 719–729 (2003).
21. Bachelet, I., Munitz, A., Moretta, A., Moretta, L. & Levi-Strauss, F. The inhibitory receptor IRp60 (CD300a) is expressed and functional on human mast cells. *J. Immunol.* **175**, 7989–7995 (2005).
22. Jurgens, L., Arndt-Jovin, D., Pecht, I. & Jovin, T.M. Proximity relationships between the type I receptor for Fcε (FcεRI) and the mast cell function-associated antigen (MAFA) studied by donor photobleaching fluorescence resonance energy transfer microscopy. *Eur. J. Immunol.* **26**, 84–91 (1996).
23. Xu, R., Abramson, J., Fridkin, M. & Pecht, I. SH2 domain-containing inositol polyphosphate 5'-phosphatase is the main mediator of the inhibitory action of the mast cell function-associated antigen. *J. Immunol.* **167**, 6394–6402 (2001).
24. Lienard, H., Bruhns, P., Malbec, O., Fridman, W.H. & Daeron, M. Signal regulatory proteins negatively regulate immunoreceptor-dependent cell activation. *J. Biol. Chem.* **274**, 32493–32499 (1999).
25. Aizawa, S. *et al.* Hematopoietic supportive function of human bone marrow stromal cell lines established by a recombinant SV40-adenovirus vector. *Exp. Hematol.* **22**, 482–487 (1994).
26. Jacobs, K.A. *et al.* A genetic selection for isolating cDNAs encoding secreted proteins. *Gene* **198**, 289–296 (1997).
27. Pirozzi, G., Terry, R.W., Epstein, D. & Labow, M.A. Identification and characterization of a novel surface antigen gene induced in mast cells activated through the high affinity IgE receptor. *J. Immunol.* **155**, 5811–5818 (1995).
28. Shibuya, A., Nakahashi-Oda, C. & Tahara-Hanaoka, S. Regulation of immune responses by the activating and inhibitory myeloid-associated immunoglobulin-like receptors (MAIR) (CD300). *Immune Netw.* **9**, 41–45 (2009).
29. Clark, G.J., Ju, X., Tate, C. & Hart, D.N. The CD300 family of molecules are evolutionarily significant regulators of leukocyte functions. *Trends Immunol.* **30**, 209–217 (2009).
30. Gebhardt, T. *et al.* Growth, phenotype, and function of human intestinal mast cells are tightly regulated by transforming growth factor β1. *Gut* **54**, 928–934 (2005).
31. Royer, B. *et al.* Inhibition of IgE-induced activation of human mast cells by IL-10. *Clin. Exp. Allergy* **31**, 694–704 (2001).
32. Hjertson, M. *et al.* Retinoic acid inhibits *in vitro* development of mast cells but has no marked effect on mature human skin tryptase- and chymase-positive mast cells. *J. Invest. Dermatol.* **120**, 239–245 (2003).
33. Ishida, S., Kinoshita, T., Sugawara, N., Yamashita, T. & Koike, K. Serum inhibitors for human mast cell growth: possible role of retinol. *Allergy* **58**, 1044–1052 (2003).
34. Gebhardt, T. β2-Adrenoceptor-mediated suppression of human intestinal mast cell functions is caused by disruption of filamentous actin dynamics. *Eur. J. Immunol.* **35**, 1124–1132 (2005).
35. Daeron, M., Malbec, O., Latour, S., Arock, M. & Fridman, W.H. Regulation of high-affinity IgE receptor-mediated mast cell activation by murine low-affinity IgG receptors. *J. Clin. Invest.* **95**, 577–585 (1995).
36. Uehara, T. *et al.* Inhibition of IgE-mediated mast cell activation by the paired Ig-like receptor PIR-B. *J. Clin. Invest.* **108**, 1041–1050 (2001).
37. Castells, M.C. *et al.* gp49B1-α₃ interaction inhibits antigen-induced mast cell activation. *Nat. Immunol.* **2**, 436–442 (2001).
38. Zhang, S., Cherwinski, H., Sedgwick, J.D. & Phillips, J.H. Molecular mechanisms of CD200 inhibition of mast cell activation. *J. Immunol.* **173**, 6786–6793 (2004).
39. Masuda, A., Nakamura, A., Maeda, T., Sakamoto, Y. & Takai, T. Cis binding between inhibitory receptors and MHC class I can regulate mast cell activation. *J. Exp. Med.* **204**, 907–920 (2007).
40. Ujike, A. *et al.* Modulation of immunoglobulin (Ig)E-mediated systemic anaphylaxis by low-affinity Fc receptors for IgG. *J. Exp. Med.* **189**, 1573–1579 (1999).
41. Daheshia, M., Friend, D.S., Grusby, M.J., Austen, K.F. & Katz, H.R. Increased severity of local and systemic anaphylactic reactions in gp49B1-deficient mice. *J. Exp. Med.* **194**, 227–234 (2001).
42. Ono, M., Bolland, S., Tempst, P. & Ravetch, J.V. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor FcγRIIB. *Nature* **383**, 263–266 (1996).
43. Bruhns, P., Marchetti, P., Fridman, W.H., Vivier, E. & Daeron, M. Differential roles of N- and C-terminal immunoreceptor tyrosine-based inhibition motifs during inhibition of cell activation by killer cell inhibitory receptors. *J. Immunol.* **162**, 3168–3175 (1999).
44. Daeron, M. *et al.* The same tyrosine-based inhibition motif, in the intracytoplasmic domain of FcγRIIB, regulates negatively BCR-, TCR-, and FcR-dependent cell activation. *Immunity* **3**, 635–646 (1995).
45. Mukai, K. *et al.* 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 (2005).
46. Obata, K. *et al.* Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood* **110**, 913–920 (2007).
47. Matozaki, T., Murata, Y., Okazawa, H. & Ohnishi, H. Functions and molecular mechanisms of the CD47-SIRPα signalling pathway. *Trends Cell Biol.* **19**, 72–80 (2009).

ONLINE METHODS

Cloning of Allergen-1 cDNA. Human Allergen-1 cDNA was isolated from a cDNA library in which cDNA fragments derived from the HAS303 cell line were ligated downstream of the promoter of the gene encoding alcohol dehydrogenase in the *Saccharomyces cerevisiae* signal-sequence-trap vector pSUC2T7M13ORI²⁶. The cDNA library was used to transform the invatase gene-deficient *SUC2⁻* yeast strain YTK12, and transformed yeast were subjected to invertase selection as described²⁶. Plasmids were isolated from 89 recovered colonies as described⁴⁸. For amplification of the cDNA inserts, the plasmids were then analyzed by PCR with primer pairs specific to the pSUC2T7M13ORI vector²⁶. A cDNA fragment (clone OAF038) was selected for use in isolating full-length cDNA because it had an immunoglobulin-like domain. Full-length cDNA sequences were obtained from the HAS303 cDNA library by 3' rapid amplification of cDNA ends with a Marathon cDNA amplification kit (Clontech); this PCR used an adaptor sequence-specific reverse primer (AP2; Clontech) and an OAF038 cDNA-specific forward primer containing the predicted start codon of *ALLERGIN-1* (5'-ATATCTCGAGGGAG AATGTGGAGCCATTGGAACA-3').

Generation of Allergen-1-deficient mice. For construction of the *Allergen-1*-targeting vector, the first exon of *Allergen-1*, which contains the start codon, was replaced with a neomycin-resistance gene cassette (Supplementary Fig. 3a). Embryonic stem cells from the C57BL/6N mouse strain were transfected by electroporation with the linearized *Allergen-1*-targeting vector and cells were selected for resistance to the aminoglycoside G418. Correctly targeted embryonic stem cell clones with normal karyotypes were injected into 3.5-day-old C57BL/6N blastocysts to create chimeric mice (*Allergen-1^{+/-}*). *Allergen-1^{+/-}* mice were intercrossed to generate homozygous *Allergen-1^{-/-}* mice, which were obtained at the expected Mendelian frequencies and developed normally. Offspring were screened by PCR and Southern blot analysis of DNA extracted from tail tissue (Supplementary Fig. 3b). *Allergen-1^{-/-}* mice and control wild-type mice were bred under specific pathogen-free conditions in the same room of the animal facility of University of Tsukuba. All experiments were done according to the guidelines of the animal ethics committee of the University of Tsukuba.

Antibodies. Mouse-specific mAb to Allergen-1 (TX83; mouse IgG1) was generated in our laboratory at the University of Tsukuba by immunization of Allergen-1-deficient mice with BW5147 transfectants expressing Flag-tagged Allergen-1 as described¹⁹; antibody to human Allergen-1 (EX33; rat IgG2b) was generated by immunization of a rat with BW5147 transfectants expressing Flag-tagged Allergen-1L (specificities of TX83 and EX33, Figs. 2a and 3a, respectively). Monoclonal antibodies to mouse and/or human CD3 (145-2C11 and UCHT1), CD8 (HIT8a), CD19 (HIB19), CD56 (B159), B220 (RA3-6B2), NK1.1 (PK136), CD14 (M5E2), CD11b (M1/70), CD11c (HL3 and B-ly6), Gr-1, CD66b (G10F5), c-Kit (2B8), FcεRI (MAR-1) and DX5 were from BD Biosciences. Monoclonal antibodies to human CD1c (AD5-8E7), CD304 (AD5-17F6) and CD203c (FR3-16A11) and to human FcεRI (CRA1) were from Miltenyi Biotec and CosmoBio, respectively.

Cells and transfectants. Mouse BMMCs were prepared as described¹⁹. Human mast cells were generated by culture of peripheral CD34⁺ cells from healthy donors (collected after informed consent was obtained) as described⁴⁹. Wild-type Allergen-1 cDNA was subcloned into the pMX-Flag retrovirus vector to produce a vector with immunoglobulin κ-light chain signal sequence, followed by sequence encoding Flag-fused Allergen-1. For the generation of site-specific Allergen-1 mutants with substitution of tyrosine, sense PCR

primers were designed that contained the codon for phenylalanine (TTT) instead of tyrosine (TAT; Tyr216) and/or phenylalanine (TTC) instead of tyrosine (TAC; Tyr241). PCR was done with a QuickChange Site-Directed Mutagenesis kit according to the manufacturer's instructions (Stratagene). All mutant cDNAs were verified by sequencing. RBL-2H3 cells, Ba/F3 and BW5147 cells stably expressing Allergen-1 were established as described¹⁹.

Biochemistry. For analysis of the tyrosine phosphorylation of Allergen-1, RBL-2H3 transfectants were stimulated for 15 min at 37 °C with 100 mM sodium pervanadate and were lysed with 1% (wt/vol) digitonin buffer as described¹⁹. Flag-tagged proteins were immunoprecipitated with anti-Flag M2 agarose (A2220; Sigma-Aldrich), then were analyzed by immunoblot with mAb 4G10 to phosphorylated tyrosine (05-321; Upstate Biotechnology) followed by horseradish peroxidase-conjugated TrueBlot ULTRA anti-mouse IgG (18-8817-33; Bay Bioscience). Otherwise, immunoprecipitates were analyzed by immunoblot with polyclonal rabbit anti-SHP-1, anti-SHP-2 and anti-SHIP (sc-287, sc-280 and sc-6244, respectively; Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated anti-rabbit immunoglobulin (NA934; GE Healthcare). Proteins were detected by enhanced chemiluminescence (Thermo Fisher Scientific).

Degranulation assay. RBL-2H3 transfectants or BMMCs were incubated for 4 h at 37 °C with 0.5 μg/ml of IgE anti-TNP in 200 μl culture medium and then were stimulated for 30 min at 37 °C with TNP-conjugated F(ab')₂ fragments of the TX83 antibody to Allergen-1 (5 μg/ml) or control immunoglobulin in 200 μl HEPES-Tyrode's buffer, pH 7.4. Culture supernatants of stimulated or unstimulated cells were collected and β-hexosaminidase was assayed as described⁵⁰. For analysis of the total cell content of β-hexosaminidase, cells were lysed with 0.5% (vol/vol) Triton X-100 in HEPES-Tyrode's buffer. Percent degranulation was calculated as follows: absorbance of culture supernatants at 405 nm × 100 / absorbance of total cell lysate supernatants at 405 nm.

Passive systemic anaphylaxis. Wild-type mice, Allergen-1-deficient mice, mast cell-deficient (*W^{sh}/W^{sh}*) mice or *W^{sh}/W^{sh}* mice injected intravenously with 1 × 10⁷ wild-type or Allergen-1-deficient BMMCs 3 months before were sensitized by intravenous injection of 20 μg mouse IgE anti-TNP (C38-2; BD Biosciences). Then, 24 h later, mice were challenged intravenously with 1 mg OVA or TNP-OVA. After antigen challenge, rectal temperatures were measured every 6 min for 180 min with a digital thermometer (Shibaura Electronics).

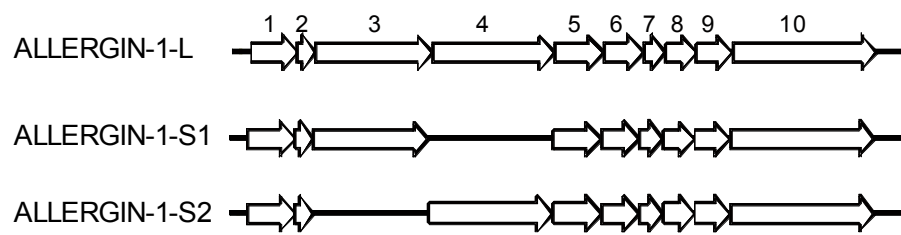
Passive cutaneous anaphylaxis. Mice were passively sensitized by intravenous injection with 2 μg mouse IgE mAb to dinitrophenol (SPE-7; Sigma-Aldrich). Then, 24 h later, mice were challenged by epicutaneous application of acetone/olive oil (4:1) alone to the right ear and 20 μl DNFB (0.6% wt/vol) in acetone and olive oil to the left ear. Ear-swelling responses were assessed by measurement of ear thickness with a digital thickness gauge (G-1A). Net ear swelling was calculated as the difference in the thickness of the right and left ear.

Statistical analyses. The unpaired Student's *t*-test was used for statistical analyses. *P* values of less than 0.05 were considered statistically significant.

48. Blanchard, M.M. & Nowotny, V. High-throughput rapid yeast DNA extraction. Application to yeast artificial chromosomes as polymerase chain reaction templates. *Genet. Anal. Tech. Appl.* **11**, 7–11 (1994).

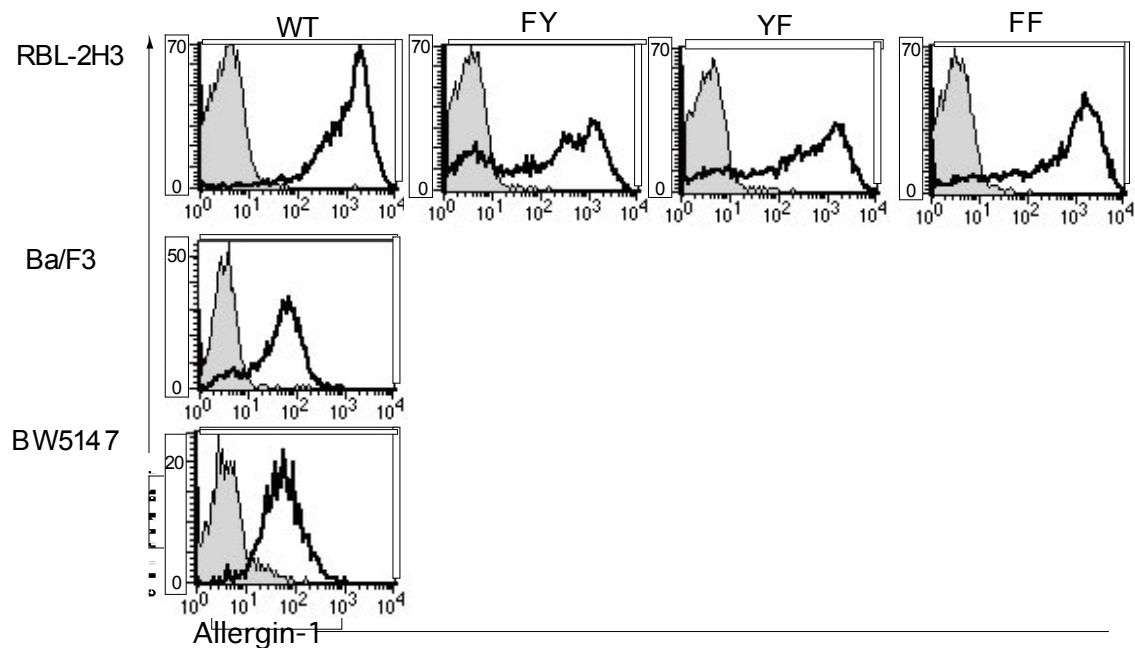
49. Saito, H., Kato, A., Matsumoto, K. & Okayama, Y. Culture of human mast cells from peripheral blood progenitors. *Nat. Protocols* **1**, 2178–2183 (2006).

50. Oda, H. *et al.* RhoH plays critical roles in FcεRI-dependent signal transduction in mast cells. *J. Immunol.* **182**, 957–962 (2009).



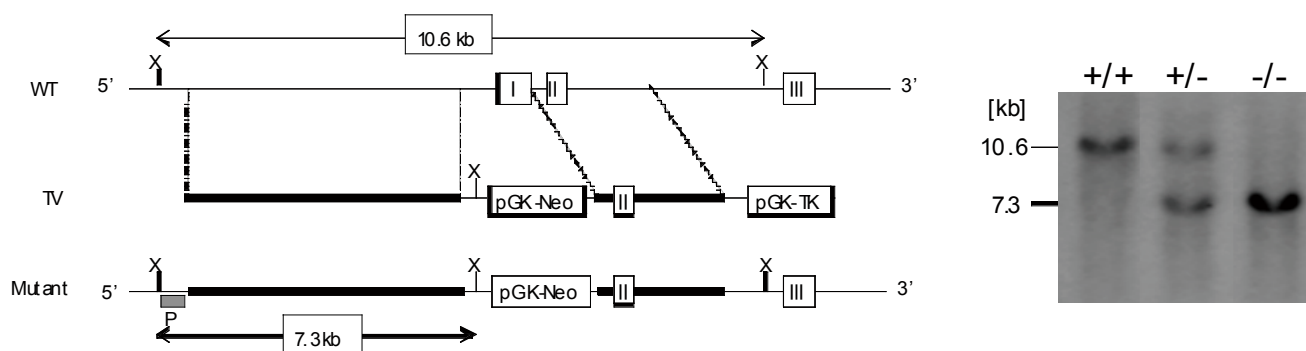
Supplementary Figure 1. Alignment of the exon structures of human *ALLERGIN-1-L*, *ALLERGIN-1-S1* and *ALLERGIN-1-S2*.

Human *ALLERGIN-1-L* consists of ten exons, whereas *ALLERGIN-1-S1* and *ALLERGIN-1-S2* lack exons 4 and 3, respectively.



Supplementary Figure 2. Establishment of transfectants expressing wild-type or mutant Allergin-1.

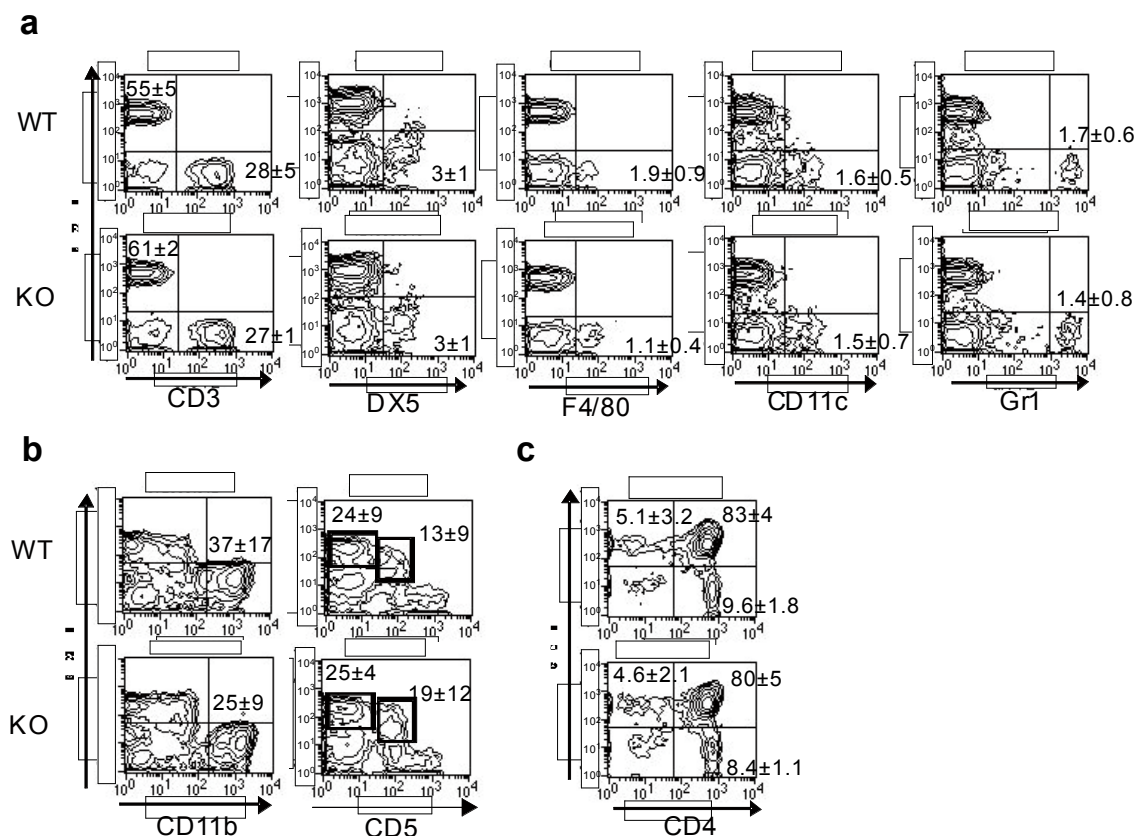
RBL-2H3, BW5147, and Ba/F3 transfectants expressing Flag-tagged WT or mutant (FY, Y-F²¹⁶; YF, Y-F²⁴¹; and FF, Y-F^{216, 241}) Allergin-1 were established, as described in **Methods** and **Fig. 3a**. The transfectants were stained with Allergin-1 mAb (TX83) (open histogram) or isotype control antibody (shaded histogram), and analyzed by flow cytometry. Data are representative of more than two independent experiments.



Supplementary Figure 3. Generation of Allergin-1-deficient mice.

(a) A targeting vector was designed to disrupt the Allergin-1 gene by homologous recombination. The WT *Allergin-1* allele (WT), the targeting vector (TV), and the targeted allele (Mutant) are shown. The first exon (I) containing the start codon was replaced by a gene for neomycin resistance (pGK-Neo). X marks the cleavage sites for *Xho* I restriction enzyme.

(b) Southern blot analysis of mouse genomic DNA digested with *Xho* I. DNA fragments from the WT (~10.6 kb) and targeted (~7.3 kb) alleles are shown. +/+, +/- and -/- represents C57BL/6N mice that are WT, chimeric, or homozygous negative for *Allergin-1*, respectively.



Supplementary Figure 4. Flow cytometry analyses of hematopoietic cells in Allergin-1-deficient mice.

Splenocytes (**a**), peritoneal exudative cells (**b**) and thymocytes (**c**) from WT ($n = 5$) and *Allergin-1*^{-/-} KO mice ($n = 5$) were stained with the antibodies indicated and analyzed by flow cytometry. Numbers in the quadrants and the boxes indicate the percentages of cell populations (mean ± SD). Data are representative of two independent experiments.

Supplementary Table

	WT	KO	<i>P</i> value
Splenocytes ($\times 10^7$)	10.0 $\pm$ 5.3	12.6 $\pm$ 1.3	0.07
CD3+ ($\times 10^7$)	2.3 $\pm$ 0.8	2.5 $\pm$ 0.8	0.74
B220+ ($\times 10^7$)	5.8 $\pm$ 1.3	7.0 $\pm$ 0.8	0.22
CD11b+ ($\times 10^6$)	3.5 $\pm$ 2.3	3.7 $\pm$ 2.6	0.93
CD11c+ ($\times 10^6$)	1.8 $\pm$ 8.9	1.6 $\pm$ 1.3	0.88
Gr1+ ($\times 10^6$)	1.5 $\pm$ 0.8	1.3 $\pm$ 0.8	0.76
DX5+ ($\times 10^5$)	7.7 $\pm$ 5.1	6.8 $\pm$ 3.2	0.84
PECs ($\times 10^6$)	2.6 $\pm$ 0.7	3.6 $\pm$ 1.0	0.15
CD5+B220+ ($\times 10^5$)	4.8 $\pm$ 2.2	3.4 $\pm$ 1.9	0.43
CD5-B220+ ($\times 10^5$)	4.6 $\pm$ 1.8	6.3 $\pm$ 1.5	0.29
CD11b+ ($\times 10^5$)	9.1 $\pm$ 5.1	14.8 $\pm$ 6.2	0.28
BM cells ($\times 10^7$)	3.4 $\pm$ 1.3	4.0 $\pm$ 0.4	0.47
B220+ ($\times 10^6$)	5.8 $\pm$ 4.2	5.7 $\pm$ 3.0	0.95
Gr1+ ($\times 10^6$)	7.1 $\pm$ 5.6	13.9 $\pm$ 6.9	0.60
CD11b+ ($\times 10^6$)	5.7 $\pm$ 4.2	4.6 $\pm$ 2.1	0.81
Thymocytes ($\times 10^7$)	8.2 $\pm$ 0.7	7.2 $\pm$ 1.7	0.15
CD4+ ($\times 10^6$)	6.6 $\pm$ 1.6	6.6 $\pm$ 1.3	0.99
CD8+ ($\times 10^6$)	4.0 $\pm$ 3.5	3.8 $\pm$ 0.5	0.92
CD4+CD8+ ($\times$			

Splenocytes peritoneal exudative cells (PECs), bone marrow (BM) cells and thymocytes from wild-type (WT, $n = 5$) and Allergin-1-deficient mice (KO, $n = 5$) were stained as described in **Supplementary Fig. 4**, and the absolute cell number of each population (mean $\pm$ SD) was determined.