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

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Abstract

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 identified a novel immunoglobulin-like receptor, Allergin-1, that contained immunoreceptor tyrosine-based inhibitory motif (ITIM)-like motifs, and showed it to be preferentially expressed on mast cells. Mouse Allergin-1 recruited *src* homology 2 (SH2) domain-containing protein tyrosine phosphatase (SHP)-1 and SHP-2, and SH2-domain-containing inositol 5-phosphatase (SHIP). Co-ligation of Allergin-1 and FcεRI suppressed IgE-mediated degranulation of bone marrow-derived cultured mast cells. Moreover, mice deficient in Allergin-1 showed significantly enhanced passive systemic and cutaneous anaphylaxis. Thus, Allergin-1 suppresses IgE-mediated, mast cell-dependent anaphylaxis in mice.

INTRODUCTION

Anaphylaxis is a life-threatening allergic reaction that is rapid in onset[1, 2]. The frequency of anaphylaxis is estimated to be 50–2000 episodes per 100 000 people[3]. Anaphylaxis can occur after exposure to certain antigens, called allergens, such as foods, drugs, insect venoms, latex, and immunotherapy injections[2]. Mast cells and basophils express the high affinity receptor for IgE (FcεRI) on the cell surface, and play 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 the 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 pro-inflammatory lipid mediators, such as prostaglandins and leukotrienes, and platelet-activating factor (PAF) 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 a type 1 immediate hypersensitivity.

Studies of mice lacking mast cells (c-kit–deficient W/W^v mice), FcεRI, or IgE provide evidence for the existence of an alternative pathway for anaphylaxis [7-10].

Immunoglobulin G (IgG)-allergen immune complexes mediate their activation signal via the low-affinity IgG receptor (FcγRIII) on basophils, inducing the release of PAF, rather than histamine. The release of PAF is the primary cause of the development of IgG-dependent systemic anaphylaxis in mice[11], although the mechanism underlying this type of anaphylaxis in humans remains unclear.

The signaling cascades mediated by FcεRI in mast cells have been extensively investigated[12]. 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 FcεRI-bound IgE induces activation of the Src family protein tyrosine kinase (PTK) Lyn that is bound to the FcεRI β subunit and activation of the nonreceptor PTK Syk. The activated Syk then phosphorylates tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM) of the β- and γ-subunits of FcεRI[13]. This early step in the activation signal leads to phosphorylation of adaptor proteins, including LAT, SLP76 and VAV, and to the activation of downstream signaling pathways[6].

FcεRI-mediated mast cell activation is modulated by several cell surface inhibitory receptors[14], including FcγRIIB[15], paired Ig-like receptor (PIR)-B[16, 17], gp49B1 [18], myeloid associated Ig-like receptor (MAIR)-I (also called CLM-8, LMIR1 or

Cd300a)[19-21], mast-cell function-associated antigen (MAFA)[22, 23], and signal regulatory protein (SIRP)-α [24]. The cytoplasmic portion of these inhibitory receptors commonly contains immunoreceptor tyrosine-based inhibitory motif(s) (ITIM). When these inhibitory receptors are co-ligated with FcεRI, the tyrosine residue in the ITIM is phosphorylated and recruits *src* homology 2 (SH2) domain-containing protein tyrosine phosphatase (SHP)-1, SHP-2 and/or SH2 domain-containing inositol 5-phosphatase (SHIP), 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 prevention or treatment of anaphylaxis has not been identified.

In the present study, we identified a novel inhibitory immunoglobulin (Ig)-like receptor, designated Allergy Inhibitory Receptor (Allergin)-1, that is preferentially expressed on mast cells, and explored its role in mast cell-mediated allergic responses in vivo and in vitro.

RESULTS

Identification of the gene encoding Allergin-1

To study the role of cell surface receptors in the regulation of differentiation and activation of hematopoietic cells, we cloned several cDNA fragments encoding putative novel transmembrane proteins from a cDNA library derived from the human bone marrow stromal cell line, HAS303[25], by using the signal sequence trap method[26]. Full-length cDNAs were isolated from a random-primed cDNA library derived from the HAS303 cell line. We identified a cDNA encoding an Ig-like receptor, designated Allergy Inhibitory Receptor-1 (Allergin-1), which contained a 19-amino acid (aa) leader sequence, a 208-aa extracellular region composed of two Ig-like domains, a 21-aa transmembrane domain, and a 95-aa cytoplasmic domain (**Fig. 1a, b**). We also identified two cDNAs encoding Allergin-1 isoforms, which lacked the first or second Ig-like domain in the extracellular portion and were designated Allergin-1 short form 1 (Allergin-1-S1) and Allergin-1 short form 2 (Allergin-1-S2), respectively (**Fig. 1b).** The extracellular portions of mouse allerging-1 and human Allergin-1L and those of human Allergin-1S1 and Allergin-1S2 contained six and three potential N-linked glycosylation sites, respectively **(Fig. 1a, b)**. Genomic DNA database analyses demonstrated that *ALLERGIN-1* consists of ten exons, but *ALLERGIN-1-S1* and *ALLERGIN-1-S2* lack

exons 4 and 3, respectively (**Supplementary Fig. 1 online**). A database search revealed mouse and rat homologues of Allergin-1 that contains a single Ig-like domain with 50% and 52% aa identities with that of human Allergin-1-S1 (**Fig. 1a, b).** Although the rat *Allergin-1* cDNA was previously reported to be *MCA-32*[27] , the molecular and functional characteristics have not been reported. The gene encoding Allergin-1 is located on chromosome 17q23.3 in humans and chromosome 11E1 in mouse, near *CD300* (or *Cd300*) family genes that encode Ig-like receptors mediating positive or negative signals in myeloid cells (**Fig. 1c**)[28, 29]. *Allergin-1* was also close to the gene, which encodes PECAM-1 (CD31 or Cd31), an ITIM-bearing Ig-like receptor expressed on myeloid cells and platelets. Genomic DNA database analysis demonstrated that, like PECAM-1 but not CD300, Allergin-1 is encoded by a single gene in both human and mouse (data not shown).

Biochemical characteristics of Allergin-1

To study the biochemical characteristics of Allergin-1, we established RBL-2H3 and Ba/F3 transfectants stably expressing mouse Allergin-1 tagged with Flag protein at the N-terminus (**Supplementary Fig. 2 online**). Immunoprecipitation of Allergin-1 protein from the lysate of RBL-2H3 transfectants followed by immunoblotting with anti-Flag

antibody revealed that the mouse Allergin-1 has a molecular weight of ~60 kDa under both reducing and non-reducing conditions (**Fig. 1d)**, suggesting that mouse Allergin-1 was a monomeric protein. On the other hand, the molecular weights of human Allergin-1L, Allergin-1S1 and Allergin-1S2 seemed to be ~70 kDa, ~40 kDa and ~40 kDa, respectively, under the reducing condition (**Fig. 1e**), although we also observed the bands with higher molecular weight in human Allergin-1S1 and Allergin-1S2 as well as mouse Allergin-1 **(Fig. 1d, e)**. Since both mouse and human Allergin-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 significantly decreased the weight of mouse and human Allergin-1, consistent with that of the polypeptide predicted from the *Allergin-1* cDNA and the presence of potential N-linked glycosylation sites in the extracellular portion (**Fig. 1d, e**).

Expression of Allergin-1

To investigate the cellular distribution of Allergin-1 expression, we generated monoclonal antibodies (mAb) against mouse Allergin-1 (**Fig. 2a**). Flow cytometry analyses of spleen cells demonstrated that Allergin-1 is expressed on myeloid lineage

cells, including dendritic cells, macrophages and neutrophils, but not on T, B or natural killer (NK) cells (**Fig. 2b**). However, Allergin-1 was most strongly expressed on mast cells in the peritoneal cavity (**Fig. 2c**). Bone marrow-derived cultured mast cells (BMMC) also expressed significant amounts of Allergin-1 (**Fig. 2d**). In contrast, Allergin-1 was not detected on basophils in the bone marrow (**Fig. 2e**). These results suggest that Allergin-1 may be involved in immune responses mediated by myeloid cells.

We also generated a mAb that specifically recognized human Allergin-1L and Allergin-1S1, but not Allergin-1S2 (**Fig. 3a)**. In agreement with mouse Allergin-1, human Allergin-1L and/or Allergin-1S1 were also expressed on myeloid cell lineages, including monocytes, neutrophiles, and dendritic cells, in the peripheral blood (**Fig. 3b)**. Although T cells and NK cells did not express Allergin-1L and Allergin-1S1, the weak expression of Allergin-1L and/or Allergin-1S1 were detected on B cells. It was noted that, unlike mouse Allergin-1, human Allergin-1L and/or Allergin-1S1 were considerably expressed on peripheral blood basophiles (**Fig. 3b)**. To examine whether Allergin-1 was expressed on human mast cells, we generated cultured mast cells derived from the $CD34^{+}$ cells in the peripheral blood mononuclear cells. These cultured human mast cells also expressed Allergin-1L and/or Allergin-1S1 (**Fig. 3c)**, consistent

with mouse Allergin-1.

Mouse Allergin-1 is tyrosine phosphorylated and recruits phosphatases.

The cytoplasmic regions of the human and mouse Allergin-1 proteins contains six and five tyrosines, respectively. The tyrosine residues at positions 313 and 338 in human, and positions 216 and 241 in mouse, may form part of immunoreceptor tyrosine-based inhibitory motif (ITIM)-like sequences (**Figs. 1a, 4a**), suggesting that Allergin-1 is tyrosine phosphorylated and recruits the SH2-containing phosphatases, such as SHP-1, SHP-2 or SHIP. To test this hypothesis, RBL-2H3 transfectants expressing mouse Allergin-1 were treated with pervanadate, which stimulates protein tyrosine phosphorylation. Immunoprecipitation of Allergin-1 with anti-Flag mAb demonstrated that Allergin-1 was tyrosine phosphorylated and recruited SHP-1, SHP-2 and SHIP (**Fig. 4b**). To determine which tyrosines were phosphorylated and responsible for recruitment of the phosphatases, we established RBL-2H3 transfectants that stably expressing Flag-tagged mutant Allergin-1 in which the tyrosines at residue 216 and/or 241 were replaced with phenylalanines (FY, Y-F^{216}; YF, Y-F^{241}; and FF, Y-F$^{216, 241}$, **Supplementary Fig. 2 online)**. As demonstrated in Figure 4b, all the mutant Allergin-1 proteins, as well as wild-type (WT) Allergin-1, were tyrosine phosphorylated, suggesting that tyrosines at

positions other than 216 and 241 were also phosphorylated. Allergin-1 mutated at residues 216 and 241 did not recruit SHP-1 or SHP-2 (**Fig. 4b**), indicating that the presence of tyrosines at these positions is required for the recruitment of SHP-1 and SHP-2. In contrast, SHIP was recruited by each of the mutant allergin-1 proteins (**Fig. 4b**), suggesting that the tyrosines at position(s) 175, 200 and/or 239 might be involved in the recruitment of SHIP.

Mouse Allergin-1 inhibits FcεRI-mediated degranulation

Because Allergin-1 is strongly expressed on mast cells, we next examined whether mouse Allergin-1 mediates an inhibitory signal against FcεRI-mediated degranulation of RBL-2H3 cells. RBL-2H3 transfectants expressing WT Allergin-1 were stimulated with anti-2,4,6-trinitrophenyl (TNP) IgE together with either TNP-conjugated control antibody or anti-Allergin-1 mAb. Co-ligation of Allergin-1 with FcεRI significantly decreased degranulation, as determined by β-hexosaminidase release from the transfectants, when compared with FcεRI stimulation alone ($p=0.001$) (**Fig. 4c**), suggesting that mouse Allergin-1 inhibits IgE-mediated degranulation of RBL-2H3 cells. However, degranulation is not affected in RBL-2H3 transfectants expressing Allergin-1 mutated at residue 216 and/or 241 (i.e., FY, YF, and FF). This finding indicates that both

tyrosines at positions 216 and 241 were involved in the inhibitory effect on degranulation. Taken together, these results suggest that mouse Allergin-1 recruits SHP-1 and SHP-2 through the phosphorylation of tyrosines at positions 216 and 241, and mediates an inhibitory signal for FcεRI-mediated degranulation of RBL-2H3 cells.

Generation of Allergin-1-deficient mice

To study the function of Allergin-1 in immune responses, we generated mice lacking the *Allergin-1* gene (**Supplementary Fig. 3 online**). Naïve *Allergin-1*$^{-/-}$ mice had normal compositions of lymphocyte and myeloid cell populations in the spleen, bone marrow, thymus, and peritoneal cavity (**Fig. 5a, Supplementary Fig. 4 online, and Supplementary Table online**). Moreover, we did not observe any difference between WT and *Allergin-1*$^{-/-}$ mice in the development of mast cells cultured from bone marrow cells in the presence of the cytokines interleukin-3 (IL-3) and stem cell factor (SCF) (**Fig. 5b**). These results suggest that disruption of *Allergin-1* did not affect differentiation of hematopoietic cells, including mast cells.

To examine whether Allergin-1 inhibits FcεRI-mediated degranulation of mast cells, as well as RBL-2H3 cells, BMMC from WT and *Allergin-1*$^{-/-}$ mice were generated (**Fig. 5c**) and stimulated with anti-TNP IgE together with either TNP-conjugated control

antibody or anti-Allergin-1 mAb. Stimulation with anti-Allergin-1 mAb significantly decreased FcεRI-mediated β-hexosaminidase release from WT BMMC (p=0.0008), but not from *Allergin-1*$^{-/-}$ BMMC (p=0.074) (**Fig. 5d**). These results indicate that Allergin-1 inhibits FcεRI-mediated degranulation of BMMC, as well RBL-2H3 cells.

Mouse Allergin-1 suppresses passive systemic anaphylaxis.

Since Allergin-1 inhibited FcεRI-mediated degranulation of mast cells in vitro, we next examined whether Allergin-1 was involved in passive systemic anaphylaxis (PSA), an IgE-mediated type 1 immediate hypersensitivity reaction. WT and *Allergin-1*$^{-/-}$ mice were passively sensitized with anti-TNP IgE mAb and then intravenously (i.v.) injected with ovalbumin (OVA) or TNP-conjugated OVA (TNP-OVA). The rectal temperature, a measure of passive systemic anaphylaxis, was determined every 6 min after the injection. Both WT and *Allergin-1*$^{-/-}$ mice injected with OVA did not show any change in rectal temperatures. In contrast, WT mice challenged with TNP-OVA showed a progressive decrease in rectal temperature to 4℃ below the basal temperature by 18 min after injection, followed by a gradual recovery to the 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 min

observation period (**Fig. 6a**). Moreover, although mast cell-deficient W^{sh}/W^{sh} mice did not show the decrease in rectal temperature, those mice that had been transplanted with BMMC derived from *Allergin-1*$^{-/-}$ mice showed significantly enhanced PSA, compared with those that had been transplanted with BMMC 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 1 immediate hypersensitivity reaction.

Mouse Allergin-1 suppresses passive cutaneous anaphylaxis.

We next examined whether Allergin-1 was also involved in IgE-mediated passive cutaneous anaphylaxis (PCA) reaction in the ear, which typically develops in the late phase (2–6 h) after allergen exposure. WT and *Allergin-1*$^{-/-}$ mice were passively sensitized by intravenous injection of anti-dinitrophenol (DNP) IgE mAb and then challenged with epicutaneous application of dinitrofluorobenzene (DNFB) in acetone/olive oil in the left year and acetone/olive oil alone in the right year. 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 the ear swelling in WT mice during the entire 50 h observation period after

the antigen challenge. Supporting these results, histological analysis demonstrated that the ear swelling in *Allergin-1*$^{-/-}$ mice 24 h after application of DNFB was considerably greater than that in WT mice (**Fig. 7b**).

DISCUSSION

Recent progress on mast cell biology demonstrated that mast cell activation is negatively regulated by several inhibitory mechanisms, including cell surface inhibitory receptors, the anti-inflammatory molecules such as TGF-β[30], IL-10[31], retinol[32, 33], and β2-adrenoreceptor agonists[34]. In the present study, we have identified a novel inhibitory Ig-like receptor Allergin-1, which contains the ITIM-like motif in the cytoplasmic portion and inhibits IgE-mediated degranulation of BMMC. IgE-mediated mouse mast cell degranulation can be inhibited by cross-linking FcεRI to an ITIM-containing cell surface receptor, including FcγRIIB[35], PIR-B[36], gp49B1[37], MAIR-I (CLM-8, LMIR1 or Cd300a)[19, 21], MAFA[22, 23] and SIRP-α[24]. Although CD200 does not bear ITIM, it is still able to inhibit FcεRI-mediated mouse mast cell activation by binding SHIP[38]. Among these inhibitory receptors, FcγRIIB, SIRP-α, CD300a (also named CMRF35) and leukocyte Ig-like receptor (LILR) B2, a human orthologue of PIR-B[39], were also demonstrated to inhibit IgE-mediated degranulation of human mast cells[14]. In vivo studies have shown that IgE-mediated systemic or cutaneous anaphylaxis are enhanced in mice deficient in FcγRIIB[40], PIR-B[39], and gp49B1[41], demonstrating 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, upon tyrosine

phosphorylation and these phosphatases are essential for the inhibition of FcεRI-mediated mast cell activation[42]. In contrast, although PIR-B constitutively associates with SHP-1, it is dispensable for PIR-B-mediated inhibition of FcεRI-mediated mast cell activation[36]. An ITIM-recruiting molecule responsible for 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 when stimulated with pervanadate. Allergin-1 mutated at either the tyrosine residue 216 or 241 recruited neither SHP-1 nor SHP-2, indicating that SHP-1 and SHP-2 require both the tyrosines to bind to Allergin-1. These results are in agreement with the functional analyses that showed that the inhibitory effect of Allergin-1 on degranulation of mast cells required both tyrosines at positions 216 and 241. The inhibitory NK cell receptors killer cell Ig-like receptors (KIR) were also reported to require two ITIMs for recruitment of tyrosine phosphatases and inhibitory signaling[43]. Allergin-1 mutated at residues 216 and 241 was still tyrosine phosphorylated and recruited SHIP when stimulated with pervanadate, suggesting that tyrosines other than those at positions 216 and 241 (i.e., 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 PSA as early as 6 min after antigen challenge, suggesting 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. Since PIR-B, which binds to the ligand MHC class I molecules[39], was constitutively tyrosine phosphorylated[36], it might be possible that PIR-B modulates basal activation of mast cells. On the contrary, FcγRIIB might downmodulate FcεRI-mediated signaling after binding to their ligands IgG immune complex[44]. We observed neither tyrosine phosphorylation of Allergin-1 expressed on BMMC as well as RBL-2H3 cell transfectant nor recruitment of phosphatases by Allergin-1 before stimulation of Allergin-1 with mAb in vitro. However, it has remained 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 is essentially required to identify a ligand for Allergin-1.

Since Allergin-1 is expressed on mast cells, but not on basophils, in mice, the enhanced IgE-mediated allergic inflammations in Allergin-1-deficient mice was

dependent on mast cells. However, we demonstrated that Allergin-1 is considerably expressed on basophils as well as mast cells in human. Recent studies demonstrated that basophils play an important role in IgG-mediated systemic anaphylaxis and IgE-mediated chronic allergic inflammation in mice[11, 45, 46]. Although the role of basophils in allergic inflammations in human has remained undetermined, Allergin-1 might be involved not only in mast cell-dependent but also in basophil-dependent allergic responses in human.

We demonstrated that both human and mouse Allergin-1 were expressed on macrophages, neutrophils and dendritic cells as well as mast cells and/or basophils. Human Allergin-1 was also expressed on B cells. This expression pattern on the broad cell types is similar to those of other Ig-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 mast cell or basophil activation. For example, FcγRIIB interferes with B cell 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 manner[14]. SIRP-α binds CD47, which is expressed on many cell populations including red blood cells. SIRP-α and CD47 interaction constitutively inhibits FcγR-dependent

and FcγR-independent phagocytosis by macrophages [47]. At present, the functional role of Allergin-1 in immune responses that involve myeloid cells other than mast cells has remained to be defined. Further studies are required to determine the role of Allegin-1 in allergic and non-allergic immune responses.

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Author contribution

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

Competing interest statement

The authors declare no conflicting financial interests.

Figure Legends

Figure 1. Molecular characteristics of Allergin-1. (a) Amino acid sequences of mouse and human Allergin-1. Amino acid (aa) residues that are identical in the two species are shaded. The putative leader and transmembrane sequences are underlined. Potential N-linked glycosylation sites in the extracellular domain are circled in red. Cysteine residues that are potentially involved in disulfide bonding of the Ig-like domains are marked with an asterisk. ITIM-like motifs are boxed. The cDNA sequence data for human *ALLERGIN-1-L, -S1, -S2,* and mouse *Allergin-1* is available in the Genbank database (www.ncbi.nlm.nih.gov/) under accession numbers AB542950, AB542951, AB542952, and AB542953, respectively. **(b)** Schematic diagram of mouse and human Allergin-1 proteins. The transmembrane domain (TM) and the potential N-linked glycosylation sites are indicated. The amino acid sequence of the first Ig-like domain (bold) in human allergin-1-L is 50% identical to the Ig-like domain in mouse Allergin-1. The aa sequences of the first (bold) and second (double lined) Ig-like domain in human Allergin-1-L is identical to that of Allergin-1-S1 and Allergin-1-S2. **(c)** Chromosomal location of the genes of *Allergin-1* and *ALLERGIN-1*. **(d, e)** RBL-2H3 cells and RBL-2H3 transfectants expressing Flag-tagged mouse Allergin-1 (d) and BW5147

transfectants expressing Flag-tagged human Allergin-1 (e), as demonstrated in Figure 3a, were lysed and immunoprecipitated with anti-Flag mAb and immunoblotted with anti-Flag polyclonal Ab. Ba/F3 cells, BW5147 cells and their transfectants expressing Flag-tagged mouse or human Allergin-1 were lysed and immunoprecipitated with anti-Flag mAb. Immunoprecipitates were treated (+) or not (-) with N-glycosidase F and immunoblotted with anti-Flag antibody.

Figure 2. Expression of mouse Allergin-1 protein. Ba/F3 (shaded) or Ba/F3 transfectants expressing Flag-tagged mouse Allergin-1 (thick line) (a) were stained with biotinylated control mouse Ig, anti-mouse Allergin-1 (TX83), or anti-Flag mAb, followed by allophycocyanin (APC)-conjugated streptavidin and analyzed by flow cytometry. Splenocytes (b), peritoneal exudative cells (c), bone marrow-derived cultured mast cells (d), and bone marrow cells (e) from C57BL/6N mice were stained with biotinylated $F(ab')_2$ fragment of anti-Allergin-1 (open histogram) or isotype control Ab (shaded histogram), and the Fluorescence isothiocyanate (FITC)-conjugated mAbs and Phycoerythrin (PE)-conjugated mAbs indicated in x- and y-axis of the left panel, respectively, followed by APC-conjugated streptavidin, and were analyzed by flow cytometry. Each boxed cells of the left panel was gated and analyzed for the

expression of Allergin-1 in the center and right panels. Data are representative of three independent experiments.

Figure 3. Expression of human Allergin-1 protein. (a) BW5147 transfectants expressing mock or Flag-tagged human Allergin-1 were stained with biotinylated control mouse Ig (shaded), anti-human Allergin-1 (EX33) (thick line, lower panel), or anti-Flag mAb (thick line, upper panel), followed by allophycocyanin (APC)-conjugated streptavidin and analyzed by flow cytometry. (**b, c**) Peripheral blood mononuclear cells (b) or cultured mast cells (c) were simultaneously stained with unlabelled anti-human Allergin-1 (shaded histogram) or control Ig (open histogram), and the FITC-conjugated and PE-conjugated mAbs indicated in x- and y-axis of the left panel, respectively, followed by Alexa 647-conjugated anti-human Allergin-1 and were analyzed by flow cytometry. Each boxed cells of the left panel was gated and analyzed for the expression of Allergin-1 in the center and right panels. Data are representative of three independent experiments.

Figure 4. Signal transduction via ITIM-like motifs of mouse Allergin-1. (a) Establishment of RBL-2H3 transfectants expressing Flag-tagged WT and mutant

Allergin-1. WT Allergin-1 contains tyrosine residues (Y) in the cytoplasmic domain at positions 175, 200, 216, 239 and 241. The tyrosines within ITIM-like motifs are marked by boxes. Site-specific mutant Allergin-1 constructs, in which one or two tyrosine residues were replaced with phenylalanine residues, are shown. (b) Tyrosine phosphorylation of Allergin-1 and recruitment of phosphatases. RBL-2H3 transfectants expressing WT or mutant Allergin-1 were stimulated (+) or not (–) with pervanadate (VO_4) and lysed with 1% digitonin buffer. Allergin-1 was immunoprecipitated (IP) with anti-Flag mAb and immunoblotted (IB) with the antibodies indicated. Data are representative of five independent experiments. (c) RBL-2H3 cells (RBL) or RBL-2H3 transfectants expressing WT or mutant Allergin-1 were sensitized with anti-TNP mouse IgE and then stimulated with TNP-conjugated $F(ab')_2$ fragments of control Ig or anti-Allergin-1 mAb (TX83). The % β -hexosaminidase release of cells that were stimulated with anti-TNP mouse IgE followed with TNP-conjugated anti-Allergin-1 (closed bars) was compared to that in cells that were stimulated with anti-TNP mouse IgE followed with TNP-conjugated control Ig (open bars), as described in the Method. Data are means of triplicate and representative of five independent experiments. Error bars show SD (n = 3). NS; not statistically significant.

Figure 5. Normal development of mast cells in Allergin-1-deficient mice. (a, b) Peritoneal exudative cells (a) or BMMC (b) derived from WT or *Allergin-1*$^{-/-}$ knockout (KO) mice were stained with the indicated mAb and analyzed by flow cytometry. Numbers in quadrants indicate the percentages of mast cells. (c) BMMC derived from WT and *Allergin-1*$^{-/-}$ KO mice were stained with anti-Allergin-1 mAb, TX83 (thick lines) or isotype control antibody (shaded). (d) BMMC derived from WT or *Allergin-1*$^{-/-}$ KO mice were stimulated as described in Fig 4c. The % β -hexosaminidase release of cells that were stimulated with anti-TNP mouse IgE followed with TNP-conjugated anti-Allergin-1 (closed bars) was compared to that in cells that were stimulated with anti-TNP mouse IgE followed with TNP-conjugated control Ig (open bars), as described in the Method. Error bars show SD (n = 3). NS; not significant. Data are representative of five independent experiments (a–d).

Figure 6. Enhanced systemic anaphylaxis in Allergin-1-deficient mice. (a) *Allergin-1*$^{+/+}$ mice (WT; □ or ○) and *Allergin-1*$^{-/-}$ (KO; ■ or ●) mice (n = 3, for each type) were i.v. injected with 20 μg of anti-TNP mouse IgE mAb. After 24 h, mice were challenged with 1 mg of OVA or TNP_6-conjugated OVA, and rectal temperatures were measured every 6 min. Data is the mean ± SEM (n = 3). Representative data of

five independent experiments are shown. (b) Mast cell deficient mice (W^{sh}/W^{sh}) were adoptively transferred with BMMC derived from WT (○) or KO (●) mice. Three months later, PSA was performed as described above. Naïve W^{sh}/W^{sh} mice that did not receive BMMC were also subjected to the PAS (△, n=2). Representative data of two independent experiments is shown. Data is the mean ± SEM (n = 5). *P < 0.05, ** P < 0.01, *** P<0.005 for the comparison of WT versus KO mice challenged with TNP-OVA.

Figure 7. Enhanced passive cutaneous anaphylaxis in Allergin-1-deficient mice. *Allergin-1*$^{+/+}$ mice (WT; ○, n = 5) and *Allergin-1*$^{-/-}$ (KO; ●, n = 5) mice were i.v. injected with 2 μg of anti-DNP mouse IgE mAb. After 24 h, mice were challenged by epicutaneous application of DNFB in acetone/olive oil to the left ear, and acetone/olive oil alone to the right ear. Net ear swelling was calculated as the difference between the thickness of the right and left ears of each mouse (a). Data are representative from three independent experiments. Error bars show SEM (n = 5). *P < 0.05, **P < 0.001, ***P<0.0005, WT versus KO mice. Histological sections of the right (control) and left (DNFB-treated) ears in WT and KO mice stained with Hematoxilin-Eosin are shown (b). Data are representative of three independent experiments.

Methods

Cloning of *Allergin-1* cDNA

Human *Allergin-1* cDNA was isolated from a cDNA library, in which cDNA fragments derived from a human bone marrow stromal cell line, HAS303, were ligated downstream of the alcohol dehydrogenase (ADH) promoter in the yeast signal sequence trap (SST) vector, pSUC2T7M13ORI[26]. The cDNA library was used to transform the *SOC2*$^{-}$ yeast strain, YTK12, and the transformed yeast was subjected to invertase selection, as described[26]. Plasmids were isolated from 89 recovered colonies, as described in[48]. To amplify the cDNA inserts, the plasmids were then subjected to PCR analysis using primer pairs specific to the pSUC2T7M13ORI vector[26]. A cDNA fragment (Clone OAF038) was selected for use in the isolation of full-length cDNA because it possessed an immunoglobulin-like domain. Full-length cDNA sequences were obtained from the HAS303 cDNA library by the 3′ rapid amplification of cDNA ends (RACE) method using a Marathon cDNA amplification kit (Clontech, CA, USA). 3′ RACE-PCR was performed with an adaptor sequence-specific reverse primer (AP2; Clontech) and an OAF038 cDNA-specific forward primer containing the predicted start codon of *ALLERGIN-1* (5′ –ATATCTCGAGGGAGAATGTGGAGCCATTTGAACA–3′).

Generation of Allergin-1-deficient mice

To construct the *Allergin-1* targeting vector, the first exon of the *Allergin-1* gene, which contains the start codon, was replaced with a neomycin-resistance gene cassette (**Supplementary Fig. 3a**). Embryonic stem (ES) cells from the C57BL/6N mouse strain were electroporated with the linearized *Allergin-1* targeting vector, and cells were selected for G418 antibiotic resistance. Correctly targeted ES cell clones with normal karyotypes were injected into 3.5-day–old C57BL/6N blastocysts to create chimeric mice (*Allergin-1*$^{+/-}$). *Allergin-1*$^{+/-}$ mice were intercrossed to generate homozygous *Allergin-1*$^{-/-}$ mice. Homozygous mice 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 (Data not shown, **Supplementary Fig. 3b online**). *Allergin-1*$^{-/-}$ mice and control WT mice were bred under specific pathogen-free conditions in the same room of our animal facility. All experiments were performed according to the guidelines of the animal ethics committee of the University of Tsukuba Animal Research Center, Japan.

Antibodies

The anti-mouse Allergin-1 mAb (TX83; mouse IgG1) was generated in our laboratory by immunizing Allergin-1-deficient mice with BW5147 transfectants expressing Flag-tagged Allergin-1, as previously described[19]. The anti-human Allergin-1 (EX33; rat IgG2b) was also generated by immunizing a rat with BW5147 transfectants expressing Flag-tagged Allergin-1L. Anti-mouse Allergin-1 $F(ab')_2$ fragments were prepared as described[19]. MAbs against mouse and/or human CD3, CD8, CD19, CD56, B220, NK1.1, CD14, CD11b, CD11c, Gr-1, CD1c, CD304, CD66b, CD203c, c-Kit, FcεRI and DX5 were purchased from BD Biosciences (San Jose, CA, USA).

Cells and transfectants

RBL-2H3 is a rat basophil leukemia cell line. Ba/F3 and BW5147 cells are mouse proB and T lymphoma cell lines, respectively. Mouse BMMCs were prepared, as described previously[19]. Human mast cells were generated by culture of peripheral $CD34^+$ cells, as described previously [49]. WT *Allergin-1* cDNA was subcloned into the pMX-Flag retrovirus vector to produce a vector with immunoglobulin kappa (Igk) chain signal sequence, followed by *Flag*-fused *Allergin-1*. To generate site-specific Allergin-1 mutants at the tyrosine residues at positions 216 (Y^{216}) and/or 241 (Y^{241}), we designed sense PCR primers that contained the codon for phenylalanine (TTT) instead of Y^{216}

(TAT) and/or phenylalanine (TTC) instead of Y^{241} (TAC). PCR was performed using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. All mutant cDNAs were verified by sequencing. RBL-2H3 cells, Ba/F3 and BW5147 cells stably expressing Allergin-1 were established, as described[19].

Biochemistry

To examine tyrosine phosphorylation of Allergin-1, RBL-2H3 cells expressing WT or mutant Allergin-1 were stimulated with 100 mM sodium pervanadate for 15 min at 37°C. Cells were lysed with buffer containing 1% digitonin, as described[19]. Flag-tagged proteins were immunoprecipitated with anti-Flag M2 agarose (Sigma-Aldrich) and immunoblotted with 4G10 mAb (Upstate Biotechnology, NY, USA), followed by HRP-conjugated TrueBlot ULTRA anti-mouse IgG (Bay Bioscience, Kobe, Japan). Otherwise, immunoprecipitates were immunoblotted with rabbit anti-SHP-1, anti-SHP-2, and anti-SHIP polyclonal antibodies (Santa Cruz Biotechnology, CA, USA), followed by HRP-conjugated anti-rabbit Ig (GE Healthcare, Chicago, USA). Proteins were detected by enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Rockford, TN, USA).

Degranulation assay

RBL-2H3 transfectants expressing WT or mutated Allergin-1 or BMMCs were incubated with 0.5 μg/ml of anti-TNP IgE in 200 μl culture medium for 4 h at 37℃. The cells were then stimulated with TNP-conjugated $F(ab')_2$ fragments of the anti-Allergin-1 antibody (TX83) (5 μg/ml) or control Ig in 200 μl HEPES-Tyrode's buffer for 30 min at 37°C. Culture supernatants of stimulated or unstimulated cells were collected and β-hexosaminidase was assayed, as described [50]. To determine the total cell content of β-hexosaminidase, cells were lysed with 0.5% Triton X-100 in HEPES-Tyrode's buffer. The degranulation (%) was calculated as follows: absorbance at 405 nm of culture supernatants × 100/absorbance at 405 nm of supernatants of total cell lysates.

Passive systemic anaphylaxis

WT, Allergin-1-deficient, Mast cell-deficient (W^{sh}/W^{sh}), or W^{sh}/W^{sh} mice that had been i.v. injected with 1 x 10^7 BMMC derived from WT or Allergin-1-deficient mice three months before were sensitized by an intravenous injection of 20 μg of anti-TNP mouse IgE mAb (BD Biosciences). 24 h later, mice were intravenously challenged with 1 mg of OVA or TNP_6-unconjugated OVA proteins. After antigen challenge, the rectal

temperature of the mice was measured with a digital thermometer (Shibaura Electronics, Saitama, Japan) every 6 min for 180 min.

Passive cutaneous anaphylaxis

Mice were passively sensitized by i.v. injection with 2 μg of anti-DNP mouse IgE mAb (Sigma-Aldrich). Twenty-four hours later, mice were challenged with epicutaneous application of 20 μl of DNFB (0.6% wt/vol) in acetone/olive oil (4:1) to the left ear and acetone/olive oil application to the right ear. The ear swelling response was assessed by measuring the ear thickness using a digital thickness gauge (G-1A, Ozaki, Tokyo). Net ear swelling was calculated as the difference between the thickness of the right and left ears of each mouse.

Statistical analyses

Statistical analyses were performed using the unpaired Student's *t*-test. P values < 0.05 were considered statistically significant.

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