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Signal-Transducing Adaptor Protein-2 Controls the IgE-Mediated, Mast Cell–Mediated Anaphylactic Responses

Yuichi Sekine,^{*,1} Keigo Nishida,^{†,1} Satoru Yamasaki,[†] Ryuta Muromoto,^{*} Shigeyuki Kon,^{*} Jun-ichi Kashiwakura,[‡] Kodai Saitoh,^{*} Sumihito Togi,^{*} Akihiko Yoshimura,[§] Kenji Oritani,[¶] and Tadashi Matsuda^{*}

Signal-transducing adaptor protein-2 (STAP-2) is a recently identified adaptor protein that regulates immune and inflammatory responses through interactions with a variety of signaling and transcriptional molecules. In the current study, we clarified the physiological role of STAP-2 in mast cell function, a key mediator of IgE-associated allergic responses. STAP-2 is constitutively expressed in mast cells. STAP-2 deficiency in mast cells greatly enhances FcεRI-mediated signals, resulting in the increased tyrosine phosphorylation of the phospholipase C-γ isoform, calcium mobilization, and degranulation. Of importance, STAP-2-deficient mice challenged with DNP-BSA after passive sensitization with anti-DNP IgE show more severe rectal temperature decrease than do wild-type mice. STAP-2-deficient mice also show increased vascular permeability and more severe cutaneous anaphylaxis after DNP-BSA injection. These regulatory functions performed by STAP-2 indicate that there is an interaction between STAP-2 and FcεRI. In addition, our previous data indicate that STAP-2 binds to the phospholipase C-γ isoform and IκB kinase-β. Therefore, our data described in this article strongly suggest that manipulation of STAP-2 expression in mast cells may control the pathogenesis of allergic diseases and have the potential for treating patients with allergy. *The Journal of Immunology*, 2014, 192: 000–000.

Mast cells play a central role in IgE-dependent allergic diseases. They constitutively express the high-affinity IgE receptor, FcεRI, on their surface (1, 2). Aggregation of FcεRI eventually leads to the release of granule components such as histamine and the change in adhesive property, cell shape, and surface topography, as well as the de novo synthesis of lipid mediators and cytokines (3). To date, there are several molecules that are important for the earliest activation steps of the FcεRI-mediated signaling pathways (4, 5). For example, Lyn associates with FcεRI and initiates signaling cascades. Syk is essential for the phosphorylation of multiple protein substrates such as phospholipase C-γ isoform (PLC-γ) (6, 7). PLC-γ catalyzes the hydrolysis of phospholipids with the formation of

diacylglycerol and inositol 1,4,5-triphosphate that is required for calcium mobilization (3, 6). These activation signals are subsequently inhibited by negative signals that are required for mast cells to return to their basal resting condition (8). Thus, FcεRI-mediated activation and subsequent inhibition are highly ordered, sequential molecular events.

We have recently cloned a novel adaptor molecule, signal-transducing adaptor protein-2 (STAP-2), whose human homolog is identical to a recently cloned adaptor molecule, BKS, a substrate of Brk (breast tumor kinase) (9, 10). Structurally, it contains an N-terminal pleckstrin homology domain and a region weakly related to an Src homology 2 domain. In addition, a proline-rich region and a YXXQ motif are observed in its C-terminal region. STAP-2 is expressed in a variety of tissues and cells, such as lymphocytes, macrophages, and hepatocytes (9), and its abundant expression pattern suggests that STAP-2 influences a variety of signaling or transcriptional molecules. Indeed, we previously reported that STAP-2 can modulate the transcriptional activity of STAT3 and STAT5, as well as TLR-mediated signals in macrophages and integrin- or SDF1α-mediated signals in T cells (9, 11–14). Through these interactions, STAP-2 plays physiological roles in immune responses.

We previously reported that overexpression of STAP-2 in a rat basophilic leukemia cell line, RBL-2H3, shows a dramatic suppression of FcεRI-mediated tyrosine phosphorylation of PLC-γ, calcium mobilization, and degranulation (15). Although overexpression experiments are sometimes artificial, these facts suggest that STAP-2 may have a potential to negatively regulate FcεRI-mediated signals. In the current study, we investigated physiological roles of STAP-2 in anaphylactic responses using STAP-2-deficient cells and mice. STAP-2-deficient bone marrow–derived mast cells (BMMCs) had more calcium entry, degranulation, and cytokine secretion than did wild-type (WT) BMMCs after FcεRI stimulation. Furthermore, STAP-2-deficient mice showed more severe IgE-mediated anaphylactic responses.

^{*}Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan; [†]Laboratory for Homeostatic Network, RIKEN Center for Integrative Medical Sciences (IMS-RCAI), Yokohama, Kanagawa 230-0045, Japan; [‡]Laboratory for Allergic Disease, RIKEN Center for Integrative Medical Sciences (IMS-RCAI), Yokohama, Kanagawa 230-0045, Japan; [§]Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo 160-8582, Japan; and [¶]Department of Hematology and Oncology, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan

¹Y.S. and K.N. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Tadashi Matsuda, Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6, Sapporo 060-0812, Japan. E-mail address: tmatsuda@pharm.hokudai.ac.jp

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Abbreviations used in this article: BMMC, bone marrow–derived mast cell; Dok, downstream of kinase; Gab, Grb2 associated binder; IKK-β, IκB kinase-β; KO, knockout; PLA, proximity ligation assay; PLC-γ, phospholipase C-γ isoform; PY, phosphorylation; SCF, stem cell factor; STAP-2, signal-transducing adaptor protein-2; WT, wild-type.

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Therefore, our results are likely to establish physiological functions of STAP-2 as a critical regulator of FcεRI-mediated signaling in mast cells.

Materials and Methods

Reagents, Abs, and mice

Recombinant mouse IL-3 was purchased from Wako Chemicals (Osaka, Japan). DNP-BSA was purchased from Cosmo Bio (Tokyo, Japan). Mouse IgE anti-DNP [2-9 (4)] was obtained from Yamasa (Tokyo, Japan). Mouse anti-DNP IgE (SPE-7), anti-actin, anti-phosphotyrosine (PY) mAb (PY20), and anti-FLAG Abs were from Sigma-Aldrich (St. Louis, MO). Anti-phospho-Syk (Tyr³⁵²), anti-phospho-p38 (Tyr¹⁸²), anti-p38, anti-ERK, anti-phospho-IκBα, and anti-Myc Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti-Syk (N-19), anti-phospho-ERK (E4), anti-PLC-γ1, and anti-IκBα Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-PLC-γ1 (Tyr⁷⁸³) Ab was from Epitomics (Burlingame, CA). The generation of STAP-2-deficient mice was described previously (9). STAP-2-deficient mice were housed and bred in the Pharmaceutical Sciences Animal Center of Hokkaido University. All animals were maintained under pathogen-free conditions and in compliance with national and institutional guidelines. All protocols were approved by the Hokkaido University animal ethics committee.

Cell culture, transfection, and Western blotting

BMMCs were prepared as described previously (16). Briefly, 6- to 8-wk-old C57/BL6 WT and STAP-2 knockout (KO) mice were sacrificed, and their bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50 μM 2-ME, and 10 ng/ml recombinant mouse IL-3 in 5% CO₂ at 37°C. After 4–5 wk of culture, the cells were confirmed to show the cell surface expression of FcεRI and c-Kit and were used for

experiments (<98% mast cells). NIH3T3 was maintained in DMEM medium supplemented with 10% FCS. Retroviral transfection was performed as previously described (17, 18). Western blotting assays were performed as described earlier (11). Briefly, BMMCs (1×10^6) were sensitized with anti-DNP IgE (5 μg/ml) for 12 h in a 12-well plate. After washing, the cells were stimulated with DNP-BSA (1 μg/ml) for the indicated periods. Then, the cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4; 0.15 M NaCl, containing 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF). The total cell lysate was resolved on SDS-PAGE and transferred to a polyvinylidene difluoride transfer membrane (PerkinElmer, Boston, MA). The filters were then immunoblotted with each Ab. Immunoreactive proteins were visualized using an ECL detection system (Millipore; Bedford, MA). Densitometric analysis for Western blotting was performed with an LAS-1000 Fluorescence Image Analyzer (Fujifilm). Cell proliferation was determined by CellTiter-Glo assays. BMMCs (1×10^5 per well) were cultured in 96-well plates with increasing amounts of IL-3 or stem cell factor (SCF). The cells were cultured for 24 h and then were added with CellTiter-Glo Reagent (Promega). The generation of ATP was determined using a luminometer (Promega).

Flow cytometric analysis

The following mAbs were used for cell surface protein staining: PE-conjugated anti-c-Kit (BD Pharmingen) or Alexa 647-conjugated anti-FcεRIα (Invitrogen) Ab. Single-cell suspensions were incubated for 30 min at room temperature with an appropriate Ab in 50 μl PBS. The cytofluorometric analysis was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA) using CellQuest Software (Becton Dickinson). A minimum of 10,000–20,000 cells per sample was analyzed.

Ca²⁺ mobilization and degranulation assay

Measurements of intracellular free Ca²⁺ concentration were performed using the Calcium Kit II-Fluo 4 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. BMMCs (1×10^5)

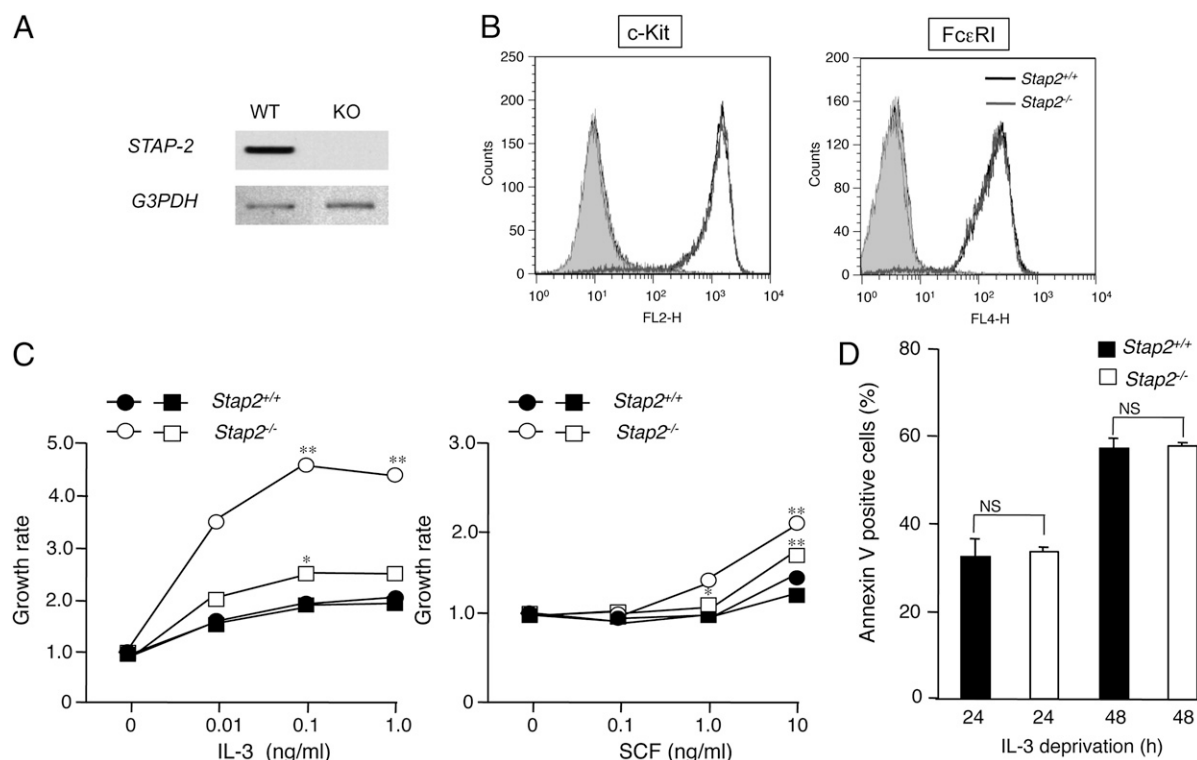


FIGURE 1. Involvement of STAP-2 in the production and survival of mast cells. **(A)** Expression of STAP-2 mRNA in BMMCs from WT and STAP-2 KO mice was monitored by RT-PCR. **(B)** The surface expression of c-Kit and FcεRI on BMMCs from WT (black line) and STAP-2 KO (gray line) mice was determined by flow cytometry. Filled histograms are isotype controls. Data shown are representative of five independent lines from each genotype. **(C)** WT (filled circle and square) and STAP-2 KO (open circle and square) BMMCs (1×10^5) in a 96-well plate were cultured with increasing amounts of IL-3 (0, 0.01, 0.1, 1 ng/ml) or SCF (0, 0.1, 1, 10 ng/ml) for 24 h. Cell growth was determined by CellTiter-Glo assays. Data shown are two independent lines from each genotype (* $p < 0.05$, ** $p < 0.01$, unpaired Student t test). The luminescent signal of nonstimulation was set as 1. **(D)** WT and STAP-2 KO BMMCs (1×10^5) were cultured without IL-3 for the indicated periods. Cells were stained with Annexin V and analyzed with flow cytometry. Similar results were obtained in three independent experiments.

were sensitized with anti-DNP IgE (0.5 $\mu\text{g/ml}$) for 12 h and incubated with 100 μl Fluo-4 AM in loading buffer at 37°C for 1 h. The fluorescence of the solution was monitored for 1 min before stimulation; then the cells were stimulated with DNP-BSA (100 $\mu\text{g/ml}$) and 1 min after stimulation by a microplate fluorometer (excitation at 480 nm and emission at 518 nm; Tecan). The assay conditions for degranulation were described previously (15). Degranulation of BMMCs was determined by measurement of β -hexosaminidase release. Cells (5×10^5) were sensitized with anti-DNP IgE (5 $\mu\text{g/ml}$) for 12 h in a 12-well plate. After washing, the cells were stimulated with DNP-BSA (1 $\mu\text{g/ml}$), calcium ionophore A23187 (500 ng/ml), or ionomycin (1 μM) to determine their maximal ability to degranulate. After incubation for 1 h at 37°C, assay buffer was recovered for analysis of β -hexosaminidase release. Total cell lysates were obtained by treatment with 0.2% Triton X-100. The medium and total cell lysates were incubated with *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside (Wako Chemicals) for 20 min at 37°C. The reaction was terminated by the addition of 0.2 M glycine buffer (pH 10.4). The release of the product *p*-nitrophenol was monitored by absorbance at 405 nm. The released β -hexosaminidase activities were expressed as a percentage of the total.

Measurement of cytokines in culture supernatant

BMMCs (1×10^6) were sensitized with anti-DNP IgE (5 $\mu\text{g/ml}$) for 12 h in a 12-well plate. After washing, the cells were stimulated with DNP-BSA (1 $\mu\text{g/ml}$) for the indicated periods. Concentrations of TNF- α , IL-4, or IL-6 in culture supernatants were measured by ELISA according to the manufacturer's instructions (TNF- α and IL-6: BioSource, Camarillo, CA; IL-4: BioLegend San Diego, CA).

RNA isolation and quantitative real-time PCR

BMMCs (1×10^6) were sensitized with anti-DNP IgE (5 $\mu\text{g/ml}$) for 12 h in a 12-well plate. After washing, the cells were stimulated with DNP-

BSA (1 $\mu\text{g/ml}$) for the indicated periods. The stimulated cells were harvested, and total RNAs were prepared using TRI reagent (Sigma-Aldrich) and used in RT-PCR. RT-PCR was performed using the RT-PCR High Plus Kit (Toyobo, Tokyo, Japan). Quantitative real-time PCR analyses of TNF- α , IL-4, or IL-6, as well as the control G3PDH mRNA transcripts, were carried out using the Assay-On-Demand gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 Sequence Detection System (Applied Biosystems).

Cell adhesion assays

Cell adhesion assays were performed as described (13) using flat-bottom 96-well plates coated with fibronectin (10 $\mu\text{g/ml}$). BMMCs (1×10^6) were sensitized with anti-DNP IgE (5 $\mu\text{g/ml}$) for 12 h in a 12-well plate. After washing, the cells were stimulated with the indicated concentration of DNP-BSA for 1 h. Then the stimulated BMMCs (1×10^5) were carried out for adhesion assay to fibronectin for 1 h incubation at 37°C, and after washing, the attached cells left in each well were estimated using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay (Cell Counting Kit-8; Wako Pure Chemical). Into each well 10 μl WST-8 solution was added, and the cells were incubated for another 1 h. The absorbance was measured at a test wavelength of 450 nm (OD 450) and a reference wavelength of 595 nm, using a microplate reader (Bio-Rad Laboratories). Percent binding was calculated as (OD 450, attached cells/OD 450, total number of cells to well).

Proximity ligation assay

Proximity ligation assay (PLA) detection was performed according to the manufacturer's protocol (Olink Bioscience, Uppsala, Sweden). Briefly, STAP-2- or mock-transfected BMMCs on coverslips were fixed using 4% paraformaldehyde for 30 min at room temperature. Subsequently, BMMCs were washed three times with PBS and blocked for 30 min at 37°C in

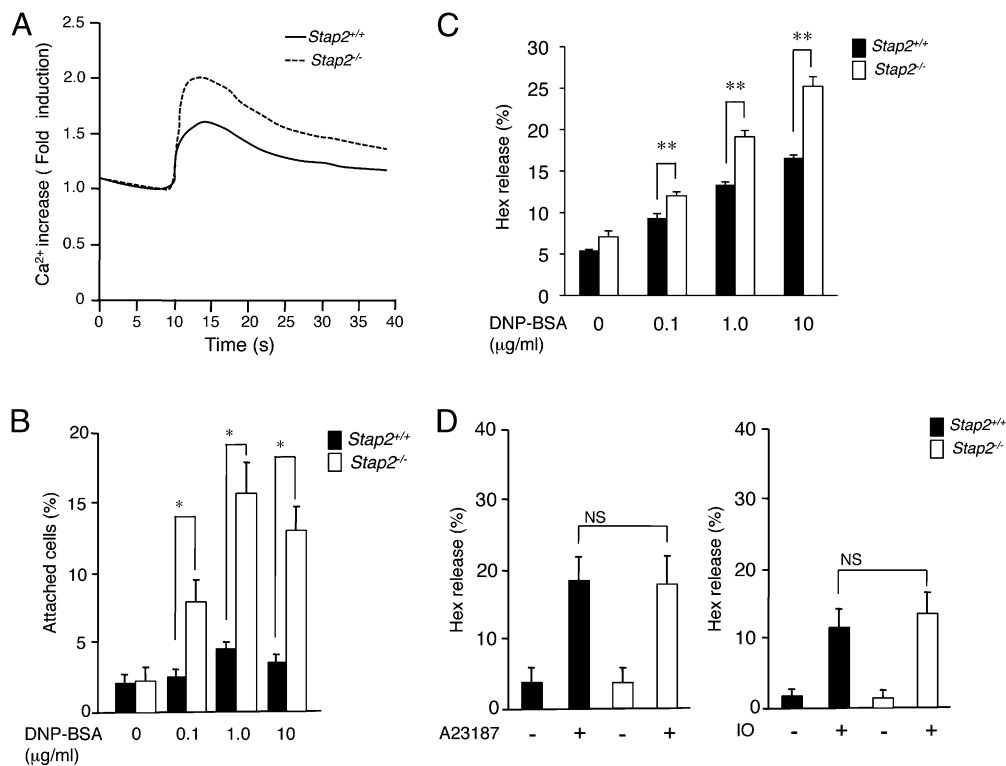


FIGURE 2. Involvement of STAP-2 in in vitro functions of mast cells. **(A)** WT and STAP-2 KO BMMCs (1×10^7) were sensitized with anti-DNP IgE (5 $\mu\text{g/ml}$) for 12 h. Sensitized mast cells were loaded with Fluo-4 AM and stimulated with DNP-BSA (5 $\mu\text{g/ml}$). The fluorescence emission ratio at 480–518 nm was monitored by a Tecan microplate fluorometer. Similar results were obtained in three independent experiments. **(B)** WT and STAP-2 KO BMMCs (5×10^5) were sensitized with anti-DNP IgE (5 $\mu\text{g/ml}$) for 12 h. Sensitized mast cells were plated in a 96-well plate. Adhesion assays were carried out for 1 h at 37°C, and the attached cells were stained with WST and quantified by reading the absorbance at 450 nm. Results are representative of three independent experiments, with standard deviations. * $p < 0.05$. **(C)** WT and STAP-2 KO BMMCs (5×10^5) were sensitized with anti-DNP IgE (5 $\mu\text{g/ml}$) for 12 h, and then stimulated without (–) or with (+) DNP-BSA (0, 0.1, 1.0, 10 $\mu\text{g/ml}$) for 0.5 h at 37°C. β -Hexosaminidase in the supernatant was measured as a percentage of total β -hexosaminidase. ** $p < 0.01$. **(D)** WT and STAP-2 KO BMMCs (5×10^5) were cultured with IL-3, and further stimulated without (–) or with (+) A23187 (500 ng/ml) or ionomycin (1 μM) for 1 h at 37°C. β -Hexosaminidase in the supernatant was measured as a percentage of total β -hexosaminidase. The results are presented as mean $\pm$ SD of triplicate experiments. Similar results were obtained when the other lines were examined.

blocking solution. After blocking, the cells were incubated for 30 min at 37°C with mouse anti-Myc (1:20; Santa Cruz) and rabbit anti-FcεRIγ Abs (1:50; Upstate) in Ab dilution solution. After washes, the cells were incubated for 60 min at 37°C with appropriate PLA probes, consisting of secondary Abs (anti-mouse and anti-rabbit) conjugated to oligonucleotides. Following washes, circularization and ligation of appropriate oligonucleotides were performed in ligase-containing solution for 30 min at 37°C. Cells were then rinsed briefly and incubated for 100 min at 37°C with the amplification solution prior to hybridizing the amplified product with a complementary probe labeled with Alexa 568. The stained cells were analyzed with the TCS SL system (Leica). Numbers of PLA-positive cells were counted using the TCS SL system (Leica); ≥ 100 independent STAP-2- or mock-transfected BMMCs were counted for each experiment.

Passive systematic anaphylaxis

WT and STAP-2 KO mice were sensitized by i.v. injection of 2 μ g mouse anti-DNP IgE Ab (SPE-7) in 100 μ l PBS via the tail vein for 16 h. After sensitization, mice were i.v. challenged with 0.5 mg DNP-BSA in 100 μ l PBS via the tail vein. Body temperature was monitored using a rectal probe before and at every 5 min for 90 min after Ag challenge.

Passive cutaneous anaphylaxis

A total of 2 μ g IgE in 20 μ l saline was injected s.c. into the ears of WT and STAP-2 KO mice for 16 h. After this sensitization, the mice were then challenged with an i.v. injection of 1 μ g polyvalent DNP-BSA in 250 μ l saline and 5 mg/ml Evans blue dye. The extravasation of Evans blue into

the ear was monitored for 30 min. The mice were then sacrificed, both ears were dissected, and Evans blue dye was extracted in 500 μ l formamide at 63°C for 5 h. The absorbance of Evans blue-containing formamide was then measured at 620 nm.

Statistical methods

The significance of differences between group means was determined by the Student *t* test.

Results

Involvement of STAP-2 in the proliferation of mast cells

We first examined the expression level of STAP-2 in BMMCs, using RT-PCR and real-time PCR. As shown in Fig. 1A, STAP-2 mRNA expression was observed in WT, but not STAP-2-deficient, BMMCs. To study the physiological function of STAP-2 in the FcεRI-mediated signaling pathway, we used STAP-2-deficient BMMCs. BM cells from WT and STAP-2-deficient mice were cultured in the presence of IL-3. At 4 wk later, highly purified mast cell populations (>98%) were generated as determined by flow cytometric analysis for cell surface expression of c-Kit, FcεRI, and IL-3R. Two types of cultured BMMCs showed a similar intensity of c-Kit, FcεRI, and IL-3R staining (Fig. 1B, Supplemental Fig. 1). Microscopic analysis of toluidine blue-stained cultured cells revealed an indistinguishable metachromatic

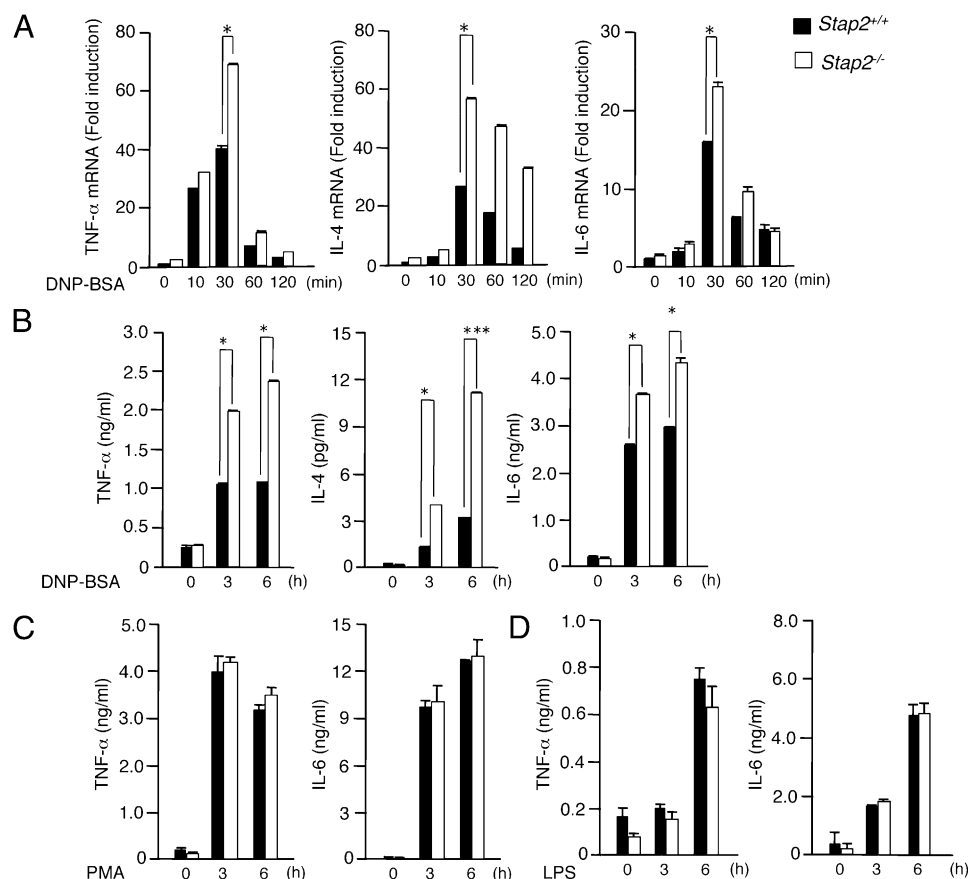


FIGURE 3. FcεRI-mediated cytokine production in WT and STAP-2 KO BMMCs. **(A)** WT and STAP-2 KO BMMCs (1×10^6) were sensitized with anti-DNP IgE (5 μ g/ml) and then stimulated with DNP-BSA (1 μ g/ml) for the indicated periods. Total RNAs were extracted, and the TNF- α , IL-4, and IL-6 mRNA expression levels were quantified by RT and quantitative real-time PCR analysis. Data represent the levels of TNF- α , IL-4, and IL-6 mRNA normalized by that of G3PDH mRNA as an internal control and are expressed relative to the values at time zero. Data represent the means $\pm$ SD of more than three different experiments. * $p < 0.05$. **(B)** WT and STAP-2 KO BMMCs (1×10^6) were sensitized with anti-DNP IgE (5 μ g/ml) and then stimulated with DNP-BSA (1 μ g/ml) for the indicated periods. Concentrations of TNF- α , IL-4, and IL-6 in culture supernatants were measured with ELISA. All values are the mean $\pm$ SD of duplicate cultures from three different lines. * $p < 0.05$, *** $p < 0.001$. **(C and D)** WT and STAP-2 KO BMMCs (1×10^6) were stimulated with PMA (50 ng/ml) (C) or LPS (1 μ g/ml) (D) for the indicated periods. Concentrations of TNF- α and IL-6 in culture supernatant were measured with ELISA. All values are the mean $\pm$ SD of triplicate cultures from three different experiments.

cell morphology (data not shown). Thus, STAP-2 does not seem to influence the mast cell differentiation program. However, cultures of STAP-2-deficient BM cells yielded larger numbers of mast cells than did WT BM cell cultures (data not shown). The proliferation response of STAP-2-deficient BMMCs to IL-3 and SCF was significantly higher than that of WT BMMCs (Fig. 1C). In contrast, IL-3 deprivation induced comparable levels of apoptosis in both WT and STAP-2-deficient BMMCs (Fig. 1D). Therefore, STAP-2 negatively regulates proliferation, but not the development or survival of BMMCs.

Involvement of STAP-2 in *in vitro* functions of mast cells

Stimulation of BMMCs with DNP-BSA after IgE-sensitization induces the elevation of calcium influx and degranulation. As shown in Fig. 2A, FcεRI-mediated elevation of calcium influx was higher in STAP-2-deficient BMMCs than in WT BMMCs. STAP-2-deficient BMMCs also showed a higher ability to attach to fibronectin than WT BMMCs (Fig. 2B). In addition, STAP-2-deficient BMMCs released larger amounts of β-hexosaminidase in response to DNP-BSA stimulation than did WT BMMCs (Fig. 2C). However, similar β-hexosaminidase release was observed when WT and STAP-2-deficient BMMCs were stimulated by A23187 or ionomycin (Fig. 2D). Therefore, STAP-2 deficiency selectively enhances FcεRI-mediated elevation of calcium influx and degranulation.

Secretion of cytokines and degranulation are important features of FcεRI-mediated mast cell activation. We then tested the effects of STAP-2 on cytokine production. As shown in Fig. 3A and 3B,

expression levels of mRNAs and proteins of FcεRI-related cytokines, TNF-α, IL-4, and IL-6 were significantly upregulated in STAP-2-deficient BMMCs after DNP-BSA stimulation, compared with WT BMMCs. In contrast, PMA- or LPS-induced cytokine production was not affected by STAP-2 deficiency (Fig. 3C, 3D). Therefore, STAP-2 selectively influences FcεRI-induced cytokine production.

Modification of FcεRI-mediated signals by STAP-2

To investigate how STAP-2 negatively regulates FcεRI-mediated signaling, we analyzed signaling events in more detail. Immunoblot analysis revealed increased tyrosine phosphorylation of several signaling molecules, including Syk and PLC-γ1, in STAP-2-deficient BMMCs before and after stimulation with DNP-BSA, compared with WT BMMCs. Syk is an essential protein tyrosine kinase required for mast cell activation. In STAP-2-deficient BMMCs, activation of Syk was enhanced when we evaluated its activation using phospho-Syk Ab (Fig. 4A, 4C). Downstream of Syk in the FcεRI-mediated signaling in mast cells, PLC-γ1 and MAPKs (MAPKs: ERK and p38), as well as NF-κB, showed modestly enhanced activation in STAP-2-deficient BMMCs (Fig. 4A, 4C). Therefore, STAP-2 negatively regulates FcεRI-mediated activation of Syk, PLC-γ1, and MAPKs, as well as NF-κB in mast cells.

STAP-2 directly interacts with FcεRI

STAP-2 has an ability to bind to a variety of cellular proteins, including transmembrane receptors (19–23). As shown in Fig. 4,

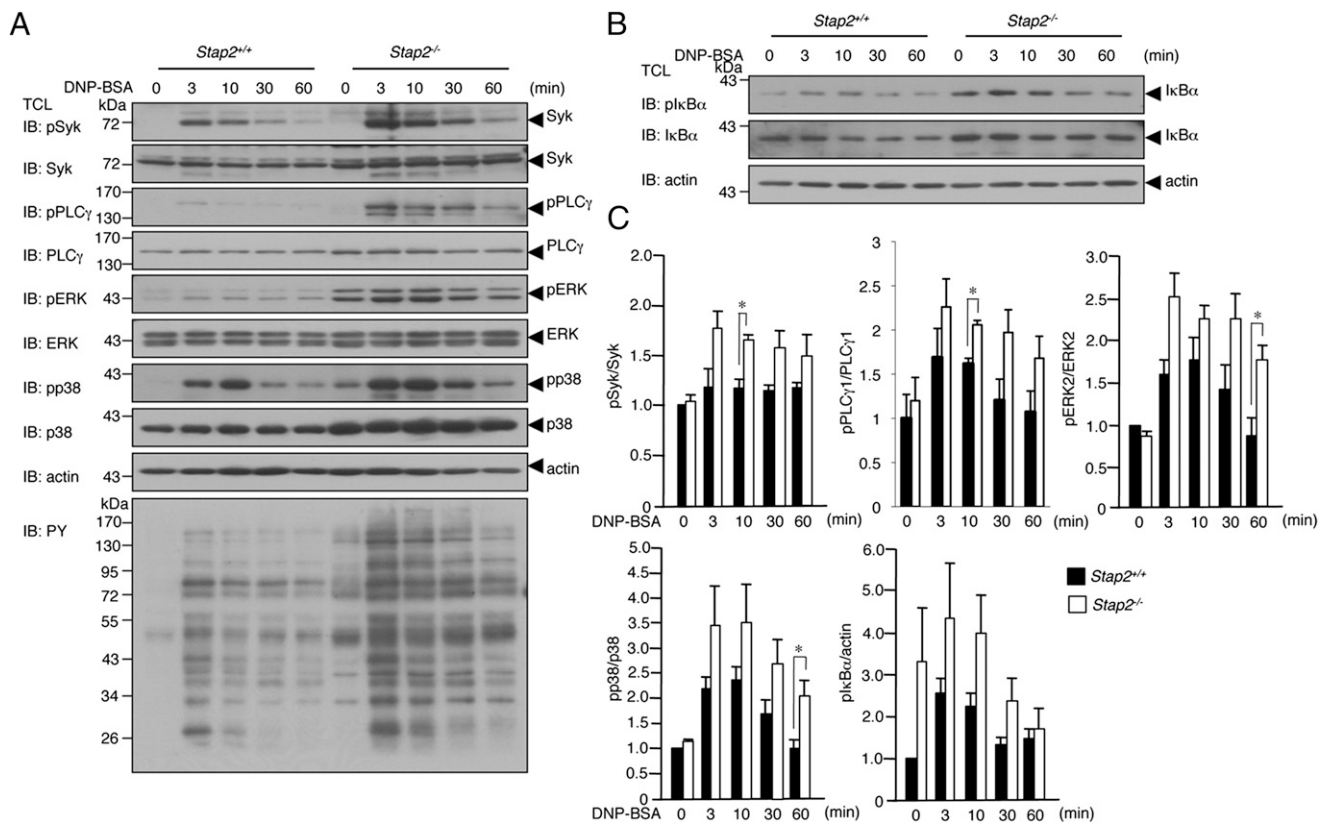


FIGURE 4. FcεRI-mediated proximal signaling in WT and STAP-2 KO BMMCs. (A) WT and STAP-2 KO BMMCs (1×10^6) were sensitized with anti-DNP IgE (5 μg/ml) for 12 h. After washing, the cells were stimulated with DNP-BSA (1 μg/ml) for the indicated periods. The cells were then lysed, and total cell lysate was analyzed by immunoblotting with anti-pSyk, anti-Syk, anti-pPLC-γ1, anti-PLC-γ1, anti-pERK, anti-ERK, anti-pp38, anti-p38, anti-actin, and anti-PY Abs. These experiments were performed three times independently, and representative data are shown. (B) WT and STAP-2 KO BMMCs (1×10^6) were sensitized with anti-DNP IgE (5 μg/ml) for 12 h. After washing, the cells were stimulated with DNP-BSA (1 μg/ml) for the indicated periods. The cells were then lysed, and total cell lysate was analyzed by immunoblotting with anti-pIkBα, anti-IkBα, and anti-actin Abs. (C) Densitometric analysis for (A) and (B) was performed using an LAS-1000 fluorescence image analyzer. The value of nonstimulation in WT BMMCs was set as 1. Data are representative of three independent experiments (mean ± SEM). * $p < 0.05$.

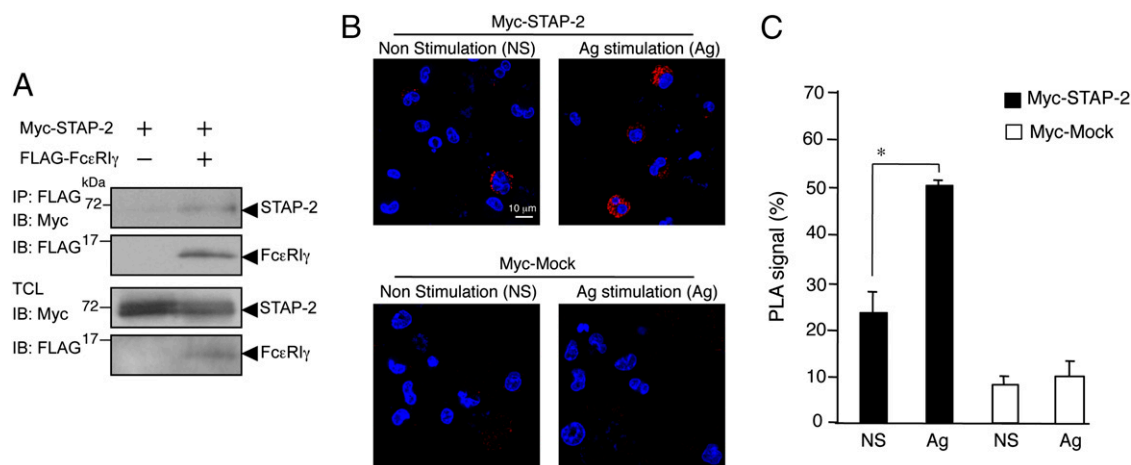


FIGURE 5. STAP-2 can associate with FcεRIγ in BMMCs. **(A)** NIH3T3 cells in a six-well plate were retrovirally transfected with Myc-STAP-2 together without or with FLAG-FcεRIγ. At 36 h after transfection, the cells were lysed and immunoprecipitated with anti-FLAG Ab and immunoblotted with anti-Myc or anti-FLAG Ab. Total cell extracts were blotted with anti-Myc or anti-FLAG Ab. These experiments were performed three times independently, and representative data are shown. **(B and C)** STAP-2 and FcεRIγ interaction in transfected BMMCs was visualized using in situ PLA. Cells were fixed, and protein-protein interactions were visualized utilizing anti-Myc (detection in STAP-2) and anti-FcεRIγ (detection in FcεRIγ) Abs, as described in *Materials and Methods*. Punctate staining (red) indicates the STAP2-FcεRIγ interaction as detected. * $p < 0.05$.

activation of Syk is markedly affected by STAP-2 deficiency. We speculated that STAP-2 may be a component of the IgE receptor complex upstream of Syk. We investigated whether STAP-2 interacts with FcεRIγ. NIH3T3 cells were transfected with Myc-STAP-2 and FLAG-FcεRIγ. As shown in Fig. 5A, immunoprecipitation of FcεRIγ with anti-FLAG Ab pulled down STAP-2 proteins (recognized by anti-Myc Ab). To further determine whether STAP-2 directly associated with FcεRIγ, we performed a PLA (Duolink), in which a fluorescent signal appears wherever two molecules are within 40 nm, indicative of their physical interaction. Although the association of transfected Myc-STAP-2 with endogenous FcεRIγ in BMMCs was observed without Ag stimulation (Fig. 5B, upper panel, NS), the interaction was greatly enhanced after Ag stimulation (Fig. 5B, upper panel, Ag). The specificity of this interaction was confirmed by the absence of PLA signals in cells

mock transfected with Myc alone (Fig. 5B, lower panel). Quantification of these interactions revealed a significant increase of cells showing the PLA signals for the association between STAP-2 and endogenous FcεRIγ at 5 min after Ag stimulation (Fig. 5C). These data indicated that STAP-2 physically associates with FcεRIγ in BMMCs.

In vivo effects of STAP-2 in anaphylactic responses

As STAP-2 inhibited FcεRI-mediated degranulation of mast cells in vitro, we next determined whether STAP-2 was involved in an IgE-mediated type I immediate hypersensitivity reaction and passive systemic anaphylaxis. We first examined whether STAP-2 was involved in IgE-mediated passive cutaneous anaphylaxis reactions in the ear. We do not observe different numbers of mast cells in the skin between WT and STAP-2-deficient mice under

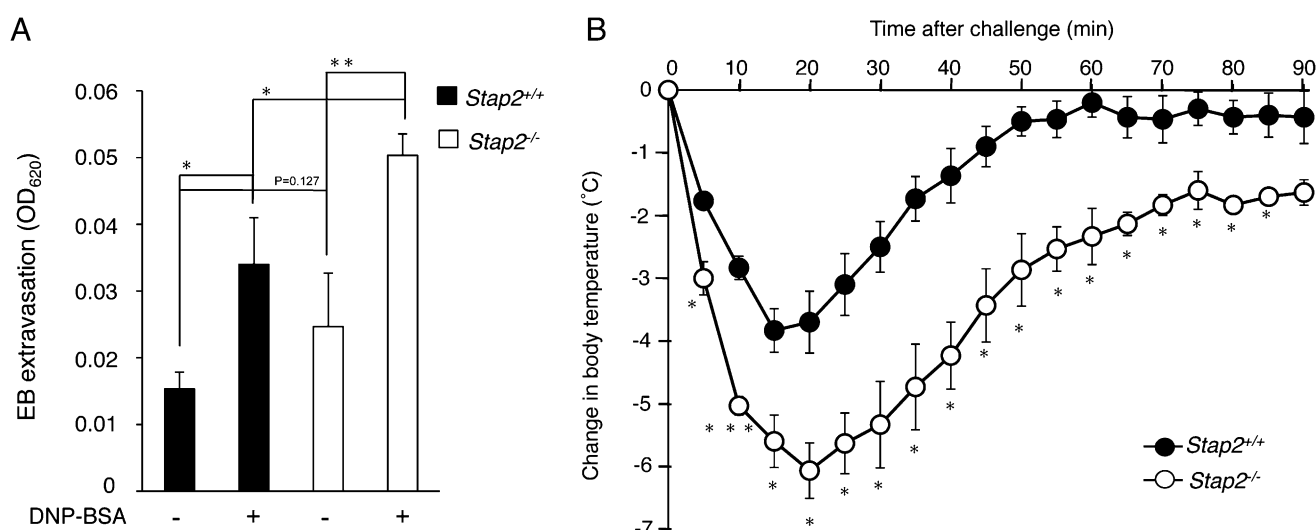


FIGURE 6. In vivo effects of STAP-2 in anaphylactic responses. **(A)** WT or STAP-2 KO mice received intradermal injections of IgE anti-DNP (2 μg) into the right ear and of saline into the left ear. After 16 h, mice were challenged by i.v. injection of Ag DNP-BSA (1 μg) along with Evans blue for 30 min. From each ear, Evans blue was extracted in formamide, and the intensity of the dye was measured by absorption at 620 nm. Data are representative of three independent experiments (means + SD of the OD at 620 nm; $n = 3$ mice per group). * $p < 0.05$, ** $p < 0.01$ (unpaired Student t test). **(B)** Rectal temperatures of WT (filled circle) and STAP-2 KO (open square) mice ($n = 3$ per genotype) injected i.v. with 2 μg of mouse anti-DNP IgE mAb, then challenged 16 h later with 0.5 mg DNP-BSA, assessed every 5 min for 90 min. Data are representative of three independent experiments (mean ± SEM). * $p < 0.05$, *** $p < 0.001$, WT versus STAP-2 KO mice challenged with DNP-BSA (unpaired Student t test).

steady state conditions (Supplemental Fig. 2A, 2B). We passively sensitized WT and STAP-2-deficient mice by intradermal injection of anti-DNP IgE and then challenged them by i.v. injection of DNP-BSA with Evans Blue. STAP-2-deficient mice tended to show more dye extravasation even before DNP-BSA injection than did WT mice, although the difference was not statistically significant. Sensitization with anti-DNP IgE significantly enhanced extravasation of Evans blue in both STAP-2-deficient and WT mice, but the level was still higher in STAP-2-deficient mice (Fig. 6A). We next examined whether STAP-2 was also involved in passive systemic anaphylaxis. We passively sensitized WT and STAP-2-deficient mice with anti-DNP IgE and then injected DNP-BSA into the presensitized mice. We measured rectal temperature, an indicator of passive systemic anaphylaxis, every 5 min after the injection. As shown in Fig. 6B, WT mice challenged with DNP-BSA showed a progressive decrease in rectal temperature to 4°C below the basal temperature by 20 min after injection, followed by a gradual recovery to basal temperature. STAP-2-deficient mice challenged with DNP-BSA showed a more severe rectal temperature decrease than did WT mice during the entire 90-min observation period. Therefore, STAP-2 is likely to suppress passive systemic anaphylaxis reactions. Taken together, these data suggest that STAP-2 negatively regulates IgE-mediated mast cell activation *in vivo* and suppresses type I immediate hypersensitivity reactions.

Discussion

Mast cells act as major effector cells of type I hypersensitivity reactions. In this study, we show that STAP-2 negatively controls mast cell functions *in vitro* and *in vivo*. To evaluate its physiological roles, we used STAP-2-deficient BMMCs and mice. STAP-2 deficiency greatly enhanced FcεRI-mediated signals, resulting in the increased tyrosine phosphorylation of PLC-γ, calcium mobilization, and degranulation. Of importance, STAP-2-deficient mice showed a more severe passive systemic anaphylaxis reaction than did WT mice. Taken together with the fact that expression of STAP-2 was constitutively observed, STAP-2 appears to be an intrinsic negative regulator of allergic responses in mast cells.

Mast cells express the high-affinity FcεRI receptor for IgE. Activation of FcεRI activates several signaling pathways, which leads to degranulation and release of histamine (1). The tyrosine kinases, including Lyn, Fyn, and Syk, are involved in initiating the assembly of large signaling complexes (2, 3). In turn, this leads to diacylglycerol production and inositol-1,4,5-triphosphate-induced calcium mobilization. In contrast, IKK-β in mast cells was reported to be recruited into the lipid raft fractions and to phosphorylate SNAP-23 upon FcεRI stimulation (24). This IKK-β-mediated pathway also leads to increased degranulation. Among these molecules, STAP-2 is a substrate for Syk (15). In addition, we have reported that STAP-2 interacts with PLC-γ and IKK-β (12, 15). In this study, we also identified that STAP-2 can physically bind to FcεRI. Therefore, STAP-2 is likely to modify FcεRI-mediated signals at multiple levels.

The overall structure of STAP-2 resembles some common features of docking proteins such as insulin receptor substrates (25), Grb2 associated binder (Gab) (26, 27), and downstream of kinase (Dok) (28), because these docking molecules contain a pleckstrin homology domain at the N terminus, a PY binding domain in the middle, and tyrosine phosphorylation sites in the C-terminal region. Gab2-deficient BMMCs most likely have defective FcεRI-mediated signaling owing to the lack of activation of PI3K (29). Dok has been reported to be essential for FcγRIIB-mediated inhibition of the FcεRI-mediated signaling pathway (30). It could be possible that STAP-2 may cooperate with Gab and/or Dok in

FcεRI-mediated signaling; however, it remains to be determined whether STAP-2 has some direct interaction with Gab or Dok.

Type I hypersensitivity reactions clinically cause allergic asthma, allergic skin inflammation, and food and drug allergy, some of which can lead to life-threatening anaphylaxis (31, 32). These conditions are often triggered by FcεRI-mediated signals in mast cells (1, 2). Thus, the inhibition of FcεRI has led to various therapeutic approaches for allergies. For example, mAbs specific for IgE are effective tools in treating patients with asthma (33) and peanut allergy (34). Inhibition of downstream signaling molecules, such as Syk, may also be a target of this treatment. Our data described in this article strongly suggest that manipulation of STAP-2 expression levels in mast cells could control pathogenesis of allergic diseases. Hence, we propose that STAP-2 could be a novel target molecule for developing drugs to treat asthma and allergic diseases.

Disclosures

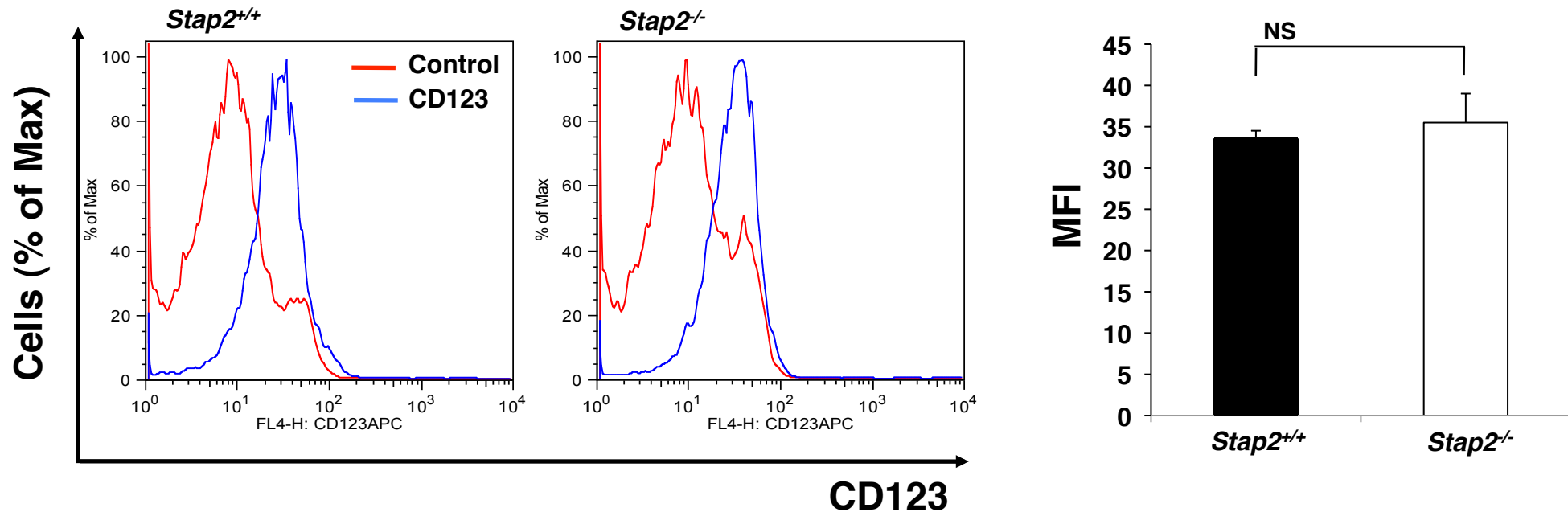
The authors have no financial conflicts of interest.

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Supplemental Figure 1

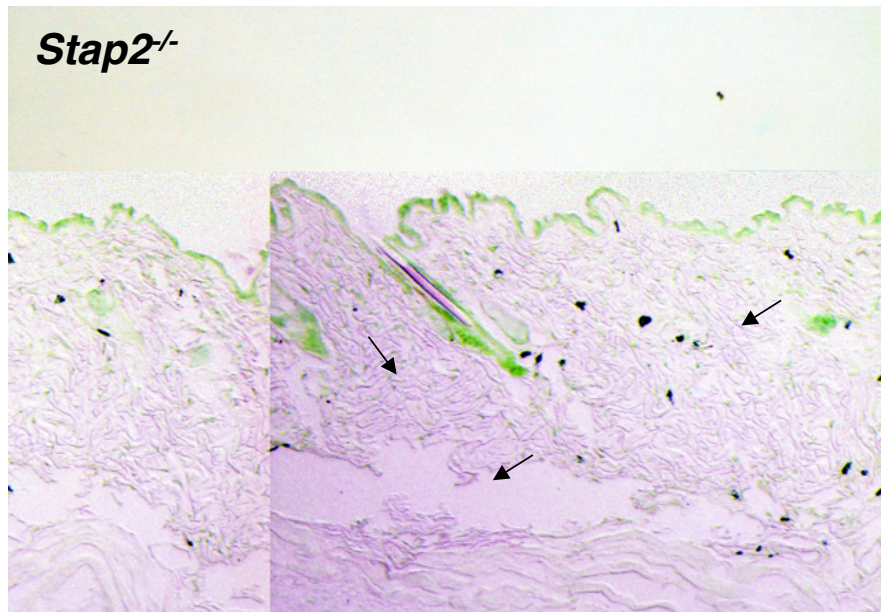
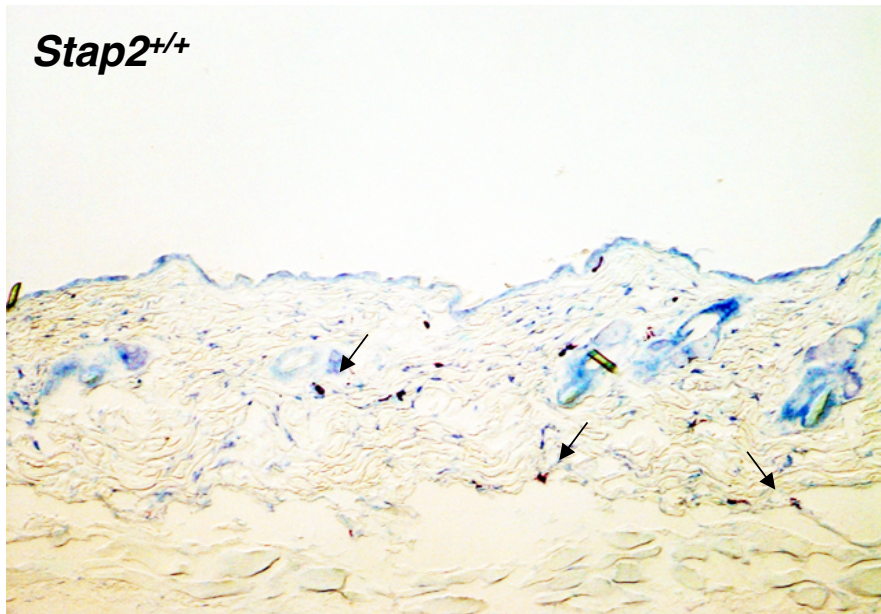


Supplemental Figure 1. Normal expression level of IL-3 receptor on *Stap2*^{-/-} BMMCs.

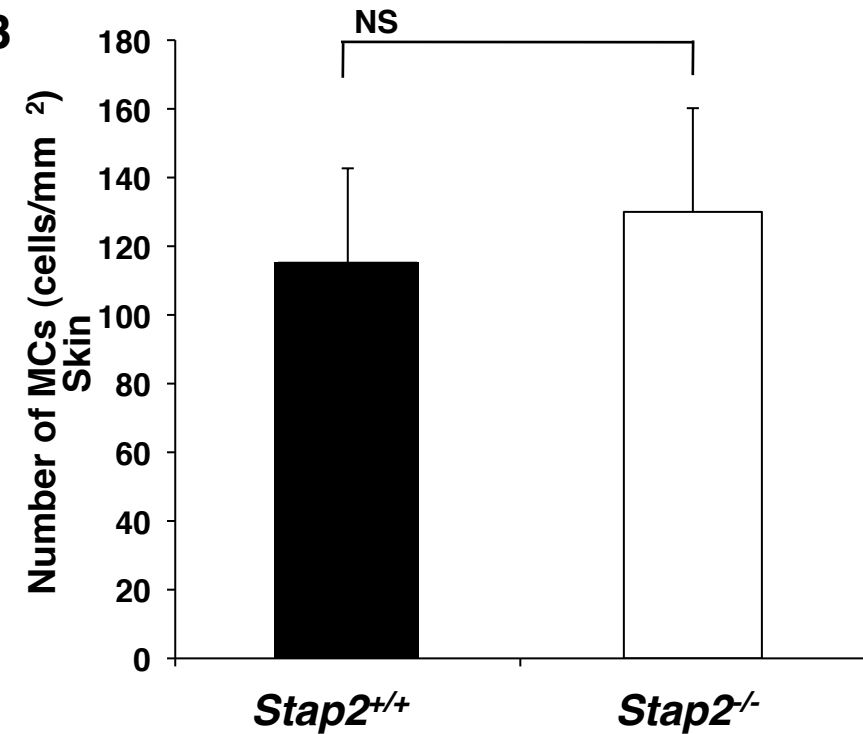
Surface expression of IL-3R alpha chain (CD123) on mast cell prepared from bone marrow. Histograms show mean fluorescent intensity (right panel). NS, not significant. (unpaired Student's *t*-test.). These experiments were performed three times independently, and representative data was shown.

Supplemental Figure 2

A



B



Supplemental Figure 2. Normal development of *Stap2^{-/-}* mast cells. (A) Histological analysis of mast cells from the skin of *Stap2^{+/+}* (n = 5) and *Stap2^{-/-}* (n = 4) mice. Skin samples were fixed in 4% paraformaldehyde, embedded in an OCT compound, snap frozen in liquid nitrogen and stored at -80°C until use. Frozen sections cut at 5 micrometer. The sections were stained with toluidine blue (pH 2.5). Arrows indicate mast cells. (B) Mast cell numbers were counted under a microscope at a magnification of x400 and expressed as the total number of the cells in two high power fields per section. Absolute numbers of mast cells expressed as number per mm² of skin. NS, not significant. (unpaired Student's *t*-test.)