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Short Communications

STAP-2 positively regulates FcεRI-mediated basophil activation and
basophil-dependent allergic inflammatory reaction

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Abstract (<250 words)

Basophils are an important cell type in the regulation of Th2 immune responses. Recently, we revealed that STAP-2 negatively regulates mast cell activation via FcεRI. However, the role of STAP-2 in basophil maturation and activation remained unclear. In this study, we demonstrated the normal development of basophils in STAP-2 deficient (STAP-2^{-/-}) mice. We also demonstrated *in vitro* normal basophil differentiation and FcεRI expression in STAP-2^{-/-} mice, suggesting that STAP-2 is dispensable for basophil maturation. Using bone marrow-derived cultured basophils (BMBs), we showed that degranulation and cytokine production of STAP-2^{-/-} BMBs were lower than those of wild-type (WT) BMBs upon stimulation with IgE/Ag. In accordance with the reduction of degranulation and cytokine production, phosphorylation of several signal molecules such as Lyn, PLC-γ2 and Erk was reduced in STAP-2^{-/-} BMBs after stimulation via FcεRI. Finally, it was observed that IgE-dependent chronic allergic inflammation of STAP-2^{-/-} mice was significantly inhibited compared with WT mice. Taken together, we conclude that STAP-2 is an adaptor molecule that positively regulates FcεRI-mediated basophil activation and basophil-dependent allergic inflammatory reactions.

Introduction

Basophils have been recognized as crucial granulocytes in allergic inflammation and in the protection against helminth infection although small numbers of basophils are found in blood. It was thought that basophils and mast cells were functionally similar because both cells express Fc ϵ RI, a high affinity IgE receptor, on their surface. However, several recent studies have reported that basophils have nonredundant functions in certain immune reactions. Mukai and colleagues found that the Fc ϵ RI-mediated chronic inflammatory reaction is dependent on basophils and that mast cells are dispensable for the reaction but not IgE-dependent acute and late-phase reactions (1). Basophils produce abundant IL-4 after stimulation with IL-33 and exposure of hapten/peptide, contributing to the initiation of Th2 immune responses (2,3). In addition, basophils, but not mast cells, are essential for secondary immune responses against helminth (4). These findings indicate that basophils are functionally distinguishable from mast cells. Signal-transducing adaptor protein-2 (STAP-2), originally identified as a substrate of breast tumor kinase, has been reported to be involved in crucial cellular events, such as cytokine-dependent STAT activation, SDF-1 α /CXCR4-mediated T cell chemotaxis, Fas/FasL-mediated T cell

apoptosis, integrin-dependent T cell adhesion, and LPS-induced macrophage activation (5-11). STAP-2 has also been reported to negatively regulate IgE/antigen (Ag)-dependent Fc ϵ RI signaling in mast cells. IgE/Ag-induced degranulation and cytokine production are significantly increased in STAP-2^{-/-} bone marrow-derived cultured mast cells (BMMCs) compared with wild type (WT) BMMCs. Using *in vivo* passive cutaneous/systemic anaphylactic models, it is suggested that STAP-2 is an important adaptor molecule for homeostatic regulation of Fc ϵ RI signaling in mast cells (12).

Although STAP-2 negatively regulates Fc ϵ RI signaling in mast cells its role in basophil development and IgE/Ag-induced basophils activation events remain unknown. We herein report that STAP-2 is essential for IgE-mediated basophil activation, but not basophil generation, both *in vitro* and *in vivo*.

Materials and Methods

Mice

Balb/c and C57BL/6 mice were purchased from SANKYO LABO SERVICE CO. Inc. (Hokkaido, Japan). C57BL/6-background STAP-2-deficient (STAP-2^{-/-}) mice as described previously (5) were backcrossed to Balb/c more than ten times. All

animal studies were approved by the Hokkaido University animal ethics committee. All mice were housed and bred in the Pharmaceutical Sciences Animal Center of Hokkaido University under specific pathogen-free conditions.

Antibodies

Anti-DNP IgE mAb (clone: DNP H1- ϵ -206) was described previously (13). PE anti-mouse CD200R3 (clone: Ba13), PE anti-mouse c-Kit (clone: 2B8), PerCP/Cy5.5 anti-mouse CD49b (clone: DX5), APC anti-mouse Fc ϵ RI α (clone: MAR-1) and anti-Lyn (clone: LYN-01) mAbs were purchased from Biolegend (San Diego, CA). APC anti-mouse CD49b mAb (clone: HM α 2) was purchased from BD Biosciences (San Jose, CA). Anti-phospho Src, anti-phospho Erk and anti-phospho PLC- γ 2 mAbs were purchased from Cell Signaling Technology (Beverly, MA). Other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Flowcytometric analysis

The cells were first incubated with 10 μ g/ml 2.4G2 (TONBO biosciences, San Diego, CA) at 4 ° C for 15 min. The cells were then incubated with

fluorochrome-conjugated Abs at 4 ° C for 30 min. Fluorescence of the stained cells was detected using FACSCalibur (BD Biosciences) and analyzed using FlowJo software version 10 (FlowJo, LLC, Ashland OR).

Generation and purification of bone marrow-derived basophil

Femoral bone marrow cells derived from Balb/c WT and STAP-2^{-/-} mice on Balb/c background were cultured in 10% FCS RPMI1640 containing 1 ng/ml recombinant mouse IL-3 (TONBO biosciences) for 9 days to generate bone marrow-derived cultured basophils (BMBs). To obtain the BMBs, CD49b⁺ cells were purified by magnetic separation system with biotinylated anti-mouse CD49b mAb (Biolegend) and Streptavidin Particle Plus – DM (BD Biosciences). The purity of CD49b⁺ BMBs was more than 95 % after the purification.

Degranulation

Degranulation of BMBs was determined by measurement of β -hexosaminidase release as described previously (12). Briefly, IgE-sensitized BMBs ($5 \times 10^4/100 \mu\text{l}$) were stimulated with indicated concentrations of DNP₂₃-HSA (BIOSEARCH TECHNOLOGIES, Inc, Novato, CA) for 30min. The supernatants and cell pellet

lysates were incubated with 10 mM *p*-nitrophenyl N-acetyl β -D-glucosaminide (Sigma-Aldrich, St. Louis, MO) for 90 min at 37 ° C and the reaction was stopped by addition of 0.4 M Glycine (pH = 10.7). The release of the product, *p*-nitrophenol, was monitored by measuring absorbance at 405 nm.

Cytokine production

IgE-sensitized BMBs were stimulated with indicated concentrations of DNP₂₃-HSA for 24 h. IL-4, IL-6 and IL-13 levels in supernatants were measured using ELISA kits (IL-4 and IL-6; Biolegend, IL-13; Affimetrix Inc., San Diego, CA).

mRNA expression analysis

Briefly, total RNA was purified using TRI Reagent (Sigma-Aldrich). An equal amount of total RNA (1 μ g) was used for reverse transcription reaction. The cDNA was amplified in a 20- μ l reaction volume using KOD FX Neo DNA polymerase (TOYOBO, Osaka, Japan). PCR was performed under the following conditions: predenaturation at 94 ° C for 2 min, 30 (GAPDH) and 35 (STAP-2) cycles of DNA amplification (GAPDH: 10 s at 98 ° C, 30 s at 57 ° C, 20 s at 68 ° C; STAP-2: 10 s at 98 ° C, 30 s at 55 ° C, 20 s at 68 ° C. PCR products were

analyzed by electrophoresis on 1.5% agarose gels. Quantitative PCR analysis for detection of cytokine expression was performed using KAPA SYBR FAST qPCR Master Mix (NIPPON Genetics Co., Ltd, Tokyo, Japan). Expression of mcpt-8 and mcpt-11 was analyzed by an RT-PCR technique using specific primers as previously described (14). Other PCR primer sets were shown as follows;

STAP-2	(forward	TGCCTCAGTTACCAGACACG,	reverse
CTGGTCCAAACGCTGGTAAT),		GAPDH	(forward
GAAATCCCATCACCATCTTCCAGG,			reverse
CAGTAGAGGCAGGGATGATGTTC),		IL-4	(forward
CATCGGCATTTTGAACGAG,	reverse	CGAGCTCACTCTCTGTGGTG),	IL-6
(forward		CCAAACTGGATATAATCAGGAAAT,	reverse
CTAGGTTTGCCGAGTAGATCTC),		IL-13	(forward
AGGAGCTGAGCAACATCACA,	reverse	GGTTACAGAGGCCATGCAAT),	
β -Actin	(forward	TGACAGGATGCAGAAGGAGA,	reverse
CGCTCAGGAGGAGCAATG).			

Western blot

For detection of phosphorylated proteins, cell lysates were prepared using lysis

buffer (50 mM Tris-HCl (pH7.4), 0.15 M NaCl, 1% NP-40, 1 mM PMSF, 25 μ M 4-nitrophenyl 4-guanidinobenzoate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 μ M pepstatin, 1 mM Na_3VO_4 , 2 mM NaF). The samples were boiled with SDS sample buffer and were analyzed by SDS-PAGE and transferred to PVDF membranes. The membranes were first blocked and incubated with primary Abs. Proteins reactive with the primary Abs were visualized with HRP-conjugated secondary Abs (GE Healthcare Bio-Sciences) and Immobilon Western Chemiluminescent HRP Substrate (Millipore, Bedford, MA). Densitometrical analysis was performed using ImageJ program (NIH, Bethesda, MD)

IgE-CAI

IgE-CAI was performed as described previously with some modification (1). Briefly, mice were intravenously sensitized with 100 μ g anti-DNP-IgE. After 24 h, mice were intradermally challenged with 10 μ g DNP₁₁-OVA into the right ear and OVA as control into the left ear. In some experiments, a basophil-depleted antibody, Ba103 (Hycult Biotech, Wayne, PA), or an isotype-matched antibody (25 μ g/mouse) was intravenously injected 24 h before antigen challenge (15). Ear thickness was monitored once daily for 6 days. To analyze ear skin

inflammation, formalin-fixed paraffin-embedded ear sample specimens (5 μm) were stained with hematoxylin and eosin.

Statistical analysis

Statistical analysis was performed using Mann-Whitney test and Tukey's multiple comparisons test.

Results and Discussion

STAP-2 deficiency influences neither basophil generation nor FcεRI expression

We previously reported that STAP-2 negatively regulates FcεRI signaling in mast cells (12). However, it remained to be determined whether STAP-2 was involved in basophil generation and maintenance. First, to analyze the role of STAP-2 in basophil differentiation *in vivo*, we monitored basophil percentages and absolute numbers in lympho-hematopoietic organs of STAP-2^{-/-} mice on Balb/c background and compared these of Balb/c WT mice. The basophil percentages in spleen and bone marrows of STAP-2^{-/-} mice were comparable to those of WT mice (STAP-2 KO 0.59 ± 0.20 vs WT 0.34 ± 0.21% in spleen, STAP-2 KO 1.08 ± 0.29 vs WT 0.95 ± 0.20% in bone marrow). On the other hand, the blood basophil percentages in STAP-2^{-/-} mice were significantly higher compared to those of WT mice (STAP-2 KO 0.94 ± 0.17 Vs WT 0.47 ± 0.04% in blood). ~~Similarly,~~ We measured the absolute numbers of basophils in these organs and showed no significant differences between WT and STAP-2^{-/-} mice (Figure 1A-C). These results suggested that STAP-2 is dispensable for the basophil development *in vivo*. Next, to investigate the effect of STAP-2 on FcεRI

expression of basophil *in vivo*, we detected the expression of FcεRI on basophils by FACS analysis. The expression levels of FcεRI on basophils of STAP-2^{-/-} mice on Balb/c background were similar to those of Balb/c WT basophils (Figure 1D and E), suggesting that STAP-2 is not essential for the regulation of FcεRI expression on basophils. We also measured total IgE levels, an important immunoglobulin that affects FcεRI expression, in the sera of WT and STAP-2^{-/-} mice and no difference was observed (data not shown). Taken together, these results indicated that the basophil phenotypes of STAP-2^{-/-} mice were similar to those of WT mice.

To test whether STAP-2 affects IL-3-dependent basophil generation *in vitro*, we cultured bone marrow cells with IL-3 to generate bone marrow-derived basophils (BMBs). After culturing for 9 days, we compared CD49b⁺CD200R3⁺ basophil percentages between cultured Balb/c WT cells and STAP-2^{-/-} cells (Figure 2A). The basophil percentages in the cultured STAP-2^{-/-} cells were similar levels to those in the cultured Balb/c WT cells (39.48 ± 4.14 % in Balb/c vs 36.91 ± 3.19 % in STAP-2^{-/-}, Figure 2B left panel). We also observed similar mast cell percentages between Balb/c WT and STAP-2^{-/-} cell cultures (Figure 2B right panel). When we investigated the expression levels of FcεRI on the surface

of both Balb/c WT and STAP-2^{-/-} BMBs, there was no significant difference between the WT and STAP-2^{-/-} BMBs (Figure 2C and D). We also found similar expression levels of mcpt-8 and mcpt-11 genes, which are basophil specific proteases, between WT and STAP-2^{-/-} BMBs (Figure 2E and F). These results suggested that STAP-2 is dispensable for IL-3-dependent basophil differentiation, and the expression of FcεRI and basophil-specific proteases *in vitro*.

Previously, it has been reported that STAP-2 constitutively interacts with STAT-5 in several cell types and this interaction is dissociated by stimulation with some cytokines such as IL-2, EPO and IL-3 (6,16). STAP-2 overexpression inhibits EPO- and IL-3-induced STAT-5 phosphorylation in transfected HEK293T and Ba/F3 cells, indicating that STAT5 phosphorylation and STAT5-mediated cytokine signaling are negatively controlled by STAP-2. STAT5 and GATA-2 are involved in the IL-3-induced generation of basophils (17). In the present , we found that deficiency of STAP-2 didn't result in inhibition of IL-3-induced basophil generation. In this regard, we hypothesize that STAP-1, another STAP family member, may be involved in IL-3 signaling for basophil development in STAP-2^{-/-} mice. Indeed, STAP-1 is truly expressed in basophils (data not shown).

The contribution of STAP-1 in IL-3-induced basophil maturation will be the subject of a future study.

STAP-2 positively regulated FcεRI-mediated basophil activation

It has been reported that STAP-2 negatively regulates FcεRI-mediated mast cells activation in vitro and in vivo (12). However, the effect of STAP-2 on basophil activation through FcεRI remains unknown. To investigate whether STAP-2 is involved in FcεRI-mediated basophil activation, we analyzed degranulation and cytokine production by STAP-2^{-/-} BMBs. First, we analyzed expression of STAP-2 in WT and STAP-2^{-/-} BMBs by RT-PCR. We observed the expression of STAP-2 in WT, but not STAP-2^{-/-}, BMBs (Fig. 3A). Next, we analyzed the biological functions of STAP-2 in basophils. Upon DNP₂₃-HSA Ag stimulation, β-hexosaminidase (β-hex) release was observed in IgE-sensitized WT BMBs. Degranulation level of STAP-2^{-/-} BMBs was lower than the WT BMBs and the statistical significance was observed when the STAP-2^{-/-} BMBs were stimulated with 100 ng/ml Ag (Figure 3AB). We next compared cytokine expression/production in STAP-2^{-/-} BMBs than WT BMBs. The levels of IL-4 and IL-6 production in STAP-2^{-/-} BMBs were lower than these in WT BMBs and we

observed the statistical differences at 10 ng/ml (only IL-4) and 100 ng/ml Ag stimulation (both IL-4 and IL-6). The level of IL-13 production in STAP-2^{-/-} BMBs also tended to be reduced, but the reduction was not statistical significance (Figure 3C) We also tested the expression of cytokine mRNA in BMB after stimulation with Ag. The expression of IL-4 and IL-6 was down-regulated in STAP-2^{-/-} BMBs compared with WT BMBs and statistical differences were observed at 100 ng/ml Ag stimulation. Also, it was observed that IL-13 expression level tended to be reduced, however, we observed no significant difference between WT and STAP-2^{-/-} (Figure 3D). We next investigated the effect of STAP-2 on FcεRI-mediated signal transduction in basophils after stimulation with Ag. After stimulation of IgE-sensitized WT BMBs with Ag, phosphorylation of Lyn, PLC-γ2 and Erk was induced. This increase in molecule phosphorylation was suppressed in Ag-activated STAP-2^{-/-} BMBs (Figure 3E). By densitometrical analysis, we found that Lyn phosphorylation at 1 minute after stimulation was significantly reduced in STAP-2^{-/-} BMBs compared WT BMBs. Also, PLC-γ2 phosphorylation at 10 minutes after stimulation was significantly inhibited in STAP-2^{-/-} BMBs. Erk phosphorylation in STAP-2^{-/-} BMBs was lower than that in WT BMBs although it was not significantly reduced (Figure 3F).

Taken together, these data suggested that STAP-2 positively regulates FcεRI-mediated basophil activation *in vitro*.

In mast cells, STAP-2 is an adaptor protein that negatively regulates FcεRI signaling (12). However, we here revealed that IgE/Ag-induced degranulation and cytokine production were significantly inhibited in STAP-2^{-/-} basophils compared with WT basophils. Although the reason for this remains unknown, there are several suggestive reports. Xiao and colleagues reported that Lyn positively and negatively regulates FcεRI signaling in mast cells depending on the strength of the stimulus via FcεRI (18). Lyn has a positive effect in mast cells when the cells are weakly stimulated such as by monomeric IgE and low Ag concentration. Conversely, when mast cells are stimulated with a high Ag concentration, Lyn^{-/-} mast cells increase cytokine production and degranulation, suggesting that Lyn also has negative regulatory functions. It has previously reported that PLC-β3 constitutively bind to Lyn/SHP-1/STAT-5 and this complex is essential for Lyn-mediated suppression of FcεRI signaling (19). Our preliminary data revealed that under negative regulation condition (high Ag stimulation), Erk phosphorylation was increased in STAP-2^{-/-} BMMCs although Lyn phosphorylation was normal (data not shown). This implicated that STAP-2

might be involved in the PLC- β 3/Lyn/SHP-1/STAT-5 complex formation for negative regulation of Fc ϵ RI-mediated mast cells activation. Although there is no report showing that the Lyn-mediated positive and negative regulation of Fc ϵ RI in basophil, we guess ~~We therefore propose~~ that the contribution of Lyn to Fc ϵ RI signaling in basophils may be different from mast cells or the threshold of stimulation in basophils may differ from that in mast cells. This will be the subject of future studies. The linker for activation of B cells (NTAL/LAT2) was reported to play a role in negative regulation of IgE/Ag- and monomeric IgE-induced Fc ϵ RI signaling (20-22). Recently, Suzuki and colleagues revealed that although both high and low affinity stimuli elicit similar receptor phosphorylation, the association of Src family kinase Fgr with NTAL/LAT2 was increased in response to a low-affinity stimulus compared with a high-affinity stimulus, resulting in lower degranulation, leukotriene secretion and cytokine production, but higher chemokine production (23). Thus, it is interesting to compare the expression of NTAL and Fgr as well as the colocalization of Fgr with NTAL between mast cells and basophils after stimulation with different antigen concentration. This complex formation may be involved in the discriminatory roles of STAP-2 in mast cells and basophils.

IgE-dependent chronic allergic inflammation was inhibited in STAP-2^{-/-} mice

IgE-dependent chronic allergic inflammation (IgE-CAI) is an experimental system to test the chronic phase response, which starts on day 2 and peaked on day 4 after Ag challenge (1). IgE-CAI is accompanied by massive infiltration of leukocytes including neutrophils and eosinophils in the ear skin lesion, and is dependent on basophils, but not mast cells. To investigate the role of STAP-2 in FcεRI-mediated basophil activation in vivo, we employed the IgE-CAI system. Before IgE-CAI induction, the ear thickness of STAP-2^{-/-} mice were comparable to those of WT mice. After IgE-sensitized mice were administered with Ag, Balb/c WT mice showed significantly increased ear thickness at the chronic phase. This increase in ear thickness was significantly inhibited in STAP-2^{-/-} mice (Figure 4A). We also observed increased inflammatory cell infiltration in the Ag-injected ears of Balb/c WT mice and this increase was attenuated in STAP-2^{-/-} mice (Figure 4B). Because we used DNP/anti-DNP IgE for the reaction, which differed from the original paper (1), we tested whether this reaction was depend on basophil or not. Basophil depletion

antibody (Ba103)-treated mice showed no IgE-CAI response, suggesting that basophil was also necessary for our IgE-CAI reaction condition (Figure 4C). Taken together, these results suggested that STAP-2 is necessary for FcεRI-mediated basophil activation *in vivo*.

Basophils have specific functions, which do not always match with those of mast cells. Upon stimulation, basophils can readily secrete large amount of IL-4, a multifunctional cytokine in immunity. STAP-2 plays an essential role in FcεRI-mediated basophil activation *in vitro* and *in vivo*, and may be a new therapeutic candidate for patients with allergic diseases, such as food allergy and bronchial asthma.

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Figure legends

Figure 1 Normal in vivo basophil development in STAP-2^{-/-} mice

(A-C) Basophil population in spleens (Sp), bone marrows (BM) and peripheral blood (Blood) of WT and STAP-2^{-/-} mice on Balb/c background. Three independent experiments were performed and pooled data were shown mean + SEM (WT n = 4, STAP-2^{-/-} n = 4). *:p<0.05 by Mann-Whitney test. (D & E) FcεRI expression on basophils of WT and STAP-2^{-/-} mice. Data were shown mean + SEM (WT n = 4, STAP-2^{-/-} n = 4). Black line and gray filled histograms indicate anti-FcεRIα mAb-treated and untreated basophils (CD49b⁺CD200R3⁺ cells in blood). ns = no significance

Figure 2 Normal in vitro basophil development in STAP-2^{-/-} mice

(A & B) Bone marrow-derived cultured basophil (FcεRI⁺c-Kit⁺) and mast cell (FcεRI⁺c-Kit⁺) populations of WT and STAP-2^{-/-} mice on Balb/c background. Six independent experiments were performed and pooled data were shown mean + SEM (WT n = 12, STAP-2^{-/-} n = 12). (C & D) FcεRI expression on bone marrow-cultured basophils of WT and STAP-2^{-/-} mice. Four independent experiments were performed and pooled data were shown mean + SEM (WT n = 7, STAP-2^{-/-} n = 7). Black line and gray filled histograms indicate anti-FcεRIα

mAb-treated and untreated basophils (CD49b⁺CD200R3⁺ cells in blood) (E & F)

Expression of mcpt-8 and mcpt-11 genes in bone marrow-cultured basophils of

WT and STAP-2^{-/-} mice. ns = no significance

Figure 3 Suppression of degranulation, cytokine production and signal molecule phosphorylation in IgE/Ag-stimulated STAP-2^{-/-} basophils

(A) Expression of STAP-2 in WT and STAP-2 KO BMB. (B) Degranulation of basophil of WT and STAP-2^{-/-} mice upon stimulation with IgE/Ag. Two independent experiments were performed and pooled data were shown mean + SEM (White bars; WT n = 4, Black bars; STAP-2^{-/-} n = 4). *:p<0.05 by Mann-Whitney test. (C) Cytokine production of WT and STAP-2^{-/-} basophils upon stimulation with IgE/Ag. Three independent experiments were performed and pooled data were shown mean + SEM (White bars; WT n = 8-10, White bars; STAP-2^{-/-} n = 8-10). ~~**p<0.01~~ *:p<0.05 by Mann-Whitney test. (D) Cytokine mRNA expression of WT and STAP-2^{-/-} basophils 2 h after stimulation with Ag. Two independent experiments were performed and pooled data were shown mean + SEM (White bars; WT n = 6, White bars; STAP-2^{-/-} n = 5). *:p<0.05, **:p<0.01 by Mann-Whitney test. (E) Phosphorylation of Lyn, PLC-γ2 and Erk in

IgE/Ag-stimulated basophils of WT and STAP-2^{-/-} mice. (F) Quantification of phosphorylation levels of Lyn, PLC-γ2 and Erk. Data were shown mean + SEM (White bars; WT n = 5-7, White bars; STAP-2^{-/-} n = 5-7). *:p<0.05, **:p<0.01 by Mann-Whitney test. ns = no significance

Figure 4 IgE-CAI in STAP-2^{-/-} mice

(A) IgE-CAI reactions of WT and STAP-2^{-/-} mice. Two independent experiments were performed and pooled data were shown mean +/- SEM (WT n = 12, STAP-2^{-/-} n = 13). *:p<0.05, ***:p<0.001 by Tukey's multiple comparisons test.

(B) H&E staining of ear tissues obtained from IgE-CAI WT and STAP-2^{-/-} mice on day 4. line = 100 μm (C) IgE-CAI reactions of Ba103-treated WT mice. Data were shown mean +/- SEM (rIgG n = 4, Ba103 n = 4). ***:p<0.001 by Tukey's multiple comparisons test.







