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Signal-transducing adaptor protein-2 has a nonredundant role for IL-33-triggered mast cell activation.

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This manuscript contains 26 pages with 3 figures.

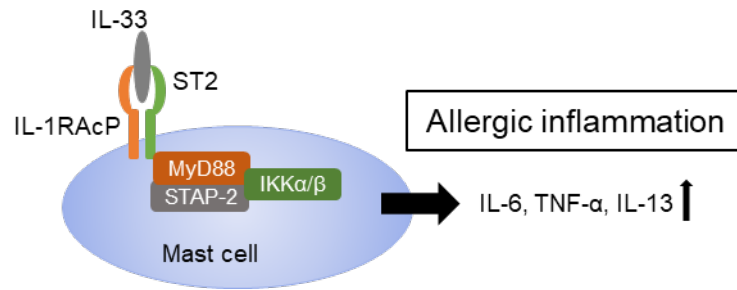
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Abstract

Signal-transducing adaptor protein (STAP)-2 is one of the STAP family adaptor proteins and ubiquitously expressed in a variety types of cells. Although STAP-2 is required for modification of FcεRI signal transduction in mast cells, other involvement of STAP-2 in mast cell functions is unknown, yet. In the present study, we mainly investigated functional roles of STAP-2 in IL-33-induced mast cell activation. In STAP-2-deficient, but not STAP-1-deficient, mast cells, IL-33-induced IL-6 and TNF-α production was significantly decreased compared with that of wild-type mast cells. In addition, STAP-2-deficiency greatly reduced TLR4-mediated mast cell activation and cytokine production. For the mechanisms, STAP-2 directly binds to IKKα after IL-33 stimulation, leading to elevated NF-κB activity. In conclusion, STAP-2, but not STAP-1, participates in IL-33-induced mast cells activation.

Keywords: Mast cells, IL-33, Signal-transducing adaptor molecule-2, ST2, NF-κB

Graphic abstract



Introduction

IL-33, one of the IL-1 family members, is a key cytokine for the development of Th2 immune responses. IL-33 is produced by damaged/stressed epithelial cells and cooperates with IL-25 to activate innate lymphoid cells (ILC2) [1-3]. Activated ILC2 then produce IL-5 and IL-13, resulting in driving generation of Th2 immune responses [1, 4, 5]. IL-33-deficient mice have been reported to be resistant to *Schistosoma mansoni*-induced pulmonary granuloma formation, which is dependent on a typical Th2-like immune responses [6]. The mice also show less severity of allergic immune responses, such as allergic rhinitis and airway inflammation [7]. Thus, IL-33 is likely to be a suitable target to generate a new therapeutic strategy for allergic disorders.

Mast cells (MCs) are an important cell type for IgE-mediated allergic inflammatory reactions, such as anaphylaxis. High affinity IgE receptor (FcεRI) is expressed on cell surface of MCs, and aggregation of IgE-bound FcεRI results in the secretion of granules containing histamine and proteases as well as the production of cytokines, such as IL-6 and TNF-α [8]. MCs are also activated by some stimuli except IgE, including neuropeptides, bacterial/virus components, and allergic inflammation-related cytokines [9]. IL-33 receptor, ST2, is expressed on MCs, and IL-33 accelerates mast cell cytokine production and mast cell-mediated allergic inflammatory reaction

[10-14]. IL-33 also activates human mast cells to promote maturation, survival, adhesion, and cytokine production [15, 16]. In addition, IL-33 is a key cytokine to amplify both IgE-dependent and -independent human mast cell activation [17].

Signal-transducing adaptor protein (STAP) family consists of two members, STAP-1 and STAP-2 [18-21]. STAP-2 is an important protein to regulate immune responses and tumorigenesis [22]. STAP-2, which is expressed in mast cells, negatively regulates degranulation and cytokine production through affecting FcεRI signal transduction [23]. STAP-2 also participates in TLR4 signal transduction and LPS-induced macrophage activation through direct binding to MyD88 [24], which is also an important adaptor protein for the IL-33 signal pathway [25, 26]. However, it remains unknown whether STAP-2 is involved in IL-33-induced mast cell activation and how STAP-2 affects ST2 signal transduction in mast cells. The aims of this study are to investigate effects of STAP-2 on IL-33-induced mast cell activation.

Materials and Methods

Mice

Balb/c and C57BL/6 mice were purchased from SANKYO LABO SERVICE CO. Inc. (Hokkaido, Japan). Balb/c-background STAP-2-deficient (STAP-2 KO) and C57BL/6-

background STAP-1 deficient (STAP-1 KO) mice were previously generated [27, 28]. 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

APC anti-mouse c-Kit (clone: 2B8), FITC anti-mouse FcεRIα (clone: MAR-1), PE anti-mouse ST2 (clone: DIH4) and PE anti-mouse TLR4 (clone: MTS510) mAbs were purchased from BioLegend (San Diego, CA). Anti-phospho Akt, anti-Akt, anti-phospho IKKα/β and anti-phospho NF-κB p65 Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti-Myc (clone: 9E10) and anti-FLAG (clone: M2) mAbs as well as anti-Myc polyclonal Ab were purchased from Sigma-Aldrich (St. Louis, MO). Other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Generation of bone marrow-derived cultured mast cells

Femoral bone marrow cells were cultured in 10% FCS RPMI1640 containing 3 ng/mL recombinant mouse IL-3 (TONBO biosciences, San Diego, CA, USA) for 4-6 weeks to generate bone marrow-derived cultured mast cells (BMMCs). The purity of FcεRI⁺c-Kit⁺

BMMCs was more than 90 % after culturing.

Cell culture and establishment of stable transfectants

Ba/F3 cells are cultured in RPMI1640 containing 10% FCS and 10% IL-3 condition medium. ST2-, STAP-2- and ST2/STAP-2-overexpressing Ba/F3 cells were generated using pEC-ST2-FLAG and pBabe-Myc-STAP-2 plasmids. After selection using puromycin (3 µg/mL) and G418 (1 mg/mL), drug-resistant Ba/F3 cells were cloned by limiting dilution methods. Expression FLAG-tagged ST2 and Myc-tagged STAP-2 in the clones was detected by FACS analysis and western blotting, and generated clones were used for the experiments.

Flowcytometric analysis

Flowcytometric analysis was performed as previously described [27].

Cytokine production

BMMCs were stimulated with indicated concentrations of IL-33 (TONBO biosciences) and LPS (Sigma-Aldrich) for 24 h. IL-6, TNF- α and IL-13 levels in supernatants were measured using ELISA kits (IL-6 and TNF- α ; BioLegend, IL-13; Affymetrix, San Diego,

CA).

Western blotting

Western blot analysis was performed as previously described [27]. For immunoprecipitation, cell lysates were incubated with primary Ab, followed by incubation with nProtein A Sepharose™ 4 Fast Flow (GE Healthcare Bio-Sciences) or Protein G Resin (GenScript Japan Inc., Tokyo, Japan). After beads were washed, the beads were boiled in 1x SDS sample buffer and co-immunoprecipitated proteins were detected by western blotting. Actin was detected as a loading control.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.02. Mann-Whitney *U*-test was employed. Data were considered significant at $p < 0.05$. Data were shown mean + SEM.

Results

STAP-2, but not STAP-1, is important for IL-33-induced mast cells activation.

We first confirmed surface expression levels of ST2 on cultured mast cells by FACS analysis. STAP-2 KO BMDCs expressed similar levels of ST2 on their surface to

WT BMMCs (Figs. 1A, B). To investigate effects of STAP-2 on IL-33-induced cytokine production, we stimulated WT and STAP-2 KO BMMCs with IL-33, and measured IL-6, TNF- α and IL-13 levels in supernatant. Although BMMCs produced these cytokines after IL-33 stimulation, the levels of IL-6 and TNF- α were significantly reduced in IL-33-stimulated STAP-2 KO BMMCs compared with WT BMMCs (Figs. 1C, D). Also, IL-13 production tended to reduce in STAP-2 KO BMMCs (Fig. 1E). Because STAP-1 is another STAP family member and involved in certain immune responses [28], we compared cytokine production of STAP-1 KO BMMCs with WT BMMCs to figure out the contribution of STAP-1 for IL-33-induced mast cell activation. Levels of IL-6, TNF- α and IL-13 in culture supernatant of IL-33-activated STAP-1 KO BMMCs were comparable to those of WT BMMCs (Figs 1F-H). Taken together, these results indicated that STAP-2 has an unique function for IL-33-induced mast cell activation.

STAP-2 has nonredundant role for TLR4-mediated mast cells activation.

IKK α underlies in both ST2- and TLR4-signaling pathways. Because STAP-2 binds to IKK α and because STAP-2 deficiency results in reduction of LPS-mediated macrophage activation [24], we evaluated the role of STAP-2 for LPS-induced mast cell activation. We first confirmed that the expression levels of TLR4 on STAP-2 KO

BMMCs were same as those on WT BMMCs (Figs. 2A, B). To investigate effects of STAP-2 on LPS-induced cytokine production, we stimulated WT and STAP-2 KO BMMCs with LPS, and measured IL-6, TNF- α and IL-13 levels in supernatant. The production of IL-6 was significantly reduced in STAP-2 KO BMMCs compared with WT BMMCs (Fig. 2C). The levels of TNF- α and IL-13 tended to reduce in STAP-2 KO BMMCs compared with WT BMMCs (Figs. 2D, E). In contrast, STAP-1 KO BMMCs produced similar levels of IL-6, TNF- α and IL-13 in supernatant compared with WT BMMCs (Figs 2F-H). Taken together, these results indicated that STAP-2 has a nonredundant function for TLR4-mediated cell activation in mast cells.

STAP-2 enhances for IL-33-induced signal transduction.

To investigate molecular mechanisms by which STAP-2 regulates IL-33 signaling, we compared ST2 signal transduction between IL-33-stimulated WT and STAP-2 KO BMMCs by western blotting. Phosphorylation levels of NF- κ B p65, IKK α / β and Akt were reduced in STAP-2 KO BMMCs compared with WT BMMCs after stimulation with IL-33 (Fig. 3A). To investigate whether STAP-2 directly regulates ST2 signal transduction or not, we prepared ST2-, STAP-2- and ST2/STAP-2- overexpressing Ba/F3 cells whose ST2 expression was confirmed by FACS analysis.

ST2 was expressed on ST2- and ST2/STAP-2-overexpressing, but not mock-transfected, Ba/F3 cells (Fig. 3B). Using these transfectants, we found that NF- κ B signal pathway was further enhanced in ST2/STAP-2-overexpressing Ba/F3 cells compared with ST2- and STAP-2-overexpressing Ba/F3 cells after IL-33 stimulation (Fig. 3C).

We next analyzed direct interaction of STAP-2 with ST2 or IRAK4, which is involved in IL-33 signal transduction. As shown in Fig. 3D, neither ST2 nor IRAK4 were immunoprecipitated with STAP-2 in 293T cell preparations, although a positive control BRK was immunoprecipitated with STAP-2 as previously shown [29]. This result suggested that STAP-2 has no ability to directly bind to ST2 or IRAK4. We finally examined the interaction of STAP-2 with IKK α using ST2- and ST2/STAP-2-overexpressing Ba/F3 cells to investigate whether STAP-2 had ability to bind to IKK α in response to IL-33. Without the IL-33 stimulation, no association of STAP-2 with IKK α was observed. When the cells were stimulated with IL-33, we observed direct interaction of STAP-2 with IKK α in ST2/STAP-2-, but not ST2-, overexpressing Ba/F3 cells (Fig. 3E). Correctively, these results suggested that STAP-2 enhances IL-33-induced activation of NF- κ B signal pathway by inducing interaction of STAP-2 with IKK α after IL-33 stimulation.

Discussions

In this study, we described that STAP-2, but not STAP-1, is involved in IL-33-induced mast cell cytokine production. Mechanistic analysis revealed that STAP-2 interacts with IKK α in IL-33-stimulated cells. These results indicate that STAP-2 has a nonredundant role for ST2-mediated mast cell activation.

Mast cells are one of the important cell types for pathogenesis of allergic diseases, such as bronchial asthma and food allergies. IL-33 is critical for mast cell activation and mast cell-mediated allergic inflammation. Indeed, IL-33 induces cytokine production in mast cells, and this IL-33/mast cell/cytokine axis plays an important role for airway inflammation [11, 13, 14, 16]. Proportions of IL-33-positive cells in lungs from patients with bronchial asthma are higher than normal subjects [30]. Thus, IL-33 is believed to be a suitable therapeutic target for allergic diseases. In the present study, we demonstrated that STAP-2 deficiency results in a decrease of IL-33-induced mast cell activation. Although details of the inducing machinery are not figured out yet, STAP-2 may directly affect canonical NF- κ B signaling because 1) phosphorylation of NF- κ B p65 and IKK α/β is reduced in STAP-2 KO BMMCs, 2) phosphorylation of these molecules is increased in ST2/STAP-2-overexpressing Ba/F3 cells, 3) STAP-2 binds to IKK α in IL-33-stimulated ST2/STAP-2-overexpressing Ba/F3 cells.

STAP family consists of two members, STAP-1 and STAP-2. We previously reported that STAP-2 is required for FcεRI-mediated signal transduction and basophil activation [27]. However, our preliminary results suggest that STAP-1 is dispensable for FcεRI-mediated basophil activation (data not shown), implicating that STAP-2 has nonredundant function of FcεRI signaling in basophils. The effects on B cell receptor-mediated B cell activation are also limited to STAP-2, but not STAP-1 (In preparation). In this study, IL-33-induced mast cell activation is reduced by STAP-2-, but not STAP-1-, deficiency, suggesting that STAP-2 has a nonredundant role for IL-33 signaling in mast cells. STAP-2-restricted function is also observed when BMDCs were stimulated with LPS. Although it is unknown yet why only STAP-2 affects ST2 and TLR4 signal pathways in mast cells, a proline-rich domain within STAP-2 might be involved in its nonredundant function in mast cells because STAP-1 has no proline-rich domain [18-21]. This will be the subject of future studies.

In this report, we showed direct interaction of STAP-2 with IKKα in ST2/STAP-2-overexpressing Ba/F3 cells upon stimulation with IL-33. We have previously reported that STAP-2 phosphorylation is important for regulation of STAT3 and STAT5 activity [21, 29, 31, 32], BCR-ABL-dependent cell growth [33], its interaction with Pyk2 [34] and EGFR-mediated tumor growth [35]. Although we examined phosphorylation of

STAP-2 in IL-33-stimulated ST2/STAP-2-overexpressing Ba/F3 cells, no phosphorylation of STAP-2 in the cells was observed (data not shown), suggesting that STAP-2 phosphorylation is not required for IL-33-induced NF- κ B signal transduction.

In summary, our study demonstrated that STAP-2 deficiency results in reduction of cytokine production from mast cells upon IL-33 stimulation. For the mechanisms, we propose that STAP-2 interacts with IKK α after IL-33 stimulation. Thus, we suggest that STAP-2 is essential for promoting IL-33-induced mast cell activation and allergic inflammation.

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Conflict of Interest (COI)

The authors declare no conflict of interest.

References

[1] P. Licona-Limon, L.K. Kim, N.W. Palm, R.A. Flavell, TH2, allergy and group 2 innate lymphoid cells, *Nature immunology*, 14 (2013) 536-542.

[2] H. Spits, D. Artis, M. Colonna, A. Diefenbach, J.P. Di Santo, G. Eberl, S. Koyasu, R.M. Locksley, A.N. McKenzie, R.E. Mebius, F. Powrie, E. Vivier, Innate lymphoid cells--a proposal for uniform nomenclature, *Nature reviews. Immunology*, 13 (2013) 145-149.

[3] M. Salimi, J.L. Barlow, S.P. Saunders, L. Xue, D. Gutowska-Owsiak, X. Wang, L.C. Huang, D. Johnson, S.T. Scanlon, A.N. McKenzie, P.G. Fallon, G.S. Ogg, A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis, *J Exp Med*, 210 (2013) 2939-2950.

[4] K. Moro, T. Yamada, M. Tanabe, T. Takeuchi, T. Ikawa, H. Kawamoto, J. Furusawa, M. Ohtani, H. Fujii, S. Koyasu, Innate production of T(H)2

cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells, *Nature*, 463 (2010) 540-544.

[5] D.R. Neill, S.H. Wong, A. Bellosi, R.J. Flynn, M. Daly, T.K. Langford, C. Bucks, C.M. Kane, P.G. Fallon, R. Pannell, H.E. Jolin, A.N. McKenzie, Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity, *Nature*, 464 (2010) 1367-1370.

[6] M.J. Townsend, P.G. Fallon, D.J. Matthews, H.E. Jolin, A.N. McKenzie, T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses, *J Exp Med*, 191 (2000) 1069-1076.

[7] Y. Haenuki, K. Matsushita, S. Futatsugi-Yumikura, K.J. Ishii, T. Kawagoe, Y. Imoto, S. Fujieda, M. Yasuda, Y. Hisa, S. Akira, K. Nakanishi, T. Yoshimoto, A critical role of IL-33 in experimental allergic rhinitis, *J Allergy Clin Immunol*, 130 (2012) 184-194 e111.

[8] T. Kawakami, S.J. Galli, Regulation of mast-cell and basophil function and survival by IgE, *Nature reviews. Immunology*, 2 (2002) 773-786.

[9] F.A. Redegeld, Y. Yu, S. Kumari, N. Charles, U. Blank, Non-IgE mediated mast cell activation, *Immunol Rev*, 282 (2018) 87-113.

[10] B. Griesenauer, S. Paczesny, The ST2/IL-33 Axis in Immune Cells during

Inflammatory Diseases, *Front Immunol*, 8 (2017) 475.

[11] L.H. Ho, T. Ohno, K. Oboki, N. Kajiwara, H. Suto, M. Iikura, Y. Okayama, S. Akira, H. Saito, S.J. Galli, S. Nakaie, IL-33 induces IL-13 production by mouse mast cells independently of IgE-FcepsilonRI signals, *J Leukoc Biol*, 82 (2007) 1481-1490.

[12] A.J. Hueber, J.C. Alves-Filho, D.L. Asquith, C. Michels, N.L. Millar, J.H. Reilly, G.J. Graham, F.Y. Liew, A.M. Miller, I.B. McInnes, IL-33 induces skin inflammation with mast cell and neutrophil activation, *Eur J Immunol*, 41 (2011) 2229-2237.

[13] L.C. Sjoberg, J.A. Gregory, S.E. Dahlen, G.P. Nilsson, M. Adner, Interleukin-33 exacerbates allergic bronchoconstriction in the mice via activation of mast cells, *Allergy*, 70 (2015) 514-521.

[14] K.A. Cho, J.W. Suh, J.H. Sohn, J.W. Park, H. Lee, J.L. Kang, S.Y. Woo, Y.J. Cho, IL-33 induces Th17-mediated airway inflammation via mast cells in ovalbumin-challenged mice, *Am J Physiol Lung Cell Mol Physiol*, 302 (2012) L429-440.

[15] M. Iikura, H. Suto, N. Kajiwara, K. Oboki, T. Ohno, Y. Okayama, H. Saito, S.J. Galli, S. Nakaie, IL-33 can promote survival, adhesion and cytokine

production in human mast cells, *Lab Invest*, 87 (2007) 971-978.

[16] Z. Allakhverdi, D.E. Smith, M.R. Comeau, G. Delespesse, Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells, *J Immunol*, 179 (2007) 2051-2054.

[17] M.R. Silver, A. Margulis, N. Wood, S.J. Goldman, M. Kasaian, D. Chaudhary, IL-33 synergizes with IgE-dependent and IgE-independent agents to promote mast cell and basophil activation, *Inflamm Res*, 59 (2010) 207-218.

[18] K. Ohya, S. Kajigaya, A. Kitanaka, K. Yoshida, A. Miyazato, Y. Yamashita, T. Yamanaka, U. Ikeda, K. Shimada, K. Ozawa, H. Mano, Molecular cloning of a docking protein, BRDG1, that acts downstream of the Tec tyrosine kinase, *Proc Natl Acad Sci U S A*, 96 (1999) 11976-11981.

[19] M. Masuhara, K. Nagao, M. Nishikawa, M. Sasaki, A. Yoshimura, M. Osawa, Molecular cloning of murine STAP-1, the stem-cell-specific adaptor protein containing PH and SH2 domains, *Biochem Biophys Res Commun*, 268 (2000) 697-703.

[20] P.J. Mitchell, E.A. Sara, M.R. Crompton, A novel adaptor-like protein which is a substrate for the non-receptor tyrosine kinase, BRK, *Oncogene*, 19

(2000) 4273-4282.

[21] M. Minoguchi, S. Minoguchi, D. Aki, A. Joo, T. Yamamoto, T. Yumioka, T. Matsuda, A. Yoshimura, STAP-2/BKS, an adaptor/docking protein, modulates STAT3 activation in acute-phase response through its YXXQ motif, *J Biol Chem*, 278 (2003) 11182-11189.

[22] T. Matsuda, K. Oritani, STAP-2 Adaptor Protein Regulates Multiple Steps of Immune and Inflammatory Responses, *Biological & pharmaceutical bulletin*, 44 (2021) 895-901.

[23] Y. Sekine, K. Nishida, S. Yamasaki, R. Muromoto, S. Kon, J. Kashiwakura, K. Saitoh, S. Togi, A. Yoshimura, K. Oritani, T. Matsuda, Signal-transducing adaptor protein-2 controls the IgE-mediated, mast cell-mediated anaphylactic responses, *J Immunol*, 192 (2014) 3488-3495.

[24] Y. Sekine, T. Yumioka, T. Yamamoto, R. Muromoto, S. Imoto, K. Sugiyama, K. Oritani, K. Shimoda, M. Minoguchi, S. Akira, A. Yoshimura, T. Matsuda, Modulation of TLR4 signaling by a novel adaptor protein signal-transducing adaptor protein-2 in macrophages, *J Immunol*, 176 (2006) 380-389.

[25] J. Schmitz, A. Owyang, E. Oldham, Y. Song, E. Murphy, T.K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D.M. Gorman, J.F. Bazan, R.A.

Kastelein, IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines, *Immunity*, 23 (2005) 479-490.

[26] Z. Yang, R. Sun, V. Grinchuk, J.A. Fernandez-Blanco, L. Notari, J.A. Bohl, L.P. McLean, T.R. Ramalingam, T.A. Wynn, J.F. Urban, Jr., S.N. Vogel, T. Shea-Donohue, A. Zhao, IL-33-induced alterations in murine intestinal function and cytokine responses are MyD88, STAT6, and IL-13 dependent, *Am J Physiol Gastrointest Liver Physiol*, 304 (2013) G381-389.

[27] J.I. Kashiwakura, S. Yamashita, M. Yoshihara, K. Inui, K. Saitoh, Y. Sekine, R. Muromoto, Y. Kitai, K. Oritani, T. Matsuda, STAP-2 positively regulates FcepsilonRI-mediated basophil activation and basophil-dependent allergic inflammatory reactions, *Int Immunol*, 31 (2019) 349-356.

[28] J.I. Kashiwakura, K. Saitoh, T. Ihara, Y. Sasaki, K. Kagohashi, S. Enohara, Y. Morioka, H. Watarai, R. Muromoto, Y. Kitai, K. Iwabuchi, K. Oritani, T. Matsuda, Expression of signal-transducing adaptor protein-1 attenuates experimental autoimmune hepatitis via down-regulating activation and homeostasis of invariant natural killer T cells, *PLOS ONE*, 15 (2020) e0241440.

[29] O. Ikeda, Y. Miyasaka, Y. Sekine, A. Mizushima, R. Muromoto, A. Nanbo, A. Yoshimura, T. Matsuda, STAP-2 is phosphorylated at tyrosine-250 by Brk and modulates Brk-mediated STAT3 activation, *Biochem Biophys Res Commun*, 384 (2009) 71-75.

[30] M. Kurowska-Stolarska, B. Stolarski, P. Kewin, G. Murphy, C.J. Corrigan, S. Ying, N. Pitman, A. Mirchandani, B. Rana, N. van Rooijen, M. Shepherd, C. McSharry, I.B. McInnes, D. Xu, F.Y. Liew, IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation, *J Immunol*, 183 (2009) 6469-6477.

[31] O. Ikeda, A. Mizushima, Y. Sekine, C. Yamamoto, R. Muromoto, A. Nanbo, K. Oritani, A. Yoshimura, T. Matsuda, Involvement of STAP-2 in Brk-mediated phosphorylation and activation of STAT5 in breast cancer cells, *Cancer science*, 102 (2011) 756-761.

[32] Y. Sekine, S. Tsuji, O. Ikeda, M. Kakisaka, K. Sugiyama, A. Yoshimura, T. Matsuda, Leukemia inhibitory factor-induced phosphorylation of STAP-2 on tyrosine-250 is involved in its STAT3-enhancing activity, *Biochem Biophys Res Commun*, 356 (2007) 517-522.

[33] Y. Sekine, O. Ikeda, A. Mizushima, Y. Ueno, R. Muromoto, A. Yoshimura,

Y. Kanakura, K. Oritani, T. Matsuda, STAP-2 interacts with and modulates BCR-ABL-mediated tumorigenesis, *Oncogene*, 31 (2012) 4384-4396.

[34] K. Saitoh, T. Tsuchiya, J.I. Kashiwakura, R. Muromoto, Y. Kitai, Y. Sekine, K. Oritani, T. Matsuda, STAP-2 interacts with Pyk2 and enhances Pyk2 activity in T-cells, *Biochem Biophys Res Commun*, 488 (2017) 81-87.

[35] Y. Kitai, M. Iwakami, K. Saitoh, S. Togi, S. Isayama, Y. Sekine, R. Muromoto, J.I. Kashiwakura, A. Yoshimura, K. Oritani, T. Matsuda, STAP-2 protein promotes prostate cancer growth by enhancing epidermal growth factor receptor stabilization, *J Biol Chem*, 292 (2017) 19392-19399.

Figure Legends

Fig. 1. Cytokine production in STAP-2 KO mast cells upon stimulation with IL-33.

(A) Expression of ST2 on WT and STAP-2 KO BMMCs. Gray-filled and black line histograms are unstained and stained BMMCs, respectively. (B) Mean fluorescence intensity of the ST2 expression on WT and STAP-2 KO BMMCs. White and black bars are WT and STAP-2 KO BMMCs, respectively. Data shown are mean + SEM of 4 independent experiments (WT = 4, KO = 6). (C-E) Levels of IL-6 (C), TNF- α (D) and IL-13 (E) in supernatants of WT and STAP-2 KO BMMCs after stimulation with IL-33

for 24 h. White and black bars are WT and STAP-2 KO BMMCs, respectively. Data shown are mean + SEM of 5 (IL-6), 4 (TNF- α) and 6 (IL-13) independent experiments (IL-6; n = 10, TNF- α ; n = 8, IL-13; n = 11). (F-H) Levels of IL-6 (F), TNF- α (G) and IL-13 (H) in supernatants of WT and STAP-1 KO BMMCs after stimulation with IL-33 for 24 h. White and gray bars are WT and STAP-1 KO BMMCs, respectively. Data shown are mean + SEM of 3 (IL-6) and 2 (TNF- α & IL-13) independent experiments (IL-6; n = 6, TNF- α ; n = 4, IL-13; n = 3). *, p<0.05, **, p<0.01 by Mann-Whitney *U*-test. ns = no significance

Fig. 2. Cytokine production in STAP-2 KO mast cells after LPS stimulation.

(A) Expression of TLR4 on WT and STAP-2 KO BMMCs. Gray-filled and black line histograms are unstained and stained BMMCs, respectively. (B) Mean fluorescence intensity of the LPS expression on WT and STAP-2 KO BMMCs. White and black bars are WT and STAP-2 KO BMMCs, respectively. Data shown are mean + SEM of 3 independent experiments (n = 3). (C-E) Levels of IL-6 (C), TNF- α (D) and IL-13 (E) in supernatants of WT and STAP-2 KO BMMCs after stimulation with LPS for 24 h. White and black bars are WT and STAP-2 KO BMMCs, respectively. Data shown are mean + SEM of 4 (IL-6 & TNF- α) and 5 (IL-13) independent experiments (IL-6; n = 6, TNF- α ;

n = 6, IL-13; n = 7). (F-H) Levels of IL-6 (F), TNF- α (G) and IL-13 (H) in supernatants of WT and STAP-1 KO BMMCs after stimulation with LPS for 24 h. White and gray bars are WT and STAP-1 KO BMMCs, respectively. Data shown are mean + SEM of 4 (IL-6 & TNF- α) and 3 (IL-13) independent experiments (IL-6; n = 8, TNF- α ; n = 8, IL-13; n = 6). *, p<0.05, **, p<0.01 by Mann-Whitney *U*-test. ns = no significance

Fig. 3. STAP-2 enhances NF- κ B signaling by direct binding to IKK α in response to IL-33

(A) Comparison of activation of NF- κ B and Akt signaling between WT and STAP-2 KO BMMCs upon stimulation with IL-33. (B) Expression of ST2 on transfected Ba/F3 cells. Gray-filled, black normal line and black thick line histograms are mock-transfected, ST2- and ST2/STAP-2-overexpressing Ba/F3 cells, respectively. (C) Comparison of activation of NF- κ B pathway among mock-transfected, ST2-, STAP-2- and ST2/STAP-2-overexpressing Ba/F3 cells after IL-33 activation. (D) Myc-tagged STAP-2 vector was cotransfected with either FLAG-tagged IRAK4, ST2 or BRK vector into 293T cells. Binding of STAP-2 to FLAG-tagged proteins was analyzed by immunoprecipitation, followed by western blotting. (E) Interaction of Myc-tagged STAP-2 with endogenous IKK α in ST2- and ST2/STAP-2-overexpressing Ba/F3 cells in the presence or absence of

IL-33 stimulation. Arrowhead shows coimmunoprecipitated IKK α . All data shown are representative of 2-3 independent experiments.

Figure 1

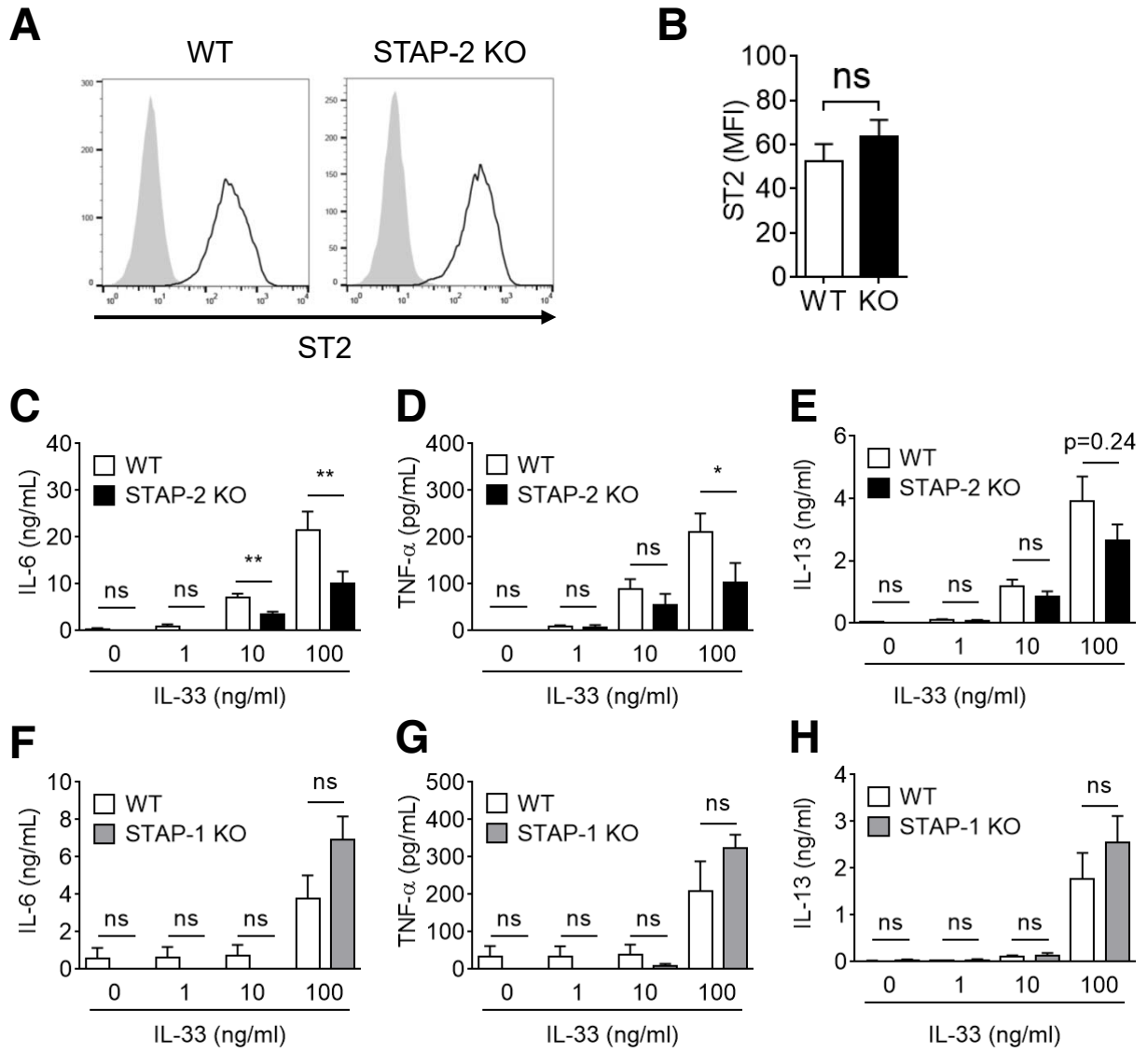


Figure 2

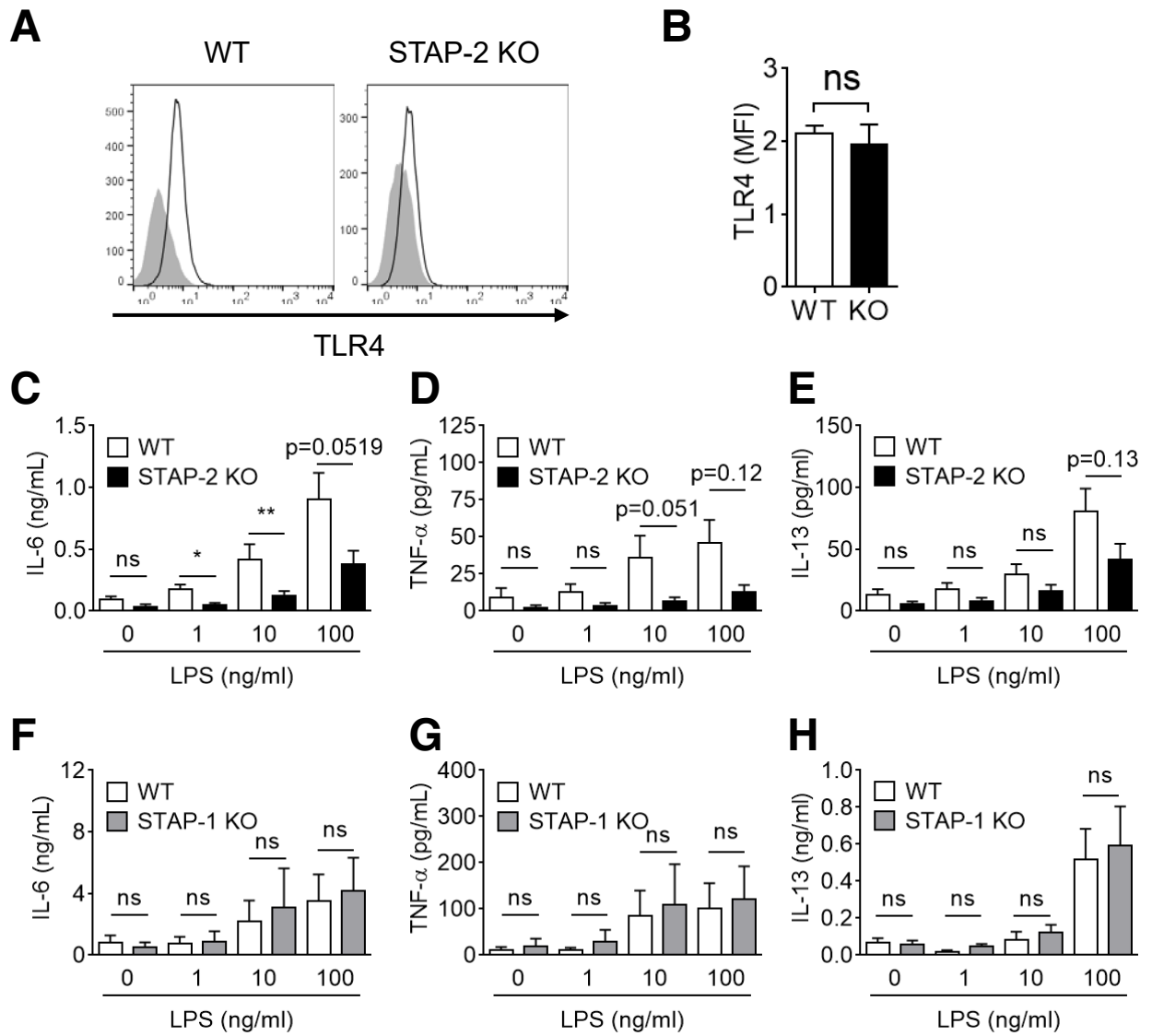


Figure 3

