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

Jun-ichi Kashiwakura[a,*], Nao Koizumi[a], Kodai Saitoh[a,†], Kota Kagohashi[a], Yuto Sasaki[a], Fuki Kobayashi[a], Shoya Kawahara[a], Yukie Yamauchi[a], Yuichi Kitai[a], Ryuta Muromoto[a], Kenji Oritani[b], Tadashi Matsuda[a,*]

[a]Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, Hokkaido 060-0812, Japan

[b]Department of Hematology, International University of Health and Welfare, 4-3 Kouzunomori, Narita, Chiba 286-8686, Japan

[†]KS's present address: HEALIOS K.K. Kobe Research Institute, 1-5-2 Minatojima-Minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan

[*]Correspondence: Jun-ichi Kashiwakura, Ph.D., Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, Hokkaido 060-0812, Japan, E-mail: junkashi@pharm.hokudai.ac.jp, Tel: +81-

11-706-3920, FAX: +81-11-706-4990 and Tadashi Matsuda, Ph.D., Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, Hokkaido 060-0812, Japan, E-mail: tmatsuda@pharm.hokudai.ac.jp, Tel: +81-11-706-3243, FAX: +81-11-706-4990

This manuscript contains 26 pages with 3 figures.

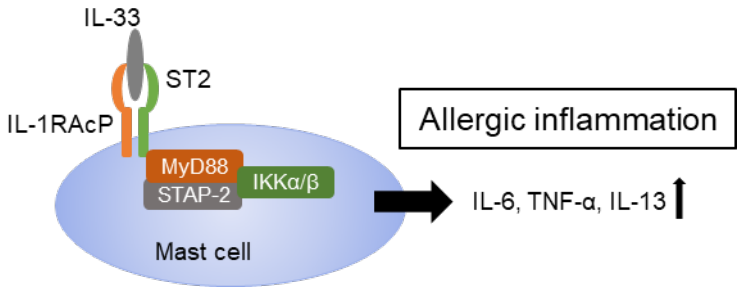
The text contains 4,118 words.

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 $Fc\varepsilon RI^{+}$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^{TM} 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 BMMCs 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.

IKKα underlies in both ST2- and TLR4-signaling pathways. Because STAP-2 binds to IKKα 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-κB p65, IKKα/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-κB 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 IKKα using ST2- and ST2/STAP-2-overexpressing Ba/F3 cells to investigate whether STAP-2 had ability to bind to IKKα in response to IL-33. Without the IL-33 stimulation, no association of STAP-2 with IKKα was observed. When the cells were stimulated with IL-33, we observed direct interaction of STAP-2 with IKKα 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-κB signal pathway by inducing interaction of STAP-2 with IKKα 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. Mehanistic analysis revealed that STAP-2 interacts with IKKα in IL-33-stimulated cells. These results indicate that STAP-2 has a nonredundant roles 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 important 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 decrease of IL-33-induced mast cell activation. Although details inducing machinery are not figured out yet, STAP-2 may directly affect canonical NF-κB signaling because 1) phosphorylation of NF-κB p65 and IKKα/β is reduced in STAP-2 KO BMMCs, 2) phosphorylation of these molecules are increased in ST2/STAP-2-overexpressing Ba/F3 cells, 3) STAP-2 binds to IKKα 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 BMMCs were stimulated with LPS. Although it is unknow 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-κB 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 IKKα after IL-33 stimulation. Thus, we suggest that STAP-2 is essential for promoting IL-33-induced mast cell activation and allergic inflammation.

Acknowledgements

We appreciate Drs. Masaaki Hashiguchi (Dokkyo Medical University) and Shintaro Sato (Osaka University) for providing the ST2-FLAG and FLAG-IRAK vectors, respectively. We thank all members of Hokkaido University, Global Facility Center (GFC), Pharma Science Open Unit (PSOU) funded by MEXT under “Support Program for Implementation of New Equipment Sharing System”.

Funding

This study was partly supported by JSPS KAKENHI Grants 19H03364 (T.M.) and

21K08451 (J.K.).

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. Nakae, 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. Nakae, 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. Sugiyma, 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-κB signaling by direct binding to IKKα in response to IL-33

(A) Comparison of activation of NF-κB 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-κB 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 IKKα in ST2- and ST2/STAP-2-overexpressing Ba/F3 cells in the presence or absence of

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

Figure 1

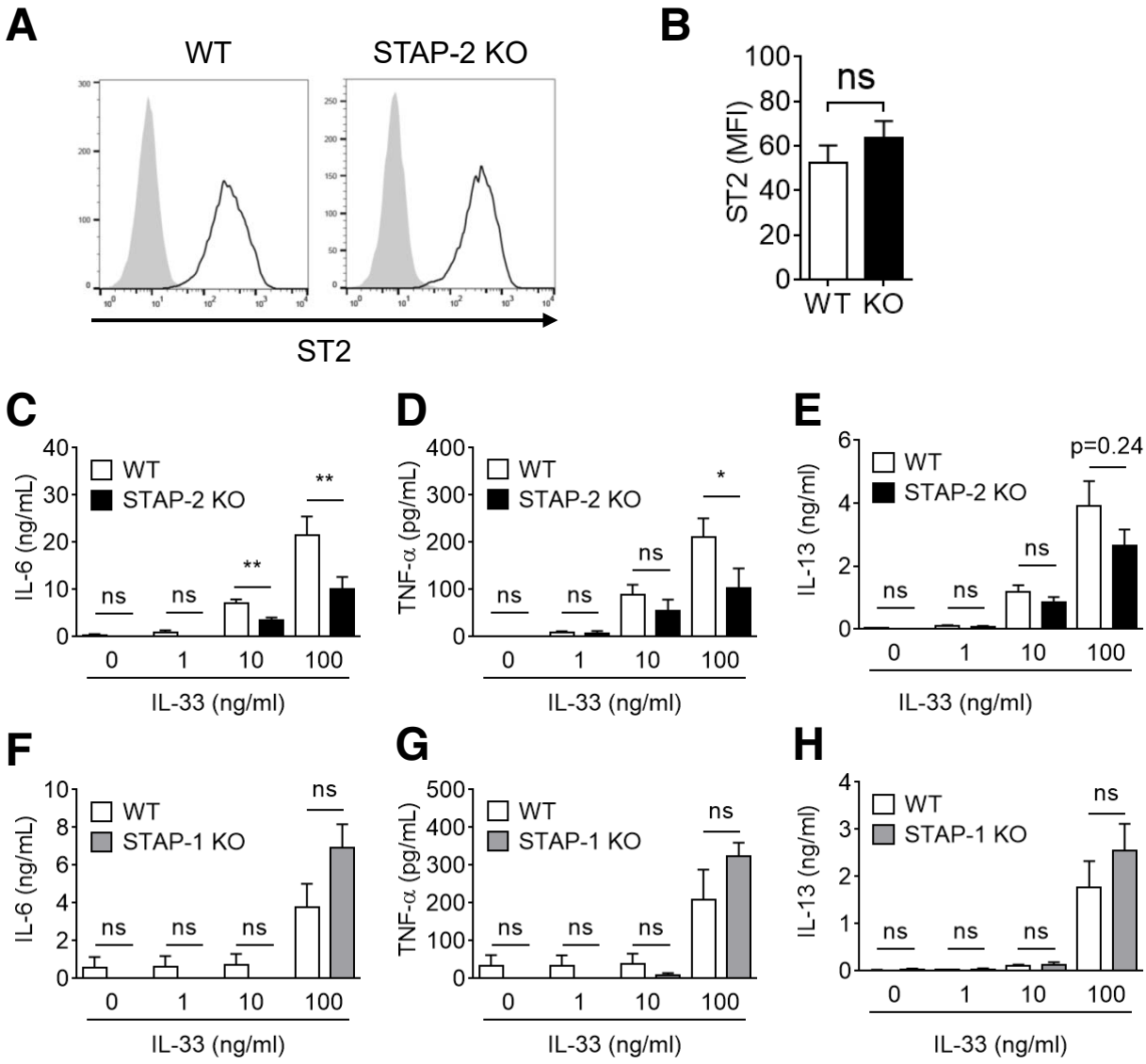

A
WT
STAP-2 KO
ST2
B
ST2 (MFI)
ns
WT KO
C
IL-6 (ng/mL)
WT
STAP-2 KO
IL-33 (ng/ml)
D
TNF-α (pg/mL)
E
IL-13 (ng/ml)
p=0.24
F
STAP-1 KO
G
H

Figure 2

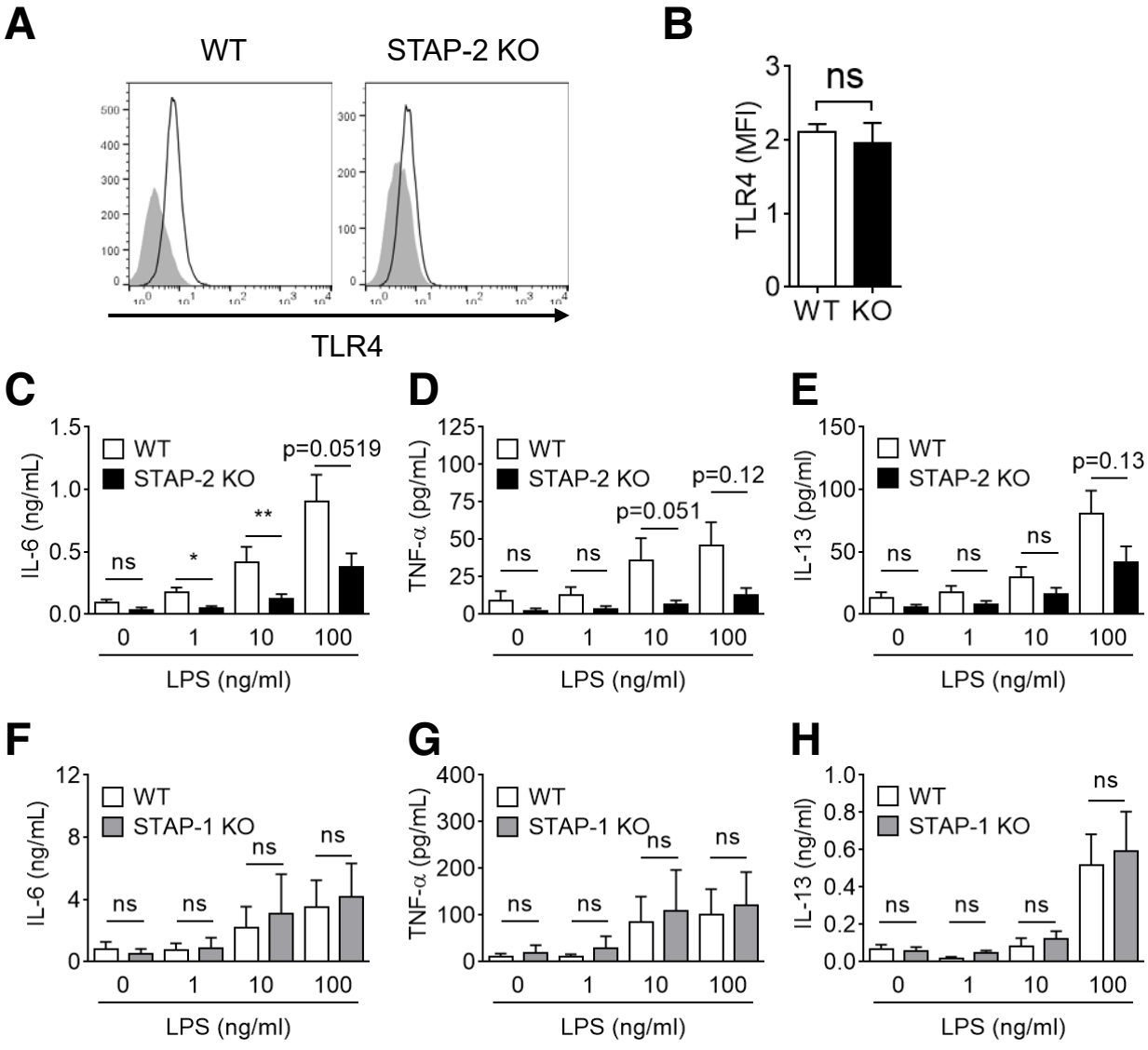
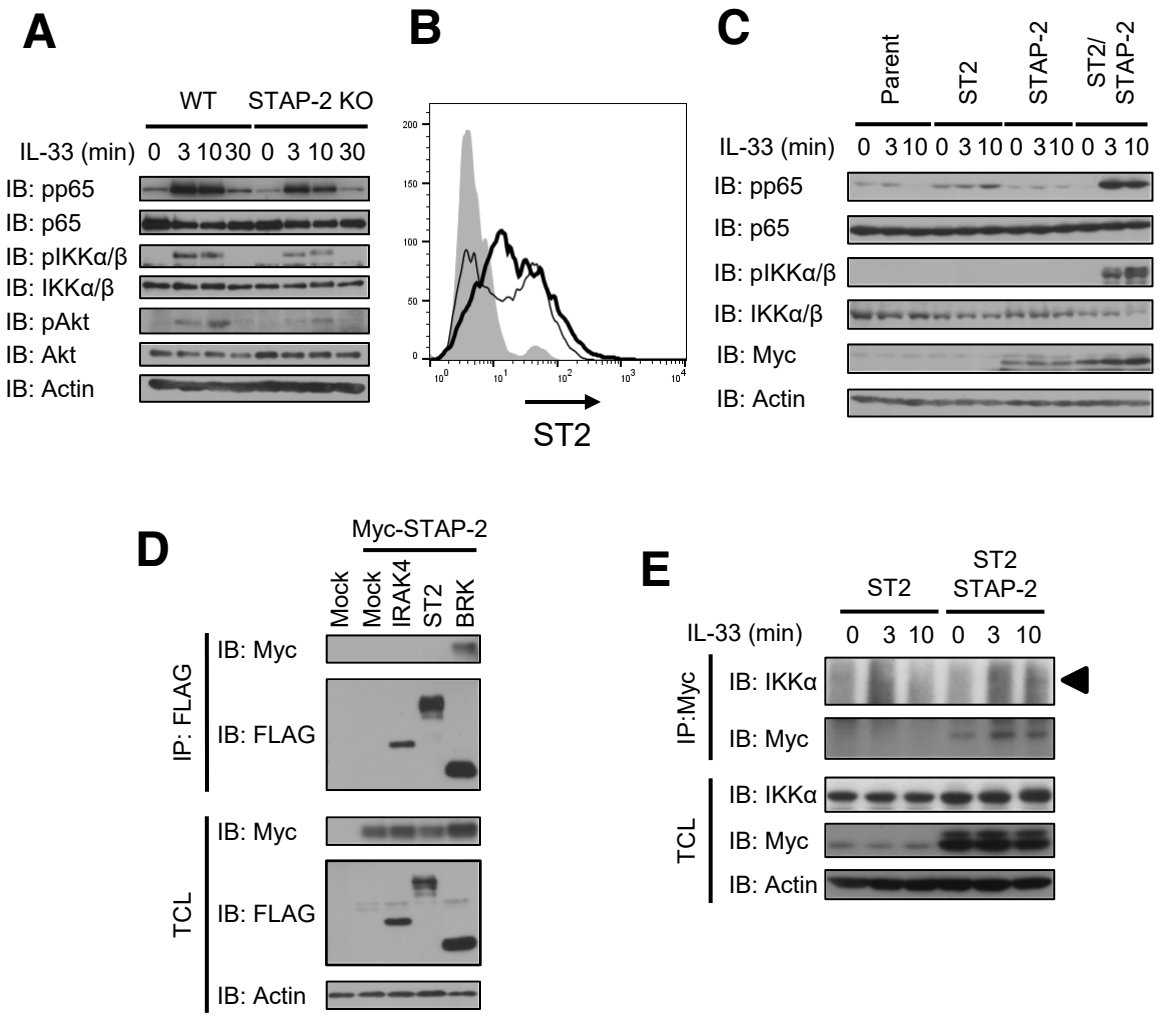

A
WT
STAP-2 KO
TLR4
B
TLR4 (MFI)
ns
WT KO
C
IL-6 (ng/mL)
WT
STAP-2 KO
p=0.0519
ns
*
**
LPS (ng/ml)
D
TNF-α (pg/mL)
p=0.12
p=0.051
E
IL-13 (pg/ml)
p=0.13
F
IL-6 (ng/mL)
STAP-1 KO
G
TNF-α (pg/mL)
H
IL-13 (ng/ml)

Figure 3


A
WT
STAP-2 KO
IL-33 (min) 0 3 10 30 0 3 10 30
IB: pp65
IB: p65
IB: pIKKα/β
IB: IKKα/β
IB: pAkt
IB: Akt
IB: Actin
B
ST2
C
Parent
ST2
STAP-2
ST2/ STAP-2
IL-33 (min) 0 3 10 0 3 10 0 3 10 0 3 10
IB: pp65
IB: p65
IB: pIKKα/β
IB: IKKα/β
IB: Myc
IB: Actin
D
Myc-STAP-2
Mock
Mock
IRAK4
ST2
BRK
IP: FLAG
IB: Myc
IB: FLAG
TCL
IB: Myc
IB: FLAG
IB: Actin
E
ST2
ST2 STAP-2
IL-33 (min) 0 3 10 0 3 10
IP:Myc
IB: IKKα
IB: Myc
TCL
IB: IKKα
IB: Myc
IB: Actin