STAP-2 negatively regulates BCR-mediated B cell activation by recruiting tyrosine-protein kinase CSK to LYN

Jun-ichi Kashiwakura[a,b], Shoya Kawahara[a], Iori Inagaki[a], Kyosuke Inui[a], Kodai Saitoh[a], Kota Kagohashi[a], Yuto Sasaki[a], Fuki Kobayashi[a], Yuichi Kitai[a], Ryuta Muromoto[a], Kenji Oritani[c], Tadashi Matsuda[a]

[a]Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, Hokkaido 0600812, Japan

[b]Department of Life Science, Faculty of Pharmaceutical Sciences, Hokkaido University of Science, 15-4-1, Maeda 7-jo, Teine-ku, Sapporo, Hokkaido 0068585, Japan

[c]Department of Hematology, International University of Health and Welfare, 4-3 Kouzunomori, Narita, Chiba 2868686, Japan

Kashiwakura and Kawahara are equally contributed to this work.

Correspondence: Jun-ichi Kashiwakura, Ph.D., Department of Life Science, Faculty of Pharmaceutical Sciences, Hokkaido University of Science, 15-4-1, Maeda 7-jo, Teine-Ku,

Sapporo, Hokkaido 0068585, Japan, E-mail: kashiwakura-j@hus.ac.jp, Tel: +81-11-676-8738, FAX: +81-11-676-8666, ORCID 0000-0002-7863-6696,and Tadashi Matsuda, Ph.D., Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, Hokkaido 0600812, Japan, E-mail: tmatsuda@pharm.hokudai.ac.jp, Tel: +81-11-706-3243, FAX: +81-11-706-4990, ORCID 0000-0002-3089-3757

Abstract

Although signal-transducing adaptor protein-2 (STAP-2) acts in certain immune responses, its role in B cell receptor (BCR)-mediated signals remains unknown. In this study, we have revealed that BCR-mediated signals, cytokine production and antibody production were increased in *STAP-2* knockout (KO) mice compared with wild-type (WT) mice. Phosphorylation of tyrosine-protein kinase LYN Y508 was reduced in STAP-2 KO B cells after BCR stimulation. Mechanistical analysis revealed that STAP-2 directly binds to LYN, dependently of STAP-2 Y250 phosphorylation by LYN. Furthermore, phosphorylation of STAP-2 enhanced interactions between LYN and tyrosine-protein kinase CSK, resulting in enhanced CSK-mediated LYN Y508 phosphorylation. These results suggest that STAP-2 is crucial for controlling BCR-mediated signals and antibody production by enhanced CSK-mediated feedback regulation of LYN.

Keywords: Signal-transducing adaptor protein-2, B cells, Lyn, Csk

Abbreviations

STAP, signal-transducing adaptor protein

BCR, B cell receptor

KO, knockout

WT, wild-type

ITAMs, immunoreceptor tyrosine-based activation motifs

BTK, Bruton's tyrosine kinase

PLC, phospholipase C

IP_3, inositol triphosphate

DAG, diacylglycerol

GRP, guanine nucleotide releasing protein

MAPK, mitogen-activated protein kinase

CIN85, Cbl-interacting 85-kDa protein

Csk, C-terminal Src kinase

mAb, monoclonal antibody

RW, ragweed pollen

GC B, germinal center B cells

Tfh, T follicular cells

PC, plasma cells

FL, full-length

Introduction

The signal-transducing adaptor protein (STAP) family, which consists of two members, STAP-1 and STAP-2, functions as an adaptor protein in several immune signaling pathways. STAP-2 has been identified as a substrate of breast tumor kinase (BRK) and is known to regulate the STAT3 signaling[1,2]. Subsequently, STAP-2 was also reported to bind to STAT5 and modulate STAT5-mediated T cell proliferation[3]. STAP-2 is involved in certain immune cell responses such as FcεRI-mediated mast cell/basophil activation[4,5], integrin-mediated T cell adhesion[6], SDF-1α-induced T cell migration[7] and Fas-mediated T cell apoptosis[8]. We have previously reported that STAP-2 is involved in TCR-mediated T cell activation and the pathogenesis of experimental autoimmune encephalomyelitis, a T cell-dependent autoimmune diseases model of multiple sclerosis[9]. However, the contribution of STAP-2 to other immune responses, particularly acquired immune reactions, is not fully understood.

B cells are essential for acquired immune responses as they produce antibodies, and aberrant B cell activation results in antibody-mediated immune diseases, such as autoimmune diseases and allergies. Engagement of BCR on B cells initiates a complex cascade of signaling events that result in B cell proliferation and maturation and immunoglobulin production. BCR aggregation triggers the phosphorylation of BCR-

78 associated Igα and Igβ immunoreceptor tyrosine-based activation motifs (ITAMs) by the
79 Src tyrosine kinase Lyn[10-12]. Phosphorylated Igα/β ITAMs then recruit additional Lyn
80 and Syk[13,14], resulting in Syk activation. After Syk is phosphorylated, Bruton's
81 tyrosine kinase (BTK) is recruited to activate Syk, and BTK-phospholipase C (PLC)-γ2
82 signaling leads to the production of inositol triphosphate (IP_3) and diacylglycerol (DAG)
83 from membrane-bound phosphatidylinositol 4, 5-bisphosphate. IP_3 increases the
84 intracellular calcium levels, resulting in increased activity and gene expression of the
85 nuclear factor of activated T cells. DAG is required for nuclear factor-κB signal
86 transduction and RasGRP (guanine nucleotide releasing protein)/Ras-mediated mitogen-
87 activated protein kinase (MAPK) activation in B cells.

88 Several mechanisms negatively regulate the BCR signaling cascade and B cell
89 activation. Cbl-interacting 85-kDa protein (CIN85) plays an important role as a negative
90 regulator of several receptor tyrosine kinase-dependent signal transduction pathways.
91 CIN85 was originally identified as a negative regulator of the EGF receptor signaling
92 cascade, and a later study showed that CIN85 is required for ligation-dependent IgE
93 receptor internalization in mast cells[15-17]. It has been reported that CIN85 is expressed
94 in B cells and interacts with SHIP-1, a major negative element of the
95 phosphatidylinositol-3-kinase pathway in lymphocytes[18]. NTAL plays an important

role in both positive and negative regulation of BCR signal transduction in B cells. It is phosphorylated by several Src family tyrosine kinases such as Lyn and Lck. [19]. NTAL-deficient B cells show slightly higher BCR-mediated B cell proliferation and calcium mobilization as well as an increased humoral immune response[20]. In contrast, BCR-mediated calcium mobilization and ERK activation were decreased in NTAL-knock down A20 B cell lines[21]. Cathlin et al. revealed that NTAL deficiency results in the reduction of BCR internalization after activation[22].

Lyn activity in B cells after aggregation of BCR is tightly regulated by CD45 and C-terminal Src kinase (Csk). Phosphorylation of Lyn Y508, a C-terminal tyrosine residue, is essential for the negative regulation of Lyn activity. Once BCR is crosslinked, CD45 selectively associates with Lyn and dephosphorylates phosphorylated Lyn Y508, leading to increased Lyn Y397 phosphorylation, which is required for the initiation of BCR signal transduction by autophosphorylation machinery[23,24]. After Lyn-mediated BCR signaling is initiated, Csk also participates in the modification of BCR signal transduction. Csk phosphorylates Lyn Y508 and induces conformational changes that inhibit Lyn activity. Indeed, Csk deficiency results in extensive phosphorylation of Lyn Y397 and constitutive activation of Lyn[25]. However, the detailed mechanisms for the regulation of Lyn activity and/or BCR signals are unknown.

In this study, we sought to investigate functional role of STAP-2 in BCR-mediated B cell activation and antibody production.

Materials and Methods

Mice and cells

Female Balb/c mice (8-12 weeks old) were purchased from SANKYO LABO SERVICE CO. Inc. (Hokkaido, Japan). Female Balb/c-background STAP-2 knockout mice (8-12 weeks old) were described previously[5]. All animal experiments were approved by the Institutional Animal Care and Use Committee of Hokkaido University (Approval No. 18-0024). The review of animal use protocol complies with the Japanese Act on Welfare and Management of Animals. All mice were housed and bred in the Pharmaceutical Sciences Animal Center of Hokkaido University under specific pathogen-free conditions. HEK293T (RRID: CVCL_0063) cell line was obtained from ATCC and maintained in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum. The cells have been authenticated by examining the expression of large T antigen and the competence to replicate vectors carrying the SV40 region of replication. In addition, the cells also were authenticated to show characteristics of HEK293T cells such as epithelial phenotype. All experiments were performed with mycoplasma-free cells.

132

133 *Antibodies*

134 Goat anti-mouse IgM F(ab')$_2$ fragment (anti-IgM) was purchased from Jackson
135 ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). FITC-anti-mouse CD3ε
136 (clone: 145-2C11), FITC-anti-mouse IgM (clone: RMM-1), APC-anti-mouse B220
137 (clone: RA3-6B2), PE-anti-mouse B220 (clone:RA3-6B2), FITC-anti-mouse CD4,
138 (clone: H129.19), FITC-anti-mouse IgD (clone: 11-26c.2a), APC-anti-mouse CD138
139 (clone: 281-2) mAbs , FITC-anti-mouse GL7 (clone: GL7) mAbs and PE-anti-mouse PD-
140 1 (clone: RMP1-30) mAbs were purchased from BioLegend (San Diego, CA, USA). APC
141 anti-mouse CXCR5 (clone: SPRCL5), APC anti-mouse Fas (clone: 15A7) and PE-anti-
142 mouse CD93 (clone: AA4.1) mAbs were purchased from eBioscience (San Diego, CA).
143 PE-anti-mouse CD43 (clone: S7) mAb was purchased from BD Biosciences (San Jose,
144 CA). Anti- phospho Lyn Y508, anti-phospho PLC-γ2, anti-phospho Akt, anti-phospho
145 ERK, anti-Lyn, anti-Akt and anti-Csk Abs were purchased from Cell Signaling
146 Technology (Beverly, MA, USA). Other Abs were purchased from Santa Cruz
147 Biotechnology (Santa Cruz, CA, USA). Anti-phospho STAP-2 Y250 Ab was generated
148 as described previously[26].

149

B cell isolation, plasmablast generation and cell activation

Splenic B cells were purified by magnetic separation system using biotinylated anti-mouse CD43 mAb (Miltenyl Biotec Inc., Auburn, CA, USA) and Streptavidin Particle Plus–DM (BD Biosciences, San Jose, CA, USA). In the experiment to detect *Stap2* expression, splenic B cells were further purified using anti-B220 magnetic beads and more than 96% highly purified B cells were obtained. *Stap2* mRNA expression was detected as previously described[5]. For cytokine production study, purified B cells were cultured with LPS (1 μg/ml, Sigma-Aldrich) for 48 hours to generate B cell plasmablasts and the B cell plasmablasts were stimulated with anti-IgM (10 μg/ml) for 24 h. IL-6 and IL-10 levels in supernatants were measured using ELISA kits (BioLegend). IgM levels on B cells and proportions of B cells in spleens were analyzed by FACS as previously described[5].

Flowcytometric analysis

Flowcytometric analysis was performed as previously described [5].

Plasmids

Myc-DDK-tagged Lyn plasmid was purchased from Origene (Rockville, MD). HA-

tagged Lyn and FLAG-tagged Csk plasmids were kindly provided by Dr. Okada (Osaka University). STAP-2 plasmids were generated as described previously[9,27].

Transfection

Human embryonic kidney carcinoma cell line, HEK293T was maintained in DMEM containing 10% FCS, and the indicated plasmids were transfected by a calcium phosphate method[3].

Immunoblotting and Immunoprecipitation

Immunoblotting, Immunoprecipitation and GST pull down assay were performed as previously described[9]. Densitometrical analysis was performed using ImageJ program (NIH, Bethesda, MD). Phos-tag^{TM}-based western blot analysis was performed using Phos-tag^{TM} Acrylamide ALL-107 (NARD institute, Ltd., Hyogo, Japan) as previously described[28].

Immunization

Mice were immunized with NP-Ficoll (5 μg, BIOSEARCH TECHNOLOGIES, Inc, Novato, CA, USA) and sera were collected at days 0, 4 and 14. For analysis of ragweed

pollen (RW)-specific antibody production, it was employed repeated RW administration mouse model as described previously with some modification[29]. Briefly, mice were intranasally treated with RW (0.1 mg in 20 μl PBS) for six consecutive days in a week for 3 weeks. Total IgE and IgG1 levels were measured as described previously[30]. For antigen-specific antibody detection, 10 μg/ml RW extract (Cosmo Bio Co., Ltd, Tokyo, Japan) and NP-BSA (BIOSEARCH TECHNOLOGIES, Inc) were coated in 96-well plates for overnight at 4 °C. After the wells were blocked with 10% FCS-PBS, diluted serum samples were incubated, and bound antibodies were detected by HRP conjugated anti-mouse IgM (BETHYL LABORATORIES, Inc., Montgomery, TX) and anti-mouse IgG1 Abs (BioLegend), followed by SA-HRP. Color was developed and absorbance at 450 nm was measured.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.02. Unpaired Student's *t*-test and Mann-Whitney *U*-test were employed. Data were considered significant at $p<0.05$. Data were shown mean + SEM.

Results and Discussion

STAP-2 is dispensable for B cell maturation

First, we compared B cell maturation between WT and STAP-2 KO Balb/c background mice. As shown in Table 1, the numbers and proportions of Pro B cells, Pre B cells and immature B cells in bone marrows of STAP-2 KO mice are similar levels compared with WT mice. We also observed normal numbers and proportions of T1/T2/T3 as well as follicular/marginal zone B cells in spleen of STAP-2 KO mice compared with WT mice. No difference of IgM^+/IgD^+ B cell proportions and numbers between WT and STAP-2 KO mice was observed. These results indicated that STAP-2 has no effect on B cell maturation similar to previous report using STAP-2 KO C57BL/6 background mice [31]. We next tested *Stap2* mRNA expression and IgM surface expression using splenic B cells of wild-type (WT) and STAP-2 knockout (KO) mice. *Stap2* expression was observed in WT but not in STAP-2 KO B cells (Figure 1A). Surface IgM expression levels in B cells of STAP-2 KO mice were similar to those of WT mice (Figure 1B). To investigate the role of STAP-2 in B cell differentiation and maintenance, we compared the proportions and absolute numbers of splenic B cells in WT and STAP-2 KO mice. Splenocytes were isolated from WT and STAP-2 KO mice, and $CD3^+$ T cells and $B220^+$ B cells were detected by FACS. As shown in Figure 1C-D, the proportions of splenic $B220^+$ B cell populations in STAP-2 KO mice were comparable to those in WT mice. The

numbers of total splenocytes and splenic $B220^+$ B cells in STAP-2 KO mice were also similar to those in WT mice (Figure 1E–F). We have also analyzed proportions of germinal center B cells (GC B; $B220^+Fas^+GL7^+$), T follicular cells (Tfh; $CD4^+CXCR5^+PD\text{-}1^+$) and plasma cells (PC; $B220^{low}CD138^+$) in spleens and compared the proportions of STAP-2 KO mice with WT mice. We observed no differences of the proportions in STAP-2 KO mice compared with WT mice under steady state conditions (Figure 1G-I). Taken together, STAP-2 is not essential for the differentiation and maintenance of B cells in peripheral lymphoid organs or for the expression of BCRs on the surface.

STAP-2 deficiency enhances BCR-mediated B cell activation in vitro and in vivo

We investigated the role of STAP-2 in B cell activation in vitro and in vivo. WT and STAP-2 KO splenic B cells were cultured with LPS for 48 h to generate B cell plasmablasts. Next, the B cell plasmablasts were stimulated with anti-IgM for 24 h, followed by measuring the cytokine levels in the culture supernatants by ELISA. Although anti-IgM stimulation induced the production of both IL-6 and IL-10, cytokine production was significantly higher in STAP-2 KO B cells than that in WT B cells (Figure 2A–B). To analyze the influence of STAP-2 deficiency on anti-IgM-induced BCR signal

transduction in splenic B cells, phosphorylation of downstream signaling molecules was analyzed by western blotting after isolated WT and STAP-2 KO splenic B cells were stimulated with anti-IgM. Phosphorylation levels of PLC-γ2, Akt, and ERK were significantly higher in STAP-2 KO B cells than in WT B cells after anti-IgM stimulation; the total protein levels were comparable (Figure 2C–D). Collectively, these results suggest that STAP-2 supports the downregulation of BCR signal transduction.

To investigate the effects of STAP-2 on antibody production *in vivo*, we employed two murine models; one was immunized with nitrophenyl (NP)-Ficoll, while the other was subjected to repeated intranasal administration of RW. WT and STAP-2 KO mice were immunized with NP-Ficoll, and serum anti-NP IgM levels were measured before and after immunization by ELISA. Although anti-NP IgM production was induced in both WT and STAP-2 KO mice, anti-NP IgM levels were significantly higher in STAP-2 KO mice than in WT mice 14 days after immunization (Figure 2E). We next analyzed whether STAP-2 affected antibody production in T cell antigen-immunized mice. As shown in Figure 2F, levels of anti-NP IgM, IgG1 and IgG2b in NP-CGG-immunized STAP-2 were comparable to those in WT mice. The differences of producing antibodies we observed between NP-Ficoll-immunized and NP-CGG-immunized mice may be due to T cell involvement because we have previously

reported that TCR-mediated T cell functions are suppressed in STAP-2 KO T cells[9]. Subsequently, we intranasally administered the WT and STAP-2 with a low dose (0.1 mg/mouse) of RW (Figure 2G). Serum analysis showed that total IgE and IgG1 levels as well as RW-specific IgG1 levels were significantly higher in RW-treated STAP-2 KO mice than in RW-treated WT mice (Figure 2H-J). We couldn't figure out which cell types are source of IL-4 for IgE class switching in a RW-induced pollinosis mouse model. STAP-2 negatively regulates FcεRI signal transduction in mast cells [4] and we observed that STAP-2 KO mast cells produce higher levels of IL-4 in response to IgE/Ag compared with WT mast cells (data not shown). It has also been reported that STAP-2 is an important for optimal TCR signal transduction in T cells because STAP-2 deficiency results in suppression of TCR signal pathway and IL-2 production in T cells [9]. Therefore, we guess higher IgE production may be due to the STAP-2 deficiency in not only B cells but also mast cells. Further experiments will be necessary for demonstrating detail mechanisms of two cell type cooperation. Collectively, these results suggested that STAP-2 is negatively involved in BCR-induced antibody production.

STAP-2 binds to Lyn through its PH and SH2 domains

Next, we compared phosphorylation levels of Lyn Y508, which is a key tyrosine residue for suppression of Lyn activity, in activated WT and STAP-2 KO B cells. As shown in Figure 3A and B, Lyn Y508 phosphorylation was lower in STAP-2 KO B cells than in WT B cells after anti-IgM stimulation, although no statistical significance was observed. We examined the interaction of STAP-2 with Lyn to clarify whether STAP-2 functions as a scaffold protein for the regulation of Lyn activity. Later, 293T cells were transfected with GST-tagged STAP-2 full-length (FL) plasmid together with Myc-DDK-tagged Lyn-expression plasmids. Then, GST-tagged STAP-2 protein was precipitated with GSH-Sepharose, and Lyn coprecipitation was detected by western blotting. Lyn was coprecipitated with STAP-2, suggesting that STAP-2 binds to Lyn directly (Figure 3C). To determine which domain of STAP-2 interacts with Lyn, we performed a coprecipitation experiment using GST-tagged STAP2 FL- and domain-expression plasmids, as shown in Figure 3D. Then, 293T cells were transfected with GST-tagged STAP-2 FL- or domain-expression plasmids together with HA-tagged Lyn-expression plasmids, and the Lyn-binding domain within STAP-2 was identified by coprecipitation using GSH-Sepharose beads, followed by western blotting using anti-HA antibody. As expected, Lyn coprecipitated with STAP-2 FL and with the PH and SH2 domains but not with the proline-rich domain (Figure 3E), indicating that STAP-2 PH and SH2 are

294 responsible for binding to Lyn. These results suggest that STAP-2 constitutively binds to
295 Lyn, and this interaction may be involved in the STAP-2-dependent modification of BCR
296 signaling in B cells.
297
298 *STAP-2 also binds to Csk through its PH and SH2 domains*
299 Because Csk is known to phosphorylate Lyn Y508, the binding between STAP-
300 2 and Csk was analyzed. When 293T cells were co-transfected with Myc-tagged STAP-2
301 FL- and FLAG-tagged Csk-expression plasmids, Csk was co-immunoprecipitated with
302 STAP-2 (Figure 3F), suggesting that STAP-2 also interacts with Csk. To determine which
303 domain of STAP-2 interacts with Csk, 293T cells were transfected with FLAG-tagged
304 Csk together with either GST-tagged STAP-2 FL- or domain-expression plasmids,
305 followed by co-precipitation using GSH-Sepharose. As shown in Figure 3E, Csk
306 coprecipitated with STAP-2 FL, as expected. In addition, the precipitation of Csk
307 contained the PH and SH2 but not the C domains of STAP-2 (Figure 3G). Collectively,
308 these results suggest that STAP-2 interacts with Lyn and Csk through its PH and SH2
309 domains and may function as a scaffold protein, recruiting Csk to Lyn.
310
311 *Formation of Lyn/STAP-2/Csk complex enhances the phosphorylation of Lyn Y508*

312 To confirm the complex formation of Lyn, Csk, and STAP-2, 293T cells were
313 transfected with expression plasmids for these three molecules. The interaction between
314 Csk and STAP-2 was enhanced in 293T cells co-transfected with Lyn in a dose-dependent
315 manner (Figure 4A). The interaction between Lyn and Csk was also enhanced in 293T
316 cells co-transfected with STAP-2 in a dose-dependent manner (Figure 4B). In accordance
317 with the increasing interaction of Lyn with Csk through STAP-2, Csk-mediated tyrosine
318 phosphorylation of Lyn Y508 was increased in Lyn/Csk-expressing 293T cells when the
319 cells were co-expressed with STAP-2 in a dose-dependent manner (Figure 4B). Therefore,
320 STAP-2 enhanced the phosphorylation of the negative regulatory tyrosine residue Y508
321 within Lyn by increasing the recruitment of Csk to Lyn.
322 Lyn is a unique kinase with both negative and positive functions[32]. In addition
323 to Lyn deficiency, active Lyn mutations also result in autoimmune disease phenotypes,
324 such as autoreactive antibody-mediated glomerulonephritis[33,34]. In the present study,
325 it was investigated that STAP-2 directly interacts with Lyn. Lyn Y508 phosphorylation is
326 involved in inhibiting Lyn Y397 phosphorylation. Once Lyn Y508 is dephosphorylated
327 by CD45, a structural change occurs to expose the catalytic domain of Lyn, and
328 autophosphorylation of Lyn Y397, a positive regulatory tyrosine, is promoted. After
329 activation of the BCR signal cascade, the phosphorylation of Lyn Y508 was reinduced by

330 Csk, and Lyn became inactive due to the closed conformation of Lyn [24,32]. We also
331 revealed that STAP-2 binding to Csk and Csk-induced Lyn Y508 phosphorylation was
332 increased in the presence of STAP-2 in HEK293T cells. Csk-deficient DT40 cells showed
333 constitutive phosphorylation of Lyn, higher Syk phosphorylation and calcium
334 mobilization[25]. Previous studies correspond to our present data, suggesting that STAP-
335 2 is necessary for appropriate BCR-mediated B cell activation by functioning as a scaffold
336 protein for Lyn and Csk to induce Csk-mediated suppression of Lyn kinase activity after
337 BCR aggregation.
338
339 *STAP-2 phosphorylation is required for increasing the interaction of Csk with Lyn*
340 A previous study has demonstrated that STAP-2 is phosphorylated by v-Src[2],
341 suggesting that Lyn phosphorylates STAP-2 after BCR stimulation, and that STAP-2
342 phosphorylation may be required for the negative regulation of Lyn. To investigate the
343 significance of STAP-2 phosphorylation, we analyzed whether STAP-2 was directly
344 phosphorylated by Lyn, and which tyrosine residue within STAP-2 was phosphorylated
345 using 293T cells transfected with STAP-2 WT and YF mutants in the presence or absence
346 of Lyn by Phos-tag-based western blotting, in which a shifted band was observed when a
347 molecule was phosphorylated[28]. We found that the bands of STAP-2 WT, Y22F, Y310F,

348 and Y322F were shifted in 293T cells co-transfected with Lyn. When 293T cells were
349 transfected with STAP-2 Y250F and Lyn, no difference in STAP-2 Y250 mobility
350 indicated the negligible effect of Lyn (Figure 4C). In contrast, we observed no effect of
351 Csk on the phosphorylation of STAP-2 (data not shown). These results suggest that
352 tyrosine 250 within STAP-2 is a Lyn-mediated phosphorylation site. To investigate
353 whether Lyn-mediated phosphorylation of STAP-2 Y250 enhanced the interaction of
354 STAP-2 with Lyn, we transfected 293T cells with HA-Lyn with Myc STAP-2 WT or
355 Y250F mutants and analyzed their interaction by co-immunoprecipitation. Lyn bound to
356 STAP-2 WT as well as the Y250F mutant (YF) (Figure 4D), suggesting that
357 phosphorylation of STAP-2 by Lyn is not essential for the association of STAP-2 with
358 Lyn. To determine whether Lyn-mediated phosphorylation of STAP-2 is required to
359 enhance the interaction of Csk with Lyn, 293T cells were transfected with Lyn and Csk
360 together with a STAP-2 WT or Y250F mutant, and the association of Csk with Lyn was
361 analyzed by co-immunoprecipitation. Although the association of Csk with Lyn was
362 observed in the absence of STAP-2, this association was enhanced in 293T cells co-
363 transfected with STAP-2 WT (Figure 4E). However, this enhanced interaction was
364 partially suppressed when 293T cells were co-transfected with STAP-2 YF (Figure 4E),
365 suggesting that Lyn-mediated STAP-2 phosphorylation is involved in the binding of Csk

366 to Lyn. We demonstrated that STAP-2 functions as a scaffold protein for negative
367 regulation of Lyn kinase activity by recruiting Csk to Lyn in activated wild-type B cells,
368 resulting in normal BCR signal transduction although this regulation was disrupted in
369 STAP-2 KO B cells (Figure 4F).

370 STAP-2 contains four putative phosphorylated tyrosine residues[2]. STAP-2
371 Y250 has been reported to be phosphorylated by intracellular tyrosine kinases, such as v-
372 Src, Jak2, Brk, BCR-ABL, and Pyk2. Brk- and v-Src-mediated STAP-2 phosphorylation
373 results in increased STAT3 and STAT5[2,26,35,36]. The proliferation of BCR-ABL-
374 expressing Ba/F3 cells was further enhanced by co-transfection with STAP-2 WT but not
375 with the Y250F mutant[37]. Phosphorylation of STAP-2 Y250 is required for interaction
376 of STAP-2 with Pyk2 because this interaction is disrupted when the STAP-2 Y250F
377 mutant is transfected instead of STAP-2 WT[27]. In the present study, Lyn, a Src family
378 tyrosine kinase, also phosphorylated the STAP-2 Y250 residue. Ingley et al. demonstrated
379 that Lyn phosphorylates Csk-binding protein (CBP/PAG1) Y314, which recruits Csk/Ctk
380 to inhibit Lyn kinase activity. Tyrosine phosphorylation is also involved in the recruitment
381 of suppressor of cytokine signaling 1 (SOCS1) for Lyn degradation[38]. Although our
382 present study suggests that Lyn-dependent STAP-2 Y250 phosphorylation is involved in
383 the upregulation of association of the STAP-2 with Csk, but not Lyn, in HEK293T cells,

it is still controversial whether the phosphorylation of STAP-2 increases the association of STAP-2 with Lyn in B cells. We previously reported that LCK phosphorylates STAP-2 Y250, and this tyrosine phosphorylation of STAP-2 enhances its association with LCK, resulting in an increase in ZAP-70 phosphorylation and IL-2 production in T cells[9]. As a subject of a future study, we need to investigate the role of STAP-2 Y250 phosphorylation by Lyn in enhancing the association of STAP-2 with Lyn in B cells. In addition, we need to investigate whether STAP-2 Y250 phosphorylation is required for its association with other regulatory proteins, such as Ctk, for inhibitory regulation of Lyn activity in B cells.

In summary, we revealed the important role of STAP-2 in the negative regulation of BCR signaling and B cell function such as antibody production. STAP-2 binds to Lyn and Csk and functions as a scaffold protein to inhibit Lyn activity. Therefore, STAP-2 plays a key role in the pathogenesis of antibody-mediated disorders such as allergies and autoimmune diseases and may be used as a possible target for the development of novel therapeutic drugs.

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Disclosures

The authors declare no financial conflicts of interests.

Data accessibility

The data supporting the findings of this study are available within the paper or can be obtained from the corresponding author, kashiwakura-j@hus.ac.jp or tmatsuda@pharm.hokudai.ac.jp, upon reasonable request.

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529

Table 1. Composition of B cell subpopulation in bone marrows and spleens

		WT		STAP-2 KO	
		%	# (x 10^5)	%	# (x 10^5)
Bone marrow	Pro	6.35 ± 0.33	9.0 ± 3.1	5.98 ± 0.36	8.9 ± 2.7
	Pre	26.4 ± 3.0	36.9 ± 12.1	24.7 ± 1.8	34.8 ± 6.0
	immature	5.06 ± 0.78	7.3 ± 3.7	3.82 ± 0.66	5.7 ± 2.8
Spleen	T1	2.27 ± 0.47	21.7 ± 11.6	1.79 ± 0.12	18.0 ± 5.0
	T2	2.26 ± 0.88	22.3 ± 19	1.34 ± 0.2	13.2 ± 2.9
	T3	1.35 ± 0.53	13.3 ± 11.4	1.12 ± 0.16	11.2 ± 3.4
	FO	13.9 ± 2.7	123.7 ± 31.8	13.9 ± 1.5	140.0 ± 43.0
	MZ	3.04 ± 0.15	27.7 ± 2.4	2.41 ± 0.18	23.8 ± 0.6
	IgM^+IgD^+	15.3 ± 0.4	123 ± 11.6	16.0 ± 1.0	154.4 ± 27.2

Data are shown mean ± SD of 2 independent experiments (n=3).

Population gating strategy: Pro B cells (Pro); $CD43^{high}B220^{mod}$, Pre B cells (Pre); $CD43^{low}B220^{mod}$, immature B cells (immature), $CD43^{low}B220^{high}$, T1; $AA4.1^-$ $B220^+IgM^{high}CD23^-$, T2; $AA4.1^-B220^+IgM^{high}CD23^+$, T3; $AA4.1^-B220^+IgM^{low}CD23^+$, follicular B cells (FO); $AA4.1^+B220^+IgM^{low}CD23^+$, marginal zone B cells (MZ); $AA4.1^+B220^+IgM^{high}CD23^-$. IgM^+IgD^+; $B220^+IgM^+IgD^+$

Figure legends

FIGURE 1. STAP-2 is dispensable for B cell maturation.

(A) *Stap2* mRNA expression in B cells from WT and STAP-2 KO mice. *G3pdh* was detected as a loading control. Two independent experiments were performed. (B) Surface IgM expression on WT and STAP-2 KO B cells. Black lines show FITC-conjugated anti-IgM mAb-treated B cells. Gray-filled histograms are unstained B cells. Right bar graph shows mean of MFI values + SEM of 2 independent experiments (WT & STAP-2 KO n = 4). (C) Populations of T cells ($CD3^+$ cells) and B cells ($B220^+$ cells) in spleens of WT and STAP-2 KO mice. Data shown are representative of 6 independent experiments (WT & STAP-2 KO n = 12). (D) B cell proportions in spleens of WT and STAP-2 KO mice. Blue and pink bars show WT and STAP-2 KO mice, respectively. Data were shown mean + SEM of 6 independent experiments (WT & STAP-2 KO n = 12). (E) Total splenocyte numbers of WT and STAP-2 KO mice. Blue and pink bars show WT and STAP-2 KO mice, respectively. Data were shown mean + SEM of 6 independent experiments (WT & STAP-2 KO n = 12). (F) Absolute numbers of splenic B cells of WT and STAP-2 KO mice. Blue and pink bars show WT and STAP-2 KO mice, respectively. Data were shown mean + SEM of 6 independent experiments (WT & STAP-2 KO n = 12). (G-I) Proportions of germinal center B cells (G), follicular helper T cells (H) and plasma cells (I) of WT

555 and STAP-2 KO mice. Blue and pink bars show WT and STAP-2 KO mice, respectively.

556 Data were shown mean + SEM of 3 independent experiments (WT & STAP-2 KO n = 5).

557

558 FIGURE 2. STAP-2 deficiency results in enhancing BCR-mediated B cell activation

559 and antibody production.

560 (A and B) IL-6 (A) and IL-10 (B) levels in supernatants of activated WT (Blue bars) and

561 STAP-2 KO (Pink bars) B cell plasmablasts. Data are shown mean + SEM of 3

562 independent experiments (WT & STAP-2 KO n = 6) *; $p<0.05$ by Unpaired Student's *t*-

563 test. (C) Phosphorylation of PLC-γ2, Akt and ERK. Data shown are representative of 6

564 independent experiments. (D) Quantification of phosphorylation levels of PLC-γ2, Akt

565 and ERK in WT (Blue bars) and STAP-2 KO (Pink bars) B cells. Data were shown mean

566 + SEM (WT & STAP-2 KO n = 6). *:$p<0.05$, **:$p<0.01$ by Mann-Whitney *U*-test. (E)

567 Serum anti-NP IgM levels in WT (Blue bars) and STAP-2 KO (Pink bars) mice after

568 immunization with NP-Ficoll. Data are shown mean + SEM of 3 independent experiments

569 (WT & STAP-2 KO n = 11). **; $p<0.01$ by Unpaired Student's *t*-test. (F) Serum anti-NP

570 IgM, IgG1 and IgG2b levels in WT (Blue bars) and STAP-2 KO (Pink bars) mice after

571 immunization with NP-CGG shown mean + SEM of 3 independent experiments (WT &

572 STAP-2 KO n = 11). (G) Procedure scheme of repeated intranasal RW administration.

573 Top arrows show intranasal (in) RW administration. (H-J) Serum levels of total IgE (J),
574 total IgG1 (I) and RW-specific IgG1 (J). Blue and pink bars show WT and STAP-2 KO
575 mice, respectively. Data were shown mean + SEM of 2 independent experiments (WT &
576 KO n = 6). *; $p<0.05$ by Mann-Whitney *U*-test.
577
578 FIGURE 3. STAP-2 interacts with Lyn and Csk through its PH and SH2 domains.
579 (A) Phosphorylation of Lyn Y508. Data shown are representative of 5 independent
580 experiments. (B) Quantification of phosphorylation levels of Lyn Y508 in WT (Blue bars)
581 and STAP-2 KO (Pink bars) B cells. Data were shown mean + SEM (WT & STAP-2 KO
582 n = 5). (C) GST-tagged STAP-2 vector was cotransfected with Myc-DDK-tagged Lyn
583 vector into 293T cells. Binding of STAP-2 to Lyn was analyzed by GST pulldown assay,
584 followed by western blotting. Two independent experiments were performed. (D) Scheme
585 of full length and truncated forms of GST-tagged STAP-2 (FL = Full length, PH = PH
586 domain, SH2 = SH2-like domain, C = C-terminal domain containing proline rich region).
587 (E) GST-tagged STAP-2 FL or truncated STAP-2 vector were cotransfected with HA-
588 tagged Lyn vector into 293T cells. Lyn-binding domains within STAP-2 was analyzed by
589 GST pulldown assay, followed by western blotting. Two independent experiments were
590 performed. (F) Myc-tagged STAP-2 vector was cotransfected with FLAG-tagged Csk

591 vector into 293T cells. Binding of STAP-2 to Csk was analyzed by immunoprecipitation,
592 followed by western blotting. Two independent experiments were performed. (G) GST-
593 tagged STAP-2 FL or truncated STAP-2 vector was cotransfected with FLAG-tagged Csk
594 vector into 293T cells. Csk-binding domains within STAP-2 was analyzed by GST
595 pulldown assay, followed by western blotting. Three independent experiments were
596 performed.
597
598 FIGURE 4. STAP-2 is phosphorylated by Lyn for regulation of Lyn activation by
599 recruiting Csk.
600 (A) GST-tagged STAP-2 vector was cotransfected with FLAG-tagged Csk vector in the
601 presence or absence of HA-tagged Lyn vector (1, 3 or 10 μg) into 293T cells. Binding of
602 STAP-2 to Csk was analyzed by GST pulldown assay, followed by western blotting. Four
603 independent experiments were performed. (B) HA-tagged Lyn vector was cotransfected
604 with FLAG-tagged Csk vector in the presence or absence of Myc-tagged STAP-2 vector
605 (1, 3 or 10 μg) into 293T cells. Binding of Lyn to Csk and Csk-associated Lyn
606 phosphorylation at Y508 were analyzed by immunoprecipitation, followed by western
607 blotting. Three independent experiments were performed. (C) Myc-tagged STAP-2 WT
608 or mutant vector was cotransfected with or without HA-tagged Lyn vector into 293T cells.

609 Phosphorylation of STAP-2 WT and YF mutants was detected by Phos-tag-based western
610 blotting. Two independent experiments were performed. (D) Myc-tagged STAP-2 WT or
611 STAP-2 Y250F (YF) vector was cotransfected with HA-tagged Lyn vector into 293T cells.
612 Binding of STAP-2 to Lyn was analyzed by immunoprecipitation, followed by western
613 blotting. Two independent experiments were performed. (E) HA-tagged Lyn vector was
614 cotransfected with FLAG-tagged Csk vector in the presence or absence of Myc-tagged
615 STAP-2 WT or Y250F mutant (YF) vector (0.3 or 1 μg) into 293T cells. Binding of Lyn
616 to Csk was analyzed by immunoprecipitation, followed by western blotting. Three
617 independent experiments were performed. (F) A model of STAP-2-mediated regulation
618 of Lyn Y508 phosphorylation by Csk. Top panel: mechanism in a wild-type B cell.
619 Bottom panel: mechanism in a STAP-2 deficient B cell.

Figure 1

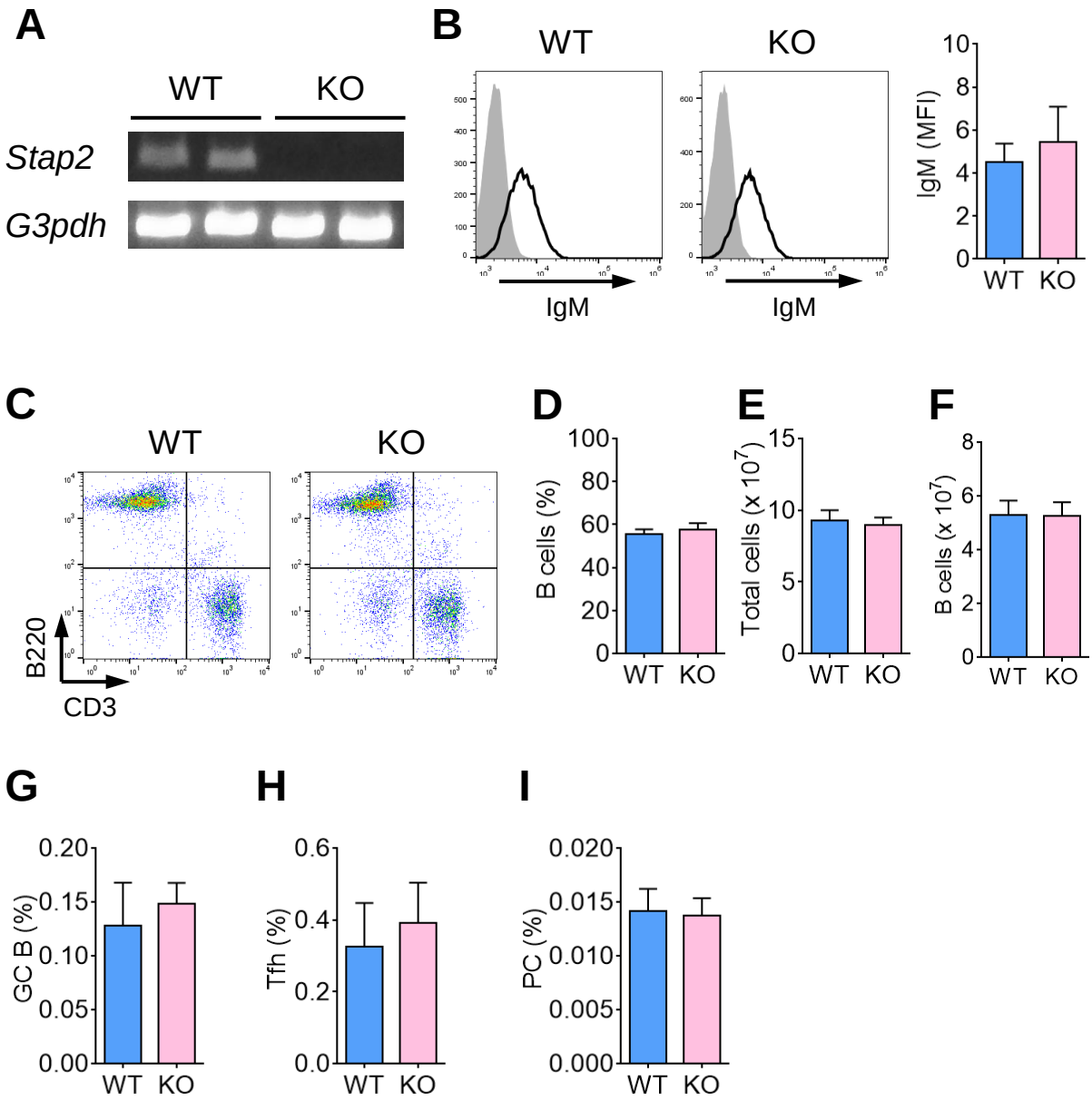

A
WT
KO
Stap2
G3pdh
B
WT
KO
IgM
IgM
IgM (MFI)
WT KO
C
WT
KO
B220
CD3
D
B cells (%)
WT KO
E
Total cells (x 10^7)
WT KO
F
B cells (x 10^7)
WT KO
G
GC B (%)
WT KO
H
Tfh (%)
WT KO
I
PC (%)
WT KO

Figure 2

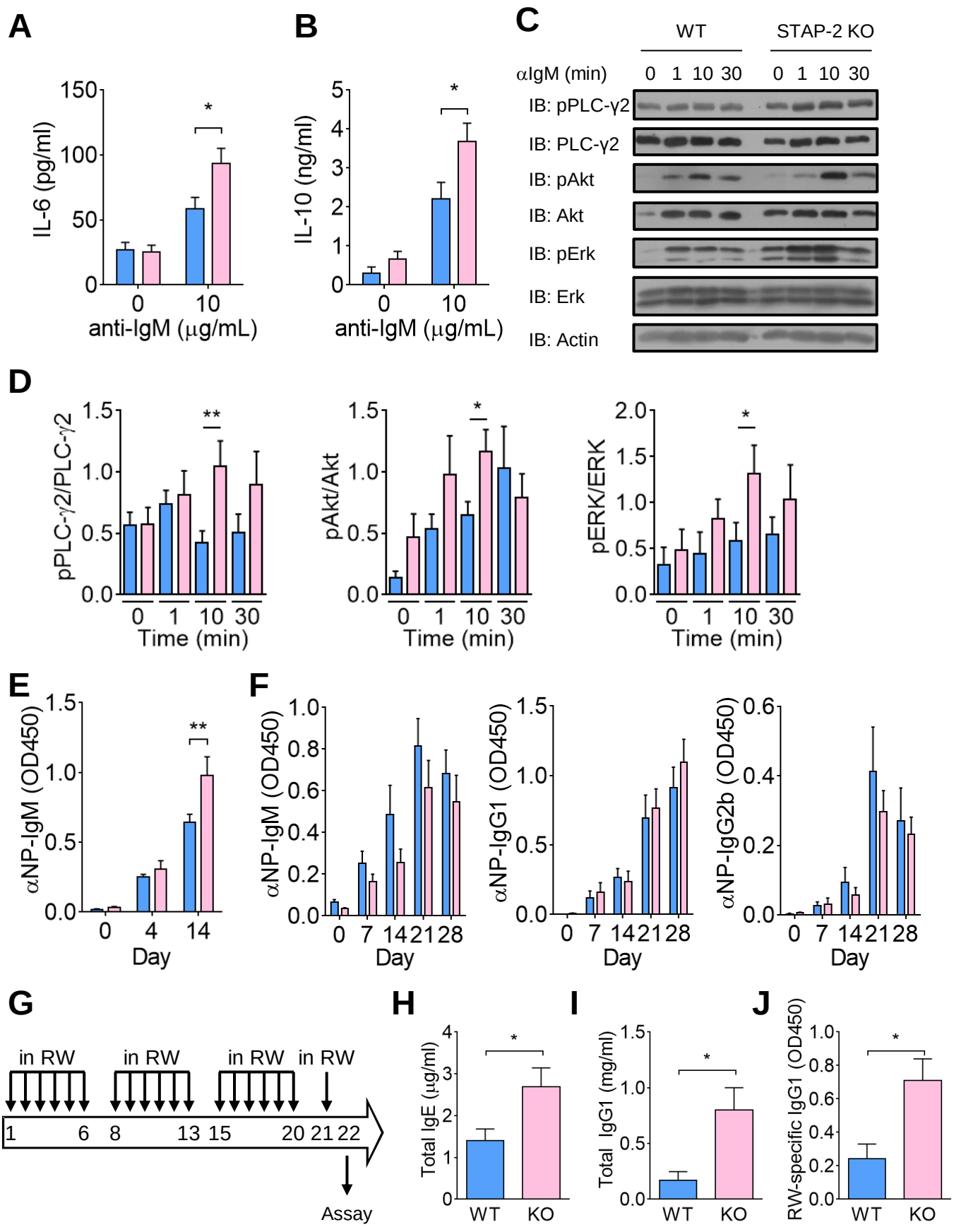

A
IL-6 (pg/ml)
150
100
50
0
*
0
10
anti-IgM (μg/mL)
B
IL-10 (ng/ml)
5
4
3
2
1
0
*
0
10
anti-IgM (μg/mL)
C
WT
STAP-2 KO
αIgM (min)
0 1 10 30 0 1 10 30
IB: pPLC-γ2
IB: PLC-γ2
IB: pAkt
IB: Akt
IB: pErk
IB: Erk
IB: Actin
D
pPLC-γ2/PLC-γ2
1.5
1.0
0.5
0.0
**
0 1 10 30
Time (min)
pAkt/Akt
1.5
1.0
0.5
0.0
*
0 1 10 30
Time (min)
pERK/ERK
2.0
1.5
1.0
0.5
0.0
*
0 1 10 30
Time (min)
E
αNP-IgM (OD450)
1.5
1.0
0.5
0.0
**
0 4 14
Day
F
αNP-IgM (OD450)
1.0
0.8
0.6
0.4
0.2
0.0
0 7 14 21 28
Day
αNP-IgG1 (OD450)
1.5
1.0
0.5
0.0
0 7 14 21 28
Day
αNP-IgG2b (OD450)
0.6
0.4
0.2
0.0
0 7 14 21 28
Day
G
in RW
in RW
in RW
in RW
1 6 8 13 15 20 21 22
Assay
H
Total IgE (μg/ml)
4
3
2
1
0
*
WT
KO
I
Total IgG1 (mg/ml)
1.5
1.0
0.5
0.0
*
WT
KO
J
RW-specific IgG1 (OD450)
1.0
0.8
0.6
0.4
0.2
0.0
*
WT
KO

Figure 3

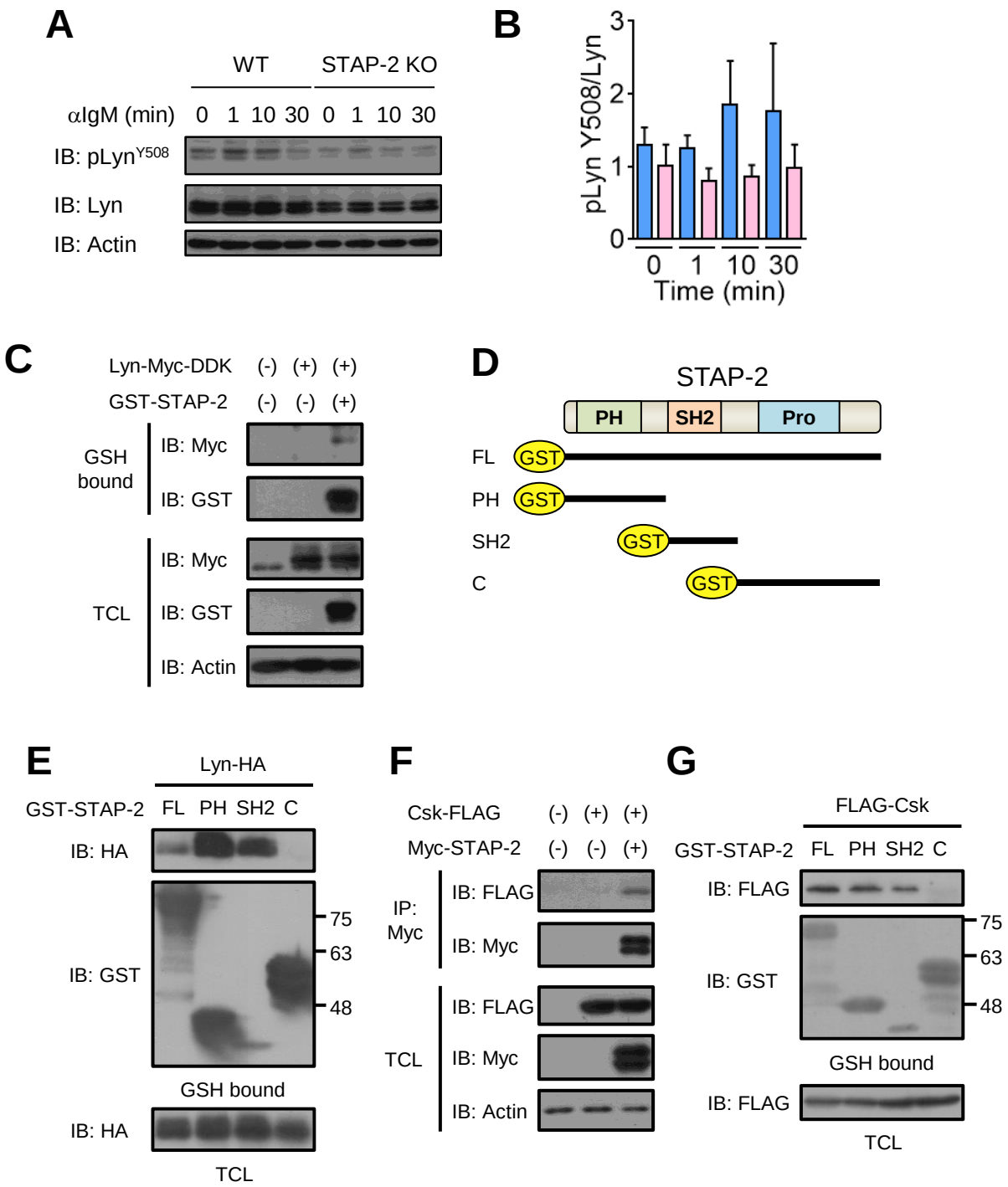
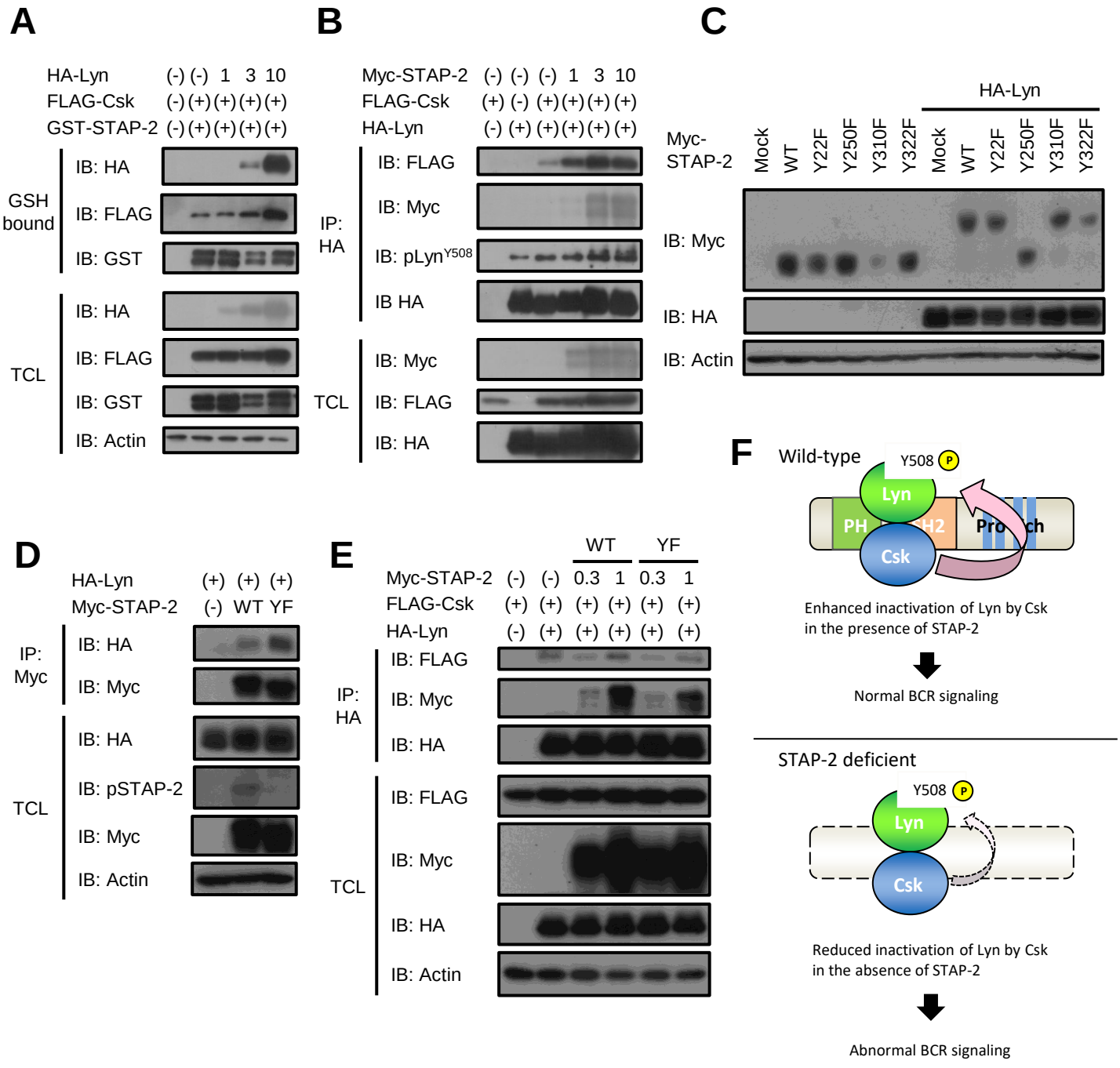

A
WT
STAP-2 KO
αIgM (min) 0 1 10 30 0 1 10 30
IB: pLyn Y508
IB: Lyn
IB: Actin
B
pLyn Y508/Lyn
3
2
1
0
0 1 10 30
Time (min)
C
Lyn-Myc-DDK (-) (+) (+)
GST-STAP-2 (-) (-) (+)
GSH bound
IB: Myc
IB: GST
TCL
IB: Myc
IB: GST
IB: Actin
D
STAP-2
PH
SH2
Pro
FL
PH
SH2
C
GST
E
Lyn-HA
GST-STAP-2 FL PH SH2 C
IB: HA
IB: GST
75
63
48
GSH bound
IB: HA
TCL
F
Csk-FLAG (-) (+) (+)
Myc-STAP-2 (-) (-) (+)
IP: Myc
IB: FLAG
IB: Myc
TCL
IB: FLAG
IB: Myc
IB: Actin
G
FLAG-Csk
GST-STAP-2 FL PH SH2 C
IB: FLAG
IB: GST
75
63
48
GSH bound
IB: FLAG
TCL

Figure 4


A
HA-Lyn (-) (-) 1 3 10
FLAG-Csk (-) (+) (+) (+) (+)
GST-STAP-2 (-) (+) (+) (+) (+)
GSH bound
IB: HA
IB: FLAG
IB: GST
TCL
IB: HA
IB: FLAG
IB: GST
IB: Actin
B
Myc-STAP-2 (-) (-) (-) 1 3 10
FLAG-Csk (+) (-) (+) (+) (+) (+)
HA-Lyn (-) (+) (+) (+) (+) (+)
IP: HA
IB: FLAG
IB: Myc
IB: pLyn Y508
IB HA
TCL
IB: Myc
IB: FLAG
IB: HA
C
HA-Lyn
Myc-STAP-2
Mock WT Y22F Y250F Y310F Y322F Mock WT Y22F Y250F Y310F Y322F
IB: Myc
IB: HA
IB: Actin
D
HA-Lyn (+) (+) (+)
Myc-STAP-2 (-) WT YF
IP: Myc
IB: HA
IB: Myc
TCL
IB: HA
IB: pSTAP-2
IB: Myc
IB: Actin
E
WT YF
Myc-STAP-2 (-) (-) 0.3 1 0.3 1
FLAG-Csk (+) (+) (+) (+) (+) (+)
HA-Lyn (-) (+) (+) (+) (+) (+)
IP: HA
IB: FLAG
IB: Myc
IB: HA
TCL
IB: FLAG
IB: Myc
IB: HA
IB: Actin
F
Wild-type
Y508 P
Lyn
PH
SH2
Csk
Enhanced inactivation of Lyn by Csk in the presence of STAP-2
Normal BCR signaling
STAP-2 deficient
Y508 P
Lyn
Csk
Reduced inactivation of Lyn by Csk in the absence of STAP-2
Abnormal BCR signaling