scientific reports

OPEN

Control of germinal center B cell survival and IgE production by an adaptor molecule containing PH and SH2 domains, Aps/Sh2b2

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The germinal centers (GCs) are structure found within secondary lymphoid organs and are important for the antibody-producing response against foreign antigens. In GCs, antigen-specific B cells proliferate intensely, inducing immunoglobulin class switching. Recent studies have shown that GCs are also an important site for class switching to IgE, which is implicated in allergy. However, the mechanisms by which IgE production is regulated in GCs remain unclear. Here, we found impairment in IgE-specific production and a reduction of GC B cells after immunization in mice deficient in the *Aps/Sh2b2* gene encoding the Lnk/Sh2b family adaptor protein Aps. GC B cells express higher levels of the *Aps* gene than non-GC B cells, and cell death of *Aps*$^{-/-}$ GC B cells is enhanced compared to wild-type GC B cells. An in vitro culture system with purified *Aps*$^{-/-}$ B cells induced the same level of IgE production and frequencies of IgE$^+$ B cells as wild-type B cells. We found that *Aps* deficiency in B cells resulted in augmented depletion of IgE$^+$ blasts by B cell receptor crosslinking with anti-CD79b antibodies compared to wild-type IgE$^+$ cells. These results suggest that Aps regulates IgE production by controlling the survival of GC B cells and IgE$^+$ plasma cells and may serve as a potential therapeutic target to control IgE production.

Secondary lymphoid organs such as the spleen and lymph nodes (LNs) are central sites for acquired immune responses. The germinal centers (GCs) are structure found within secondary lymphoid organs and are important for the antibody-producing response against foreign antigens[1]. In GCs, antigen-specific B cells proliferate intensely, inducing immunoglobulin (Ig) class switching[2,3]. This process is aided by follicular helper T (Tfh) cells, a special type of helper T cell in GCs. Ig class switching is required to produce IgG, IgA, and IgE[3], and antibodies of each isotype are involved in biological immune defense responses through isotype-specific mechanisms. IgE is found at low levels in healthy individuals, and the low percentage of IgE-positive cells makes it challenging to study the mechanisms of IgE synthesis. Recent technological advances have revealed the complex mechanisms behind IgE production. Class switching to IgE is induced by Tfh-derived interleukin (IL)-4 and inhibited by IL-21[4–7], and IgE$^+$ B cells rapidly differentiate after class switching into antibody-producing plasma cells (PCs). This process is driven by antigen-independent signaling of the IgE-B cell receptor (BCR)[8,9], and IgE$^+$ PCs are short-lived[10–13]. The expression of BCRs on the cell surface of IgE$^+$ PCs is higher than that of IgG$^+$ PC isotypes, and strong signals from the BCRs induce cell death, resulting in the elimination of IgE$^+$ PCs[14].

IgE is an allergy-associated Ig isotype that binds to Fcε receptors on mast cells and basophils. IgE induces degranulation to release various cytokines, chemokines, and lipid mediators after crosslinking with specific antigens[15]. Elevated serum IgE levels are associated with severe disease in patients with allergies. Inhibition of IgE function is considered an effective therapy for allergies, and anti-IgE antibodies have been used as a treatment for severe asthma[16–18]. Therefore, clarifying the mechanisms behind IgE production and controlling in vivo IgE levels are important goals in developing therapies against allergic diseases.

The adaptor molecule containing PH and SH2 domains (Aps)/Sh2b2 is an intracellular adaptor protein and a member of the Lnk/Sh2b adaptor family comprising Lnk/Sh2b3 and Sh2-b/Sh2b1. These molecules share several protein interaction domains such as the dimerization domain, proline-rich region, pleckstrin homology (PH)

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domain, src homology-2 (SH2) domain, and conserved tyrosine at the C-terminus[19–22]. We and other groups have reported that Lnk and Sh2-b regulate the signaling of various cytokines and growth factors, such as stem cell factor, thrombopoietin, and erythropoietin for Lnk[22–25]; and growth hormone, insulin-like growth factor, insulin, and leptin for Sh2-b[26–29]. We have also shown that Aps is expressed in B cells and tyrosine-phosphorylated after BCR stimulation[21]. We generated *Aps*-deficient mice and showed that Aps regulates the actin cytoskeleton, the number of peritoneal B-1 cells, and IgM and IgG3 production against thymus-independent type 2 (TI-2) antigens *in vivo*[30]. It has also been reported that Aps regulates BCR signaling through cytoskeletal remodeling in mature B cells[31]. Furthermore, we showed that Aps is expressed in mast cells, and that *Aps*-deficient bone marrow (BM)-derived mast cells (BMMCs) increase degranulation after high-affinity IgE receptor (FcεRI) crosslinking with IgE and antigen[32]. However, the precise molecular function of Aps in vivo remains largely unknown.

In this study, we examined the effects of *Aps*-deficiency in allergic reactions. Unexpectedly, we found that *Aps* deficiency results in an impairment of IgE production and a decrease in GC B cells after immunization. *Aps*$^{-/-}$ GC B cells showed decreased cell survival, although their cell cycling was normal. BCR-crosslinking of *Aps*$^{-/-}$ in vitro differentiated B cells with GC B-like phenotypes (iGB cells)[33], enhanced the reduction of IgE^{+} cells compared with wild-type (WT) iGB cells. Our results indicate that Aps controls GC B cell survival and IgE production, and that further analysis may help elucidate the mechanisms of IgE production.

Results

Aps-deficient mice have a defect in IgE production

In a previous report, we found that Aps is expressed in mast cells and that *Aps*$^{-/-}$ mast cells have increased degranulation after FcεRI crosslinking with IgE and antigen[32]. These results prompted us to examine the function of Aps in allergic inflammation, in which mast cells play important roles. We backcrossed *Aps*$^{-/-}$ mice with mice having a BALB/c background and performed contact hypersensitivity (CHS) experiments using fluorescein isothiocyanate (FITC) or 1-fluoro-2,4-dinitrobenzene (DNFB) as the hapten. *Aps*$^{-/-}$ mice showed almost equivalent thickening of the ear after hapten immunization and challenge as WT mice (Supplementary Fig. S1A). Unexpectedly, however, serum from challenged *Aps*$^{-/-}$ mice contained lower levels of total IgE antibodies than those from WT mice but showed no differences in IgG1 and other Ig isotypes (Fig. 1A and Supplementary Fig. S1B). We hypothesized that impairment of IgE production was observed only after challenge by hapten applied to the skin. To examine whether IgE is produced normally after systemic immunization, we immunized mice subcutaneously or by intraperitoneal (*i.p.*) injection with ovalbumin (OVA) mixed with alum adjuvant and found that serum IgE levels in *Aps*$^{-/-}$ mice were lower than those in WT mice, but that other Ig classes were present at similar levels (Fig. 1B). These data indicate that *Aps*$^{-/-}$ mice show selective impairment in the production of IgE, but not other Ig isotypes, after immunizations with various antigens.

Aps expression in B cells is important for IgE production

Aps/Sh2b2 transcripts were detected in various immune cells, including macrophages and B cells, but not T cells[21]. We observed that dendritic cell (DC) migration and activation and Th2 differentiation occurred normally in *Aps*$^{-/-}$ LNs after immunization with FITC (Supplementary Fig. S1C and S1D). To clarify whether Aps in B cells is important for optimal IgE production in mice, we generated BM chimeric mice by transferring WT or *Aps*$^{-/-}$ BM cells mixed with BM from B cell-deficient Jh mice at a ratio of 1:9. In these mice, B-lineage cells were either WT or *Aps*-deficient, whereas other hematopoietic cells were a mixture of WT and *Aps*$^{-/-}$ cells. We analyzed peripheral blood cells from reconstituted mice by flow cytometry and confirmed that B cell compartments were normal and there was no difference between WT and *Aps*$^{-/-}$ (data not shown). After reconstitution, mice were immunized with OVA plus alum, and serum Ig levels were determined using ELISA. Chimeric mice reconstituted with cells from *Aps*$^{-/-}$ and Jh mice showed decreased levels of IgE compared with WT plus Jh chimeric mice (Fig. 2A). Both chimeric mice types mounted normal and comparable IgG1 or IgM production against OVA (Fig. 2B). Our results suggest that Aps expressed in B cells is indispensable for optimal IgE production in vivo after immunization.

Aps$^{-/-}$ B cells have the ability to produce IgE normally in vitro

We hypothesized that Aps may regulate signals required for IgE production and examined whether *Aps*$^{-/-}$ B cells could produce normal levels of IgE in vitro. We purified naive B cells from WT or *Aps*$^{-/-}$ mouse spleens, and then stimulated these cells with LPS or anti-CD40 antibodies and IL-4. After 5 days of culture, we found that the supernatant from the *Aps*$^{-/-}$ B cell culture contained almost the same levels of IgE as that from the WT B cell culture (Fig. 3A). Other Ig isotypes were also detected at the same level in both cultures (data not shown). We also assessed another in vitro culture system for surface IgE^{+} B cells, the iGB cell culture, with CD40 ligand and BAFF-expressing fibroblasts as feeder cells[33]. Again, in the *Aps*$^{-/-}$ naive B cell culture, we detected almost comparable frequencies of surface IgE^{+} cells as in the culture with WT B cells after 4 and 7 days of culture (Fig. 3B). These data indicate that *Aps*$^{-/-}$ B cells can produce IgE normally in vitro, and signals required for IgE production in B cells were not perturbed in the absence of Aps.

Reduced numbers of GC B cells in *Aps*-deficient mice

Previous experiments showed discrepancies between in vivo and in vitro IgE production by *Aps*$^{-/-}$ B cells. We hypothesized that the in vivo environment, which is important for IgE production, was affected in *Aps*$^{-/-}$ mice, and that Aps in B cells plays a role in the interaction and/or maintenance of a conducive environment for IgE production. Recent reports have revealed that IgE^{+} B cells are present in the GCs in secondary lymphoid organs of immunized mice[10,11,34], and IL-4 secreted from Tfh cells is indispensable for IgE class switching at this location[35,36]. First, we purified GC and non-GC B cells and found that *Aps* expression was higher in GC B cells than in non-GC B cells (Fig. 4A). We focused on GC formation and/or maintenance and found that the ratio

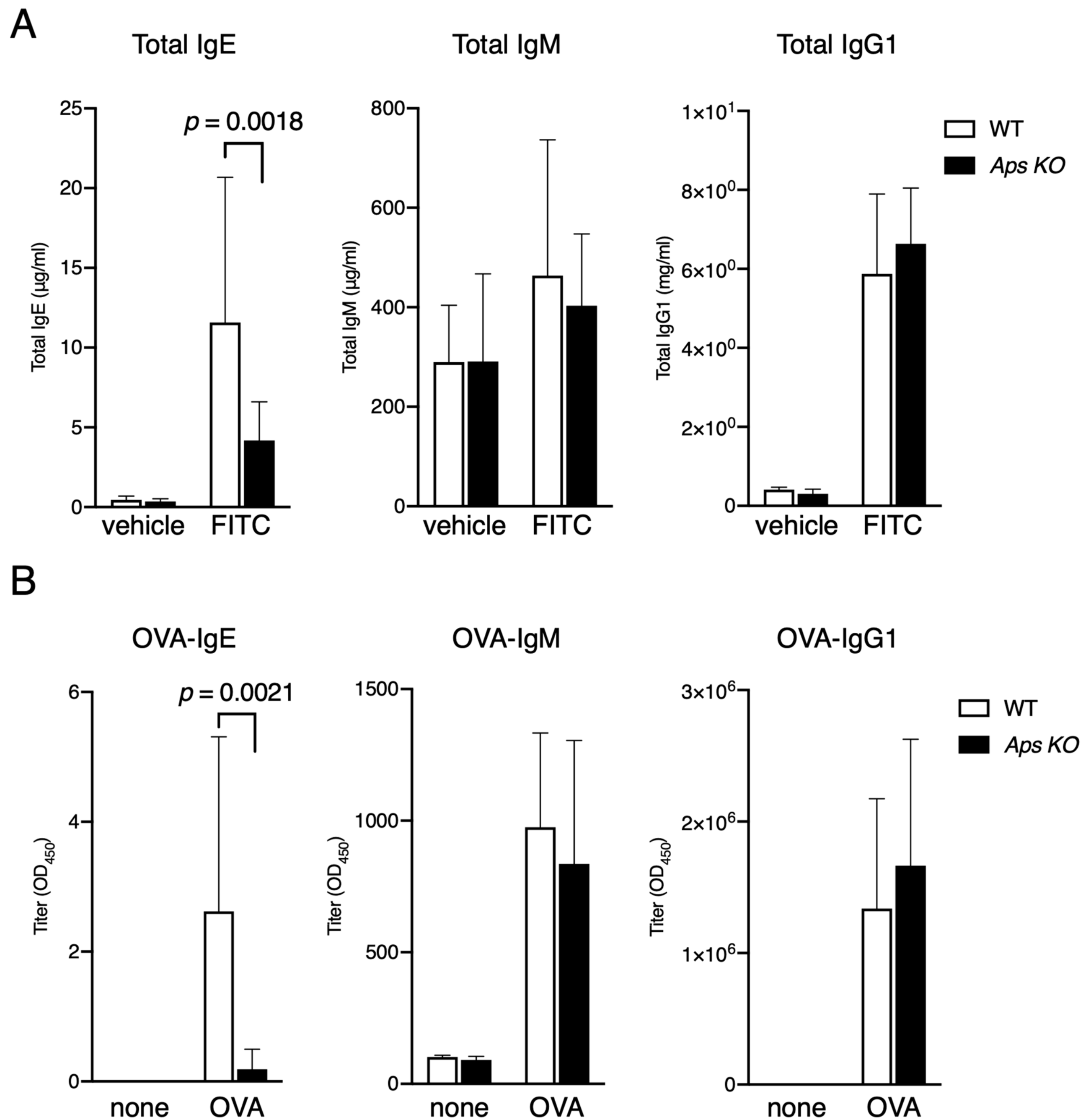

Figure 1. *Aps*-deficient mice show specific impairment of IgE production. (**A**) Total immunoglobulin concentration in serum from WT or *Aps*$^{-/-}$ mice immunized and challenged with FITC in a 1:1 solution of dibutylphthalate:acetone 24 h before drawing of blood samples. Each Ig concentration was determined by isotype-specific ELISA. (**B**) Ig titers of OVA-specific Igs in sera from OVA-immunized WT or *Aps*$^{-/-}$ mice. Blood samples were collected 14 days after challenge.

and number of GC B cells was significantly decreased in the spleens and LNs of immunized *Aps*$^{-/-}$ mice (Fig. 4B and data not shown). Next, we examined whether *Aps*$^{-/-}$ GC B cells entered the cell cycle by labeling cells with 5-bromo-2'-deoxyuridine (BrdU). Seven hours after *i.p.* injection of BrdU, spleen cells were analyzed by flowcytometry and cycling (BrdU-incorporated) cells were detected by anti-BrdU antibodies. No difference was found between WT and *Aps*$^{-/-}$ GC B cells (Fig. 4C). In addition, approximately 2% of non-GC B cells from both WT and *Aps*$^{-/-}$ spleens were found to annexin-V- and 7AAD-positive, indicating dead cells. In contrast, however, the dead cell rate within GC B cells from *Aps*$^{-/-}$ spleens was significantly higher than the GC B cells in spleens of WT mice (Fig. 4D). These results suggest that GC B cells decreased due to cell death, and that surviving GC B cells could enter the cell cycle and proliferate normally in *Aps*$^{-/-}$ mice.

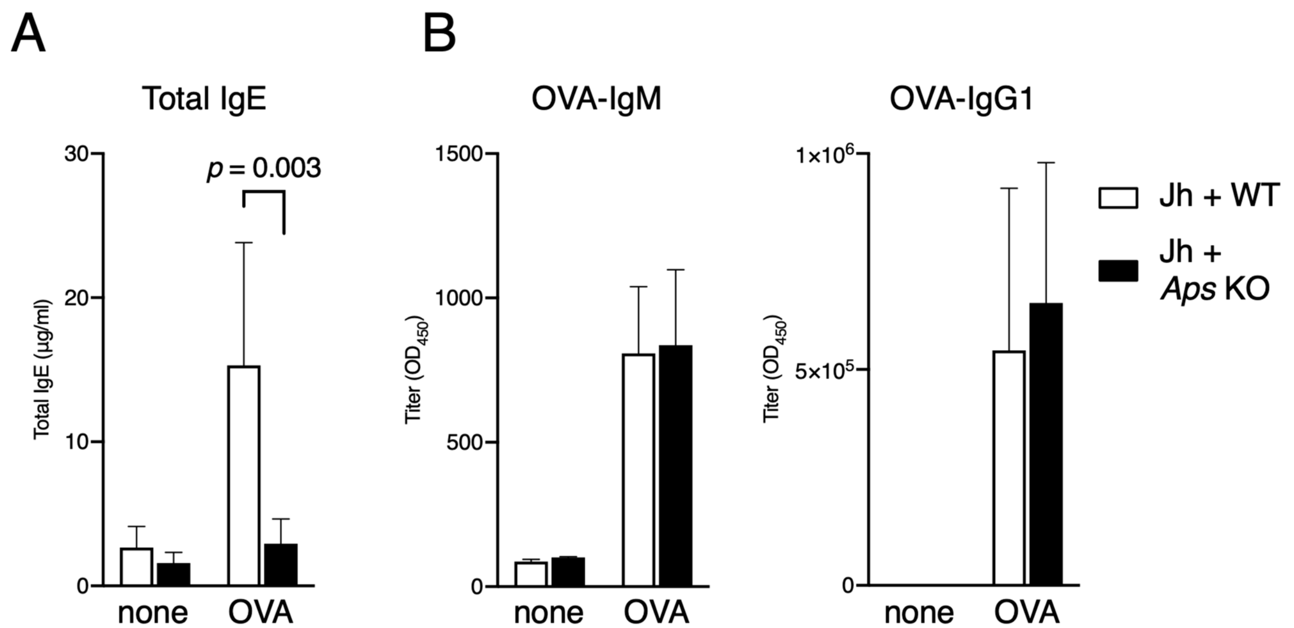

Figure 2. Aps expression in B cells is required for optimal IgE production. (**A**) IgE production from BM chimeric mice. B cell-specific *Aps*$^{-/-}$ mice were generated by transfer of WT or *Aps*$^{-/-}$ BM cells mixed with BM from Jh mice. After reconstitution, mice were immunized with OVA plus alum, and sera were taken 14 days after the OVA challenge. (**B**) Titers of OVA-specific IgG1 and IgM in sera from OVA-immunized BM chimera mice as described under (A). Ig levels were determined by ELISA.

The percentage of IgE-switched cells is not lower in *Aps*-deficient GCs

The results of the previous analysis prompted us to focus on IgE production in *Aps*$^{-/-}$ GC B cells. Recent reports have shown that IgE^{+} cells are present in GCs but are short-lived[10,11,34]. We attempted IgE staining of GC B cells to clarify whether *Aps*$^{-/-}$ GC B cells could produce IgE normally in vivo following a published protocol[10]. However, the proportion of these cells among total B cells was relatively low, as established by our staining, and there were no significant differences between WT and *Aps*$^{-/-}$ cells (Supplementary Fig. S2A). To obtain more reliable results, we purified GC B cells 7 days after immunization and aimed to examine post-switch transcripts from the switched ε locus (PSTε) and switched γ1 locus (PST γ1) to determine the percentage of cells in GC B cells that have completed the class switch recombination. PSTε and PST γ1 were detected at comparable levels from both WT and *Aps*$^{-/-}$ GC B cells (Supplementary Fig. S2B). The PSTε/PSTγ1 ratio revealed that GC B cells that class switched to IgE exhibited no significant differences between WT and *Aps*$^{-/-}$ (Fig. 4E). These results suggest that decreased survival in GC B cells contributed partially to lower serum IgE production in *Aps*$^{-/-}$ mice, but generation of IgE^{+} cells in GC was relatively maintained.

Tfh cell differentiation and function are normal in *Aps*-deficient mice

Impaired GCs in immunized *Aps*$^{-/-}$ mice prompted us to examine the differentiation and function of Tfh cells in *Aps*$^{-/-}$ GCs, because previous reports have shown that IL-4 from Tfh cells is indispensable for optimal IgE production after immunization[35,36]. We previously showed that the *Aps* gene is not expressed in T cells[21]; however, some reports have indicated that interaction between GC B cells and Tfh cells is essential not only for GC B cells but also for Tfh cell responses and GC reactions[37–41]. First, GC B cells were purified from OVA-immunized mice, and their antigen-presenting abilities were examined by in vitro co-culture with 5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE)-labeled CD4^{+} T cells from DO11.10 T cell receptor (TCR) transgenic/*Rag2*-deficient mice with or without OVA antigen (Supplementary Fig. S3A). *Aps*$^{-/-}$ GC B cells have an almost comparable antigen-presenting ability to WT GC B cells, and DO11.10 CD4^{+} T cells proliferate normally in co-culture with *Aps*$^{-/-}$ GC B cells and OVA antigen (Supplementary Fig. S3A). Next, to investigate their helper function, Tfh cells were sorted by FACS from spleen cells taken from OVA-immunized WT or *Aps*$^{-/-}$ mice and co-cultured with similarly FACS-sorted WT or *Aps*$^{-/-}$ GC B cells with or without OVA antigen (Supplementary Fig. S3B). After 1 week of culture, IgE concentrations were determined by ELISA, and Tfh cells from OVA-immunized *Aps*$^{-/-}$ mice showed equivalent helper abilities as WT Tfh cells (Supplementary Fig. S3B). Consistent with this result, *Aps*$^{-/-}$ Tfh cells showed almost normal *Bcl6*, *Il21*, and *Il4* gene expression levels (Supplementary Fig. S3C and S3D). Thus, Tfh cells function normally in *Aps*$^{-/-}$ GCs and there are intrinsic defects in *Aps*$^{-/-}$ GC B cells.

Augmented BCR-induced elimination of IgE^{+} B cells in the absence of Aps

A recent study reported that IgE-producing plasma cells (PCs) have high surface BCR expression, and their ligation induces apoptosis and elimination of IgE PCs[14]. We examined whether IgE-specific elimination augments *Aps*$^{-/-}$ IgE^{+} PCs and GC B cells. Employing an in vitro iGB culture system, WT and *Aps*$^{-/-}$ iGB cells were generated, stimulated with anti-CD79b for BCR ligation, and analyzed with flow cytometry after 18–20 h. WT iGB

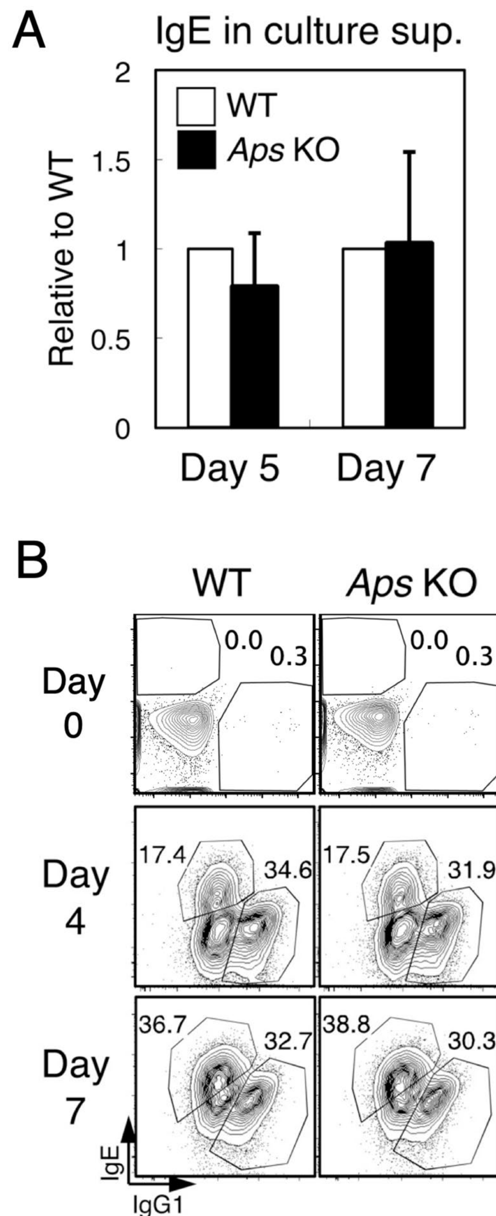

Figure 3. *Aps*$^{-/-}$ B cells can produce IgE in vitro. (**A**) Naive B cells were purified from WT or *Aps*$^{-/-}$ spleens by MACS and cultured with LPS and IL-4 stimulation for the indicated number of days. IgE concentrations were measured by ELISA. Multiple experiments were performed, and values relative to that of the WT cells were calculated in each set and summarized on the graph. (**B**) WT or *Aps*$^{-/-}$ naive B cells were cultured with CD40 ligand and BAFF-expressing feeder cells for the indicated number of days with IL-4. Cells were harvested and analyzed by flow cytometry. Numbers indicate percentages of the IgG1$^+$ or IgE$^+$ populations. The plotted data represent multiple experiments.

cells showed a reduction of the IgE$^+$ population in an anti-CD79b-dose dependent manner, which is consistent with the previous report[14] (Fig. 5A, C, white circles). The IgG1$^+$ population slightly decreased in the presence of 0.1—2.5 μg/ml of anti-CD79b; however, these levels were restored in the presence of a high degree of BCR ligation. In *Aps*$^{-/-}$ iGB cells, the IgE$^+$ population decreased more severely by BCR ligation than in WT cells (Fig. 5A, C, black circles). The IgG1$^+$ population also tended to decrease more than WT cells in the presence of 0.1–2.5 μg/ml of anti-CD79b, even though it restored comparably to WT cells in higher concentrations of anti-CD79b. The same experiment was conducted with the anti-κ antibody, resulting in a more pronounced reduction in *Aps*$^{-/-}$ IgE$^+$ iGB cells compared to WT cells, as well as with the anti-CD79b antibody (Supplementary Fig. S4). Following BCR cross-linking stimulation, the percentage of CD138$^+$ cells among IgE$^+$ cells exhibited a tendency to decrease compared to unstimulated cells in both WT and *Aps*$^{-/-}$ cells, indicating that enhanced reduction of *Aps*$^{-/-}$ IgE$^+$ cells after BCR ligation occurred in CD138$^+$ cells as well as in CD138$^-$ iGB cells (Fig. 5B). We calculated the ratio of IgE$^+$ versus IgG1$^+$ in *Aps*$^{-/-}$ cells and normalized the ratio to those of WT cells in the same condition

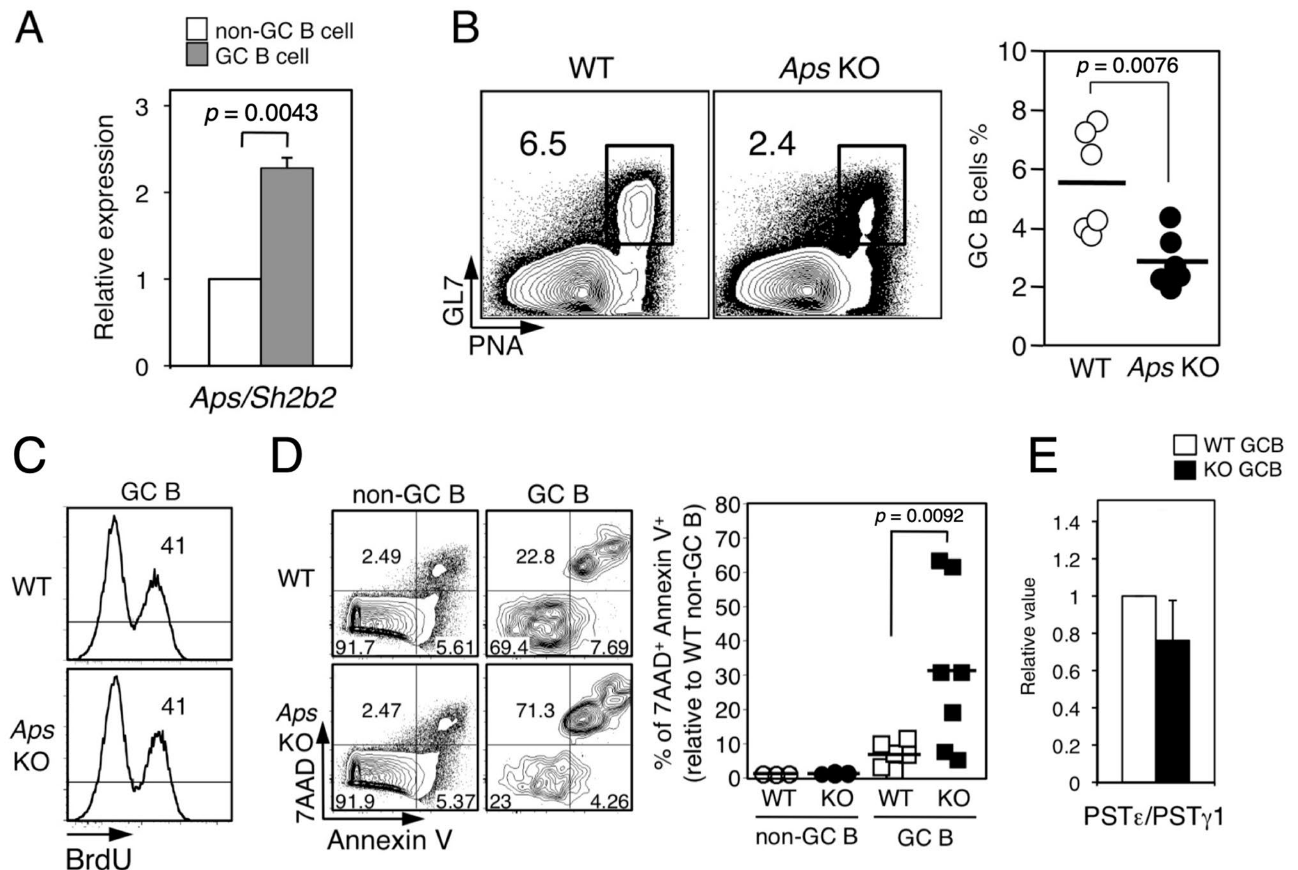

Figure 4. *Aps*$^{-/-}$ mice have lower proportions of GC B cells. (**A**) *Aps* gene expression in GC and non-GC B cells. GC and non-GC B cells were isolated from spleens of OVA-immunized WT mice by FACS sorting. Total RNA was purified, and *Aps/Sh2b2* gene expression was examined by real-time PCR. *Aps* gene expression was normalized by *Hprt* expression. (**B**) Skin-draining lymph node cells were analyzed 7 days after subcutaneous immunization with OVA plus alum. The FACS data shown represent multiple experiments, and summaries are displayed in the dot graph. (**C**) OVA-immunized WT or *Aps*$^{-/-}$ mice were intraperitoneally injected with BrdU 7 h before analysis. Surface antigens on splenocytes were stained, and cells were fixed, permeabilized, and stained with anti-BrdU antibodies. BrdU-incorporated cells were analyzed by FACS. (**D**) Splenocytes from OVA-immunized WT or *Aps*$^{-/-}$ mice were stained with 7AAD and annexin V. Cells were analyzed by flow cytometry. The left panel represents multiple experiments, and summaries of experiments are displayed in the right graph. (**E**) Post-switch transcripts (PST) detected by real-time PCR in non-GC or GC B cells isolated from spleens of WT or *Aps*$^{-/-}$ mice immunized with OVA and alum. The bar graph represents three independent experiments with similar results. The ratio of PSTε/PSTγ1 in WT or *Aps*$^{-/-}$ GC B cells.

and found that the reduction of *Aps*$^{-/-}$ IgE$^+$ iGB cells was statistically significant (Fig. 5D). It has been recently reported that calcium signaling is important for IgE$^+$ cell survival[42,43]. Additionally, Aps has been suggested to function downstream of the BCR[21,30,32,44]. Therefore, we examined intracellular Ca^{2+} levels after BCR stimulation using splenic B cells and iGB cells. Our findings revealed no difference in calcium elevation after BCR stimulation between WT and *Aps*$^{-/-}$ (Supplementary Fig. S5). These results suggest that *Aps*-deficient IgE$^+$ cells are more easily eliminated by BCR ligation than WT cells, which may be due to enhanced signaling other than calcium.

Discussion

We have studied *Aps/Sh2b2* and clarified that this adaptor protein functions in various signaling pathways, including BCR signaling[21,30,32,44]. In a previous report, we found that *Aps* regulates FcεRI-induced degranulation in mast cells, and this led us to hypothesize that *Aps* may control the effector phase in allergic diseases. In this study, we examined the in vivo roles of the adaptor protein Aps in hapten-induced CHS models with *Aps*$^{-/-}$ mice we have generated. We found that *Aps*-deficiency did not significantly affect inflammatory responses, such as CHS ear swelling after challenge with hapten FITC and DNFB. We revealed that serum IgE levels are lower in CHS-induced *Aps*$^{-/-}$ mice than in WT mice. Previous reports indicate that contact hypersensitivity induced by FITC is Th2-dependent, whereas contact hypersensitivity induced by DNFB is mediated by CD8$^+$ T cells[45,46]. Therefore, it is suggested that IgE production is decreased in *Aps*$^{-/-}$, regardless of the type of T cells activated. IgE production was impaired in not only CHS-induced, but also OVA-alum immunized *Aps*$^{-/-}$ mice.

Although the importance of Aps in B cells for IgE production was clearly demonstrated by our BM chimera experiments, *Aps*$^{-/-}$ naive B cells produced normal levels of IgE in two different culture systems with CD40/LPS

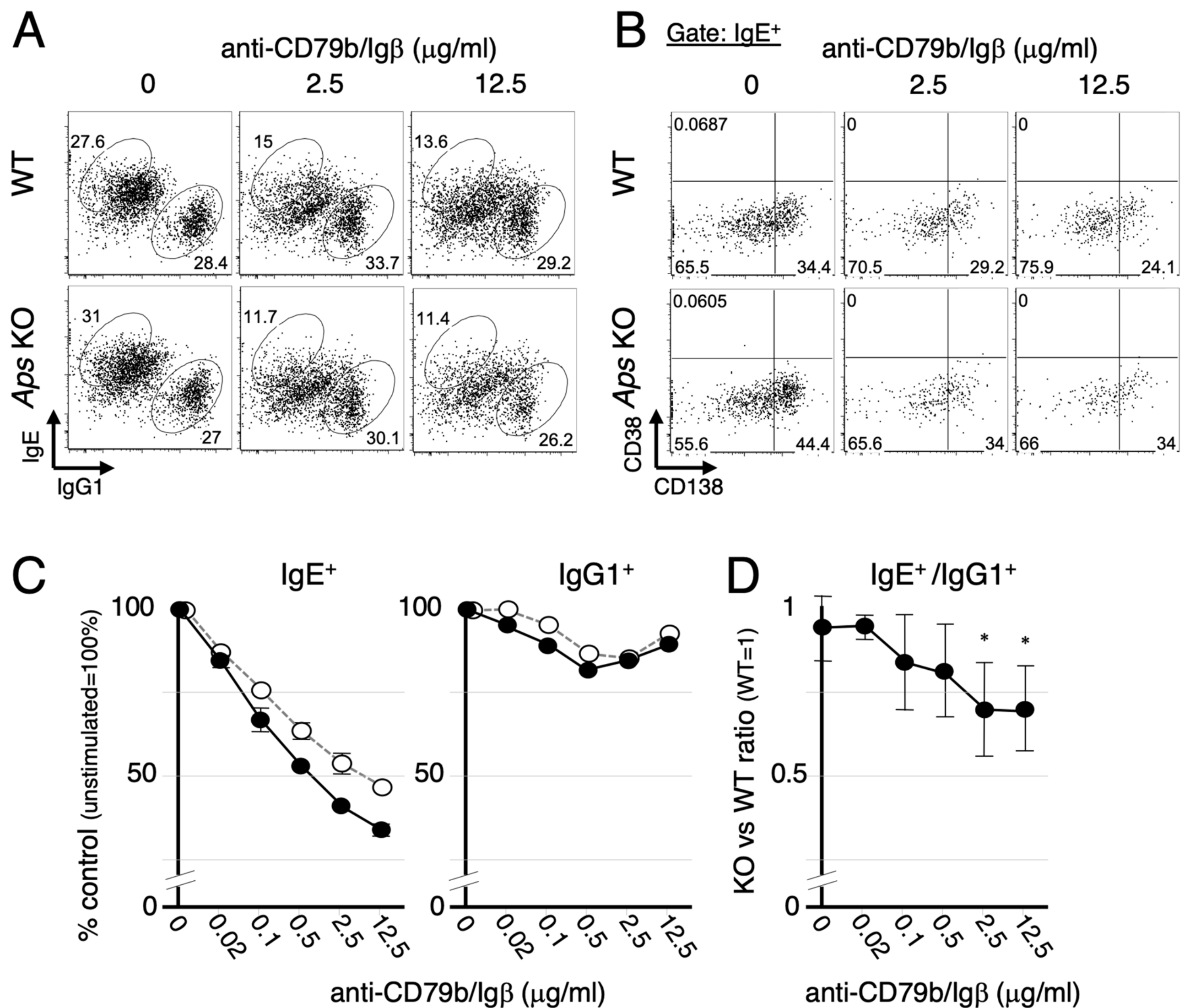

Figure 5. Augmented reduction of IgE⁺ iGB cells in response to BCR ligation in the absence of Aps. (**A**) iGB cells induced from WT or *Aps*$^{-/-}$ spleen B cells were stimulated with the indicated amount of anti-CD79b antibodies for 18 h and analyzed with FACS to determine the IgE⁺ and IgG1⁺ populations. Numbers represent the percentages of the IgG1⁺ or IgE⁺ populations. The plot data shown represent multiple experiments. (**B**) Expression of CD38 and CD138 on IgE⁺ iGB cells in the experiment shown in (**A**). Numbers represent the percentages of the population within each gate. The plot data shown represent multiple experiments. (**C**) Values of percent control of the IgE⁺ or IgG1⁺ population were calculated and plotted in each stimulation. WT: white circle, *Aps*$^{-/-}$: black circle. IgE⁺ or IgG1⁺ percentages in unstimulated samples were set as 100%. The data shown represents multiple experiments. (**D**) The ratio of IgE⁺/IgG1⁺ in each stimulation was calculated and compared by dividing the *Aps*$^{-/-}$ value by the value obtained for WT. Data from multiple experiments were analyzed and plotted. * $p < 0.05$.

and IL-4 stimulation. We have previously shown that the proliferation of *Aps*$^{-/-}$ B cells in response to CD40, LPS, and IL-4 stimulation is normal[30], which is consistent with the results of in vitro experiments in the present study. CD40/LPS and IL-4 are the minimal required signals to induce IgE production in B cells, and we speculate that various other signals in the in vivo environment could modulate IgE production. Although IgE⁺ cells are challenging to detect in vivo, recent reports using IgE reporter mice showed that IgE-switched B cells are present in GCs and can further differentiate into short-lived plasma cells[10,11,34]. Other reports have shown that IL-4 from Tfh cells is indispensable for IgE production[35,36]. We found that *Aps* expression increased in GC B cells, and that *Aps*$^{-/-}$ GC B cells showed decreased cell survival compared to WT GC B cells. Increased expression of Aps in GC B cells protects these cells from cell death, maintaining GCs at an optimal size and duration, which are all required for normal IgE production.

Aps-deficient iGB cells showed an augmented reduction of IgE⁺ cells by BCR ligation in a dose-dependent manner. IgG1⁺ iGB cells also showed a slight reduction in the presence of a low concentration of anti-CD79b stimulation, and the reduction was slightly enhanced in *Aps*$^{-/-}$ iGB cells, although the mild reduction in IgG1⁺ cells was masked by BCR-induced proliferation in response to a high degree of BCR ligation. Serum immunoglobulin

levels represent the cumulative production of the immune response over several weeks, including the GC reaction. The reduction of GC B cells was significant in *Aps*$^{-/-}$ mice compared to WT mice. Both IgE^{+} and IgG1^{+} GC B cells might be involved in the reduction; however, IgE^{+} GC B cells would be eliminated more by BCR ligation where Aps mediated the signals (see discussion below). While IgE levels were severely affected by the absence of Aps, IgG1 levels were relatively maintained even in the mice with reduced proportions of GC B cells. Reductions in GC B cells do not necessarily correlate with reduced IgG1 production. For example, mice deficient in Bam32, an adaptor molecule downstream of the BCR, show normal antigen-specific IgG1 production despite decreased levels of GC B cells and affinity maturation of IgG1 and IgG2a[47]. Cyclin D3-deficient mice also show a significant loss of GC B cells, but normal levels of antigen-specific IgG are produced in these mice[48]. Mutant mice that lack Tfh cell-specific IL-4 production show a lower IgG1 and IgE response after immunization, and IgE is more severely affected than IgG1[35,36]. Thus, IgE production might be more dependent on GCs than IgG1 production, and we speculate that only IgE was impaired in *Aps*$^{-/-}$ mice.

In GC, Tfh-GC B cell interactions are important for B cell activation and antibody production, and GC B cells also help the optimal activation of Tfh cells through their surface molecules[39,41]. Co-culture experiments using WT or *Aps*$^{-/-}$ GC B cells and CD4^{+} T cells from DO11.10 TCR transgenic/*Rag2* KO mice revealed that *Aps*$^{-/-}$ B cells have normally functioning antigen presentation. Furthermore, Tfh cells from immunized *Aps*$^{-/-}$ mice also induced IgE production activity in GC B cells, similarly to WT Tfh cells. Although GC B cells are reduced in *Aps*$^{-/-}$ mice, they are still capable of inducing Tfh proliferation, and there are enough Tfh cells in *Aps*$^{-/-}$ GCs to produce similar amounts of IgE as in WT mice, if enough GC B cells are present.

A recent study revealed that BCR-crosslinking induces apoptosis in IgE^{+} plasma cells due to higher surface BCR expression[14]. Interestingly, BCR-stimulated *Aps*$^{-/-}$ iGB cells showed an enhanced reduction only in the IgE^{+} population compared with WT iGB cells. IgE surface expression was comparable in WT and *Aps*$^{-/-}$ iGB cells. These results indicate that Aps may function downstream of IgE-BCR signaling. Both CD138^{+} and CD138^{-} cells among IgE^{+} iGB cells decreased after BCR cross-linking, indicating that IgE^{+} plasma cells are also susceptible to BCR stimulation-induced cell death as well as IgE^{+} GC B cells in the *Aps*$^{-/-}$ mice. The anti-CD79b-stimulated IgE^{+} population decreased in an antibody dose-dependent manner. On the other hand, the IgG1^{+} population was reduced at lower (0.1—0.5 μg/ml) antibody concentrations but retained at higher (12.5 μg/ml) concentrations. These results indicate that intracellular signals in IgE^{+} and IgG1^{+} cells are different, and *Aps*-deficiency might affect only IgE-BCR signaling. Comparing WT and *Aps*$^{-/-}$ mice in the reduction of the IgE^{+} population, a greater reduction of *Aps*$^{-/-}$ iGB cells is observed with higher concentrations of anti-CD79b stimulation, whereas in the IgG1^{+} population, *Aps*$^{-/-}$ tends to be reduced only with lower concentrations of anti-CD79b stimulation. It is likely that the IgG1^{+} population is also affected by *Aps*$^{-/-}$ at low concentrations of stimulation, but that cells may survive by canceling the effects of *Aps*$^{-/-}$ when stimulation from IgG1-BCR exceeds a certain threshold. This could be one of the mechanisms causing the reduction in *Aps*$^{-/-}$ GC B cells, where antigen-dependent selection is constantly occurring for weeks after immunization. Calculating the ratio of IgE^{+} to IgG1^{+} populations showed that only the IgE population was significantly reduced after high-concentration stimulus. The iGB experiments with anti-CD79b stimulation shown in this study resulted from overnight stimulation, and longer stimulation is expected to occur in vivo since GC formation and IgE production in mice takes about 7–10 days after antigen immunization. The stimuli that induce GC formation and IgE production on day 7–10 post-immunization are considered to be an accumulation of short-term stimuli used in in vitro experiments. Therefore, in addition to a more significant decrease in the IgE^{+} population, the IgG1^{+} population may also be affected by stimulation of the BCR, leading to a decrease in the *Aps*$^{-/-}$ whole GC population.

In this study, we showed that IgE production was specifically impaired and that GC B cells decreased in *Aps*$^{-/-}$ mice. The molecular mechanism of how Aps regulates IgE-BCR downstream signaling remains unclear and should be clarified in the future. In previous reports, we have shown that Aps can regulate the actin cytoskeleton and cell survival in peritoneal B-1 cells and degranulation from mast cells[30,44]. Another report has shown that Aps regulates BCR signaling through cytoskeletal remodeling in mature B cells[31]. The actin cytoskeleton is a crucial factor in the intracellular signaling of various surface receptors, including the BCR[49]. Aps might regulate IgE-BCR signaling by controlling the cytoskeleton in GC B cells and PCs. Although more detailed molecular mechanisms need to be clarified, these results suggest a novel regulatory system of IgE production and B cell survival. Because *Aps*$^{-/-}$ mice are healthy, with no severely impaired phenotypes except slight increases in the number of peritoneal B-1 cells and responses to TI-2 antigen[30], Aps would be a good therapeutic target to control serum IgE levels and allergic inflammation.

Methods
Mice
WT BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). *Aps/Sh2b2*-deficient mice were generated as described previously[30], and crossed with BALB/c mice at least ten times. Jh (B cell-deficient) mice and DO11.10 TCR transgenic/*Rag2*-deficient mice were purchased from Taconic Biosciences (Hudson, NY). Mice were maintained under specific pathogen-free conditions and used for experiments at 6–14 weeks of age. This study was approved by the Animal Care and Use Committee of the Research Institute, National Center for Global Health and Medicine (approval No. 2023-A039). All mice were handled in accordance with the Guidelines for Animal Experiments of the Research Institute, National Center for Global Health and Medicine and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Sevoflurane was used as an inhalation anesthetic to anesthetize mice.

Antibodies and reagents

The following antibodies were used: FITC- or allophycocyanin (APC)-Cy7-conjugated anti-B220/CD45R (clone RA3-6B2, BioLegend, San Diego, CA), Alexa Fluor 647-conjugated anti-GL7 (clone GL7, BD Biosciences, San Jose, CA), biotin-conjugated anti-CD95 (clone Jo2, BD Biosciences), fluorescein- or biotin-conjugated peanut agglutinin (PNA, Vector Laboratories, Burlingame, CA), FITC-conjugated anti-IgG1 (clone A85-1, BD Biosciences), phycoerythrin (PE)- or biotin-conjugated anti-IgE (clone RME-1, BioLegend), biotin-conjugated anti-MHC class II (clone M5/114.15.2, BioLegend), FITC- or biotin-conjugated anti-CD4 (clone GK1.5, eBioscience, San Diego, CA), PE-Cy7-conjugated anti-PD-1 (clone J43, eBioscience), PE-conjugated anti-CXCR5 (clone 2G8, BD Biosciences), PE-conjugated anti-CD38 (clone 90, BD Biosciences), biotin-conjugated anti-CD8α (clone 53–6.7, eBioscience), biotin-conjugated anti-CD11b (clone M1/70, BioLegend), biotin-conjugated anti-CD11c (clone N418, BioLegend), biotin-conjugated anti-Gr-1 (clone RB6-8C5, BioLegend), and biotin-conjugated anti-IgD (clone 11-26c.2a, BioLegend). Biotinylated cells were visualized by incubation with PE-, PE-Cy7, APC-, or APC-Cy7-conjugated streptavidin (BD Biosciences, eBioscience. or BioLegend). To detect dead cells, a FITC Annexin V Apoptosis Detection Kit II (BD Biosciences) was used according to the manufacturer's instructions, with one exception: instead of propidium iodide (PI), 7-amino-actinomycin D (7AAD) was used. To detect BrdU-incorporated cells, a BrdU Flow Kit (BD Biosciences) was used according to the manufacturer's instructions.

Immunization

Contact hypersensitivity was induced with the hapten FITC (Sigma-Aldrich, St. Louis, MO) or DNFB (Sigma-Aldrich) as described previously[50]. A blood sample was collected from each mouse 24 h after challenge by the hapten solution applied to both sides of the right ear. For antigen-specific antibody production, mice were immunized with 0.2 ml of 100 µg OVA (Sigma-Aldrich) in PBS mixed with Imject Alum (Pierce Biotechnology, Rockford, IL) by intraperitoneal injection on day 0. Serum was collected on day 14, when mice were challenged with 0.2 ml of 100 µg OVA in PBS. Serum was also collected on day 28.

Mixed bone marrow chimera mice

BM cells were isolated from femurs and tibias of WT, *Aps*$^{-/-}$, or Jh mice. WT or *Aps*$^{-/-}$ BM was mixed at a 1:9 ratio with Jh BM, and 2×10^6 mixed BM cells in PBS were intravenously injected into WT BALB/c mice that were lethally X-ray-irradiated (9.5 Gy). 8 weeks after transfer, reconstituted mice were immunized with OVA and alum as described above.

In vitro culture

Red blood cell-lysed splenocytes were incubated with 5 µg/ml of biotinylated anti-CD43 (clone S7, BD Biosciences) on ice, washed, and incubated with 20 µl of anti-biotin MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) per 10^7 total cells at 4 ℃ for 20 min. The CD43$^-$ fraction was isolated from naive B cells using MACS LD columns (Miltenyi Biotech) and cultured in a 24-well plate at 1×10^6 cells/ml with various stimulants. Induced GC B (iGB) cell culture was performed as described previously[33] with 40LB cells that were kindly provided by Dr. Kitamura (Tokyo University of Science). Naive CD4$^+$ T cells were purified from DO11.10 TCR transgenic/*Rag2*-deficient mouse spleens using a naive CD4$^+$ CD62L$^+$ Cell Isolation Kit (Miltenyi Biotech) and labeled with CFSE (Invitrogen, Waltham, MA). Labeled T cells were cultured with GC B cells isolated from WT or *Aps*$^{-/-}$ mice with or without OVA peptide for 3 days, after which a CFSE dilution was analyzed by flow cytometry. Generated iGB cells were partially purified by depletion of CD40L$^+$ 40LB cells using a magnetic sorter system, and stimulated with various concentrations of anti-CD79b (clone HM79)[51,52] without 40LB feeder cells for 20 h. Stimulated iGB cells were analyzed by flow cytometry for IgE$^+$ and IgG1$^+$ populations.

Flow cytometry

Red blood cell-lysed single-cell suspensions were prepared from spleens and LNs. After Fc-blocking with culture supernatant from a 2.4G2 hybridoma, cells were incubated in staining buffer (PBS containing 2% fetal calf serum) with an antibody cocktail for 20 min on ice in the dark. After washing twice with staining buffer, cells were suspended in staining buffer containing 1 µg/ml 7AAD, and data were acquired using a FACSCanto II (BD Bioscience) and analyzed with FlowJo software (BD Bioscience).

FACS sorting

For GC B cell sorting, pooled splenocytes from immunized mice were incubated with biotinylated anti-CD4, anti-CD8, anti-CD11b, anti-CD11c, anti-Gr-1, and anti-IgD on ice for 30 min, and then washed and incubated with BD IMag Streptavidin Particles Plus-DM (BD Biosciences) at 4 ℃ for 20 min. The negative fraction was collected with a BD IMagnet (BD Biosciences) and then stained with anti-B220, anti-CD38, and anti-GL7. The B220$^+$ CD38$^-$ GL7$^+$ fraction was sorted as GC B cells with a FACS Aria III. For Tfh sorting, anti-CD19, anti-B220, anti-CD8, anti-CD11b, anti-CD11c, and anti-Gr-1 were used to deplete other linage cells, and anti-CD4, anti-PD-1, and anti-CXCR5 were used to stain the remaining cells. CD4$^+$ PD-1$^+$ CXCR5$^+$ cells were sorted as Tfh cells.

ELISA

Flat-bottom 96-well plates (Maxisorp, Nunc, Rochester, NY) were coated with 5 µg/ml anti-mouse Igs (AbD Serotec, Oxford, UK) in antibody-coating buffer (pH 9.5) at 4°C overnight. Plates were washed using an ImmunoWash 1575 Microplate Washer (Bio-Rad, Hercules, CA) and blocked with 1% BSA PBS for 1 h at room temperature (RT). Mouse sera or culture supernatant was diluted with 1% BSA PBS and incubated in the wells of 96-well plates at RT for 2 h with shaking. After washing, plates were incubated with anti-isotype antibodies conjugated with

horseradish peroxidase (HRP) (AbD Serotec) at RT for 1 h with shaking. Finally, the plates were incubated with 3,3′,5,5′ -Tetramethylbenzidine (TMB) substrate (Nacalai Tesque, Kyoto, Japan) at RT for 15 min, and then stop solution was added. The absorbances at 450 nm and 570 nm were measured using an iMark Microplate Absorbance Reader (Bio-Rad), and the data were analyzed with Microplate Manager software (Bio-Rad). To determine total IgE, a Mouse IgE ELISA Max Kit (BioLegend) was used according to the manufacturer's instructions. For OVA-specific Igs, except IgE, plates were coated with OVA in the same antibody-coating buffer. To detect OVA-specific IgE, plates were coated with anti-IgE (clone RME-1, BioLegend), and specific antibody was detected by using biotin-labeled OVA generated with the EZ-Link Sulfo-NHS-Biotinylation Kit (Thermo Scientific/Pierce) and streptavidin-HRP (BioLegend) with TMB substrate.

Real-time PCR

RNA was isolated from purified cells using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the instructions from the manufacturer, and cDNA was synthesized using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Waltham, MA). Real-time PCR was performed with a Fast SYBR Green Master Mix (Applied Biosystems) and StepOnePlus Real-Time PCR System (Applied Biosystems). Data were analyzed with StepOne software (Applied Biosystems). The following primers were used: *Aps/Sh2b2*: forward 5′ -TGCTGA CCTTCAACTTCCAGG-3′, reverse 5′ -CGGAGCATATCAAACACGGAC; post-switch transcript from the switched ε locus (PSTε): forward 5′ -CTCTGGCCCTGCTTATTGTTG-3′, reverse 5′ -AGTTCACAGTGCTCA TGTTCAG-3′; post-switch transcript from the switched γ1 locus (PST γ1): forward 5′ -CTCTGGCCCTGCTTA TTGTTG-3′, reverse 5′ -GGATCCAGAGTTCCAGGTCACT-3′, and *Hprt*: forward 5′ -GTTGGATACAGGCCA GACTTTGTTG-3′, reverse 5′ -GATTCAACTTGCGCTCATCTTAGGC-3′.

Statistical analysis

All statistical analyses were performed using Prism 6 software (GraphPad Software, San Diego, CA, USA). Two groups were compared using Student's t-test. Multiple groups were compared using one-way ANOVA. *P* values of < 0.05 were considered statistically significant.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Received: 21 December 2023; Accepted: 26 July 2024
Published online: 01 August 2024

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Acknowledgements

We thank Dr. Daisuke Kitamura (Tokyo University of Science) for the protocol for iGB cell culture and for providing 40LB cells; N. Suzuki-Yamazaki, T. Mori, and other colleagues in the National Center for Global Health and Medicine for helpful discussions and advice; and N. Ikenoya, M. Takayashiki, Y. Suzuki, R. Hidaka, T. Norose, and M. Anraku for technical assistance.

Author contributions

M.I. and S.T. designed the study; M.I., S.H., F.K., and S.T. performed experiments; M.I. and S.T. analyzed the data; M.I. and S.T. wrote the manuscript; all authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-68739-3.

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