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Contribution of DOCK11 to the Expansion of Antigen-Specific Populations among Germinal Center B Cells

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ABSTRACT

Germinal centers (GCs) are a structure in which B cell populations are clonally expanded, depending on their affinities to Ag. Although we previously isolated a characteristic protein called dedicator of cytokinesis 11 (DOCK11) from GC B cells, limited information is available on the roles of DOCK11 in GC B cells. In this study, we demonstrate that DOCK11 may contribute to the expansion of Ag-specific populations among GC B cells upon immunization of mice. The lack of DOCK11 in B cells resulted in the lower frequency of Ag-specific GC B cells along with enhanced apoptosis upon immunization. Under competitive conditions, DOCK11-deficient B cells were dramatically prevented from participating in GCs, in contrast to DOCK11-sufficient B cells. However, minor impacts of the DOCK11 deficiency were identified on somatic hypermutations. Mechanistically, the DOCK11 deficiency resulted in the suppression of B cell–intrinsic signaling in vitro and in vivo. Although DOCK11 expression by B cells was required for the induction of T follicular helper cells at the early stages of immune responses, minor impacts were identified on the expansion of Ag-specific populations among GC B cells. Thus, DOCK11 appears to contribute to the expansion of Ag-specific populations among GC B cells through the stimulation of B cell–intrinsic signaling.


INTRODUCTION

Germinal centers (GCs) are a structure in which B cell populations are clonally expanded, depending on their affinities to Ag (1–4). Upon Ag binding, B cells are activated and proliferate to form GCs. Ig genes are somatically hypermutated, resulting in the generation of BCR with high affinities to Ag. High-affinity B cell clones are selectively expanded with help from T follicular helper (Tfh) cells (5–11). Thus, GCs are an important structure for the selection of high-affinity B cell clones.

We previously isolated a characteristic protein called dedicator of cytokinesis 11 (DOCK11, also known as Zizimin2) from GC B cells (12). As a guanine nucleotide exchange factor, DOCK11 activated the rho family GTPase cell division cycle 42 (CDC42), resulting in cytoskeletal reorganization (13, 14). Because the cytoskeleton plays an important role in humoral immune responses (15–18), DOCK11, similar to CDC42 (19–22), may play some roles in B cells.

Among DOCK-D family proteins (23–25), DOCK10 and DOCK11 are expressed by B cells (12, 26). We and other groups previously reported that the lack of DOCK10 caused mild to no defects in the development of B cells or humoral immune responses (26–29). Similarly, the lack of DOCK11 caused only mild, if any, defects in the development of B cells (28). Limited information is currently available on the roles of DOCK11 in humoral immune responses, including the clonal expansion of GC B cell populations.
In the current study, we examined the impact of the DOCK11 deficiency on the formation of GC B cells upon immunization. Because DOCK11 was found to contribute to the expansion of Ag-specific populations among GC B cells, the underlying mechanisms were examined in more detail.

**MATERIALS AND METHODS**

**Mice**

All mice were maintained on a C57BL/6 background under specific pathogen-free conditions. Dock11 knockout (KO) (28), Cdh9-Cre (30), IgG1 C region (Cyl)-Cre (31), Dock11 flox (28), Cdc42 flox (32), and B6-8f mice (33) were described previously. Ly5.1 mice were obtained from Sankyo Labo Service (Tsukuba, Japan). Dock11 flox mice were recovered from frozen embryos by the German Research Center for Environmental Health (Neuherberg, Germany) and backcrossed to C57BL/6 mice for more than 10 generations. Each genotype was elucidated by PCR. All mice were maintained on a C57BL/6 background under specific pathogen-free conditions.

**Immunization**

Primary immunization was performed by an i.p. injection of 50 μg of 4-hydroxy-3-nitrophenylacetyl (NP) coupled to Ficoll (NP33-CGG; LGC Biosearch Technologies, Teddington, Middlesex, U.K.), or 100 μg of alum-precipitated chicken γ globulin (CGG) (Calbiochem, currently Sigma-Aldrich, St. Louis, MO), or 100 μg of alum-precipitated NP33-CGG (LGC Biosearch Technologies). Secondary immunization was performed by an i.p. injection of 50 μg of NP33-CGG.

**MACS**

A single-cell suspension was prepared by passing tissues through a 100-μm nylon cell strainer. RBC lysis was performed in lysis buffer (150 mM NH4Cl, 14 mM NaHCO3, and 2 mM EDTA). To isolate B cells, cells were incubated with anti-CD43 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). More than 90% purity was achieved, as measured by flow cytometry. To enrich GC B cells, cells were incubated with anti-CD44 biotin (clone no. GLK1.5, 0.63 μg/ml; BioLegend, San Diego, CA), anti-CD8a biotin (clone no. 53-6.7, 1:800; Tonbo Biosciences, San Diego, CA), anti–TER-119 biotin (clone no. TER-119, 0.63 μg/ml; BioLegend), anti-CD38 biotin (clone no. 90, 1.25 μg/ml; BD Biosciences, San Jose, CA), and, in some cases, anti–IgG L chain κ (Igk) biotin (clone no. RMK-12, 1.25 μg/ml; BioLegend) and anti-CD45.1 (Ly5.1) biotin (clone no. A20, 1.25 μg/ml; BioLegend), followed by an incubation with antibiotin microbeads (Miltenyi Biotec).

**Flow cytometry**

The following Abs and reagents were used for flow cytometry: anti-BCL6 BV421 (clone no. K112-91, 1:400; BD Biosciences), anti-CD4 PE-Cy7 (clone no. GLK1.5, 0.25 μg/ml; BioLegend), anti-CD9 allophycocyanin-Cy7 (clone no. 6D5, 0.25 μg/ml; BioLegend), anti-

**Quantitative RT-PCR**

RNA was extracted with a TRI reagent (Molecular Research Center, Cincinnati, OH), followed by reverse transcription to cDNA with ReverTra Ace transcriptase (Toyobo, Osaka, Japan). Quantitative PCR was performed using a THUNDERBIRD SYBR mix (Toyobo). The primers used were as follows: 5′-GCTTGACAG CATGGCCAAAA-3′ and 5′-AACGTCGAGGACCCACTAGG-3′ for Dock11 (34); 5′-TAGTGCCACCTCGCTGACT-3′ and 5′-CAAATAAACGCCAAGC-3′ for Aicda (35); and 5′-AGTCCTGGCCCTTTGTACACA-3′ and 5′-GATCCGGAGGGCCCTACTA AAC-3′ for 18S ribosomal RNA (34). All samples were run in duplicate on a PikoReal real-time PCR system (Thermo Fisher Scientific). The ΔΔ cycle threshold method was applied for the relative quantification of RNA expression levels.

**Preparation of NP-BSA**

For ELISA of anti-NP Abs, varied concentrations of 4-hydroxy-3-nitrophenylacetic acid succinimide ester (LGC Biosearch Technologies) were conjugated to BSA, according to the manufacturer’s instructions. The resultant NP-BSA conjugates were purified...
through a PD-10 desalting column (Cytiva, Marlborough, MA). The conjugation ratio was determined by the absorbance value at 430 nm.

**ELISA**
Serum was collected from mice under anesthesia with isoflurane. For sandwich ELISA, 96-well plates were coated with 2 μg/ml NP20-BSA or NP4-BSA. Serially diluted samples were loaded, followed by incubations with 1 μg/ml anti-IgG1 biotin (clone no. A85-1; BD Biosciences) and streptavidin HRP (1:3000; Cytiva). Color development was performed with tetramethylbenzidine and stopped by acidification. Absorbance at 450 nm was measured on a 680 microplate reader (Bio-Rad Laboratories, Hercules, CA). Dose-response curves were analyzed, using R software (R Foundation for Statistical Computing, Vienna, Austria) (36). Ab concentrations were determined, based on the EC_{50} values of dilution factors. NIG9 anti-NP IgG1 (37) was used as a standard.

**Adoptive transfer**
B1-8 IgH-bearing B cells were isolated by MACS using spleens pooled from three or more mice. The numbers of NP-binding cells were enumerated by flow cytometry. Adoptive transfer was performed by an i.v. injection of B cells, including 1 × 10^5 of NP-specific cells per recipient.

**BCR sequencing**
BCR sequencing was performed, as previously described (38), with some modifications. Briefly, NP-specific IgG1+ GC B cells were single-cell sorted into 10 μl of RNase-free water containing 50 ng of carrier RNA (Thermo Fisher Scientific). Gene segments of IgH V region 186.2 (V_{H}186.2) linked to Cγ1 were amplified, using a SuperScript IV one-step RT-PCR system (Thermo Fisher Scientific). Primers for nested PCR were as follows: 5'-TTCTTGGCAGCAA CAGCTACA-3' (V_{H}186.2 sense), 5'-GGATCCAGAGTTCCAGGT CACT-3' (Cγ1 external antisense), and 5'-GGAGTTAGTTTGGG CAGCAG-3' (Cγ1 internal antisense).

**Cell stimulation**
To stimulate B cells, DMEM was supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 10 mM HEPES, 1% nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 52 μM 2-ME. B1-8 IgH-bearing B cells were incubated at 37°C with or without 0.2 μg/ml NP20-Ficoll. The stimulation was stopped by directly adding twice the volume of ice-cold fixation/permeabilization working solution in a Foxp3/ transcription factor staining buffer set.

**Statistical analysis**
Statistical analyses were performed using R software. A p value <0.05 was considered to be significant.

**RESULTS**

**Downregulation of DOCK11 in GC B cells**
To identify the roles of DOCK11 in GC B cells, we compared the expression levels of DOCK11 in GC B cells with those in naive follicular B cells. Naive follicular B cells were isolated from spleens of naive mice (Supplemental Fig. 1A). B1-8 IgH forms an NP-specific BCR when combined with an Ig L chain λ (39). To obtain Ag-specific GC B cells, B1-8 IgH-carrying mice (33) were i.p. immunized with alum-precipitated NP-CGG. NP-specific IgG1+ GC B cells were then isolated from spleens (Supplemental Fig. 1B). The isolation of GC B cells was confirmed by the upregulation of activation-induced cytidine deaminase (40) (Fig. 1A). In contrast, the expression levels of DOCK11 in NP-specific IgG1+ GC B cells were decreased to 27% of those in naive follicular B cells (Fig. 1B).

**Impact of the DOCK11 deficiency on the frequency of Ag-specific GC B cells**
Although DOCK11 was originally isolated from GC B cells (12), the expression levels of DOCK11 were rather downregulated in GC B cells as compared with those in naive follicular B cells. Thus, DOCK11 seemed to be dispensable in GC B cells. To examine whether the remaining expression of DOCK11 is still required in GC B cells, we generated mice lacking DOCK11 in B cells. Dock11 flox mice (28) were crossed with Cd19-Cre mice (30). After

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**FIGURE 1. Downregulation of DOCK11 in GC B cells.**
The expression levels of activation-induced cytidine deaminase (A) and DOCK11 (B) by the indicated splenic B cells were measured by quantitative RT-PCR and normalized to those of 18S ribosomal RNA. GC B cells were isolated from the spleens of B1-8 IgH-carrying mice 14 d postimmunization with alum-precipitated NP-CGG. Gating strategies for naive follicular B cells (B220⁺CD19⁺CD23⁻CD21⁻) and NP-specific IgG1⁺ GC B cells (B220⁺CD19⁺CD95⁺CD38⁺NP⁺IgG1⁺Igc⁺) are shown in Supplemental Fig. 1A, 1B, respectively. Each symbol represents an individual mouse. Horizontal lines represent geometric means. Data are pooled from two independent experiments, using six or more mice per experimental group. ***p < 0.001, **p < 0.01 (Welch t test).

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immunization with NP-CGG, the numbers of NP-specific IgG1+ GC B cells were enumerated by flow cytometry (Fig. 2A). NP-specific IgG1+ GC B cells were similarly formed to the levels of those in Cd19-Cre mice (Fig. 2B). In contrast, the frequencies of NP-specific cells among IgG1+ GC B cells were decreased in DOCK11-deficient mice (Fig. 2C). Thus, the DOCK11 deficiency appeared to affect the frequency of Ag-specific populations among GC B cells.

The frequencies of Ag-specific GC B cells were further examined using mice lacking other DOCK11-related proteins, including another DOCK-D family protein DOCK10 and the DOCK11 substrate CDC42 (12, 14). Dock10 and Cdc42 flox mice (32) were crossed with Cd19-Cre mice. After immunization with NP-CGG, NP-specific IgG1+ GC B cells were similarly formed in these mice (Supplemental Fig. 2A). The frequencies of NP-specific cells among IgG1+ GC B cells were decreased in CDC42-deficient mice but not in DOCK10-deficient mice (Supplemental Fig. 2B).

FIGURE 2. Impact of the DOCK11 deficiency on the specificity of GC B cells.
(A) Gating strategies for NP-specific IgG1+ GC B cells. The left contour plots are gated on B220+ cells. Numbers show the percentages of cells in each gate. (B) Numbers of NP-specific and whole IgG1+ GC B cells (B220+CD19+CD95+CD38–IgG1+) in the spleens of the indicated strains 14 d postimmunization with NP-CGG. Each symbol represents an individual mouse. Horizontal lines represent geometric means. Data are pooled from four independent experiments, using eight or more mice per experimental group. (C) Frequencies of NP-specific cells among IgG1+ GC B cells in (B). (D) Gating strategies for apoptotic cells among NP-specific IgG1+ GC B cells. The contour plots are gated on B220+CD19+CD95+CD38–NP+IgG1+ cells. Numbers show the percentages of cells in each gate. (E) Frequencies of apoptotic cells among NP-specific IgG1+ GC B cells in (D). Each symbol represents an individual mouse. Horizontal lines represent means. Data are pooled from two independent experiments, using nine or more mice per experimental group. *p < 0.05, **p < 0.01, ***p < 0.001 (Welch t test). cKO, conditional KO.

FIGURE 3. Impact of the DOCK11 deficiency after the B cell activation stage.
(A) Numbers of NP-specific and whole IgG1+ GC B cells (B220+CD19+CD95+CD38–IgG1+) in the spleens of the indicated strains 14 d postimmunization with NP-CGG. Each symbol represents an individual mouse. Horizontal lines represent geometric means. Data are pooled from four independent experiments, using eight or more mice per experimental group. (B) Frequencies of NP-specific cells among IgG1+ GC B cells in (A). (C) Levels of NP-specific IgG1 in serum of the indicated strains 14 d postimmunization with NP-CGG, as measured by ELISA. Each symbol represents an individual mouse. Horizontal lines represent means. Data are pooled from two independent experiments, using nine or more mice per experimental group. **p < 0.01, *p < 0.05 (Welch t test). cKO, conditional KO.

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Collectively, the DOCK11-CDC42 axis may contribute to the frequency of Ag-specific GC B cells.

We then examined the impact of the DOCK11 deficiency on the apoptosis of Ag-specific GC B cells. Apoptotic cells were identified by binding of a fluorophore-conjugated VAD-FMK inhibitor to activated caspases (Fig. 2D). After immunization of DOCK11-deficient mice with NP-CGG, NP-specific IgG1+ GC B cells contained larger numbers of apoptotic cells than those from Cd19-Cre mice (Fig. 2E). Thus, DOCK11 may be required for the survival of Ag-specific GC B cells.

Impact of the DOCK11 deficiency after the B cell activation stage

We then investigated the stage at which the DOCK11 deficiency may affect the frequency of Ag-specific GC B cells. Cy1-Cre mice are a strain in which the expression of Cre recombinase is induced by transcription of the Cy1 (31). Reflecting the finding that Ig class-switching starts as early as the B cell activation stage (41), Cre-mediated recombination occurs within a few days after immunization (31). Therefore, by crossing Dock11lox mice with Cy1-Cre mice instead of Cd19-Cre mice, one would be able to examine the impact of the DOCK11 deficiency in GC B cells while DOCK11 expression is maintained in naive B cells. After immunization of the resultant mice with NP-CGG, NP-specific IgG1+ GC B cells were similarly formed to the levels of those in Cy1-Cre mice (Fig. 3A). In contrast, the frequencies of NP-specific cells among IgG1+ GC B cells were decreased in mice lacking DOCK11 by Cy1-Cre recombinase (Fig. 3B). These results indicate that the lack of DOCK11 after the B cell activation stage still affects the frequency of Ag-specific populations among GC B cells.

We next examined whether the serum Abs were reflected by the frequency of Ag-specific GC B cells. After immunization with NP-CGG, serum levels of both high- and low-affinity Abs were decreased in mice lacking DOCK11 by Cy1-Cre recombinase (Fig. 3C). Additionally, the ratios between the levels of high- and low-affinity Abs were also decreased, suggesting that DOCK11 expression after the B cell activation stage may contribute to the affinity maturation of Abs.

Impact of the DOCK11 deficiency on the expansion of Ag-specific populations among GC B cells

The specificities of GC B cells are assessed through competition among B cell clones (3). Because DOCK11 appeared to contribute to independent experiments, using five mice per experimental group. (F) Frequencies of B cell clones carrying the indicated numbers of nucleotide mutations on the Vλ185.2 region among donor-derived NP-specific IgG1+ GC B cell clones in (E). Numbers in the centers represent the numbers of clones sequenced. Means and SEM of the nucleotide mutations are shown below the pie chart. p = 0.070 (Welch t test). (G) Frequencies of high-affinity clones carrying a W33L mutation among donor-derived NP-specific IgG1+ GC B cell clones in (E). Numbers in the centers represent the numbers of clones sequenced. p = 0.38 (Fisher exact test). ***p < 0.001 (Welch t test). WT, wild-type.

FIGURE 4. Impact of the DOCK11 deficiency on the expansion of Ag-specific populations among GC B cells.

(A) Experimental outline to examine the impact of the DOCK11 deficiency on the selection of GC B cells. (B) Contour plots showing donor-derived NP-specific cells (B220+CD38+CD95+NP-Ly5.1+) among naive B cells from mice given transfers with B1-8 IgH-bearing B cells of the indicated Dock11 genotypes. Contour plots are gated on B220+CD38+CD95+ cells. Detailed gating strategies are shown in Supplemental Fig. 3B. Numbers show the percentages of cells in each gate. (C) Frequencies of donor-derived NP-specific cells among naive B cells in (B). Each symbol represents an individual mouse. Horizontal lines represent means. Data are from four independent experiments, using five or more mice per experimental group. (D) Contour plots showing donor-derived IgG1+ GC B cells (B220+CD38+CD95+NP-IgG1+Ly5.2+Ly5.1+) from mice given the transfer with the B1-8 IgH-bearing B cells of the indicated Dock11 genotypes, followed by an immunization with NP-CGG. Contour plots are gated on B220+CD38+CD95+NP-IgG1+ cells. Detailed gating strategies are shown in Supplemental Fig. 3C. Numbers show the percentages of cells in each gate. (E) Frequencies of donor-derived cells among NP-specific IgG1+ GC B cells in (D). Each symbol represents an individual mouse. Horizontal lines represent means. Data are from two
the frequency of Ag-specific GC B cells, we examined the impact of the DOCK11 deficiency on the competition among B cell clones. To generate DOCK11-deficient B cells with specificity to NP, Dock11 KO mice (28) were crossed with B1-8 IgH-carrying mice. As a source of a DOCK11-sufficient control, B1-8 IgH-carrying mice were used. The numbers of NP-specific B cells were enumerated by flow cytometry (Supplemental Fig. 3A). B cells from these mice, including $1 \times 10^5$ of NP-specific cells, were transferred into congenic wild-type mice (Fig. 4A). The resultant chimeras would contain donor-derived NP-specific B cells with or without DOCK11 expression and endogenous DOCK11-sufficient B cells. NP-specific B cells accounted for 0.06% of splenocytes, irrespective of DOCK11 expression on the transferred cells (Fig. 4B, 4C, Supplemental Fig. 3B). After immunization with NP-CGG, DOCK11-deficient donor cells accounted for 89% of NP-specific IgG1+ GC B cells (Fig. 4D, Supplemental Fig. 3C). In contrast, DOCK11-deficient counterparts accounted for only 15% of NP-specific IgG1+ GC B cells (Fig. 4E). Thus, DOCK11-deficient clones were outcompeted by endogenous DOCK11-sufficient clones in a B cell–intrinsic manner.

Ig genes are somatically hypermutated in GCs, contributing to the generation of high-affinity B cell clones. To examine the impact of the DOCK11 deficiency on somatic hypermutations, the donor-derived NP-specific IgG1+ GC B cells described above were single-cell sorted, followed by sequencing of the V_{H}186.2 region, a region corresponding to specificity to NP (42, 43). Although statistical significance was not identified ($p = 0.070$ in Welch $t$ test), smaller numbers of mutations were accumulated among DOCK11-deficient GC B cells (Fig. 4F). Similarly, smaller numbers of high-affinity clones with a W33L mutation (42) were identified among DOCK11-deficient GC B cells, as compared with those among DOCK11-sufficient counterparts (Fig. 4G). However, no statistical significance was identified ($p = 0.38$ in Fisher exact test). Thus, the impact of the DOCK11 deficiency on somatic hypermutations, if any, may be minor.

**Impact of the DOCK11 deficiency on B cell–intrinsic signaling**

DOCK11 was found to contribute to the expansion of Ag-specific populations among GC B cells in a cell-intrinsic manner. To examine the impact of the DOCK11 deficiency on B cell–intrinsic signaling (44), splenic B cells were isolated from B1-8 IgH-carrying naïve mice or DOCK11-deficient counterparts, followed by stimulation with T cell–independent type II Ag NP-Ficoll. In DOCK11-sufficient B cells, the stimulation induced the phosphorylation of

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**FIGURE 5. Impact of the DOCK11 deficiency on B cell–intrinsic signaling.**

(A) Histograms of B1-8 IgH-bearing B cells (wild type [WT]) or DOCK11-deficient counterparts (KO) expressing the indicated phosphorylated (p) kinases after the stimulation with or without NP-Ficoll for 5 min. Histograms are gated on B220+Igκ– cells. (B) Mean fluorescence intensity (MFI) for the indicated phosphorylated kinases after the stimulation with NP-Ficoll in (A). Each symbol represents an individual mouse from which B cells were isolated. Horizontal lines represent means. Data are pooled from two independent experiments, using six or more mice per experimental group. (C) Gating strategies for NP-specific B cells with GC phenotypes. The left contour plot is gated on CD19+ cells. Numbers show the percentages of cells in each gate. (D and E) Numbers of NP-specific GC-like B cells (D) and frequencies of GC-like cells among NP-specific B cells (E) in spleens from B1-8 IgH-carrying mice (WT) or DOCK11-deficient counterparts (KO) 7 d postimmunization with NP-Ficoll. Each symbol represents an individual mouse. Horizontal lines represent geometric means (D) and means (E). Data are pooled from two independent experiments, using six to seven mice per experimental group. **$p < 0.01$, *$p < 0.05$ (Welch $t$ test).
kinases, including SYK and BTK (Fig. 5A, 5B). In contrast, the phosphorylation of these kinases was suppressed in DOCK11-deficient B cells. Thus, the DOCK11 deficiency appeared to suppress B cell–intrinsic signaling in vitro.

The impact of the DOCK11 deficiency was then examined in vivo. B1-8 IgH-carrying mice or DOCK11-deficient counterparts were i.p. immunized with NP-Ficoll. As previously reported (45), NP-specific B cells with GC phenotypes were induced in the spleens of DOCK11-sufficient mice (Fig. 5C–E). However, the numbers and frequencies of these GC-like B cells were decreased in DOCK11-deficient mice. Collectively, DOCK11 may contribute to B cell–intrinsic signaling in vitro and in vivo.

**Contribution of DOCK11 expression by B cells to the induction of Tfh cells**

B cell–intrinsic signaling appeared to be responsible for the impaired expansion of Ag-specific populations among GC B cells in DOCK11-deficient mice. However, the selection of GC B cells is generally dependent on help from Tfh cells (46–48). Therefore, we examined the impact of the DOCK11 deficiency on the induction of Tfh cells. B cell–specific DOCK11-deficient mice and Cd19-Cre mice as a control were immunized with NP-CGG. The numbers of Tfh cells were enumerated in the spleens of these mice (Fig. 6A). The numbers (Fig. 6B) and frequencies of Tfh cells (Fig. 6C) were both lower in DOCK11-deficient mice than in Cd19-Cre mice. In contrast, similar numbers and frequencies of CD4+ effector T cells were identified in these mice (Supplemental Fig. 4). These results suggest that DOCK11 expression by B cells is required for the differentiation of Tfh cells among Th cells.

To further examine at which stage the induction of Tfh cells was influenced, mice lacking DOCK11 expression by Cyl1-Cre recombinase were used. As mentioned above, DOCK11 expression would start to be deleted at the B cell activation stage (31). Cyl1-Cre mice were used as a control. After immunization with NP-CGG, Tfh cells were similarly formed in these mice (Fig. 6D, 6E). Given the timing of the induction of Cyl1-Cre recombinase (31), these results indicate that DOCK11 expression by B cells is required for the induction of Tfh cells at the early stages of immune responses.

**Impact of decreased Tfh cells on the expansion of Ag-specific GC B cells**

Because the induction of Tfh cells was suppressed in DOCK11-deficient mice, we examined the competition of GC B cell clones in these mice. B cell–specific DOCK11-deficient mice and Cd19-Cre mice as a control were immunized with alum-precipitated CGG, followed by the transfer of naive B cells from B1-8 IgH-carrying congenic mice (Fig. 7A). Before secondary immunization, 3–4% of NP-specific cells were identified among the transferred cells (Fig. 7B, 7C). Because GCs are an open structure (49, 50), Ag-specific naive B cells potently participate in ongoing GC reactions (51). After the secondary immunization of Cd19-Cre recipients with NP-CGG, NP-specific cells accounted for 87% of donor-derived IgG1+ GC B cells (Fig. 7D, 7E). Although the frequencies of NP-specific cells were significantly decreased to 79% in DOCK11-deficient recipients, the difference may be minor. Thus, although the induction of Tfh cells was suppressed in DOCK11-deficient mice (Fig. 6B, 6C), the impact on the competition of GC B cell clones, if any, appeared to be minor.

High-affinity B cell clones are selectively expanded with help from limited numbers of Tfh cells (46–48). Because the formation of Tfh cells was suppressed in DOCK11-deficient mice, the expansion of B cell clones was examined in these mice. The donor-derived NP-specific IgG1+ GC B cells described above were single-cell sorted, followed by sequencing of the V_{H}186.2 region. Although slightly smaller numbers of mutations accumulated in donor-derived GC clones, if any, appeared to be minor.
B cells from DOCK11-deficient recipients (Fig. 7F), similar changes did not always occur in the frequency of high-affinity clones with the W33L mutation (42) (Fig. 7G). Thus, although the induction of Tfh cells was suppressed in DOCK11-deficient mice, the maturation of GC B cells was not affected as much as originally expected.

**DISCUSSION**

In this study, we examined the contribution of DOCK11 to the expansion of Ag-specific populations among GC B cells. Immunization of conditional KO strains revealed that DOCK11 appeared to be required for the survival of Ag-specific GC B cells. Through adoptive transfer experiments, DOCK11 was found to contribute to the expansion of Ag-specific populations among GC B cells in a cell-intrinsic manner. The DOCK11 deficiency resulted in the suppression of B cell–intrinsic signaling in vitro and in vivo. Although DOCK11 expression by B cells was required for the induction of Tfh cells at the early stages of immune responses, minor impacts were identified on the expansion of Ag-specific populations among GC B cells. Thus, DOCK11 may contribute to the expansion of Ag-specific populations among GC B cells through the stimulation of B cell–intrinsic signaling.
Although DOCK11 was originally isolated from GC B cells (12), DOCK11 expression was rather downregulated in GC B cells as compared with that in naïve follicular B cells. In our previous finding, the expression levels of DOCK11 in GC B cells were higher than those in non-GC B cells from immunized mice (12). Under this condition, non-GC B cells are comprised of varied types of cells, including activated B cells and Ag-experienced memory B cells. Collectively, immunization appeared to induce the downregulation of DOCK11 in B cells, but the relative expression levels were higher in GC B cells than in non-GC B cells.

The DOCK11 deficiency in B cells resulted in the impaired expansion of Ag-specific populations among GC B cells. Because the selection of GC B cells is generally dependent on help from Tfh cells (46–48), we focused on the induction of Tfh cells as well as B cell–intrinsic signaling. After immunization, the induction of Tfh cells was suppressed in mice lacking DOCK11 in B cells. However, unlike Ag-specific GC B cells, no influence was identified on the induction of Tfh cells in mice lacking DOCK11 by Cyl-Cre recombinase. Although B cells are dispensable for priming of T cells, interactions between B and T cells are critical for the maintenance of Tfh cells (52–54). Collectively, DOCK11 expression by B cells may be required for the interactions between B and Tfh cells at the early stages of immune responses.

The present results strengthen the current model in which cytoskeletal reorganization plays an important role in humoral immune responses (15–18). In our previous studies, the over-expression of DOCK11 resulted in the activation of CDC42 (12) and subsequent cytoskeletal reorganization (13). Other groups reported that CDC42 was required for Ag recognition and presentation by B cells (19, 21). Accordingly, the DOCK11 deficiency resulted in the suppression of B cell–intrinsic signaling upon Ag recognition. Furthermore, DOCK11 expression by B cells was required for the induction of Tfh cells, which depend on Ag presentation by B cells (52–56). Thus, the DOCK11-CDC42 axis may contribute to humoral immune responses.

Although we identified some roles for DOCK11 in GC B cells, its biological significance currently remains unclear. To the best of our knowledge, the role of DOCK11 in diseases has not yet been examined. According to the Oncomine database provided by Thermo Fisher Scientific, expression levels of DOCK11 appear to be downregulated in several types of lymphomas, including Burkitt lymphoma (57) and diffuse large B cell lymphoma, not otherwise specified (58). Therefore, further studies are required in the future.

DISCLOSURES

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