Zinc transporter SLC39A10/ZIP10 controls humoral immunity by modulating B-cell receptor signal strength

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The humoral immune response, also called the antibody-mediated immune response, is one of the main adaptive immune systems. The essential micronutrient zinc (Zn) is known to modulate adaptive immune responses, and dysregulated Zn homeostasis leads to immunodeficiency. However, the molecular mechanisms underlying this Zn-mediated modulation are largely unknown. Here, we show that the Zn transporter SLC39A10/ZIP10 plays an important role in B-cell antigen receptor (BCR) signal transduction. *Zip10*-deficiency in mature B cells attenuated both T-cell–dependent and –independent immune responses in vivo. The *Zip10*-deficient mature B cells proliferated poorly in response to BCR cross-linking, as a result of dysregulated BCR signaling. The perturbed signaling was found to be triggered by a reduction in CD45R phosphatase activity and consequent hyperactivation of LYN, an essential protein kinase in BCR signaling. Our data suggest that ZIP10 functions as a positive regulator of CD45R to modulate the BCR signal strength, thereby setting a threshold for BCR signaling in humoral immune responses.

B lymphocyte | acquired immunity | germinal center | antigen-presenting cell | zinc signaling

The humoral immune response is a major arm of the adaptive immune systems, in which B cells play a key role (1, 2). In the bone marrow (BM), the initial commitment to pro-B cells occurs, followed by their differentiation into pre-B cells and then into immature (IMM) B cells, which express the B-cell antigen receptor (BCR) on their surface. The IMM B cells then migrate to the spleen as transitional B cells, and further differentiate into follicular (FO) or marginal zone (MZ) mature B cells. FO B cells are a highly recirculating population, and are essential for T-cell–dependent (TD) immune responses, in which BCR-activated B cells enter the germinal center (GC), where they undergo massive expansion and Ig class-switch recombination (CSR). In contrast, MZ B cells are noncirculating and mediate rapid T-cell–independent (TI) immune responses against blood-borne pathogens. In addition to the conventional B-2 cell subset described above, a distinct B-1 cell subset resides mainly in the peritoneal cavity and produces natural antibodies (1, 2).

The BCR is composed of membrane-bound Ig and associated Igα and Igβ subunits. Following BCR binding to its cognate antigen, the Igα and Igβ subunits are phosphorylated on tyrosines within their immunoreceptor tyrosine-based activation motifs (ITAMs) by SRC family kinases, including LYN (3). The SYK tyrosine kinase is then recruited to the phosphorylated ITAMs, resulting in SYK's activation, and the subsequent activation of downstream molecules, such as ERK, PI3K, and NF-κB. BCR activation not only drives adaptive immune responses, but also mediates a "tonic" signal together with B-cell–activating factor (BAFF) receptor (BAFFR) signaling to help maintain immunocompetent mature B-cell pools in the steady state (4, 5). Thus, BCR signaling critically regulates the activation status and fate decisions of B cells.

Zinc (Zn) deficiency leads to lymphopenia and to attenuations of both cellular and humoral immunity, resulting in an increased susceptibility to various pathogens (6, 7). Zn is reported to function as a signaling factor (8–11) and its homeostasis is tightly controlled by Zn transporters, the SLC39/ZIP and SLC30/ZnT family members, which contribute to Zn influx and efflux, respectively (12, 13). Notably, it was shown that Zn transferred by a specific Zn transporter can selectively fine-tune distinct intracellular signaling events (14) by targeting specific signaling molecules (15–20). Moreover, the disruption of a given Zn signaling axis can have pathogenic consequences in the absence of redundant machinery (21). However, the specific mechanisms involved in Zn and Zn transporter modulation of the immune system—in particular, the humoral immune response—are not well understood.

In this study, we showed that the Zn transporter, ZIP10 (Zrt- and Irt-like protein 10), is required for proper antibody responses following BCR activation. Mice with a conditional knockout of ZIP10 in mature B cells showed dramatic attenuations of TD and TI antibody responses. In addition, GC development

Significance

The essential micronutrient zinc is known to modulate adaptive immune responses and dysregulated zinc homeostasis leads to immunodeficiency. However, the molecular mechanisms underlying this zinc-mediated modulation are unknown. We show that the zinc transporter ZIP10 plays an important role in B-cell receptor (BCR) signaling. *Zip10*-deficiency in mature B cells attenuated both T-cell–dependent and –independent immune responses. *Zip10*-deficient mature B cells proliferated poorly in response to BCR cross-linking, as a result of dysregulated BCR signaling. Our data establish that ZIP10 functions as a cellular regulator to modulate BCR signaling in humoral immune responses.

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failed in these mice, resulting in a marked reduction in antigen-specific IgG1 responses. Moreover, ZIP10 deficiency led to hyperactivated BCR signaling, which reduced cell proliferation because of decreased CD45R protein tyrosine phosphatase (PTPase) activity. Our results establish a link between ZIP10 and humoral immunity, in which ZIP10 controls the BCR signal strength as a positive regulator of CD45R, thereby setting a threshold for B-cell signaling.

Results

Conditional Ablation of ZIP10 in Cells Involved in the Humoral Immune Response. We first investigated the role of ZIP10 (22, 23), a Zn transporter whose physiological functions were unknown, in immune cells involved in the humoral immune response. *Zip10* was ubiquitously but differentially expressed in various tissues, including immune tissues (24). Among the splenic immune cell populations, B cells expressed the highest level of *Zip10* mRNA (*SI Appendix*, Fig. S1). To generate mice with the conditional deletion of ZIP10 in antigen-presenting cells (APCs), which regulate the humoral immune response, we used invariant chain (*Cd74*; *Ii*)-*Cre* transgenic mice, in which transgene-encoded Cre recombinase is expressed concurrently with the *Ii* locus, which is constitutively activated in MHCII$^+$ APCs, such as the B-cell and dendritic cell (DC) populations (*SI Appendix*, Fig. S2 *A–C*). *Zip10* was reduced by 70–80% in the splenic B cells and BM-derived DCs from the *Ii-Cre*/*Zip10*-conditional knockout (*Ii-Cre*-cKO) mice (*SI Appendix*, Fig. S2*D*).

ZIP10 Deficiency Reduces the Mature B-Cell Populations. The *Ii-Cre*-cKO mice exhibited reduced numbers of splenic DCs and B cells (Fig. 1*A*). Among the B-cell subsets, most of the splenic, but not BM B-cell populations expressed *Ii* promoter-driven Cre recombinase (Fig. 1*B*). The number of FO B cells was significantly reduced in the *Ii-Cre*-cKO mice (Fig. 1*B* and *SI Appendix*, Fig. S3). The reduction in FO B cells was confirmed by examining the population of IgD^{hi}IgM^{lo} cells in the spleen and blood (Fig. 1*C*), and the recirculating mature B-cell pool in the BM from *Ii-Cre*-cKO mice (Fig. 1*D*). Similar data were obtained using *Cd21-Cre* transgenic mice (5), in which *Zip10* was specifically deleted in mature B cells and follicular DCs, but not using *Cd11c-Cre* transgenic mice (25), in which *Zip10* was deleted in DCs (*SI Appendix*, Fig. S4). Thus, the B-cell production appeared to be normal in *Ii-Cre*-cKO mice, but the homeostasis of conventional B-2 cells in the periphery was impaired. Furthermore, the number of B-1 cells and IgG3 natural antibody level were also significantly reduced in the *Ii-Cre*-cKO mice (*SI Appendix*, Fig. S5).

***Zip10*-Deficient Mature B Cells Have a Shortened Lifespan.** To determine whether the lymphopenic phenotype in *Ii-Cre*-cKO mice was the result of a cell-autonomous defect, we performed the adoptive transfer of splenic B cells into *Rag1*-KO mice. The resident IgD^{hi}IgM^{lo} B-cell population in the recipient spleen remained constant after the transfer of control cells, but decreased over time after the transfer of *Ii-Cre*-cKO B cells (Fig. 2*A*), suggesting that the decreased number of mature B cells is a B-cell–autonomous defect, and that ZIP10 is required for the persistence of mature B cells in the spleen.

Next, we examined the possibility that a substantial portion of the newly generated peripheral B cells failed to enter the long-lived B-cell pool in the spleen, which would be reflected in an increased rate of B-cell turnover. Mice were fed BrdU, and the BrdU incorporation was measured in their B cells. Higher levels of BrdU were incorporated into the mature B cells of the spleen and BM from *Ii-Cre*-cKO than from control mice (Fig. 2*B*). It was possible that the higher BrdU incorporation rate in *Zip10*-deficient mature B cells was caused by the activation of the homeostatic proliferation machinery, because naïve B cells introduced into a lymphopenic host undergo antigen-independent proliferation (26). To exclude this possibility, we generated a mixed BM chimera by injecting wild-type (CD45.1) and *Ii-Cre*-cKO (CD45.2) BM cells at various ratios into lethally irradiated

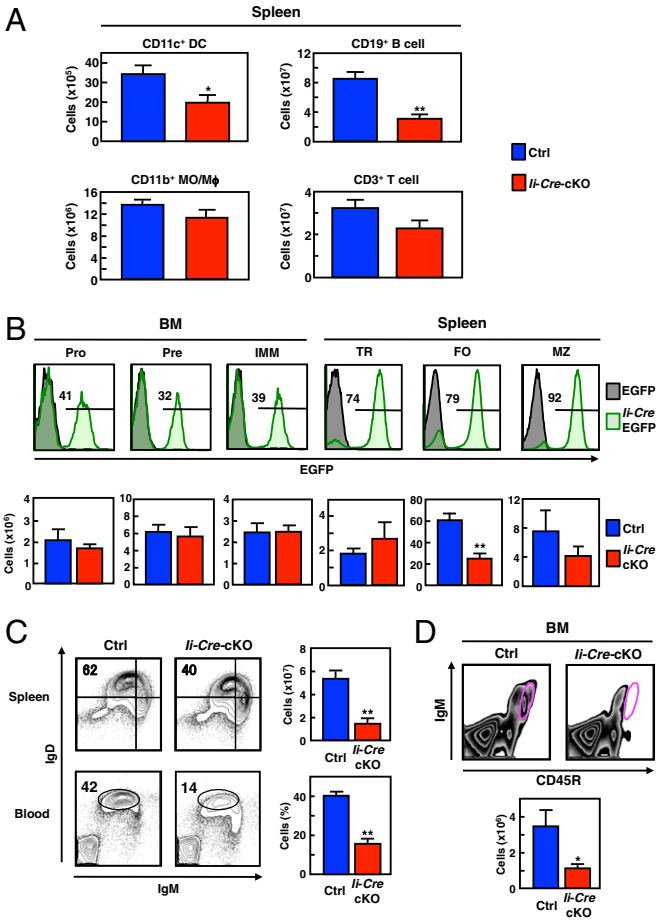

Fig. 1. ZIP10 deficiency leads to the loss of mature B cells. (*A*) The numbers of CD11c$^+$ DC, CD19$^+$ B cell, CD11b$^+$ MO/Mϕ, and CD3$^+$ T cell in the spleen (11- to 12-wk-old males, $n = 4$ for each). Data represent the mean ± SEM (*$P < 0.05$, **$P < 0.01$). (*B*, *Upper*) EGFP reporter expression driven by Cre recombinase activity in B-cell subsets. (*Lower*) Bar charts representing the cell numbers in each B-cell subset (11- to 12-wk-old males, $n = 4$ for each). Data represent the mean ± SEM (**$P < 0.01$). (*C*) IgD^{hi}IgM^{lo} FO B-cell number and population in the spleen and blood, respectively (spleen; 11- to 12-wk-old males, $n = 4$ for each, blood; 17-wk-old males, $n = 5$ for each). Data represent the mean ± SEM (**$P < 0.01$). (*D*) CD45R^{hi}IgM$^+$ recirculating mature B-cell number in the BM (11- to 12-wk-old males, $n = 4$ for each). Data represent the mean ± SEM (*$P < 0.05$).

CD45.1 host mice. High levels of BrdU were incorporated into most of the reconstituted *Zip10*-deficient mature B cells compared with the reconstituted wild-type B cells, independent of the ratio of cells transferred (Fig. 2*C*), suggesting that the *Zip10*-deficient mature B cells were not influenced by homeostatic regulation mechanisms; rather, they failed to enter the long-lived pool and showed rapid turnover. To confirm that the *Ii-Cre*-cKO B cells underwent increased turnover, we measured the turnover rate of the blood-circulating B-cell pool, which mostly consists of recirculating mature B cells. As expected, BrdU-labeled *Ii-Cre*-cKO cells rapidly disappeared from the blood (Fig. 2*D*).

Mature B-cell homeostasis depends on BAFFR- (4) and BCR-mediated signaling (5, 27). Although BAFFR was expressed at lower levels (~20%) in the *Ii-Cre*-cKO compared with control B cells, there was no significant difference in the living B-cell population compared with control cells at 4 d after BAFF treatment (*SI Appendix*, Fig. S6), suggesting that the loss of ZIP10 did not critically affect the BAFF dependency.

Impaired TD and TI Responses in the Absence of ZIP10. We next asked whether BCR signaling was impaired in *Zip10*-deficient

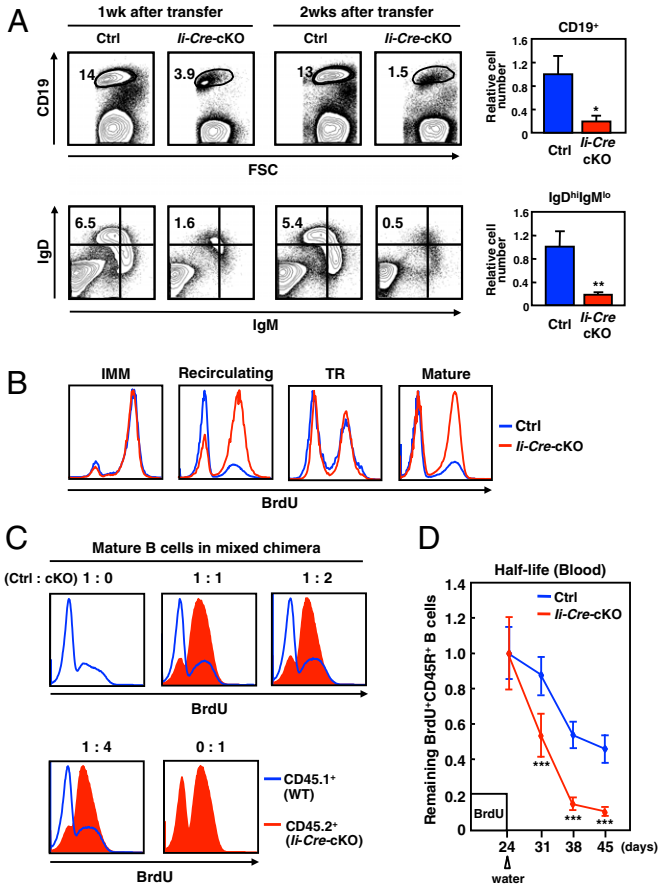

Fig. 2. *Zip10*-deficient mature B cells have a shortened lifespan. (*A*) Resident IgD^{hi}IgM^{lo} FO B-cell population in the spleen from *Rag*-1 KO mice transferred purified splenic B cells. Bar charts representing the relative cell numbers 10 d posttransfer ($n = 3$ for each). Data represent the mean ± SD (*$P < 0.05$, **$P < 0.01$). (*B*) BrdU incorporation in B-cell subsets in the BM and spleen. IMM: CD45R^{lo}IgM^{+}; recirculating: CD45R^{hi}IgM^{+}; TR: CD45R^{+}AA4.1^{+}; mature: CD45R^{+}AA4.1^{-}. (*C*) BrdU incorporation in mature (CD45R^{+}AA4.1^{-}) B cells from mixed BM chimeras. (*D*) Lifespan of peripheral B cells. The relative percentage of CD45R^{+}BrdU^{+} cells in the blood is shown (8 wk, $n = 4$ for Ctrl, $n = 9$ for *Ii-Cre*-cKO). Data represent the mean ± SD (***$P < 0.001$).

B cells. We found that the BCR- but not Toll-like receptor-mediated proliferative activity was severely diminished in these cells (Fig. 3*A*), indicating that ZIP10 selectively regulates BCR signaling in vitro. *Ii-Cre*-cKO mice immunized with a TD antigen, 4-hydroxy-3-nitrophenylacetyl-conjugated chicken γ-globulin (NP-CGG), showed the dramatic reduction of anti-NP–specific IgM and IgG1 productions in a cell-intrinsic manner (Fig. 3*B*). Intriguingly, the secretion of IgG1 was considerably decreased (Fig. 3*B*), suggesting that GC formation, which is required for the generation of high-affinity IgG1 antibodies (28), might have been abrogated in the *Ii-Cre*-cKO mice. In fact, the generation of GC B cells was severely impaired in these mice (Fig. 3*C*), although the capacity for CSR was unaffected (Fig. 3*D*). These data collectively suggest that ZIP10 controls the TD immune response by regulating the persistence of GC B cells.

TI antigens are classified into type 1 (TI-1) and type 2 (TI-2). TI-1 antigens stimulate antibody production in all B cells in a polyclonal manner, whereas TI-2 ones primarily promote MZ B-cell activation, leading to robust IgM and IgG3 antibody productions. Immunization with 2, 4, 6-trinitrophenyl (TNP)-Ficoll (TI-2) or TNP-LPS (TI-1) resulted in anti-TNP IgM and IgG3 secretions that were both severely reduced in *Ii-Cre*-cKO mice (Fig. 4 *A* and *B*), and the impaired TI-2 response was because of a cell-intrinsic defect of *Zip10*-deficient B cells (Fig. 4*C*). Thus, ZIP10 contributes to both TD and TI immune responses, and its absence may result in reduced BCR-dependent cellular activity.

ZIP10 Controls the BCR Signal Transduction Pathway Through CD45R PTPase Activity. We next examined the molecular mechanisms involved in the ZIP10-mediated modulation of BCR signaling. In B cells, ZIP10 was predominantly localized to the plasma membrane and was expressed with modifications such as glycosylation and truncation (*SI Appendix*, Figs. S7 *A–C* and S8), as previously described (23). Reflecting these observations, Zn uptake capacity was significantly lower in *Zip10*-deficient B cells (*SI Appendix*, Fig. S7*D*). However, intriguingly, inductively coupled plasma-atomic emission spectroscopy (ICP-AES) and flow cytometric analysis with Zn indicators showed little alteration in the total intracellular Zn content in the *Zip10*-deficient B cells

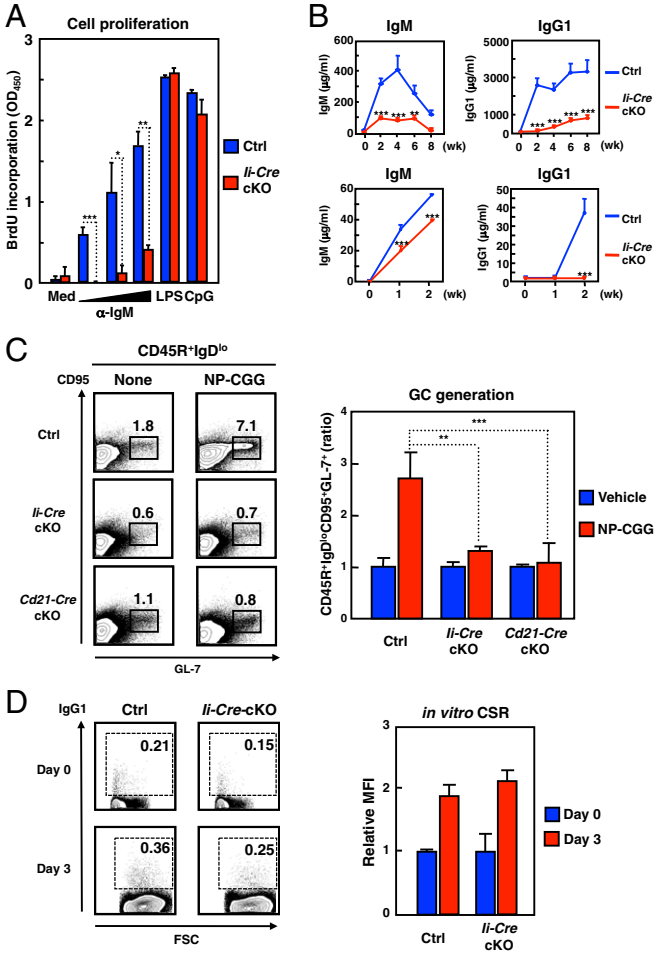

Fig. 3. Impaired antibody production in the absence of ZIP10. (*A*) Cell proliferation assay. Data represent the mean ± SD (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (*B*, *Upper*) TD response in mice ($n = 8$ for each) immunized intraperitoneally with NP-CGG in alum. Data represent the mean ± SEM (**$P < 0.01$, ***$P < 0.001$). (*Lower*) TD response in *Rag1*-KO mice transferred purified wild-type or *Ii-Cre*-cKO CD43^{-} splenic B cells with wild-type splenic CD4^{+} T cells, and immunized with NP-CGG. Data represent the mean ± SEM (***$P < 0.001$). (*C*) GC (CD45R^{+}IgD^{lo}CD95^{+}GL-7^{+}) B-cell formation. Bar charts representing the relative GC B-cell populations (Nontreated control; $n = 6$, NP-CGG-treated control; $n = 5$, Nontreated *Ii-Cre*-cKO; $n = 4$, NP-CGG-treated *Ii-Cre*-cKO; $n = 4$, Nontreated *Cd21-Cre*-cKO; $n = 3$, NP-CGG-treated *Cd21-Cre*-cKO; $n = 4$). Data represent the mean ± SEM (**$P < 0.01$, ***$P < 0.001$). (*D*) CSR in vitro. Bar charts representing the relative mean fluorescence intensity of surface IgG1 expression. Data represent the mean ± SD.

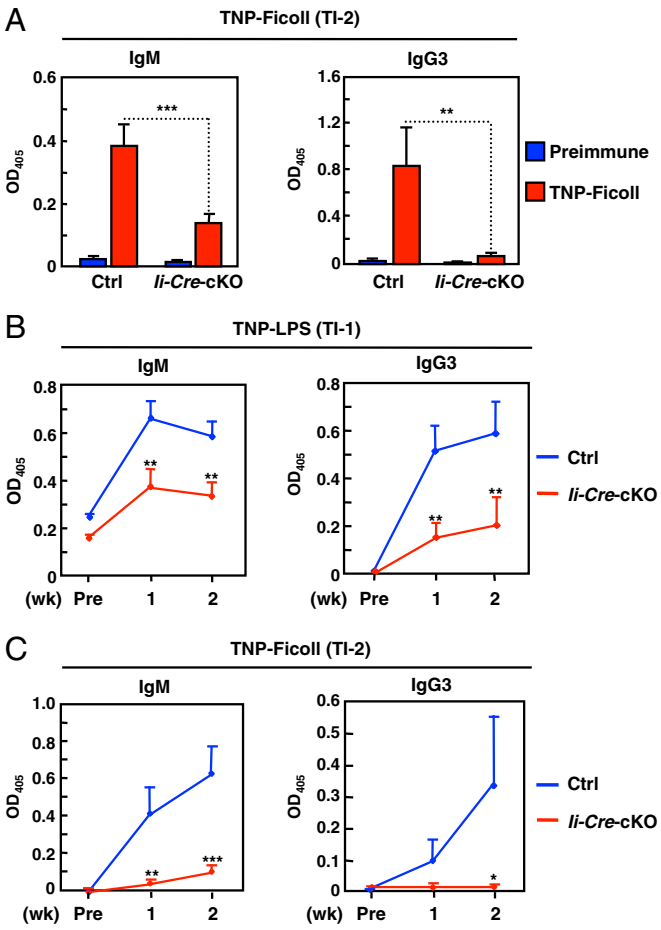

Fig. 4. ZIP10 deficiency results in impaired TI responses. (*A*) TI-2 response in mice (n = 5 for each) immunized intraperitoneally with TNP-Ficoll. Data represent the mean ± SEM (**P < 0.01, ***P < 0.001). (*B*) TI-1 response in mice (n = 5 for each) immunized intraperitoneally with TNP-LPS. Data represent the mean ± SEM (**P < 0.01). (*C*) TI-2 response in *Rag1*-KO mice transferred with purified CD19$^+$ splenic B cells and immunized intraperitoneally with TNP-Ficoll. Data represent the mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001).

(*SI Appendix*, Fig. S7 *E–G*), suggesting that ZIP10 may transport Zn from extracellular fluid under spatio-temporally restricted conditions, rather than affecting the overall intracellular Zn homeostasis in these cells.

Because the BCR-induced cell proliferation was impaired in *Zip10*-deficient B cells (Fig. 3*A*), we speculated that BCR signaling is dysregulated in the absence of ZIP10. Indeed, *Ii-Cre*-cKO B cells showed hyperactivations of ERK, AKT, and NF-κB pathways after BCR cross-linking (Fig. 5 *A* and *B*). Ig stimulation also led to augmented activation of the upstream kinase, SYK (Fig. 5*C*). The induction of the activity of SRC-family kinases, such as LYN, via BCR is essential for coupling BCR stimulation to the activation of downstream pathways (29, 30). The phosphorylation of the stimulatory tyrosine residue (Y397) of LYN was up-regulated in *Zip10*-deficient B cells (Fig. 5*C* and *SI Appendix*, Fig. S9).

CD45R is proposed to exert a negative effect on LYN activity in the lipid rafts (31). BCR stimulation temporarily excludes CD45R from the lipid rafts, releasing CD45R's inhibitory effect on LYN and initiating signaling, but CD45R immediately reassociates with the lipid rafts (31). Thus, the spatiotemporal positioning of CD45R after BCR cross-linking dictates the status of LYN activity. Although the CD45R expression was slightly decreased in the mature B-cell subsets from *Ii-Cre*-cKO mice (Fig. 5 *D* and *E*), its PTPase activity was reduced to nearly half the level of control cells (Fig. 5*F*), indicating that ZIP10 positively regulates CD45R activity in mature B cells.

Notably, under normal conditions, LYN was not constitutively activated in *Zip10*-deficient B cells (*SI Appendix*, Fig. S10), suggesting that the partial reduction of CD45R does not significantly affect LYN's activity in the steady state; rather, it may preferentially abrogate the negative regulatory effect on LYN after BCR cross-linking, leading to the augmentation of LYN activity. Indeed, the forced introduction of Zn ex vivo revealed that the BCR-induced LYN activation was suppressed by Zn in *Zip10*-deficient B cells (Fig. 5*G*). Intriguingly, the reduced CD45R PTPase activity was inclined to recover by forced Zn introduction ex vivo in *Zip10*-deficient B cells (Fig. 5*H*). Furthermore, the activity of an active form of CD45R recombinant protein containing two PTP domains (amino acids 592–1291) was not up-regulated at any Zn concentrations, but was strongly suppressed only in the presence of an unphysiologically high concentration of Zn (1 mM), to the same extent as with orthovanadate (Na_3VO_4), a general PTPase inhibitor, in vitro (*SI Appendix*, Fig. S11). Thus, undefined mechanisms may exist by which ZIP10 positively and indirectly modulates CD45R PTPase activity at the restricted region, such as lipid rafts, where a small amount of CD45R dynamically interacts with LYN (31), perhaps through a Zn-requiring process.

Zn-Deficient Mice Display an Impaired TD Antibody Response. Zn deficiency is known to attenuate the humoral immunity (6, 7). We finally asked how Zn is required for the antibody-mediated immune response in a mouse model. The mice were fed Zn-adequate (ZnA) or Zn-deficient (ZnD) chow for 4 wk, and were then immunized with NP-CGG in alum. The ZnD mice displayed a clear growth retardation phenotype and reduced serum Zn 2 wk after the start of Zn-deficient diet (*SI Appendix*, Fig. S12 *A* and *B*), as previously reported (32). Unexpectedly, the number and intracellular free Zn level of the FO B cells were not altered between the two groups (*SI Appendix*, Fig. S12 *C* and *D*). In ZnD splenic B cells, the expressions of some Zn transporters and metallothioneins were changed (*SI Appendix*, Fig. S13). These findings suggest that the unknown resistant system against ZnD condition by the alteration of Zn transporter expressions maintains Zn homeostasis and alleviates the effect of Zn deficiency on the cell death of this population.

This hypothesis is supported by the finding that the forced chelation of Zn in splenic B cells induced cell death with the induction of *Zip10* transcription (*SI Appendix*, Fig. S14), suggesting that ZIP10 may be involved in cell survival in the critical ZnD environment. Although the previous report described that ZnD up-regulates ZIP10 expression (33), we could not observe it in splenic B cells from 4-wk ZnD mouse in vivo (*SI Appendix*, Fig. S13). In future studies, a more detailed time-course analysis will be required. Notably, we found that the GC B-cell population and antigen-specific IgG1 response were significantly reduced in the immunized ZnD-fed mice (*SI Appendix*, Fig. S12 *E* and *F*). Because GC B cells contained a higher amount of Zn than FO B cells (*SI Appendix*, Fig. S12*G*), they may be more susceptible to the ZnD condition. Thus, the ZnD-induced abrogation of GC formation and the antibody response partly mimicks the phenotypes in *Zip10*-deficient mice (Fig. 3 *B* and *C*), and suggests that Zn is a critical regulator in BCR-mediated cell activation process.

Discussion

In the present study, we found that a ZIP10 deficiency in mature B cells leads to their reduction (Fig. 1) and impaired TD and TI antibody responses (Figs. 3 and 4). Therefore, ZIP10 is important for both mature B-cell maintenance and humoral immune responses.

TD immune responses were considerably attenuated in *Ii-Cre*-cKO mice, and this defective response occurred in a cell-intrinsic manner (Fig. 3*B*). Notably, the secretion of antigen-specific IgG1

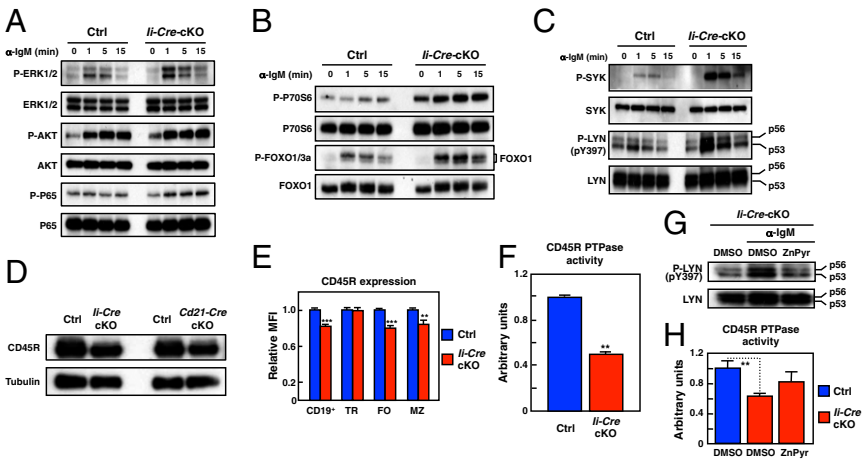

Fig. 5. ZIP10 deficiency dysregulates BCR signaling. (*A*) BCR-induced ERK, AKT, and NF-κB P65 activation. (*B*) BCR-induced P70S6 and FOXO activation. (*C*) BCR-induced SYK and LYN activation. (*D*) Immunoblot for CD45R expression in purified splenic B cells. (*E*) Relative mean fluorescence intensity of CD45R surface expression in splenic B-cell subsets. Data represent the mean ± SD (**$P < 0.01$, ***$P < 0.001$). (*F*) Measurement of CD45R PTPase activity. Data represent the mean ± SD (**$P < 0.01$). (*G*) Effect of Zn introduction on LYN activation. The cells were stimulated with anti-IgM F(ab′)$_2$ with or without Zn plus pyrithione for 5 min. (*H*) Effect of Zn on CD45 PTPase activity assayed ex vivo. The cells were treated with Zn plus pyrithione for 5 min. Data represent the mean ± SD (**$P < 0.01$).

was almost completely abrogated because of the impaired GC formation in *Ii-Cre*- and *Cd21-Cre*-cKO mice (Fig. 3*C*). These findings suggest that the signals produced by ZIP10-deficient mature B cells are not sufficient for their proliferation, resulting in impaired antibody responses. This notion is supported by the impaired BCR-induced cell proliferation (Fig. 3*A*) and the marked attenuation of the TI response in *Ii-Cre*-cKO mice (Fig. 4). These data indicate that the reduced number of mature B cells (Fig. 1) cannot fully account for the suppressed TD and TI responses, and that impaired BCR signaling is likely to contribute to these attenuated responses in *Ii-Cre*-cKO mice. Thus, ZIP10 contributes appreciably to the humoral immune responses by regulating the cellular signaling after antigen exposure.

Because BCR-mediated signaling controls B-cell activation (3), we speculated that the loss of ZIP10 might affect this signaling cascade. Surprisingly, *Zip10*-deficient B cells exhibited enhanced overall BCR signaling (Fig. 5 *A*–*C*). Although this result seems to be paradoxical with the impaired proliferation of *Zip10*-deficient B cells (Fig. 3*A*), LYN actually plays a critical role not only in activating BCR-mediated signals, but also in simultaneously generating inhibitory signals mediated by FCγRIIB1, CD22, and paired immunoglobulin-like receptor-B (PIR-B), subsequently leading to recruitment of the SH2-containing inositol-5′-phosphatase 1 (SHIP-1) and SHP-1 PTPases, which down-regulate BCR signaling (30). Thus, the reduced Ig-stimulated proliferation of *Zip10*-deficient mature B cells may have been because of a rapid negative-feedback loop elicited by inhibitory signals, which led to a shortened signal that was insufficient to promote proliferation. This notion is supported by the observations that LYN$^{up/up}$ mice, which express a constitutively active form of LYN, display the spontaneous and simultaneous activation of positive (SYK) and negative (CD22, SHP-1, SHIP-1) regulators, and impaired cell proliferation upon BCR cross-linking (29).

We found that CD45R, which negatively regulates LYN activity, exhibited reduced PTPase activity in *Zip10*-deficient B cells (Fig. 5*F*). However, the partial reduction of CD45R PTPase activity did not affect the LYN activity in resting *Zip10*-deficient B cells (*SI Appendix*, Fig. S10), in agreement with a previous report that the stimulatory tyrosine of LYN has a normal phosphorylation level in B cells carrying mutant CD45R alleles that reduce CD45R's expression (34). Given the BCR-dependence of the dynamic behavior of CD45R (31), the BCR-induced augmentation of LYN activity in *Zip10*-deficient B cells may result from an impairment of the poststimulatory negative feedback on LYN by CD45R. In support of this scenario, *Zip10*-deficient B cells showed lower uptake of Zn from extracellular space (*SI Appendix*, Fig. S7*D*), and Zn plus pyrithione treatment ex vivo suppressed the LYN activation after BCR stimulation with the up-regulation of CD45R PTPase activity in *Zip10*-deficient B cells (Fig. 5 *G* and *H*). In addition, the coincubation of Zn with CD45 protein in vitro did not up-regulate the PTPase activity, but rather suppressed it at the unphysiologically high Zn concentration (*SI Appendix*, Fig. S11). Thus, regarding how ZIP10 positively regulates the CD45R PTPase activity, our results suggest that ZIP10 acts through a Zn-requiring process rather than by directly affecting PTPase, as previously reported for the receptor PTPase-β (35). In general, PTPase activity is inhibited by oxidants (36), and the Zn's ability to function as an antioxidant is well established (37). Thus, it is possible that the decrease in CD45R PTPase activity in *Zip10*-deficient mature B cells was because of reduced Zn-mediated antioxidant effects in redox signaling. Based on the finding that the cell membrane-localized ZIP10 transports Zn (*SI Appendix*, Figs. S7 *A*–*D* and S8) (22), ZIP10 may positively regulate the CD45R PTPase activity through Zn uptake from the extracellular space to participate in the negative feedback of BCR signaling.

Notably, neither conventional ICP-AES nor a fluorescent method could detect a difference in intracellular Zn content between the control and *Zip10*-deficient B cells (*SI Appendix*, Fig. S7 *E*–*G*). Furthermore, we could not observe the significant changes of expression patterns of other Zn transporters between the two groups (deposited microarray data in RefDIC; see *SI Appendix*). These findings suggest that ZIP10 is not a major contributor to the overall intracellular Zn homeostasis, but may dynamically affect BCR signaling in a local manner by transporting a subtle amount of Zn from the extracellular fluid. This could indeed be the case. ZIP10 is expressed at a rather low level in splenic B cells and even in its ectopically expressed-293T cells (*SI Appendix*, Figs. S7 *A*–*C* and S15), implicating its rapid protein turnover and spatiotemporal expression. Nevertheless, ZIP10 deficiency leads to a striking loss of FO B cells and marked impairment of the antibody response. Given that a redundant system does not appear to be functional in *Zip10*-deficient B cells, these data collectively suggest that ZIP10 has a more efficient Zn-transporting ability than other Zn transporters, and is critically involved in mature B-cell functions. Therefore, ZIP10 may function as a Zn importer in restricted regions, such as the lipid rafts required for BCR signaling, rather than broadly over the entire cell.

In light of the enhanced BCR signaling described above, it appears that the *Zip10*-deficient phenotype is a partial phenocopy of the LYN$^{up/up}$ phenotype, in which a substantial fraction of resting mature B cells is deleted, probably because of enhanced signaling above a certain threshold (29). Anergic B cells have a shortenend half-life in the presence of competitive B cells, and exhibit impaired proliferation in response to antigen-induced BCR aggregation (38). Thus, B-cell anergy may account for the increased B-cell turnover in *Ii-Cre*-cKO mice. However, we could not detect an up-regulation of LYN activation in *Zip10*-deficient B cells in the steady state (*SI Appendix*, Fig. S10), suggesting that ZIP10 controls mature B-cell maintenance by a LYN-independent mechanism.

How similar is the immunological abnormalities in *Zip10*-deficient mice to those in animals under Zn deficiency? Zn deficiency attenuates the Th1 response, which promotes Ig CSR to noncytophilic IgG2, such as IgG2a (IgG2c in C57BL/6), without affecting the Th2 response, which does promote CSR toward cytophilic IgG1 and IgE (39). Given that ZIP10 deficiency significantly attenuated the level of IgG2c but not IgG1 and IgE in steady state (*SI Appendix*, Fig. S5), it would be interesting to hypothesize that the loss of ZIP10 may also affect signal transduction mediated by Th1 cytokines, such as IFN-γ, while remaining intact with one by Th2 cytokines in resting B cells. Nevertheless, our data showed that both *Zip10*-deficient and ZnD mice displayed the reduced GC B-cell number followed by the attenuated antigen-specific IgG1 response upon antigen exposure (Fig. 3*C* and *SI Appendix*, Fig. S12). Because membrane Zn concentrations are strongly influenced in dietary Zn deficiency (40), these findings collectively suggest that the spatiotemporal Zn uptake by cell membrane-localized ZIP10 plays more important role in the activated B-cell function via BCR signaling, rather than in that of the resting B cells.

In conclusion, this study provides novel insight into the function of the Zn transporter ZIP10 in antibody-mediated immune response. Our findings establish that ZIP10 functions as a previously unidentified regulator of BCR signaling by setting its threshold, and is thus required for regulating the humoral immune response. Notably, we recently found that ZIP10 also has an important role in preventing apoptosis at the pro–B-cell stage (24), suggesting that ZIP10 plays distinct roles in the early and late B-cell developmental stages by regulating different signaling cascades. In this regard, *Zip10*-deficient mice may be a useful animal model for studying B-cell homeostasis and function in vivo. Future studies investigating the molecular details involving ZIP10 will improve our understanding of Zn's role in lymphocyte biology.

Materials and Methods

Cell Sorting. The primary splenic B-cell, MO/Mϕ, DC, $CD4^+$ T-cell, and $CD8^+$ T-cell populations were sorted by autoMACS or LS columns (Miltenyi Biotech), using CD19 or CD43, CD11b, CD11c, CD4, and CD8 microbeads (Miltenyi Biotech), respectively. BM and splenic B-cell subsets were sorted by FACSAria II.

Statistical Analysis. Differences among multiple groups were compared by one-way ANOVA followed by a post hoc comparison using Fisher's protected least-significant difference test. The two-tailed Student *t* test was used to analyze the difference between two groups. Detailed descriptions of all of the materials and methods are provided in the *SI Appendix, SI Materials and Methods*.

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