Molecular immunology and signaling

Short Communication

Plasma membrane Ca$^{2+}$ ATPase 1 (PMCA1) but not PMCA4 is critical for B-cell development and Ca$^{2+}$ homeostasis in mice

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The amplitude and duration of Ca$^{2+}$ signaling is crucial for B-cell development and self-tolerance; however, the mechanisms for terminating Ca$^{2+}$ signals in B cells have not been determined. In lymphocytes, plasma membrane Ca$^{2+}$ ATPase (PMCA) isoforms 1 and 4 (PMCA1 and PMCA4, aka ATP2B1 and ATP2B4) are the main candidates for expelling Ca$^{2+}$ from the cell through the plasma membrane. We report here that Pmca4 (Atp2b4) KO mice had normal B-cell development, while mice with a conditional KO of Pmca1 (Atp2b1) had greatly reduced numbers of B cells, particularly splenic follicular B cells, marginal zone B cells, and peritoneal B-1a cells. Mouse and naïve human B cells showed only PMCA1 expression and no PMCA4 by western blot, in contrast to T cells, which did express PMCA4. Calcium handling was normal in Pmca4$^{-/-}$ B cells, but Pmca1 KO B cells had elevated basal levels of Ca$^{2+}$, elevated levels in ER stores, and reduced Ca$^{2+}$ clearance. These findings show that the PMCA1 isoform alone is required to ensure normal B-cell Ca$^{2+}$ signaling and development, which may have implications for therapeutic targeting of PMCAs and Ca$^{2+}$ in B cells.

Keywords: B cells · Ca$^{2+}$ · PMCA1 · PMCA4 · development

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

B-cell receptor (BCR) signaling thresholds are tightly regulated during development to ensure that signal strength is sufficient for positive selection yet not as high as would be induced by an auto-reactive BCR encountering self-antigen. This tight control of signaling requires precise adjustment of changes to cytosolic Ca$^{2+}$ concentration, and consequently mutations in proteins...
that affect Ca\textsuperscript{2+} levels are often associated with autoimmune disease [1]. Cytosolic Ca\textsuperscript{2+} levels are maintained in a balance with stored levels in organelles by channels and pumps including sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPases (SERCAs) that transfer Ca\textsuperscript{2+} to the endoplasmic reticulum (ER) and by plasma membrane Ca\textsuperscript{2+} ATPases (PMCA) that expel Ca\textsuperscript{2+} outside the cell through the plasma membrane [2, 3]. PMCA activity is regulated by multiple mechanisms, many converging on the C-terminal tail that mediates binding to proteins that localize it to specific microdomains and that inhibits ATPase activity until released by Ca\textsuperscript{2+}-bound calmodulin [4, 5]. In humans, PMCAs are associated with cardiovascular disease, hypertension, and malaria resistance [6].

PMCs consist of four isoforms, PMCA1-4: PMCA1 is expressed in a wide range of tissues, PMCA2 and PMCA3 are largely expressed in the nervous system, and PMCA4 expression is less broad than PMCA1 and may be controlled by differentiation [6]. PMCA isoforms are also variably expressed during development according to requirements for Ca\textsuperscript{2+} signal strength and duration [2–7]. As a result, diseases due to PMCA mutations are isoform specific. In mice, a complete deletion of PMCA1 is lethal, but deletions of PMCA2, 3, and 4 induce diseases including deafness, ataxia, and sterility [6]. Both PMCA1 and PMCA4 are expressed in mouse T cells [8]. Moreover, in human Jurkat T cells, PMCA4 is the leading isoform for calcium clearance and NFAT transcriptional activity [9]. However, mouse B cells can differ from T cells in their requirements for various Ca\textsuperscript{2+} handling proteins; for example, the mitochondrial Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger NCLX is required for B-cell migration but not T-cell migration [10]. Therefore, identifying which PMCA isoforms are present in B cells is necessary for specifically targeting those isoforms for therapeutic benefit. In mouse B cells, one study reported that PMCA4 negatively regulates BCR signaling to Ca\textsuperscript{2+} clearance by associating with CD22 [11]. Here, we compare the roles of PMCA1 and PMCA4 in B cells and report that PMCA1 is the main isoform expressed in B cells and that reductions in PMCA1 compromised B-cell development and Ca\textsuperscript{2+} efflux.

Results and discussion

B-cell development requires the PMCA1 isoform but not PMCA4

We previously showed that neuroplastin is a chaperone-like molecule required for PMCA stability, and T cells lacking neuroplastin have a profound reduction in both PMCA1 and PMCA4 leading to failures in Ca\textsuperscript{2+} clearance and NFAT signaling [8]. We wanted to extend these studies to the roles of PMCAs in B cells; therefore, we first compared the effects of loss of either Pmca1 or Pmca4 on B-cell development. Surprisingly, Pmca4 knockout (KO) B cells were present in normal numbers in every population in the BM, spleen, and peritoneal cavity, with the exception of a reduction in peritoneal B-1b cells (Fig. 1A). Similarly, mice with a heterozygous B-cell-specific mutation of the Pmca1 gene, Pmca1\textsuperscript{+/-} CD19-Cre\textsuperscript{+} mice, also had normal B-cell development, with only a slight reduction in B-1 cells that may be due to CD19-Cre effects [12] (Fig. 1B). In contrast, mice with a homozygous conditional KO (cko) of Pmca1 in B cells, Pmca1\textsuperscript{-/-} CD19-Cre\textsuperscript{+} mice, showed strongly decreased numbers and relative proportions for most populations subsequent to early pre-pro B cells and in splenic follicular B cells (FOBs), although marginal zone B cell (MZB) numbers were normal (Fig. 1C). In the peritoneum, B-1a cells in particular showed a strong relative reduction (Fig. 1C). Gating strategies and representative flow cytometric plots are shown in Fig. 1D and Supporting Information Fig. 1. Thus, peripheral B-cell differentiation requires PMCA1 but not PMCA4.

Comparison of PMCA1 and PMCA4 expression in T and B cells

Using western blots, we were unable to detect PMCA4 protein in B-cell lysates from either WT or Pmca4 KO mice (Fig. 2A). PMCA4 was found in T-cell lysates and in total splenocyte lysates from WT but not Pmca4 KO mice, confirming the specificity of the PMCA4 antibody (Fig. 2A). We next checked expression of PMCA1 in lysates from control and from Pmca1 cko B cells mice and observed that the CD19-Cre-mediated deletion of Pmca1 was inefficient [12–15], as PMCA1 expression was reduced but not completely absent (Fig. 2B). The remaining PMCA1 expression suggested that WT B cells were still present. Using genomic PCR, we confirmed the presence of WT configuration alleles (with Flip and LoxP sites at the Pmca1 locus) along with Cre-deleted Pmca1 alleles (Supporting Information Fig. 2A–C). The ratio of Pmca1 cko to WT configuration alleles increased gradually in BM B cells subsets and paralleled development, likely due to the increased CD19 expression during development [12, 15]. One exception was noted in MZBs as there was a decrease in the proportion of Pmca1 cko alleles in this population compared to FOBs, indicating that there was a strong selection against the Pmca1 mutation in MZBs (Supporting Information Fig. 2C).

PMCA1 was clearly detected in all human cell lines, but PMCA4 signals were only strong in Jurkat T cells and Raji lymphoma B cells and were present at relatively low levels in Ramos and BJAB B-cell lines. The results from primary human naïve B cells were consistent with mouse B cells, as only PMCA1 protein was detected in the lysates and not PMCA4. A slight amount of PMCA4 was detectable in human memory B cells, possibly due to upregulation of PMCA4 during B-cell differentiation (Fig. 2C, Supporting Information Fig. 2D). A PCR analysis of the relative levels of Pmca1 and Pmca4 mRNA showed only trace amounts of Pmca4 and markedly stronger levels of Pmca1 mRNA in B cells, in line with the western blot results (Fig. 2D and E). Together, these data show that mouse and human B cells differ from T cells in that they express substantially less PMCA4 and a much higher proportion of PMCA1.

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PMCA1 promotes B cell Ca²⁺ clearance

To test the extent of PMCA involvement in Ca²⁺ extrusion in B cells, we compared Ca²⁺ entry and clearance using a flow cytometric store-operated calcium entry (SOCE) protocol [8, 16] (typical WT B cell trace shown in Fig. 3A). Addition of thapsigargin (Tg) depletes Ca²⁺ stores in the ER by irreversibly inhibiting SERCA pumps, resulting in an increase in cytosolic Ca²⁺ ([Ca²⁺]ᵢ) before levels return to baseline. Subsequent addition of Ca²⁺ then causes a large increase in [Ca²⁺], via store-operated opening of ORAI1 channels, and a stable plateau of the [Ca²⁺]ᵢ trace is established due to simultaneous influx and extrusion of Ca²⁺.

Removal of extracellular Ca²⁺ stops the Ca²⁺ influx, resulting in a biphasic fast and slow response of Ca²⁺ clearance (Fig. 3A).

To test the requirement for PMCASEs in Ca²⁺ clearance in B cells, we added sodium orthovanadate (Na₃VO₄), an inhibitor of
**Figure 2.** Specific expression of PMCA1 versus PMCA4 in B cells. (A and B) Lysates (30 μg/lane) from (A) WT and Pmca4−/− B cells (n = 10 and 4), WT CD4+ T cells (n = 4), WT and Pmca4−/− splenocytes (n = 4 each), and (B) Pmca1f/f CD19-Cre+ and Cre− control B cells (n = 4 each) were immunoblotted for PMCA1, PMCA4, and actin as loading controls. (C) Lysates (30 μg/lane) from Jurkat and Raji cell lines (n = 4 each) and from purified human naïve and memory B cells (n = 4) were blotted as in (A) with ERK1/2 as a loading control. One representative image from three independent experiments for (A), two experiments for (B), and two experiments for (C) is shown. (D and E) Relative mRNA expression levels of Pmca1 and Pmca4 isoforms in murine B cells (D) and human B cells (E) were analyzed by RT-PCR using the relative PCR strategy (diagrams, left) showing the set of PCR primers (forward primer, FP; reverse primer, RP) used to amplify a 1 kb region from both Pmca1 and Pmca4 cDNA. (D) The resulting PCR products were digested with ClaI (C), BglII (B), both (CB) or neither (uncut, U), yielding two ∼0.4 and ∼0.6 kb fragments for Pmca1 and two ∼0.5 fragments for Pmca4. Fragments were subsequently separated by electrophoresis (n = 5 from 5 experiments). For B cells, an additional image of a longer exposure of the gel shows some Pmca4 product. (E) Relative PCR as in (D) but with the indicated human cell lines and primary human B cells. PCR fragments were digested with Psil/Aanl (A) and BglII (B), both (AB), or neither (uncut, U), yielding two fragments of ∼0.7 and ∼0.3 kb for Pmca1 and two ∼0.5 fragments for Pmca4 (n = 2 from two experiments).
Figure 3. Roles for PMCA1 in B-cell calcium clearance. Cytosolic Ca\textsuperscript{2+} levels in follicular B cells (FOBs) labeled with Ca\textsuperscript{2+}-sensitive dyes Fluo-3 and FuraRed measured by ratiometric flow cytometry. (A) Diagram showing complete kinetics of SOCE experiment in WT FOBs; see text for details. (B) Ca\textsuperscript{2+} clearance after SOCE in the absence of SERCAs from 1 min before Ca\textsuperscript{2+} removal, as shown in diagram in (A), in WT FOBs treated with PMCA blocking reagent VO\textsubscript{4}\textsuperscript{3-} at 2 mM or 10 mM (representative plot from three independent experiments with one WT mouse per experiment). (C–E) Depletion of Ca\textsuperscript{2+} from ER stores by treatment with 1 μM thapsigargin (Tg) to block SERCAs in the absence of extracellular Ca\textsuperscript{2+} (left panel) and Ca\textsuperscript{2+} clearance after SOCE (right panel), as shown in diagram (A). (F–H) Bar graph of baseline cytosolic Ca\textsuperscript{2+} levels show mean ± SD of Fluo-3/FuraRed ratios normalized to the corresponding WT value in each experiment (left) and representative trace of Ca\textsuperscript{2+} kinetics upon 10 μg/mL anti-IgM F(ab\textsuperscript{'})\textsubscript{2}, in the presence of 1 mM extracellular Ca\textsuperscript{2+} (right panel). Student’s t-test, ***p < 0.001. (C and F) WT and Pmca4<sup>−−</sup> FOBs (one representative experiment of five for release of ER Ca\textsuperscript{2+}, one representative experiment of five for Ca\textsuperscript{2+} clearance, mean ± SD from six experiments for baseline Ca\textsuperscript{2+}, one representative experiment of six for BCR-induced Ca\textsuperscript{2+}; n = 1 mouse per genotype in each experiment). (D, and G) Control (Pmca1<sup>−−</sup> CD19-Cre<sup>−</sup>) and Pmca1<sup>−−</sup> CD19-Cre<sup>−</sup> FOBs (one representative experiment of five for depletion of ER Ca\textsuperscript{2+}, one representative experiment of three for Ca\textsuperscript{2+} clearance, one representative experiment of five for release of ER Ca\textsuperscript{2+}, one representative experiment of five for Ca\textsuperscript{2+} clearance, mean ± SD from six experiments for baseline Ca\textsuperscript{2+}).
phosphate-dependent enzymes such as phosphatases and PMCA. We observed a strong decrease in Ca\textsuperscript{2+} clearance from B cells in a dose-dependent manner (Fig. 3B), accompanied by cells with extremely high [Ca\textsuperscript{2+}], (Supporting Information Fig. 4D), demonstrating the importance of PMCA to Ca\textsuperscript{2+} clearance. In Pmca4 KO B cells, all Ca\textsuperscript{2+} signals were normal including Ca\textsuperscript{2+} release from intracellular stores and the activation of Ca\textsuperscript{2+} clearance (Fig. 3C), as well as basal [Ca\textsuperscript{2+}], and BCR-activated [Ca\textsuperscript{2+}], (Fig. 3F and Supporting Information Fig. 3B). Additionally, in heterozygous Pmca1\textsuperscript{+/-} CD19-Cre\textsuperscript{+} B cells, Ca\textsuperscript{2+} release from intracellular stores was modestly elevated and Ca\textsuperscript{2+} clearance was reduced (Fig. 3D) but basal and IgM-induced Ca\textsuperscript{2+} were normal (Fig. 3G and Supporting Information Fig. 3C). In comparison, Ca\textsuperscript{2+} was strongly affected in Pmca1 cko B cells, with greatly increased Ca\textsuperscript{2+} levels in ER stores and reduced Ca\textsuperscript{2+} clearance after SOCE (Fig. 3E) and increases in basal [Ca\textsuperscript{2+}], and in BCR-activated [Ca\textsuperscript{2+}], (Fig. 3H and Supporting Information Fig. 3D). Interestingly, the strongly increased [Ca\textsuperscript{2+}], following Tg in Pmca1 cko cells (Fig. 3E) suggests that the SERCA pumps were able to counteract the increased [Ca\textsuperscript{2+}], in the cko cells by storing it in the ER. This Ca\textsuperscript{2+} removal by the SERCAs from the cytoplasm may keep the cko cells alive longer, as prolonged high levels of Ca\textsuperscript{2+} are cytotoxic and may cause cell death during the SOCE assay.

We also tested Ca\textsuperscript{2+} in separate B-cell subsets from Pmca1 cko mice, and found that [Ca\textsuperscript{2+}], was elevated in all subsets and so was not specific to any stage of development (Supporting Information Fig. 3F–G), with one exception. In splenic MZBs, [Ca\textsuperscript{2+}] levels were not as increased as in the other subsets, and the response to Tg was only slightly elevated (Supporting Information Fig. 3A and E). These findings are consistent with the genomic PCR that showed a higher proportion of WT configuration MZB cells in the Pmca1 cko B cells (Supporting Information Fig. 2C), indicating that the [Ca\textsuperscript{2+}] levels were almost normal because there were more WT B cells in the subset. Thus, the high Ca\textsuperscript{2+} levels in the other subsets from Pmca1 cko mice correlate with lack of PMCA1 expression. Flow cytometry density plots of [Ca\textsuperscript{2+}], in single Pmca1 cko B cells also show a higher fraction of cells with extreme [Ca\textsuperscript{2+}], upon Tg treatment and after SOCE when compared to WT, Pmca4\textsuperscript{+/-}, or heterozygous Pmca1\textsuperscript{+/-} CD19-Cre\textsuperscript{+} B cells (Fig. 3I), and similar to WT B cells treated with high doses of VO\textsubscript{3}– (Supporting Information Fig. 4D). We quantified the frequency of Pmca1 cko FOBs that showed extreme [Ca\textsuperscript{2+}], upon Tg treatment (Supporting Information Fig. 4A–C) and found that there was about a five- to tenfold increase in these cells in Pmca1 cko samples compared to controls (Supporting Information Fig. 4C). Together, these data show that PMCA1 is essential for restraining Ca\textsuperscript{2+} levels in B cells while PMCA4 is not.

**Concluding remarks**

Here, we characterized expression of PMCA isoforms and found that PMCA1 is the main isoform in mouse and human B cells and is required for normal mouse B-cell development and for inhibiting Ca\textsuperscript{2+} levels. We used the CD19-Cre system to analyze Pmca1 cko B cells because CD19 expression rises throughout B-cell development, thus relatively inefficient deletion in earlier B cell stages allows for development of PMCA1+ cells that progress to subsequent stages. Loss of PMCA1 affected all B-cell subsets but in particular MZB cells and B-1a cells. These observations are supported by the “strength of signaling” model for the role of BCR signaling in the development of FOB and MZB cells [17, 18]. This model predicts that only MZB precursors with BCR that is weakly reactive to self-antigen will become MZB cells, while intermediate signaling through BCR would lead to FOBS, and strong BCR signals to B-1 cells. As Pmca1 cko MZB precursors would have higher levels of Ca\textsuperscript{2+} signaling, they would not be selected as mature MZBs. The WT configuration MZBs present in the mix would then expand to fill the MZB niche [18, 19], supporting our genomic PCR finding of more WT configuration alleles in the MZBs and an almost normal Ca\textsuperscript{2+} phenotype. Moreover, Pmca1 cko mice lack B-1a cells while B-1b cells are present. B-1b cells represent the activated form of B-1a cells [20]. The high levels of intracellular Ca\textsuperscript{2+} in Pmca1 cko B-1 cells may lead to increased activation of the cells, subsequent downregulation of CD5, and the switch to the CD5− B-1b cells.

In contrast to a previous study reporting that PMCA4 is active in B cells [11], we found no protein expression of PMCA4 in mouse B cells and no defect in Ca\textsuperscript{2+} clearance in Pmca4\textsuperscript{+/-} B cells. However, we do not rule out a potential role for PMCA4 in BCR-induced differentiation of B cells, which would require strong BCR activation to achieve sustained levels of cytosolic Ca\textsuperscript{2+} signaling and then possibly a specific isoform of PMCA to shape and reduce it. This would be consistent with our finding that human memory B cells and lymphoma cells express low levels of PMCA4, with ImmGen data [21], and with previous reports that PMCA4 expression is upregulated during differentiation of cancer cell lines and in B cells from chronic lymphocytic leukemia patients [22–24]. In Jurkat T cells and in a mouse B-cell line, Ca\textsuperscript{2+} clearance is achieved only through PMCA, with no contribution from the Na+-Ca\textsuperscript{2+} exchanger NCX [11, 16]. Given that we found no PMCA4 in primary mouse B cells, a lack of NCX would mean that PMCA1 is the sole mechanism for Ca\textsuperscript{2+} extrusion. Interestingly, B cells with knockouts of key regulators of SOCE—STIM1 and STIM2, or ORAI1—have severe defects in SOCE and BCR signaling to NFAT, but still develop normally [25, 26]. We show here, though, that Pmca1 cko B cells do not develop...
normally, suggesting that developing B cells must keep Ca\(^{2+}\) levels relatively low by refraining from SOCE and by expressing PMCA1.

### Materials and methods

#### Mouse and cell lines

*Pmca1*\(^{1/1}\) (Atp2b1\(^{-}\)) mice were generated from C57BL/6 ES cells provided by UC Davis Knockout Mouse Project (www.komp.org; project ID CSD77635) as described [8]. CD19-Cre\(^+\) mice were previously described and on a C57BL/6 background\(^{11}\). *Pmca4\(^{-}\)/- (Atp2b4) mice [27] (MMRRC stock #36807) and corresponding WT controls were on a FVB/N background. All mouse studies were approved by the Landesverwaltungsamt Halle, Germany (representing the state of Saxony-Anhalt) (License: 2-1181). Jurkat T cells, Raji, Ramos, and BJAB cell lines were from ATCC.

#### Lymphocyte preparation and flow cytometry

Mouse T and B cells were prepared as described [8]. For Western blots, splenic T cells and B cells were enriched using the MACS CD4\(^+\) T cell isolation kit and CD43 microbeads, respectively (MACS; Miltenyi Biotec). Human PBMCs were obtained from healthy volunteers and isolated using a leukocyte reduction filter (Sepacell RZ-2000; Asahi Kasaei Medical) supplied by the Institute of Transfusions Medicine (University of Magdeburg). Experiments were approved by the Ethics Committee of the Otto-von-Guericke University (EK28/08 to ACZ and EK06/11 to MBW). Informed individual donors provided written consent prior to sampling. Human naive and memory B cells were enriched from PBMC preparations using the MACS B cell isolation kit II in combination with CD27 MicroBeads for human tissues (Miltenyi). For flow cytometry, experiments were performed in accordance with established guidelines [28]. Leukocyte suspensions were incubated with the indicated fluorochrome conjugated antibodies (see Supporting Information Table S1 for all antibodies) and acquired on a BD FACS Canto II flow cytometer. Cell populations were analyzed using BD FACS Diva software and FlowJo (Treestar) according to gating strategies as shown in Supporting Information Fig. S1.

#### Flow cytometry of ratiometric Ca\(^{2+}\) measurements

B cells from different sources were labeled separately with anti-B220 using donor-specific fluorescent conjugates (V450 or APC/Cy7) and mixed for further treatments and Ca\(^{2+}\) measurements within the same tube. Cells were additionally labeled with anti-CD21 (APC) to discriminate follicular from marginal zone B cells as shown in Supporting Information Fig. 1. Mixed cells were loaded with 1.3 \(\mu\)g/mL Fluo-3 and 2.7 \(\mu\)g/mL FuraRed (Life Technologies) in RPMI for 30 min at 37°C, then washed and incubated in standard Ringer’s solution (155 mM NaCl, 4.5 mM KCl, 2 mM MgCl\(_2\), 10 mM D-glucose, 5 mM HEPES, pH 7.4), which either contained 1 mM CaCl\(_2\) or 1 mM EGTA instead for measurements of Ca\(^{2+}\) release from internal stores. Fluo-3/FuraRed ratio was acquired on a BD FACS Canto II. BCR-specific response was induced after baseline recording in Ca\(^{2+}\)-containing buffer by addition of 10 \(\mu\)g/mL of anti-IgM F(ab’)\(_2\). Ca\(^{2+}\) release from the ER was induced by 1 \(\mu\)M thapsigargin (Millipore) to irreversibly block SERCAs in Ca\(^{2+}\)-free buffer. To measure Ca\(^{2+}\) clearance following SOCE, thapsigargin-treated cells were washed to remove EGTA and respended in Ca\(^{2+}\)-containing Ringer’s solution. High Ca\(^{2+}\) levels resulting from SOCE were recorded for 3 min. To monitor Ca\(^{2+}\) clearance, cells were centrifuged and respended in Ca\(^{2+}\)-free Ringer’s solution and acquired immediately for additional 5 min. Ca\(^{2+}\) kinetics were analyzed using FlowJo kinetics tool and Excel.

For Ca\(^{2+}\) measurements in BM B-cell populations, total BM cells were labeled with anti-IgM APC, anti-B220 V450, and a dump mix containing anti-CD11b and anti-TCR-\(\beta\) APC/Cy7 antibodies. In this assay, cells from different donors could not be mixed, and were separately labeled with Fluo3 and FuraRed, and washed and respended in Ca\(^{2+}\)-free Ringer solution for measurement of ER Ca\(^{2+}\) release by addition of Tg as described above. BM B-cell populations were gated as depicted in Supporting Information Fig. 1. Area under the curve (AUC) either after addition of anti-IgM F(ab’)\(_2\) for 10 min, after addition of Tg for 10 min or after removal of extracellular Ca\(^{2+}\) for 5 min was calculated from the resulting mean kinetics curve of each donor cell population in each experiment using Excel software. AUC values in single experiments were normalized to the AUC of the corresponding WT control cells in the same experiment. In order to estimate the frequency of cells with extreme Ca\(^{2+}\) load, cells treated with Tg were first gated in a 3-min time interval around peak levels (1–4 min after addition of Tg). Cells with extreme Ca\(^{2+}\) load were then defined by having Fluo3/FuraRed ratios larger than 99.0% of the corresponding control cells for each experiment, and quantified as the mean frequency ± SD.

#### Preparation of protein extracts from B cells and western blotting

Cell pellets were homogenized by incubation at 4°C for 30 min in Triton-homogenization buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton-X-100, 2 mM MgCl\(_2\), 750 U/mL Benzonase (Sigma), and protease inhibitors (protease inhibitor cocktail tablets, Roche)), centrifuged at 15 000 \(g\) for 20 min, and the resulting supernatants were used for analysis. Protein content was determined using a bicinchoninic acid kit according to the manufacturer’s instructions (ThermoFisher Pierce). Western blots were performed as described [8]. In brief, anti-PMCA1 and anti-PMCA4 antibodies (see Supporting Information Table S1) were used at 1:1000 and immunoreactivity was detected using an ECL imager (GeneGnome XRQ, Syngene, Cambridge, UK).
Estimation of relative levels of \textit{Pmca1} and \textit{Pmca4} mRNAs

Total RNA from mouse tissues was isolated using peqGold TriFastTM (peqGold, Erlangen, Germany) according to the manufacturer’s instructions. Total RNA from purified mouse or human cells and cell lines was isolated using the NucleoSpin RNA Kit according to the manufacturer’s instructions (Macherey Nagel, Düren, Germany). The analysis was performed according to a method published by Okunade et al. [27]. This strategy is based on first simultaneously amplifying \textit{Pmca1} and \textit{Pmca4} cDNA fragments, followed by gene-specific restriction digest of PCR products (as shown in Fig. 2D and E). First-strand cDNA was prepared using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Briefly, 500 ng total RNA was reverse transcribed using random primers, followed by PCR using either the primers described in reference [27] (for mouse samples), or primers adapted to human sequences (forward primer 5′-GGTACCCATGTATGGGAAGTTCTGG-3′ corresponding to \textit{Pmca1} codons 363–371 and \textit{Pmca4} codons 259–267 and reverse primer 5′-ATGATCTGATGCGGCTTCTGAAATG-3′ corresponding to \textit{Pmca1} codons 598–607 and \textit{Pmca4} codons 588–597 for X-splice variants in splice site A or 576–585 for Z-splice variants, respectively). The primers have two mismatches relative to each mRNA to allow for efficient amplification of \textit{Pmca1} and \textit{Pmca4}, but not \textit{Pmca2} or \textit{Pmca3}. PCR products were purified using the NucleoSpin Gel and PCR clean-up Kit (Macherey Nagel), followed by restriction digests of 400 ng purified PCR product with the enzymes specified in Fig. 2 (Fast Digest Enzymes, Thermo Fisher Scientific).

PCR of genomic DNA from sorted B-cell populations and DCs

Splenic FOBs and MZBs and BM B cell populations were sorted on BD FACS Aria III according to gating strategies shown in Supporting Information Fig. 1A and D. BM-derived DCs were on BD FACS Aria III according to gating strategies shown in Supporting Information Fig. 1A and D. BM-derived DCs were sorted by flow cytometry, and Stefanie Holze and Christoph Schwarzer for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft grant SFB854-B08 to K.D.F. and U.T., SFB854-B14 to M.B.-W., and by the ABINEP graduate school funded by the Ministry for Economics, Science, and Digitization of the State Sachsen-Anhalt to K.D.F. Open access funding enabled and organized by Projekt DEAL.

Statistical analysis

Statistical significance was determined by unpaired Student’s two-tailed \(t\)-tests or one-way ANOVA tests as indicated using Excel or GraphPad Prism version 7, respectively.

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Abbreviations: FOB: follicular B cell • MZB: marginal zone B cell • PMCA: plasma membrane Ca(2+) ATPase • SERCA: sarco/endoplasmic reticulum Ca(2+) ATPase • SOCE: store-operated calcium entry • Tg: thapsigargin

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