The H2B deubiquitinase Usp22 promotes antibody class switch recombination by facilitating non-homologous end joining

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Class switch recombination (CSR) has a fundamental function during humoral immune response and involves the induction and subsequent repair of DNA breaks in the immunoglobulin (Ig) switch regions. Here we show the role of Usp22, the SAGA complex deubiquitinase that removes ubiquitin from H2B-K120, in the repair of programmed DNA breaks in vivo. Ablation of Usp22 in primary B cells results in defects in γH2AX and impairs the classical non-homologous end joining (c-NHEJ), affecting both V(D)J recombination and CSR. Surprisingly, Usp22 depletion causes defects in CSR to various Ig isotypes, but not IgA. We further demonstrate that IgG CSR primarily relies on c-NHEJ, whereas CSR to IgA is more reliant on the alternative end joining pathway, indicating that CSR to different isotypes involves distinct DNA repair pathways. Hence, Usp22 is the first deubiquitinase reported to regulate both V(D)J recombination and CSR in vivo by facilitating c-NHEJ.
**Results**

**Generation of B-cell-specific Usp22 KO mice.** Previous work with the CH12 cell line suggested that Usp22 has a critical role in DSB repair pathways that govern CSR (Fig. 1a). To test whether Usp22 is necessary to repair programmed DSBs that occur during B-cell development in vivo (i.e., V(D)J recombination and CSR), we employed the Cre-LoxP system to generate Usp22 conditional KO mice, as Usp22 is essential for mouse embryogenesis. Usp22flox/fox mice (Fig. 1b) were crossed with CD19-cre mice to knock out Usp22 in pre-B cells. Quantitative PCR (qPCR) analysis showed that Usp22 messenger RNA was reduced in spleen B cells from CD19-cre-Usp22 KO mice compared with wild-type (WT) littermates (Fig. 1c). Lipopolysaccharide (LPS) stimulation resulted in a decrease in Usp22 mRNA level in WT B cells when compared to the unstimulated cells (Supplementary Fig. 1a). Consistent with its role in deubiquitinating H2B, we found that the level of H2Bub was markedly increased in splenic B cells from CD19-cre-Usp22 KO mice (Fig. 1d and Supplementary Fig. 1c-e).

To evaluate whether B-cell deletion of Usp22 affects CSR in vivo, we first assayed the levels of various Ig isotypes in the serum of Usp22 KO or control mice. We found that the levels of polyclonal IgG1 and IgG3 isotypes were reduced and IgM was increased in the serum of naive CD19-cre-Usp22 KO mice compared with naive WT littermates (Fig. 1f). Interestingly, no defect in the serum IgA level was observed in CD19-cre-Usp22 KO mice (Fig. 1f). As IgA has an essential role in the control of intestinal microbiota, we also tested the level of polyclonal IgA in the feces and also found no defect in fecal IgA levels in CD19-cre-Usp22 KO mice (Fig. 1f). These data demonstrated that Usp22 is critical for H2B deubiquitination in primary B cells and is required for CSR to IgG subclasses but not to IgA, under homeostatic conditions.

Usp22 KO mice exhibited defects in IgG but not IgA responses. To further evaluate the role of Usp22 on CSR, we first utilized Nitrophenyl Chicken Gamma Globulin (NP-CGG) as a model antigen to immunize mice. At day 22 post-NP immunization, we found that anti-NP IgG levels were reduced, whereas anti-NP IgM levels were increased in the serum of naive CD19-cre-Usp22 KO mice compared with naïve WT littermates (Fig. 1f). Importantly, no defect in the serum IgA level was observed in CD19-cre-Usp22 KO mice (Fig. 1f). As IgA has an essential role in the control of intestinal microbiota, we also tested the level of polyclonal IgA in the feces and also found no defect in fecal IgA levels in CD19-cre-Usp22 KO mice (Fig. 1f). These data demonstrated that Usp22 is critical for H2B deubiquitination in primary B cells and is required for CSR to IgG subclasses but not to IgA, under homeostatic conditions.

**Supporting Information**

- Supplementary Fig. 1a: Consistent with its role in deubiquitinating H2B, we found that the level of H2Bub was markedly increased in splenic B cells from CD19-cre-Usp22 KO mice (Fig. 1d and Supplementary Fig. 1c-e).
- Supplementary Fig. 1b: We found that deletion of Usp22 did not markedly affect various spleen B-cell subsets (Fig. 1e and Supplementary Fig. 1c-e).
- Supplementary Fig. 1c: We found that deletion of Usp22 did not markedly affect various spleen B-cell subsets (Fig. 1e and Supplementary Fig. 1c-e).

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Fig. 1 Spleen B-cell profiles and total Ig isotype analysis of Usp22 KO or WT mice. 

a Schematic showing the dynamic regulation of histone H2B ubiquitination (Ub). Addition of ubiquitination to H2B is mediated by RNF20/RNF40, while the removal of ubiquitination from H2B is mediated by SAGA complex that is composed of Usp22, Eny2, Atxn7, and Atxn7L3. 

b The structure of targeted usp22 locus. The protein-encoding exon 2 (133 bp) of the usp22 gene was flanked by loxP sites (triangles). Usp22\textsuperscript{fl/fl} mice are bred to CD19-cre or Mb1-cre mice to delete exon 2, leading to disruption of usp22 gene expression specifically in B cells. 

c Spleen B cells were purified from CD19-cre-Usp22 KO mice or WT littermates. qPCR analysis of Usp22 mRNA was performed with resting spleen B cells or B cells that were stimulated with LPS for 3 days ex vivo (n = 3 mice per group). Data represent two independent experiments. 

d Spleen B cells from CD19-cre-Usp22 KO and WT littermate mice were stimulated with LPS for 2.5 days, exposed to 8 Grays of γ-radiation, and then collected at various time points for western blot analysis of H2B monoubiquitination (H2BK120Ub). Data represent four independent experiments. 

e Absolute numbers analysis of spleen B cells from CD19-cre-Usp22 KO and WT littermates. FO, follicular B cells; MZ, marginal zone B cells. Data represent two independent experiments each with three to four mice per group. 

f Serum analysis of various Ig isotypes in unimmunized CD19-cre-Usp22 KO and WT littermates, as well as fecal IgA from the same mice (n = 10 mice per group). Data in c, e, and f were presented as mean ± SEM and were analyzed using two-tailed unpaired Student’s t-test. *p < 0.05 and **p < 0.01; NS, not significant.
**Fig. 2** Usp22 KO mice exhibited defective IgG/IgE CSR but not IgA CSR. **a** NP25-CGG plus alum was intraperitoneally injected into CD19-cre Usp22 KO or WT littermate mice. At d22 post immunization, mice were subjected to NP-specific IgG+ ELISPOT assay to measure the amount of NP-specific IgG+ antibody secreting cells (ASC) in the spleen (n = 7 mice per group). Data were combined from two independent experiments. **b** Rotavirus (RV) was orally administered to CD19-cre-Usp22 KO or WT littermates. Fecal samples were collected at the indicated time-points post-RV infection, followed by fecal supernatant preparation. The 1:2 dilution of fecal supernatant was used for fecal anti-RV IgA ELISA assay. Data was analyzed by two-way ANOVA. **c** ELISPOT assays were performed at d23 post-RV infection, to measure the numbers of IgA+ RV-specific antibody secreting cells in lamina propria (LP) and bone marrow (BM), respectively. Data in **b** and **c** represent two independent experiments each with three mice per group. **d** Splenic B cells from CD19-cre-Usp22 KO or WT littermates were ex vivo induced to switch to IgG1, IgG2b, IgG3, IgE, and IgA (n = 3 mice per group). **e** Same as **d**, except that Mb1-cre Usp22 KO mice were used (n = 3–4 mice per group). Data in **d** and **e** represent three independent experiments; data in **a** and **c–e** were analyzed using two-tailed unpaired Student’s t-test. Data were presented as mean ± SEM. *p < 0.05 and ***p < 0.001; NS, not significant.
To determine whether the defect in CSR in Usp22-deficient B cells is cell intrinsic, we induced CSR to various Ig isotypes ex vivo using purified spleen B cells. We found that CSR to IgG1, IgG2b, IgG3, and IgE were markedly reduced in spleen B cells from CD19-cre-Usp22 KO mice, compared with littermate controls (Fig. 2d and Supplementary Fig. 3c). Again, surprisingly, IgA CSR was normal in CD19-cre-Usp22 KO splenic B cells (Fig. 2d). To eliminate the possibility that this phenomenon was due to the CD19-cre transgene, we crossed Usp22lox/lox mice with Mbl-cre mice to delete Usp22 in pro-B cells and induced CSR ex vivo. We again observed defects in IgG and IgE CSR, but not in IgA CSR in splenic B cells. In addition, we isolated B cells from lymph nodes (LN) and found that CD19-cre-Usp22 KO LN B cells were also defective in IgG1 CSR ex vivo when compared with littermate control (Supplementary Fig. 2d). Interestingly, we observed that Usp22 KO LN B cells exhibited a minor defect in IgA CSR (Supplementary Fig. 2d). The minor deficiency in IgA CSR between spleen and LN B cells may be due to the possibility that mature B cells are more predominant in LN than in the spleen.

As Mbl-cre-mediated gene deletion can occur in early pro-B cells before V(D)J rearrangement, we tested whether Usp22 is involved in the repair of DSBs that occur during V(D)J recombination. Hence, we analyzed B-cell profiles in the BM using the "Hardy" staining scheme. The early B-lineage cells (Hardy Fractions A and B) actively undergo D to J rearrangement (in Fraction B); up to one quarter of these early B cells also undergo V to (D)J recombination in their IgH alleles. We observed that early pro-B cells (R200+/CD43+), especially the Fraction B subset (Fr B: CD43+/R200+/CD24+/BP-1−), were markedly increased in the BM of Mbl-cre-Usp22 KO mice, compared with WT littermates (Supplementary Fig. 2a-c), indicative of a blockade in B-cell development in Usp22 KO mice. We evaluated V(D)J recombination at the molecular level and found that distal V3H1 to DJH1 segment recombination, but not proximal V3H2 to DJH1 or D-to-J recombination, seemed to be impaired in pro-B cells from Mbl-cre-Usp22 KO mice (Supplementary Fig. 3d-e). These data suggest that Usp22 is involved in the long-range V(D)J recombination and B-cell development.

Impaired \( \gamma H2AX \) levels in Usp22 KO spleen B cells. To investigate the potential mechanisms for IgG/IgE CSR defects in Usp22-deficient B cells, we first assessed whether AID expression was impaired, as AID is essential for CSR. We found that AID was increased at both mRNA and protein levels in spleen B cells from CD19-cre-Usp22 KO mice compared with WT littermates (Fig. 3a and Supplementary Fig. 4a), which might be due to the role of Usp22 in regulating the gene transcription. However, these data are inconsistent with a reduction in CSR to IgG/IgE in Usp22-deficient B cells. One possibility that could explain the CSR defect to IgG/IgE, but not IgA in Usp22-deficient B cells, is the reduction of germine transcripts in \( \gamma H2AX \) switch regions, but not \( \mu H \) switch region. By evaluating the germine transcripts at day 2 post-CSR stimulation, we found comparable levels of \( \mu \), \( \gamma H2AX \), and \( \gamma H2AX \) transcripts between Usp22 KO and WT splenic B cells (Supplementary Fig. 4b-c). To test whether AID function at the \( \mu \) and \( \gamma H2AX \) switch region was affected, we sequenced the regions upstream of the \( \mu \) and \( \gamma H2AX \) switch region, respectively. However, we found no significant difference in AID mutation frequency in splenic B cells between CD19-cre-Usp22 KO and WT group (Table 1), which is consistent with our previous studies with Usp22 KO CH12 cells. Together, these data indicate that different effects of Usp22 deficiency on IgG/IgE vs IgA CSR in primary B cells are independent of AID activity, suggesting that repair of DSBs downstream of AID is different when leading to IgG/IgE CSR than IgA.

\( \gamma H2AX \) has a critical role in DSB repair by recruiting various DNA repair factors to DSB regions, and it has been shown that premature degradation of \( \gamma H2AX \) signaling leads to impaired DNA damage repair. To test whether enhanced H2Bub has an impact on \( \gamma H2AX \) kinetics in mice, splenic B cells were stimulated with LPS and then exposed to \( \gamma \)-radiation, followed by \( \gamma H2AX \) immunofluorescent staining. We found that \( \gamma H2AX \) kinetics were altered in CD19-cre-Usp22 KO B cells, with a marked decrease in \( \gamma H2AX \) four hours post irradiation (Fig. 3b). We also observed a premature degradation of \( \gamma H2AX \) in Usp22 KO group under conditions that lead to IgA CSR (Fig. 3b), which is consistent with our previous data in IgA-switched CH12 cells.

As a defect in \( \gamma H2AX \) signaling could manifest in a defect in DSB repair that can impair cell cycle progression during CSR, we evaluated whether the cell cycle was altered in Usp22 KO primary B cells. By pulsing cells with EdU and then staining with propidium iodide (PI), we found that LPS-stimulated spleen B cells from CD19-cre-Usp22 KO mice exhibited an increased proportion of G2-M phase cells and a decreased proportion of G1 and G2-M phases compared with controls (Fig. 3c and Supplementary Fig. 3d). Consistent with this finding, proliferation assays showed that LPS-stimulated Usp22-KO B cells proliferated more quickly than WT B cells (Fig. 3d). However, the IgG/IgE CSR defects in Usp22-deficient spleen B cells are unlikely due to alterations in cell cycle, as CSR to IgG1 is defective in CD19-cre-Usp22 KO B cells at various stages of division (Fig. 3e and Supplementary Fig. 4e). Collectively, these data show that Usp22 deficiency results in premature degradation of \( \gamma H2AX \) signaling and a potentially disrupted G1-S checkpoint, which may manifest in impaired DNA repair during CSR.

IgA CSR is more dependent on A-EJ than IgG CSR. As V(D)J recombination requires c-NHEJ, the impaired pro-B-cell development in Mbl-cre-Usp22 KO mice suggests that Usp22 is critical for c-NHEJ. The repair of DSBs during CSR is mediated by both the c-NHEJ pathway and A-EJ pathway. Unlike DSBs repaired by A-EJ, DSBs repaired by c-NHEJ typically have little to no impact on cell survival and development. As Usp22 KO splenic B cells exhibited defective CSR to IgG and IgE but not IgA, we hypothesized that CSR to various Ig isotypes may rely on different DNA repair pathways to various degrees. To test this hypothesis, we examined switch junctions from IgG1- vs IgA-switched spleen B cells, as analysis of MH usage at switch junctions can reflect the DNA repair pathways that were utilized during CSR. Sequencing of IgG1-switched spleen B cells revealed that the MH length between the \( \mu \) and \( \gamma H2AX \) switch regions is comparable between CD19-cre-Usp22 KO and WT littermates (MH: 2.4 vs 2.2, \( P = 0.75 \) (Fig. 4a)), although a trend of increased 6 + bp MH usage was observed in Usp22-KO IgG1 B cells (9%) when compared with Usp22-WT B cells (2%). In contrast, we found a significantly longer MH between the \( \mu \) and \( \gamma H2AX \) switch regions in IgA-switched CD19-cre-Usp22 KO spleen B cells, compared with WT littermates (MH: 3.0 vs 1.9, \( P < 0.05 \) (Fig. 4b). The percentage of insertions was slightly decreased in Usp22 KO B cells when compared with WT control (Fig. 4a,b). The pattern of breakpoint locations, which were distributed across the switch regions, were comparable between Usp22-WT and -KO groups (Fig. 4c,d).

The increased MH usage in \( \mu \)–\( \gamma H2AX \) switch regions but not \( \mu \)–\( \gamma H2AX \) switch regions in Usp22-deficient B cells has been observed before in DNA Ligase IV- and DNA-PKcs-deficient human B cells (i.e., defective in c-NHEJ) and suggests that CSR to IgA...
employs A-EJ more efficiently when c-NHEJ is impaired, while CSR to IgG1 does not. These results further suggest that Usp22 promotes the c-NHEJ but not the A-EJ pathway. To further validate the distinct dependence of IgG and IgA CSR on c-NHEJ vs A-EJ, we evaluated the surface Ig isotype in spleen B cells from 53BP1-deficient mice, which are deficient in c-NHEJ\cite{14}, after ex vivo CSR stimulation. We found that 53BP1\textsuperscript{−/−} B cells exhibited a less severe defect in IgA CSR (~ 60% reduction, compared with controls) than CSR to other Ig isotypes (~ 80–90% reduction) (Fig. 5a). In addition, we used the SUV4-20 inhibitor
A-196 (A-197 as the control compound) to suppress c-NHEJ during ex vivo CSR of WT spleen B cells. Inhibition of c-NHEJ caused a defect in IgG1 CSR (~ 50% reduction), but not IgA CSR (Fig. 5b).

To determine why CSR to IgA is more dependent on the A-EJ pathway than switching to other isotypes, we analyzed the sequence homology between switch regions within the mouse IgH locus. We reasoned that since A-EJ utilizes microhomologies during the reaction, recombining sequences with increased homology might favor A-EJ over c-NHEJ. We found that in both C57BL/6 and BALB/c mice, a higher degree of homology was observed between Sμ and Sa, when compared with that between Sa and Sy1, Sy2b, and Sy3 switch regions (Fig. 5c,d and Supplementary Fig. 5-6). It is notable that Sa and Sμ switch regions also had a relatively high degree of nucleotide identity, although to a lesser degree as compared with the Sa and Sμ switch regions. Hence, the high degree of primary DNA sequence identity between Sμ and Sα switch regions might explain why A-EJ is more efficient during IgA CSR at the molecular level.

Discussion

By generating the first KO mouse of the SAGA complex, we identified Usp22 as a new factor that is essential to repair programmed DNA breaks that occur during B-cell ontogeny. We demonstrate that Usp22 promotes c-NHEJ and CSR in vivo. Interestingly, B-cell-specific ablation of Usp22 resulted in defective CSR to various IgG/IgE isotypes but not to IgA. We further demonstrated that CSR to IgG/IgE is primarily mediated by c-NHEJ, whereas CSR to IgA is more dependent on MH-mediated A-EJ. Indeed, consistent with a previous report that measured serum Ig isotypes in 53BP1−/− mice, we found by measuring surface Ig isotypes that 53BP1−/− B cells exhibited a less severe defect in IgA CSR than CSR to other Ig isotypes. In addition, we observed that chemical inhibition of c-NHEJ has strong effects on IgG CSR but not IgA CSR, which is similar to the CSR phenotype in Usp22-deficient B cells. The difference in IgA CSR between Usp22 KO mice and 53BP1−/− mice may be due to the fact that c-NHEJ is essentially null in 53BP1−/− mice but is likely only reduced in Usp22 KO mice. In addition to IgA CSR, it has been reported that A-EJ pathways are also preferentially utilized for CSR to IgD37,38. As μ and a switch regions from both human and mouse share higher sequence identity compared with other switch regions, this might be the reason for the increased dependence of A-EJ for IgA CSR, which in turn would suggest that primary DNA sequences can partly dictate the usage of DNA repair pathways.

Ubiquitination-mediated signaling transduction has important roles in many biological processes, including various aspects of immune functions. In contrast to ubiquitinating enzymes, the roles of deubiquitinases are less well studied, especially in the context of B cells. Histone H2B has been implicated in DNA damage response, as H2Bub at lysine 120 is thought to induce changes in chromatin conformation, making damaged DNA accessible by various repair factors. Usp22 has been shown to deubiquitinate both histone H2A and H2B in biochemical assays. A recent report showed that H2Bub levels are not increased in Usp22-deficient HEK293T cells due to redundancy with other deubiquitinases. However, we found that Usp22 deletion in primary spleen B cells resulted in a marked increase in H2Bub, which is consistent with our findings in Usp22-KO CH12 cells, indicating that Usp22 is essential for H2B deubiquitination in B cells, and that other deubiquitinase have significant backup roles to Usp22 in these cells.

V(D)J recombination is essential for the surface expression of BCR and T-cell receptor. We found that Usp22 deletion in early B cells mediated by Mb1-cre caused a block at the Fraction B stage during B-lymphopoiesis. At the molecular level, we observed that long-range V-(D)J rearrangement seems to be impaired in the Mb1-cre-Usp22 KO mice, which is similar to the defective V-(D)J recombination phenotype observed in Pax5−, IL-7R−, or 53BP1-deficient mice or patients with NIPBL mutations. Nevertheless, more work needs to be carried out to establish a role for Usp22 in V(D)J recombination, and whether the defect in B cell development is due to altered cell cycle and/or cell death in the Usp22 KO mice. It also remains to be investigated why distal vs proximal V-to-DJ recombination has different requirements for certain c-NHEJ factors, such as 53BP1 and Usp22.

Phosphorylation of H2AX has a critical role in the DNA damage response by recruiting various DNA repair factors to damage regions and it has been shown that premature degradation of γH2AX signaling impairs DNA repair. We found that ablation of Usp22 in B cells resulted in a defect in irradiation-induced γH2AX levels at later time-points (i.e., 4 h post irradiation). The potential mechanisms by which excessive H2Bub affects γH2AX signaling are unknown. It is possible that excessive H2Bub inhibits the recruitment/stability of γH2AX kinase (i.e., ATM and DNAPK) via steric hindrance and/or preferentially recruits certain γH2AX phosphatases to sites of DNA damage.

IgA is the most abundant antibody isotype in the body and has an essential role in the control of pathogen invasion and commensal bacteria at mucosal surfaces, such as the gastrointestinal tract. Many recent studies including our own have demonstrated that dysregulation of the microbiome are linked to many diseases, such as inflammatory bowel disease, neurologic diseases, and various cancers. In our previous report, we found that knockdown or KO of Usp22 caused a defect in IgA CSR in the CH12 cell lines. Here, by generating B-cell-specific Usp22 KO mice, we found that Usp22 ablation led to defects in CSR to...
Table 1 Frequency of mutations upstream of switch μ or γ1 regions

|                | Usp22 WT |          | Usp22 KO |
|----------------|----------|----------|----------|
|                | S’μc     | S’γ1d    |          |
| Sequences (#)  | 54       | 47       |          |
| Nucleotides sequenced (#) | 30,742  | 24,974   |          |
| Mutations (#)  | 14       | 5        |          |
| Mutation frequencya | 4.55 × 10⁻⁴ | 2.00 × 10⁻⁴ |          |
| Mutations at G/C (%) | 86      | 100      |          |
| AID hotspot mutations (%)b | 79      | 100      |          |
| Deletions/insertions (#) | 0       | 0        |          |

MH: 2.22

MH: 2.4

IgG1 P<0.05

MH: 1.89

MH: 2.98

4% Frequency is defined as unique mutations/nucleotide sequenced. aMutations at WRc and QYW motifs, where W = A/T, R = A/G, and Y = T/C. cCells stimulated to IgA (n = 4 mice per group). dCells stimulated to IgG1 (n = 3 mice per group).

Fig. 4 Microhomology analysis of switch junctions of IgG1- or IgA-switched spleen B cells. Spleen B cells isolated from CD19-cre-Usp22 KO or WT littermates were induced for IgG1 (a and c) or IgA (b and d) CSR ex vivo for 4 days. IgG1 and IgA switch junctions were cloned from these cells, and subjected to sequencing. Approximately 50–60 unique switch junction sequences were analyzed per group. a Sμ/Sγ1 or b Sμ/Sα junctions were categorized based on their microhomology usage: near-blunt joins (0–2 bp), joins with microhomology (3–5 bp or 6+ bp), or insertions. The microhomology length (MH) used by each unique switch junction sequence are summarized (right panel). c Distribution of breakpoints across μ or γ1 switch regions for IgG1-switched Usp22-WT or KO cells. d Distribution of breakpoints across μ or α switch regions for IgA-switched Usp22-WT or KO cells. Data in a-d were combined from three mice per group; data in a and b were presented as mean ± SEM and were analyzed using two-tailed unpaired Student’s t-test. NS, not significant
Fig. 5 IgG1 CSR primarily relies on c-NHEJ, whereas IgA CSR is more dependent on A-EJ. a Spleen B cells isolated from 53BP1 KO or WT littermates were induced for ex vivo IgG1, IgG2b, IgG3, or IgA CSR for 4 days. 53BP1−/− B cells (deficient in c-NHEJ) exhibited a less severe defect in IgA CSR (~60% reduction) compared with IgG CSR (~80–90% reduction). Data represent two independent experiments each with three to four mice per group. b A-196 (10 µM) was used to inhibit c-NHEJ during the ex vivo CSR of WT spleen B cells and A-197 is the control compound. Inhibition of c-NHEJ markedly reduced IgG1 CSR, but not IgA CSR (n = 4 samples per group). Data represent three independent experiments. c, d The switch region sequence of Sμ on the IgH locus of C57BL/6 background mice were compared with that of c Sα or d Sγ1. Dot Matrix analysis of the mouse switch regions. The dots represent homologies between two switch regions with a search length of 30 bp and defined percentage of identity. Data in a and b were presented as mean ± SEM and were analyzed using two-tailed unpaired Student’s t-test. ***p < 0.001; NS, not significant
various Ig isotypes except IgA in vivo. The ex vivo CSR assays revealed that Usp22 KO splenic B cells are defective in almost all the isotypes, except IgA. However, Usp22 KO LN B cells are defective in both IgG1 and, to a lesser extent, IgA CSR, which is similar to the CSR phenotype with 53BP1−/− mice. The discrepancy between these two studies is unclear. It is notable that CH12 B-cells have a B1 B-cell phenotype, whereas the vast majority of splenic B cells have a B2 B-cell phenotype, and recent studies have reported major differences between B1 and B2 B cells regarding their CSR toward IgA50,52. Nevertheless, as Usp22 deletion in vivo caused strong defects in various Ig isotypes but less so to IgA. Usp22 could be exploited as a therapeutic target in IgG or IgE-mediated autoimmune diseases, such as systemic lupus erythematosus, autoimmune thrombocytopenia, and asthma. It is expected that Usp22 inhibitors have the potential of ameliorating the pathogenesis of these antibody-mediated autoimmune diseases, without disturbing the homeostasis of the gut microbiota. Furthermore, our findings, that IgA CSR can utilize two DNA repair pathways while IgG CSR only employs one, highlight the biological need for IgA production as observed in μMT mice, which can produce IgA but not other isotypes53.

In summary, we report that Usp22 is the first deubiquitinationase that regulates both V(D)J recombination and CSR in vivo by promoting c-NHEJ. We found that IgG CSR is primarily mediated by c-NHEJ, while IgA CSR is more dependent on A-EJ, indicating that CSR to different isotypes involve distinct DNA repair pathways.

Methods

Mice. C57BL/6 WT mice (Charles River Laboratory, St. Constant, QC, Canada; 6–8-week-old females) were bred in our Animal Vivarium facility at the University of Toronto. Mice containing two flox sites in the upstream of exon 2 of Usp22 and two loxP sites flanking exon 2 of Usp22 were purchased from UC DAVIS KOMP Repository (Davis, CA) and bred with FLPl-o deleter mice (The Jackson Laboratory), to generate Usp22fl/fl mice. Usp22−/− mice were then bred with CD19Cre (The Jackson Laboratory) or MbrCre mice, respectively. CD19−/−Usp22fl/fl mice were bred with Usp22fl/fox mice, to generate Usp22 KO (CD19−/−Usp22fl/fox and WT littermates (i.e., Usp22fl/fox)) for experimental use (sex-matched). Mice were bred with Usp22−/− mice, to generate Usp22−/− with CD19−/−Cre (CD19−/−CreUsp22−/−) or Mbr−/−Cre mice, respectively. CD19−/−CreUsp22−/−fl/fox mice were bred with Usp22fl/fox mice, to generate Usp22 KO (CD19−/−CreUsp22fl/fox and WT littermates (i.e., Usp22fl/fox)) for experimental use (sex-matched; 12–16 weeks old). Mbr−/−CreUsp22−/−fl/fox mice were bred with Usp22fl/fox mice, to generate Usp22 KO (Mbr−/−CreUsp22−/−fl/fox and WT littermates (i.e., Usp22fl/fox)) for experimental use (sex-matched; 6–7 weeks old). 53BP1−/− mice were bred with 53BP1fl/fl to generate 53BP1−/−/− and WT littermates for experimental use (sex-matched; 6–8 weeks old). All the mice were maintained under pathogen-free conditions. The experimental procedures were approved by the Animal Care Committee of University of Toronto.

Usp22 and AID qPCR. Spleen B cells were purified from CD19−/−CreUsp22 KO or WT littermates (around 12–16 weeks old), using the EasySep Mouse B-cell isolation Kit (Stemcell Technologies). For LPS-stimulated group, spleen B cells (0.2 × 10^6/mL) were cultured in complete RPMI with 50 μg/mL LPS for 4 days. For IgG1/IgG2 switching, 25 ng/mL of interleukin (IL)-4 (R&D Systems) was added; for IgG2b switching, 30 μg/mL dextran-sulfate (Sigma Aldrich) was added; for IgG3 switching, 3 or 10 ng/mL anti-IgG dextran (SouthernBiotech) or 10 ng/mL of anti-IgG dextran (Bioxcell) was added, for B-cell CSR. For IgA CSR, the following antibodies were added: 10 ng/mL of anti-IgA dextran (SouthernBiotech), 10 ng/mL of anti-IgA dextran (Bioxcell); for IgA CSR, the following antibodies were added: 10 ng/mL of anti-IgA dextran (SouthernBiotech), 10 ng/mL of anti-IgA dextran (Bioxcell), and anti-IgG2b (SouthernBiotech) or anti-IgG2a (SouthernBiotech) antibodies were added. For A-EJ inhibition assay, splenic B cells were isolated from WT mice (around 6–10 weeks old) and cultured in complete RPMI media with the inhibitor, as previously described53. Spleen B cells (0.2 × 10^6/mL) were first stimulated with LPS (25 μg/mL) in the presence of 10 μM A-196 or control compound A-197 for 1 day. For IgG1 CSR, the cells were changed with new media containing LPS (50 μg/mL) plus IL-4, and fresh A-196 or A-197 for 4 days. For IgA CSR, splenic B cells were stimulated with LPS (50 μg/mL), IL-4, TGf-β, IL-5, and anti-IgD dextran for 4 days.

AID and H2Bub western blotting. Purified spleen B cells were stimulated with 50 μg/mL LPS for 3 days and then collected for western blotting with mouse anti-AID monoclonal antibody (Cell Signaling Technology, clone: L7E7, catalog number: 4975; 1/1000 dilution), or rabbit polyclonal antibody to total H2B (Abcam, catalog number: ab118977; 1/1000 dilution). For H2Bub western blotting, the purified spleen B cells were first stimulated with LPS for 2.5 days, exposed to 8 Grays of γ-radiation, and then collected at various time points for western blotting with mouse monoclonal antibody against H2BK120ub (Millipore, clone: 56, catalog number: 05-1312; 1/1000 dilution), or rabbit polyclonal antibody against total H2B (Abcam, catalog number: ab118977; 1/1000 dilution).

Spleen and BM B-cell profiling. Single-cell suspension of spleen was prepared from CD19−/−CreUsp22 KO or WT littermate mice (around 12–16 weeks old), whereas BM cells were extracted from hind legs of Mbr−/−CreUsp22 KO or WT littermates (around 6–7 weeks old), followed by red blood cell lysis. The cells were stained and resuspended in fluorescence-activated cell sorting (FACS) buffer (2% fetal bovine serum in phosphate-buffered saline (PBS)), and incubated with mouse anti-CD11b (BioLegend; 2.4G2 mAb; 1/100 dilution). As previously described54, splenic cells were stained with rat alkaline phosphocynase (APC)-conjugated anti-CD45R/B220 (RA3-6B2; SouthernBiotech; 1/150 dilution), rat eFluor 450-conjugated IgM (J11/41; eBioscience; 1/150 dilution), rat phycocerythrin (PE)-conjugated CD3 (AA4.1; eBioscience; 1/50 dilution), and rat fluoroscint isothiocyanate (FITC)-conjugated CD23 (B384; eBioscience; 1/100 dilution); BM cells were stained with APC-conjugated anti-CD45R/B220, eFluor 450-conjugated IgM, rat PE Cy7-conjugated CD43 (S7; BD Pharmingen; 1/150 dilution); rat FITC-conjugated CD24 (30-F1; eBioscience; 1/100 dilution), and rat PE-conjugated BP-1 (6C3; BioLegend; 1/100 dilution). The stained cells were incubated with PI, acquired on a BD LSR Fortessa X-20 flow cytometer (BD Biosciences), and analyzed by Flowjo software (Treestar Inc.).
which MultiScreen-HTS-HA filter plates were coated with NP32-BSA. Later steps were performed as previously described13,14.

**Rotavirus-specific ELISA and ELISPOT assay.** CD19-cre Usp22 KO or WT littermates (around 12–16 weeks old) were infected with rotavirus as we previously described15,16. Cells were flushed out from the femurs and tibia of CD19-cre Usp22 KO or WT littermates, followed by red blood cell lysis. MultiScreen-HTS-HA filter plates were coated overnight with inactive rotavirus antigen (Microtix) and then blocked with complete RPMI media. The plates were incubated with twofold serial dilutions of BM cells or lymphocytes from lamina propria overnight, followed by detection with horseradish peroxidase (HRP)-conjugated anti-mouse IgA Ab (SouthernBiotech, catalog number: 1040–05; 1/500 dilution). Then the colorimetric precipitating substrate for HRP, AEC (Vector Laboratories), was used to develop the plate. Positive dots on the membrane within wells were counted with a Nikon stereomicroscope.

**V(D)J and D-J PCR.** BM cells from C57BL/6 and BALB/c background mice were subsequently compared by the dot-plot analysis (percentage: 50–70%) for the distal and proximal V(D)J PCR. DNA from Fraction C of BM cells was used and two rounds of PCR amplification were performed as previously described17,18, the first round of PCR reaction containing both Vβ5.888 and Vβ7.183, and the Jμ 4E primer, was performed over 30 cycles (1 min at 95 °C, 1 min at 60 °C, and 1.5 min at 72 °C); for the second round of PCR, 1 μl aliquots of the first round PCR reaction was used, containing either Vβ5.888 or Vβ7.183, with the nested Jμ 4A primer (30 cycles: 1 min at 95 °C, 1 min at 63 °C, and 1.5 min at 72 °C). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control14.

**Cell cycle analysis and CFSE dilution assay.** The cell cycle analysis was performed with the use of Click-it Plus Edu Alexa Fluor 647 kit (ThermoFisher Scientific), Edu is the modified analog of thymidine and gets incorporated into newly synthesized DNA. In brief, purified spleen B cells from CD19-cre Usp22 KO or WT littermates were stimulated with LPS for 3 days, before pulsing with Edu for 4 h. The cells were fixed with 4% formaldehyde, permeabilized with Perm buffer, and stained with Click-it reaction cocktail. After washing, the cells were treated with 100 μg/ml RNase A for 10 min and then stained with PI. The stained cells were acquired on a BD LSRFortessa X-20 flow cytometer and analyzed by Flowjo software.

CSF assay. Spleen B cells were pulsed with 5 μM CSF (Celltrace, Life Technologies), washed, and cultured in the presence of LPS plus IL-4. Cells were collected 4 days after CSF pulsing and IgG1 CSR stimulation, stained with PE anti-mouse IgG1, and acquired with a BD LSRFortessa X-20 flow cytometer and analyzed by Flowjo software. The concentration of pulsed CSF decreases as the cells divide, and thus the intensity of CSF reflects the stages of cell division.

**hH2AX immunofluorescence staining.** Spleen B cells were purified from CD19-cre Usp22 KO or WT littermates, and stimulated with LPS for 2.5 days, before 0 or 8 Gy γ-irradiation (Gammacell 1000). At 1 h or 4 h post irradiation, the LPS-stimulated cells were cytospin onto poly-t-lysine slides, followed by Triton X-100 permeabilization. The slides were blocked with 10% BSA (BioShop) plus goat f(ab)2 polyclonal antibody against mouse IgG (Abcam, catalog number: ab6668; 1/100 dilution), to decrease nonspecific staining. The hH2AX staining using mouse monoclonal antibody against hH2AX (Millipore, clone: JBW301, catalog number: 05–636; 1/250 dilution) and quantification of foci formation were performed as we previously described14.

**Germline transcripts and switch junction analysis.** For germline transcript PCR, splenic B cells from CD19-cre Usp22 KO or WT mice were used for IgG1 or IgA CSR ex vivo for 2 days. RNA was extracted and cDNA was synthesized as described previously19. Semi-quantitative PCR was performed to amplify γH2AXExon1 and Iα-Ca germline transcripts using the primers as previously described17,18, and GAPDH was used as an internal control15.

For switch junction analysis, spleen B cells from CD19-cre Usp22 KO or WT littermates were induced for IgG1 or IgA CSR ex vivo for 4 days. Genomic DNA from these cells was extracted by chloroform/isoamyl alcohol precipitation and purified with ethanol. Switch μ-α and μ-γ1 junctions were PCR amplified with Q5 polymerase (New England Biolabs) using primers as we and others described17,18, then subsequently cloned using Zero Blunt TOPO (Life Technologies). Sequencing was carried out at The Centre for Applied Genomics (Toronto, Canada), and switch junction sequences were analyzed as we previously described14. To quantify AID activity at switch regions, we repeated the same procedure for switch junction analysis but instead using primers targeting the region upstream of switch μ or γ1.

**Switch region homology analysis.** Mouse immunoglobulin gene sequences for C57BL/6 were obtained from NCBI database (NC_000786.6 Ch12 and GRCh38.p4 C57BL/6J) and the switch regions, based on their highly repetitive nature were defined by dotplot analysis using the DNASTar software (percentage: 60 or 70, Window: 30 bp, Min Quality: 1). Switch regions for BALB/c mice were described previously20. The sequence homology of μ and downstream switch regions of C57BL/6 and BALB/c background mice were subsequently compared by the dot-plot analysis (percentage: 50–90, Window: 30 bp, Min Quality: 1).

**Statistical analysis.** Data were presented as mean ± SEM and were analyzed using two-tailed unpaired Student’s t-test or two-way analysis of variance as indicated. *p < 0.05, **p < 0.01, and ***p < 0.001.

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

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Author Contributions

C.L. designed the experiments, performed the research, analyzed data, and wrote the manuscript. T.I., C.S., M.B., E.L., and A.L. performed the research. Q.P., L.D., and J.L.G. analyzed data and edited the manuscript. A.M designed the experiments, analyzed data, and wrote the manuscript.

Additional information

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