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Increased Targeting of Donor Switch Region and IgE in S γ 1-Deficient B Cells

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Ab class switch recombination involves a recombination between two repetitive DNA sequences known as switch (S) regions that vary in length, content, and density of the repeats. Abs expressed by B cells are diversified by somatic hypermutation and class switch recombination. Both class switch recombination and somatic hypermutation are initiated by activation-induced cytidine deaminase (AID), which preferentially recognizes certain hot spots that are far more enriched in the S regions. We found that removal of the largest S region, S γ 1 (10 kb), in mice can result in the accumulation of mutations and short-range intra-S recombination in the donor S μ region. Furthermore, elevated levels of IgE were detected in trinitrophenol-OVA-immunized mice and in anti-CD40 plus IL-4-stimulated B cells in vitro. We propose that AID availability and targeting in part might be regulated by its DNA substrate. Thus, prominently transcribed S regions, such as S γ 1, might provide a sufficient sink for AID protein to titrate away AID from other accessible sites within or outside the Ig locus. *The Journal of Immunology*, 2010, 185: 000–000.

B cells produce a diverse repertoire of Abs to recognize foreign Ags. Early in development, through the function of RAG-1, and RAG-2, the V region of Ab gene is assembled by joining of one of many distant V, D, and J segments to form the V (D)J coding region (1). B cells with a properly assembled coding exon can migrate from the bone marrow to the periphery in the form of naive IgM-producing B cells, generally believed to produce the low-affinity pool of primary Abs (2). After encountering an Ag, activated B cells are able to further diversify the V coding region to generate high-affinity Abs to mount an effective immune response, a process known as somatic hypermutation (SHM) (3). In addition, B cells can replace the default C μ with a set of downstream C H regions via a mechanism known as class switch recombination (CSR) (4).

SHM is mediated by activation-induced cytidine deaminase (AID) (5, 6), which deaminates cytidine in DNA to uracil, with resulting mismatches ultimately processed by base excision and/or mismatch repair to generate point mutations at V regions at a high rate (10^{-3} – 10^{-4} nucleotides per cell division) (7). Mutations within the assembled V region extend from 200 bp downstream of the V promoter, within the leader intron, and continue through the V gene and ~1.5 kb into the intron (8). A consensus sequence DGYW (G:C is the

mutable position; D = A/G/T, Y = C/T, W = A/T) has been designated as a favored mutational hot spot based on compiled mutation spectra of endogenous V genes and transgenes (9). Among DGYW variants, AGCT is the preferred hot spot for SHM in vivo (9), which is also evolutionarily conserved in S regions (10, 11). S regions (see below) and their surrounding sequences can also undergo AID-dependent mutations during CSR, although at lower frequency, compared with the observed mutations within V regions (12).

CSR is also dependent on AID (5, 6), which targets specialized sequences, known as S regions, to ultimately generate DNA double-strand break (DSB) intermediates (13, 14). During CSR, the default C μ is replaced with other downstream C H exons (15). CSR enables the Ab to maintain its Ag-binding V region while switching its C H -encoded effector portion. In mice, S regions are organized in the following order: 5'-S μ -S γ 3-S γ 1-S γ 2b-S γ 2a-S ϵ -S α -3' and consist of a tandem array of short repeats ranging from 1 to 10 kb in length (11). Each S region is preceded by intronic (I) promoters (16). I μ (5' of S μ) is constitutively transcribed, whereas transcription and accessibility of downstream S regions depend on the particular stimuli that B cells receive (16). After binding to DNA (17, 18), AID is activated by phosphorylation through functions of prelocalized catalytic and regulatory subunits of protein kinase A (19). Long-range joining of AID-mediated DSBs in S μ and other S regions is carried out by either classical nonhomologous end joining or alternative end-joining pathways (20).

There are two types of recombination in the mouse C H locus. CSR is a long-range recombination (65–200 kb) between S μ and a downstream S region, whereas intraswitch recombination (ISR) is a short-range recombination (1–10 kb) within the same S region (21). It has been shown that the frequency of recombination directly correlates with the distance of the two DSBs (22). Therefore, the likelihood of joining two DSBs that are near each other is greater than with two that are far apart. Although AID-mediated ISR is particularly high in the S μ region (~15% in IgM-producing hybridomas) (21, 23–25) (26), its occurrence is independent of location within the IgH locus and can occur in randomly integrated S regions within the genome (21). Furthermore, in B cells in which

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Abbreviations used in this paper: AID, activation-induced cytidine deaminase; B, BamHI; CSR, class switch recombination; DSB, double-strand break; GL, germline; GLT, germline transcription; H, HindIII; I, intronic; ISR, intraswitch recombination; R1, EcoRI; SHM, somatic hypermutation; TNP, trinitrophenol; WT, wild-type; X, XbaI.

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the accessibility of AID to $\text{S}\mu$ region is suppressed, efficient recombination within $\text{S}\gamma 1$ was observed, suggesting that S regions might function independently of each other (27). Although CSR is abolished in 53BP-1-deficient B cells, an increased ISR is detected in both $\text{S}\mu$ and $\text{S}\gamma 1$, indicating that 53BP-1 might play a role in the synapsis/joining of distant DSBs (28). B cell hybridomas derived from AID-deficient B cells rarely exhibit ISR, demonstrating the essential role of AID in ISR (21). Although both ISR and CSR are AID dependent (5, 6), the exact relationship between the two is not well understood.

S regions are essential for CSR (29–32) and contain tandem repetitive hot spots, such as TGGGG, GGGGT, GGGCT, GAGCT, and AGCT (11). The numbers of tandem repetitive sequences within S regions determine how efficiently a particular S region can recruit AID to carry out CSR (33). In addition, mammalian S regions are G rich on the coding strand, a feature that is considered important for formation of R-loops and DNA/RNA hybrid strands, making them even better AID hot spots (30, 34). In the mouse, $\text{S}\mu$ has the highest number of tandem repeats, whereas $\text{S}\gamma 1$ is the largest (10 kb) and Se is the shortest S region with the fewest tandem repeats (11). Shortening the size of a particular S region (29, 33, 35) or replacing it with a less efficient one (31) reduces the rate of switching to the modified S region. Analysis of various species, ranging from amphibians to mammals, suggests that during the course of evolution S regions have increased their number and specificity of tandem repeats, G richness, and size to recruit AID more efficiently (11). These features are particularly improved in mammals, which can use the adaptive branch of the immune system very efficiently to clear foreign pathogens.

To address whether removal of $\text{S}\gamma 1$ would alter the targeting of other accessible S regions, we examined AID-associated events (CSR, ISR, SHM) in mice deficient in $\text{S}\gamma 1$ ($\Delta\text{S}\gamma 1$) B cells. We found that the absence of $\text{S}\gamma 1$ results in the accumulation of mutations and intra-S recombination in the donor $\text{S}\mu$ region. Furthermore, elevated levels of IgE were detected in immunized mice and in stimulated B cells in vitro. Our findings suggest that although $\text{S}\gamma 1$ primarily functions to serve as a prominent AID DNA target, it can also titrate AID activity away from other accessible sites in the IgH locus.

Materials and Methods

Primers, DNA constructs, oligo probes, and Abs

PCR conditions and primers used for germline transcription (GLT), CSR, SHM, and junction sequencing are described below. Mouse mAb anti-AID (39-2500; Zymed/Invitrogen, Carlsbad, CA) was used to detect AID by Western blot. Monoclonal anti-mouse Abs (BD Pharmingen, San Diego, CA) were used to detect Ig levels followed with alkaline phosphatase-conjugated goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL) as the detection Ab. Purified mouse Ig (BD Pharmingen) was used as the standard. Surface Ig staining was performed using PE anti-mouse Ig (BD Pharmingen) Abs.

Generation of $\text{S}\gamma 1$ mutant mice

The generation of mutant embryonic stem cells has been described previously (22). Mutant mice were generated by injecting modified embryonic stem cells into C57B6 blastocysts, which were maintained in 129sv background. Heterozygous mice were bred to generate homozygous or wild-type (WT) controls for different studies.

Hybridoma and B cell cultures

Spleen cells from 6- to 8-wk-old mice were stimulated in vitro with anti-CD40 (1 $\mu\text{g}/\text{ml}$; HM40-3; Pharmingen) plus IL-4 (25 ng/ml), with LPS (20 $\mu\text{g}/\text{ml}$) plus IL-4 (25 ng/ml), or just with LPS (22). A total of 0.5 million cells were seeded in one well of six-well plates in RPMI 1640 media supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin-streptomycin, and 100 μM β -mercaptoethanol. After stimulation, activated B cells were used to generate hybridomas in accordance with standard methods at day 4 (22). Briefly, splenocytes were fused with NS1 cells to make hybridomas.

After selection, hybridomas were tested by ELISA for expression of different Ab isotypes, and clonal hybridomas were expanded for making genomic DNA.

In vivo immunization

Trinitrophenol (TNP)-OVA 50 $\mu\text{g}/\text{alum}$ 2 mg was injected i.p. in 100 μl sterile PBS, followed by a second injection at day 28. Serum samples were collected (60 μl per time point) on days 3 (baseline), 7, 14, 28, and 42 via tail vein for Ab isotype measurements.

Isotype switching assay

Millipore (Bedford, MA) Beadlyte Mouse Immunoglobulin Isotyping Kit (catalogue no. 48-300) was used as recommended by the manufacturer to determine concentrations of different Ab isotypes. For in vitro stimulations, splenocyte Ig levels were measured at day 6, and for in vivo studies, the serum samples were collected at indicated dates post immunization. FACS analyses were done at day 4 of stimulation. Samples were collected on a FACSscan (BD Biosciences, San Jose, CA) and analyzed using Flowjo software (Tree Star, Ashland, OR). To measure TNP-OVA-specific IgG1 or IgE, ELISA plates (Nunc, Naperville, IL) were coated with TNP-OVA (Biosearch Technologies, Novato, CA). After blocking with BSA, mouse serum samples were added, and bound IgG1 and IgE were detected using specific Abs (BD Biosciences). Concentrations were determined using TNP-OVA-specific IgG1 and IgE isotype mAbs as standards.

Southern blot

Genomic DNA obtained from hybridoma clones was used to assess DNA rearrangement and CSR in the IgH locus. Genomic DNA was digested overnight with the indicated restriction enzymes (New England Biolabs, Beverly, MA), and the digest was resolved by applying the samples to a 0.8% agarose gel. The resolved samples were then transferred to a ζ -probe blotting membrane (Bio-Rad, Hercules, CA) and fixed by ultraviolet cross-linking and/or baking in an 80°C vacuum oven (20 min). [^{32}P] labeled $\text{S}\mu$ and Se oligo probes or $\text{C}\mu$ (21) and Ce probes were used following standard Southern blotting protocols. The labeled DNA was visualized by exposure to x-ray films (Kodak, Rochester, NY).

SHM in the Jh4-E μ region of immunized WT littermate and $\Delta\text{S}\gamma 1$ mice

WT littermate or $\Delta\text{S}\gamma 1$ mice were immunized with human CD4-FC (CD4 molecule fused to the FC region of Ab; Genentech, San Francisco, CA) once a week, and 42 d later IgM $^{+}$ germinal center cells were isolated from spleen of these mice by FACS sorting (GL7 $^{+}$, IgM $^{+}$, B220 $^{+}$). DNA was prepared from the sorted cells, using standard protocols. Extracted DNA was then used as a template to amplify the Jh4-E μ region of IgM $^{+}$ germinal center cells using the following primers and PCR protocol: Forward: 5'-GGTGGAGTCCC-TGGATGATGGGATAGGG-3'; Reverse: 5'-AAAGATTGTGTGAAGCCG-TTTTGACCAGAATGTC-3'; 95°C for 2 min; (95°C for 30 s, 60°C for 30 s, and 72°C for 90 s) $\times$ 35 cycles, 72°C for 7 min. The PCR product was cloned into 2.1-TOPO vector using a TOPO-TA cloning kit according to the manufacturer's protocol (Invitrogen, catalogue no. 45-0641). Vectors with Jh4-E μ inserts were propagated and purified using standard protocols and were sequenced with following primers: M13Forward, M13Reverse, internal forward: 5'-GGAGGCTCATTGAGGGAGATGCTA-3', and internal reverse: 5'-GTCAAGATGGCCGATCAGAAC-3'. Nonredundant mutations observed in this region were recorded as SHM.

PCR primers and oligo probes

Primers used to measure mouse GLT by TaqMan (Applied Biosystems, Foster City, CA) and RT-PCR were as follows: I $\gamma 1$ Forward (F): 5'-GGCCCTTCCAGATCTTTGAG-3', C $\gamma 1$ Reverse (R): 5'-GGATCCAGA-GTTCCAGGTAAGT-3', I $\gamma 3$ F: 5'-TGGGCAAGTGGATCTGAACA-3', C $\gamma 3$ R: 5'-CTCAGGGAAGTAGCCTTTGACA-3', I ϵ F: 5'-TGGGAT-CAGACGATGGAGAATAG-3', C ϵ R: 5'-CCAGGGTCATGGAAGCAG-TG-3', β -actin F: 5'-TACCTCATGAAGATCCTCA-3', β -actin R: 5'-TT-CGTGGATGCCACAGGAC-3', GAPDH F: 5'-GTGGCAAGTGGAGAG-TTGTGGCC-3', GAPDH R: 5'-GATGATGACCCGTTGGCTCC-3'.

The following primers were used in nested PCR for detection of mouse e CSR: E μ F: 5'-CTCTGGCCCTGCTTATTGTTG-3', Ce exon-I R1: 5'-CCTGATAGAGGCTGTGAGAAAGGAAGGACC-3', I μ F: 5'-AGACC-TGGGAATGTATGGTT-3', Ce exon-I R2: 5'-TAGGTTAGACTTATTTAT-ATCACTGCATGC-3'.

The following internal oligo probes were used in Southern blot to confirm $\text{S}\mu$ -Se CSR in amplified PCR products: Ce oligo probe: 5'-TGCCCCCT-

CGCTGGCTTAGCACAGGGGCTTC-3', S μ oligo probe: 5'-GTTGAG-AGCCCTAGTAAGCG-3'.

Primers used to sequence S μ -Se junctions in IgE⁺ clones were the following: 1 F: 5'-AATGGATACCTCAGTGGTTTTAATGGTGG-3', 2 F: 5'-GAGAAAGAATAGAGACCTGCAGTTGA-3', 3 F: 5'-CTAAACTAGGCTGGCTTAACCGAGATGA-3', 1 R: 5'-CCCACAGGGCTTGGTGGCCAGAGATCCGA-3', 2 R: 5'-CCCCGTGATTCTTACACCCATCAGGCTT-3', 3 R: 5'-GCCCGATTGGCTCTACCTACCCAGTCTGGC-3', 4 R: 5'-ACAGCCTAGAATAGTCTAGAACTGCCAAC-3'.

Primers used to amplify I ϵ to the C ϵ region (~4.5 kb) in IgM⁺ clones to detect Se internal deletion are the same as the ones used for GLT: I ϵ F and C ϵ R (see above). Primers used to amplify an ~1-kb region of Se to analyze internal deletions are as follows: Se F: 5'-AGCTAGACTACACTGAGTTAACCTG-3', Se R: 5'-GTTTAGCCCAATCCAGTCCAGTCCAACCAATCTAT-3'. In both cases genomic DNA was used as a template.

PCR primers that were used to amplify Jh4 to the S μ region to analyze the rate of SHM in IgM⁺ clones were the following: Jh4 F: 5'-GCTTGAGTAGTTCTAGTTTCCCAAAC-3', C μ R: 5'-GCCTTCTAGCCACAGGACAGTGACTT-3'.

Primers used to sequence Jh4-S μ PCR products are as follows: Jh4 F: 5'-GCTTGAGTAGTTCTAGTTTCCCAAAC-3', 3p.J4-C μ F1: 5'-CTGAGCTAGCATGGGCTG-3', 3p.J4-C μ R1: 5'-ACCAGGCCCTACTCAGCC-3', 3p.J4-C μ F2: 5'-AGCCTAGCCTGAGCTGGAC-3', 3p.J4-C μ R2: 5'-GGTCCTAACTGGTCCAGGC-3', 5p.J4-C μ F1: 5'-CCACTTCTGAGTATTCATTTAGG-3', 5p.J4-C μ R1: 5'-ATAAGCAGGCCAGAGGG-3', 5p.J4-C μ F2: 5'-TGTGTGTCTTTGAGTACCGTTG-3', 5p.J4-C μ R2: 5'-CAGCATCAGTACCTCACTAATACAC-3', 5p.J4-C μ

F3: 5'-GTGAGAGGACAGGGGCTG, 5p.J4-C μ R3: 5'-GGCCTTCTCTGTCTGGGTC-3', C μ R: 5'-GCCTTCTAGCCACAGGAACGTGACTT-3'.

Germline transcription by RT-PCR and TaqMan

For RT-PCR, we isolated total RNA from the stimulated splenocytes (day 2) and prepared cDNA from mRNA (300 ng total RNA was used), as explained previously (23). Prepared cDNA samples along with listed primers (see above) were then used to measure GLT. PCR cycles were at 94°C for 3 min; (94°C for 30 s, 59°C for 30 s, and 72°C for 60 s) $\times$ 27 cycles, 72°C for 5 min. For GAPDH, 55°C annealing temperature and 32 cycles were used. For e, 39 cycles were used. For TaqMan, splenocytes from 3 WT and 3 Δ Sy1 mice were stimulated for 2 d, and RNA was prepared from these samples. Then, 50 ng prepared RNA was used with Promega Access RT-PCR System (A1250) (Promega, Madison, WI) following the manufacturer's protocol with inclusion of SYBR Green (S7563 Invitrogen). All samples were in triplicates, and Applied Biosystem's 7500 Real Time PCR System was used to collect and analyze the read-outs.

Determining S μ -Se CSR and sequencing S μ -Se junctions by PCR

To sequence S μ -Se junctions in IgE-positive Hybridoma clones, we use nested PCR to amplify and sequence this region. First, we used E μ F and C ϵ exon-IR1 primers to amplify this region from genomic hybridoma DNA. PCR cycles were at 94°C for 2 min; (94°C for 10 s, 60°C for 30 s, and 68°C for 150 s) $\times$ 35 cycles, 68°C for 7 min. The product from this reaction was used as a template (2 μ l) for a second PCR reaction using I μ F and C ϵ exon-I R2 primers. PCR cycles were the same as above, except the annealing temperature was lowered to 55°C. PCR products were then gel purified (Qiagen, Valencia, CA) and directly sequenced.

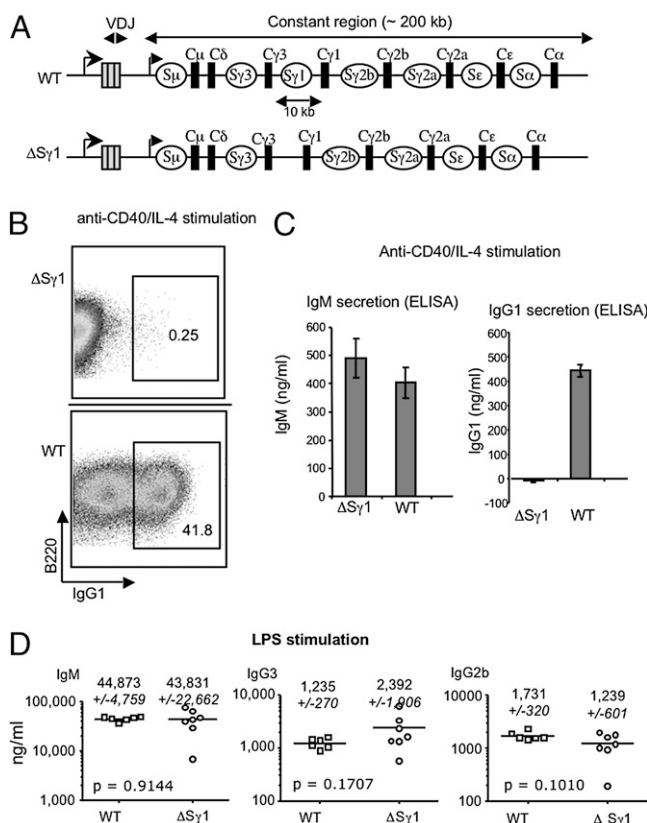


FIGURE 1. Isotype switching to IgG1 is abolished in Δ Sy1 splenocytes. *A*, Schematic presentation of IgH locus C region in WT and Δ Sy1 mice. Note that in Δ Sy1 mice, Sy1 is replaced with a 0.5-kb inert DNA insert (22). *B*, Splenocytes from WT and Δ Sy1 mice were stimulated with anti-CD40/IL-4, and 4 d post stimulation, B cells were analyzed for expression of surface IgG1 by FACS. *C*, Secretion of IgG1 and IgM Abs were measured by ELISA 6 d post stimulation. WT and Δ Sy1 splenocytes were stimulated with anti-CD40/IL4. Data shown are the average value obtained from three (WT) and six (Δ Sy1) mice. Error bars represent SD. *D*, Splenocytes from WT ($\square$) or Δ Sy1 ($\circ$) mice were stimulated with LPS, and 6 d post stimulation, levels of secreted IgM, IgG3, and IgG2b were measured by ELISA. Each circle or square represents data obtained from one mouse.

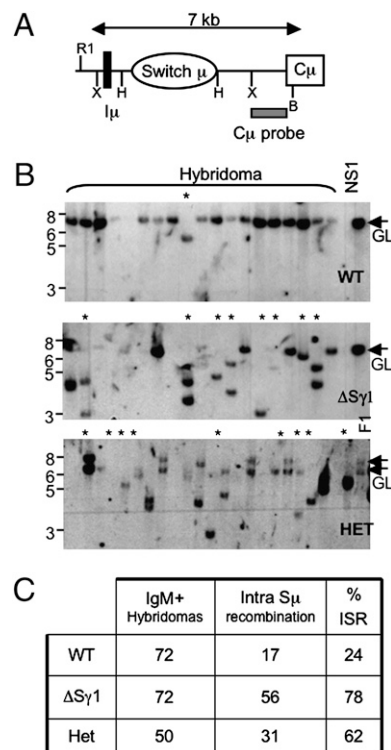


FIGURE 2. Increased internal S μ recombination in Δ Sy1 splenocytes. *A*, Schematic representation of the μ region in mouse IgH. C μ DNA probe used for Southern blot analysis in *B* is depicted. *B*, Southern blot analysis of internal S μ deletion in IgM⁺ hybridomas from WT, heterozygote, and Δ Sy1 mice. Genomic DNA was digested with EcoRI and BamHI, and a C μ DNA probe was used to analyze deletions in the S μ region of NS1, as this particular region is deleted in the NS1 cell line. *C*, Table summarizing IgM⁺ hybridomas analyzed for number of alleles displaying internal S μ recombination. *B*, BamHI; GL, germline; H, HindIII; R1, EcoRI; X, XbaI. MW marker unit is kb.

Results

Abolished IgG1 isotype switching in Δ S γ 1 B cells

To generate S γ 1 mutant mice (Δ S γ 1), the S γ 1 region (10 kb) was replaced with a 0.5-kb inert I sequence containing two ISceI sites (Fig. 1A). B cells generated from Δ S γ 1 blastocyst chimeras in a RAG-deficient background cannot support CSR to IgG1 (22); however, the generation of germline mice enabled us to dissect the role of S γ 1 in detail. In splenic B cells from WT and Δ S γ 1 mice were stimulated with indicated stimuli and were analyzed on day 4 by FACS and day 6 by ELISA (Fig. 1B–D). FACS analysis showed that levels of IgG1⁺ B cells in WT splenocytes stimulated with anti-CD40/IL4 is ~40%, whereas IgG1⁺ B cells in Δ S γ 1 splenocytes is ~0.25%. This finding shows a 160-fold decrease in levels of IgG1⁺ B cells in WT compared with Δ S γ 1 splenocytes (Fig. 1B). Analyzing secreted IgG1 and IgM levels in anti-CD40/IL4-stimulated splenocytes via ELISA showed that although default IgM Ab levels were comparable in both WT and Δ S γ 1 B cells, Δ S γ 1 B cells could not secrete any detectable levels of IgG1 (Fig. 1C).

To show that the IgH locus is intact and functions normally, we stimulated WT or Δ S γ 1 B cells with LPS, which does not result in IgG1 CSR, and monitored isotype switching to IgG3, and IgG2b. We found that levels of CSR to IgG3, and IgG2b are not significantly different between WT and Δ S γ 1 B cells (Fig. 1D). Furthermore, AID expression levels (Supplemental Fig. 1A), or GLT levels (Supplemental Fig. 1B, 1C) remain intact in Δ S γ 1 splenocytes, suggesting that lack of S γ 1 region is the only factor that contributes to abolishment of CSR to IgG1.

Enhanced intra-S μ recombination in Δ S γ 1 B cells

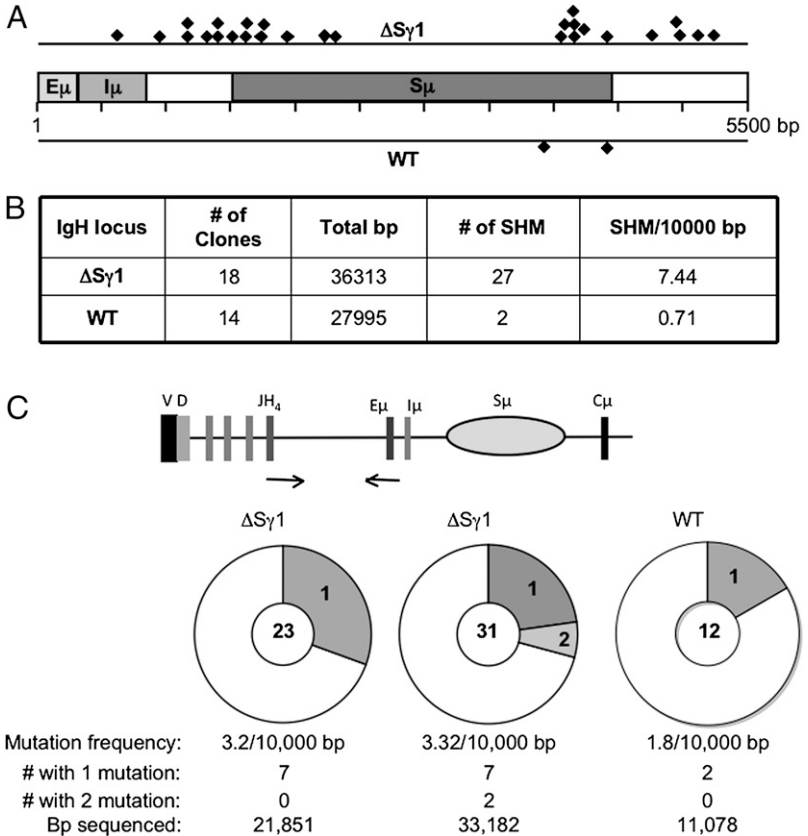
The S γ 1 region represents the largest (10 kb) S region in the mouse IgH locus (11). Therefore, we asked whether removal of S γ 1 could influence other accessible AID targets in the locus. To answer this

question, we generated hybridomas from anti-CD40/IL4-stimulated WT or Δ S γ 1 splenocytes and looked for the frequency of ISR in different S regions that are transcribed under this stimulation. ISR is often seen at low frequency (~15–20%) in S μ but usually not other downstream S regions (21, 25). Such deletions manifest themselves as smaller DNA fragments when analyzed by Southern blotting.

Genomic DNA from WT, Δ S γ 1, and heterozygous IgM⁺ hybridomas was digested with EcoRI and BamHI restriction enzymes and subjected to Southern blot using a C μ probe (Fig. 2A, 2B) to analyze ISR in S μ . We observed 56 and 31 internal S μ deletions, respectively, out of 72 Δ S γ 1 and 50 heterozygous hybridomas, whereas only 17 internal S μ deletions were observed among 72 WT hybridomas analyzed (Fig. 2B, 2C). Furthermore, larger internal S μ deletions were detected in homozygous Δ S γ 1 (15 and 6 hybridomas with deletions >1 kb and 2 kb, respectively) and heterozygous Δ S γ 1 (8 and 2 hybridomas with deletions >1 kb and 2 kb, respectively) relative to WT (only 7 hybridomas with deletions <1 kb), as inferred by Southern blot analysis (Fig. 2B, 2C). Overall, we observed ~3.3- and 2.6-fold higher levels of intra-S μ deletions in Δ S γ 1 and heterozygous hybridomas, respectively, relative to WT.

To analyze ISR in the S ϵ region of WT and Δ S γ 1 splenocytes, we used PCR to amplify I ϵ -C ϵ region of IgH (4.5 kb) that encompasses S ϵ . We did not find any ISR in the S ϵ region of WT or Δ S γ 1 hybridomas (Supplemental Fig. 2A). To rule out small internal S ϵ deletions, we used PCR primers from 5' and 3' boundaries of S ϵ to amplify a 1-kb span of this region. Again we failed to detect any reduction in the size of PCR products that could be indicative of ISR in the S ϵ region (Supplemental Fig. 2B). This could result from the fact that S ϵ is a very poor AID hot spot and therefore it is very unlikely for S ϵ to undergo large ISRs even in Δ S γ 1 splenocytes (11). However, we cannot rule out the possibility that microdeletions may be generated in this region. It is also possible that S μ is prone to frequent DSBs, consistent with the finding that under

FIGURE 3. Δ S γ 1 splenocytes display higher number of mutations. **A**, PCR was used to amplify a region of IgH locus spanning downstream of Jh4 to S μ from IgM⁺ hybridomas (no amplification from NS1 host cells). The PCR products were gel purified and sequence analyzed for mutations occurring from SHM. The approximate position of mutations observed in WT and Δ S γ 1 colonies is represented by ♦. **B**, Table comparing and summarizing the number of mutations in hybridomas that gave positive PCR products. $p = 0.0014$, using Poisson generalized linear model analysis. **C**, Increase in rate of SHM in the Jh4-E μ region of Δ S γ 1 mice compared with WT littermates. The Jh4-E μ region was amplified by PCR from IgM⁺ germinal center cells of mice 42 d post immunization with human CD4 extracellular domain fusion protein. Nonredundant and unique mutations in this region are considered SHM. Mutation frequency is denoted as number of mutations per 10,000 bp. Two Δ S γ 1 mice and one WT mouse were used for this analysis.



normal conditions large deletions are not usually detected in downstream acceptor S regions (28) (21).

SHM is increased in ΔSγ1 B cells

We postulated that lack of Sγ1 might alter the availability of AID and therefore increase SHM. To analyze the rate of SHM in WT versus ΔSγ1 splenocytes, we designed PCR primers downstream of JH4 and upstream of the Cμ region to look at mutations in the vicinity of the Sμ region (within the Cμ probe, Fig. 2A). Because the region encompassing the Cμ probe is deleted in NS1 cells (see Fig. 2B legend), the entire DNA amplified by PCR must originate from the primary B cells. PCR products from IgM⁺ WT or ΔSγ1 hybridomas were sequenced and analyzed for mutations against the germline JH4-Cμ of IgH. Fig. 3A illustrates a schematic of the PCR-amplified region and approximate location of mutations recorded from WT or ΔSγ1 samples. We observed a 10-fold increase for mutations in ΔSγ1 hybridomas (7.44×10^{-4}), compared with WT (0.71×10^{-4}) hybridomas (Fig. 3B).

To determine whether removal of Sγ1 can change the rate of SHM in the vicinity of V regions, we analyzed mutations observed in the Jh4-Eμ region of the IgM⁺ germinal center in immunized WT littermate or ΔSγ1 mice (Fig. 3C). The Jh4-Eμ region is normally intact in all B cells and can be used as a surrogate to study the rate of SHM (3). Generally, the rate of SHM in IgM⁺ B cells is low (2). We analyzed the rate of SHM 42 d post immunization in two ΔSγ1 and one WT littermate mice. Our result shows a modest increase (almost 2-fold) in SHM in ΔSγ1 mice (3.2 mutations/10,000 bp) compared with WT littermates (1.8 mutations/10,000 bp) (Fig. 3C). Similar results were observed when the Jh4-Eμ regions of IgM⁺ ΔSγ1 and WT hybridomas were analyzed (Supplemental Table II). Therefore, in the absence of the Sγ1 region, our data support a clear increase in the rate of mutation in the Iμ-Sμ region and a modest increase in the rate of SHM in Jh4-Eμ region. This observation suggests that removal of Sγ1 can redirect AID to other available targets, resulting in an increase in the rate of CSR, SHM, and internal switch deletion.

Sμ-Se recombination is increased in the nonproductive alleles of ΔSγ1 B cells

Although RAG1/2-mediated V(D)J recombination involves the process of allelic exclusion, AID-mediated CSR occurs at the IgH locus of both chromosomes (4). Only the IgH locus with properly rearranged VDJ can produce IgM or other isotype switched Abs. We reasoned that if the removal of Sγ1 results in more frequent AID targeting at other available hot spots, then the nonproductive allele provides a valid target for analyzing AID activities within the IgH locus. We analyzed Sμ-Se CSR in nonproductive alleles of IgG1⁺ heterozygous (ΔSγ1/WT) or WT hybridomas. Because, in the IgG1⁺ heterozygous colonies, the WT allele must be the productive allele, the rate of Sμ-Se CSR can be analyzed in a setting where one ΔSγ1 and one WT allele can be compared (Fig. 4A).

We used PCR primers to amplify the Iμ-Cε region of the IgH locus, which can be amplified only if Sμ-Se CSR has occurred in the nonproductive allele of IgG1⁺ WT or heterozygous hybridomas. The PCR products were further analyzed by Southern blotting using a Cε oligo probe, confirming that they represent Sμ-Se CSR (Fig. 4B). Approximately 50% of the analyzed IgG1⁺ heterozygous colonies showed Sμ-Se CSR in their nonproductive allele, whereas we did not see any Sμ-Se CSR in WT samples. We also screened the IgM⁺ WT and ΔSγ1 hybridomas for Sμ-Se CSR by PCR. Our data show that ~30% of ΔSγ1 B cells undergo Sμ-Se CSR in their nonproductive alleles, a marked increase when compared with WT splenocytes (1.4%) (Fig. 4C).

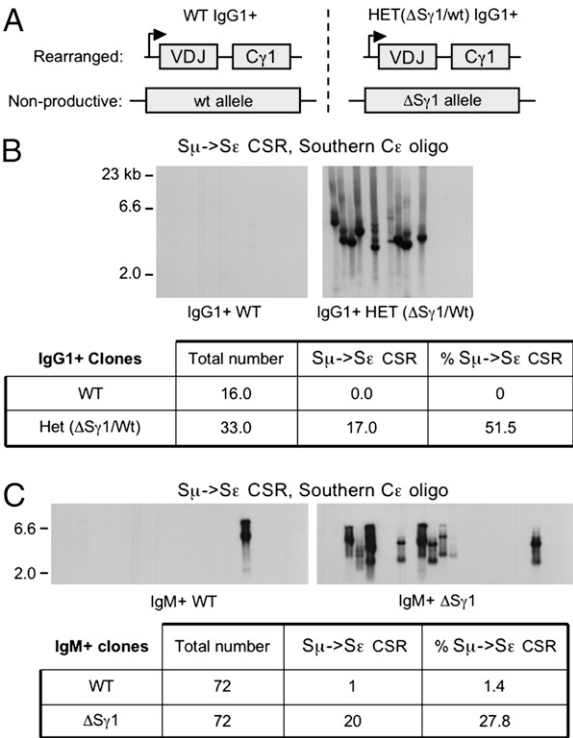
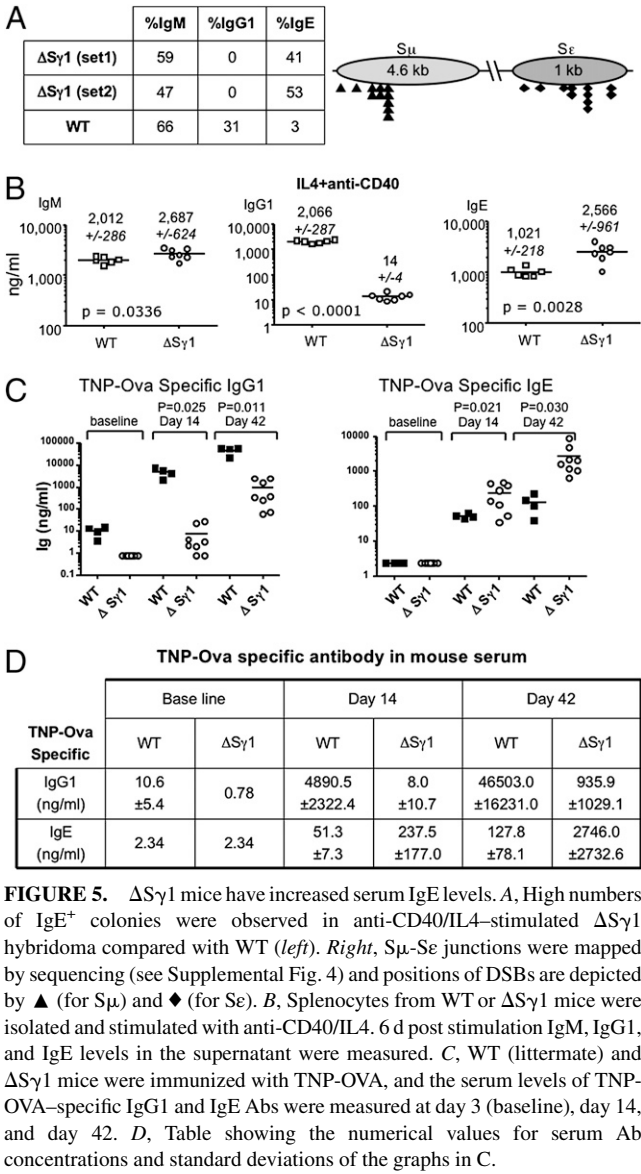


FIGURE 4. Increased Sμ-Se CSR in nonproductive alleles of ΔSγ1 splenocytes. **A** Illustrates that both WT and heterozygous (ΔSγ1/WT) IgG1⁺ hybridomas have a single nonproductive allele that can also undergo CSR. **B**, PCR was used to analyze the nonproductive alleles of IgG1⁺ WT and heterozygous hybridomas for Sμ-Se CSR. Amplified PCR products were further analyzed by Southern blotting, using an internal Cε oligo probe, to confirm Sμ-Se CSR. Table summarizes the number of colonies screened and the number of Sμ-Se CSR observed in nonproductive alleles of IgG1⁺ colonies. **C**, As for **B**, except IgM⁺ WT and ΔSγ1 hybridomas were screened for Sμ-Se CSR in their nonproductive alleles.

Serum IgE levels are increased in ΔSγ1 mice

When stimulated with anti-CD40/IL4, more hybridomas derived from ΔSγ1 splenocytes were IgE⁺ compared with WT splenocytes (Fig. 5A, left panel). To ensure that these colonies are indeed IgE⁺, we used PCR analysis to amplify Sμ-Se CSR products using 5'-Iμ and 3'-Cε primers, followed by Southern blotting using internal Sμ and Cε oligo probes (Supplemental Fig. 3A). We further confirmed Sμ-Se CSR by digesting genomic DNA from IgE⁺ hybridomas with EcoRI followed by Southern blotting using a Cε probe (Supplemental Fig. 3B, 3C). Results from all of these experiments clearly show that anti-CD40/IL4 stimulated ΔSγ1 IgE⁺ hybridomas have indeed undergone Sμ-Se CSR. We further mapped the recombination sites of Sμ-Se by sequencing Sμ-Se junctions from IgE⁺ hybridomas (Supplemental Fig. 4). Fig. 5A, right panel, depicts approximate locations of junctional breakpoints within Sμ and Se regions mapped by sequencing.

On the basis of these results, we reasoned that the total in vitro IgE Ab levels in anti-CD40/IL4-stimulated ΔSγ1 splenocytes should be higher than in WT splenocytes. To address this question, we stimulated WT and ΔSγ1 splenocytes with LPS (control) or anti-CD40/IL4 for 6 d and measured levels of relevant Ab isotypes in the supernatant. Because stimulation with LPS does not result in transcription from Sγ1, this region remains inaccessible and should not have any effects on isotype switching from IgM to IgG3 or IgG2b. For samples stimulated with LPS, we measured levels of IgM, IgG3, and IgG2b, and, as expected, found no significant differences in Ab levels between WT and ΔSγ1 splenocytes (Fig. 1D). Interestingly, in



anti-CD40/IL4-stimulated samples we noticed a 2.5-fold increase in the levels of IgE Ab in $\Delta S\gamma 1$ samples compared with WT (Fig. 5B, *right panel*). As expected, $\Delta S\gamma 1$ samples did not express any significant amounts of IgG1 Ab, whereas the levels of IgM Ab were comparable between WT and $\Delta S\gamma 1$ splenocytes stimulated with anti-CD40/IL4 (Fig. 5B, *middle and left panels*).

To test whether IgE levels are higher in vivo, we immunized $\Delta S\gamma 1$ and WT littermate mice with TNP-OVA to invoke a T cell-mediated response, mimicking anti-CD40/IL4 stimulation. Serum samples were collected on days 3 (baseline), 14, and 42 post immunization. We measured both total (IgM, IgG1, and IgE) and TNP-OVA-specific (IgG1 and IgE) Ab levels in these mice. Although the total IgM Ab levels were comparable, $\Delta S\gamma 1$ mice had ~3-fold higher levels of total IgE Ab and no significant IgG1 expression, compared with WT mice, on day 42 (Supplemental Fig. 5, Supplemental Table I). More importantly, $\Delta S\gamma 1$ mice had almost 20-fold higher levels of TNP-OVA-specific IgE Ab, compared with WT mice, on day 42. Again, $\Delta S\gamma 1$ mice failed to produce any significant levels of TNP-OVA-specific IgG1 Ab ~50-fold lower than that of the WT at day 42 (Fig. 5C, 5D). The low levels of IgG1 isotype switching in $\Delta S\gamma 1$ mice is likely due to AID targeting of DGYW sites (e.g., AGCT) within the inert DNA sequence (nine AGCT sites were identified by

sequencing; data not shown). These inert sequences are able to support extremely low (1–2%) levels of CSR in vitro (31). To measure the magnitude of IgE induction in a disease model, we used TNP-OVA experimental murine model of airway lung inflammation. In this model, after sensitization of mice in the periphery, challenge by multiple intratracheal instillations of OVA results in induction of IgE, as measured on day 42 compared with baseline levels at day 33. Consistent with our general TNP-OVA immunization model, higher levels of IgE were seen in the $\Delta S\gamma 1$ mice than in the WT group, suggesting that lack of S $\gamma 1$ affects the level of IgE isotype switching (Fig. 6A).

Discussion

We have shown that removal of a prominent AID target (S $\gamma 1$) results in more frequent AID targeting of donor S μ regions and increased IgE levels. Availability of S $\gamma 1$ significantly influences the targeting of participating donor S μ regions on both chromosomes in *cis* and *trans*. In addition, we have observed a high incidence of S μ -S ϵ recombination in the nonproductive alleles of $\Delta S\gamma 1$ B cells. It appears that AID mediates numerous and frequent DSBs in the S μ region, many of which are resolved by short-range joining of the breaks that are manifested as intra-S μ deletions. Meanwhile, AID targets downstream S regions (such as S ϵ) less

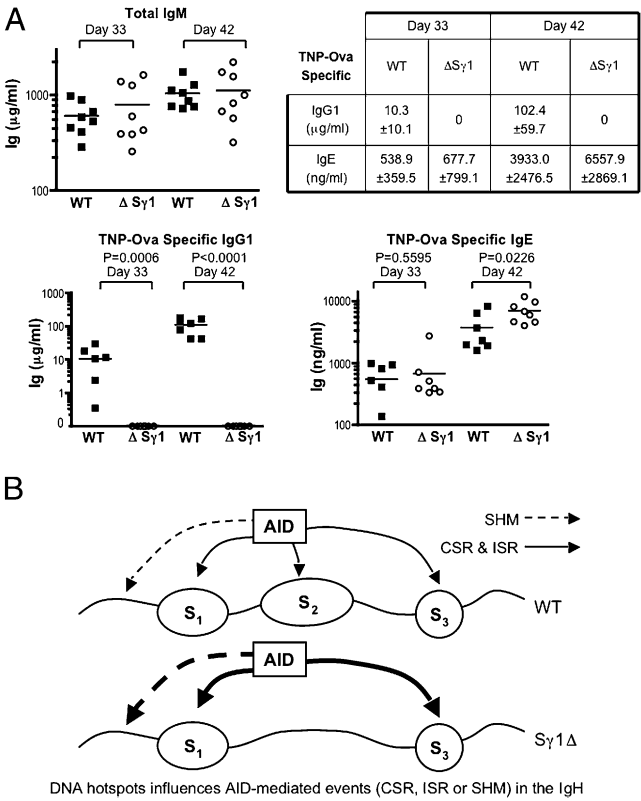


FIGURE 6. **A**, TNP-OVA-specific IgG1 and IgE titers in lung airway murine inflammation model. Serum samples were collected at day 42 post TNP-OVA challenge. Baseline levels at day 33 prechallenge were identical for both WT and $\Delta S\gamma 1$ groups. **B**, A proposed model for the role of AID hot spots in AID targeting. To carry out its function, AID needs to be targeted to proper sites in the genome. Targeting of AID to the VDJ region results in SHM, whereas in the S region AID creates DSBs followed by CSR or ISR. Removal of an AID target (in this case, the transcribed S region, S₂) can retarget AID to other hot spots, resulting in an increased SHM, CSR, and ISR in these sites (depicted by thicker arrow lines). Dashed and solid arrow lines represent SHM and CSR/ISR, respectively.

frequently, creating single DNA breaks (or microdeletions) that can support CSR when joined to S μ by long-range recombination.

Before the discovery of AID, utilizing a Δ S γ 1 mouse in which the promoter element of S γ 1 region was deleted, Radbruch's group (36, 37) reported that frequency of CSR to the S ϵ locus is independent of prior switching to other S regions. Interestingly, Δ S γ 1 mice, which should, to a certain degree, phenocopy Δ S γ 1 mice (inaccessible S γ 1 vs. deleted S γ 1), also showed a higher level of IgE than that in WT in in vitro stimulated B cells (37). The current paper, together with the previous study, suggests that sequential switching in S μ -S γ 1-S ϵ does not play an important role in IgE isotype switching because in the absence of S γ 1, IgE levels, if anything, are elevated (38–40). By competing for AID, an accessible S γ 1 region may reduce the switching to S ϵ rather than enhancing it. This raises the possibility that size/accessibility of S regions and their ability to recruit AID can play a role in the efficiency of S μ -S ϵ CSR and therefore IgE levels. In humans, the size of the S γ 4 region has been reported to be polymorphic (41). In this context, it is tempting to postulate that the size and accessibility of certain S regions might impact isotype switching to other isotypes, such as IgE.

CSR and SHM share several features, and therefore it is possible that at some stage during B cell activation, the availability of a CSR substrate might influence SHM frequency. First, a single enzyme, AID, is essential for both of these processes and mutation of AID leads to complete abrogation of SHM and CSR (5, 6). Second, the targeting mechanism of V or S regions during both SHM and CSR is facilitated by transcription likely to provide AID access to these DNA regions (16). Third, the DNA target motifs within V and S regions overlap and predominantly consist of DGYW hot spots (11). Fourth, fish AID, which is presumably evolved to mediate only SHM, can efficiently rescue CSR of AID^{-/-} mouse B cells, suggesting that AID presumably evolved only to undergo SHM and had the potential to catalyze CSR before the teleost and tetrapod lineages diverged (42, 43). Fifth, the size of AID target hot spots directly determines CSR frequency in a linear fashion, demonstrating that DNA substrates dictate AID targeting (33). Sixth, ancient amphibian S regions resemble SHM substrates, except for the density of hot spots, which is far more enriched in the S regions (31). Finally, CSR and SHM share several repair mechanisms, such as base excision and mismatch repair (11).

Given all these known common features between CSR and SHM and our current data, we propose that S region substrate availability impacts the frequency of DSBs in other S regions as well as SHM (Fig. 6B). This model suggests that removal of an AID target site with enriched hot spots, in this case a S region (S $_2$), can result in more frequent targeting of other available hot spots by AID. This underlines the importance of S regions to recruit AID and prevent its off-target activities. In support of this model, lupus-prone mice display high levels of AID and extensive DNA lesions, constituting S region deletions and mutations at least in the IgH locus (44). In addition, higher instances of mutations have been reported in IgM⁻IgD⁺CD38⁺ germinal center B cells, which have lost their S μ region (45). It is possible that under conditions of chronic inflammation or infection where B cells undergo rounds of activation, a higher chance of ISR might predispose aberrant AID-associated events elsewhere in the genome.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental figure legends

Supplemental figure 1

A) AID expression is normal in both WT and $\Delta S\gamma 1$ splenocytes. WT and $\Delta S\gamma 1$ splenocytes were obtained and a fraction (WT) was collected as non-stimulated while the rest were stimulated with LPS, LPS/IL4, or anti-CD40/IL4 for 48 hours. Cells were then lysed and anti AID antibody (1:1000) was used to monitor expression of AID by western blotting.

B) Measuring germline transcription by RT-PCR. Splenocytes from WT or $\Delta S\gamma 1$ mice were stimulated with LPS, or anti-CD40/IL4 for 2 days. RNA was isolated from these cells in order to make cDNA, which was subsequently diluted (1, 1/5, and 1/25) and used in PCR reactions to amplify the intended transcription targets (see materials and methods).

C) Germline transcription measured by Taqman. Splenocytes from 3 WT and 3 $\Delta S\gamma 1$ mice were stimulated with LPS, or anti-CD40/IL4 for 2 days and RNA was isolated from them. 50 ng of RNA from each mouse was used per reaction and in triplicates for measuring each transcript. The average values and standard deviations for each group are represented.

Supplemental figure 2

A) Detection of internal Se deletions. PCR primers were used to amplify a region of IgH (about 4.5 kb) corresponding to I ϵ to C ϵ , from $\Delta S\gamma 1$ IgM⁺ hybridomas. No obvious changes in size that can be attributed to internal Se was observed.

B) Same as A, except PCR primers from 5' and 3' boundaries of S ϵ were used to amplify a 1 kb region of S ϵ from WT and Δ S γ 1 IgM⁺ hybridomas. No changes in size, suggestive of any internal S ϵ , was observed in WT or Δ S γ 1 samples.

Supplemental figure 3

A) Confirming S μ -S ϵ CSR in Δ S γ 1 IgE⁺ hybridomas. PCR primers from 5' E μ and I μ and 3' C ϵ were used in nested PCR to amplify a product that can only be observed if S μ -S ϵ CSR has occurred (top). Southern blot analysis, using C ϵ probe (middle) and S μ probe (bottom), was further utilized to confirm that S μ -S ϵ CSR has indeed occurred in Δ S γ 1 IgE⁺ hybridomas.

B) Schematic representation of C ϵ region in mouse IgH locus. C ϵ probe used in southern blot analysis has been depicted. E= EcoRI sites, and H= HindIII sites.

C) Southern blot analysis further confirms S μ -S ϵ CSR in Δ S γ 1 IgE⁺ hybridomas.

Genomic DNA from Δ S γ 1 IgE⁺ colonies was digested with EcoRI and a C ϵ probe was used to analyze S μ -S ϵ CSR. GL= germline.

Supplemental figure 4

Amplified PCR products from some of the Δ S γ 1 IgE⁺ hybridomas (see supplemental figure 3A) were sequenced in order to map S μ -S ϵ junctions. Sequencing data depicting S μ -S ϵ junctions from 15 colonies are shown. S μ sequence is shown on the top while S ϵ sequence is shown at the bottom. The region of S μ or S ϵ sequence that matches with the sequenced clone is shown in bold. For each clone, the sequence that matches with S μ is shown in red while the sequence that matches with S ϵ is shown in blue. Approximate

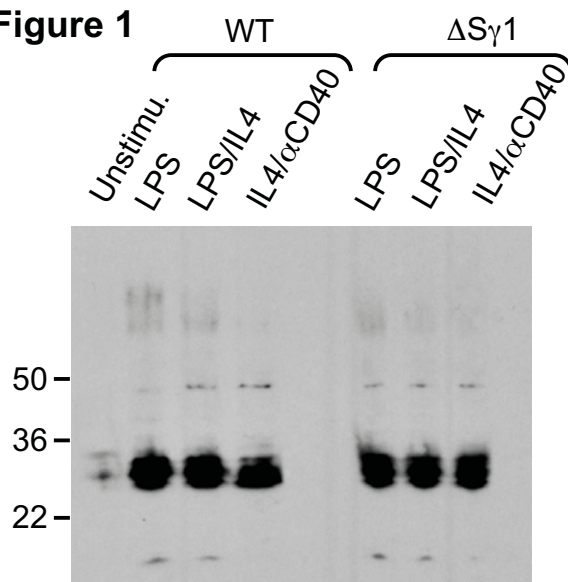
location of DSBs in S μ and S ϵ , based on sequencing the junctions, is shown in figure 5A, right panel.

Supplemental figure 5

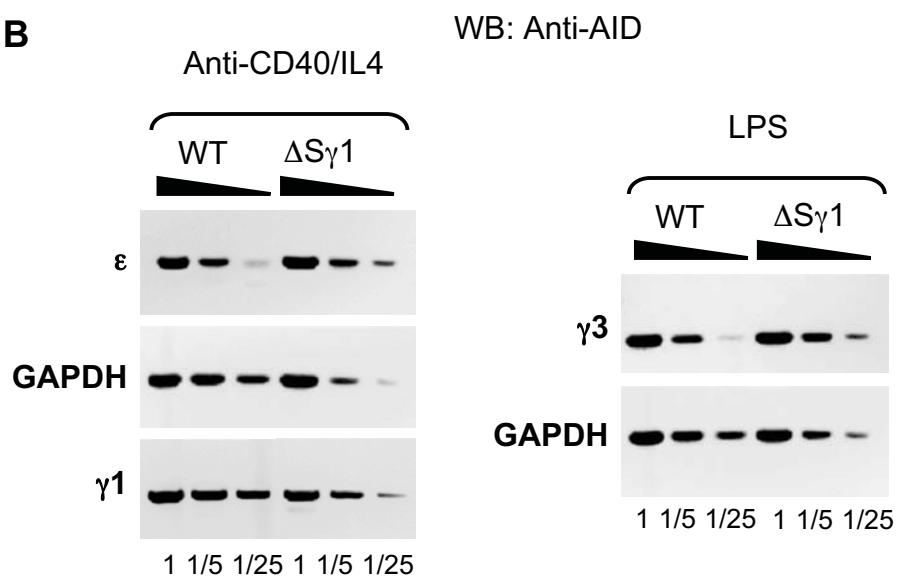
WT (littermate) and Δ S γ 1 mice were immunized with TNP-Ova and the total serum levels of IgM, IgG1 and IgE antibodies were measured at day 3 (base line), day 14, and day 42.

Supplemental Figure 1

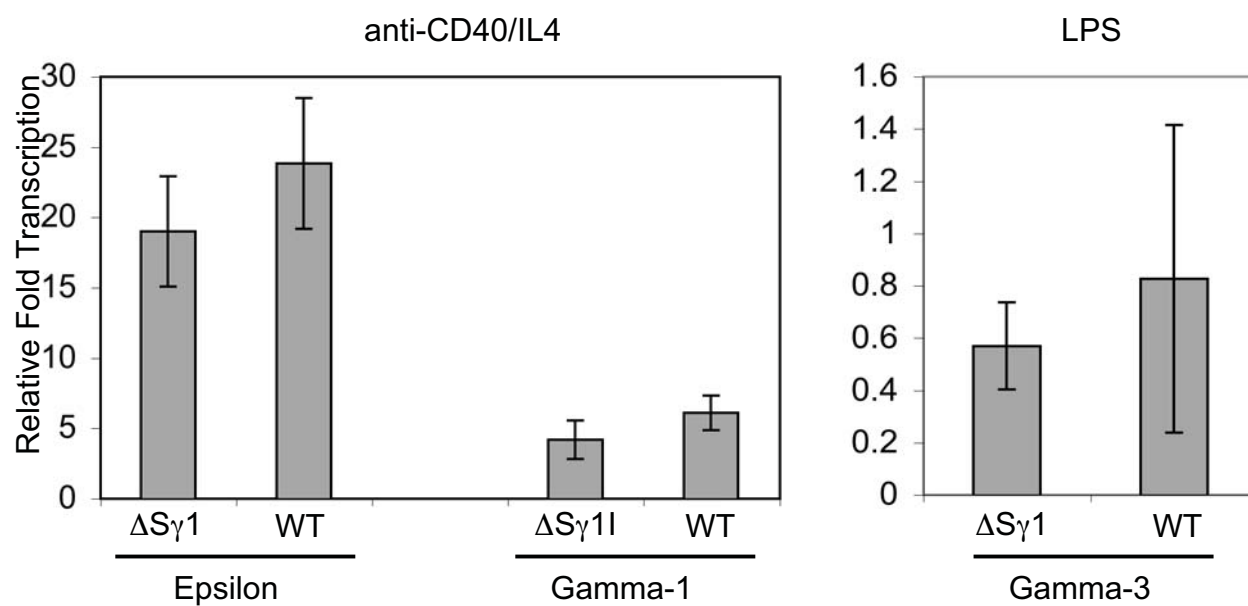
A



B

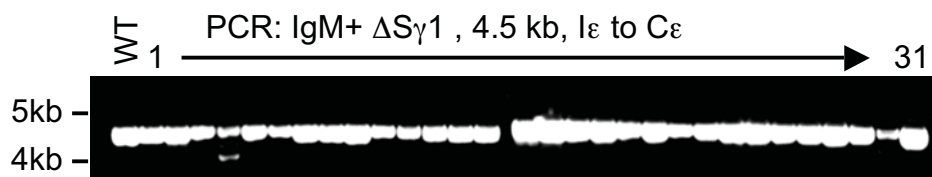


C

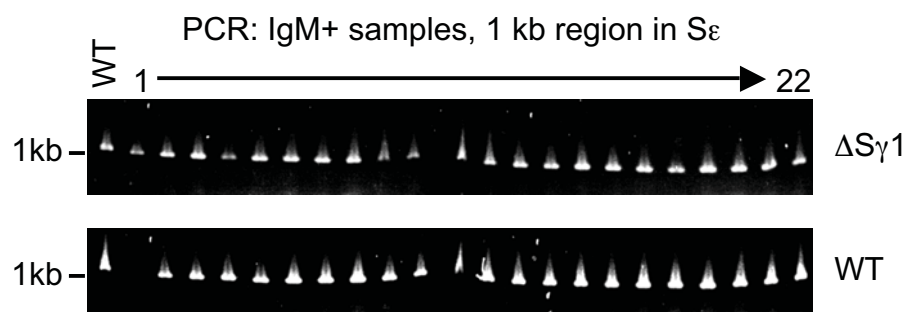


Supplemental Figure 2

A

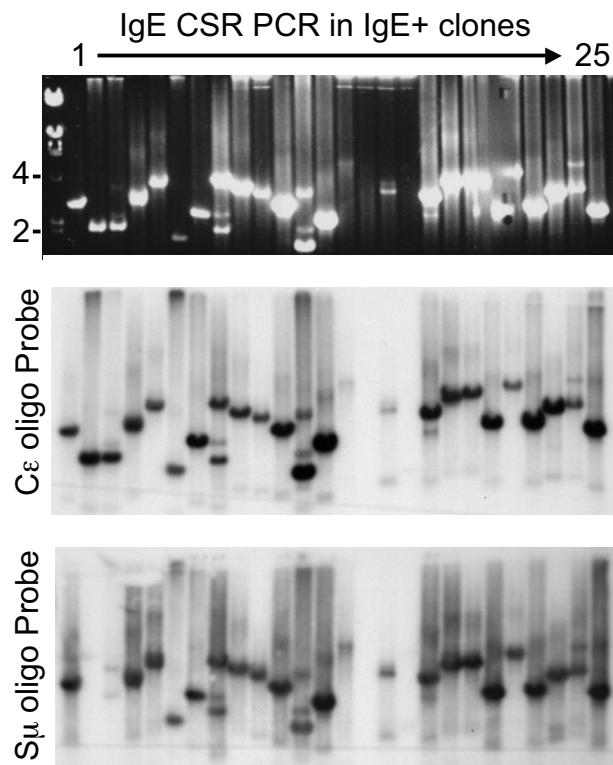


B

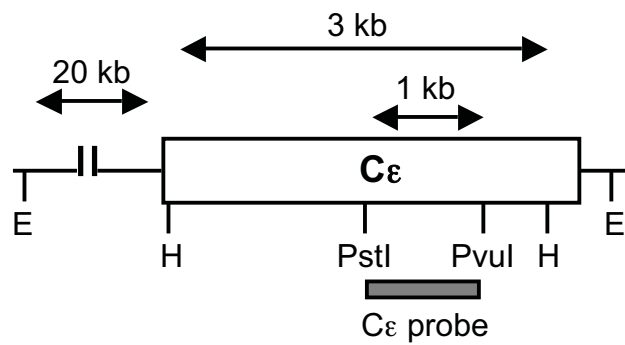


Supplemental Figure 3

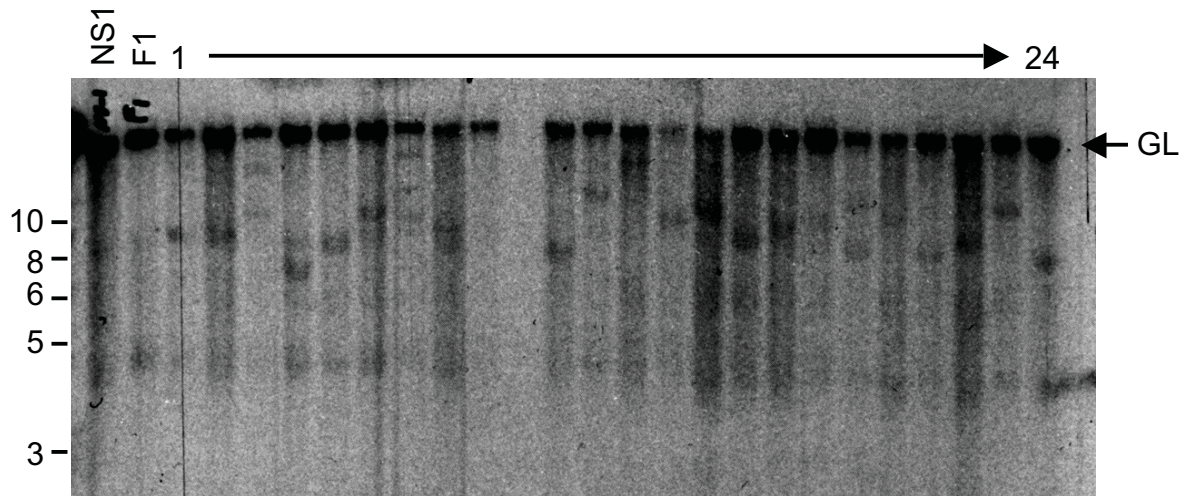
A



B



C



Supplemental Figure 4

TTGATCTACAACCTCAATGTGGTTTAATGAATTTGAAGTTGCCAGTAAATG	Sμ
TTGATCTACAACCTCAATGTGG : TTAATGTGCTTTCTGGGATCCACGGAGG	214
GGCTTCTGAAGTGGTTGTATTCTCTATGTGCTTTCTGGGATCCACGGAGG	Sε
GAGCTGAGCTAAGCTGGGGTGAGCTGAGCTGAGCTTGACTGAGCTAGGGT	Sμ
GGGGTGAGCTGAGCTGGGGTGAGCGGCTAGCCTGAGCTCGATGGGGGGT	130
GGTCTGGACTGAACTGACTGAGCTGGGCTAGCCTGAGCTCGATGGGGGGT	Sε
CCTTCTCTGTCTGGGTCATTACTGTTGCTCAGCAAGCTTTATGAGTCTGG	Sμ
CCTTCTCTGTCTGGGTCATTACTGTTCAGCTCAGCTCAGCTCAGCTCAGC	145
CCAGCCTCACTCAGCTCCTCTTAGCCAGTTTAGCCTAGCTCATTCCAGC	Sε
AGCTG : AGCTGGGGTGAGCTGAGCT : GAGG : TGAGCTGAGCTGAGCTGGG	Sμ
AGCTGAAGCTGGGTTGAGCTGAGCTATAGATTTGGTTGGACTGGACTGGA	195
CCTTGGGTGGACTAGGCTGAGCTG : ATAGATTTGGTTGGACTGGACTGGA	Sε
AATG : A : : : : CT : : G : : : A : : : A : GACTCAGTCCCTCTTAAGCAGAAAC	Sμ
CTAGTCCACCCAAGGTAGCTCAGTTGACTCAGTCCCTCTTAAGCAGAAAC	42
CTAGTCCACCCAAGGTAGCTCAGTTGAGCTCAGCTCAGCTCAGCTCAG	Sε
GCTGTGTTGGGGTGAGCTGATCT : GA : AATGAGCTACTCTGGA : GTAGCT	Sμ
GGTGAATTGGGCTG : GGTGAGCTGGGACTGAGCTTAGCTGGACCTGGCTG	98
TCTGGGCTGAACCAGAT : TGCCTAGACTGAGCTTAGCTGGACCTGGCTG	Sε
TTTGGCTCATCTCGGTTAAGCCAGCCTAGTTTAGCTTAGCGGCCAGCTC	Sμ
CTCATCCCAGCCTACCCCAAACAGCTAGTTTAGCTTAGCGGCCAGCTC	201
CTCATCCCAGCCTACCCCAAACAGTTTCAGTTTAGCCCAATCCAGTCCAG	Sε
GTTCAATTACAGTCTACTTCATTTTGCTCAAGCCAGCTTTGCTTACCTC	Sμ
AGCCACCTTATAGAAGTCTAGCCCGCTCAAGCCAGCTTTGCTTACCTCA	273
AGCCACCTTATAGAAGTCTAGCCAGC : CCCTCTCAGCTAAACTAAGCTT	Sε
GAGCTGAGCTGAGCTTGACTGAGCTAGGGTGAGCTGGACTGAGCTGGGGT	Sμ
GAGCTGAGCTGAGCTTGACTGAGCTAGCTCATGCCAGGTCAATCCAGGCC	310
GACT : AGTCTAG : TCAAGTT : A : : : AGCTCATGCCAGGTCAATCCAGGCC	Sε
AGAATTGAGAAAGAATAGAGACCTGCAGTTGAGGCCAGC : AGG : TCGGCT	Sμ
AGAATTGAGAAAGAATAGAGACCTGGTTTGGGGTAGGCTGGGATGAGCTG	353
TGGATTGGGCTAAACT : GA : AC : TGGTTTGGGGTAGGCTGGGATGAGCTG	Sε
CCCTAGCTCAG : CT : CAGTCCAGCTCAGCCAGCTCAGCTCAGCTCAGCTC	Sμ
ACAGTGTTCTGCCTCCAAGATGACCCAGCCAGCTCAGCTCAGCTCAGCTC	381
ACAGTGTTCTGCCTCCAAGATGACCTCCGTGGATCCAG : AAAGCACATA	Sε

 S_μ is Red

 S_ε is Blue

Supplemental Figure 4 continues

AAC**T**AATACACTTAACTGAAATAAT**TCCAGACAATTGTATACACGTTTTC** S_μ
ACTGTAGTATGACTCAGCCACCTTATCCAGACAATTGTATACACGTTTTC 358
ACTGTAGTATGACTCAGCCACCTTATAGAAGTCTAGCCCAGCCCCTCTCA S_ε

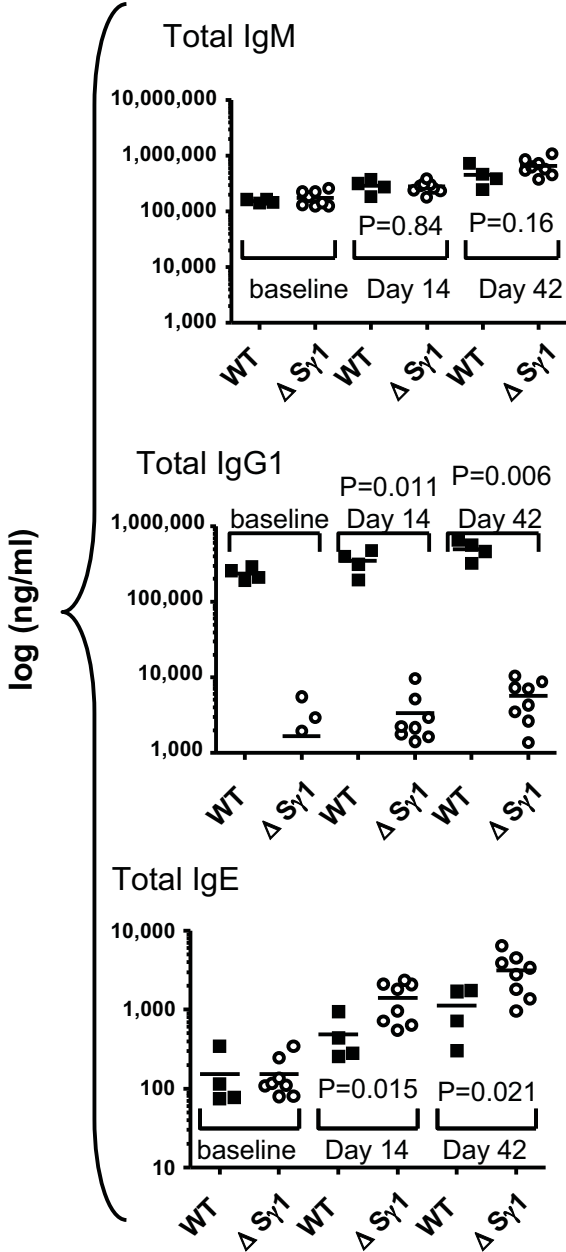
CTTCTATATTAAACCCACCATTAA**AAACCACTGAGGTATCCATTTTATCT** S_μ
GCTCAACCCAGCTCAGCTCAGCCTAAACCACTGAGGTATCCATTTTATCT 135
GCTCAACCCAGCTCAGCTCAGCCTAGTCCA:CCCAAGGTAGCTCAGTTCA S_ε

ATGCTACGCTGTGTTGGGGTGAGCTGATCTGAAATGAGCTACTCTGGAGT S_μ
ATGCTACGCTGTGTTGGGGTGAGCTAAGTTGCACTGGGCAGGGGTGGGCT 166
AGGGTAGGCTGGACTGGGCTGAGCTAAGTTGCACTGGGCAGGGGTGGGCT S_ε

GGTGAGCTGAGCTGGGTGAGCTGAGCTAAGCTGGGGTGAGCTGAGCTGAG S_μ
GGTGAGCTGAGCTGGGTGAGCTGAGA:TGAGCTAGCTTTTGTATATTTCGG 264
G:G:GCTAGGCTGGGCAGGGCTG:GACTGAGCTAGCTTTTGTATATTTCGG S_ε



Supplemental Figure 5



Supplemental table 1

Total antibody in mouse serum

Total antibody	Base line		Day 14		Day 42	
	WT	$\Delta S\gamma 1$	WT	$\Delta S\gamma 1$	WT	$\Delta S\gamma 1$
IgM ($\mu\text{g/ml}$)	155.7 ± 13.7	179.2 ± 55.2	289.4 ± 80.8	279.3 ± 63.2	458.2 ± 201.6	664.4 ± 232.0
IgG1 ($\mu\text{g/ml}$)	238.2 ± 44.6	1.7 ± 1.8	346.9 ± 120.8	3.4 ± 2.8	501.5 ± 142.6	5.7 ± 3.2
IgE (ng/ml)	152.5 ± 128.5	152.9 ± 94	483.5 ± 325.4	1407.4 ± 756.6	1122.7 ± 724.9	3158.8 ± 1831.9

Supplemental table 2

Hybridoma Jh4-Emu sequencing

Jh4-Emu	# of Clones	Total bp	# of SHM	SHM/10000 bp
$\Delta S_{\gamma 1}$	12	13467	15	11.1
WT	13	11935	8	6.72