

Polyclonal hyper-IgE mouse model reveals mechanistic insights into antibody class switch recombination

Shahram Misaghi¹, Kate Senger¹, Tao Sai¹, Yan Qu, Yonglian Sun, Kaja Hamidzadeh, Allen Nguyen, Zhaoyu Jin, Meijuan Zhou, Donghong Yan, Wei Yu Lin, Zhonghua Lin, Maria N. Lorenzo, Andrew Sebrell, Jiabing Ding, Min Xu, Patrick Caplazi, Cary D. Austin, Mercedes Balazs, Merone Roose-Girma, Laura DeForge, Søren Warming, Wyne P. Lee, Vishva M. Dixit², and Ali A. Zarrin²

Genentech, Inc., South San Francisco, CA 94080

Edited by Frederick W. Alt, Program in Cellular and Molecular Medicine, Boston Children's Hospital; and Howard Hughes Medical Institute, Harvard Medical School, Boston, MA, and approved August 9, 2013 (received for review December 18, 2012)

Preceding antibody constant regions are switch (S) regions varying in length and repeat density that are targets of activation-induced cytidine deaminase. We asked how participating S regions influence each other to orchestrate rearrangements at the *IgH* locus by engineering mice in which the weakest S region, S_ε, is replaced with prominent recombination hotspot S_μ. These mice produce copious polyclonal IgE upon challenge, providing a platform to study IgE biology and therapeutic interventions. The insertion enhances ϵ germ-line transcript levels, shows a preference for direct vs. sequential switching, and reduces intraswitch recombination events at native S_μ. These results suggest that the sufficiency of S_μ to mediate *IgH* rearrangements may be influenced by context-dependent cues.

immunoglobulin | AICDA | asthma | allergy | germline transcription

Switch (S) regions are essential and specialized targets of activation-induced cytidine deaminase (AID) (1–3) that are ordered 5'-S_μ-S_{γ3}-S_{γ1}-S_{γ2b}-S_{γ2a}-S_ε-S_α-3' (4) in the mouse *IgH* locus (Fig. 1A). Joining of distant dsDNA breaks (DSBs) between donor S_μ and any downstream S region constitutes class switch recombination (CSR). CSR to specific constant heavy-chain genes is subject to tight transcriptional regulation, which increases the accessibility of a given S region before CSR (5, 6). The primary role of S regions is to seed DSBs (7), which are repaired by nonhomologous end joining (8) predominately during the G1 phase of the cell cycle (9), whereas homologous recombination is dispensable in CSR (8, 10, 11). AID initiates CSR by targeting cytidines in transcribed repeat-rich S regions (12–14). Limited amounts of active AID in B cells can be inferred from recent studies that showed that AID heterozygous mice have reduced levels of somatic hypermutation (SHM) and CSR (15–18). Epigenetic modifications during CSR are emerging as an important regulatory mechanism to influence CSR (19). Thus, complex regulatory mechanisms act in concert to ensure appropriate AID regulation, likely to limit its off-target activity (20).

S regions have acquired intrinsic properties to make them the ultimate substrate for AID within the genome (21). Ancient S regions resemble SHM substrates, except they have a higher density of hotspots. The density of hotspots in S regions is significantly higher than in V regions (4), potentially creating areas highly susceptible to DSBs (4). In mammals, S regions appear to have further diverged by incorporating features such as the ability to form R-loops, which are single-stranded DNA loops formed by the association of an RNA transcript with a DNA template (22) and G-quartets, which are four-stranded structures of guanine-rich DNA (23), to maximize them as targets for AID (24). S region length enhances CSR (25), and there is an inverse correlation between the distance of DSBs and recombination frequency (7). In mice, S_ε is one of the shortest and least repetitive S regions, and, with the exception of S_α, it is the farthest from S_μ (4). CSR to S_ε involves sequential CSR between S_μ and S_{γ1} before combining with S_ε (26–29); however, a sequential pathway is not required, as direct CSR between S_μ and S_ε occurs

when S_{γ1} is genetically ablated (30, 31). It is possible for multiple DSBs to occur within a single S region, which leads to intraswitch recombination (ISR). This phenomenon is seen more frequently in S_μ (28, 32, 33) than in other S regions in the context of the normal *IgH* locus, possibly because it is enriched in AID target motif sites (4) or because context-dependent cues regulate the targeting of the donor S_μ region. ISR in downstream acceptor S regions are more abundant in S_μ^{−/−} (28) or transcriptionally inactive S_μ mutants (34), suggesting acceptor S regions are able to mount ISR in the absence of S_μ.

To generate a polyclonal “hyper-IgE” mouse model and to gain insights into how S regions work outside their native context, we created a mouse model in which the weakest S region, S_ε, was replaced with the strongest AID hotspot, S_μ. S_μ knock-in (S_μKI) mice produce abundant IgE at the expense of other isotypes. S_μKI IgE is antigen (Ag)-specific and produced in response to a variety of local and systemic stimuli. On a mechanistic level, the presence of S_μ in place of S_ε enhances ϵ germ-line transcript (GLT), suggesting its presence influences accessibility of the locus. Circle transcript studies reveal a preference for direct CSR vs. sequential in S_μKI mice. The knocked-in switch also negatively affects ISR of endogenous S_μ. Taken together, these results suggest the S_μ sequence has properties that are at least in part context-dependent.

Results

S_μKI Modified ϵ Allele Outcompetes S_{γ1} to Produce High Amounts of IgE in Stimulated B Cells. An overview of the *IgH* locus is shown in

Significance

Switch (S) regions are repetitive DNA sequences. During an immune response, one of several S regions recombine with a donor switch (S_μ) that is constitutively “on,” resulting in the production of antibodies with new functions. Donor S_μ is large and very repeat-rich, while another switch, S_ε, is less than half its size with a low density of repeats. We replaced S_ε with S_μ in mice. These mice switch to S_ε more effectively and produce high levels of IgE antibodies implicated in asthma, making this a useful model to study disease. In addition, placing S_μ outside of its native context revealed insights into how switches work.

Author contributions: S.M., K.S., V.M.D., and A.A.Z. designed research; S.M., K.S., T.S., Y.Q., Y.S., K.H., A.N., Z.J., M.Z., D.Y., W.Y.L., Z.L., A.S., J.D., M.X., P.C., C.D.A., M.B., W.P.L., and A.A.Z. performed research; T.S., M.N.L., M.R.-G., and S.W. contributed new reagents/analytic tools; S.M., K.S., T.S., Y.Q., Y.S., K.H., A.N., Z.J., W.Y.L., Z.L., J.D., M.X., P.C., M.B., L.D., W.P.L., V.M.D., and A.A.Z. analyzed data; and S.M., K.S., V.M.D., and A.A.Z. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

¹S.M., K.S., and T.S. contributed equally to this work.

²To whom correspondence may be addressed. E-mail: dixit.vishva@gene.com or zarrin.ali@gene.com.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1221661110/-DCSupplemental.

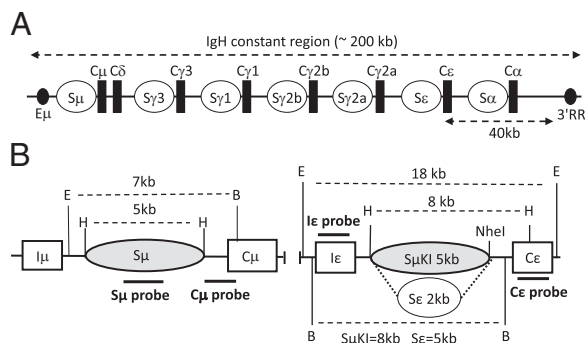


Fig. 1. Targeting strategy and generation of $S\mu$ KI mice. B, BamHI; E, EcoRI; H, HindIII. (A) Schematic representation of the *IgH* constant region in mice. (B) Schematic representation of $S\mu$, $S\epsilon$, and $S\mu$ KI within the *IgH* locus. $S\mu$, $C\mu$, $I\epsilon$, and $C\epsilon$ probes are depicted. Estimated sizes of $S\mu$ vs. $S\mu$ KI after HindIII digestion and $S\mu$ KI vs. $S\epsilon$ after BamHI digestion are indicated.

Fig. 1A. A diagram of probes used in Southern blotting analyses is shown in Fig. 1B. Gene targeting was used to replace murine $S\epsilon$ (~2 kb) with $S\mu$ (~5 kb) in C57BL/6 ES cells (Fig. S1A and B). Homozygous knock-in mice are referred to hereafter as $S\mu$ KI mice, and all animals were maintained in a C57BL/6 background. A phenotypic analysis of the $S\mu$ KI mice did not reveal any defects in lymphocyte development (Fig. S1C). The effect of inserted $S\mu$ on CSR was assessed by FACS and ELISA analysis of splenocytes stimulated with LPS plus IL-4 or LPS alone for 4 d (Fig. 2). In cells stimulated with LPS and IL-4 (Fig. 2A), WT and $S\mu$ KI cultures contained similar numbers of IgM^+ B cells. However, in the $S\mu$ KI cultures, $IgG1^+$ B cells were decreased ~11-fold, whereas the level of IgE^+ B cells in the $S\mu$ KI cultures increased approximately sixfold relative to WT. As expected, this effect was AID-dependent, as AID-deficient splenocytes (35) yielded negligible $IgG1^+$ or IgE^+ B cells. In response to LPS stimulation, WT and $S\mu$ KI cells produced comparable percentages of IgM^+ B cells (Fig. 2B). A ~50% decrease in $IgG3$ levels was seen in the $S\mu$ KI, whereas an approximately fourfold increase in IgE^+ cells was seen in $S\mu$ KI cultures relative to WT cultures. ELISAs conducted 6 d after stimulation showed similar tradeoffs in secreted IgG and IgE (Fig. 2C and D). With LPS/IL-4 stimulation (Fig. 2C), IgM levels were comparable among WT, heterozygous, and $S\mu$ KI cultures; however, IgE was far more abundant when cells carried an $S\mu$ KI allele. With LPS treatment (Fig. 2D), a ~50% decrease in secreted $IgG3$ was observed in the KI, as well as an enhanced level of secreted IgE .

DNA rearrangements of spleen cell-derived *IgH* loci in hybridomas reflect CSR events in normal splenic B cells at a single-cell level. To quantitate CSR, hybridomas were generated from splenocytes stimulated for 2 and 4 d with LPS/IL-4 (Table 1). On day 4, $IgG1^+$ clones were reduced ~10-fold in the KI relative to WT, and IgE^+ clones increased approximately sevenfold. Heterozygous hybridomas showed an intermediate phenotype, with threefold fewer $IgG1^+$ clones and fivefold more IgE^+ clones relative to WT. The increased number of IgE^+ clones in the KI does not appear to result from sequential switching, as more IgE^+ B-cells are already evident in $S\mu$ KI compared with WT only 2 d after stimulation. PCR and sequencing analysis confirmed that IgE^+ hybridomas contained $S\mu$ - $S\epsilon$ (WT) or $S\mu$ - $S\mu$ ($S\mu$ KI) junctions (Figs. S2 and S3). These results, together with the aforementioned FACS and ELISA data, are consistent with AID targeting to ϵ being enhanced substantially by substitution of $S\mu$ for $S\epsilon$.

$S\mu$ KI Mice Produce Copious IgE in Response to a Variety of Systemic and Local Challenges. We determined whether the IgE produced by $S\mu$ KI mice is Ag-specific or merely reflects a nonspecific surge in CSR to the modified ϵ locus by applying several systemic and

local challenges. Mice were immunized with T-cell-dependent antigen 2,4,6-trinitrophenyl ovalbumin (TNP-OVA) (31) and IgE titer was measured. No TNP-specific IgE was present before immunization (Fig. 3A), although slightly enhanced total serum IgE was seen in $S\mu$ KI (Fig. S4A). On days 14 and 28 postimmunization, TNP-specific IgE titers in $S\mu$ KI mice were approximately fivefold higher than in WT mice. $S\mu$ KI mice were also locally challenged with various model allergens to see if they would produce serum IgE in excess. $S\mu$ KI mice immunized with TNP-OVA were challenged intranasally with aerosolized TNP-OVA to model asthma (Fig. 3B). Challenged $S\mu$ KI mice produced 8- to 10-fold higher TNP-specific serum IgE than their WT counterparts, whereas baseline TNP-specific IgE was undetectable. Remarkably, $S\mu$ KI mice accumulated significant levels of Ag-specific IgE (~100,000 ng/mL) within 4 d after challenge. Induction of IgE in $S\mu$ KI mice was similarly robust following intranasal delivery of an Ag mixture in the absence of systemic sensitization (Fig. 3C and Fig. S4B), or following s.c. infection with *Nippostrongylus brasiliensis* larva (Fig. 3D). By contrast, IgE titers in WT mice postinfection barely reached the basal IgE titers seen in the $S\mu$ KI mice. In sum, our in vivo data demonstrate that the $S\mu$ KI allele supports robust IgE isotype switching in response to a variety of challenges.

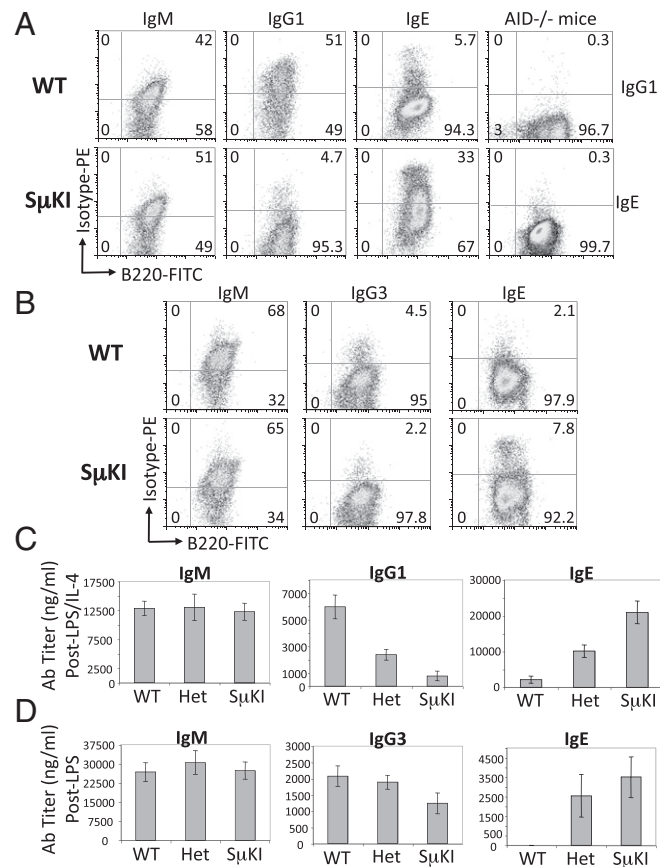


Fig. 2. In vitro stimulated $S\mu$ KI B cells produce higher levels of IgE and lower levels of $IgG1$ antibodies compared with WT B cells. (A) Splenocytes were stimulated 4 d with LPS/IL-4 and the indicated antibody isotypes measured by FACS. B220 was used as a B-cell marker, and AID^{-/-} mice were used as negative control. (B) Splenocytes were stimulated 4 d with LPS, and the indicated antibody isotypes measured by FACS. B220 was used as a B-cell marker. (C) Splenocytes from WT, heterozygous, and $S\mu$ KI mice ($n = 5$) were stimulated with LPS/IL-4 for 6 d, and the indicated antibody isotypes measured by ELISA. (D) Splenocytes from WT, heterozygous, and $S\mu$ KI mice ($n = 5$) were stimulated with LPS for 6 d, and indicated antibody isotypes measured by ELISA.

Table 1. Quantification of isotype switching by B-cell hybridomas

Day/hybridoma	No. of clones	IgM ⁺ , %	IgG1 ⁺ , %	IgE ⁺ , %
Day 2				
WT	562	95	3	2
KI	594	88	1	11
Day 4				
WT (fusion 1)	1,440	32	57	11
WT (fusion 2)	946	21	68	11
HET	1,379	20	21	59
KI (fusion 1)	1,444	14	6	80
KI (fusion 2)	1,141	18	4	78

At 2 or 4 d after LPS/IL-4 stimulation, splenocytes from WT and μ KI (i.e., heterozygous or homozygous) mice were fused to the NS-1 host to create hybridomas. ELISAs were used to obtain percent IgM⁺, IgG1⁺, and IgE⁺ hybridomas.

Replacement of ϵ with μ Enhances Epsilon GLT. To explore the mechanistic basis for enhanced IgE production in μ KI mice, we analyzed GLT levels under various stimuli. Germ-line transcripts are non-coding RNAs that begin at a short exon upstream of each S region termed the I exon, and proceed through the constant regions. Purified B cells were activated 2 d with LPS or LPS plus IL-4 (Fig. 4*A* and *B* and Fig. S5*A*), LPS plus IFN- γ , or IL-4 plus α CD-40 and TGF- β (Fig. S5*B* and *C*). The LPS-treated samples showed comparable levels of GLT induction for μ , γ 2b, and γ 3 between WT and μ KI B cells. As expected, WT cells did not produce ϵ transcripts before or after LPS treatment. In contrast, μ KI cells showed enhanced basal ϵ transcription without stimulation, and with LPS treatment this was slightly enhanced (Fig. 4*A*). Likewise, in independent stimulations that used LPS plus IL-4 (Fig. 4*B*), ϵ GLT levels were again high in resting μ KI cells and were induced by approximately twofold in response to activation. GLT levels for μ and γ 1 appeared unaltered by the μ insertion. Northern blot analysis consistently showed enhanced ϵ GLT (Fig. S5*A*) (36). GLT levels for μ , γ 2a, and α appeared comparable between WT and μ KI (Fig. S5*B* and *C*). The enhanced ϵ GLT seen in the knock-in suggests that, in addition to providing a longer and more motif-rich region for CSR, the insertion alters ϵ locus accessibility.

CSR to the μ KI Locus Is Largely Direct Instead of Sequential. The enhanced CSR to the μ KI locus raises the question of whether switching occurs sequentially via γ 1 or is direct. Sequential switching is a two-part mechanism in which CSR first occurs between μ and γ 1, which subsequently switches to ϵ , or between γ 1 and ϵ , which then switches to μ (27, 28). To address this question, we looked for the presence of circle transcripts in LPS/IL-4 activated B cells over time (Fig. 5). Circle transcripts are produced when the intervening genomic DNA between two connecting S regions is looped out, forming an episome in which the I promoter of the downstream S region ends up driving transcription of the constant (C) region adjacent to the upstream S region.

We analyzed RNA from resting and activated WT and μ KI B cells by using RT-PCR and primer sets capable of detecting direct and sequential switch products (Fig. 5*A*). The product of primers I ϵ FW and C μ RV (Fig. 5*B*, lanes 1–5) displays switching to ϵ , which could be direct or sequential via an μ /S γ 1 or S γ 1/S ϵ intermediate. Visualized with a probe to C μ , this product appears after 48 h and appears significantly more robust in μ KI cells, consistent with their enhanced IgE production. The combination of primers I ϵ FW and C γ 1RV identifies CSR between γ 1 and ϵ , which is a unique sequential switch product (Fig. 5*B*, lanes 6–10). Highlighted by a C γ 1 probe, this product appears after 72 h in WT cells, becoming more abundant by 96 h. Its levels are significantly lower in μ KI cells, suggesting the cells favor a more direct mechanism of CSR over a sequential pathway. Equivalent

RNA inputs were used in this experiment, as shown by β -actin RT-PCR (Fig. 5*C*).

To determine what fraction of B cells undergo sequential switching, we generated a panel of IgM⁺ and IgE⁺ hybridomas from WT and μ KI B cells stimulated with LPS/IL-4 (Fig. S6). Genomic DNA from the IgM⁺ hybridomas was digested with EcoRI to release a fragment containing S ϵ /S μ KI (Fig. S6*A* and *B*; see also Fig. 1*B*) and hybridized to the C ϵ probe. In the event of recombination between S γ 1 and S ϵ /S μ KI, the size of the EcoRI fragment would be altered (28). We did not detect changes in band size in hybridomas from 2- or 4-d stimulated B cells, suggesting the frequency of sequential switching is low in both genotypes. We also used a nested PCR strategy to amplify S μ -S ϵ junctions from IgE⁺ hybridomas and probed for the presence of S γ 1 in Southern blots (Fig. S6*C* and *D*). We detected seven S μ -S ϵ junctions containing S γ 1 among 57 WT hybridomas (12.2%), vs. 1 of 58 hybridomas containing S γ 1 for S μ KI (1.7%), suggesting sequential switching is less favored in μ KI B cells.

Endogenous μ ISR Is Reduced in the Presence of Knocked-In μ . To view the impact of the μ KI on ISR, IgM⁺ hybridomas were analyzed for deletions within endogenous μ by Southern blot (Fig. 6*A* and *B*). ISR was detected in 60% of WT hybridomas, which decreased to 38% and 25% of heterozygous and homozygous μ KI hybridomas, respectively. The observation that μ KI negatively impacts ISR at upstream endogenous μ implies that the insertion competes away factors necessary for ISR or that μ KI channels endogenous μ region breaks into a productive CSR reaction with higher frequency. It should be noted that Southern blotting is a low-resolution assay, and so may reflect only a fraction of total events (32).

We next examined whether μ KI could undergo ISR in the context of the ϵ locus. The I ϵ probe was used to analyze ISR within S ϵ and inserted μ in hybridomas created from IgM⁺ B cells (Fig. 6*C*). Among 96 hybridomas, S ϵ did not display any internal recombination events and μ KI displayed only one event,

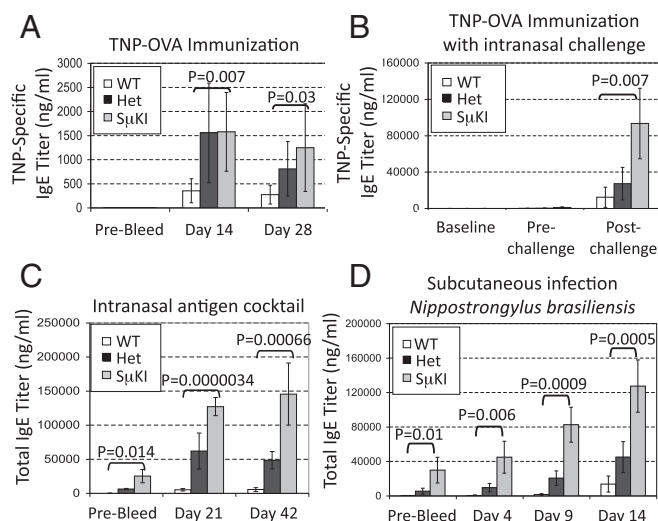


Fig. 3. μ KI mice express higher levels of IgE compared with WT mice in vivo. (A) WT ($n = 3$), heterozygous (Het; $n = 3$), and μ KI ($n = 7$) mice were immunized with TNP-OVA. TNP-OVA-specific serum IgE levels were measured by ELISA before and at 14 and 28 d postimmunization. (B) Mice ($n = 5$ per group) were immunized with TNP-OVA/alum and challenged with aerosolized 1% TNP-OVA. Baseline, prechallenge and postchallenge TNP-OVA-specific serum IgE levels were measured by ELISA. (C) WT, Het, or μ KI mice ($n = 6$ per group) were sensitized to multiple allergens as explained in *Materials and Methods*, and total serum IgE levels were measured by ELISA before and on days 21 and 42 postsensitization. (D) Total serum IgE levels in WT, Het, and μ KI mice ($n = 5$ per group) were measured by ELISA before and 4, 9, and 14 d postinfection with 500 *N. brasiliensis* larvae. P values were calculated between WT and μ KI samples by using a *t* test assuming unequal variances.

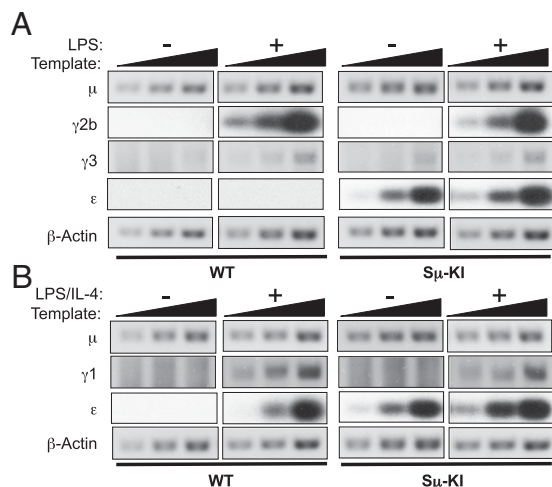


Fig. 4. GLT levels in WT vs. S_{μ} -KI mice. At least three independent mice per genotype were tested with similar results. (A) Semiquantitative RT-PCR with RNA from WT or S_{μ} -KI B cells that were freshly isolated or stimulated with LPS for 2 d. Starting amounts of RNA were 2 ng, 10 ng, and 50 ng. A fixed cycle number in the linear range for the specific product was used. Gel images are shown for μ (25 cycles) and β -actin (25 cycles). Southern blot autoradiographs are shown for $\gamma 2b$ (30 cycles), $\gamma 3$ (30 cycles), and ϵ (45 cycles for WT, 30 cycles for S_{μ} -KI). (B) Similar to A but with LPS/IL-4 stimulation. Gel images are shown for μ , $\gamma 1$ (30 cycles), and β -actin. A Southern blot autoradiograph is shown for ϵ .

suggesting the primary sequence of S_{μ} is not efficient at ISR. Instead, context-dependent cues such as the presence of regulatory elements (e.g., enhancer E_{μ} or I_{μ}), rate of transcription, or local chromatin modifications may have influence.

The differences in mechanism by which ISR, CSR, and SHM occur are not well understood and the processes can be temporally distinct (37). We hypothesized that, if S_{μ} -KI impacts other S regions in the context of CSR, it might also affect SHM in S_{μ} -KI B cells. We sorted IgM^{+} or $IgG1^{+}$ germinal center B cells in response to TNP-OVA to scan for mutations downstream of JH4 and upstream of endogenous S_{μ} (Fig. S7A) (31). Our analysis revealed that the rate of SHM in S_{μ} -KI B cells was not significantly different from WT. Rather, there was a slight trend toward lower mutation frequency in the S_{μ} -KI allele. To document the impact on SHM directly in V regions, a panel of $IgG1$ -producing TNP-OVA specific hybridomas was generated after TNP-OVA immunization, and heavy chain variable region (VH) gene sequences were aligned with germ-line counterparts to locate mutations (Fig. S7B). We did not detect a major difference in mutation frequency between WT and S_{μ} -KI. Thus, alteration of the IgH locus in S_{μ} -KI impacts CSR and ISR, but does not appear to impact SHM.

Discussion

S_{μ} is a constitutively transcribed donor S region that is unusually dense with AID target hotspots (4). S region size linearly correlates with enhanced CSR (25). In our model, we replaced the 2-kb S_{ϵ} with a larger ~ 5 -kb core S_{μ} . The GLT of the S_{μ} -KI locus is enhanced relative to unmodified S_{ϵ} . Thus, increased CSR seen in the S_{μ} -KI could be a result of the sequence of S_{μ} or the increased S region length combined with greater transcriptional accessibility. The result is a dramatic increase in the magnitude of IgE isotype switching, even exceeding the $IgG1$ levels associated with the largest S region, $S_{\gamma 1}$.

It is not understood to what degree the context of S_{μ} influences its ability to undergo CSR and ISR. We provide data supporting the notion that S_{μ} inserted in place of S_{ϵ} is insufficient to carry out certain functions it would have in its endogenous location. The knocked-in sequence is able to reduce ISR of endogenous S_{μ} from a distance, either by competing for

limited factors or through providing DSBs leading to productive CSR with its upstream twin. However, despite the known recombination of S_{μ} , the KI is insufficient to generate levels of ISR comparable to its upstream counterpart, suggesting the sequence itself is insufficient or the ϵ locus is not conducive to ISR events (28, 31). This is also unusual given that ISR is found for endogenous S_{ϵ} (28, 31) as well as other acceptor S regions (28, 32, 33). The robust targeting of native S_{μ} might facilitate productive CSR by making targeting of downstream S regions rate-limiting, which ultimately may limit the duration and production of unnecessary DSBs (32). Although AID is capable of initiating DSBs in all S regions, AID-mediated ISR may be influenced by regional DNA architecture and recruited DNA repair machinery. Our finding suggests that the frequent targeting of endogenous S_{μ} is context-dependent.

The mechanism of direct vs. sequential CSR to produce IgE is not well understood, and this process might be developmentally regulated (38). Our analysis of circle transcripts indicates that direct and sequential switching can mediate CSR. Sequential switching might involve $S_{\mu}/S_{\gamma 1}$ junctions joining to S_{ϵ} or switching between $S_{\gamma 1}/S_{\epsilon}$ before joining S_{μ} (27, 39). In our assay, we observed reduced levels of $S_{\gamma 1}/S_{\epsilon}$ products in S_{μ} -KI relative to WT. These data indicate that, in S_{μ} -KI cells, switching is more direct. In addition, our ISR data suggests that a prominent, active downstream S substrate prefers direct switching vs. indirect with donor S_{μ} , in which there is a higher chance to generate two simultaneous DSBs (7). This interpretation agrees with the fact the sequential switching is mainly observed for S_{ϵ} and not for other S regions (27, 39).

It is intriguing that steady-state GLT at the ϵ locus is positively affected by the S_{μ} insertion. This observation could explain the low but detectable levels of IgE observed in LPS-stimulated S_{μ} -KI B cells. It could be that the ϵ locus is an inherently difficult

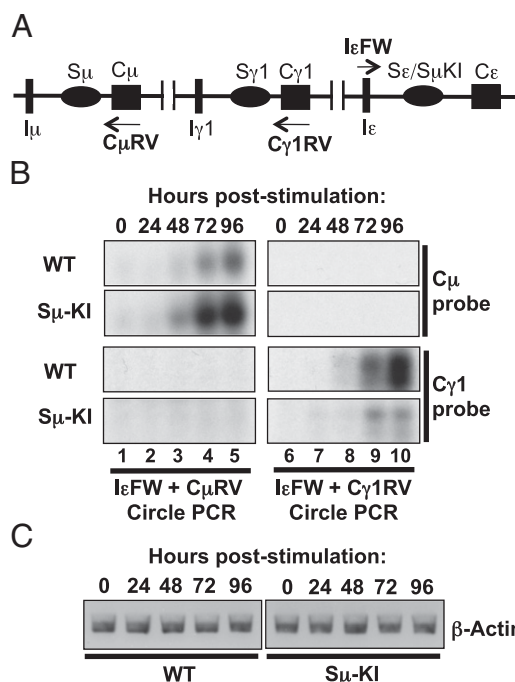
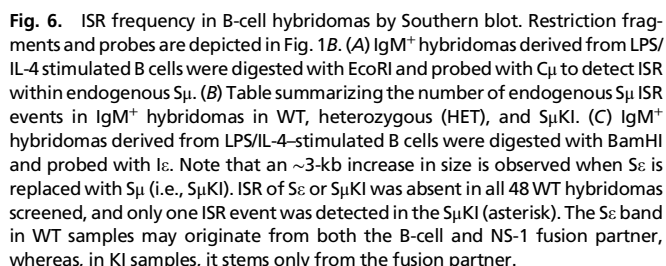


Fig. 5. Contribution of direct vs. sequential switching to IgE . (A) Diagram of the IgH locus and relevant features and primers indicated. (B) RNA was isolated from WT or S_{μ} -KI B cells at the indicated time points after LPS/IL-4 stimulation. RT-PCR was performed by using primers specific to I_{ϵ} and C_{μ} (result of circles formed from S_{μ} - S_{ϵ} junctions, lanes 1–5), and primers specific to I_{ϵ} and $C_{\gamma 1}$ (result of circles formed from $S_{\gamma 1}$ - S_{ϵ} junctions, lanes 6–10). The resulting products were hybridized to probes corresponding to C_{μ} and $C_{\gamma 1}$. (C) β -Actin at 20 cycles is shown as a loading control.



the epsilon locus, replacement of Cε exons with a *Pgk-neomycin* cassette in an antisense orientation can inhibit the GLT of Cγ3, Cγ2b, and Cγ2a genes (36). According to our study, however, the enhanced activity of the Iε promoter at the SμKI allele does not appear to appreciably affect the GLT of other heavy chain constant (C_H) genes. In contrast to the *Pgk-neomycin* insertion model, the sense orientation of the endogenous I epsilon promoter in our SμKI model is conserved and does not include additional sequences such as a *neomycin* cassette. Our finding demands additional studies to elucidate the mechanism behind this observation.

The $\text{S}\mu\text{KI}$ hyper-IgE mouse model is unique for the study of IgE biology and its associated pathology. Allergen-bound IgE activates mast cells via the $\text{Fc}\epsilon$ receptor and releases a spectrum of inflammatory mediators (44). Poor CSR potential and short $\text{IgE } t_{1/2}$ might have been selected during evolution to limit IgE abundance (4) and the danger of unwanted effector functions associated with anaphylaxis and allergies (44). Not only are acute asthmatic patients successfully treated with IgE neutralizing antibodies (45), new indications for this therapeutic intervention include allergic rhinitis, food allergy, atopic dermatitis, chronic urticaria, and allergic bronchopulmonary aspergillosis (45). *IgE* transgenic mice have the major shortcoming of being unable to mount a polyclonal Ag-specific IgE response (46–48). By altering de novo CSR, the resultant $\text{S}\mu\text{KI}$ mice are able to mount diverse and polyclonal Ag-specific IgE responses to local or systemic challenges, thereby providing a unique experimental model for the accelerated assessment of future therapies in a facile system that more closely mimics the human condition.

CSR/ISR/SHM Assays. Splenocytes were isolated and stimulated as previously explained (31). ELISAs and surface staining of B cells for IgM, IgG1, IgG3, and B220 were performed as explained (31). To visualize IgE, cells were blocked with anti-mouse IgE blocking antibody (eBioscience) and then stained with biotin-isotype or anti-mouse IgE-biotin (eBioscience) followed by streptavidin-phycoerythrin (Pharmingen). Southern blot methods were described previously (31). Southern probes included C μ (XbaI-BamHI, 0.8 kb), I ϵ (BamHI-NcoI, 1 kb), S μ (HindIII, 5 kb), and C δ (PstI-PvuII, 1 kb; Fig. 1B). For GLT and circle transcripts, RT-PCR reactions were carried out by using the Access RT-PCR kit (Promega). Cycling parameters were 45 °C for 45 min and 94 °C for 2 min, then repeating cycles of 94 °C for 30 s, 60 °C for 30 s, and 68 °C for 30 s. GLT amplification primers were reported elsewhere (24). Circle transcripts were detected with GLT primers except for CuRV 5'-AATGGTGCTGGGCAGGAAGT-3'.

Hybridomas. The procedure for generating hybridomas have been described elsewhere (49). Briefly, splenocytes from 6- to 8-wk-old mice (WT, heterozygous, and KI) were stimulated *in vitro* with LPS (20 ng/mL) and IL-4 (25 ng/mL). After 4 d, 20 million B cells were fused with NS1 cells (American Type Culture Collection) at a 1:1 ratio by using the Cyto Pulse CEEF-50 apparatus (Cyto Pulse Sciences). After 7 to 10 d selection in hypoxanthine-aminopterin-thymidine (HAT) media (StemCell Technologies), the single hybridoma clones were screened for IgE-, IgM- or IgG1-specific hybridomas by Velocity 11 Biocel 1200 Automation System (Agilent Technologies). TNP-OVA specific hybridomas were generated from splenocytes at day 42 after repeated immunization (as described later) and screened by ELISA.

In Vivo Studies and Animal Care. TNP-OVA immunization (T-cell-dependent immune response) was performed by i.p. injection of TNP-OVA/Alum adjuvant at day 0, followed by another injection of TNF-OVA at day 28. Blood samples were collected on days 3, 7, 14, 21, 28, 35, and 42 for antibody isotype measurements. For the asthma model, mice were sensitized with an i.p. injection of TNP-OVA/Alum on day 0. Mice were challenged with aerosolized 1% TNP-OVA on day 35 after sensitization, followed by seven consecutive days of challenging as indicated earlier. Blood samples for antibody isotype measurements were collected at day 2 (baseline bleed), day 34 (prechallenge), and day 42 (postchallenge). Mice infected with *N. brasiliensis* larvae s.c. were placed on polymyxin B (110 mg/L; Calbiochem) and neomycin (1.1 g/L; Sigma-Aldrich) medicated water for 5 d. Serum samples were collected before and 4, 9, or 14 d after infection for antibody isotype measurements. For the multiple Ag allergen model, mice were sensitized and exposed to multiple allergens intranasally twice per week for 6 wk. Each mouse was exposed intranasally to extracts of *Dermatophagoides farinae* (5 µg), *Dermatophagoides pteronyssinus* (5 µg), *Ambrosia artemisiifolia* (50 µg), and *Aspergillus fumigatus* (5 µg) (Greer Laboratories) mixed in 50 µL PBS solution. One week after the last exposure, total IgE and IgG1 were measured by ELISA. SuK1 and WT mice were generated

at Genentech and maintained in accordance with American Association of Laboratory Animal Care guidelines. The experiments were conducted in compliance with National Institute of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Genentech Institutional Animal Care and Use Committee.

ACKNOWLEDGMENTS. The authors thank Eric Pinaud and Ming Tian for providing technical advice and insightful discussions; Mariela del Rio, Ben Grellman, and Vida Asghari for managing the mouse colony; and Kim Newton, Flavius Martin, Harinder Singh, and Menno Van Lookeren Campagne for carefully reviewing the manuscript.

- Khamlichi AA, et al. (2004) Immunoglobulin class-switch recombination in mice devoid of any S mu tandem repeat. *Blood* 103(10):3828–3836.
- Luby TM, Schrader CE, Stavnezer J, Selsing E (2001) The mu switch region tandem repeats are important, but not required, for antibody class switch recombination. *J Exp Med* 193(2):159–168.
- Zarrin AA, et al. (2004) An evolutionarily conserved target motif for immunoglobulin class-switch recombination. *Nat Immunol* 5(12):1275–1281.
- Hackney JA, et al. (2009) DNA targets of AID evolutionary link between antibody somatic hypermutation and class switch recombination. *Adv Immunol* 101:163–189.
- Yancopoulos GD, et al. (1986) Secondary genomic rearrangement events in pre-B cells: VHDJH replacement by a LINE-1 sequence and directed class switching. *EMBO J* 5(12):3259–3266.
- Stavnezer-Nordgren J, Sirlin S (1986) Specificity of immunoglobulin heavy chain switch correlates with activity of germline heavy chain genes prior to switching. *EMBO J* 5(1):95–102.
- Zarrin AA, et al. (2007) Antibody class switching mediated by yeast endonuclease-generated DNA breaks. *Science* 315(5810):377–381.
- Yan CT, et al. (2007) IgH class switching and translocations use a robust non-classical end-joining pathway. *Nature* 449(7161):478–482.
- Schrader CE, Guikema JE, Linehan EK, Selsing E, Stavnezer J (2007) Activation-induced cytidine deaminase-dependent DNA breaks in class switch recombination occur during G1 phase of the cell cycle and depend upon mismatch repair. *J Immunol* 179(9):6064–6071.
- Robert I, Dantzer F, Reina-San-Martin B (2009) Parp1 facilitates alternative NHEJ, whereas Parp2 suppresses IgH/c-myc translocations during immunoglobulin class switch recombination. *J Exp Med* 206(5):1047–1056.
- Hasham MG, et al. (2012) Activation-induced cytidine deaminase-initiated off-target DNA breaks are detected and resolved during S phase. *J Immunol* 189(5):2374–2382.
- Petersen-Mahrt SK, Harris RS, Neuberger MS (2002) AID mutates E. coli suggesting a DNA deamination mechanism for antibody diversification. *Nature* 418(6893):99–103.
- Chaudhuri J, et al. (2003) Transcription-targeted DNA deamination by the AID antibody diversification enzyme. *Nature* 422(6933):726–730.
- Bransteitter R, Pham P, Scharff MD, Goodman MF (2003) Activation-induced cytidine deaminase deaminates deoxycytidine on single-stranded DNA but requires the action of RNase. *Proc Natl Acad Sci USA* 100(7):4102–4107.
- McBride KM, et al. (2006) Regulation of hypermutation by activation-induced cytidine deaminase phosphorylation. *Proc Natl Acad Sci USA* 103(23):8798–8803.
- Dorsett Y, et al. (2007) A role for AID in chromosome translocations between c-myc and the IgH variable region. *J Exp Med* 204(9):2225–2232.
- Takizawa M, et al. (2008) AID expression levels determine the extent of cMyc oncogenic translocations and the incidence of B cell tumor development. *J Exp Med* 205(9):1949–1957.
- Sernández IV, de Yébenes VG, Dorsett Y, Ramiro AR (2008) Haploinsufficiency of activation-induced deaminase for antibody diversification and chromosome translocations both in vitro and in vivo. *PLoS ONE* 3(12):e3927.
- Daniel JA, et al. (2010) PTIP promotes chromatin changes critical for immunoglobulin class switch recombination. *Science* 329(5994):917–923.
- Vuong BQ, Chaudhuri J (2012) Combinatorial mechanisms regulating AID-dependent DNA deamination: Interacting proteins and post-translational modifications. *Semin Immunol* 24(4):264–272.
- Zhu C, et al. (2012) Origin of immunoglobulin isotype switching. *Curr Biol* 22(10):872–880.
- Yu K, Chedin F, Hsieh CL, Wilson TE, Lieber MR (2003) R-loops at immunoglobulin class switch regions in the chromosomes of stimulated B cells. *Nat Immunol* 4(5):442–451.
- Dempsey LA, Sun H, Hanakahi LA, Maizels N (1999) G4 DNA binding by LR1 and its subunits, nucleolin and hnRNP D, A role for G-G pairing in immunoglobulin switch recombination. *J Biol Chem* 274(2):1066–1071.
- Muramatsu M, et al. (2000) Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102(5):553–563.
- Zarrin AA, Tian M, Wang J, Borjeson T, Alt FW (2005) Influence of switch region length on immunoglobulin class switch recombination. *Proc Natl Acad Sci USA* 102(7):2466–2470.
- Yoshida K, et al. (1990) Immunoglobulin switch circular DNA in the mouse infected with *Nippostrongylus brasiliensis*: Evidence for successive class switching from mu to epsilon via gamma 1. *Proc Natl Acad Sci USA* 87(20):7829–7833.
- Mandler R, Finkelman FD, Levine AD, Snapper CM (1993) IL-4 induction of IgE class switching by lipopolysaccharide-activated murine B cells occurs predominantly through sequential switching. *J Immunol* 150(2):407–418.
- Zhang T, et al. (2010) Downstream class switching leads to IgE antibody production by B lymphocytes lacking IgM switch regions. *Proc Natl Acad Sci USA* 107(7):3040–3045.
- Xiong H, Dolpady J, Wabl M, Curotto de Lafaille MA, Lafaille JJ (2012) Sequential class switching is required for the generation of high affinity IgE antibodies. *J Exp Med* 209(2):353–364.
- Jung S, Siebenkotten G, Radbruch A (1994) Frequency of immunoglobulin E class switching is autonomously determined and independent of prior switching to other classes. *J Exp Med* 179(6):2023–2026.
- Misaghi S, et al. (2010) Increased targeting of donor switch region and IgE in Sgamma1-deficient B cells. *J Immunol* 185(1):166–173.
- Dudley DD, et al. (2002) Internal IgH class switch region deletions are position-independent and enhanced by AID expression. *Proc Natl Acad Sci USA* 99(15):9984–9989.
- Reina-San-Martin B, Chen HT, Nussenzweig A, Nussenzweig MC (2004) ATM is required for efficient recombination between immunoglobulin switch regions. *J Exp Med* 200(9):1103–1110.
- Gu H, Zou YR, Rajewsky K (1993) Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* 73(6):1155–1164.
- Sun Y, et al. (2013) Critical role of activation induced cytidine deaminase in experimental autoimmune encephalomyelitis. *Autoimmunity* 46(2):157–167.
- Seidl KJ, et al. (1999) Position-dependent inhibition of class-switch recombination by PGK-neor cassettes inserted into the immunoglobulin heavy chain constant region locus. *Proc Natl Acad Sci USA* 96(6):3000–3005.
- Di Noia JM, Neuberger MS (2007) Molecular mechanisms of antibody somatic hypermutation. *Annu Rev Biochem* 76:1–22.
- Wesemann DR, et al. (2011) Immature B cells preferentially switch to IgE with increased direct Sμ to Sε recombination. *J Exp Med* 208(13):2733–2746.
- Mills FC, Mitchell MP, Harindranath N, Max EE (1995) Human Ig S gamma regions and their participation in sequential switching to IgE. *J Immunol* 155(6):3021–3036.
- Morrison AM, et al. (1998) Deregulated PAX-5 transcription from a translocated IgH promoter in marginal zone lymphoma. *Blood* 92(10):3865–3878.
- Janz S (2006) Myc translocations in B cell and plasma cell neoplasms. *DNA Repair (Amst)* 5(9–10):1213–1224.
- Manis JP, Tian M, Alt FW (2002) Mechanism and control of class-switch recombination. *Trends Immunol* 23(1):31–39.
- Pinaud E, et al. (2011) The IgH locus 3′ regulatory region: Pulling the strings from behind. *Adv Immunol* 110:27–70.
- Galli SJ, Tsai M, Piliponsky AM (2008) The development of allergic inflammation. *Nature* 454(7203):445–454.
- Ben-Shoshan M (2010) Omalizumab for asthma: indications, off-label uses and future directions. *Recent Pat Inflamm Allergy Drug Discov* 4(3):183–192.
- Adamczewski M, Köhler G, Lamers MC (1991) Expression and biological effects of high levels of serum IgE in epsilon heavy chain transgenic mice. *Eur J Immunol* 21(3):617–626.
- Curotto de Lafaille MA, et al. (2001) Hyper immunoglobulin E response in mice with monoclonal populations of B and T lymphocytes. *J Exp Med* 194(9):1349–1359.
- Matsuoka K, et al. (1999) Establishment of antigen-specific IgE transgenic mice to study pathological and immunobiological roles of IgE in vivo. *Int Immunol* 11(6):987–994.
- Sun Y, et al. (2012) Evolutionarily conserved paired immunoglobulin-like receptor α (PILRa) domain mediates its interaction with diverse sialylated ligands. *J Biol Chem* 287(19):15837–15850.