

Replacement of I γ 3 germ-line promoter by I γ 1 inhibits class-switch recombination to IgG3

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Class-switch recombination (CSR) enables IgM-producing B cells to switch to the production of IgG, IgE, and IgA. The process requires germ-line (GL) transcription that initiates from promoters upstream of switch (S) sequences and is regulated by the 3' regulatory region (3'RR) located downstream of the Ig heavy chain (IgH) locus. How the 3'RR effect its long-range activation is presently unclear. We generated a mouse line in which I γ 3 GL promoter was replaced by I γ 1. We found that GL transcription could initiate from the inserted I γ 1 promoter and was induced by increased concentrations of IL-4 and that the transcripts were normally spliced. However, when compared with GL transcripts derived from the endogenous I γ 1 promoter in the same stimulation conditions, those from the inserted I γ 1 promoter were less abundant. CSR to C γ 3 was abrogated both *in vivo* and *in vitro*. The results strongly suggest that the endogenous I γ 1 promoter insulates the inserted I γ 1 from the long-range activating effect of the 3'RR. The implications of our findings are discussed in light of the prominent models of long-distance activation in complex loci.

3' regulatory region | Ig heavy chain locus | promoter competition

Two types of rearrangements take place at the Ig locus: V(D)J assembly that generates the variable (V) region genes at the IgH and IgL loci during early stages of B cell development and class-switch recombination (CSR) at the IgH locus of mature B cells. Another genetic alteration known as somatic hypermutation targets the V exons that acquire point mutations, allowing selection of mutated B cell clones that produce higher-affinity antibodies (1).

In the mouse, the constant (C) region genes are organized in the following order: 5'-C μ -C δ -C γ 3-C γ 1-C γ 2b-C γ 2a-C ϵ -C α -3'. CSR occurs between highly repetitive switch (S) sequences located upstream of all of the C genes except C δ . The S sequences differ both in size and in the nature of the repeats (2). CSR is often directed to the same S sequences on both homologous chromosomes (3–5) and is preceded by a biallelic GL transcription directed by I GL promoters (6). The transcripts run through the I exon and the S sequences and undergo polyadenylation downstream of the C exons. Splicing enables fusion of the I exon to the C region and excision of the intervening sequences, yielding sterile transcripts (1). The processing of GL transcripts is required for efficient CSR (7–11). CSR involves several ordered steps that begin with the recognition and targeting of S regions in a GL transcription- and higher-order structures-dependent manner, and the initiation of staggered DNA breaks within partner S sequences by activation-induced cytidine deaminase (AID) (12, 13), a single-stranded-DNA-specific cytidine deaminase (14–17). Studies showed that GL transcription was necessary for the accessibility of S sequences to AID through at least two mechanisms: (i) GL transcripts form RNA-DNA hybrids with the template strand (18–22), whereas the single-stranded nontemplate strand forms long and stable R-loops *in vivo*, which may serve as substrates for AID (23). The latter was shown to associate with the chromatin of the target S sequences in a GL transcription-dependent manner through a direct interaction with the transcription machinery (24). (ii) AID is phosphorylated

in a B cell-specific manner enabling an interaction with replication protein A (25–27).

Activation and targeting of CSR can be mimicked *in vitro* by a combination of certain mitogens and cytokines to induce or suppress GL transcription of specific C genes (2). *Cis*-regulatory elements located upstream of the GL promoters or downstream of the IgH locus control CSR by regulating GL transcription (28). Different knockout experiments demonstrated the importance of GL transcription for efficient CSR (29–32) and its regulation by the 3' regulatory region (3'RR), which comprises four DNase I hypersensitive sites with enhancer activity, hs3a, hs1–2, hs3b, and hs4 (28). Any attempt to tackle the role of the 3'RR in CSR *in vivo* must accommodate at least three facts: (i) GL transcription is necessary for the accessibility of S sequences to AID; (ii) although necessary, GL transcription is not sufficient and processing of GL transcripts is required for efficient CSR; (iii) GL transcription is regulated in a major part by *cis*-regulatory elements upstream of the GL promoters or downstream of the IgH locus.

How the 3'RR effects its long-range activation is presently unclear. Essentially four models have been proposed to account for long-distance interactions between enhancers and promoters. In the looping model, a physical interaction is established between the enhancer and the promoter through protein–protein interactions with a looping-out of the intervening sequences. In the scanning model, the enhancer recruits its specific factors and the complex slides along the chromatin fibre culminating in the contact with the factors bound by the target promoter and looping-out of the intervening sequences. The linking model invokes modified chromatin domains that are established between the enhancer and the promoter through a chain of higher-order complexes generated by facilitator proteins. Finally, the enhancer may direct the promoter to subnuclear compartments where high levels of transcription are achieved (33–37).

Here, we replaced I γ 3 GL promoter with I γ 1 and investigated the consequences of the mutation on GL transcription derived from the endogenous and the replacement I γ 1 promoters and on CSR to the corresponding isotypes.

Results

Replacement of I γ 3 GL Promoter by I γ 1. To generate the mutant mouse line, a 2-kb PmeI-EcoRV fragment containing the I γ 3 promoter region and the proximal part of I γ 3 exon (38, 39) was replaced by an $\approx$ 0.5-kb PCR-amplified fragment comprising

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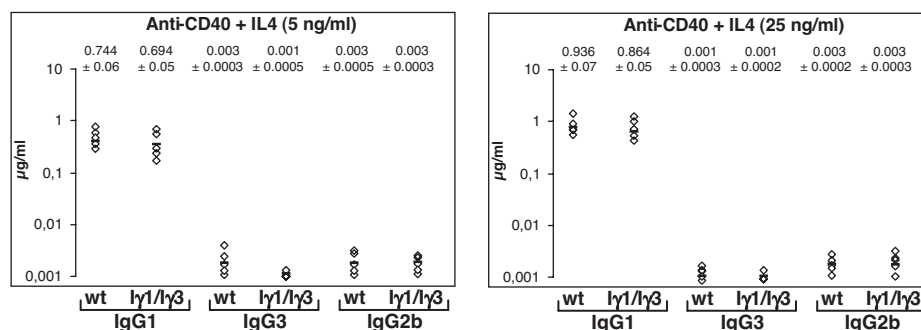


Fig. 1. Analysis of Ig production in the culture supernatants. ELISA analysis of IgG1, IgG3, and IgG2b secretion after anti-CD40+IL4-stimulation at 5 ng/ml and 25 ng/ml of IL4. Splenocytes from five littermates of WT or $\gamma 1/1\gamma 3$ mice were analyzed for anti-CD40+IL4-induced IgG1, IgG3, and IgG2b secretion 5 days after stimulation. The experiment was performed twice. Mean Ig levels from two independent experiments and mean deviations are indicated.

the Iy1 enhancer/promoter with known DNA-binding sites for inducible transcription factors (40, 41) [supporting information (SI) Scheme 1]. We chose to remove 2 kb instead of the ≈ 0.5 -kb sequence that contains Iy3 promoter to get rid of potential unidentified regulatory elements upstream of the Iy3 promoter. The targeting vector was designed so that the homologous recombination event leads to a chimeric sequence made up of the Iy1 enhancer/promoter (hereafter the inserted Iy1 promoter) and of the 368-bp-long distal part of Iy3 exon with its canonical splice donor site. A *neo^r*-specific probe allowed to exclude random integration events in the two recombinant ES clones that were injected into blastocysts (data not shown). Both clones allowed GL transmission of the mutation. The *neo^r* gene was deleted by mating homozygous

N/N mice (*neo^r*-containing alleles) with a Cre-expressing transgenic mice (the homozygous floxed mice will be referred to as I γ 1/I γ 3 mice and the heterozygous mice will be referred to as Δ /+). We amplified the I γ 1/I γ 3 chimera from genomic DNA of mutant mice and checked that no mutation occurred in the inserted sequence (SI Scheme 1).

Analysis of Serum IgG3 in $\gamma 1/\gamma 3$ Mice. To analyze the sera, $\gamma 1/\gamma 3$ mice were bred, and the progeny were bled at week 8. ELISA showed a complete absence of IgG3 in the sera of unimmunized $\gamma 1/\gamma 3$ mice, whereas IgG3 was readily detected in the sera of WT control mice. The other isotypes tested were found in comparable titers in the sera of WT and $\gamma 1/\gamma 3$ mice (SI Fig. 6A and B). Thus, replacement of $\gamma 3$ promoter by $\gamma 1$ leads to a specific shutdown of IgG3 production *in vivo*.

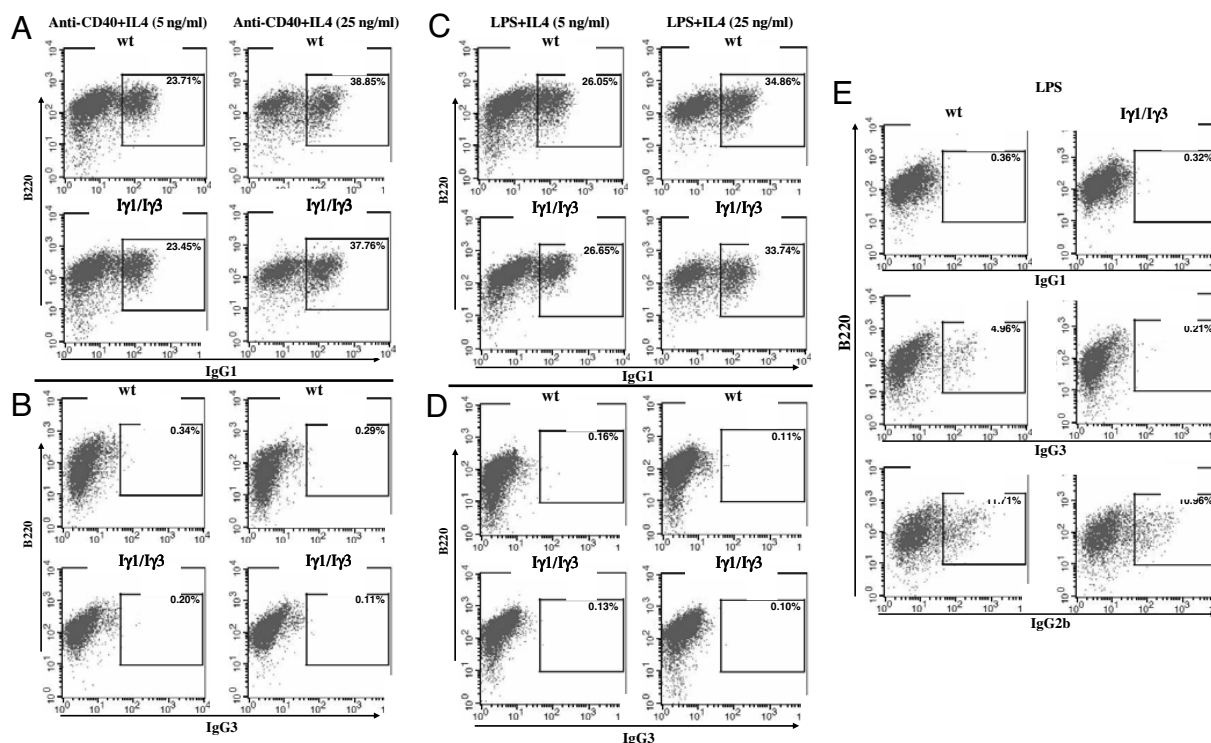


Fig. 2. Cell surface Ig expression on stimulated splenocytes. (A and B) Splenocytes from WT or $\gamma 1/1\gamma 3$ mice were cultured for 5 days with anti-CD40+IL4 at 5 or 25 ng/ml of IL4, and stained with anti-B220 and anti-IgG1 (A) or anti-IgG3 (B). (C and D) Splenocytes from WT or $\gamma 1/1\gamma 3$ mice were cultured for 5 days with LPS+IL4 at 5 or 25 ng/ml of IL4 and stained with anti-B220 and anti-IgG1 (C) or anti-IgG3 (D). (E) Splenocytes from WT or $\gamma 1/1\gamma 3$ mice were cultured for 5 days with LPS and stained with anti-B220 and anti-IgG1, anti-IgG3, or anti-IgG2b. The percentages of switched splenic B cells among the B220+ populations are indicated. The data shown are representative of two independent experiments.

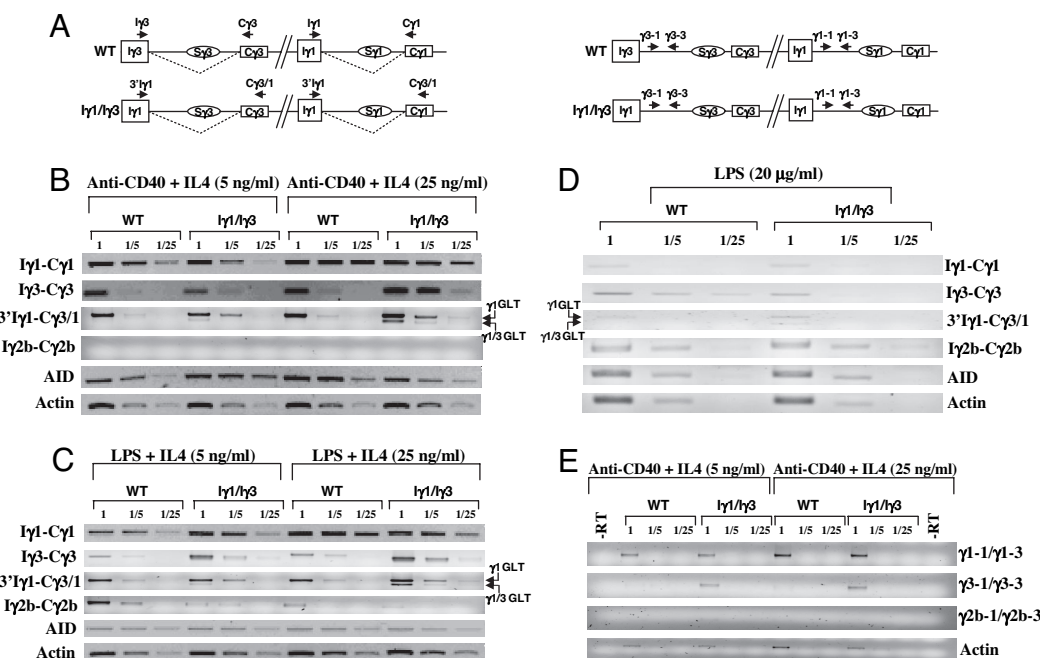


Fig. 3. Analysis of GL transcription. (A) Relative position of some primers used for RT-PCR on the spliced (dotted line) $\gamma 3$ and $\gamma 1$ transcripts (Left) and on unspliced transcripts (Right) from WT and $I\gamma 3/I\gamma 1$ alleles respectively (not to scale). (B) RT-PCR was performed on WT or $I\gamma 1/I\gamma 3$ GL transcripts from anti-CD40+IL4-activated splenocytes RNA (day 3) for $I\gamma 1$ -C $\gamma 1$, $I\gamma 3$ -C $\gamma 3$, $I\gamma 2b$ -C $\gamma 2b$, 3'I $\gamma 1$ -C $\gamma 3/1$ GL, or AID and actin transcripts, respectively. Single-stranded cDNAs or dilutions thereof (1/5 and 1/25) were subjected to PCR, using appropriate primers. Arrows indicate transcripts initiating from the endogenous $I\gamma 1$ promoter (upper band, referred to as $\gamma 1$ GLT) or from the replacement promoter (lower band, $\gamma 1/\gamma 3$ GLT). (C) RT-PCR was performed as in A on GL transcripts from LPS+IL4-activated splenocytes. (D) RT-PCR was performed as in A on GL transcripts from LPS-activated splenocytes. (E) RT-PCR was performed on WT or $I\gamma 1/I\gamma 3$ RNAs from nuclei of anti-CD40+IL4-activated splenocytes for $\gamma 1$, $\gamma 3$, $\gamma 2b$, or β -actin unspliced transcripts, respectively. Single-stranded cDNAs or dilutions thereof (1/5 and 1/25) were subjected to PCR, using appropriate primers.

IgG3 Production by *in Vitro*-Activated Splenocytes. To check that the lack of IgG3 production by homozygous mice is a B cell autonomous process, we resorted to *in vitro* activation of splenocytes. CSR to IgG1 can be mimicked *in vitro* by culturing splenic B cells in the presence of anti-CD40+IL4 or LPS+IL4. The treatments activate $I\gamma 1$ GL promoter, which contains NF- κ B and IL4-responsive motifs (SI Scheme 1), thus enhancing GL transcription from $I\gamma 1$ promoter and subsequent switching to IgG1. In the $I\gamma 1/I\gamma 3$ mouse line, the treatment should enable transcription and hence the accessibility of the S $\gamma 1$ region downstream of the endogenous $I\gamma 1$ promoter and of S $\gamma 3$ sequences downstream of the inserted $I\gamma 1$ promoter, potentially leading to the production of IgG1 and IgG3, respectively.

Total splenocytes from littermates were stimulated with anti-CD40+IL4 for 5 days, and supernatants were analyzed by ELISA. At 5 ng/ml of IL4, we found comparable IgG1 secretion in $I\gamma 1/I\gamma 3$ supernatants and WT controls. An increase in IgG1 production was detected for both genotypes by increasing the concentration of IL4 to 25 ng/ml (Fig. 1). In contrast, IgG3 production in $I\gamma 1/I\gamma 3$ supernatants was at the background level at both concentrations of IL4 (Fig. 1). The same pattern was found for IgG3 production when $I\gamma 1/I\gamma 3$ splenocytes were stimulated with LPS+IL4 at both IL4 concentrations (SI Fig. 7). When splenocytes were cultured in the presence of LPS alone, a treatment that induces switching to IgG3 and IgG2b, no IgG3 was detected in the supernatants of $I\gamma 1/I\gamma 3$ splenocytes in contrast to WT and IgG2b controls (SI Fig. 7).

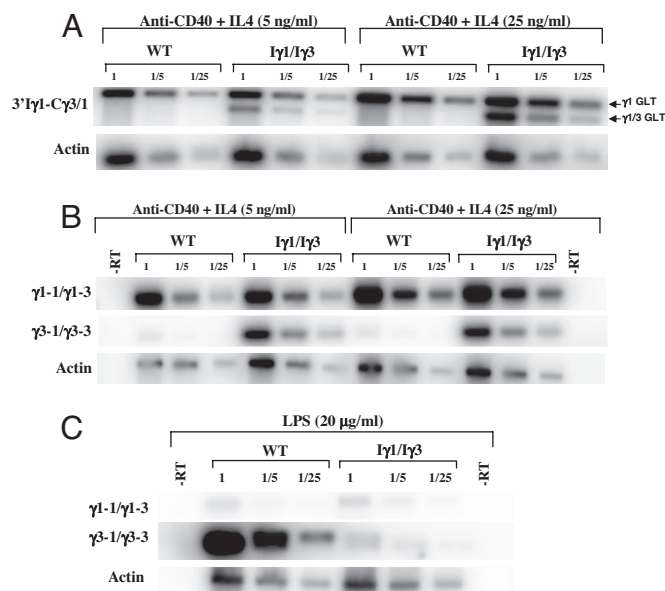
Surface Expression on *in Vitro*-Activated Splenocytes. Surface expression of IgG3, IgG1, IgG2b, and IgA on LPS-, anti-CD40+IL4-, LPS+IL4-, or LPS+TGF- β -activated splenocytes was monitored by flow cytometry, using an anti-B220 antibody and anti-IgG3, anti-IgG1, anti-IgG2b, or anti-IgA antibodies. Surface expression of IgG1 was comparable in WT and $I\gamma 1/I\gamma 3$ B220⁺ splenocytes stimulated with anti-CD40+IL4 and similarly increased with IL4 concentration ($\approx 23\%$ at 5 ng/ml of IL4 and $\approx 38\%$ at 25 ng/ml of

IL4 for both genotypes) (Fig. 2A). Such increase in surface expression was not observed for IgG3, IgG2b, or IgA, which remained at the background level in the same stimulation conditions for both WT and $I\gamma 1/I\gamma 3$ B cells (Fig. 2B and SI Fig. 8). A similar pattern was found with LPS+IL4 stimulation (Fig. 2C and D and SI Fig. 8). In contrast, LPS stimulation allowed surface expression of IgG2b to comparable levels between WT and $I\gamma 1/I\gamma 3$ B220⁺ splenocytes but failed to induce surface expression of IgA, the latter being induced by LPS+TGF- β (Fig. 2E and SI Fig. 8). IgG3-surface expression was induced in the WT- but not in the mutant LPS-activated splenocytes (Fig. 2E).

Thus, increasing IL4 concentration leads to a parallel increase in IgG1 but not in IgG3 surface expression, again indicating a shut-down of IgG3 production in $I\gamma 1/I\gamma 3$ splenocytes. In addition, LPS and LPS+TGF- β stimulations clearly show that the defect does not target downstream isotypes.

Analysis of GL Transcription. Given the abrogation of IgG3 production *in vitro* and *in vivo* and the extinction of IgG3 surface expression on the $I\gamma 1/I\gamma 3$ splenocytes, it was critical to check the GL transcription that initiates from the inserted $I\gamma 1$ promoter. Total RNAs from anti-CD40+IL4- or LPS+IL4-activated WT and $I\gamma 1/I\gamma 3$ splenocytes were reverse-transcribed and amplified in semiquantitative conditions, using isotype-specific GL transcript primers. For the inserted $I\gamma 1$ promoter, we used a pair of primers specific for the distal part of $I\gamma 3$ exon and the C $\gamma 3$ -1 exon ($I\gamma 3$ -C $\gamma 3$) and a primer in the 3' part of $I\gamma 1$ promoter, which should, in combination with a primer whose sequence is common to C $\gamma 3$ and C $\gamma 1$, amplify both the hybrid $\gamma 3$ (797 bp) and the native $\gamma 1$ (900 bp) transcripts (3'I $\gamma 1$ -C $\gamma 3/1$ primers) (Fig. 3A).

By using isotype-specific primers, we noted an increase in the abundance of the $I\gamma 1$ -C $\gamma 1$ transcripts for both genotypes by increasing the concentration of IL4 to 25 ng/ml. A different picture emerged for $I\gamma 3$ -C $\gamma 3$ transcripts, where the increase in IL4 led to



Our results do not disprove the subnuclear redirection model (see below); however, it is difficult to figure out why the 3'RR should direct the endogenous Iy1 but not the inserted Iy1 promoter to the transcription factories (48). Although the large distance between the Iy1 promoters may be invoked to explain their different outcomes, the phenotype of mice bearing insertions of the *neo'* gene at the IgH constant locus suggests that the distance between the promoters might not be the real issue, because transcription from upstream promoters was severely impaired regardless of their distance from the *neo'* gene.

Our data support the notion that an activation signal originating from the 3'RR is interrupted by the active endogenous I γ 1 promoter and hence does not reach the inserted I γ 1 promoter. Although our current data do not argue against the linking model, we favor the view that the active endogenous I γ 1 promoter somehow insulates the upstream I γ 1 promoter from the activating effect of the 3'RR, perhaps through a tracking/scanning model. Based on the model of West and Fraser (37), one possibility would be that, in response to the appropriate stimulation, the 3'RR directs the IgH constant locus to a transcription factory where some opening of the constant locus occurs. The signal-mediated selection of I γ 1 GL promoters would allow recruitment of specific factors that bind to

proximal sequences, enabling a 3'RR-independent transcription to occur. The 3'RR-transcription factors complex would slide along the chromatin fiber; alternatively, the latter is reeled in until a stable contact is established between the 3'RR and the endogenous I γ 1 promoter, allowing a high level of transcription. The "sequestration" of the 3'RR by the endogenous I γ 1 promoter would hamper interaction between the 3'RR and the upstream I γ 1 promoter. Transcription activation from the upstream promoter would thus rely mainly on proximal sequences that seem insufficient for CSR. This model would predict that insertion of I γ 1 promoter downstream of the endogenous I γ 1 promoter would lead to a decrease of GL transcription derived from the endogenous promoter. This hypothesis is currently being tested.

An important finding in this study is the complete and specific inhibition of CSR to C γ 3 in I γ 1/I γ 3 mice both *in vivo* and *in vitro*. Given the requirement of GL transcription for CSR, one potential explanation is that, despite induced GL transcription from the inserted I γ 1 promoter, the minimal threshold of GL transcription required for detectable CSR has not been reached. In this scenario, by sequestering the 3'RR, the endogenous I γ 1 promoter would transcriptionally out-compete the inserted I γ 1 promoter leading to CSR to IgG1 but not to IgG3. Alternatively, one might speculate that the severity of the inhibition is such that it cannot be explained solely on the basis of lesser GL transcription from the inserted I γ 1 promoter and that insulation of the 3'RR may have profound effects on CSR to S γ 3 in a more complex way than by simply impairing its GL transcription. In this scenario, although decreased, GL transcription derived from the inserted I γ 1 promoter would be sufficient to ensure some CSR to C γ 3; however, retention of the 3'RR by the downstream promoter may somehow block the recombination step at S γ 3. An intriguing possibility would be that, in addition to its role as a transcriptional control element, the 3'RR may act as a switch recombination enhancer that might play a role in conferring isotype specificity. How the 3'RR achieves this function is presently unclear, a situation that is reminiscent of E μ enhancer, which acts both as a transcriptional and a recombinational control element during V(D)J recombination (49, 50).

Materials and Methods

Targeting Vector and Mice. The $\gamma 1$ -targeting construct was generated by using a plasmid containing an ≈ 8 -kb XhoI-BamHI fragment spanning $\gamma 3$ and $5\gamma 3$. A ClaI linker was inserted in PmeI and EcoRV sites and the resulting plasmid was digested with ClaI and self-ligated. The $\gamma 1$ promoter was PCR-amplified by using ZO $\gamma 1$ -1 and ZO $\gamma 1$ -2 primers. The PCR product was checked by sequencing and inserted downstream of the *neo^r* gene. The whole cassette was then excised as a ClaI fragment and inserted in ClaI site of the targeting construct. An HSV *tk* gene was inserted in the NotI site for negative selection. The ES cell line CK35 [kindly provided by C. Kress (Institut Pasteur, Paris, France)] was transfected by electroporation and selected by using G418 (300 μ g/ml) and gancyclovir (2 μ M). Recombinant clones were identified by Southern blot analysis after an EcoRI digest with external probes: a 1.0-kb EcoRI-XhoI fragment as a 5' probe and a 1.7-kb BamHI-SphI as a 3' probe. Two ES clones showing homologous recombination were injected into C57BL/6 blastocysts and the male chimeras were then mated with C57BL/6 females. GL transmission of the mutation was checked by Southern blot, using the same digest and probes. Homozygous N/N mutant mice were mated with Ella-cre transgenic mice [a kind gift of H. Westphal (National Institute of Child Health and Human Development, Bethesda, MD), used under a noncommercial research license agreement from Dupont Pharma]. The progeny was checked by Southern blot for Cre-mediated deletion, using a Δ probe and a 440-bp PCR-amplified fragment, using $\gamma 3f$ and $\gamma 3-4$ primers. The fragment spans the 3' part of $\gamma 3$ exon and the adjacent downstream intronic sequences. The experiments on mice have been carried out according to the Centre National de la Recherche Scientifique Ethical Committee guidelines and approved by the Committee.

Spleen Cell Cultures. Single-cell suspensions of splenocytes from 6- to 8-week-old mice were activated *in vitro* at a density of 10^6 cells per ml in RPMI medium 1640 supplemented with 10% FCS, 50 μ M 2-ME, and 20 μ g/ml of LPS (*S. typhimurium*; Sigma) or 500 ng/ml of anti-CD40 (R&D Systems). IL4

(R&D Systems) was added at 5 ng/ml or at 25 ng/ml. TGF- β (R&D Systems) was added at 1 ng/ml. At day 3, aliquots of cells were removed for RNA preparation.

Flow Cytometry Analysis. At day 5 after stimulation, splenocytes (5×10^5 cells per assay) were labeled by using spectral red-conjugated anti-B220 and FITC-conjugated anti-IgG3, anti-IgG2b, anti-IgG1, or anti-IgA (BD Pharmingen). Isotype controls were included in each experiment for each stimulation condition. Data were obtained on 1.5×10^4 viable cells by using a Beckman Coulter XL apparatus.

ELISAs. Sera or supernatants from spleen cell cultures (harvested at day 5 after stimulation) were analyzed for the presence of IgM, IgG3, IgG1, IgG2a, IgG2b, and IgA by ELISA as described in ref. 46. Serum analysis was performed on the progeny of two independent breedings. For each progeny, six mice per genotype were independently analyzed twice.

Oligonucleotides and RT-PCR Analysis of GL Transcription. The oligonucleotides are listed in SI Fig. 10. The RT-PCR conditions for spliced transcripts and the expected sizes of the PCR products have been described (46, 51). After agarose gel electrophoresis, the PCR products were transferred to nylon membranes (Perkin-Elmer) and hybridized to 5' end-radiolabeled Cyt3/1 or mActi-5 oligonucleotides. The hybridization signals were quantified by a phosphorimager (Molecular Dynamics). To avoid potential PCR saturation problems with undiluted samples, the comparisons were based on the diluted samples' signals and corrected to the corresponding actin signals.

For unspliced transcripts, splenic B cells from 6-week-old mice were purified as the negative fraction, using anti-CD43 beads and LS columns (Miltenyi) according

to the supplier's protocol. B cells were stimulated with LPS (20 μ g/ml) or anti-CD40 (500 ng/ml) and IL4 (at 5 or 25 ng/ml) at a density of 10^6 cells per ml. After 48 h, the cells were washed twice with ice-cold PBS, gently resuspended in 10 mM Tris (pH 7.4), 10 mM NaCl, 2.5 mM MgCl₂, and 0.5% Nonidet P-40 and left on ice for 5 min. The cell lysates were carefully added on the top of an equal volume of 10 mM Tris (pH 7.4), 10 mM NaCl, 2.5 mM MgCl₂, 10% sucrose, and 0.5% Nonidet P-40 and centrifuged for 10 sec at 4,800 rpm (A4-44 rotor; Eppendorf). The nuclear pellets were lysed in TRIzol (Invitrogen), and RNA was purified according to the supplier's instructions. The contaminating DNA was removed by adding DNase I (Invitrogen), the purified RNA was reverse-transcribed following the manufacturer's protocol, and the resulting single-strand cDNA was subjected to PCR, using $\gamma 1-1/\gamma 1-3$, $\gamma 3-1/\gamma 3-3$, or Acti6/Acti7 primer pairs. All of the expected PCR products are 246 bp long, and the corresponding sequences are located upstream of the 5' sequences ($\gamma 3$, $\gamma 1$, and $\gamma 2b$) and within the first intron of the β -actin gene, respectively. PCR controls included RNA substrates without reverse transcription and water with the primers. PCR conditions were as follows: 94°C for 5 min, 35 cycles (94°C for 2 min, 55°C for 30 sec, and 72°C for 1 min). The RT-PCR products were transferred to nylon membranes and hybridized to the corresponding amplicons that were radiolabeled by random-priming. The hybridization signals were quantified as described above.

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- Chaudhuri J, Alt FW (2004) *Nat Rev Immunol* 4:541–552.
- Stavnezer J (2000) *Curr Top Microbiol Immunol* 245:127–168.
- Radbruch A, Muller W, Rajewsky K (1986) *Proc Natl Acad Sci USA* 83:3954–3957.
- Hummel M, Berry JK, Dunnick W (1987) *J Immunol* 138:3539–3548.
- Winter E, Krawinkel U, Radbruch A (1987) *EMBO J* 6:1663–1671.
- Delpy L, Le Bert M, Cogné M, Khamlichi AA (2003) *Eur J Immunol* 33:2108–2113.
- Lorenz M, Jung S, Radbruch A (1995) *Science* 267:1825–1828.
- Hein K, Lorenz MG, Siebenkotten G, Petry K, Christine R, Radbruch A (1998) *J Exp Med* 188:2369–2374.
- Qiu G, Harriman GR, Stavnezer J (1999) *Int Immunol* 11:37–46.
- Kuzin II, Ugine GD, Wu D, Young F, Chen J, Bottaro A (2000) *J Immunol* 164:1451–1457.
- Samara M, Oruc Z, Dougier HL, Essawi T, Cogné M, Khamlichi AA (2006) *Int Immunol* 18:581–589.
- Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T (2000) *Cell* 102:553–563.
- Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O, Catalan N, Forveille M, Dufourcq-Lapelouse R, Gennery A, et al. (2000) *Cell* 102:565–575.
- Chaudhuri J, Tian M, Khuong C, Chua K, Pinaud E, Alt FW (2003) *Nature* 422:726–730.
- Dickerson SK, Market E, Besmer E, Papavasiliou FN (2003) *J Exp Med* 197:1291–1296.
- Pham P, Bransteitter R, Petruska J, Goodman MF (2003) *Nature* 424:103–107.
- Ramiro AR, Stavropoulos P, Jankovic M, Nussenzweig MC (2003) *Nat Immunol* 4:452–456.
- Reaban ME, Griffin JA (1990) *Nature* 348:342–344.
- Reaban ME, Lebowitz J, Griffin JA (1994) *J Biol Chem* 269:21850–21857.
- Daniels GA, Lieber MR (1995) *Nucleic Acids Res* 23:5006–5011.
- Tian M, Alt FW (2000) *J Biol Chem* 275:24163–24172.
- Mizuta R, Iwai K, Shigeno M, Mizuta M, Uemura T, Ushiki T, Kitamura D (2003) *J Biol Chem* 278:4431–4434.
- Yu K, Chedin F, Hsieh CL, Wilson TE, Lieber MR (2003) *Nat Immunol* 4:442–451.
- Nambu Y, Sugai M, Gonda H, Lee CG, Katakai T, Agata Y, Yokota Y, Shimizu A (2003) *Science* 302:2137–2140.
- Basu U, Chaudhuri J, Alpert C, Dutt S, Ranganath S, Li G, Schrum JP, Manis JP, Alt FW (2005) *Nature* 438:508–511.
- Pasqualucci L, Kitaura Y, Gu H, Dalla-Favera R (2006) *Proc Natl Acad Sci USA* 103:395–400.
- Chaudhuri J, Khuong C, Alt FW (2004) *Nature* 430:992–998.
- Khamlichi AA, Pinaud E, Decourt C, Chauveau C, Cogné M (2000) *Adv Immunol* 75:317–345.
- Jung S, Rajewsky K, Radbruch A (1993) *Science* 259:984–987.
- Zhang J, Bottaro A, Li SC, Stewart V, Alt FW (1993) *EMBO J* 12:3529–3537.
- Bottaro A, Lansford R, Xu L, Zhang J, Rothman P, Alt FW (1994) *EMBO J* 13:665–674.
- Harriman GR, Bradley A, Das S, Rogers-Fani P, Davis AC (1996) *J Clin Invest* 97:477–485.
- Blackwood EM, Kadonaga JT (1998) *Science* 281:61–63.
- Bulger M, Groudine M (1999) *Genes Dev* 13:2465–2477.
- Engel JD, Tanimoto K (2000) *Cell* 100:499–502.
- Francetel C, Schubeler D, Martin DJ, Groudine M (2000) *Nat Rev Mol Cell Biol* 1:137–143.
- West AG, Fraser P (2005) *Hum Mol Genet* 14:R101–111.
- Rothman P, Lutzker S, Gorham B, Stewart V, Coffman R, Alt FW (1990) *Int Immunol* 2:621–627.
- Gerondakis S, Gaff C, Goodman DJ, Grumont RJ (1991) *Immunogenetics* 34:392–400.
- Lin SC, Stavnezer J (1996) *Mol Cell Biol* 16:4591–4603.
- Xu MZ, Stavnezer J (1992) *EMBO J* 11:145–155.
- Hu X, Eszterhas S, Pallazzi N, Bouhassira EE, Fields J, Tanabe O, Gerber SA, Bulger M, Engel JD, Groudine M, Fiering S (2007) *Blood* 109:2210–2216.
- Cogné M, Lansford R, Bottaro A, Zhang J, Gorman J, Young F, Cheng HL, Alt FW (1994) *Cell* 77:737–747.
- Manis JP, van der Stoep N, Tian M, Ferrini R, Davidson L, Bottaro A, Alt FW (1998) *J Exp Med* 188:1421–1431.
- Seidl KJ, Manis JP, Bottaro A, Zhang J, Davidson L, Kisselgof A, Oettgen H, Alt FW (1999) *Proc Natl Acad Sci USA* 96:3000–3005.
- Pinaud E, Khamlichi AA, Le Morvan C, Drouet M, Nalesso V, Le Bert M, Cogné M (2001) *Immunity* 15:187–199.
- Dunnick WA, Shi J, Graves KA, Collins JT (2005) *J Exp Med* 201:1459–1466.
- Osborne CS, Chakalova L, Brown KE, Carter D, Horton A, Debrand E, Goyenechea B, Mitchell JA, Lopes S, Reik W, Fraser P (2004) *Nat Genet* 36:1065–1071.
- Hesslein DG, Schatz DG (2001) *Adv Immunol* 78:169–232.
- Jung D, Giallourakis C, Mostoslavsky R, Alt FW (2006) *Annu Rev Immunol* 24:541–570.
- Khamlichi AA, Glaudet F, Oruc Z, Denis V, Le Bert M, Cogné M (2004) *Blood* 103:3828–3836.



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Scheme 1. (A) Structure of the targeted locus: scheme of a WT allele showing a rearranged IgH locus and highlighting the $\gamma 3$ and the $\gamma 1$ genes and the 3'RR (not to scale). (B) Structure of the targeting construct in which the *neor* cassette flanked by two loxP sites (filled triangles) followed by the I $\gamma 1$ promoter were inserted into the ClaI site used to replace PmeI and EcoRV sites, respectively. 'I $\gamma 3$ stands for the distal part of the I $\gamma 3$ exon. (C) Structure of the targeted allele. An XbaI site in the I $\gamma 3$ region is lost following replacement by I $\gamma 1$ promoter and two EcoRI sites are brought in by the *neor* cassette. The location of the 5' probe (1-kb EcoRI-XhoI fragment), the 3' probe (1.7 kb BamHI-SphI fragment) and the Δ probe (a 440-bp PCR fragment) is indicated. (D) Structure of the floxed allele. The relative position of the primers used to amplify the I $\gamma 1$ promoter from genomic DNA of floxed mice to check that no mutation was introduced during the targeting event are indicated. B, BamHI; C, ClaI; P, PmeI; R, EcoRI; RV, EcoRV; S, SphI; X, XbaI; Xh, XhoI. (E) Southern blot analysis of WT and mutant mice before Cre-mediated deletion of the selectable marker using two external probes following an EcoRI digest, and after removal of the selectable marker using an internal probe (Δ probe). (F) Nucleotide sequence of I $\gamma 1$ promoter (capital letter). The critical NF- κ B and STAT6 sites that respond to anti-CD40 and IL4 respectively are highlighted in bold and boxed. ClaI site is shown in underlined lowercase letters. The distal part of I $\gamma 3$ exon is indicated by italicized capital letter. The splice donor site is shown in bold and underlined. The 5' part of the $\gamma 3$ intron is shown in lower case letters. The primers used to check the sequence of the I $\gamma 1$ promoter in floxed mice are underlined.

SI Figure 6

Fig. 6. (A) Analysis of Ig production in the sera of unimmunized mice. Analysis of IgM, IgG1, IgG3, IgG2a, IgG2b, and IgA secretion in 8-week-old mice was done by ELISA. Six mice from each genotype were analyzed. Mean Ig levels from two independent experiments and mean deviations are indicated. (B) Analysis of Ig production in the sera of unimmunized mice. Analysis of IgM, IgG1, IgG3, IgG2a, IgG2b, and IgA secretion in 8-week-old mice was done by ELISA. Six mice from each genotype were analyzed. The results are from littermates of breedings independent from those used in SI Fig. 6A. Mean Ig levels from two independent experiments and mean deviations are indicated.

SI Figure 7

Fig. 7. Analysis of Ig production in the culture supernatants. ELISA analysis of IgG1, IgG3, and IgG2b secretion after LPS- or LPS+IL4-induced stimulation at 5 ng/ml and 25 ng/ml of IL4, or of IgA secretion after LPS+TGF- β -induced stimulation. Splenocytes from five littermates of WT or I γ 1/I γ 3 mice were analyzed. The experiment was performed twice. Mean Ig levels from two independent experiments and mean deviations are indicated.

SI Figure 8

Fig. 8. FACS analysis of stimulated splenocytes. (A-D) Cell surface IgG2b and IgA expression on stimulated splenocytes. Splenocytes from WT or I γ 1/I γ 3 mice were cultured as described in Fig. 4 and stained with anti-B220 and anti-IgG2b or anti-IgA. (E) Cell surface IgA expression on LPS+TGF- β -stimulated splenocytes. Splenocytes were stained with anti-B220 and anti-IgA. The percentages of switched splenic B cells among the B220+ populations are indicated. The data shown are representative of two independent experiments.

SI Figure 9

Fig. 9. (A) Oligonucleotides used in this study. (B) Nucleotide sequence at the junction between the I γ 3 and C γ 3-1 exons. The sequences of the primers I γ 3f and C γ 3r used to amplify the distal part of I γ 3 exon (I γ 3, in bold capital letters) and the proximal part of the first exon of C γ 3 gene (C γ 3-1, italicized capital letters) are underlined. The corresponding genomic sequences are shown below. The splice donor site (capital letters) and acceptor site (lowercase letters) are underlined. Intron sequences are shown in lower case letters. The genomic sequences are from EMBL-ID:MMD343.

SI Figure 10

Fig. 10. Quantification of GL transcription. (A-C) The hybridization signals were quantified by a phosphorImager. Total cpm of each band was quantified and the ratio for different genotypes and stimulation conditions were determined as indicated in the tables. The ratios were corrected to the corresponding actin ratios. The ratios were calculated for 1/5 and 1/25 dilutions and yielded similar results. The data from two independent experiments are shown.

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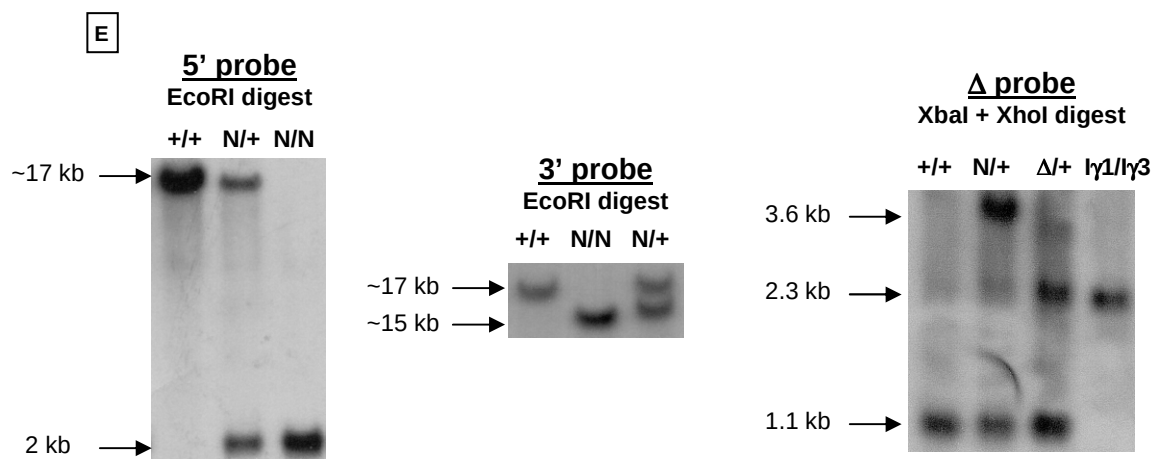
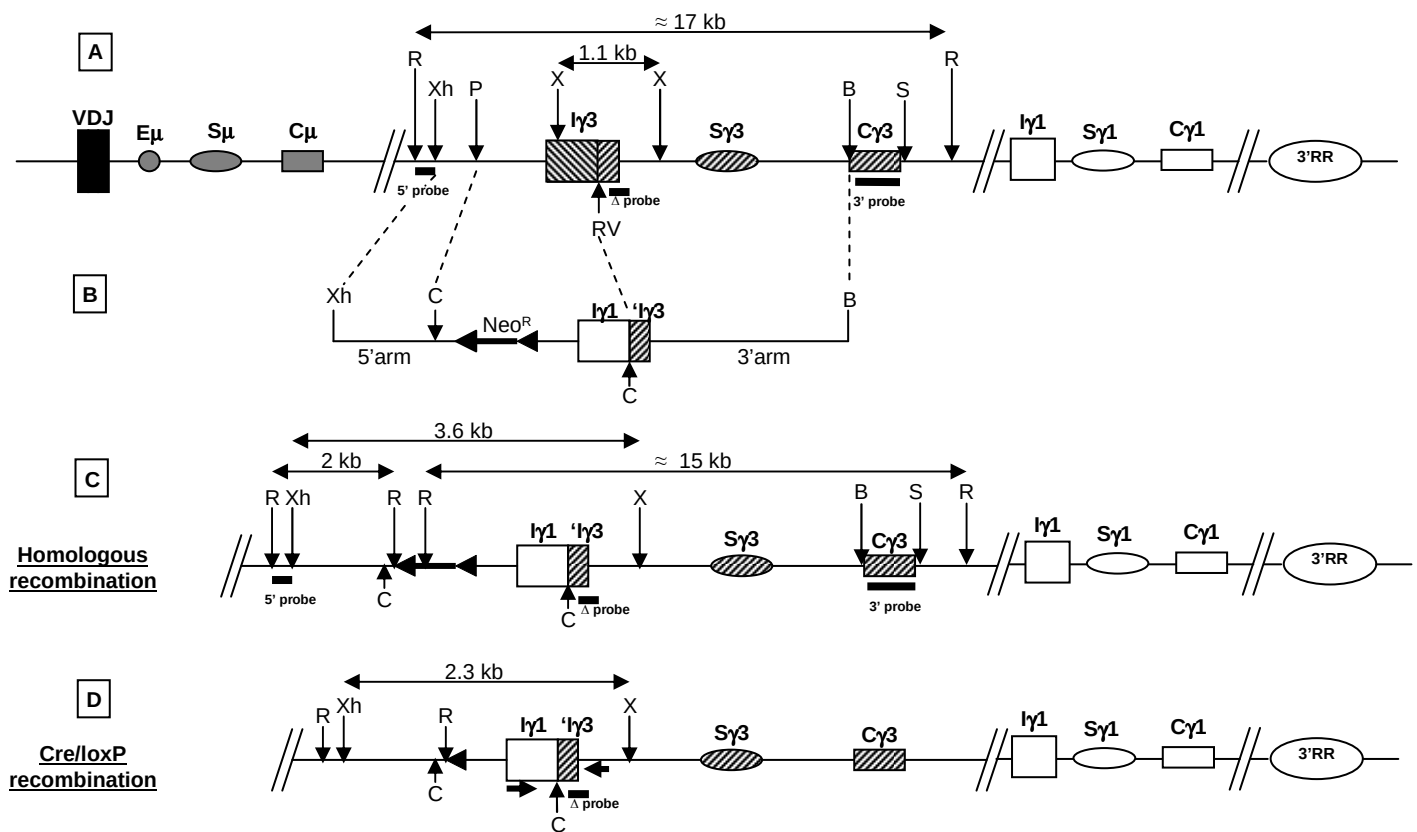
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F

ZOy1-2 primer

GGACTCTGTACTCTGTTCACAGCCCTCAGCCTCTGTGCCTCAGGCGAATCCGGGTTTTCTTGAGATAGCACCAAC
AAGATACAGGTAAAGGGAGATATAGCCTCCAAAGCTGGACTTACTTAGATCAGCCATGAATGAGAGACCCAGTT
CCTATGAAACCTCTCCAATGAGGTGGTCAGGAAAGAGTGGGCATAATCACTCAGCCTCAACCAGCCCAGGGACC
CCCAAAGGCCAGGTGCACAGAGAAACAGGAGTACAGCCAGGCTTCCTACCTTCTCCCCTGAGTCTCAAGGGTA

STAT6 site

CCGCCTCACCCTCACCACACACATTCACATGAAGTAATCTAAGTCAGGTTTGGACTCCCCCTCACCCTCTGACAC

C/EBP

κB1 site

AGAAACCCCCAGAATGAAAGGGGAACCCCTGTCAGGAAATGCCTTATGCCACCCACTGTCAATCCTGTTCTTAatc

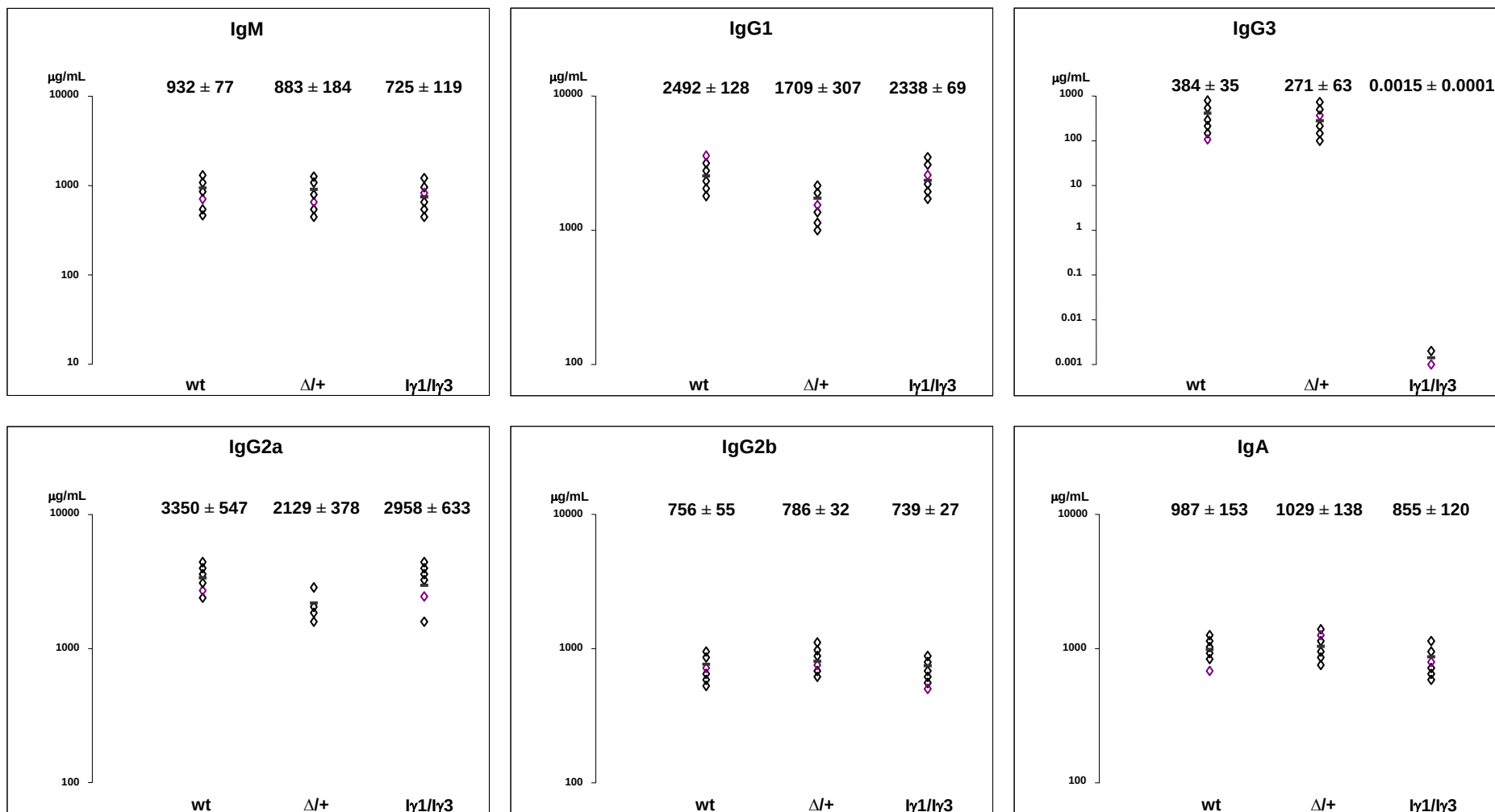
κB2 site

κB3 site

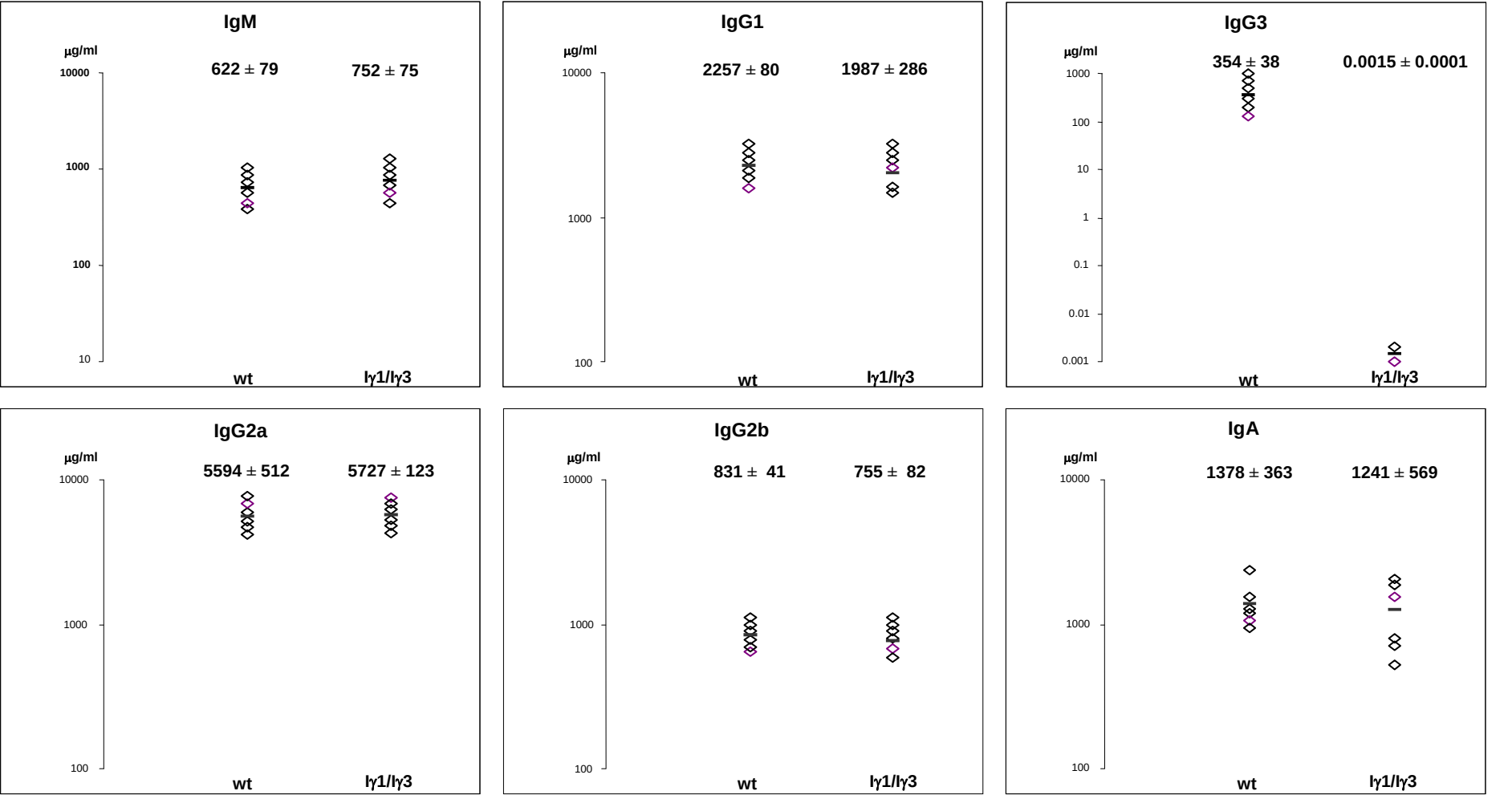
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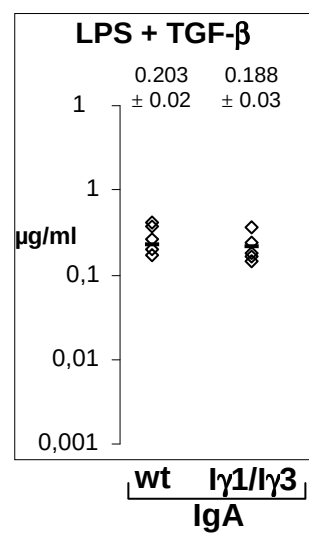
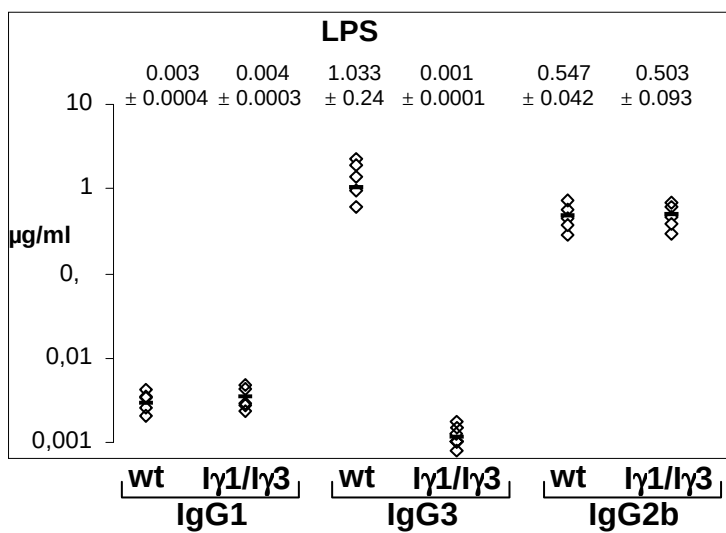
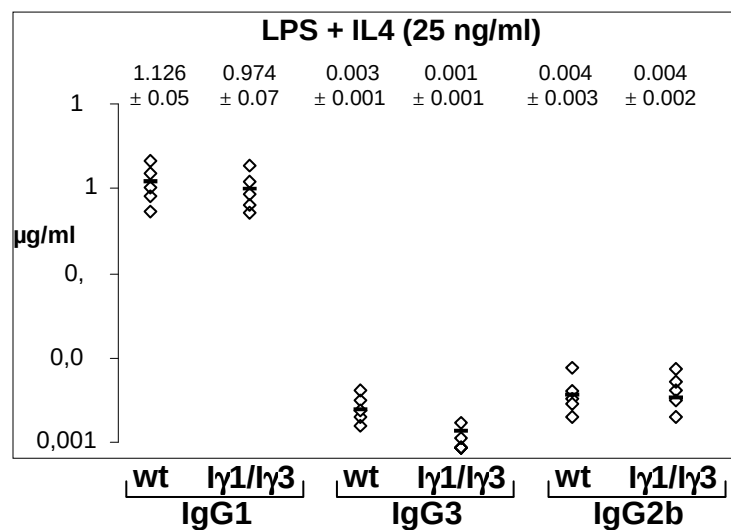
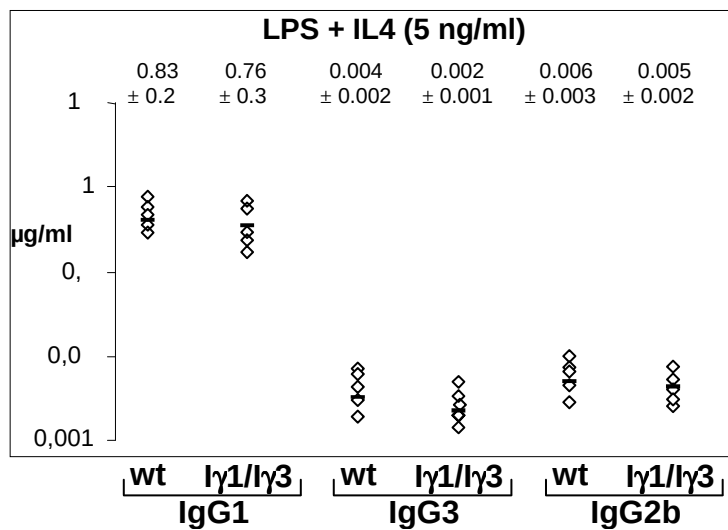
γ3-4 primer

SI Figure 6A

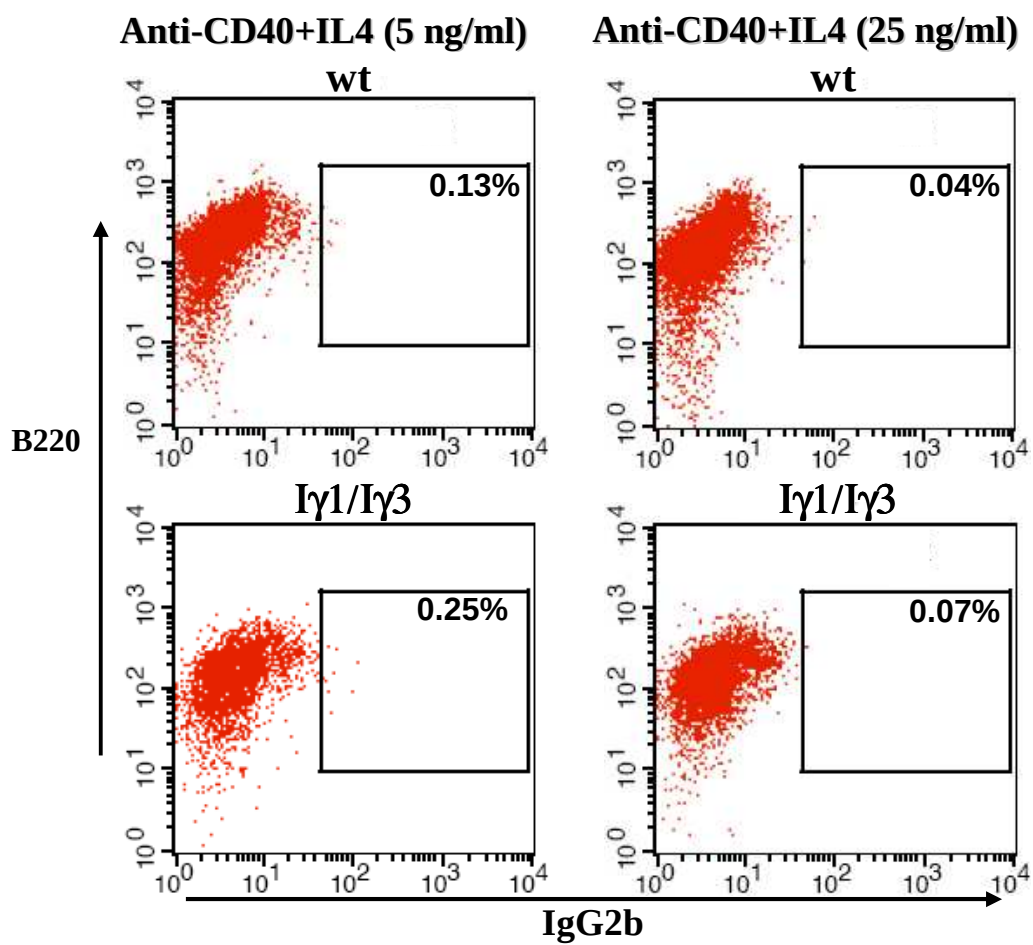


SI Figure 6B

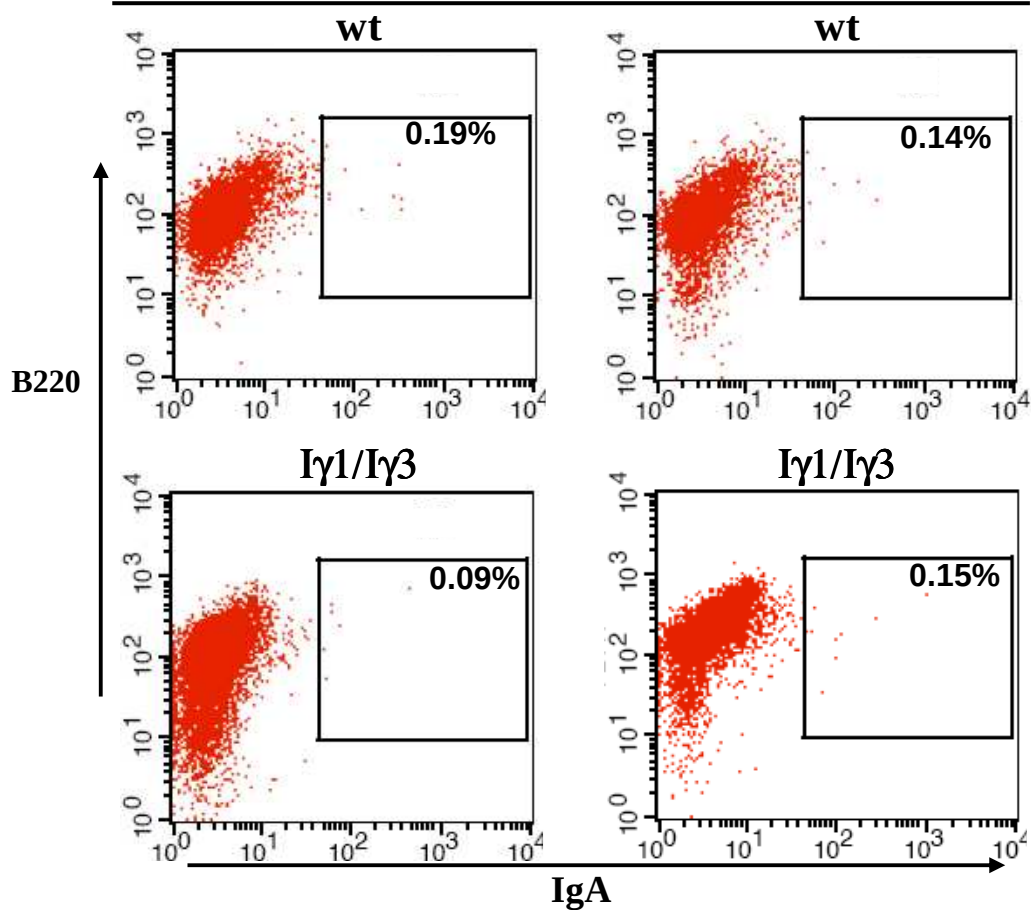




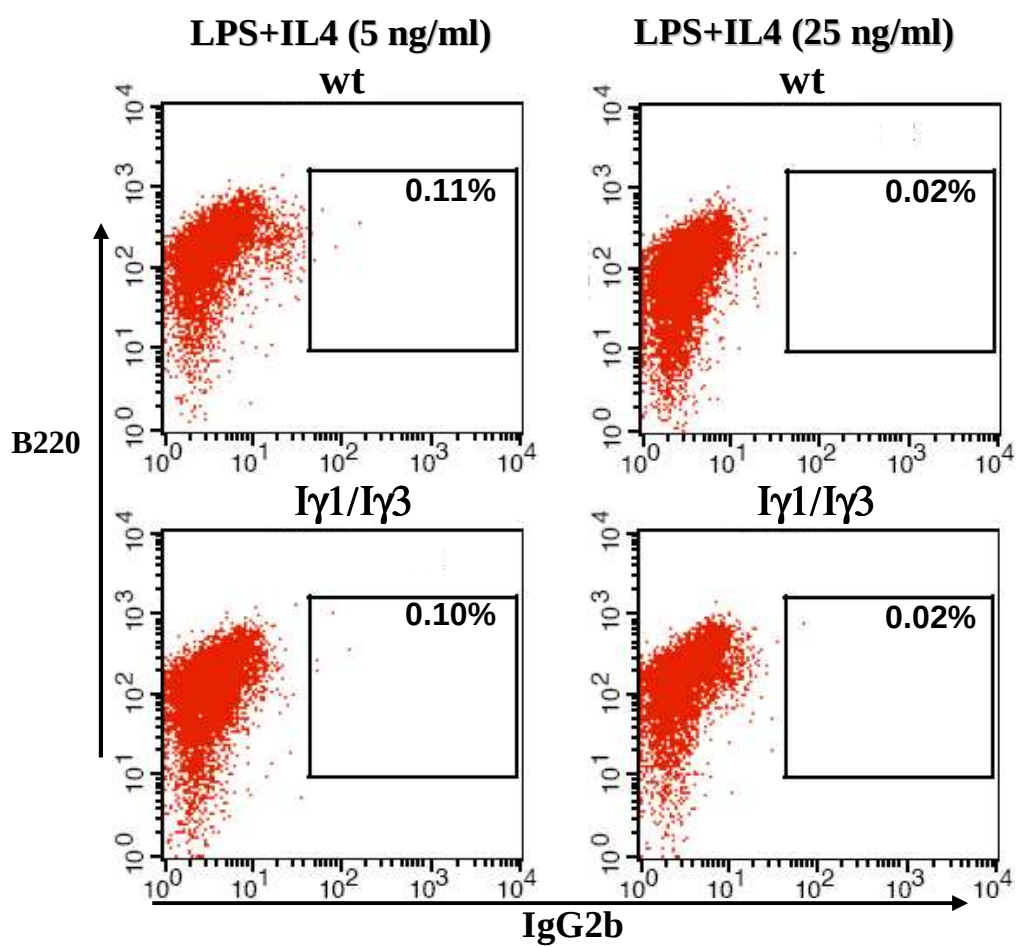
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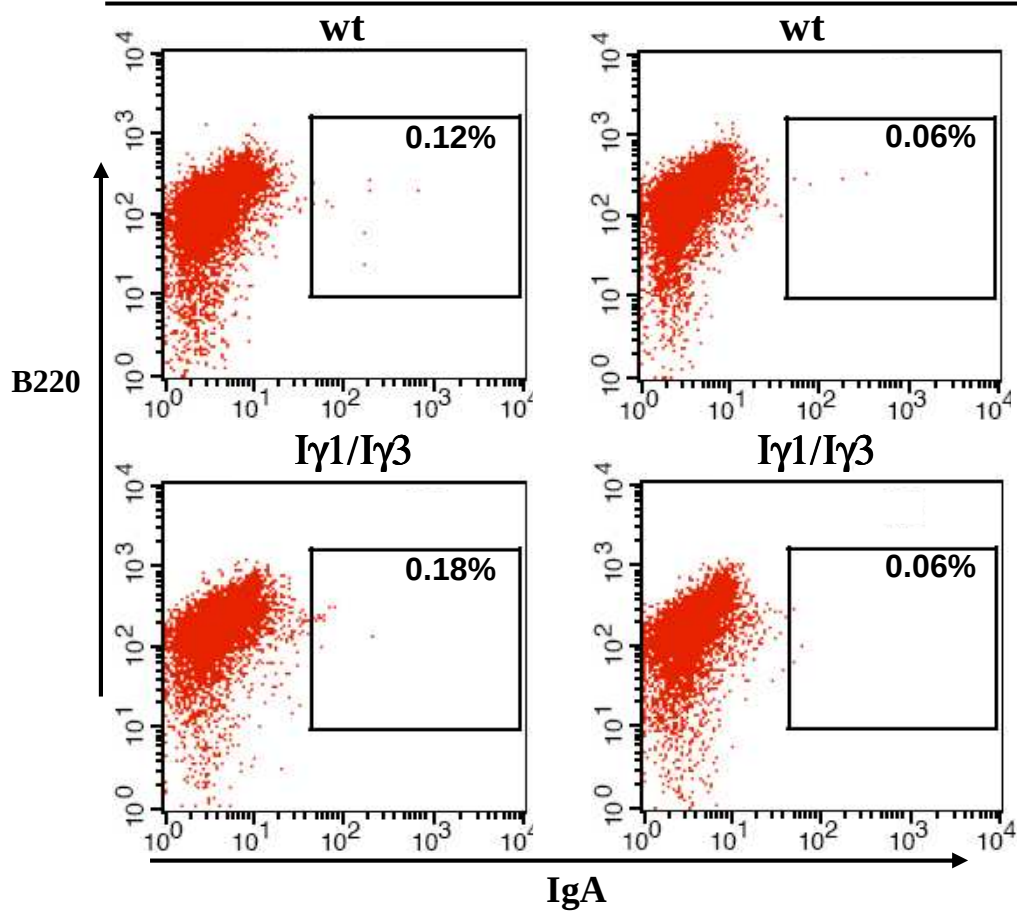
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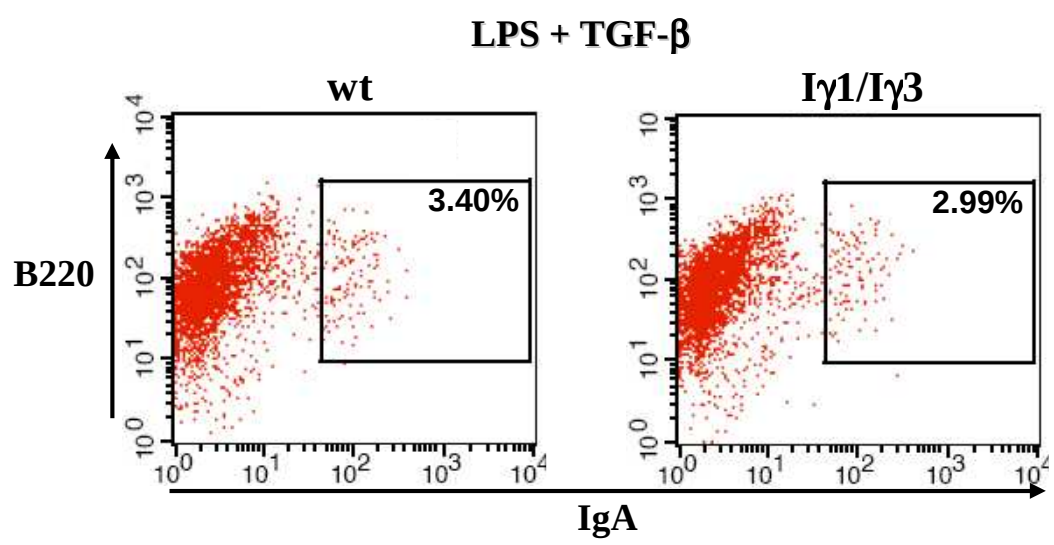
C



D



E



A

Oligonucleotides

Spliced transcripts :

3'l γ 1 : CCACCCACTGTCAATCCTGTTCTTA
C γ 3/1 : ATCCTTGGGCTTTGGGGGGAAGATGAAGAC
l γ 3f : TGGGCAAGTGGATCTGAACA
C γ 3r : CTCAGGGAAGTAGCCTTTGACA
l γ 1f : GGCCCTTCCAGATCTTTGAG
C γ 1r : GGATCCAGAGTTCCAGGTCAC
l γ 2bf : CACTGGGCCTTTCCAGAACTA
C γ 2br : CACTGAGCTGCTCATAGTGTAGAGTC
mActi-4: TACCTCATGAAGATCCTGA
mActi-5 : TTCATGGATGCCACAGGAT
Aicda1 : GACAGCCTTCTGATGAAGCAAAAG
Aicda2 : CATACGAAATGCATCTCGCAAGTC.

Unspliced transcripts :

γ 3-1 : CTGTGGCTGCTCAACTTGGTACCTTTA
 γ 3-3 : GTGGAAGCTAAGGTTTAGGAGTCAA
 γ 1-1 : CTGTGCTTGGATCACCACACTTCC
 γ 1-3 : CAGGATCAATCCCAGCCATTTGGG
 γ 2b-1 : CTTTCTTTCAGCTTCATTCATGGAAC
 γ 2b-3 : GTTGACCTGACCTAGAGACTGGTGGAC
Acti6 : GGATCACTCAGAACGGACACCATG
Acti7 : GATGGACCTAATACGGCTTTTAAC

Sequencing of the inserted promoter:

ZO γ 1-2 : GGCAACTAGTGGACTCTGTACTCTGTACAGCCCTCAGCC
 γ 3-4 : GCCTCCACAGCTCACCTGGAG

Supplementary data to Figure 5

B

'Iy3-'Cγ3-1 junction

Ig3f

TGGGCAAGTGGATCTGAACACACACAACAAAAACAACACTACTGCTACCACCACCACCAGA
GTCAGCCTCAAGGAGATGATGGGATATATCAGGATACCTAAGATAAGATGAACTTGGTCTGT
CTGGAAGCTGGCAGGACCAAATTCGCTGAGTCATCAGAGAAGAGGTGGCCAGAGGAGCAAGA
TCTCTGCAGCAGAAATCTGCAGGACTAACAAAGCTACAACAACAGCCCCATCTGTCTATCCC
TTGGTCCCTGGCTGCAGTGACACATCTGGATCCTCGGTGACACTGGGATGCCTTGTCAAAGG
CTACTTCCCTGAG

Cg3r

'Iy3 exon and γ3 intron sequence

TGGGCAAGTGGATCTGAACACACACAACAAAAACAACACTACTGCTACCACCACCACCAGA
GTCAGCCTCAAGGAGATGATGGGATATATCAGGATACCTAAGATAAGATGAACTTGGTCTGT
CTGGAAGCTGGCAGGACCAAATTCGCTGAGTCATCAGAGAAGAGGTGGCCAGAGGAGCAAGA
TCTCTGCAGCAGAAATCTGCAGGACTAACAAAGGTgagtttatagaggaaagtaggcatgga

γ3 intron and 'Cγ3-1 exon

ctgtctgataacctcactcatcctcctatcttgcagCTACAACAACAGCCCCATCTGTCTAT
CCCTTGGTCCCTGGCTGCAGTGACACATCTGGATCCTCGGTGACACTGGGATGCCTTGTCAA
AGGCTACTTCCCTGAG

A

Ratio (Spliced GLTs)	$\frac{\text{native } \gamma 1 \text{ at 5 ng/ml}}{\text{chimeric } \gamma 3 \text{ at 5 ng/ml}}$		$\frac{\text{native } \gamma 1 \text{ at 25 ng/ml}}{\text{chimeric } \gamma 3 \text{ at 25 ng/ml}}$	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
3'l γ 1-C γ 3/1	10.5	11.7	4.7	5.28

Ratio (Spliced GLTs)	$\frac{\text{wt at 25 ng/ml}}{\text{wt at 5 ng/ml}}$		$\frac{\text{l}\gamma 1/\text{l}\gamma 3 \text{ at 25 ng/ml}}{\text{l}\gamma 1/\text{l}\gamma 3 \text{ at 5 ng/ml}}$		$\frac{\text{l}\gamma 1/\text{l}\gamma 3 \text{ at 5 ng/ml}}{\text{wt at 5 ng/ml}}$		$\frac{\text{l}\gamma 1/\text{l}\gamma 3 \text{ at 25 ng/ml}}{\text{wt at 25 ng/ml}}$	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Native $\gamma 1$	2.79	2.54	2.57	2.98	0.85	0.94	1.04	0.99
Actin	1.1	1.15	0.93	0.94	1.16	1.05	0.92	1.03

B

Ratio (Unspliced GLTs)	$\frac{\text{wt at 25 ng/ml}}{\text{wt at 5 ng/ml}}$		$\frac{\text{l}\gamma 1/\text{l}\gamma 3 \text{ at 25 ng/ml}}{\text{l}\gamma 1/\text{l}\gamma 3 \text{ at 5 ng/ml}}$		$\frac{\text{l}\gamma 1/\text{l}\gamma 3 \text{ at 5 ng/ml}}{\text{wt at 5 ng/ml}}$		$\frac{\text{l}\gamma 1/\text{l}\gamma 3 \text{ at 25 ng/ml}}{\text{wt at 25 ng/ml}}$	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
$\gamma 1\text{-}1/\gamma 1\text{-}3$	2.70	3.9	2.37	2.39	1.40	1.47	0.93	1.23
$\gamma 3\text{-}1/\gamma 3\text{-}3$	1.28	0.9	1.42	1.53	67.21	70.9	69.5	84.29
Actin	1.36	1.1	1.3	0.89	1.19	1.0	0.96	1.3

C

Ratio (Unspliced GLTs)	$\frac{\text{wt}}{\text{l}\gamma 1/\text{l}\gamma 3}$	
	Exp. 1	Exp. 2
$\gamma 1\text{-}1/\gamma 1\text{-}3$	0.91	0.94
$\gamma 3\text{-}1/\gamma 3\text{-}3$	37.28	41.2
Actin	1.36	1.01