Immunity, Volume *27*

Supplemental Data

S-S Synapsis during Class Switch Recombination Is Promoted by Distantly Located Transcriptional Elements and Activation-Induced Deaminase

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Supplemental Experimental Procedures

A series of controls to assess the efficacy of the 3C method for the *Igh* locus were performed and include the efficiency of restriction site digestion (Suppl. fig. 1), determination of template concentration and exponential amplification of PCR reactions (Suppl. fig. 2). All the primer sets were designed to avoid strain specific polymorphisms (Suppl. fig. 3). To permit comparison of crosslinking and ligation efficiencies between sample preparations we measured the crosslinking frequency between two *Gapd* gene specific HindIII fragments (G.a and G.b), which are separated by 3.5 kb and are located on chromosome 2 (fig. 1b). The equation for calculating the relative crosslinking frequency is shown (fig.1c). The crosslinking frequency for the two *Gapd* fragments was arbitrarily set to a value of 1 to enable sample comparisons.

After template preparation for 3C, the efficiency of restriction site digestion was monitored by real-time PCR analysis using primers spanning the restriction sites under study. Hind III restriction sites were equivalently cleaved following overnight digestion with an excess of enzyme (Suppl. fig. 1). Second, we determined template concentration using a real-time PCR assay for the *Gapd* gene and then normalized sample DNA concentrations for all subsequent analyses (data not shown). A template concentration was chosen (~200 ng DNA template per reaction) such that all PCR reactions were in the exponential phase of amplification with all primer pairs used for these studies (Suppl. fig. 2). All the primer sets were designed to avoid strain specific polymorphisms (Suppl. fig. 3). Third, PCR amplification efficiency can vary for different primer sets in quantitative PCR. To correctly compare PCR amplification between primer sets we used a control template in which all possible ligation products are present in equimolar amounts. DNA fragments that span each of the restriction sites analyzed were prepared and mixed in equimolar concentrations. The fragments were digested with Hind III and then ligated and the mix was added to genomic DNA that had been digested and ligated, to serve as the control template. Fourth, to permit comparison of crosslinking and ligation efficiencies between sample preparations we measured the crosslinking frequency between two *Gapd* gene specific HindIII fragments (G.a and G.b), which are separated by 3.5 kb and are located on chromosome 2 (fig. 1b). This non-expressed *Gapd* gene was chosen because the expressed *Gapd* gene on chromosome 6 used in previous studies (Spilianakis and Flavell, 2004) was variably expressed following stimulation with LPS and LPS+IL4 (unpublished data). Differences in the extent of crosslinking and ligation are normalized in each sample by internally comparing the crosslinking signals of the *Igh* locus to that of the *Gapd* locus.

Supplemental Reference

Spilianakis, C.G., and Flavell, R.A. (2004). Long-range intrachromosomal interactions in the T helper type 2 cytokine locus. Nat. Immunol. *5*, 1017–1027.

Suppl. Figure 1

3'Eα

Eμ γ3 γ1 γ2b γ2a ε α 3a 1,2 3b,4

1 2 3 4 17 18 5 6 7 8 9 10 11 12

A B C D E F G

Gapd

13 14 15 16

G.a G.b

Naked U L L/I R L L/I

% Cut 100 75 50 25

2 ⟶ 18

WT (129 X C57BL/6)

U L L/I U L L/I

SuTR Δ/Δ

U L L/I U L L/I U L L/I

Aicda -/-

hs3b4 +/+ U L L/I

hs3b4 Δ/Δ U L L/I

129 Sve WT U L L/I

cEμ Δ/Δ U L L/I

U U U ConA ConA ConA

T cells

Figure S1. Analysis of Restriction Enzyme Digestion of Crosslinked Genomic Chromatin Templates from Nuclei of Different Cell Types

Formaldehyde treated nuclei of splenic B cells from WT, SμTRΔ/Δ, *Aicda*-/-, hs3b4Δ/Δ, and cEμΔ/Δ mice that were unstimulated (U), CD43- resting (R), or activated with LPS (L), or LPS+IL4 (L/I) for 48 hours and of splenic T cells from WT mice that were unstimulated (U) or activated with Concanavalin A (ConA) for 48 hours were digested with HindIII overnight at 37°C. An aliquote of each sample was used to assess the efficiency of cutting of all relevant HindIII sites prior to ligation. After reversal of crosslinks, phenol-chloroform extraction and ethanol precipitation, the DNA was analyzed in real-time PCR with primer pairs spanning each of the HindIII sites used to generate the restriction fragments examined in 3C assays. The quantitative PCR analysis used SYBR Green PCR Mix (Applied Biosystems) and an ABI7900HT system according to the manufacture's instructions. To correct for varying input DNA concentrations from sample to sample, primers amplifying the Gapd gene were used. The degree of cutting of each site was determined by comparison to undigested and fully digested naked control DNA. The y-axis represents the percentage of digestion at each Hind III site analyzed and the x-axis shows the numbers corresponding to each HindIII site shown in the diagram of the *Igh* and *Gapd* loci. Representative examples for the restriction enzyme digestion analysis of each type of chromatin sample used in 3C analysis are shown. The scheme at the top indicates the genomic organization of the loci with the Hind III restriction fragments indicated. The numbers above the Hind III fragment indicate the restriction sites checked for digestion and these sites are also indicated under the digestion maps shown in the left panel. Note that Hind III restriction sites 3,4 are deleted in SμTRΔ/Δ DNA.

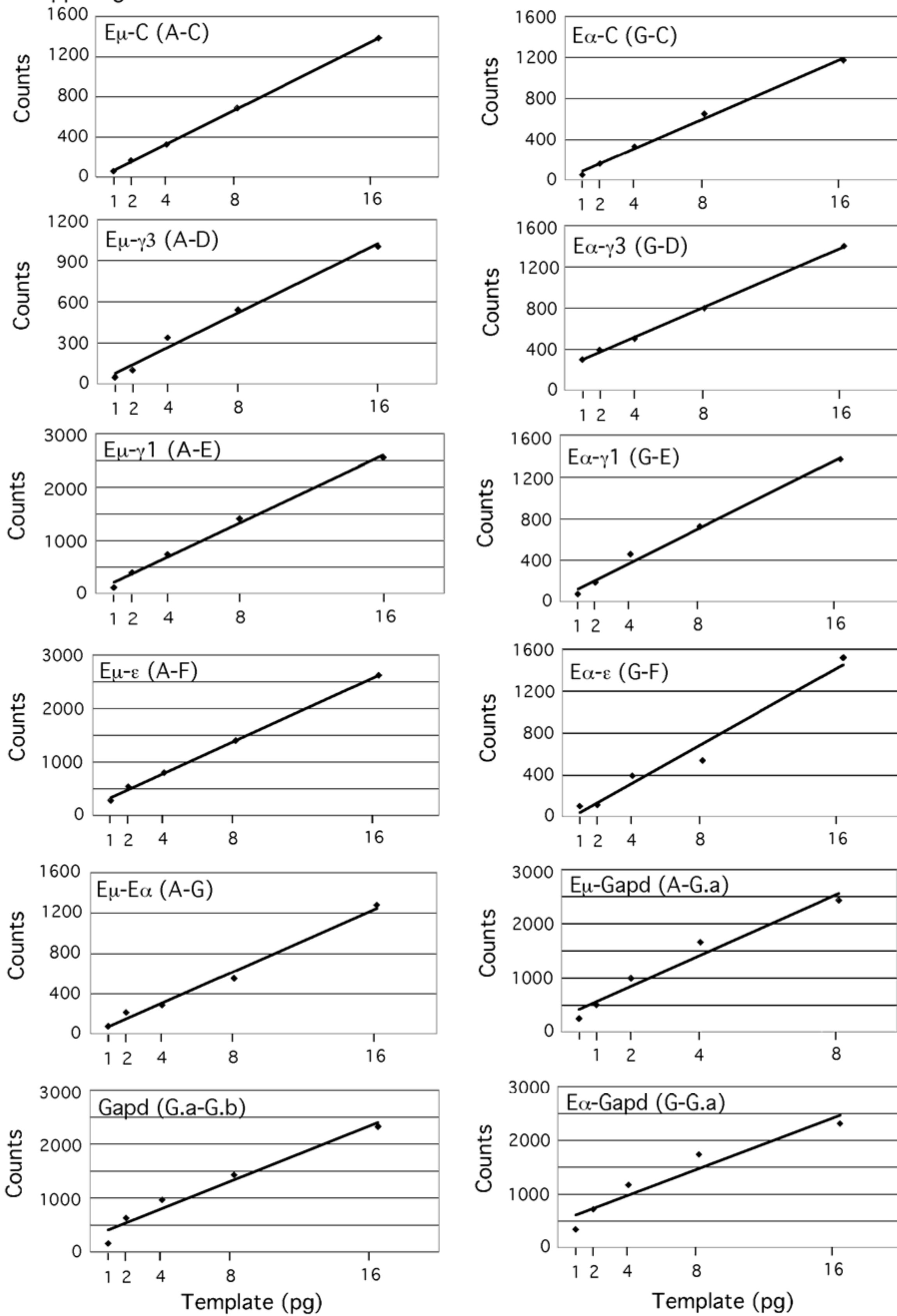
Suppl. Figure 2
Eμ-C (A-C)
Eα-C (G-C)
Eμ-γ3 (A-D)
Eα-γ3 (G-D)
Eμ-γ1 (A-E)
Eα-γ1 (G-E)
Eμ-ε (A-F)
Eα-ε (G-F)
Eμ-Eα (A-G)
Eμ-Gapd (A-G.a)
Gapd (G.a-G.b)
Eα-Gapd (G-G.a)
Counts
Template (pg)

Figure S2. All PCR Reactions Are in the Exponential Phase of Amplification with All Primer Pairs Used for 3C Analysis

Anchor primers 3'A and 3'G were paired with the primers for the other 3C fragments and used in PCR reactions with serial dilutions of the control mix to determine the relative efficiencies of each primer pair combination. Samples of the control mix were included in every 3C assay to assure that the chromatin template 3C signals were in the linear range of detection.

3C Assay primers :

3’ A	GGAACAATTCCACACAAAGACTC
3’ B	GCTGACATGGATTATGTGAGG
5’ C	GTTTTGATTATTATATGGCAGGAGAAG
3’ D	AGAGGAACCAAGTAGATAGGAC
5’ E	CGACACTGGGCAGTTCATTTTG
3’ F	TGTGATTACCTACCTGATCCC
3’ G	CAAGGTGTTAAGGAAAACTTGCTC
3’ G.a	CAGTAGACTCCACGACATAC
5’ G.b	AGTAGTGCGTTCTGTAGATTCC

Primer Pairs for Generation of Control Fragments :

3’ A.f	CTGGGAATGTATGGTTGTGG
3’ A.r	GAGCTCTATGATTATTGGTTAACAGGC
5’ C.f	TCCTGACACAAACACCTAGGA
5’ C.r	TGGCCTTTCTCCCTTGCTG
3’ D.f	GGGAGCTGGGGCTATCAGA
3’ D.r	AGTTCCTGTGCTTGACCTGGTA
5’ E.f	CTTATTATCCTCCAAGAAGGCTG
5’ E.r	GCAAACATCATTGACAGCAGTATC
3’ F.f	AAAGCACCTTCACCTGCAAG
3’ F.r	ATCCAGTCCTTGGCAACTACA
3’ G.f	TGACATCTAATCTGACATGGAGG
3’ G.r	AGGTGGAGATGTGTAGAGAC
3’ G.a.f	ATTTCTCGTGGTTCACACCCA
3’ G.a.r	AAAGTGGAGATTGTTGCCATC
5’ G.b.f	GTCCTTTAAGGATTATAGATGAGG
5’ G.b.r	GACGTCATGGCTTTGAAGTG

Figure S3. List of Primers in the 3C Assay and for Generation of Control Fragments
3C assay primers specific for each HindIII fragment are denoted by a capital letter referring to the 3C fragments shown in Figure 1A and whether it is the 5’ or 3’ site of that fragment. The forward (f) and reverse (r) primer pairs for the generation of control fragments spanning each HindIII site used in the 3C assays are also listed.

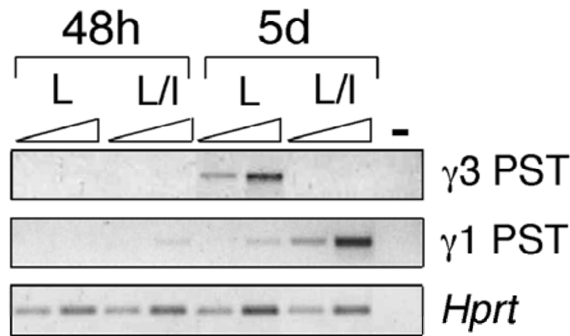

Figure S4. Post-Switch Transcripts Were Analyzed by Semiquantitative RT-PCR with cDNAs Derived from Splenic B Cells that Were Unstimulated or Activated with LPS or LPS+IL4 for 48 Hours and 5 Days

Hprt PCR products were harvested after 28 cycles (lanes 1, 3, 5, 7) and 30 cycles (lanes 2, 4, 6, 8). PST PCR products were harvested after 32 cycles (lanes 1, 3, 5, 7) and 34 cycles lanes 2, 4, 6, 8).

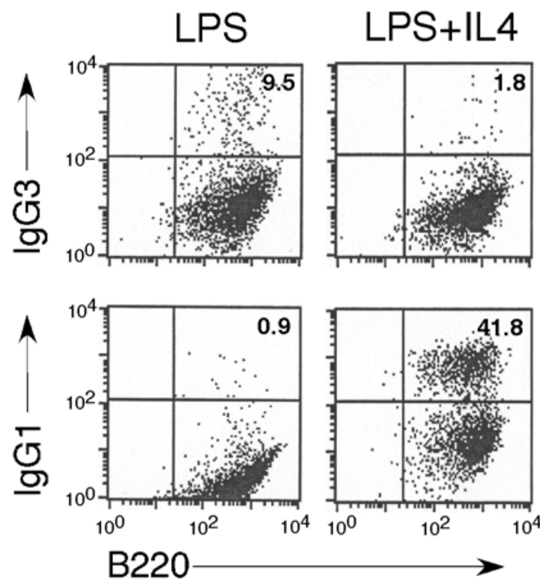

Figure S5. FACS Analysis of Surface IgG3 and IgG1 on Splenic B Cells after5 Days of LPS and LPS+IL4 Stimulation

Cells were stained with propidium idodide and dead cells were excluded. The cells activated with LPS and LPS+IL4 are shown in the left and right panel, respectively. The frequency of isotype switched B cells in the cultures are indicated. FITC labeled rat anti-mouse IgG3 and rat anti-mouse IgG1 (BD Pharmingen, catalogue numbers 553403 and 553443) were used according to the manufacture's instructions.

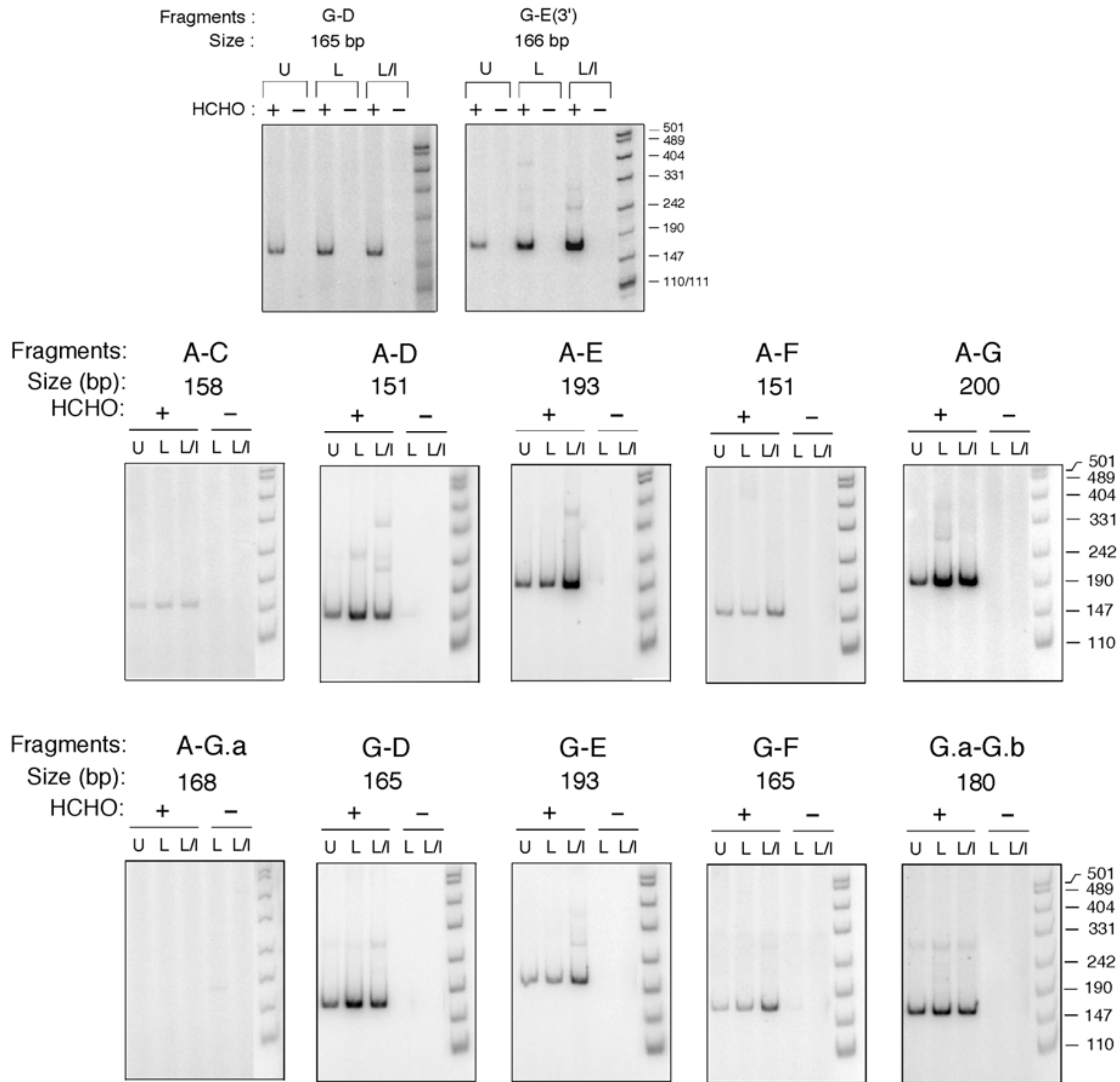

Figure S6. The Specificity of the 3C PCR Product Amplification Is Confirmed

Representative 6% PAGE resolution of radioactively labeled PCR products from 3C assays performed on chromatin templates prepared from WT B cells that were either unstimulated (U), or activated in culture with LPS (L), or LPS+IL4 (L/I) and then either treated (+) or not treated (-) with formaldehyde (HCHO). The full gel runs along with a size marker (pUC19 HpaII) are shown to demonstrate the specificity each of the PCR reactions and that the associations are dependent upon formaldehyde crosslinking. Additionally, each product was sequenced and determined to be the precise sequence predicted from a ligation of the indicated HindIII fragments. The Ga.-G.b product results from the crosslinking of neighboring fragments of the Gapd (chromosome 2) gene and is used to control for the quality of each chromatin sample. Note that no crosslinking is detected between the Gapd gene and the IgH locus.

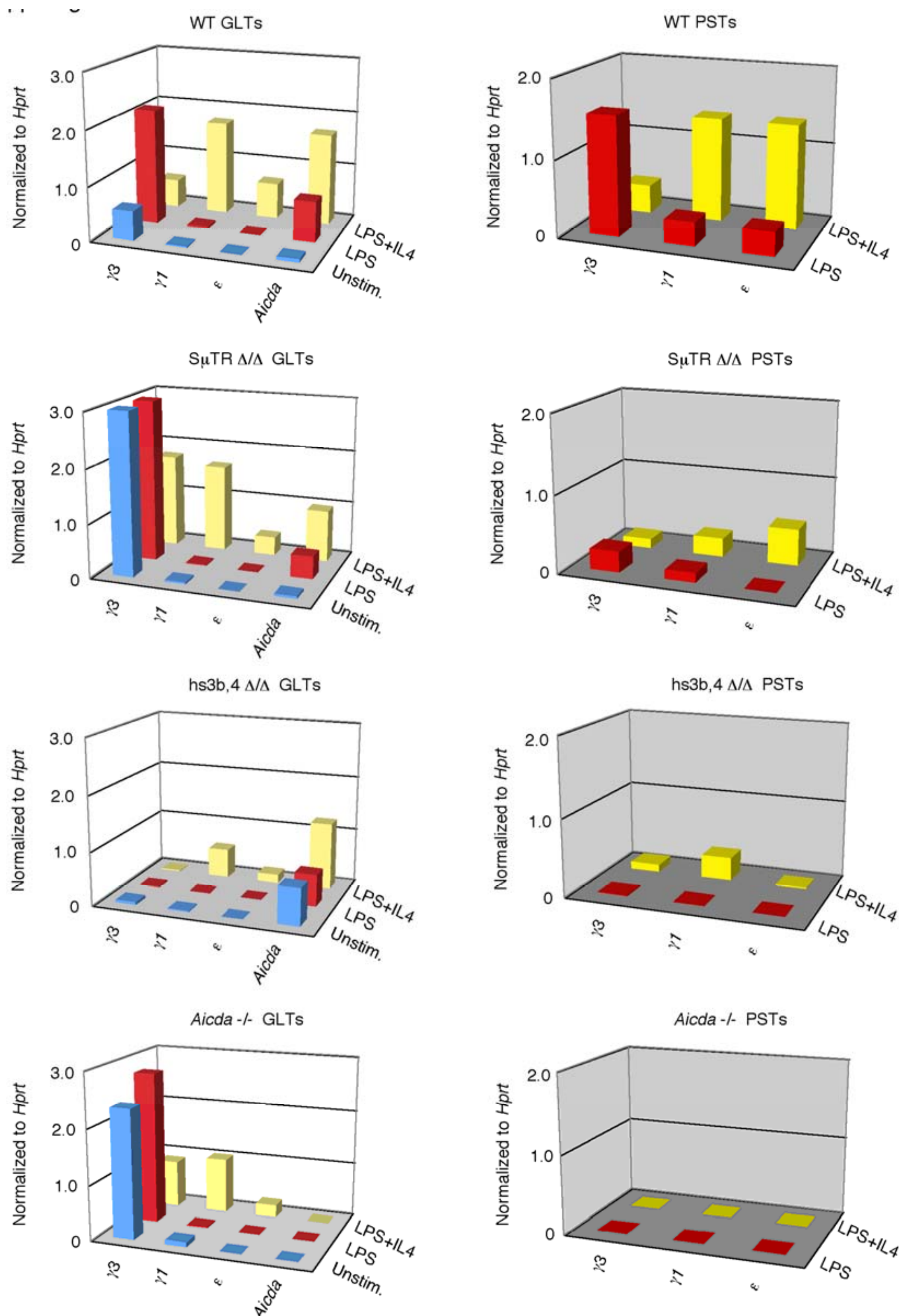

Figure S7. Real-Time RT-PCR Analysis of GLT and PST Expressed in B Cells Activated to Switch from Various Mouse Strains

Germline transcripts (GLT) (left panel) and post-switch transcripts (PST) (right panel) were analyzed by quantitative real time RT-PCR using cDNAs derived from splenic B cells that were unstimulated (Unstim) or activated with LPS and LPS+IL4 for 48 hours and 5 days,

respectively. *Hprt* PCR products were used as a loading control and used to normalize the PCR results. GLTs and PSTs were PCR amplified in the presence of SYBR Green PCR Mix (Applied Biosystems) using an ABI7900HT system according to the manufacture's instructions. Forward (f) and reverse (r) primers used in the GLT RT-PCR were: g3f 5' tgtctggaagctggcagga 3', γ3r 5' agctcagggaagtagcctttg 3', γ1f 5' aggaatgtgtttggcatggac 3', γ1 r 5' cactgtcactggctcagggaa 3', *Aicda* f 5' ccatttcaaaaatgtccgct 3', *Aicda* r 5' caggtgacgcggtaacacc 3', *Hprt* f 5' gttggatacaggccagactttgttg 3', *Hprt* r 5' tactaggcagatggccacaggacta 3'. Primers used in the PST RT-PCR were: Iμ-f 5' ctctggccctgcttattgttg 3' paired with the reverse GLT primers.

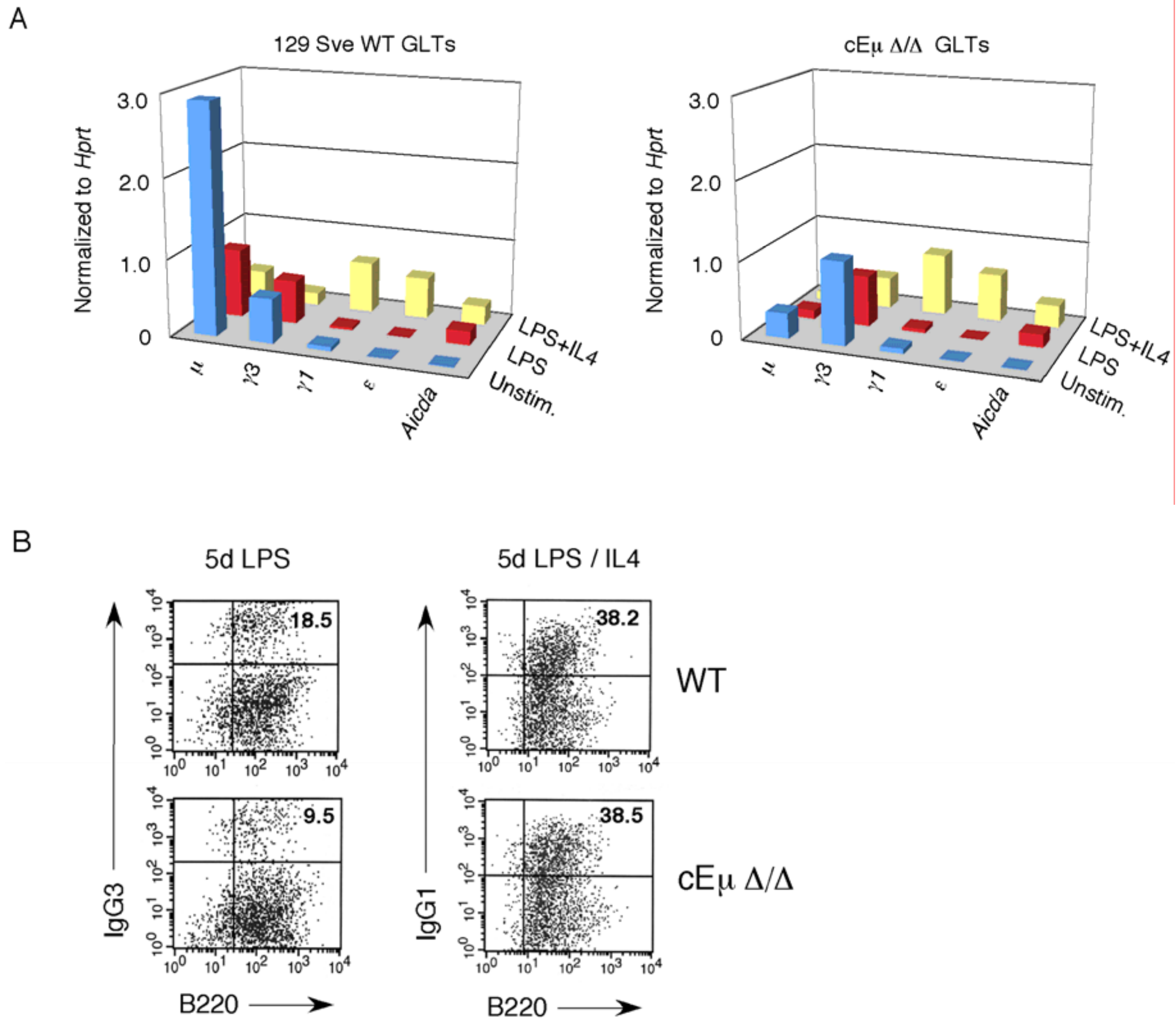

Figure S8. GLT RT-PCR and FACS Analysis of 129Sve WT and cEμΔ/Δ B Cells

A.) Germline transcripts (GLT) were analyzed by quantitative real time RT-PCR using cDNAs derived from splenic B cells that were unstimulated (Unstim) or activated with LPS and LPS+IL4 for 48 hours. *Hprt* PCR products were used as a loading control and used to normalize the PCR results. GLTs were PCR amplified in the presence of SYBR Green PCR Mix (Applied Biosystems) using an ABI7900HT system according to the manufacture's instructions. Forward (f) and reverse (r) primers used in the GLT RT-PCR were: g3f 5' tgtctggaagctggcagga 3', γ3r 5' agctcagggaagtagcctttg 3', γ1f 5' aggaatgtgtttggcatggac 3', γ1 r 5' cactgtcactggctcagggaa 3', *Aicda* f 5' ccatttcaaaaatgtccgct 3', *Aicda* r 5' caggtgacgcggtaacacc 3', *Hprt* f 5' gttggatacaggccagactttgttg 3', *Hprt* r 5' tactaggcagatggccacaggacta 3'. **B.)** FACS analysis of surface IgG3 and IgG1 expression following 5 days of LPS and LPS+IL4 stimulation. Cells were stained with propidium idodide and dead cells were excluded. The cells activated with LPS and LPS+IL4 are shown in the left and right panel, respectively. The frequency of isotype switched B cells in the cultures are indicated. FITC labeled rat anti-mouse IgG3 and rat anti-mouse IgG1 (BD Pharmingen, catalogue numbers 553403 and 553443) were used according to the manufacture's instructions.

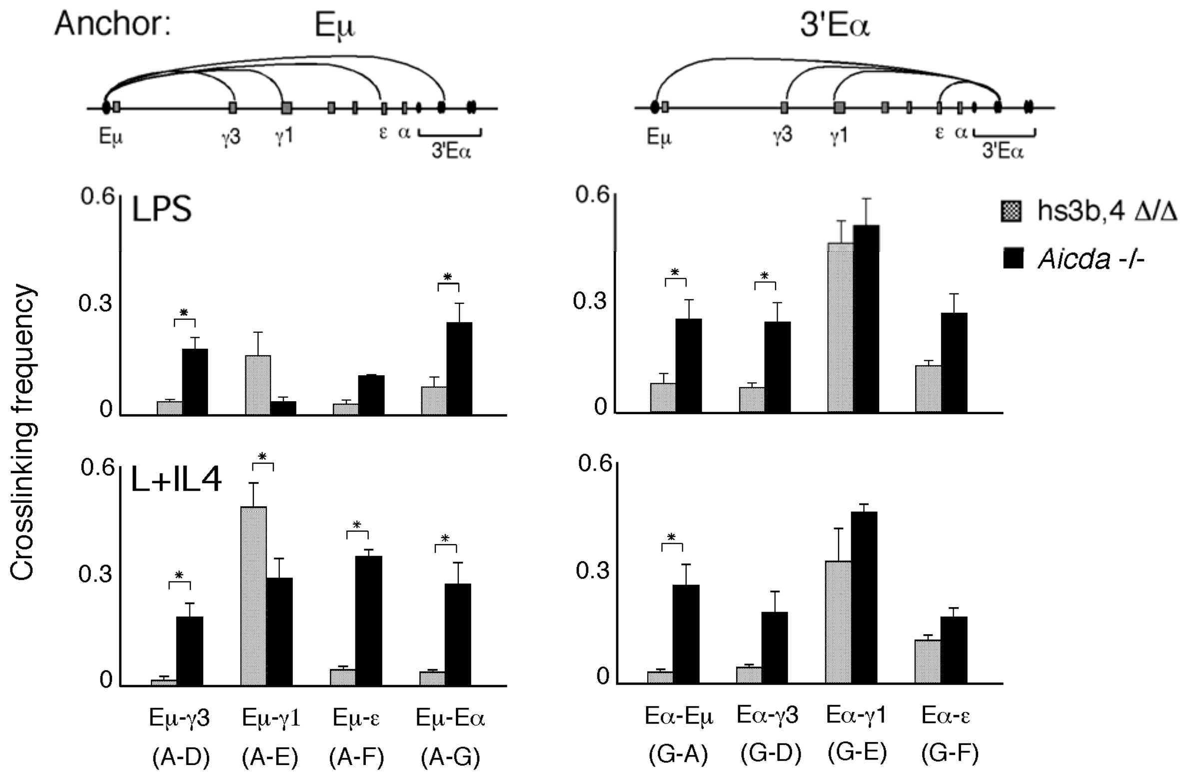

Figure S9. Comparison of Crosslinking Frequencies in hs3b,4Δ/Δ and *Aicda*$^{-/-}$ B Cells

Chromatin templates from LPS or LPS+IL4 activated splenic B cells from hs3b,4Δ/Δ and *Aicda -/-* mice were analyzed for crosslinking between anchor fragment A (Eμ) and the locus fragments D-G (left panel) and between anchor fragment G (3'Eα) and fragments (A,D-F) (right panel). The average crosslinking frequencies and SEMs are shown. Pairwise comparisons of 3C results were analyzed by the Student's t-test and those with p values $p < 0.05$ are indicated with starred brackets.