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Binding of LBP-1a to Specific Immunoglobulin Switch Regions *in vivo* Correlates with Specific Repression of Class Switch Recombination

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Abstract

Upon stimulation of mature B cells, class switch recombination (CSR) can alter the specific immunoglobulin heavy chain constant region that is expressed. In a tissue culture cell line, we previously demonstrated that inhibition of late SV40 factor (LSF) family members enhanced IgM to IgA CSR. Here, isotype specificity of CSR regulation by LSF family members is addressed in primary mouse splenic B cells. First, we demonstrate that LBP-1a is the prevalent family member in B lymphocytes. Second, we demonstrate by ChIP that LBP-1a binds genomic sequences around mouse switch regions (S) in an isotype-specific manner, in accordance with computational predictions: binding is observed to S_μ and S_α, but not to the tested S_γ1, regions. Importantly, binding of LBP-1a is tightly regulated, with occupancy at genomic S regions dramatically decreasing following LPS stimulation. Finally, the consequence of DNA-binding by LBP-1a is determined using bone marrow chimeric mice in which LSF/LBP-1 activity is inhibited in hematopoietic lineages. Upon *in vitro* stimulation of such primary B-cells, CSR occurs with a higher efficiency to IgA, but not to IgG1. These results are supportive of a model whereby LBP-1a represses CSR in an isotype-specific manner via direct interaction with switch regions involved in the recombination.

Keywords

B cells; Immunoglobulins; Molecular Biology; Recombinant Viral Vectors

Introduction

Class switch recombination (CSR) involves genomic DNA recombination in mature B cells that alters expression of the immunoglobulin heavy (H) chain constant region. The consequence is diversification of antibody effector function while preserving antigen specificity. The nonhomologous DNA recombination occurs within or near switch (S) regions, which consist of tandemly repeated DNA motifs located upstream of each H chain

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constant coding region. Antigens, bacterial products, and cytokines induce switching, with each distinct combination of signals generating expression of specific isotype(s) [1–3]. Extracellular signaling drives switching to a particular isotype by activating transcription from a promoter located 5' of the respective S region. Such germline transcription is required to facilitate accessibility of S region DNA for CSR [4;5]. The extent to which the concurrent, enhanced chromatin accessibility in S regions promotes CSR is unclear [5].

Although molecular mechanisms for CSR have been extensively studied, little is known about non-transcriptional regulatory pathways, and in particular, means to prevent inappropriate CSR. As the predominant sites of DNA recombination [4], the S regions themselves provide intriguing regulatory targets. Indeed, several proteins specifically recognize S regions and activate CSR [1;6;7]. Of these, NF- κ B/p50 can promote isotype-specific CSR without influencing germline transcription [8;9].

As we demonstrated previously, another class of transcription factors, the Late SV40 Factor (LSF) family members, binds switch region sequences *in vitro* [10]. In the I.29 μ B cell line, which undergoes IgM to IgA isotype switching *in vitro*, LSF or other family members represses CSR without altering levels of germline transcripts. This provided the first instance of non-transcriptional repression of CSR by a specific DNA-binding protein [10]. The LSF family of transcription factors includes three paralogs, which share a DNA recognition motif, CTGG N₆ CTT/GG. The two ubiquitous members of the family, LSF and LBP-1a/b (leader binding protein-1a/b), are 72% identical in aa sequence and may be functionally redundant in many tissues [11].

Here we report that LBP-1a is the dominant family member expressed in mouse primary B lymphocytes. A high density of binding sites is predicted only in particular S regions. Consistent with these predictions, in resting B lymphocytes, LBP-1a specifically binds to particular S regions (e.g. S α) but not demonstrably to others (e.g. S γ 1). Notably, LBP-1a is released from bound S regions after LPS stimulation. Finally, inhibition of LBP-1a function leads to enhanced expression of only specific isotypes (IgA but not IgG1) when mouse B lymphocytes are stimulated to undergo CSR *in vitro*, correlating with binding of LBP-1a to the relevant S region.

Results and Discussion

LBP-1a is the predominant LSF/LBP-1 family member in B-cells

In order to distinguish LSF and LBP-1a, anti-peptide antibodies were produced against divergent regions (Fig. 1A). The antibody specificities and sensitivities were tested against recombinant proteins (Fig. 1B) and mammalian cell extracts in which each protein was independently overexpressed (Fig. 1C). In both instances, each antibody was highly specific for its cognate protein. The LBP-1a antibody recognizes multiple protein species from untransfected 293T extracts (Fig. 1C); due to alternative splicing, the cellular LBP-1a gene can encode two major products: LBP-1a and the larger LBP-1b [12]. LBP-1b is expressed in a tissue-specific manner [13], and is not evident in primary B cells (Fig. 1D).

With these new reagents, we determined the relative abundance of LSF and LBP-1a in primary B-cells. By comparing the reactivity of each antibody against whole cell lysates from mouse splenic B-cells (Fig. 1D, right panels) to its reactivity against a standard curve of purified recombinant His-LSF or His-LBP-1a (Fig. 1D, left panels), we concluded that primary mouse B cells contain at least 5-fold more LBP-1a (on the order of 10⁴ molecules/cell) than LSF.

In primary, resting B-lymphocytes LBP-1a binds genomic DNA around particular S regions

We previously demonstrated *in vitro* that recombinant LSF, as well as related proteins in mouse splenic cellular extracts, could bind specific sequences within S μ , S α , and S ϵ [10]. S regions can be clustered into two groups: S μ , S ϵ , and S α contain repeated pentameric sequences, whereas S γ 1, S γ 2a, S γ 2b, and S γ 3 contain 49–52 base-pair repeats [2;3]. We computationally analyzed the likelihood of LSF/LBP-1a binding sites within each S region. In some (S μ , S ϵ , S α , and S γ 3), numerous LSF/LBP-1a recognition sites were predicted; in others (S γ 1, S γ 2a, and S γ 2b), exceedingly few (Fig. 2A and Fig. S1A in supporting material). A similar pattern was evident when human S regions were analyzed (Fig. S2).

We tested these predictions by ChIP assays in primary murine splenic B-lymphocytes, focusing on S μ , which is operative in all CSR, and S α and S γ 1, which are target switch regions of the two classes of repeats. Binding of LBP-1a, the more abundant LSF family member in B-cells (Fig. 1D), was monitored. Amplification of specific switch regions in the genomic DNA was analyzed by semi-quantitative PCR analysis (see Materials and Methods); the highly repetitive nature of these genomic sequences prevented design of real-time PCR primers. Binding *in vivo* was robust at both S μ and S α (Fig. 2B), where the amplicons were located at least 1.5 kb and 3.5 kb, respectively, downstream of transcriptional regulatory elements (the intronic promoters and the enhancer region), and either at the border of or well within S region sequences. However, as expected given the low density of predicted high affinity binding sites (roughly one per 3500 bp) LBP-1a binding was not detectable around S γ 1 in the region of the amplicon (Fig. 2B). Similarly, LBP-1a occupied S ϵ and S γ 3, the respective amplicons being at least 2.5 kb and 3.2 kb downstream of the intronic promoters, with minimal or no occupancy of S γ 2a and S γ 2b in the regions probed (Fig. S1B in supporting material). The experimental findings at all heavy chain gene loci are therefore consistent with predictions of LSF/LBP-1a binding sites within these S region sequences.

LPS stimulation of B-lymphocytes decreases binding of LBP-1a to S regions

Functionality of LBP-1a association with S regions *in vivo* was tested initially by assaying binding upon stimulation of primary B-lymphocytes. In previous studies, inherent DNA-binding activity of LSF/LBP-1a to S sites in mouse splenic extracts, assayed by EMSA, decreased after stimulation to undergo CSR [10]. Using the specific antibody (Fig. 1), levels of LBP-1a were measured in whole cell extracts of purified splenic B-lymphocytes stimulated by LPS for increasing amounts of time (Fig. 2C). Surprisingly, LBP-1a levels (and LSF protein levels; data not shown) substantially increased by 24 h after stimulation. In parallel LPS-stimulated cultures, we directly measured binding of LBP-1a to genomic S regions by ChIP. Occupancy at both S μ (Fig. 2D) and S α (Fig. 2E) was readily apparent at 0 and 10 h, but markedly decreased at 24 and 48 h post-stimulation. Thus, although protein levels increased at late time points after stimulation, binding decreased.

The decrease in DNA-binding with LPS stimulation is likely due to direct modification of LBP-1a. The closely related paralog, LSF, is directly targeted by mitogenic signal transduction pathways in multiple cell types, altering LSF DNA-binding potential, in part through modification of S291 [14;15]. LBP-1a contains an analogous serine; we hypothesize that LBP-1a is targeted downstream of cytokine signaling cascades in resting B cells, being modified to reduce binding to S regions.

The kinetics of release of LBP-1a from S region sequences following stimulation of B cells are consistent with LBP-1a inhibiting early events in CSR. Induction of AID mRNA and some germline transcripts occurs by 12 to 24 h post-stimulation [16;17], with Ig α sterile transcripts [18], formation of R-loops [19], and alterations in histone acetylation by 48 h

post-stimulation [20]. The inverse correlation between binding of LBP-1a and activation steps of CSR suggests that LBP-1a is a repressor.

LBP-1a-mediated repression of CSR to IgA, but not IgG1, in murine splenic B-cells

In a B cell line capable of undergoing CSR only from S μ to S α *in vitro*, LSF/LBP-1a did indeed repress CSR [10]. However, the question remained as to whether, in primary B cells, CSR in general would be subject to such inhibition, since all CSR in IgM-expressing B cells involves the S μ switch region with which LSF/LBP-1a interacts, or if CSR only at certain heavy chain gene loci would be inhibited. To distinguish between these possibilities, we generated bone marrow chimeric mice in which a dominant negative form of LSF (LSFdn) was expressed in donor hematopoietic cells. LSF family members oligomerize with each other and bind DNA as obligate tetramers [11]. Two aa substitutions in the DNA-binding region of LSFdn prevent DNA-binding [21]; due to oligomerization this is a dominant phenotype that blocks all LSF family members [11]. This decrease in DNA-binding of endogenous LSF family members by LSFdn expression has previously been demonstrated in multiple studies, including in a murine B cell line [10].

Bone marrow cells from donor mice were transduced *in vitro* with retrovirus capable of expressing both LSFdn and GFP. Transplantation of these cells into lethally irradiated mice repopulated the recipient hematopoietic system. The resulting transduced, GFP-expressing splenic B cells were isolated and analyzed for CSR upon stimulation *in vitro*. Two control B cell populations were used. First, non-transduced cells in the donor bone marrow gave rise to GFP-negative splenic B cells in the same mouse, providing an ideal internal control for the GFP-positive, LSFdn-expressing cells (see Fig. S3 in supporting material for demonstration of GFP-negative versus GFP-positive populations). Second, other recipient mice were transplanted with donor bone marrow transduced with parental retrovirus, capable of expressing only GFP, in order to control for the possibility that transduction with any retrovirus would alter the efficiency of CSR.

Expression of LSFdn was first validated in the LSFdn group of mice. Isolated GFP-positive and GFP-negative populations of primary splenic B-cells were harvested and immunoblotted with antibody specific to LSF. Endogenous murine LSF was detected in GFP- cells, whereas at least 50-fold higher levels of exogenous human LSFdn were detected in GFP+ cells from the same mouse (Fig. 3A). Considering relative expression levels of LBP-1a and LSF (Fig. 1D), LSFdn expression was also at least 10-fold higher than that of LBP-1a. Even equimolar concentrations, both *in vitro* and *in vivo*, are sufficient to dramatically suppress wild type DNA-binding of LSF family members [21;22].

To determine whether this expression of LSFdn affects the frequency of CSR, purified splenic B-cells from both chimeric LSFdn mice and chimeric control mice were stimulated to undergo CSR *in vitro*. Unlike results in the I.29 μ B cell line, in the absence of stimulation, there was no detectable IgA or IgG1 expression in B cells isolated from LSFdn mice (data not shown). That alleviation of repression by LBP-1a is not sufficient to induce CSR in primary resting B cells in the absence of signaling is consistent with extensive analyses indicating that specific activation signals are absolutely required.

Due to the differences in density of LBP-1a occupancy at switch regions S α vs. S γ 1 (Fig. 2), and to the relatively high efficiency of switching that can be induced *in vitro* to these two regions, we compared switching to IgA with that to IgG1 [2]. Cells were gated on the basis of GFP expression and surface levels of IgG1 and IgA expression after stimulation were monitored by flow cytometry (see Fig. S3 in supporting material for example). In initial experiments, comparison of the degree of CSR in BALB/cByJ B cells to that in the chimeric B cells indicated that CSR in the chimeric cells was less robust (approximately one fourth

the levels of CSR), suggesting that bone marrow transplantation compromises the degree of CSR achievable in primary B cells in culture. We note that the depression in frequency of CSR in chimeric mouse B cells unfortunately precluded investigation of the inherently less efficient switching *in vitro* to isotypes other than IgA and IgG1.

By normalizing the percentage of transduced cells undergoing CSR to that of nontransduced cells in the same B cell population from the same mouse, we could most accurately determine the effect of LSFdn expression on CSR. The ratios for each individual mouse are plotted for expression of IgA and IgG1 (Fig. 3B and C, respectively), compiled from multiple independent experiments. These experiments included activation of B cells using multiple protocols; the effect of LSFdn on IgA CSR induced either by LPS, IL-5 and TGF- β 1 (left panel) or by LPS and IL-4 (right panel) are shown in Fig. 3B. Importantly, for chimeric control mice, splenic B cells switched expression from IgM to either IgG1 or IgA at the same frequencies, irrespective of whether or not they expressed GFP, resulting in a ratio for each isotype of 1.0. For the chimeric LSFdn-expressing mice, expression of IgG1 was also unaltered in the transduced cells (ratio of 0.94, Fig. 3C). In stark contrast, the percentage of LSFdn-expressing cells presenting surface IgA was significantly elevated (1.8-fold, $P<0.005$) compared to their non-transduced counterparts (Fig. 3B, left panel). A distinct cytokine cocktail appeared to result in a similar derepression of IgA expression by LSFdn (1.6-fold, $P=0.067$; Fig. 3B, right panel). These data demonstrate that LSF family members do not downregulate CSR in general, but only to certain isotypes (e.g. IgA).

How LBP-1a reduces CSR requires further investigation. Effects of LBP-1a, or other LSF family members, on CSR could potentially be either indirect or direct. Indirect effects might result, for instance, from LBP-1a affecting the degree of cell cycling. However, we view this possibility as unlikely for the following reasons. First, in a B cell line capable of undergoing CSR, expression of LSFdn, although similarly affecting CSR [10], did not detectably alter cell growth properties (data not shown). Second, since induction of IgG1 and of IgA in primary B cells both require multiple cell divisions [23;24], it is difficult to conceive of how indirect effects of LBP-1a would impact switching to one, but not to the other. Our combined findings that LBP-1a binds $\Sigma\mu$ and $\Sigma\alpha$, but apparently not $\Sigma\gamma$ 1, and that inhibition of LSF/LBP-1a increases levels of immunoglobulin class switching to IgA, but not to IgG1, instead suggest that LBP-1a inhibits CSR directly, as a consequence of LBP-1a binding S region sequences upstream of the respective heavy chain coding region.

Direct regulation of CSR by LBP-1a could result from either transcriptional or non-transcriptional mechanisms. Regulation of germline transcription by LBP-1a is unlikely, given that LSFdn did not alter the levels of germline transcripts in a B cell line in which it regulated CSR [10], and that the demonstrated binding of LBP-1a at the heavy chain loci is distant from the transcriptional regulatory regions. Alternative mechanisms for regulation of CSR by LBP-1a include, although are not limited to, inhibition of S-S synapsis, counteracting AID deamination or strand breakage, or reduction in chromatin accessibility. With regards to the latter, activation of CSR correlates with histone acetylation in S regions [5;25]. We note that LBP-1a may have the capability of inhibiting chromatin accessibility, in that the highly similar LSF interacts with multiple inhibitory chromatin modifying factors including histone deacetylases, Sin3A corepressor and the polycomb protein RING [11].

Concluding Remarks

The roles of specific switch region sequences in regulation of CSR remain to be fully elucidated, especially since the specific primary sequences of S regions appear unessential for the basic mechanism of CSR [4]. We hypothesize that specific repeat sequences mediate differential regulation of CSR, by providing specific binding sites for isotype-specific

activators (e.g. NF- κ B), and isotype-specific repressors (e.g. LBP-1a) to finetune appropriate CSR in response to the complex signaling to B-lymphocytes *in vivo*.

Materials and Methods

Recombinant protein purification

6X His-tagged LSF and LBP-1a were isolated using Ni-NTA nickel charged resin from bacteria transformed with pQE30LSF and pQE30LBP-1a, respectively (see supporting material for plasmid constructions). The concentrations of each recombinant protein were determined against a standard curve of purified BSA by SDS-PAGE, staining with Commassie-G250, densitometry, and ImageQuant analysis (version 1.2).

Antibody production and immunoblotting

Peptides corresponding to aa 317–332 of LBP-1a and 314–328 of LSF (bolded, Fig. 1A) were coupled to keyhole limpet hemocyanin. Antisera were produced in rabbits (Covance Research Products Inc.). Antibodies were purified using a HiTrap protein G column followed by affinity purification against peptide immobilized to SulfoLink coupling gel.

Purified B cells ($5\text{--}20 \times 10^6$) were lysed in 2% SDS, 50 mM Tris HCl, pH 8.0, 0.1 mM DTT, 10% glycerol, subjected to SDS-PAGE, and immunoblotted with affinity-purified antibodies, followed by anti- β -actin (Sigma). 293T cells were maintained in DMEM plus 10% fetal bovine serum. Cells were transfected using lipofectamine (Invitrogen) and a total of 8 μ g DNA, including pCXLBP-1a and pCXLBP-1c (LSF) [12] as indicated, per 10 cm dish. Nuclear extracts were prepared as described previously [26] prior to immunoblotting.

Bone marrow transplantation

Male BALB/cByJ mice (6–8 weeks) from The Jackson Laboratory were handled in accordance with the National Institutes of Health and institutional guidelines. Five days prior to bone marrow harvest from femurs, donor mice were injected i.p. with 5 mg of 5-fluorouracil. Marrow was subjected to density separation using Lympholyte-M and cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, antibiotics, 100 ng/ml SCF, 100 ng/ml flt-3 ligand and 50 ng/ml Thrombopoetin (R and D Systems) for 24 h prior to retroviral transduction.

Retroviral packaging cells were maintained in DMEM plus 10% fetal bovine serum. Following transfection with pCL-Eco [27] plus either MIGW [28] or MIGW-LSFdn (see supporting material for construction) using Fugene (Roche), viral supernatants were collected. Bone marrow cells were transduced 3 times for 24 h each with 80% viral supernatant, 20% bone marrow medium and 12 μ g/ml polybrene.

Recipient mice were irradiated with 900 rads of gamma rays; 3 h later bone marrow from 1 donor (0.5×10^6 cells) was injected into the tail vein of one recipient mouse.

B cell isolation

Splenic B cells were isolated 8–10 wks after bone marrow transplantation, using negative selection [29]. Briefly, splenocytes were depleted of T cells using anti-Thy1.2 and rabbit complement. B-cells were isolated by density gradient centrifugation and cultured in RPMI plus 10% fetal bovine serum, 2 mM L-glutamine, 50 μ M 2-ME, and antibiotics. The B cells were phenotyped as containing fewer than 1% T cells (B220-CD3+) or macrophages (B220-CD14+). For chimeric mice, cells were sorted by FACS into GFP-expressing and non-GFP-expressing populations. The gate for non-GFP expressing cells was set at minimal fluorescence, to eliminate contamination with cells expressing GFP at low levels. The gate

for GFP-expressing cells was set above levels of maximal background fluorescence from BALB/cByJ splenocytes.

LBP-1a ChIP

LSF/LBP-1 binding sites were predicted with POSSUM (<http://zlab.bu.edu/~mfrith/possum>; score threshold 7, residue abundance range 1000, pseudocount 1) using the LSF binding matrix (accession number M00947) from TRANSFAC and mouse S region accession numbers S μ : J00440-J00442, S α : D11468, S γ 1: D78344.

ChIP assays were performed according to the Upstate Cell Signaling Solutions protocol. Briefly, 20×10^6 isolated, splenic B-cells were crosslinked in 1% formaldehyde for 10 min at 37°C, and quenched with 125 mM glycine. Cell lysates were sonicated to shear chromatin to around 500 bp; DNA was extracted from a fraction of the lysate for input controls. Precleared lysates were immunoprecipitated using 100 μ l preimmune or immune rabbit serum or 5.0 μ g normal rabbit IgG (Southern Biotech). Input and immunoprecipitated DNA were assayed for switch region sequences by PCR. Through amplification of increasing amounts of input DNA (e.g. Fig. 2D, 2E), a range of signals could be ascertained that was monotonically increasing with respect to the amount of input. In this manner, saturation of signals was avoided, and the results are semi-quantitative. Primer sequences included: S μ as previously described [30]; S α : AGCTTACCTAGACCGGGCAGAC and ACCTCAACTAAGTCCATCTTAGGCC; S γ 1: TATGATGGAAAGAGGGTAGCATT and CTGGGCTGGTCTGTCAACTCCTT.

CSR analysis

Isolated splenic B-cells were stimulated either 4 or 7 days with 25 μ g/ml LPS and 25 ng/ml IL-4 to assay IgG1 or IgA expression or with 25 μ g/ml LPS, 25 ng/ml IL-5 and 1 ng/ml TGF- β 1 to assay IgA expression. Surface IgG1 and IgA were measured by flow cytometry, using biotinylated IgG1 or IgA antibodies (1:500 dilution) and allophycocyanin-conjugated streptavidin (1:2000 dilution) in the presence of 2.4G2 anti-FcR antibody (BD Biosciences). The GFP-positive gate was set above the fluorescence levels of non-GFP-transduced B cells. IgA- or IgG-positive gates were set above fluorescence levels of B-cells from chimeric mice stained either with biotinylated isotype control antibody (553923-BD Biosciences) followed by allophycocyanin-conjugated streptavidin, or allophycocyanin-conjugated streptavidin alone. Analyses were performed using FlowJo software. In calculating percentage of cells undergoing CSR, the percentage of IgA+ (or IgG1+) cells in the experimental sample were first adjusted by the percentage of IgA+ (or IgG1+) cells in the parallel isotype control analysis of the same cells. Samples in which the isotype control percentages were high relative to the experimental percentages (e.g. LSFdn mice, Fig. S3B in supporting material) were removed from consideration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AID	activation-induced deaminase
CSR	class switch recombination
LSF	Late SV40 Factor
LBP-1a	Leader Binding Protein-1a
S	switch region

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Figure 1.

LBP-1a is the predominant LSF family member in primary B cells. A) Sequences of LBP-1a (aa 274–336) and LSF (aa 277–332) are aligned. Antipeptide antibodies were produced against nonconserved epitopes (bold). B) Ten ng of His-LBP-1a or His-LSF were subjected to SDS-PAGE and immunoblotted (IB) with the anti-peptide antibodies specific to LBP-1a or LSF, as indicated. C) Extracts (50 µg) from untransfected 293T cells, cells transfected with pCxLSF, or cells transfected with pCxLBP-1a were separated by SDS-PAGE through a 7.5% gel and immunoblotted (IB) with the antipeptide antibodies specific to LSF or LBP-1a, as indicated. D) Left panels: Decreasing levels of purified His-LSF or His-LBP-1a were immunoblotted with the respective antipeptide antibodies. Right panels: on the same gels,

whole cell lysates from 20×10^6 splenic mouse B-cells in a single experiment were immunoblotted with anti-peptide antibodies specific to either LSF (upper panels) or LBP-1a (lower panels), and subsequently with antibody against β -actin.

Figure 2.

LBP-1a binds to S μ and S α , but not S γ 1, regions in resting B cells and is released after LPS stimulation. A) Schematic of mouse immunoglobulin S region sequences with predicted LSF/LBP-1a binding sites. Long horizontal lines, to scale, represent each S region sequence; intersecting vertical lines represent predicted LSF/LBP-1a binding sites. Shorter horizontal lines below each S region represent regions amplified in ChIP assays. Amplicon sequences are in boxes below each S region schematic; PCR primers are in bold. Predicted LSF/LBP-1a binding sites within or flanking each amplicon are in shaded boxes. Brackets indicate overlapping sites. B) Isolated primary splenic murine B-lymphocytes were analyzed by ChIP with LBP-1a antiserum. Negative controls included non-specific rabbit IgG (IgG) and preimmune rabbit serum (PI). Input represents 1.25% of ChIP starting material. DNA

were amplified with the primers in (A). Data are representative of at least 3 independent experiments. C) Whole cell extracts from 10×10^6 B-cells, stimulated with 50 $\mu\text{g/ml}$ LPS for the indicated amounts of time, were immunoblotted (IB) with antibodies against LBP-1a or β -actin, as indicated. D–E) Resting B-cells were stimulated with 50 $\mu\text{g/ml}$ LPS for the indicated times; LBP-1a occupancy at $S\mu$ (D) and $S\alpha$ (E) were analyzed as in (B). Upper panel: ChIP analysis with nonspecific rabbit IgG (–) or LBP-1a antiserum (+) at the indicated hours post-LPS stimulation. Lower panel: Amplification of input samples for each time point representing 0.125% (odd lanes) or 0.0125% (even lanes) of the ChIP starting material. Data are representative of 4 independent experiments.

Figure 3.

LSFdn expression in B-cells increases switching to IgA, but not to IgG1, upon induction of CSR. A) Isolated B-cells (5×10^6) from LSFdn chimeric mice were sorted into transduced (GFP+) and non-transduced (GFP-) populations based on GFP levels. Cell extracts from a single experiment were immunoblotted for LSF/LSFdn, using the LSF antipeptide antibody (Fig. 1), and for β-actin. B) Efficiency of IgM to IgA switching was determined for splenic B cells isolated from two groups of mice. Control mice (each indicated by a triangle) received bone marrow transduced with parental MSCV retrovirus. LSFdn mice (each indicated by a circle) received bone marrow transduced with MSCV-LSFdn retrovirus. Following stimulation in culture, splenic B cells were simultaneously analyzed for GFP and

surface IgA expression. GFP expression levels separated transduced versus nontransduced populations. The fold change in IgA expression upon LSFdn expression, calculated as the ratio of the percentages of transduced cells vs. nontransduced cells that are IgA-positive, is plotted on the y-axis. Left panel: Cells were stimulated with LPS, IL-5, and TGF- β 1 (control: $n = 5$; LSFdn: $n = 6$). Means (horizontal lines) and SEM were: 1.05 ± 0.17 for control mice, 1.88 ± 0.19 for LSFdn mice. $*p=0.0048$ (Student's t -test). Right panel: Cells were stimulated with LPS and IL-4 (control: $n = 4$; LSFdn: $n = 2$) Means (horizontal lines) and SEM were: 0.88 ± 0.06 for control mice, 1.43 ± 0.09 for LSFdn mice. The probability that the values in these two data sets would be ordered by chance in this manner is 0.067 (Mann-Whitney U test). C) Splenic B cells derived from control and LSFdn mice characterized in (B) were analyzed for isotype switching to IgG1. Cells were stimulated with LPS and IL-4 (control: $n = 6$; LSFdn: $n = 8$). Cells were analyzed for GFP and IgG1 expression, and the fold change in IgG1 expression upon LSFdn expression calculated analogously to above (B). The means and SEM for the ratio of percentage IgG1-positive transduced cells to percentage IgG1-positive non-transduced cells were: 0.98 ± 0.11 for control mice and 0.92 ± 0.14 for LSFdn mice. The difference in means is not statistically significant.