Uncoupling Splicing From Transcription Using Antisense Oligonucleotides Reveals a Dual Role for I Exon Donor Splice Sites in Antibody Class Switching

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Class switch recombination (CSR) changes antibody isotype by replacing $C_{\mu}$ constant exons with different constant exons located downstream on the immunoglobulin heavy ($IgH$) locus. During CSR, transcription through specific switch (S) regions and processing of non-coding germline transcripts (GLTs) are essential for the targeting of activation-induced cytidine deaminase (AID). While CSR to IgG1 is abolished in mice lacking an $I\gamma_1$ exon donor splice site (dss), many questions remain regarding the importance of I exon dss recognition in CSR. To further clarify the role of I exon dss in CSR, we first evaluated RNA polymerase II (RNA pol II) loading and chromatin accessibility in S regions after activation of mouse B cells lacking $I\gamma_1$ dss. We found that deletion of $I\gamma_1$ dss markedly reduced RNA pol II pausing and active chromatin marks in the $S\gamma_1$ region. We then challenged the post-transcriptional function of I exon dss in CSR by using antisense oligonucleotides (ASOs) masking I exon dss on GLTs. Treatment of stimulated B cells with an ASO targeting $I\gamma_1$ dss, in the acceptor $S\gamma_1$ region, or $I\mu$ dss, in the donor $S\mu$ region, did not decrease germline transcription but strongly inhibited constitutive splicing and CSR to IgG1. Supporting a global effect on CSR, we also observed that the targeting of $I\mu$ dss reduced CSR to IgG3 and, to a lesser extent, IgG2b isotypes. Altogether, this study reveals that the recognition of I exon dss first supports RNA pol II pausing and the opening of chromatin in targeted S regions and that GLT splicing events using constitutive I exon dss appear mandatory for the later steps of CSR, most likely by guiding AID to S regions.

Keywords: B cells, class switch recombination, splicing, germline transcripts, I exon, antisense oligonucleotides

INTRODUCTION

During immune responses, B cells can diversify the immunoglobulin (Ig) repertoire through class switch recombination (CSR) and somatic hypermutation (SHM). SHM introduces mutations in the variable (V) regions of Ig genes modifying antibody affinity for a cognate antigen. The mouse Ig heavy chain ($IgH$) locus comprises eight constant genes ($C_{\mu}$). CSR involves long-range interactions...
Transcription through S regions is required for CSR (3). Germline (GL) transcription is initiated from intervening (I) promoters a few kilobases upstream of both the donor and the acceptor S regions. The μ I promoter drives constitutive transcription through Sμ while γ, ε, or α I promoters are inducible. Primary GL transcripts (GLTs) exhibit a structured architecture composed of a non-coding I exon, an intronic S region, and Cμ exons (4, 5). In mature GLTs, the I exon is spliced to the first exon (CH1) of the adjacent constant gene. Because multiple stop codons are present in the three reading frames of I exons, these GLTs do not encode peptides of significant lengths.

During CSR, AID initiates double-strand DNA breaks (DSBs) by deaminating cytidines inside the transcribed S regions. GL transcription through S regions of Cμ gene favors AID accessibility to S regions (3). GL transcription promotes generation of RNA:DNA hybrid structures (R-loops) (6, 7) revealing single-stranded DNA (ssDNA) that serves as a substrate for AID (8). The impairment of transcription elongation upon R-loop formation (9) may favor RNA polymerase II (RNA pol II) pausing. RNA pol II pausing then promotes AID recruitment to S regions (10, 11). Paused RNA pol II and histone modifications associated with “open” chromatin, such as histone H3 lysine 4 trimethylation (H3K4me3) and histone H3 lysine 9 acetylation (H3K9ac), are enriched in transcribed I–S regions and have been involved in AID targeting to S regions primed for CSR (12–16). Moreover, the suppressor of Ty 5 homolog (Sp5) transcription elongation factor and the RNA exosome, a cellular RNA-processing degradation complex, associate with AID together with paused RNA pol II in transcribed S regions and are required for CSR (17, 18).

Beyond the prerequisite transcription of S regions, splicing of GLTs has been proposed to be important for the CSR process. Notably, CSR to IgG1 is severely impaired in a mouse model lacking the Iγ1 exon donor splice site (dss) (19, 20). Further supporting a role for splicing of GLTs in CSR, several RNA processing and splicing factors are critical regulators of CSR (21, 22). Interestingly, it has recently been proposed that intronic switch RNAs produced by the splicing of primary GLTs act as guide RNAs and target AID to DNA in a sequence-specific manner (23). After lariat debranching by the RNA debranching enzyme (DBR1), these switch RNAs are folded into G-quadruplexes. G-quadruplexes and AID are targeted to the S region DNA through the post-transcriptional action of the DEAD-box RNA helicase 1 (DDX1) (24).

Even though these data suggest that processing of GLTs by the splicing machinery is necessary for CSR, the precise role of I exon dss recognition in antibody class switching remains largely unknown. To address this issue, we first analyzed whether the presence of Iγ1 exon dss could influence RNA pol II pausing and chromatin accessibility of the Sγ1 region, as early events leading to CSR to IgG1. For that, chromatin immunoprecipitation (ChIP) experiments were performed in stimulated B cells from the previously described human metallothionein IIα (hMT) and s-hMT (splice hMT) mouse models, lacking or harboring Iγ1 exon dss, respectively (19, 20). We next specifically evaluated the impact of GLT splicing on CSR to IgG1 by using antisense oligonucleotides (ASOs) targeting specific I exon dss on primary GLTs, from both donor and acceptor S regions. Contrary to the models used previously to study the impact of I exons on CSR, treatment of mouse B cells by ASOs masks only a short RNA sequence (23–25 nucleotides) surrounding the I exon dss on primary GLTs. This antisense strategy bypassing the impact of I exon dss recognition on transcription is very useful for studying the involvement of I exon dss recognition in CSR at the post-transcriptional level. Collectively, our data indicate that the recognition of I exon dss exerts both transcriptional and post-transcriptional roles during CSR.

**MATERIALS AND METHODS**

**Mice**

Two- to 8-month-old C57BL/6, s-hMT, and hMT mice were used. hMT and s-hMT mice were kindly obtained from Dr. A. Radbruch (Leibniz Institute, Berlin, Germany). Mice were housed, and procedures were conducted in agreement with European Directive 2010/63/EU on animals used for scientific purposes applied in France as the “Décret n°2012-118 du 1er février 2013 relatif à la protection des animaux utilisés à des fins scientifiques.” Accordingly, the present project APAFIS#15279-2018052915087229 v3 was authorized by the “Ministère de l’Education Nationale, de l’Enseignement Supérieur et de la Recherche.”

**Splenic B Cell in vitro Stimulation and ASO Treatments**

Splenic B cells isolated from C57BL/6, hMT, and s-hMT mice were purified with the EasySep Mouse B Cell Isolation Kit (Stemcell Technologies). B cells were cultured for 2–4 days in RPMI 1640 with UltraGlutamine (Lonza) containing 10% fetal calf serum (FCS) (Dominique Dutscher), 1 mM sodium pyruvate (Eurobio), 1% AANE (Eurobio), 50 U/ml penicillin/50 µg/ml streptomycin (Gibco), and 129 µM 2-mercaptoethanol (Sigma-Aldrich). Splenic B cells were stimulated with either 1 or 5 µg/ml lipopolysaccharide (LPS) (LPS-EB Ultrapure, InvivoGen), 1 µg/ml LPS + 20 ng/ml interleukin 4 (IL4) (recombinant murine IL-4, PeproTech), or 5 µg/ml anti-CD40 [mouse CD40/TNFRSF5 MAb (Clone 1C10), Bio-Techne, USA]+ 40 ng/ml IL4. For ASO treatments, vivo-morpholinio ASOs (Iγ1 dss ASO: 5'-CCCACCTCCCCCTGGTCACCTACGCCG-3'; Iε dss ASO: 5'-GGCTGCCTCTGGCTTACCATTTG-3') and an irrelevant ASO (control: 5'-CCCTTACCTACGTTACATATTTTA-3') were designed and purchased at Gene Tools, LLC (Philomath, USA). Stimulated splenic B cells were cultured in the presence of 2, 3, or 4 µM ASO, as indicated.

Culture samples were harvested at day 2, 3, or 4 for subsequent flow cytometry analysis, ChIP assays, or RNA/protein extraction, as described below and in the Supplementary Methods.
Supernatants of stimulated B cells were collected at day 3 or 4 and stored at −20°C until used for enzyme-linked immunosorbent assays (ELISAs).

Flow Cytometry
Cell suspensions of in vitro stimulated splenic B cells were washed in phosphate-buffered saline (PBS). To reduce Fc receptor-mediated binding by antibodies of interest, B cells were preincubated for 15 min with 0.5 µg/ml anti-mouse CD16/CD32 (Clone 2.4G2, BD Pharmingen, ref 553142) in FACS buffer [PBS supplemented with 2% FCS and 2 mM (ethylenedinitrilo)tetraacetic acid (EDTA) (Sigma-Aldrich)]. Cells were then labeled with 0.5 µg/ml anti-mouse B220-BV421 (clone RA3-6B2, BioLegend, ref 103240) and 0.5 µg/ml anti-mouse IgG1-FITC (clone A85-1, BD Pharmingen, ref 553443) antibodies or with 0.25 µg/ml anti-mouse IgG1-FITC (clone RA3-6B2, BD Pharmingen, ref 552094) and 0.5 µg/ml anti-mouse IgG1-FITC (clone A85-1, BD Pharmingen, ref 553443) or 0.5 µg/ml anti-mouse IgG3-FITC (clone R40-82, BD Pharmingen, ref 553403) and anti-mouse IgG2a/2b-BV421 (clone R2-40, BD OptiBuild, ref 744292) antibodies.

After 45 min, cells were washed in PBS and suspended in FACS buffer. For nonviable-cell exclusion, 7-AAD (BD Pharmingen, ref 559925) was added on 10 min before flow cytometry analysis. Data were acquired on a BD Pharmingen Fortessa LSRR (BD Biosciences, San Jose, CA, USA) and analyzed using Flowlogic™ software (Miltenyi Biotec).

ELISAs
Culture supernatants and sera were analyzed for the presence of IgM, IgG1, or IgG2b by ELISA. Blood samples were collected from 12-week-old hMT and s-hMT® mice. Serum samples were recovered by centrifugation and stored at −20°C until used. ELISAs were performed in polycarbonate 96-multiwell plates, coated overnight at 4°C (100 µl per well) with 2 µg/ml IgM, IgG1, or IgG2b antibodies (Southern Biotechnologies: goat anti-mouse IgM human ads UNLB, ref 1020-01; goat anti-mouse IgG1 human ads-UNLB, ref 1070-01; and goat anti-mouse IgG2b human ads-UNLB, ref 1090-01) in PBS. After three successive washing steps in PBS with 0.05% Tween 20, a blocking step with 100 µl of 3% bovine serum albumin (BSA) (Euromedex) in PBS was performed for 30 min at 37°C. After three washing steps, 50 µl of sera/supernatant or standards IgM, IgG1, or IgG2b (Southern Biotechnologies, 400 ng/ml) were diluted into successive wells in 1% BSA/PBS buffer and incubated for 2 h at 37°C. After three washing steps, 100 µl per well of 1 µg/l alkaline phosphatase (AP)-conjugated goat anti-mouse antibodies (Southern Biotechnologies: goat anti-mouse IgG1 human ads-AP, ref 1070-04; goat anti-mouse IgM-AP, ref 1021-04; Beckman Coulter: goat anti-mouse IgG2b human ads-AP, ref 1090-04) was incubated in PBS with 0.05% Tween 20 for 2 h at 37°C. After three washing steps, AP activity was assayed: 100 µl of substrate for AP (SIGMAFAST™ p-nitrophenyl phosphate tablets, Sigma-Aldrich) was added, and after 15 min, the reaction was blocked with the addition of 50 µl of 3 M NaOH (Sigma-Aldrich). Optic density was then measured at 405 nm on a Multiskan FC microplate photometer (Thermo Scientific).

RT-PCR and Quantitative RT-PCR
In vitro stimulated splenic B cells were harvested, and RNA was extracted using the TRizol® reagent (Invitrogen) procedure. Reverse transcription was carried out on 1 µg of DNase I (Invitrogen)-treated RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Priming for reverse transcription was done with random hexamers.

To analyze GL transcription, PCRs were performed on cDNA using the Taq Core Kit (MP Biomedicals) and appropriate primer pairs (primer pairs are provided in Supplementary Table 1). PCR amplification of the β-actin transcript was used as an internal loading control.

To determine nucleotide sequence of normal and alternative transcripts, PCRs were performed on cDNA using the Phusion® High-Fidelity DNA Polymerase (New England BioLabs) and appropriate primer pairs. After purification of the RT-PCR products using the NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel) according to the manufacturer’s instructions, sequencing was performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit on a 3130 × 1 Genetic Analyzer ABI PRISM (Applied Biosystems). Quantification of the purified RT-PCR products was also performed using ImageJ software.

Quantitative PCRs were performed on cDNA using Premix Ex Taq™ (probe qPCR), ROX Plus (Takara), or TB Green Premix Ex Taq™ II (Tli RNase H Plus), ROX Plus (Takara) on a StepOnePlus Real-Time PCR system (Applied Biosystems). Transcripts were quantified according to the standard 2−ΔΔCT method after normalization to Gapdh. Primers and probes used for determination of transcripts are listed in Supplementary Table 1.

ChIP Assays
ChIP assays were performed using anti-H3ac (Millipore, 06-599), anti-H3K4me3 (Millipore, 07-473), anti-H3K9ac (Millipore, 06-942), anti-RNA pol II (CTD4H8, Santa Cruz Biotechnology, sc-47701), anti-RNA pol II ser2P (Abcam, ab5095), and anti-RNA pol II ser5P (Abcam, ab5131) as previously described (25). In brief, 1 × 107 LPS-stimulated B cells from hMT and s-hMT® mice were harvested at day 2, washed twice in PBS, and cross-linked at 37°C for 15 min in 15 ml of PBS with 1% formaldehyde (Sigma-Aldrich). The reaction was quenched with 0.125 M glycine (Sigma-Aldrich). After lysis, chromatin was sonicated to 0.5–1 kb using a VibraCell 75043 (Thermo Scientific). To analyze GL transcription, PCRs were performed on cDNA extracted using the TRIzol® reagent (Invitrogen) procedure.

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buffer with 0.02% SDS, and genomic DNA was obtained after phenol/chloroform extraction. Analysis of immunoprecipitated DNA sequences was done by quantitative PCR using the primer pairs described in Supplementary Table 1.

Statistical Analysis
Results are expressed as means ± SEM, and overall differences between variables were evaluated by an unpaired two-tailed Student's t test using Prism GraphPad software (San Diego, CA).

RESULTS

RNA pol II Pausing and Histone Modifications Upstream of the Sy1 Region Are Altered in Mice Lacking Iy1 dss
To clarify the role of I exon dss in CSR, we first performed comparative experiments in homozygous s-hMT and hMT mice (19, 20). In these models, the promoter and Iy1 exon have been replaced by the LPS-inducible hMT promoter and an artificial hMT exon containing (s-hMT) or lacking (hMT) Iy1 dss (Figure 1A). As previously described by Lorenz and collaborators (20), IgG1 class switching was almost abolished in hMT mice, whereas CSR to other Ig isotypes remained unaffected (Supplementary Figures 1A–C). This was not associated with a difference in AID mRNA expression between splicing B cells isolated from hMT and s-hMT mice (Supplementary Figure 1D). Previous nuclear run-on assays have shown that the transcription rate of the Sy1 region was higher in LPS-stimulated B cells from s-hMT mice than that from hMT mice (20), suggesting a role for Iy1 exon dss in GL transcription of the Sy1 region. In agreement with these results, we found low levels of unspliced γ1 GLTs in LPS-stimulated B cells from hMT mice, compared to s-hMT (Figure 1B).

Paused RNA pol II and histone modifications associated with “open” chromatin are involved in AID recruitment to S regions during CSR (10, 12, 13, 15, 16, 18). To study the role of I exon dss in RNA pol II pausing and chromatin remodeling in the Sy1 region, we performed ChIP experiments in splenic B cells isolated from hMT and s-hMT mice after 2 days of LPS stimulation. First, antibodies directed against total RNA pol II, elongating RNA pol II (Ser2P), or pausing RNA pol II (Ser5P) were used. In agreement with the low level of γ1 GLTs described in Figure 1B, stimulated B cells from hMT mice displayed significantly decreased total RNA pol II loading throughout the Sy1 region, compared to s-hMT mice (Figure 1C). Interestingly, a significant decrease in Ser5P RNA pol II loading (Figure 1D), but not Ser2P RNA pol II loading (Figure 1E), was observed in the promoter–Sy1 region in stimulated B cells from hMT compared to s-hMT mice. Accordingly, stimulated B cells from s-hMT mice exhibited significantly higher Ser5P/Ser2P ratios upstream from the Sy1 region than B cells from hMT animals (Figure 1F), suggesting that the recognition of I exon dss regulated RNA pol II pausing upstream from the Sy1 region. As a control, we found similar Ser5P and Ser2P RNA pol II loading in Sp. and Sy2b regions in stimulated B cells from both hMT and s-hMT mice (Supplementary Figure 2).

We next performed similar ChIP experiments using antibodies directed against variants of histone H3. We observed very low levels of acetylated histone H3 (H3ac), a general marker of chromatin accessibility, throughout the whole promoter–Sy1 region in LPS-stimulated B cells from hMT compared to s-hMT mice (Figure 1G). In B cells from s-hMT mice, the highest levels of H3ac, H3K9ac, and H3K4me3 histone forms were observed in the region upstream from Sy1 (Figures 1G–I). In contrast, this increase was not anymore observed in B cells from hMT mice.

Collectively, our data indicate that Iy1 exon dss recognition is of key importance for transcriptional activity of the Sy1 region by supporting RNA pol II pausing and chromatin remodeling, two occurrences required for AID recruitment in S regions and efficient CSR.

ASO Strategy Targeting I Exon dss Allows Uncoupling GL Transcription and Splicing in Wild-Type B Cells
We next wanted to study the function of I exon splicing on CSR. For this purpose, we treated stimulated splenic B cells isolated from wild-type (WT) mice with vivo-morpholino ASO targeting the dss sequence of Iy1 exon (Iy1 dss ASO) (Figure 2A). Indeed, we previously demonstrated that passive administration of these phosphorodiamidate morpholino oligonucleotides complexed with an octaguanidinendendrimer could very efficiently modulate the splicing of Ig transcripts (26). First, we wanted to verify that masking the dss sequence of Iy1 exon on GLTs by ASO did not inhibit γ1 GL transcription. After 2 days of culture with LPS + IL4 and 2μM ASO, expression of unspliced γ1 GLTs in activated B cells was significantly increased after treatment with Iy1 dss ASO (Figure 2B), compared to cells treated with an irrelevant control ASO. This suggested an accumulation of unspliced γ1 GLTs due to Iy1 dss ASO-induced splicing inhibition. In order to study the consequences of Iy1 dss ASO on spliced γ1 GLTs, we next performed PCR using the Iy1–Cy1 primer pair (Iy1-for and Cy1-rev, described in Figure 2C and Supplementary Table 1) on cDNA from ASO-treated WT splenic B cells stimulated by LPS + IL4. After 2 days, the γ1 GLT profile was strikingly different in control and Iy1 dss ASO conditions (Figure 2C, top). A band corresponding to the constitutively spliced γ1 GLT (involving the constitutive Iy1 exon dss) was strongly detected in control but slightly detected upon Iy1 dss ASO treatment (Figure 2C, top, and Supplementary Figure 3). Remarkably, in both control and Iy1 dss ASO conditions, additional bands were also detected that could account for new splicing isoforms. Sanger sequencing indeed revealed alternative transcripts so far not described in the literature (alternative transcripts 1, 2, and 3; Figure 2C, middle, and Supplementary Figure 3) involving donor and acceptor splice sites internal to the Iy1 exon. The fact that these alternative splice sites were predicted with high consensus value by the HSF 3.1 tool [HSF 3.1, 07/24/2019, http://www.umd.be/HSF/HSF.shtml, (27)] (Figure 2C, bottom) indicated that the different PCR products were bona fide spliced transcripts. As a proof of ASO efficiency, treatment with Iy1 dss ASO almost abolished
the major constitutive GLT and prevented alternative transcript 1 detection (Figure 2C, top). Thus, after treatment with Iγ1 dss ASO, transcript isoforms using the constitutive Iγ1 dss (constitutive transcript and alternative transcript 1) represented only 16% of the detected transcripts (vs. 85% in control). Another consequence of Iγ1 dss ASO treatment is the detection of alternative transcript 3 and a better detection of alternative transcript 2.

Since our data showed that Iγ1 dss ASO strongly decreased γ1 GLT constitutive splicing, the ASO strategy is a good tool to study the function of Iγ1 exon splicing on CSR.

FIGURE 1 | Deletion of Iγ1 exon dss decreased RNA pol II pausing and chromatin accessibility in the Sy1 region. (A) Wild-type (WT) γ1 locus and s-hMT and hMT murine models. Black and gray elements represent gene segments of the γ1 WT locus. The white elements are gene segments specific to s-hMT and hMT models and are not present in WT animals. The red fragment corresponds to the sequence containing the I exon donor splice site (dss), retained in the s-hMT model and absent in the hMT model. P, promoter; Iγ1, γ1 I exon; Sy1, hMT I exon; Sy1, γ1 switch region; CH1, γ1 constant exon 1. (B) Splenic B cells were isolated from WT, homozygous s-hMT and homozygous hMT mice and stimulated with LPS + IL4 or LPS. After 2 days of stimulation, γ1 GLT expression relative to GAPDH mRNA expression was monitored by quantitative RT-PCR using Sy1U primers described in schema A. Expression of γ1 GLTs in B cells isolated from WT mice and stimulated by LPS + IL4 was normalized to 1. (C–I) Splenic B cells were isolated from homozygous s-hMT and hMT mice and stimulated with LPS. After 2 days of stimulation, cells were analyzed for RNA pol II (C), Ser5P RNA pol II (D), and Ser2P RNA pol II (E) levels in the γ1 locus by ChIP coupled to quantitative PCR. Ser5P RNA pol II/Ser2P RNA pol II ratios are indicated (F). Cells were also analyzed for H3ac (G), H3K9ac (H), and H3K4me3 (I) levels in the γ1 locus by ChIP coupled to quantitative PCR. Background signals from mock samples with irrelevant antibody were subtracted. Values were normalized to total input DNA. Position and sequence of primers used for quantitative PCR are described in schema A (triangles) and Supplementary Table 1. (B–I) Data are means ± SEM of at least two independent experiments, n = 3–5 for each genotype. Unpaired two-tailed Student’s t test was used to determine significance. ns: non-significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
FIGURE 2 | Treatment with γ1 exon dss ASO inhibited γ1 GLT constitutive splicing and IgG1 class switching in B cells. (A) Antisense oligonucleotide targeting the donor splice site of γ1 exon (γ1 dss ASO) was designed and synthesized as “vivo-morpholino ASO” permitting passive administration of ASO in cells (Gene Tools, LLC). Targeted γ1 GLT (uppercase: exon sequence; lowercase: intron sequence) and γ1 dss ASO sequences are indicated. γ1, γ1 I exon; Sγ1, γ1 switch region; CH1γ1, γ1 constant exon 1; dss, donor splice site.

(B–G) Splenic B cells were isolated from C57BL/6 mice, stimulated with LPS + IL4, and treated with 2 µM γ1 dss ASO (ASO) or an irrelevant ASO (control) for 2 days (B, C) or 3 days (D–G). (B) Unspliced γ1 GLT expression relative to GAPDH mRNA expression was monitored by
Treatment With \( \gamma_1 \) Exon dss ASO Specifically Inhibits IgG1 Class Switching

We next investigated the effect of ASO-mediated \( \gamma_1 \) GLT splicing inhibition on CSR to IgG1. After 3 days of culture with LPS + IL4 and 2 \( \mu \)M ASO, CSR to IgG1 was greatly diminished in B cells treated with \( \gamma_1 \) dss ASO compared to B cells treated with an irrelevant control ASO. The percentage of IgG1-positive B cells determined by flow cytometry was decreased 4-fold in \( \gamma_1 \) dss ASO-treated B cells (Figures 2D,E), and IgG1 levels determined in culture supernatants by ELISA were almost 2-fold lower in \( \gamma_1 \) dss ASO-treated cells (Figure 2F) than in control B cells. In agreement with the CSR defect, \( \mu_\gamma\)-Cy1 switched transcripts were decreased by 4-fold in \( \gamma_1 \) dss ASO-treated B cells (Figure 2G).

In order to control \( \gamma_1 \) dss ASO specificity, we realized similar experiments in B cells stimulated by anti-CD40 + IL4. Similar to what was observed in LPS + IL4-stimulated B cells, after 2 days of culture with anti-CD40 + IL4 and 2 \( \mu \)M ASO, expression of unspliced \( \gamma_1 \) GLTs was significantly increased (Figure 3B), and CSR to IgG1 was significantly diminished (Figures 3C,D) in B cells treated with \( \gamma_1 \) dss ASO compared to B cells treated with an irrelevant control ASO. However, expression of unspliced Is GLTs (Figures 3A,E) and CSR to IgE (Figures 3F,G) were similar in B cells treated with the irrelevant control ASO or \( \gamma_1 \) dss ASO. This showed that \( \gamma_1 \) dss ASO specifically decreased CSR to IgG1 by inhibiting splicing precisely on \( \gamma_1 \) GLTs.

Collectively, these data indicate that splicing of \( \gamma_1 \) GLTs is necessary for CSR to IgG1 and strongly suggest that the use of the constitutive \( \gamma_1 \) exon dss during splicing is required for efficient CSR.

Treatment With an ASO Targeting the Constitutive I exon dss in the Donor \( \mu_\gamma \) Region Inhibits IgG Class Switching

Our results showed that the ASO strategy targeting the constitutive I exon dss from the \( \mu_\gamma \) acceptor region inhibited CSR to IgG1. We next developed a similar approach to determine whether specifically masking the constitutive I exon dss from the donor \( \mu_\gamma \) region could also inhibit CSR to IgG1. Splenic B cells isolated from WT mice were stimulated by LPS + IL4 and treated with an ASO targeting the constitutive I exon dss (Is dss ASO) (Figure 4A). In order to study the consequences of Is dss ASO on spliced IgG GLTs, we performed PCR using the Is-\( \gamma_1 \)- primer pair (I-d- and C\( \gamma_1 \)-rev, described in Figure 4A and Supplementary Table 1) on cDNA from stimulated splenic B cells treated with 2 \( \mu \)M ASO for 2 days. A band corresponding to the constitutively spliced Is transcript (involving the constitutive I exon dss) was detected in control but slightly detected after Is dss ASO treatment (Figure 4B). Similar to what was observed in stimulated splenic B cells treated with \( \gamma_1 \) dss ASO, we detected the presence of alternatively spliced Is transcripts in cells treated with Is dss ASO. These alternative transcripts were indicative of the use of alternative Is dss present in the intronic S region previously described by Kuzin and collaborators (28). In addition, after 3 days of culture with LPS + IL4 and 2 \( \mu \)M ASO, CSR to IgG1 was significantly diminished in B cells treated with Is dss ASO compared to B cells treated with an irrelevant control ASO (Figures 4C,D). After 2 days of 2 \( \mu \)M ASO treatment, expression of unspliced \( \gamma_1 \) GLTs was similar in B cells treated with an irrelevant control or Is dss ASO (Figure 4E). This showed that the defect in IgG1 class switching observed after treatment by Is dss ASO was not due to an inhibition of \( \gamma_1 \) GLT splicing but to the splicing decrease of IgG GLTs. Therefore, correct splicing of GLTs produced in both the donor S\( \mu_\gamma \) and the acceptor Sy1 regions is required for efficient IgG1 class switching.

We next investigated whether masking the constitutive Is dss could also inhibit class switching to other isotypes than IgG1. Splenic B cells isolated from WT mice were stimulated by LPS and treated with Is dss ASO. After 3 days of culture with LPS and 3 or 4 \( \mu \)M ASO, the percentage of IgG3-positive B cells was significantly diminished in B cells treated with Is dss ASO compared to B cells treated with an irrelevant control ASO (Figures 4F–H). Moreover, treatment of B cells with Is dss ASO strongly decreased the level of \( \gamma_3 \) heavy-chain protein (Supplementary Figure 4). After 3 days of culture with LPS, the percentage of IgG2b-positive B cells was significantly diminished only in B cells treated with 4 \( \mu \)M Is dss ASO compared to B cells treated with an irrelevant control ASO (Figures 4I–K).

These results indicated that treatment with ASO masking the constitutive Is dss on the donor \( \mu_\gamma \) region globally...
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Impaired antibody class switching in B cells treated by \(\mu\) exon dss ASO. (A) Antisense oligonucleotide targeting the donor splice site of \(\mu\) exon (\(\mu\) dss ASO) was designed and synthesized as “vivo-morpholino ASO” permitting passive administration of ASO in the cells (Gene Tools, LLC). Targeted \(\mu\) GLT (uppercase: exon sequence; lowercase: intron sequence) and \(\mu\) dss ASO sequences are indicated. \(\mu\), \(\mu\) I exon; S, \(\mu\) switch region; CH1\(\mu\), \(\mu\) constant exon 1; dss, donor splice site.

(B–K) Splenic B cells were isolated from C57BL/6 mice, stimulated with LPS + IL4 (B–E) or LPS (F–K) and treated with 2 \(\mu\)M (B–E), 3 \(\mu\)M (F,G,J) or 4 \(\mu\)M (H,I,K) \(\mu\) dss ASO or an irrelevant control ASO for 2 days (B,E) or 3 days (C,D,F–K). (B) Constitutively and alternatively spliced \(\mu\) transcripts analyzed as described (Continued)
Ser5P RNA pol II and chromatin accessibility in the promoter–Sy1 region. Moreover, our data demonstrated that, in S regions primed for CSR, GLT splicing involving the constitutive I exon dss is an essential step for efficient CSR.

Regarding the impact of I exon dss on transcription, our comparative analysis of s-hMT and hMT B cells indicated that, in the absence of Iγ1 dss, GL transcription and RNA pol II loading were very weak at the γ1 locus. Interestingly, RNA pol II binding was strongly diminished at the hMT promoter, suggesting that the presence of Iγ1 exon dss enhanced the initiation of GL transcription. These data are consistent with the poor transcription observed upon deletion of a large portion of Iγ2b exon including dss (30). Although the function of I exon dss in transcription regulation has been overlooked, such intron-mediated enhancement of gene expression has long been described in a wide range of organisms, including mammals (31). Indeed, it has been demonstrated that the presence of a dss facilitates the transcription preinitiation complex assembly and stimulates transcription even in the absence of splicing (32, 33). Whether the recognition of I exon dss promotes the formation of pre-initiation complex (32) and involves a gene looping interaction between promoter and dss, as described in yeast cells (34), remains to be investigated. Our ChIP analysis further indicated that, in the absence of Iγ1 exon dss, RNA pol II pausing is markedly decreased in the promoter–Sy1 region, whereas the rate of RNA pol II elongation remains mostly unchanged. It is tempting to speculate that the I exon dss acts as an anchor for the co-transcriptional machinery and is required for appropriate associations of critical factors, like Spt5, with RNA pol II to enable recruitment of AID to S regions. After B cell stimulation, transcribed I–S regions are a focus for increased modified histones, such as H3ac, H3K9ac, or H3K4me3, and chromatin accessibility (13, 16). Reinforcing the idea that GL transcription, RNA pol II pausing, and the establishment of active chromatin marks in S regions are interconnected (10), we found a global reduction of active H3ac marks in the promoter–Sy1 region of stimulated B cells from hMT mice, compared to s-hMT. The H3K9ac and H3K4me3 enrichment specifically detected upstream the Sy1 region was also lost in hMT mice. Interestingly, it has been proposed that H3K4me3 serves as a mark for recruiting the recombimase machinery for CSR independently of its function in transcription (15, 35). These studies have shown that Spt5 and the histone chaperone FACTilitates Chromatin Transcription (FACT) are not required for transcription of S regions but regulate H3K4me3 modification and DNA cleavage in CSR (15, 35).

Iγ1 exon dss recognition is necessary for the regulation of GL transcription. Consequently, using classical I exon deletion and/or replacement mouse models neither allow distinguishing the roles of I exon dss in the interconnected processes of transcription and splicing nor permit distinguishing requirements of splicing per se from that of stable S GLTs (36). Several studies including ours have shown the efficacy of ASO-mediated approaches to modify RNA splicing in B-lineage cells (26, 37, 38). Here, we used ASOs targeting a short sequence (23–25 nucleotides) spanning the Iγ1 or Iμ exon dss on pre-mRNA to analyze the consequences of GL splicing defect on CSR in splenic B cells from WT mice. We showed that, in addition to its positive effect on transcription, I exon dss recognition is required at the post-transcriptional level for efficient CSR. Indeed, we demonstrated that masking Iγ1 exon dss inhibited CSR to IgG1 and masking Iμ exon dss decreased CSR to IgG1, IgG3, and, to a lesser extent, IgG2b isotypes. Further investigations in both mouse and human B cells will be necessary to check whether masking Iμ exon dss could decrease CSR to all isotypes. ASOs masking Iγ1 exon dss in other acceptor S regions than Sy1 could also be used to verify whether the recognition of I exon dss is required for efficient CSR toward each switched Ig isotype.

Our results strongly suggest that splicing of GLTs per se is not sufficient to induce CSR. Indeed, we found a marked reduction of CSR to IgG1 upon treatment with ASOs masking constitutive I exon dss on GLTs produced in the donor Sμ or the acceptor Sy1 regions, even though alternative splicing events could be readily detected. By contrast, Kuzin and collaborators showed normal surface Ig expression and serum Ig levels in ΔIμ·s−/− mice harboring a deletion of 236 bp spanning the constitutive Iμ dss (28). As expected, there were no detectable spliced Iμ transcripts in these mice. Nevertheless, alternative “Iμ-like” GLTs driven by regulatory elements other than Iμ were detected at low levels in B cells from ΔIμ·s−/− mice. The authors suggested that such alternative “Iμ-like” GLTs directly contribute to CSR activity (28). However, our results showed that these alternative “Iμ-like” GLTs might not be sufficient for efficient CSR activity in vitro. Indeed, after treatment of B cells with Iμ dss ASO, we detected Iμ·Cμ alternative transcripts indicative of the use of the same cryptic Sμ dss than described by Kuzin and collaborators whereas a drastic inhibition of CSR was observed. The regulation of μ locus is complex, and further investigations are needed to understand the discrepancies concerning the impact of Iμ dss absence at the DNA level, in genetically modified mouse model, and at the RNA level, with our ASO strategy, on CSR. Nonetheless, our data are in agreement with a
study of Ruminy and collaborators (39). They detected recurrent acquired mutations at the Iµ dss on the functional IgH allele of t(14;18) positive lymphomas cases presenting restricted IgM expression and proposed that disruption of Iµ constitutive dss, inducing the expression of abnormal GLTs, may be involved in the perturbation of CSR observed in these lymphomas. Moreover, Sprt depletion by siRNA in the CH12F3-2A B cell line severely impaired CSR to IgA despite a dramatically increased expression of cryptic transcripts initiating from the Sµ intronic region (35). As observed in the donor Sµ region, masking the constitutive I exon dss on GLTs produced in the acceptor Sy1 region with Iy1 dss ASO strongly inhibited CSR to IgG1 despite Iy1–Cy1 alternative transcripts being detectable. Additional molecular studies will be required to delineate the mechanism involved in the ASO-mediated inhibition of CSR. Several scenarios could explain the inhibition of CSR by I exon dss ASOs. First, RNA-binding proteins have been shown to interact with AID (22, 40–42), and the ASO, attached to the GLTs, could avoid fixation of such proteins necessary for CSR. Second, it has been described that, when the accumulation of intronic switch RNAs was prevented from the onset of CH12F3-2A B cell stimulation, R-loop levels were decreased by half in S regions (24). In our study, after I exon dss ASO treatment, the accumulation of unspliced GLTs indicated a strong splicing inhibition. Thus, even though low levels of constitutive and alternative spliced GLTs were detected after ASO treatment, the lariat abundance may be too weak to induce R-loop formation at S regions and efficient CSR. Third, even if alternatively spliced GLTs were detected after ASO treatments, the sequence of intronic lariat generated after alternative splicing could prevent efficient CSR. For example, alternative lariat sequence could impede the lariat debranching by DBR1 and the subsequent generation of G-quadruplex RNA structures that participate in guiding AID-debranching by DBR1 and the subsequent generation of G-quadruplex RNA structures that participate in guiding AID.

In summary, our study highlighted a dual role for I exon dss during CSR, at the DNA and RNA levels, and further paves the way for antisense strategies in the modulation of antibody class switching and immune response efficiency.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the 'Ministère de l’Education Nationale, de l’Enseignement Supérieur et de la Recherche'. APAFIS#15279-2018052915087229 v3.

AUTHOR CONTRIBUTIONS

AM, MA, and J-ML performed experiments and analyzed data. CC, SL, and NS performed experiments. LD conceived the project, designed experiments, analyzed the data, and wrote the manuscript. SP conceived the project, designed experiments, performed experiments, analyzed the data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00780/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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