Elucidation of the enigmatic IgD class-switch recombination via germline deletion of the IgH 3′ regulatory region

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Classical class-switch recombination (cCSR) substitutes the Cμ gene with Cγ, Cα, or Cε, thereby generating IgG, IgE, or IgA classes, respectively. This activation–induced deaminase (AID)–driven process is controlled by the IgH 3′ regulatory region (3′RR). Regulation of rare IgD CSR events has been enigmatic. We show that μδCSR occurs in mouse mesenteric lymph node (MLN) B cells and is AID–dependent. AID attacks differ from those in cCSR because they are not accompanied by extensive somatic hypermutation (SHM) of targeted regions and because repaired junctions exhibit features of the alternative end-joining (A-EJ) pathway. In contrast to cCSR and SHM, μδCSR is 3′RR-independent, as its absence affects neither breakpoint locations in Sμ– and Sμ-like (ρμ) nor mutation patterns at Sμ–εμ junctions. Although mutations occur in the immediate proximity of the μδ regulatory region (3′RR), is absent distal to the junctions within both Sμ and rearranged VDJ regions. In conclusion, μδCSR is active in MLNs, occurs independently of 3′RR-driven assembly, and is even dramatically increased in 3′RR-deficient mice, further showing that its regulation differs from cCSR.

After functional V(D)J recombination, bone marrow immature B cells express a BCR made up of surface IgM. In secondary lymphoid organs, mature B cells simultaneously express surface IgD of the same specificity as surface IgM through alternative splicing of a pre-mRNA composed of V(D)J and both Cγ and Cδ heavy chain constant exons (Moore et al., 1981; Preud’homme et al., 2000; Chen and Cerutti, 2010). After encountering antigen, in adequate lymphoid tissue structures providing accessory signals (direct interactions with T cells and follicular dendritic cells and binding of cytokines secreted by such cells), activated B cells undergo classical class-switch recombination (cCSR) and substitute the Cμ gene with Cγ, Cα, or Cε, thereby generating secondary IgG, IgA, and IgE BCR and antibody classes with the same antigenic specificity but new effector functions. This recombination targets specific stretches of repetitive DNA (S regions) preceding Cμ and all Cγ, Cα, and Cε genes and requires the DNA-editing enzyme activation-induced deaminase (AID; Pavri and Nussenzweig, 2011). Transcription and, to an even higher extent, accessibility of the IgH locus S regions to cCSR is under the control of the IgH 3′ regulatory region (3′RR), located downstream of Cα, and encompassing the four transcriptional enhancers hs3a, hs1.2, hs3b, and hs4 (Vincent-Fabert et al., 2010a; Pinaud et al., 2011). Genomic disruption or complete deletion of the 3′RR in the mouse abrogates cCSR to all IgG, IgA, and IgE classes (Vincent-Fabert et al., 2010a,b; Pinaud et al., 2011). Recently the 3′RR was also reported to be transcribed and itself undergo AID-mediated mutation and recombination around phylogenetically conserved switch-like DNA repeats, highlighting its close functional partnership with AID (Péron et al., 2012).

Despite the absence of a repetitive S region upstream of Cδ and similar to class switching from IgM to IgG, IgA, and IgE, some CD38+ human B cells from tonsils carry an IgH locus switched to IgD expression after an AID–mediated deletion.

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of Cµ (Arpin et al., 1998; Brandtzaeg and Johansen, 2005; Johansen et al., 2005; Chen et al., 2009). Secreted IgD originating from such cells with CSR from Cµ to Cδ (µδCSR) enhances immune surveillance and can exert proinflammatory and antimicrobial effects (Chen et al., 2009). Although there is no canonical switch region 5’ to the Cδ gene in mammals (Pireaud’homme et al., 2000; Chen and Cerutti, 2010), rudimentary Sγ-like (σγ) sequences have been described at Cµ-Cδ junctions but CSR from µ to δ stands as a rare event whose regulation is obscure (Chen and Cerutti, 2010). To explore the role of the 3’RR on such Cµ-Cδ recombinations, in the context of the endogenous locus, we analyzed IgH 3’RR-deficient mice lacking the 30-kb extent of the 3’RR, a deletion which we previously characterized as inducing a severe cCSR defect toward IgG, IgA, and IgE (Vincent-Fabert et al., 2010b), a severe somatic hypermutation (SHM) defect (Rouaud et al., 2013) but with normal V(D)J recombination (Rouaud et al., 2012).

RESULTS

IgD secretion in mice occurs at low levels and is poorly understood. Germline transcription of Iµ-Cµ enrolls the Sµ regions as a substrate for DNA modification by the cCSR machinery including AID (Chen and Cerutti, 2010). Iµ-Cµ transcripts can originate either from alternate splicing of a pre-mRNA encompassing Cµ and Cδ exons or from primary Cδ transcripts after µδCSR. Fig. 1 schematizes these processes and locates PCR primers and probes used in this study.

µδCSR occurs in normal mesenteric LNs (MLNs) of wt mice and 3’RR-deficient mice

µδCSR is currently not documented in mice (Chen and Cerutti, 2010). Although little is known about the role of soluble IgD in physiology, a well-known inflammatory disease in humans features high serum IgD levels and was thus called hyper-IgD syndrome, a disease also featuring enlargement of MLNs (Oretti et al., 2006). In a search for IgD+IgM− cells after µδCSR in wt mice, we thus focused our attention on MLN B cells. S regions consist of tandem repeats of short G-rich sequences. While readily showing Sµ repeats, dot plot analysis of the mouse DNA fragment encompassing the Eµ enhancer to Cδ only revealed few DNA repeats upstream of Cδ in a short 0.5-kb-long region that was thereby named σδ (Preud’homme et al., 2000; Fig. 2 A). Using a nested PCR-based strategy (location of primers in Fig. 1) followed by Southern blotting (location of the probe in Fig. 1) of PCR products, we were able to detect Sµ-σδ junctions as a result of Sµ to σδ CSR (Fig. 2 B). We definitely authenticated Sµ-σδ junctions by cloning them and analyzing their sequences (Fig. 2 C and Table S1). Because 3’RR-deficient mice are known to be almost completely defective for cCSR (Vincent-Fabert et al., 2010b), we also checked them for µδCSR. To our surprise, Southern blotting of PCR product revealed the presence of Sµ-σδ junctions as a result of Sµ to σδ CSR (Fig. 2 B). These junctions were again authenticated by cloning and sequencing (Fig. 2 C and Table S1).

Molecular features of µδCSR junction sequences

The locations of Sµ-σδ breaks in Sµ and σδ for wt mice and 3’RR-deficient animals are reported in Fig. 2 D. Among Sµ-σδ junctions in wt mice (Fig. 2 C and Table S1), 13/21 (61.9%) showed 1–8-bp-long junctional microhomology. Complex junctions were also frequent (6/21, 28.6%). Similarly to wt mice, junctions with short nucleotide insertions and complex junctions were found in 3’RR-deficient mice. Junctions with microhomologies 1–8 bp long represented 45.7% (16/35) of junctions (Fig. 2 C and Table S1). Complex junctions represented 31.4% (11/35) of junctions. Direct Sµ-σδ joining was slightly increased in 3’RR-deficient mice (7/35, 20%) as compared with wt mice (2/21, 9.2%). To compare µδCSR junctions cloned from MLN cells from wt mice with cCSR junctions, splenocytes from wt mice were stimulated with LPS or LPS + IL4 to generate Cµ-Cγ1, and Cµ-Cγ1 cCSR, respectively (Vincent-Fabert et al., 2009). Sequences of Sµ-Cγ1 and Sµ-Cγ1 junctions resulting from Sµ to Sγ and Sγ cCSR were reported in Fig. 2 C and Table S1. Features of Sµ-Sµ cCSR junctions (i.e., relative frequencies of direct joints, microhomologies, insertions, and complex junctions) were similar to those classically reported (Stavnezer et al., 2010). Sequences from Sµ-σδ junctions of wt mice showed strikingly different features in that they more rarely exhibited a direct joining (2/21, 9.5%).
than either S<sub>µ</sub>-Sγ<sub>1</sub> (12/46, 26.1%) or S<sub>κ</sub>-Sγ<sub>3</sub> (11/70, 15.9%) junctional sequences. Apart from that, the location of S<sub>κ</sub> breaks was similar in C<sub>µ</sub>-Cδ<sub>κ</sub>, C<sub>µ</sub>-Cγ<sub>1</sub>, and C<sub>µ</sub>-Cγ<sub>3</sub> junctions (Fig. 2 D). Finally, the cloned Sα<sub>κ</sub> breaks were mostly located in the 3′ part of the Sα<sub>κ</sub> region similar to breaks in the downstream part of Sγ<sub>1</sub> and Sγ<sub>3</sub> for cCSR junctions (Fig. 2 D). The increased representation of DNA breaks in the 5′ part of S<sub>κ</sub> and the 3′ part of Sα<sub>κ</sub> might involve a methodological bias whereby PCR cloning of the shortest recombined DNA segment would be favored.

**Identical phenotype of IgD<sup>-</sup>IgM<sup>-</sup> B cells in 3′RR-deficient and wt mice**

Flow cytometry analysis indicated that IgD<sup>-</sup>IgM<sup>-</sup> B cells accounted for only ~0.2% of total MLN cells in wt mice (Fig. 3 A). Consistent with published human data (Chen et al., 2009), a low percentage of IgD<sup>-</sup>IgM<sup>-</sup> B cells expressed CD138 (syndecan-1), a hallmark of mature plasma cells (Fig. 3 B and C). Furthermore, the vast majority of IgD<sup>-</sup>IgM<sup>-</sup> B cells did not express surface B220, CD11b, CD19, CD21, and CD23 markers which are usually down-regulated by differentiated plasma cells (Fig. 3, B and C). IgD<sup>-</sup>IgM<sup>-</sup> B cells also lacked PNA and GL7 staining and thus differed from germinal center (GC) B cells (Fig. 3 C). In contrast to human IgD<sup>-</sup>IgM<sup>-</sup> B cells (Arpin et al., 1998; Chen et al., 2009), no bias for surface λ-light chain was found (not depicted). Similarly to human IgD<sup>-</sup>IgM<sup>-</sup> B cells (Chen et al., 2009), mouse IgD<sup>-</sup>IgM<sup>-</sup> B cells expressed CD5, an activation-induced B cell molecule (Fig. 3, B and C), and had a low proliferation rate (Ki67 index of ~50%; Fig. 3 C). IgD<sup>-</sup>IgM<sup>-</sup> B cells were not detected in splenocytes, bone marrow B cells, and circulating B cells of wt mice (unpublished data). Flow cytometry analysis indicated the presence of IgD<sup>-</sup>IgM<sup>-</sup> B cells in MLNs of 3′RR-deficient mice and that they accounted for ~2% of total MLN cells (Fig. 3 A), i.e., a 10-fold increase as compared with wt mice. Increased IgD switching was also confirmed after Southern blotting of S<sub>µ</sub>-Sγ<sub>1</sub> junctions (Fig. 2 B). Besides their increased abundance, IgD<sup>-</sup>IgM<sup>-</sup> B cells from 3′RR-deficient animals qualitatively differed from those in wt mice by showing a lower expression of the B cell surface markers B220, CD19, CD21, and CD23 (Fig. 3, B and C), although
they overwhelmingly (80%) expressed CD5 and poorly proliferated (<40% were Ki67⁺) as for wt IgD⁺IgM⁻ cells. Due to their low levels in MLNs, IgD⁺IgM⁻ B cells did not show up in wt mice using immunohistochemistry but were detected in 3'RR-deficient mice. In agreement with their PNA⁻ and GL7⁻ status, IgD⁺IgM⁻ cells were located in the marginal zone of GC (Fig. 4 A) and had a typical ovaloid-elongated plasma cell shape, medium to large size, and an abundant cytoplasm containing C₅ IgH chains (Fig. 4 A). In contrast to humans (Chen et al., 2009), and confirming results with wt mice, we did not find any bias in the κ⁺/λ⁺ ratio when specifically studying those cells that express IgD in the absence of IgM expression (Fig. 4 B). Similarly to wt mice, such cells were not detected in blood, bone marrow, spleen, and Peyer's patches of 3'RR-deficient mice (unpublished data). A large fraction of B cells from the anti-mesenteric wall of the mouse small intestine displays an IgD⁺IgM⁻ phenotype (Hamada et al., 2002). We confirmed the presence of IgD⁺ B cells by immunostaining all along the mouse small intestine but, again, more noticeably in 3'RR-deficient mice than in wt mice (Fig. 4 C).

μCSR has numerous characteristics of cCSR

To ensure whether the IgD⁺ phenotype is linked to μCSR, PCR analysis of μCSR in sorted IgD⁺IgM⁻ cells (from three mice) was performed in 3'RR-deficient mice (where these cells are more abundant and accessible to cell sorting). After cloning and sequencing we detected 11 different μCSR sequences among 22 analyzed sequences (i.e., 50%). No junctions were found with sorted IgD⁻IgM⁺ cells, reinforcing the hypothesis that the IgD⁺IgM⁻ phenotype is linked to μCSR. Real-time PCR analysis indicated a dramatic decrease (62%, P = 0.005, Mann-Whitney U test) of C₅ transcripts in sorted IgD⁺IgM⁻ cells as compared with sorted IgD⁺IgM⁺ cells, reinforcing the hypothesis that the IgD⁺IgM⁻ phenotype is linked to μCSR.
with other reverse PCR primers (location in Fig. 1) corresponding to Cμ and a region between Cα and Ts. Using these primers, we were unable to detect any µδ junctions (out of 200 independent junk sequences analyzed) in MLN DNA of four 3’RR-deficient mice with detectable µδCSR, demonstrating that the joining between Sμ and Ts represents a specific recombination event. Using a PCR amplification (location of primers in Fig. 1), followed by Southern blotting (location of the probe in Fig. 1), we identified extrachromosomal αδ-Sμ circles in MLN B cells of 3’RR-deficient mice (Fig. 5 B), indicating ongoing µδCSR and a looping-out and deletion mechanism analogous to cCSR (in conditions where ongoing µδCSR was not detectable in MLN DNA from wt mice). The identity of the PCR product was confirmed by sequencing (location of primers in Fig. 1; not depicted). The hallmark of cCSR is its dependence on AID for DSBs formation in S regions. Ligation-mediated PCR (LM-PCR) highlighted the presence of DSBs in αδ in wt mice and 3’RR-deficient mice but not in AID-deficient mice (Fig. 5 C). Using the same experimental strategy as for wt and 3’RR-deficient mice, we were unable to detect any Sμ-αδ junction in genomic DNA from MLNs of AID-deficient mice by Southern blotting of PCR products designed to amplify such junctions (Fig. 5 D). Despite the lack of significant hybridization, we further tried to clone potential junctions from AID-deficient mice (by the same protocol as for wt mice) and failed to identify any (out of 288 independent junk sequences analyzed) indicating that µδCSR in mice requires AID as previously suggested by analyses in human AID-deficient patients (Arpin et al., 1998; Chen et al., 2009). Finally, an Lμ-Cα transcript was previously identified in human (Chen et al., 2009). Real-time PCR analysis confirmed its presence not only in IgD+IgM+ (where it can originate from alternate splicing of a pre-mRNA encompassing Cμ and Cα exons) but also in IgD+IgM− where it is the reflect of the post-IgD CSR transcription (Fig. 5 E).

**Mutations in IgD+IgM− cells**

Interactions with cognate antigens recruit activated B cells into GC and can induce AID-mediated modifications. These include SHM in V(DJ) exons for the generation of high-affinity antibodies and cCSR. (Henderson and Calame, 1998; Durandy, 2003; Hackney et al., 2009). cCSR and SHM are linked to transcription, dependent from some common actors, and both blocked by AID deficiency. SHM can also be considered as the usual mark for antigen-experienced B cells having participated to a GC reaction (Chan and Brink, 2012). We purified IgD+IgM− cells from 3’RR-deficient mice having participated to a GC reaction (Chan and Brink, 2012). We purified IgD+IgM− cells from 3’RR-deficient mice (where these cells are more abundant and accessible to cell sorting) to evaluate Vκκ SHM and identify them as pre- or post-GC B cells. To stimulate SHM, mice were immunized both orally with sheep red blood cells and intraperitoneally with LPS (i.e., in conditions known to yield in vivo activated spleen and Peyer’s patches B cells with Vκκ SHM both in wt and 3’RR-deficient mice; Pérón et al., 2012; Rouaud et al., 2013). Rearranged IgH VDJ and Vκκ regions from MLN IgD+IgM− cells of 3’RR-deficient mice were cloned and sequenced. No SHM

(Fig. 5 A). During CSR, there are abundant double-strand breaks (DSBs) in Sμ. A fraction of these DSBs may be joined to rare DSBs in other parts of the genome, especially within flanking regions on the same chromosome. To demonstrate a specific association of αδ with µδCSR, PCR analysis was performed...
could be documented out of 90 VDJ sequences analyzed (3 mutations among 68,582 bp analyzed, 0.0044%) and 58 κ sequences analyzed (7 mutations among 36,134 bp analyzed, 0.0194%). Although the absence of SHM in VDJ rearranged region was expected in 3′RR-deficient mice recently demonstrated with and IgH locus-specific SHM defect (Rouaud et al., 2013), the lack of SHM in the κ light chain locus clearly indicate that those B cells with μδCSR have not undergone conventional antigen-driven maturation as usually occurring into GC. By comparison, 3′RR-deficient animals recently analyzed after the same immunization protocol showed a >70-fold higher (1.39%) SHM rate in VκJκ segment from Peyer’s patches GC B cells (Rouaud et al., 2013). The lack of mutation in VDJ segments fitted well with their PNA-GL7− status and their localization in the marginal zone of GC. Contrasting with mice, human IgD−IgM− had extensive SHM in their IgV genes that indicated their clonal relatedness (Arpin et al., 1998). Mouse IgD−IgM− had no SHM in their IgV genes and the use of a JH1 probe on Southern blots from IgD−IgM− cell DNA revealed no rearranged bands in addition to the germ-line band indicating no potential clonal relatedness (unpublished data). We finally analyzed mutations during μδCSR in wt mice as compared with cCSR to find if intensity of AID attack is similar between them. The mutation frequency at Sμ breakpoints and 5′ to Sκ breakpoints were lower in μδCSR than in IgG1 and IgG3 cCSR (although the latter control was chosen using in vitro conditions poorly inducing SHM; Fig. 6, A and B). A decrease of 25 and 52% in the frequency of mutations in the Sμ donor region was found during μδCSR as compared with IgG1 and IgG3 cCSR, respectively. The numbers of mutations at Sκ breakpoints and 3′ to Sκ breakpoints were also much lower in target Sγ region of cCSR (a decrease of 89 and 86% of those found in Sγ1 and Sγ2, respectively; Fig. 6, A and B). Thus, not only the Sμ donor region but also the Sκ acceptor region implicated in μδCSR exhibited lower AID-induced mutations than Sμ, Sγ1, and Sγ3 during IgG1 and IgG3 cCSR. Interestingly, the pattern of mutations at Sμ–Sκ junctions, 5′ to Sμ, and 3′ to Sκ was similar in wt mice and 3′RR-deficient animals (Fig. 6 B), showing that the absence of the 3′RR did not affect the AID-targeting at the Sμ donor and Sκ acceptor regions during μδCSR.

**DISCUSSION**

cCSR is a very complex process during which accessibility to recombination is regulated by both cis-acting factors (such as germline I promoters and 3′RR enhancers) and trans-acting factors (most of which are induced by cytokine-dependent signals and/or B cell activation; Stavnezer et al., 2010; Boboila et al., 2012). Analysis of DNA sequence at or around the recombination sites has helped us to understand the mechanisms first generating DSBs after targeting of S regions by AID and then allowing synopsis and repair of chromosomal breaks mostly through a classical nonhomologous end joining (C-NHEJ) pathway or to a lower extend through the alternative end-joining (A-EJ) pathway (Yan et al., 2007; Boboila et al., 2012). Although various disruptions of the 3′RR partially compromised cCSR (Cogné et al., 1994; Manis et al., 1998; Pinaud et al., 2001; Vincent-Fabert et al., 2009; Bébin et al., 2010), its complete...
deletion has clarified its mandatory role for cCSR toward IgG, IgA, and IgE isotypes (Vincent-Fabert et al., 2010b) as well as for SHM in rearranged VDJ exons (Rouaud et al., 2013). Phylogenetic studies indicate that IgD is as ancient as IgM but, in contrast to IgM, IgD is evolutionarily labile showing, for instance, many duplications/deletions of domains and splice forms (Ohta and Flajnik, 2006; Rauta et al., 2012). Due to the lack of an authentic Sδ region, B cells exclusively expressing IgD are extremely rare in humans and almost absent in mice (Owens et al., 1991). Our study clearly shows that despite the lack of canonical Sδ region, CSR from μ to δ occurs in MLNs from wt mice. Sequences of μδCSR junctions reveal a lower percentage of direct end junctions in μδCSR than in cCSR together with a higher percentage of junctions with either microhomologies between recombined ends or showing insertions of various lengths at the recombination site. Notably, this pattern is strongly reminiscent of the junctions affected by the A-EJ pathway and previously reported during cCSR in B cells lacking key components of the C-NHEJ pathway: its main characteristic being a dramatic reduction of CSR direct joints and frequent junctions with microhomologies and insertions (Yan et al., 2007). Although the A-EJ pathway has only recently been shown to contribute to cCSR, it has been shown that in the absence of C-NHEJ, cCSR can be performed by A-EJ at up to 50% of wt levels (Yan et al., 2007). Notably, the same A-EJ-based pattern of junctions is also observed when referring to a few mouse IgD-secreting hybridomas documented in the past (Owens et al., 1991).

Similarly to cCSR, the absence of μδCSR in AID-deficient mice confirms it also requires AID. The hallmark of CSR is its dependence on AID for DSBs formation in S regions. LM-PCR experiments found the presence of DSBs in Sδ in wt mice and 3′RR-deficient mice but not in AID-deficient mice. μδCSR is also AID-dependent in human (Chen et al., 2009). However, this does not imply that all IgD-producing plasma cells have undergone μδCSR. Indeed, serum IgD persists and is even augmented in some AID-deficient patients, probably as a result of the activation of an alternative pathway involving the emergence of plasma cells that produce both IgM and IgD via alternative splicing, particularly at mucosal sites (Chen et al., 2009). Our data indicated that during μδCSR the Sδ and (still more clearly) the Sα acceptor region are less actively targeted by AID than Sμ, Sγ1, and Sγ3 during IgG1 and IgG3 cCSR. This suggests, aside from differences in junction diversity, that μδCSR is regulated differently from cCSR. Individual S regions range in size from 10 kb for Sγ1 (IgG1 being the most abundant Ig class) to 2.5 kb for Sδ and to only 1 kb for Sα. Studies pointed the S region size as an important factor in determining endogenous cCSR efficiency because cCSR frequencies correlated with S region length (Zarrin et al., 2005). The very low level of μδCSR in wt mice may be linked to the short length (0.5 kb) of the Sδ region and to a less active targeting by AID than Sγ1 and Sγ3 during cCSR.

An intronic (I) exon is positioned upstream of each Cδ gene except Cδ both in mice and humans. Iδ-Cδ1 germinal transcripts render Sδ regions substrate for DNA modifications by the CSR machinery, including AID. As previously identified in humans (Chen et al., 2009), an Iδ-Cδ3 transcript is identified in mice. This Iδ-Cδ3 transcript is of importance to render both Sδ and Sα regions substrate for DNA modifications, especially because no Iδ exon is documented in the mouse and the human IgH locus. In contrast to mice and humans, an Iδ exon has been recently reported in the bovine IgH locus (Xu et al., 2012). This Iδ exon derives from the recent duplication of an Iδ-Cδ1 genomic fragment (Zhao et al., 2002). Of interest, the bovine δ gene can be expressed by...
CSR; analysis of $S_\mu$-$S_\delta$ junctions demonstrated, as in mice, a preference for using the microhomology-based end-joining pathway (Xu et al., 2012). After $\mu$CSR, $I_\mu$-$C_\delta$ transcripts are still detected and are the reflection of the post-IgD CSR transcription and not simply a germinal transcript of importance to render both $S_\mu$ and $\sigma_\delta$ regions substrate to CSR-induced DNA modifications.

The 3′RR is mandatory for CSR toward IgG, IgA, and IgE isotypes (Vincent-Fabert et al., 2010b). This is obviously not the case for IgD. Patients with hyper-IgM syndromes have higher $\mu$CSR that may stand as a compensatory response due to the absence of other switched isotypes (Chen and Cerutti, 2010). It appears that deletion of the 3′RR, most probably through a compensatory response due to the absence of other switched isotypes, enhances IgD CSR without widely impacting the patterns of $S_\mu$-$\sigma_\delta$ junctions. Thus, AID-induced $\mu$CSR occurs independently of the IgH 3′RR, a result that markedly contrasts with $S_\mu$-$S_\delta$ and $S_\mu$-$\sigma_\delta$ cCSR. The lower AID-induced mutations both in $S_\mu$ and $\sigma_\delta$ region during $\mu$CSR (as compared with those in $S_\mu$, $S_\delta$, and $S_\delta$ during $S_\mu$-$S_\delta$ and $S_\mu$-$\sigma_\delta$ cCSR) suggest that AID might be recruited to the IgH locus by different means than CSR. This result partially fits with recent data showing, in addition to cCSR, that the 3′RR is also a key element for AID recruitment during AID-induced SHM both in VDJ-expressed sequences and $S_\mu$ (Rouaud et al., 2013). The analysis of SHM in VDJ expressed sequences of 3′RR-deficient B cells that have undergone $\mu$CSR confirms that the 3′RR is essential to the SHM process on V(D)J exons and that a physiological dissociation exists between SHM and CSR in primary B cells of 3′RR-deficient mice as in T cell–independent responses (Phan et al., 2005).

CSR to IgD is obviously a rare event and we now show that it occurs independently of the IgH 3′RR. It might be of interest to notice that in this special case, and in contrast to SHM of VDJ and of $S_\mu$ regions during cCSR, the rate of mutations affecting the target regions of $\mu$CSR is not affected by the 3′RR deletion. This observation, together with the unique location of IgD switching only in the mucosa-associated lymphoid tissue, argues for a specific regulation of this event, allowing AID recruitment to the IgH locus in a 3′RR-independent manner and in response to stimuli that remains to be determined. Transcription being required for AID targeting, the simultaneous accessibility of $S_\mu$ and $\sigma_\delta$ to AID likely needs constitutive $I_\mu$-$C_\delta$ transcription (encompassing both $S_\mu$ to $\sigma_\delta$). However, because $I_\mu$-$C_\delta$ transcription is common in naïve B cells and during their initial entry into GC, it is obviously not sufficient by itself for inducing accessibility to $\mu$CSR and another stimulus uniquely occurring into mouse MLNs and into human tonsils likely allows the 3′RR-independent recruitment of AID on the IgH locus in some B cells. Until now, our knowledge concerning $\mu$CSR in mice only derived from studies of myeloma and hybridoma cell lines (Owens et al., 1991; Preud'homme et al., 2000). 3′RR-deficient mice give us the first opportunity to investigate it in primary B cells.

Location of IgD$^+$IgM$^-$ cells in the marginal zone, but not in the follicular zone, together with their PNA$^-$$GL7^-$ status and their lack of SHM in the VDJ segment might be suggestive of T cell–independent non-GC origin for cells with $\mu$CSR, as previously documented in physiology for some mucosa-associated lymphoid tissue B cells undergoing T cell–independent IgA CSR (Bergqvist et al., 2010). In addition, cCSR in the absence of SHM was reported in some cases of chronic lymphocytic leukemia B cells (Oppezzo et al., 2003) upon N-terminal mutation of AID (Shinkura et al., 2004) and, recently, in $Ung^{-/-}$ mice during an acute antigenic challenge (Zahn et al., 2013).

Our data with $\mu$CSR further indicates that AID can be recruited to the IgH locus under various different forms, eventually associated with some different partners to mediate either cCSR and VDJ SHM (both processes requiring the 3′RR), or in the present case $\mu$CSR (where the 3′RR is at least superfluous; Ronai et al., 2007). According to the concept that such IgD–only cells may locally participate in immune surveillance against pathogens present at mucosal sites, their increase in 3′RR-deficient mice with an IgA production defect might be a compensatory mechanism as previously suggested in IgA-deficient patients (Brandtzaeg and Johansen, 2005).

IgD$^+$IgM$^-$ B cells in mice exhibited numerous similarities with human ones. Consistent with published human data (Chen et al., 2009), they were CD138$^+$, expressed the activation-induced (or B1 compartment-related) molecule CD5, and down-regulated several B cell–specific markers usually down-regulated by differentiated plasma cells. Whether human IgD$^+$IgM$^-$ cells show a strong bias toward the preferential expression of the $\lambda$-light chains (Arpin et al., 1998; Chen et al., 2009), it is not the case in mice, a result which might be linked to the $\kappa^{+}/\lambda^{+}$ ratio, globally reported in mouse B cells around 95/5 (Arakawa et al., 1996). Whether human IgD$^+$IgM$^-$ cells are reported to have undergone extensive SHM and display clonal relatedness (Arpin et al., 1998), mice IgD$^+$IgM$^-$ cells are unmaturated in their VDJ regions without any evident sign of clonality. These latter results are in accordance with their localization in the marginal zone of GC, a region with cells not submitted to an intensive SHM process or exhibiting a high rate of proliferation (mice IgD$^+$IgM$^-$ cells had a low Ki67 index).

In conclusion, IgD CSR occurs in B cells of MLNs of wt mice. There is extensive somatic mutation of the IgH region in human IgD$^+$ cells (Arpin et al., 1998) but not in mouse. The effects of physiological location of switching cells might explain this difference as well as the structural differences in mouse and human 3′RR. The molecular features of $S_\mu$-$\sigma_\delta$ junctions suggest a predominant involvement of the A-EJ pathway in this process. In contrast to IgG, IgA, and IgE classes, IgD CSR occurs independently of the IgH 3′RR and now stands as an amazing exception contrasting with all the other AID-dependent modifications of the IgH locus previously shown to formally involve the 3′RR in mouse B cells.

MATERIALS AND METHODS

**Mice.** Generation of 3′RR-deficient mice has been previously reported (Vincent-Fabert et al., 2010b). wt mice and 3′RR-deficient mice were 8–12 wk old except when specified. Mice were bred and maintained under specific pathogen-free conditions. Mice immunizations were done orally with sheep red blood cells for 2 wk and intraperitoneally with 10 μg LPS for 3 d.
Cell cytometry analysis. Single-cell suspensions from MLNs were fixed and permeabilized with the Intraprep permeabilization reagent (Beckman Coulter) before incubation with FITC-labeled IgD (SouthernBioTech) or irrelevant antibody and analyzed on a Fortessa LS R2 (BD; Truffinet et al., 2007; Vincent-Fabert et al., 2009). Phenotyping of IgD+IgM+ cells were made with the following antibodies: v450-IgD, APC-IgM, FITC-B220, FITC-CD5, PE-CD11b, PE-CD19, PC7-CD23, PE-CD138, PE-GL7 and FITC-PNA, and PE-Fas and FITC-Ki67.

Dot plot analysis. Dot plot analysis of the mouse DNA fragment encompassing the 5' Ex a intronic enhancer and the Cε exon 2 was done with the Nucleic Acid Dot Plot Program. Window size and mismatch limit were set to 23 and 3, respectively.

Amplification, cloning, and sequencing of S_{μ}-CSR junctions. S_{μ}-CSR junctions were studied using a touchdown PCR, followed by a nested PCR on fresh MLN cells using the following parameters. Touchdown PCR: primer 1, 5'-CAGTTGAGGCGACAGGCT-3' and primer 2, 5'-CCATTACATTACAGCCCCGGG-3' (1 cycle, 98°C for 5 min; 3 cycles, 98°C for 30 s, 60°C for 45 s, and 72°C for 90 s; 3 cycles, 98°C for 40 s, 56°C for 90 s, 62°C for 40 s, and 72°C for 90 s; 25 cycles, 98°C for 30 s, 60°C for 40 s, and 72°C for 90 s; and 1 cycle, 72°C for 7 min). Nested PCR: primer 3, 5'-GGAGTTGGCTGATGACT-3' and primer 19, 5'-TTGG-3'; and primer 4, 5'-CAACAGCCCAGGT-3' (located in-between primer 5 (5'-CAGCCCAGGTTTATCTTTTCA-3') and primer 19, 5'-TTGG-3').

CSR circles were studied using a touchdown PCR, followed by a nested PCR on S_{μ}-CSR junctions. The Cε CSR circles were amplified by PCR using the following parameters: primer 6, 5'-GCGAAGCTTARGCCTGGGRCTTCAGTGAAG-3' and primer 7, 5'-AGGACTGTTCACTCTATCTTTACCAC-3' (1 cycle, 98°C for 3 min; 30 cycles, 94°C for 30 s, 55°C 72°C for 30 s, and 72°C for 90 s), and 1 cycle at 72°C for 10 min. PCR products were cloned into the Zero Blunt TOP PCR cloning (Invitrogen) and sequenced with an ABI-PRISM sequencer (Perkin-Elmer; Fiancette et al., 2011). The circle PCR products were cloned into the Zero Blunt Topo PCR cloning (Invitrogen). Plasmids were isolated using the NucleoSpin kit (Macherey-Nagel Eurl) and sequenced using an automated laser sequencer (ABI-PRISM sequencer; Perkin-Elmer; Fiancette et al., 2011).

Amplification, Southern blotting, and sequencing of S_{δ}-CSR junctions. μCSR circles were amplified by PCR using the following parameters: primer 6, 5'-GGACTCTGTCACCAAAATTTCA-3' (located at the beginning of ηδ) and primer 7, 5'-GAAGACATTITGGGAAAGGCTGACT-3' (located in Cε exon 1: 1 cycle; 98°C for 25 min; 40 cycles, 94°C for 15 s, 55°C for 30 s, and 72°C for 120 s; and 1 cycle, 72°C for 7 min). Hybridization of μCSR circles was performed with a 240 bp probe (probe B) located in the Cε exon 1 (Péron et al., 2012). S_{δ}-CSR circles were cloned by a nested PCR fragment according to the following parameters: primer 8, 5'-CTCTGGCCCTGCTTATTGTTG-3' and primer 9, 5'-TTATCGATGAGGACCAGAGG-3' (1 cycle, 94°C for 3 min; 30 cycles, 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 1 cycle, 72°C for 7 min) of the JH4 segment using 1 cycle (98°C for 30 s), 33 cycles (98°C for 10 s, 66°C for 30 s and 72°C for 90 s), and 1 cycle (72°C for 10 min). PCR products were cloned and sequenced as described (Fiancette et al., 2011). Real-time PCR analysis of membrane C_{μ} and I_{μ}-C_{μ} transcripts. Total RNA was prepared from freshly isolated IgD+IgM+ and IgD+IgM- MLN B cells. Reverse transcription was performed with 500 ng of total RNA. RT-PCR experiments were done with SuperScript III reverse transcription (Invitrogen). Real-time PCR analysis (on an ABI Prism 7000 system; Applied Biosystems) was made with the Syber Green method using 10 ng cDNA using the following primer: I_{μ} primer 14, 5'-GCTCCAGCTGATTTTCACTG-3' and C_{μ} primer 15, 5'-GCTCCAGCTGATTTTCACTG-3' (μ membrane primer 16 (in exon μ 4), 5'-TGAANCTCCGAAGAGCTA-3' and μ membrane primer 17 (in exon μ 6 membrane), 5'-TTCCTCTCCATCCTACCTC-3'). GAPDH was used for normalization of gene expression levels (TaqMan reference Mm99999915-g1).

Spleen cells for CSR. Single-cell suspensions of spleen cells were cultured 3 days at 10^6 cells/ml in RPMI 1640 with 10% FCS and 20 µg/ml LPS, with or without addition of 20 ng/ml IL-4 (PeproTech). 3-day in vitro-stimulated splenocytes were harvested and DNA was extracted for investigation of S_{μ}-CSR junctions. (Vincent-Fabert et al., 2009).

LM-PCR. Genomic DNA from ss mice, 3′RR-deficient mice, and AID-deficient mice was extracted and purified using Phase Lock gel. 10 µg was treated with T4 DNA polymerase (New England Biolabs, Inc.), which trims back 3′ overhangs while filling in 3′ overhangs, thereby yielding blunt 5′-phosphorylated DNA ends. 1 µg DNA was then ligated with T4 DNA ligase (New England Biolabs, Inc.) in a 20-µl reaction volume with the double-strand anchor linker BW (Zan and Casali, 2008). DSBs were detected by semi-nested LM-PCR using BW1 primer targeting the BW linker sequence (5′-CGGGTGACC GGAGATCTGAATTC-3') and primer 2, specific to 3′ ss DNA (5′-CCCATTTAA GAGCAGAGCTTAC-3'), using the following parameters: 1 cycle, 94°C for 30 s; 25 cycles, 94°C for 10 s, 55°C for 20 s, and 65°C for 2 min; and 1 cycle, 65°C for 7 min. DSBS were studied after hybridization with the same 32P-labeled probe as for Southern blotting of C_{μ}-CSR junctions.

Clonality assay. Genomic DNA prepared from IgD+IgM+ cells of 3′RR-deficient mice was digested with EcoRI and analyzed by Southern blot with a 32P-labeled JH probe (Truffinet et al., 2007).

Online supplemental material. Table S1 shows S_{μ}-CSR, S_{δ}-CSR, and S_{ε}-CSR junctions. This paper is dedicated to Michael S. Neuberger in memoriam. We thank S. Desforges and B. Remberard for help with animal care.
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