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AID and APOBEC3 Involvements in Non-Conventional IgD Class Switch Recombination in Mice

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In mature B-cells, class switch recombination (CSR) substitutes the constant (C) μ gene with other C genes (such as γ, ε and α) thereby generating IgG, IgE and IgA antibodies with new effector functions but same the antigenic specificity compared to IgM. CSR is a complex process with I-promoter transcription, targeting of the DNA-editing enzyme activation-induced deaminase (AID) to specific DNA switch (S) regions preceding C regions (except C_δ), generation of double strand breaks and recruitment of DNA repair factors for the end-joining process. The classical non-homogenous end joining (c-NHEJ) pathway ligates DNA ends with little or no homology while the alternative end joining (A-EJ) pathway ligates DNA ends with microhomology [1]. Several *cis*-transcriptional enhancers located along the IgH locus have been implicated (near or far) in CSR control. Deletion of the 3' regulatory region (3'RR) (located 3' to the C_α gene) markedly reduced conventional (γ, ε and α) CSR [2]. Deletion of the 5'E_μ enhancer (located upstream of C_μ) has, at most, a modest effect on synthesis of switched Ig and no really evident role on CSR [3]. Deletion of the 3'γ1E element (located between $C_{\gamma1}$ and $C_{\gamma2b}$ genes) restricts CSR to IgG_3, IgG_{2a} and IgG_{2b} illustrating its role as an isotype specific transcriptional enhancer of CSR [4]. IgD CSR is a rare and enigmatic CSR restricted to a few B-cell subsets in specific lymphoid tissues (such as mucosa-associated tissue, mesenteric lymph nodes and the peritoneal cavity) [5,6]. A link between IgD CSR and microbiota has been reported demonstrating a role for IgD in the homeostatic regulation of the microbial community [7]. The mechanistic regulation of IgD CSR remains elusive. Transcription is a prerequisite for conventional CSR and deletion of several IgH *cis*-enhancers are reported to affect it. AID targeting on S regions is a prerequisite for conventional CSR and AID deletion abolishes it. We investigated the role of IgH *cis*-enhancers and members of the AID/APOBEC family during IgD CSR.

E_μ-deficient [3], 3'Eγ1-deficient [4], APOBEC3-deficient [8], AID-deficient (Janvier Labs, France), *wt* 129/SV *and wt* C57BL6 mice were housed and procedures were conducted in agreement (APAFIS-13855) with European Directive 2010/63/EU on animals used for scientific purposes. Peritoneal cavity B-cells were used. Mice were pristane-treated (1ml *i.p.*) for 2 weeks to induce inflammation before recovery of peritoneal cavity cells. B-cells were purified using EasySep™ mouse B-cell isolation Kit (STEMCELL Technologies). DNA was extracted by phenol/chloroform. S_μ-σ_δ (σ for S-like) junctions were amplified by nested PCR with 100 ng DNA (Phusion High-Fidelity PCR Master Mix with HF Buffer) as previously reported [9]. NGS Barcoded libraries with 200-bp read lengths were prepared using Ion Xpress plus Fragment Library Kit (Thermo Fisher Scientific) according to manufacturer's instructions. 100 pM of each barcoded library were run on the Ion Proton sequencer (Life Technologies) located in the "GénoLim platform" of the Limoges University (France). Data analysis was performed using the computational tool for automatic analysis of CSR junctions sequenced by high-throughput sequencing (CSReport) [10]. Sequenced reads were mapped to S_μ and σ_δ regions using a BLAST algorithm. The computational tool developed for experiments performs junction assembly,

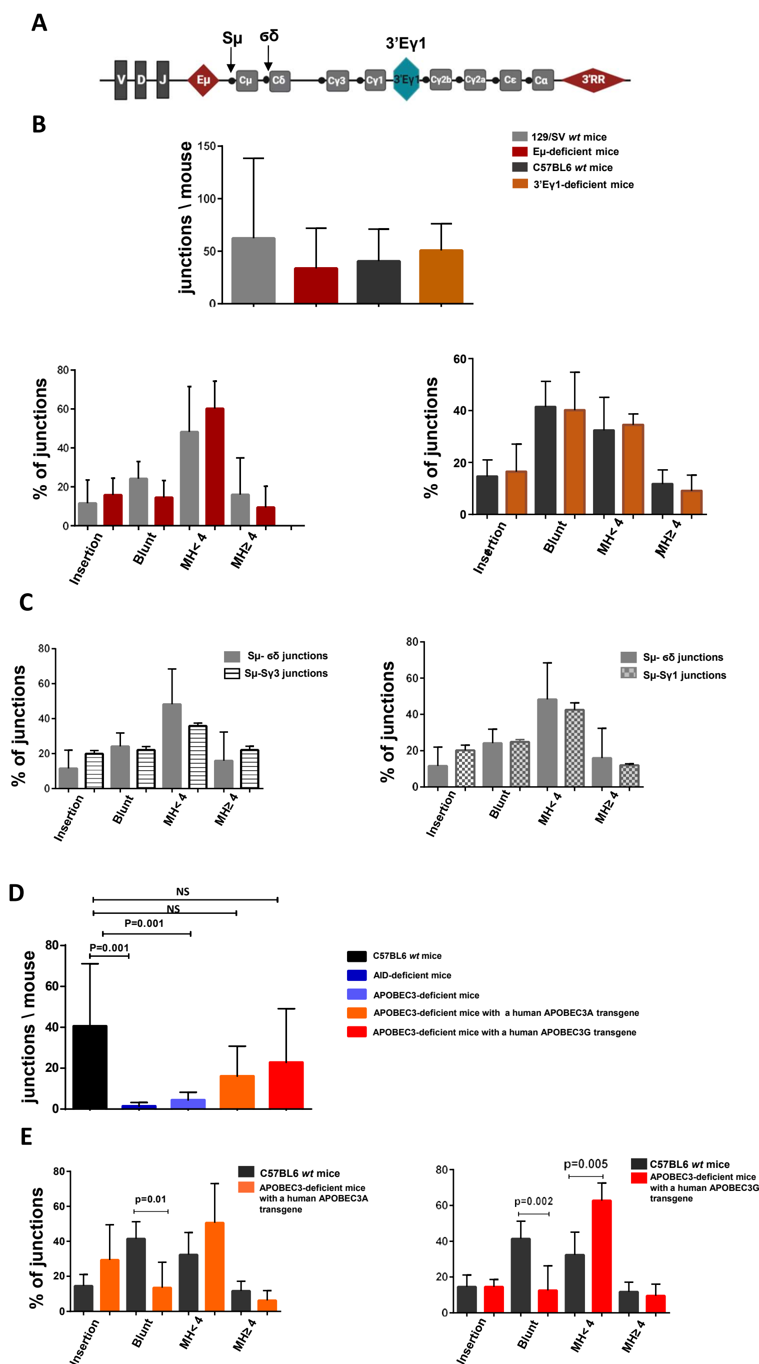

Figure 1: IgD junctions in various deficient mice. **A:** Representation (not to scale) of the IgH locus. Locations of E_μ, $3'E\gamma_1$ and 3'RR elements are indicated. **B:** Number of S_μ-σ_δ junctions in E_μ-deficient, $3'E\gamma_1$-deficient, *wt* 129/SV and *wt* C57BL6 mice. E_μ-deficient mice: 303 junctions, 8 mice; $3'E\gamma_1$-deficient mice: 203 junctions, 4 mice; *wt* 129/SV mice: 374 junctions, 6 mice; *wt* C57BL6 mice: 203 junctions, 5 mice. Junctions are classified as blunt, with insertions, with micro-homologies (<4bp) or with large-homologies (≥ 4bp). No significant difference (Mann-Whitney *U*-test) compared to *wt* mice. **C:** Structure profiles for S_μ-$S_{\gamma3}$, S_μ-$S_{\gamma1}$ and S_μ-σ_δ junctions in *wt* 129/SV mice: 12306 γ_1 junctions (6 mice), 5942 γ_3 junctions (6 mice) and 374 δ junctions (6 mice). No significant difference (Mann-Whitney *U*-test). **D:** number of S_μ-σ_δ junctions in *wt* C57BL6 mice, AID-deficient mice, APOBEC3-deficient mice, APOBEC3-deficient mice with a human APOBEC3A transgene and APOBEC3-deficient mice with a human APOBEC3G transgene. S_μ-σ_δ junctions in *wt* C57BL6 mice: 203 junctions, 5 mice; AID-deficient mice: 10 junctions, 7 mice; APOBEC3-deficient mice: 44 junctions, 10 mice; APOBEC3-deficient mice with a human APOBEC3A transgene: 209 junctions, 13 mice; APOBEC3-deficient mice with a human APOBEC3G transgene: 297 junctions, 13 mice. Significance with the Mann-Whitney *U*-test. **E:** Structure profiles for S_μ-σ_δ junctions in *wt* C57BL6 mice and APOBEC3-deficient mice with an APOBEC3A or APOBEC3G transgene. Same junctions and mice as in D. Significance with the Mann-Whitney *U*-test.

identifies breakpoints in S_μ and σ_δ, identifies junction structure (blunt, micro-homology, large-homology or junction with insertions) and outputs a statistical summarization of identified junctions.

The IgH locations of S_μ, σ_δ, C_μ, C_δ, 5'E_μ, 3'Eγ_1 and 3'RR are depicted in Figure 1A. If transcription is required for CSR, we previously reported that deletion of the 3'RR master control element of CSR had no effect on IgD CSR [6]. Since 3'RR is far from σ_δ-C_δ we investigated the impact of deletion of the two other nearby *cis*-transcriptional elements 5'E_μ (mice with a 129 background) and 3'Eγ1 (mice with a C57BL6 background). IgD CSR was previously reported in 129/SV and C57BL6 *wt* mice [6,7]. Data indicated the presence of IgD CSR in 5'E_μ- and 3'Eγ1-deficient mice (Figure 1B). Analysis of S_μ-σ_δ junctions indicated that their structural profile (blunt, micro-homology, large-homology or junction with insertions) (Figure 1B) was not significantly different from IgD junctions in 129/SV and C57BL6 *wt* mice. Thus, deletion of the 3 major *cis*-transcriptional enhancers of the IgH locus did not affect IgD CSR suggesting that *cis*-enhancer-induced transcription is not required for this non-conventional CSR. Comparison of IgD junctions with IgG_3 junctions (obtained after LPS stimulation) or IgG_1 junctions (obtained after LPS+IL4 stimulation) revealed no significant differences in their structural profile (Figure 1C) suggesting a similar end-joining process for δ, γ_3 and γ_1 CSR and that mechanistic differences between IgD CSR and conventional CSR occurred in the first step of the CSR process. AID is essential for conventional CSR and colocation of breakpoints in S_μ with AID hot spot motifs was found in IgD junctions [9]. Confirming the involvement of AID in IgD CSR, no significant numbers of S_μ-σ_δ junctions were found in AID-deficient mice (Figure 1D). Analysis of breakpoints in σ_δ collocated with motifs mostly targeted by APOBEC3 [9]. Confirming the putative involvement of APOBEC3 in IgD CSR, a significant decrease in the number of S_μ-σ_δ junctions was found in APOBEC3-deficient mice (Figure 1D). Of interest, IgD junction levels increased in APOBEC3-deficient mice expressing a human APOBEC3A or APOBEC3G transgene (a single APOBEC3 gene is present in rodents whereas seven copies are found in humans). The S_μ-σ_δ junctions in human APOBEC3A/G expressing mice were slightly different from those of *wt* control mice with less blunt junctions and more junctions with short microhomologies (Figure 1E).

In mature B-cells the expression of IgM and IgD through alternative splicing of a long mRNA transcript is constitutive indicating that S_μ and σ_δ regions are always transcribed and explaining why no *cis*-enhancer-induced transcription (3'RR, 5'E_μ, 3'E_γ1) is required for IgD CSR. Similarly, for conventional CSR, AID is clearly implicated in IgD CSR, most probably through its constant S_μ targeting. APOBEC3 proteins act in cancer and in gene editing (including Ig ones) [11,12]. When IgD CSR is almost totally abrogated in AID-deficient mice (AID is essential to target S_μ), this was not the case in an APOBEC3-deficient background where a residual level of S_μ-σ_δ junctions was found. An explanation would be that APOBEC3 hot spots in σ_δ are also AID cold spots. Expression of human APOBEC3A/G transgenes (both expressed in human B-cells) in an APOBEC3-deficient background increased levels of S_μ-σ_δ junctions but with a different panel of junctions that could be explained by the fact that mouse APOBEC3 preferentially deaminates TYC motifs (Y=T/C) whereas TC and CCC motifs are preferred by human APOBEC3A and APOBEC3G, respectively [13]. Recent discoveries of IgD in ancient vertebrates and AID/APOBEC-like cytidine deaminases as ancient innate immune mediators in invertebrates suggest that IgD has been preserved throughout evolution [14]. IgD CSR would be an evolving residue allowing the specific production of IgD, which is reactive to commensal microbiota, before expansion of the IgH locus by duplication [15], leading to the emergence of γ, α and ε isotypes and subsequently conventional AID-induced CSR induced via the appearance of conventional GC-rich S regions. Clearly, the role for APOBEC3 in the targeting of the σ_δ region during the IgD CSR deserves more studies. The difficulty to monitor it, due to the rarity of this event which is restricted to a few B-cell subsets in specific mouse lymphoid tissues and currently impossible to reproduce *in vitro*, remains an exciting challenge to be met.

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Disclosure of Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Y.D and J.C.M. designed research. M.F., N.G. and H.I. performed research. M.F., J.C.M. and Y.D. analyzed data. M.F., N.G., H.I., J.C.M., and Y.D. wrote the paper. Y.D. obtained financial grants.

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