Haploinsufficiency of Activation-Induced Deaminase for Antibody Diversification and Chromosome Translocations both In Vitro and In Vivo

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Abstract

The humoral immune response critically relies on the secondary diversification of antibodies. This diversification takes places through somatic remodelling of the antibody genes by two molecular mechanisms, Class Switch Recombination (CSR) and Somatic Hypermutation (SHM). The enzyme Activation Induced Cytidine Deaminase (AID) initiates both SHM and CSR by deaminating cytosine residues on the DNA of immunoglobulin genes. While crucial for immunity, AID-catalysed deamination is also the triggering event for the generation of lymphomagenic chromosome translocations. To address whether restricting the levels of AID expression in vivo contributes to the regulation of its function, we analysed mice harbouring a single copy of the AID gene (AID+/−). AID+/− mice express roughly 50% of normal AID levels, and display a mild hyperplasia, reminiscent of AID deficient mice and humans. Moreover, we found that AID−/− cells have an impaired competence for CSR and SHM, which indicates that AID gene dose is limiting for its physiologic function. We next evaluated the impact of AID reduction in AID+/− mice on the generation of chromosome translocations. Our results show that the frequency of AID-promoted c-myc/IgH translocations is reduced in AID+/− mice, both in vivo and in vitro. Therefore, AID is haploinsufficient for antibody diversification and chromosome translocations. These findings suggest that limiting the physiologic levels of AID expression can be a regulatory mechanism that ensures an optimal balance between immune proficiency and genome integrity.

Introduction

B cells are responsible for generating a repertoire of antibodies of virtually unlimited diversity in order to confront the antigenic universe. Antibody diversification is achieved through somatic remodelling of immunoglobulin (Ig) genes at two different stages of B cell differentiation. The first one is antigen-independent and takes place during B cell generation in the bone marrow through a site-specific recombination named V(D)J recombination, which gives rise to B cells expressing a primary repertoire of low affinity IgM antibodies [reviewed in [1]]. Upon antigen encounter B cells have as yet another chance to further diversify their antibody repertoire in germinal centers by two independent molecular mechanisms called somatic hypermutation (SHM) and class switch recombination (CSR). SHM reshapes the antigen binding site of Ig genes by introducing nucleotide changes in their variable genes. B cells where SHM gives rise to antibodies with higher affinity for their cognate antigen are positively selected, a process referred to as affinity maturation [reviewed in [2] and [3]]. CSR is a region-specific recombination reaction that replaces the primary μ constant (Cμ) region by a downstream constant region (Cγ, Cε or Cα), thereby generating antibodies endowed with new functions for pathogen neutralization while retaining the same antigen specificity. CSR takes place between highly repetitive sequences that precede the Cμ, Cγ, Cε and Cα genes, called switch regions, through the generation of double strand breaks (DSBs), ligation, and concomitant excision of the intervening sequence from the locus [reviewed in [4,5]].

Both SHM and CSR are initiated by the very same enzyme, Activation Induced Cytidine Deaminase (AID) [6]. In humans, mutations in the AID gene are associated with a rare (1/2000000) immunodeficiency called Hyper IgM Syndrome type 2 (HIGM2) [7]. HIGM2 patients display impaired CSR and SHM and are prone to bacterial infections of the respiratory and digestive tracts [7]. AID initiates SHM and CSR by deaminating cytosine residues of the variable and switch regions of the Ig genes, respectively [8,9,10,11,12,13]. Cytosine deamination on DNA converts a cytosine residue to thymine, yielding an abasic site which is repaired by uracil excision repair (UER) [14,15]. The absence of the UER pathway results in the generation of chromosome translocations that involve one of the Ig loci and a tumor suppressor gene, most frequently c-myc [16].

Most of the lymphomas diagnosed in the western world arise from mature B cells and are characterized by the presence of chromosomal translocations that involve one of the Ig loci and a proto-oncogene [17,18]. These translocations are known to play a
role in the etiology of these B cell neoplasias [17,18]. In vivo and in vitro studies have shown that AID can promote the generation of pro-lymphomagenic translocations [19,20,21,22], and that CSR and the translocation reaction are initiated by a common pathway that involves DNA deamination and UNG [20]. The impact of AID function in B cell neoplasia development has been addressed in a number of in vivo models, including IL6 [19,21] and pristane [23] promoted plasmacytomas, BCL-6-induced diffuse large B cell lymphoma [22], Ep-Myc model of B cell lymphoma [24] and a myc-induced multiple myeloma model [25]. In all the cases absence of AID either delayed the onset or shifted the nature of the neoplasia towards a more immature origin, hence reinforcing the idea that AID expression plays a role in the generation of mature B cell lymphomas by promoting DNA lesions.

Therefore, AID function, while crucial to the development of an efficient immune response, can pose a risk to DNA stability in B cells. Different regulatory mechanisms may be responsible to minimize unwanted DNA damage by AID.

First, AID mutagenic activity is mostly limited to the Ig loci (reviewed in [26]), and although AID-induced lesions in other genes have been reported [27,28], these events are rare. Second, AID accessibility to DNA is restrained by fine control of subcellular localization [29,30]. Third, the presence of AID mRNA is mainly restricted to activated mature B cells [31], thus limiting its function to the cell type and time window where it is required. Transcriptional regulation exerted by B cell specific transcription factors and cis elements (reviewed in [32]), as well as microRNA-mediated post-transcriptional regulation [33,34,35] contribute to this expression pattern.

We hypothesized that limiting physiological AID expression levels could provide an additional mechanism to restrict its deleterious activity. To address this question we analysed the impact of AID reduction in AID<sup>−/−</sup> mice on CSR, SHM and the generation of chromosome translocations.

**Results**

AID expression is limiting for its function in CSR

In order to assess whether physiologic AID expression level is limiting for its function, we evaluated AID gene dose effect in mice harbouring one or two functional alleles of the AID gene, AID<sup>+/−</sup> and AID<sup>+/+</sup> mice, respectively. B cells from congenic Balb/c ByJ animals [21] were used to minimize strain-to-strain variations. We hypothesized that limiting physiological AID expression would conversely result in an increase of CSR. To approach this issue we transduced spleen B cells from wild type mice with retroviruses encoding AID or AIDE58Q catalytically inactive mutant, together with GFP for tracking purposes. We found that AID, but not AID<sup>E58Q</sup> overexpression, promotes an increase in the efficiency of CSR, as measured by the expression of IgG1 after stimulation in the presence of LPS and IL4 (Figure 1g and not shown).

We next asked if surpassing the level of physiologic AID expression would conversely result in an increase of CSR. To approach this issue we transduced spleen B cells from wild type mice with retroviruses encoding AID or AID<sup>E58Q</sup> catalytically inactive mutant, together with GFP for tracking purposes. We found that AID, but not AID<sup>E58Q</sup> overexpression, promotes an increase in the efficiency of CSR, as measured by the expression of IgG1 after stimulation in the presence of LPS and IL4 (Figure 1g and not shown).

From these results we conclude that AID gene dose affects the efficiency of CSR in primary B cells, which implies that the AID gene is haploinsufficient for CSR.

AID expression is limiting for SHM

To assess whether AID haploinsufficiency was also evident on its activity in SHM we first analysed the accumulation of mutations in the 5′ end of the μ switch region (Spμ). AID activity during CSR leads to the induction of mutations in Spμ. We stimulated CFSE-labelled spleen B cells from AID<sup>−/−</sup> and AID<sup>−/−</sup> mice in the presence of LPS and IL4 for 96h and quantified the accumulation of mutations in cells that had undergone 5 or more divisions after sorting, DNA extraction, PCR amplification, cloning and sequencing. We found a lower mutation frequency in the Spμ region of AID<sup>−/−</sup> when compared to AID<sup>+/+</sup> cells (1.2×10<sup>−4</sup> vs 1.9×10<sup>−5</sup>) although this difference was not statistically significant (t test p = 0.171) (Figure 2a).

We next examined the SHM frequency in vivo by isolating Fas<sup>−/−</sup>GL7<sup>−</sup> germinal centre cells from AID<sup>−/−</sup> and AID<sup>−/−</sup> peyer’s patches and sequencing of the intronic region immediately downstream of the JH<sub>4</sub> gene. Our analysis showed that B cells from AID<sup>−/−</sup> mice contain fewer mutations than AID<sup>+/−</sup> cells (0.9×10<sup>−4</sup> vs 3.1×10<sup>−3</sup>) (t test p = 0.011) (Figure 2b). From these results we conclude that AID is also haploinsufficient for SHM and therefore that the physiologic level of AID expression is limiting for the diversification of antibodies.

**B cells from IL6tgAID<sup>−/−</sup> hyperplastic lymph nodes harbour fewer translocations than IL6tgAID<sup>+/−</sup> B cells**

IL6 transgenic (IL6tg) mice develop lymphoid hyperplasia, presumably resulting from IL6-induced proliferation protection of mature B cells from apoptosis. Hyperplastic lymphoid tissues from IL6tg mice are enriched in B cells that harbour chromosomal translocations involving the IgH locus and the c-myc oncogene (c-myc/IgH), analogous to those found in human Burkitt lymphoma. In the absence of AID, IL6tg B cells are devoid of c-myc/IgH translocations and the onset of lymphoid hyperplasia...
AID Haploinsufficiency

bars show standard deviation (n = 5). ND, non-detectable. (B) AID+/− spleens contain increased numbers of B cells. Bars represent the number of spleen B cells (CD43 negative) and statistical bars represent standard deviations. n (AID+/−) = 18, n (AID+/+) = 16 and n (AID+/+) = 9. t test p (AID+/− vs AID+/+) = 6 × 10−4. (C) Peyer’s patch B cells from AID+/− mice contain intermediate FasGL7 cell numbers. Spleen B cells from AID−/−, AID−/− and AID+/− were stained with anti-Fas and anti-GL7 antibodies and analysed by flow cytometry. Percentage of FasGL7 cells is indicated. One representative experiment is shown (n = 3). (D) ELISA quantification of IgG serum levels in AID−/−, AID−/− and AID+/− was stimulated for 3 days in the presence of LPS and IL4. RNA was analysed B cells from IL6tgAID−/−, AID−/− and AID+/− were transduced with retroviral vectors encoding wild type (AID) or a catalitically inactive (AIDE58Q) AID along with GFP to monitor transduction. CSR to IgG1 was measured by flow cytometry. Representative plots of CFSE labelling and IgG1 expression are shown. Percentage of IgG1 cells is indicated. (F) Time-course analysis of CSR. B cells from AID+/+, AID−/− and AID+/− were stimulated for the indicated times (X axis) in the presence of LPS and IL4. Percentage of IgG1 as measured by flow cytometry is represented (Y axis). Statistical bars show standard deviations. p values (AID+/− vs AID+/+) for IgG1: 48 h, 0.04; 72 h, 0.03; 96 h, 6 × 10−3; 120 h, 3 × 10−3; for IgG3: 48 h, 0.1; 72 h, 8 × 10−3; 96 h, 0.02; 120 h, 0.02 (unpaired two-tailed Student’s t test, n = 9). (G–H) AID overexpression increases CSR. Spleen B cells from AID−/− mice were transduced with retroviral vectors encoding wild type (AID) or a catalitically inactive (AIDE58Q) AID along with GFP to monitor transduction. CSR to IgG1 was measured by flow cytometry 2 days after transduction. (G) A representative flow cytometry plot is shown in panel G. Percentages of IgG1 cells within the GFP+ population are shown. (H) Percentage of IgG1+ cells within AID or AIDE58Q GFP+ cells determined in 4 independent experiments. Statistical bars represent standard deviations. Statistically significant differences (p < 0.05) are indicated with a (*) (A–H).

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sia is delayed [19,21]. Breakpoints of c-myc/IgH translocations found in IL6tg mice cluster in a narrow region of the c-myc gene, which encompasses part of its first exon and first intron. In contrast, translocation breakpoints have been found spreading over a large region of the IgH locus, from the V-JH region to the alpha switch (Sα) segment [19,21,37]. This distribution likely reflects the sites of AID-initiated double strand breaks during CSR and on occasion, during SHM.

To determine if AID gene dose has an influence on the frequency or distribution of c-myc/IgH translocations in vivo we analysed B cells from IL6tgAID+/− and IL6tgAID+/+ hyperplastic lymph nodes by long-range PCR, cloning and sequencing. Oligonucleotides priming at the mu switch (Sμ) region or at the Sα region of the IgH were combined with c-myc oligonucleotides to detect proximal, c-myc/IgH1a or distal, c-myc/IgH2a translocations, respectively [38] (Figure 3a). We found that the total frequency of c-myc/IgH translocations was reduced in IL6tgAID+/− (0.77 × 10−3) B cells when compared to IL6tgAID+/+ (1.95 × 10−3) (Figure 3c, left).

We readily detected proximal c-myc/IgH1a translocations in both IL6tgAID+/− and IL6tgAID+/+ B cells, in agreement with previous reports [21,37] (Figure 3b). When the frequency of c-myc/IgH1a translocations was calculated by performing serial dilutions of B cell samples from hyperplastic lymph nodes, we found that it was slightly reduced in IL6tgAID+/− when compared with IL6tgAID+/+ cells (Figure 3c, middle; 1.2 × 10−3 vs 0.8 × 10−3). Breakpoints from IL6tgAID+/− and IL6tgAID+/+ translocations were mapped and characterized by cloning and sequencing (Figure 3d and Table 1). We found that translocation breakpoints at the c-myc gene mostly clustered at the end of the first exon and the beginning of the first intron, regardless of the genotype being analysed (Figure 3d). Breakpoints at the IgH locus

Figure 1. AID is haploinsufficient for CSR. (A) AID levels are reduced in AID−/− mice. Spleen B cells from AID+/−, AID−/− and AID−/− were stimulated for 3 days in the presence of LPS and IL4. RNA was isolated and AID expression was assessed by real-time RT-PCR. Bars represent AID mRNA expression relative to AID−/− B cells. Statistical error
were slightly more proximal to E4 in the case of IL6tgAID+/− B cells (Figure 3d), although this difference was not statistically significant. Both mutation frequency nearby translocation junctions and number of microhomology nucleotides at junctions were similar in IL6tgAID+/− and IL6tgAID−/− B cells.

In contrast to c-myc/IgH proximal translocations, we found that distal c-myc/IgH translocations, while present in 20% of the IL6tgAID+/− samples, were not detected in any of the IL6tgAID−/− mice analysed (Figure 3b–c, right). We conclude that in vivo, the reduction of AID expression in IL6tgAID−/− mice results in a shifted pattern and reduced frequency of c-myc-IgH translocations.

The frequency of c-myc/IgH translocations initiated in B cells through the same molecular pathway as CSR, which involves cytosine deamination by AID, and UNG. This process can be recapitulated in vitro by stimulating spleen B cells in the presence of LPS and IL4. The frequency of these events in wild type B cells is extremely low (below one translocation every ten million cells), but it is increased in the absence of p53, p19ARF or ATM, reflecting that DNA damage and oncogenic stress pathways are activated downstream of AID function to prevent aberrantjoinings or spreading of cells that harbour lymphomagenic translocations. In particular, p53-mediated protection against AID-triggered c-myc/IgH translocations seems to require both alleles of the tumor suppressor, since p53+/− B cells display a dramatic increase in translocation frequency when compared to wild type littermates. Therefore we decided to exploit the higher frequency of c-myc/IgH translocations found in p53+/− B cells to determine whether restricting AID expression levels has an impact on the occurrence of these events.

To verify that the p53+/− genotype or the mixed strain of these mice do not interfere with AID haploinsufficiency (described above), we generated p53+/− AID+/+ and p53+/− AID−/− animals and analysed the efficiency of CSR in LPS+IL4 B cell cultures. Expectedly, we found that p53−/− AID+/− B cells show a decreased level of CSR when compared to p53+/− AID−/+ cells, as measured by the expression of IgG1 at different culture time-points (Figure 4a). This reduction is comparable to that observed in Balb/c p53−/− mice (see above).

Translocation frequency was then assessed in p53−/− AID+/+ and p53−/− AID−/− spleen B cells upon LPS+IL4 activation. Spleen B cells were isolated from 10 independent mice (5 mice per genotype), and the presence of translocations was analysed by PCR (depicted in Figure 3a, left) in 90 million cells (45 million cells per genotype) after 3 days of stimulation. Translocation identity was verified after gel analysis, southern blot with IgH- and myc-specific probes (see Figure 4b for representative gels), cloning, and sequencing. We found that the frequency of c-myc/IgH translocations is significantly reduced in p53−/− AID−/− B cells as compared to p53+/− AID+/+ B cells (0.19×10−6 vs 0.61×10−6, Fisher test p = 0.0025). We did not find significant differences regarding translocation breakpoints (mapped in Figure 4d), mutations or microhomology at junctions (Table 2) in p53−/− AID+/− vs p53−/− AID−/− translocations.

From this result we conclude that the reduced AID levels expressed by mice that harbour a single copy of the gene (AID−/−) results in a decreased frequency of c-myc/IgH translocations. Therefore, AID is haploinsufficient for the generation of these lymphomagenic lesions.

**Discussion**

The progress of the humoral immune response relies on the reshaping of the antibody repertoire upon infection by the introduction of somatic changes into the DNA of Ig genes. Higher affinity antibodies are produced by SHM and new effector capabilities are generated by CSR. SHM and CSR are initiated by AID through the deamination of cytosine residues on antibody genes [6]. Accordingly, AID is a critical enzymatic activity for the development of the adaptive immune response [6,7]. Recent reports have stated that AID-mediated deamination on DNA can also lead to the generation of unwanted lesions, namely, mutations outside the Ig genes [27,28], or DNA breaks and chromosomal translocations [19,20,21,22], whose contribution to lymphoid neoplasia has been demonstrated in several in vivo models [19,21,22,23,24,25]. Therefore AID function is expected to be tightly regulated to prevent the generation of DNA lesions.
Here, we have addressed the question of whether restriction of AID expression levels in vivo could play a regulatory role in its activity. We performed our analyses in mice that harbour a single functional allele of the AID gene, which results in a reduction of roughly 50% of AID mRNA levels. This reduction is likely due to a per-cell decrease of AID expression [36].

Figure 3. c-myc/IgH translocation frequency is reduced in IL6tgAID+/− mice. (A) Schematic representation of the IgH and c-myc genes (upper) and the derivative chromosomes (c-myc/IgHμ and c-myc/IgHa, lower) arising from proximal and distal translocations, respectively. Variable (Vμ) and constant (Cμ and Cα) genes are represented as grey and black boxes, respectively. Sμ and Sα switch regions and Eμ enhancer are shown as striped and black ellipses, respectively. C-myc exons are drawn as white boxes. Arrows show the position of primers used for PCR amplification. (B) Proximal (left) and distal (right) c-myc/IgH translocations detected in IL6tgAID+/− (upper gels) and IL6tgAID−/− (lower gels) mice. DNA from IL6tgAID+/− and IL6tgAID−/− hyperplastic lymph node B cells was amplified as described in materials and methods using the primers depicted in (A). Representative amplification products analysed in ethidium bromide stained gels are shown. Mouse identifications are shown above the lanes. (C) Frequency of c-myc/IgH translocations in IL6tgAID+/− and IL6tgAID−/− mice. Translocation frequency was determined by serial dilution of DNA samples, followed by PCR amplification, cloning and sequencing. Graphs show the overall translocation frequency (left), frequency of proximal c-myc/IgHμ translocations (middle) and frequency of distal c-myc/IgHa translocations (right). (D) Representation of translocation breakpoints at the c-myc and IgHμ genes found in IL6tgAID+/− and IL6tgAID−/− B cells. Amplification products of proximal c-myc/IgH translocations were cloned and sequenced. Translocation breakpoints at the c-myc (upper diagram) and IgH (lower diagram) genes are shown as closed (IL6tgAID+/−) and open (IL6tgAID−/−) circles. C-myc exon 1 and IgH Eμ enhancer are represented as grey boxes and distance to these elements is shown underneath (bps). Arrows on the right indicate the position of the PCR oligonucleotides used for amplification.

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Both in mice and humans, the absence of AID results in a hyper IgM immunodeficiency syndrome that is characterized by the absence of somatic mutations and of Ig isotypes other than IgM. This phenotype is accompanied by lymphoid hyperplasia and enlarged germinal centers. Although the significance of these latter features to the immunodeficiency is unclear, we found that reduction of AID expression in AID<sup>+/−</sup> mice brings about a mild increase of B cell numbers in the spleen and of germinal center cells in Peyer’s patches. More importantly, AID gene dose is limiting for the generation of switched isotypes, as B cells from AID<sup>+/−</sup> mice have an impaired ability to perform CSR in vivo, in agreement with very recently published results [36,39].

This observation is reinforced by the finding that exogenous AID targeting, rather than its specificity.

### Analysis of AID-promoted c-myc/IgH translocations in IL6tg mice

| Translocation<sup>a</sup> | ID<sup>b</sup> | Genotype | c-myc<sup>c</sup> | Jnc<sup>d</sup> | IgH<sup>e</sup> |
|--------------------------|--------------|-----------|-------------------|----------------|-------------|
| IgH<sub>H</sub>          | A1           | AID<sup>+/+</sup> | AAAACGCTCGAGGAAcTCTTTTCCAG | G          | GCTGGGGTGAGCTGAGCTGG |
| IgH<sub>H</sub>          | A2           | AID<sup>+/+</sup> | CTTCCTCTCTGTACCTGACAGAC | G          | CTGCCGCTGGACGCTGAGCTGG |
| IgH<sub>H</sub>          | A3           | AID<sup>+/+</sup> | GCTTCAACAGAGCGGAGGAGCTCT | A          | TGGGAGGTTCTGGGGTACGCTGAGCTGG |
| IgH<sub>H</sub>          | A4-1         | AID<sup>+/+</sup> | AATTCCAGGCTCAGAGGAGCTGAGAG | AGC        | CGCTGCTGGGACGCTGAGCTGG |
| IgH<sub>H</sub>          | A4-2         | AID<sup>+/+</sup> | GAAGGGAGTGGGCGGAGGCAGGCTG | A          | TGGGAGGTTCTGGGGTACGCTGAGCTGG |
| IgH<sub>H</sub>          | A10          | AID<sup>+/+</sup> | TGAGGAGTGGGGCACGCCTGGGTG | G          | GCTGGGGTGAGCTGAGCTGG |
| IgH<sub>H</sub>          | A11          | AID<sup>+/+</sup> | AAGCTGACAGACGCTGTCGTCACGC | GC         | GCTGGGGTGAGCTGAGCTGG |
| IgH<sub>H</sub>          | A13          | AID<sup>+/+</sup> | AGCGGGGAGTGGGACTCTGTCGTC | GC         | GCTGGGGTGAGCTGAGCTGG |
| IgH<sub>H</sub>          | A15          | AID<sup>+/+</sup> | TAAAGGCTCAGGGAGGTTGACGA | C          | TGGGAGGTTCTGGGGTACGCTGAGCTGG |
| IgH<sub>H</sub>          | A17          | AID<sup>+/+</sup> | ACTTCTGAGAAATGCTCAAAACAA | C          | TGGGAGGTTCTGGGGTACGCTGAGCTGG |
| IgH<sub>H</sub>          | B2           | AID<sup>+/−</sup> | CTGACCTCATGAGGAGGGCCGACAT | A          | GCTGGGGTGAGCTGAGCTGG |
| IgH<sub>H</sub>          | B5           | AID<sup>+/+</sup> | CTTTCTGAAAGGTCTCAAAACAA | A          | aGCTGGGGTGAGCTGAGCTGG |
| IgH<sub>H</sub>          | B6           | AID<sup>+/−</sup> | CACTCCAGACAGTCTTCTTACGT | GC         | GCTGGGGTGAGCTGAGCTGG |
| IgH<sub>H</sub>          | B7           | AID<sup>+/+</sup> | AAGACAGACGAGAAAAGCACCAGC | A          | GCTGGGGTGAGCTGAGCTGG |
| IgH<sub>H</sub>          | B9           | AID<sup>+/−</sup> | TCAAATGCTACTCGGCGCTTTTCCCT | T          | GGGCTAGCTGGCTGTGAGCCAT |
| IgH<sub>H</sub>          | B10          | AID<sup>+/−</sup> | CTTTCCTAGGCGAGGCTAGTCGGG | GC         | TGGGAGGTTCAAGGTGAGCTGG |
| IgH<sub>H</sub>          | B11          | AID<sup>+/−</sup> | AAAAGACAGAGAATGGAAGGAAGACT | G          | GCTGAGCTGGCTGTGAGCTGG |
| IgH<sub>H</sub>          | B13          | AID<sup>+/−</sup> | CAAGAGATCCACCGGCGCTAGGTA | G          | GAGCTGAGCTGGCTGTGAGCTGG |
| IgH<sub>x</sub>          | A11          | AID<sup>+/−</sup> | ATCTGGAAGCAGTTACCCGGA | CT         | GAGCTGAGCTGGCTGTGAGCTGG |
| IgH<sub>x</sub>          | A12          | AID<sup>+/−</sup> | GAGGGATGGGGAGCGAGCCGACCTC | C          | TGGGAGGTTCTGGGGTACGCTGAGCTGG |
| IgH<sub>x</sub>          | A15          | AID<sup>+/−</sup> | AACCGGCGCTGACATTCCACCTGAGCGA | G          | GCTGGGGTGAGCTGAGCTGG |

<sup>a</sup>Proximal (IgH<sub>H</sub>) or distal (IgH<sub>x</sub>) c-myc/IgH chromosomal translocation.

<sup>b</sup>Mouse identification as referred to in figure 2b.

<sup>c</sup>c-myc sequence adjacent to translocation junction. Mutations are shown in lowercase.

<sup>d</sup>Microhomology at junctions (overlapping sequences that cannot be assigned to a single sequence, c-myc, or Ig).

<sup>e</sup>Reverse complementary sequence adjacent to translocation junction. Mutations are shown in lowercase.

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This observation is reinforced by the finding that exogenous AID expression results in an increase of the CSR rate. In addition, our results show that AID expression is indeed limiting for SHM. Altogether, these data indicate that AID is haploinsufficient for antibody diversification. In humans, AID deficiency (HIGM2) is considered an autosomal recessive disease [7,40,41,42]. This apparent discrepancy with our data can not be discriminated. Our results show unequivocally that diminishing AID levels affects only the frequency of these events. This reduction in c-myc/IgH translocations found in AID<sup>+/−</sup> mice is not accompanied by a shift in the location or nature of translocation breakpoints, which indicates that diminishing AID levels affects only the frequency of targeting, rather than its specificity.
Conversely, we had previously observed that AID overexpression in primary B cells produces a major increase in the frequency of c-myc/IgH translocations. In particular, a ten-fold protein increase gave rise to a thousand-fold increase in translocation frequency [20]. This observation suggests that surpassing a certain level of AID expression is likely to overwhelm the cellular surveillance pathways that protect against these lesions and to result in their accumulation in a non-linear fashion. Together, these results show that AID haploinsufficiency is also revealed in its deleterious activity as promoter of illegitimate chromosome translocations. This implies that there is a gene dose effect of AID expression, and therefore, that AID is haploinsufficient. Our findings suggest that restraining the physiologic levels of AID expression can be a mechanism that allows the achievement of an optimal balance between immune proficiency and genome integrity.

**Materials and Methods**

**Mice, B cell cultures, flow cytometry and ELISA assays**

Congenic Balb/c AID<sup>+/+</sup> mice were obtained by breeding Balb/c AID<sup>−/−</sup> mice (Ramiro et al., 2004) with wild type Balb/c mice. p53<sup>+/−</sup>AID<sup>+/−</sup> mice were obtained by breeding Balb/c AID<sup>−/−</sup> mice with C57BL/6 p53<sup>−/−</sup> mice (Jackson laboratories). Lymph node samples were obtained from IL6tgAID<sup>+/+</sup>, IL6tgAID<sup>+/−</sup> and IL6tgAID<sup>−/−</sup> mice (Ramiro et al., 2004, Dorsett 2007). All experiments with mice were performed following the Animal Bioethics and Comfort Committee protocols approved by the Instituto de Salud Carlos III. B cells were purified from spleens by anti-CD43 immunomagnetic depletion (Miltenyi Biotech), labelled with 5 μm CFSE (Molecular Probes) (when indicated) and cultured in RPMI in the presence of 25 μg/ml LPS (Sigma),...
10 ng/ml IL-4 (Peprotech), 10 mM Hepes (Gibco), 50 μM β-mercaptoethanol (Gibco) and 10% fetal bovine serum (Gibco). For class switch recombination and differentiation assays BAFF (20 ng/ml R&D Systems) was also included to the previously described medium. Retroviral transductions were performed in LPS+IL4 stimulated B cells in the presence of 8 μg/ml polybrene. Flow cytometry analysis was performed after staining with anti-GL7–FITC, anti-CD95–PE, anti-CD19–APC, anti-IgG1 biotin (BD Biosciences). For determination of serum Ig titers, age-matched P-values were calculated using unpaired Student’s t test (GraphPad software). For analysis of mutations at the Sμ region, CD43 cells were purified by footpad injection with 50 μg/ml of NP-CGG (Biosearch Technologies) in complete Freund’s adjuvant. Serum was collected from blood samples 15 days after the immunization and IgG titers in AID mice were determined using a mouse IgG specific ELISA system (Roche Applied Science).

Real time quantitative PCR

Total RNA was isolated with Trizol (Invitrogen) after Ficoll gradient centrifugation (Amerham Biosciences) of stimulated B cells and converted into cDNA using random primers (Roche) and SuperScript II Reverse transcriptase (Invitrogen). AID mRNA were purified, cloned and sequenced. Sequence analysis was performed using GraphPad software.

**Mutation analysis**

For analysis of mutations at the Sμ region, CD43 splenic B cells were CFSE labelled, stimulated for 96h in the presence of LPS and IL4 and B cells that had undergone 5 or more cell divisions were isolated by sorting (FACSaria). DNA was extracted and amplified using the oligonucleotides 5’-AATGGATACCTCTGGTCTTTATATGGGTGTTTTT-3’ and 5’-GCGGCCCGGCTCA-TTTCCAGTTCAGTCGAGGC-3’ for 26 cycles (Pfu Ultra, Stratagene). For JH4 intron mutations, Fas’GL7 cells were purified by cell sorting (FACSaria), DNA was isolated and amplified with oligonucleotides 5’-ACTATGGTTATAGGAC-3’ and 5’-CTGGA-CTTTCGGGTTGTTG-3’ for 9 cycles and then 5’-GGTCAAGAGCATTGCAGCAAGGAAGCTG-3’ and 5’-TCTCTAGACAGCAACTAC-3’ for 21 cycles using Pfu Ultra (Stratagene). Amplification products were purified, cloned and sequenced. Sequence analysis was performed using Lasergene software. P-values were calculated using unpaired Student’s t test (GraphPad software).

**Translocations assays**

C-myc/IgH translocation detection by PCR was performed as previously described (Ramiro et al., 2004; Kovalchuk et al., 1997) on 1 μg (for p55−/− samples) or 500-4 ng (for IL10g samples) of genomic DNA. C-myc/IgH and c-myc/IgH translocations were amplified using two rounds of PCR with Expand Long Template PCR system (Roche). Primers for derivative 12 c-myc/IgH first round: 5’-GAGGAGAGCGAGGATGATCAGAGAGGATAAAAAGA-GAA-3’ and 5’-GGGGGAGGGGGTGTTGCAAATATAAAGA-3’; for derivative 12 c-myc/IgH second round: 5’-GAGGAGAGCAGAGGATGATCAGAGAGGATAAAAAGAGGAA-3’ and 5’-GGGGGAGGGGGTGTTGCAAATATAAAGA-3’.

| Table 2. c-myc/IgH chromosomal translocations generated in vitro |
|---------------------------------------------------------------|
| **Translocation** | **ID** | **Genotype** | **c-myc** | **Jnc** | **IgH** |
|-------------------|--------|-------------|-----------|--------|--------|
| IgH1              | 1F7    | AID+/-      | AGACGTGTGAGGCGGACTCAT | T      | GAGCAGTACTGAGGAGGAGCT |
| IgH1              | 1G5    | AID+/-      | AGACAAATCCCTCCGGGAGCC  | TG     | AGCTGGGCTGAGCTAGTGGGATA |
| IgH1              | 2G7    | AID+/-      | CGGCGAGTCCCAAGTAGGAATGaA | GG     | GGTGAGCTGAGGAGCCTGAGT |
| IgH1              | 6H5    | AID+/-      | AAATTTACTAGGATGACATTATT | TG     | TGAAGCAGTGGTGGGAGAGATGAGC |
| IgH1              | 6H8    | AID+/-      | GTCCGTGCCAGGCTGAGAAGAGT | TG     | TGAAGCAGTGGTGGGAGAGATGAGC |
| IgH1              | 8B6    | AID+/-      | AATGTTGACACGAGGGTCAGCT | TG     | TGAAGCAGTGGTGGGAGAGATGAGC |
| IgH1              | 10G5   | AID+/-      | ACAGGGGTCTTCCAAGGCTTAAGA | A      | GCTAGGCTGGGCTGAGTGGGATS |
| IgH1              | 10G12  | AID+/-      | ACAGGCTGAGGGCTCTTTCAGCT | GACTGA | GACTGAGGCTGGTGGGAGAGATGAGC |
| IgH1              | 10H5   | AID+/-      | GGTCTTCCGAAAGCAATGCGG  | T      | GAGCAGTACTGAGGAGGAGCT |
| IgH1              | 11F4   | AID+/-      | AACAGCTGAGGAGTCCCTTCTGAC | TG     | TGAAGCAGTGGTGGGAGAGATGAGC |
| IgH1              | 11H7   | AID+/-      | GAAATGGACACAGTGAATTTAAA | GGGGCAGTGGAGTGGGAGGACT | TGAAGCAGTGGTGGGAGAGATGAGC |
| IgH1              | 3C9    | AID+/-      | GTCCGTCCAGGCTGAGAAGAGG  | TG     | TGAAGCAGTGGTGGGAGAGATGAGC |
| IgH1              | 7D3    | AID+/-      | AGTAACCTCCTGAGGCTTACCA | TG     | TGAAGCAGTGGTGGGAGAGATGAGC |
| IgH1              | 8C5    | AID+/-      | TCCTTTTCAAGGAGGCTGACTGGG  | GGG    | GAGCAGTACTGAGGAGGAGCTGAGT |
| IgH1              | 8H10   | AID+/-      | AGCTCTTTCAGGAGAGCTCTGCG  | GGG    | GAGCAGTACTGAGGAGGAGCTGAGT |
| IgH1              | 9A4    | AID+/-      | GTAAACCTCCTGAGGCTTACCA | TG     | TGAAGCAGTGGGCTGAGTGGGATS |
| IgH1              | 9C3    | AID+/-      | TGGAGAACTGTGCAAAAGCAAGA | A      | GCTAGGCTGGGCTGAGTGGGATS |
| IgH1              | 10A10  | AID+/-      | GAGAGCTGATCGGGGCGGAGGAGCAG | GAGCAGT | GAGCAGTACTGAGGAGGAGCTGAGT |

*Proximal IgH1 c-myc/IgH1 chromosomal translocation.
*Mouse identification as referred to in figure 2b.
**c-myc sequence adjacent to translocation junction. Mutations are shown in lowercase.
**Microhomology at junctions (overlapping sequences that cannot be assigned to a single sequence, c-myc, or Ig).
**IgH Reverse complementary sequence adjacent to translocation junction. Mutations are shown in lowercase.

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Author Contributions

Conceived and designed the experiments: IVS VgIY ARR. Performed the experiments: IVS VgIY YD. Analyzed the data: IVS VgIY ARR. Contributed reagents/materials/analysis tools: ARR. Wrote the paper: VgIY ARR.

AID Haploinsufficiency

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