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Alternative End-Joining and Classical Nonhomologous End-Joining Pathways Repair Different Types of Double-Strand Breaks during Class-Switch Recombination

Elena M. Cortizas,* Astrid Zahn,† Maurice E. Hajjar,* Anne-Marie Patenaude,† Javier M. Di Noia,‡,‡ and Ramiro E. Verdun*§,¶

Classical nonhomologous end-joining (C-NHEJ) and alternative end-joining (A-EJ) are the main DNA double-strand break (DSB) repair pathways when a sister chromatid is not available. However, it is not clear how one pathway is chosen over the other to process a given DSB. To address this question, we studied in mouse splenic B cells and CH12F3 cells how C-NHEJ and A-EJ repair DSBs initiated by the activation-induced deaminase during IgH (Igh) class-switch recombination (CSR). We show in this study that lowering the deamination density at the Igh locus increases DSB resolution by microhomology-mediated repair while decreasing C-NHEJ activity. This process occurs without affecting 53BP1 and γH2AX levels during CSR. Mechanistically, lowering deamination density increases exonuclease I recruitment and single-stranded DNA at the Igh locus and promotes C-terminal binding protein interacting protein and MSH2-dependent DSB repair during CSR. Indeed, reducing activation-induced deaminase levels increases CSR efficiency in C-NHEJ–defective cells, suggesting enhanced use of an A-EJ pathway. Our results establish a mechanism by which C-NHEJ and this C-terminal binding protein interacting protein/MSH2-dependent pathway that relies on microhomology can act concurrently but independently to repair different types of DSBs and reveal that the density of DNA lesions influences the choice of DSB repair pathway during CSR. The Journal of Immunology, 2013, 191: 000–000.

Unrepaired DNA double-strand breaks (DSBs) are highly toxic lesions that can produce a permanent arrest of cell division or even cell death. Even though different DNA repair pathways can be recruited to sites of DNA strand breaks in a normal cell, the choice of an appropriate repair pathway is critical to avoid genomic instability that could lead to cell death or transformation.

Mammalian cells rely on nonhomologous end joining (NHEJ) and homologous recombination (HR) as the main pathways for repairing DSBs. Because HR depends on the presence of homologous sequences elsewhere in the genome, it is active during the S and G2/M phases of the cell cycle. In contrast, classical-NHEJ (C-NHEJ) can fuse DNA ends without any sequence homology and is active during all the cell-cycle phases (1, 2). Therefore, C-NHEJ is the preferred pathway for the repair of DSB in G1 cells, when sister chromatids are not available. This is illustrated by the essential role of C-NHEJ during the repair of DSBs induced by ionizing radiation (3). C-NHEJ is also critical for the repair of programmed DSBs generated during lymphocyte development. Under these circumstances, C-NHEJ repairs the DSBs that are physiologically intermediates during V(D)J recombination of B and TCRs genes and plays a dominant role during Ig class-switch recombination (CSR) in B cells (4).

In mature B lymphocytes, CSR underpins the change of the Cμ C-region of the IgH (Igh) to a downstream C-region such as Cε, Cγ, or Cδ. This process allows B lymphocytes to switch from the initially expressed IgM to a different isotype (IgA, IgG, or IgE) while keeping the same Ag specificity. At the molecular level, CSR is a deletional recombination reaction at the Igh locus between highly repetitive switch regions (S-regions), which are located just upstream of each C-region (4). CSR depends on activation-induced cytidine deaminase (AID), an enzyme that converts deoxycytidines to deoxyuridines within the donor and acceptor S-regions (5). The deaminase activity of AID in both the donor Sp and the downstream acceptor S-region initiates noncanonical repair, leading to DSBs that are recombined to resolve through one of two end-joining pathways during the G1 phase of the cell cycle (5–10). The important role of C-NHEJ during CSR is supported by the drastically reduced level of CSR observed in cells with mutations in the core components of the C-NHEJ pathway, such as the DNA-end binding complex Ku70/Ku80 heterodimer or the DNA ligase complex XRCC4/ligase-4 (Lig4) (11–15). However, C-NHEJ–defective B cells still have significant levels of CSR, in which the switch junction sites show a preference for the use of microhomologies for the joining of DNA ends (13, 15–19). This end-joining mechanism, which is loosely defined by not relying...
on core components of the C-NHEJ pathway, has been termed alternative-NHEJ, microhomology-mediated-EJ, or alternative end-joining (A-EJ). Hence, although the C-NHEJ pathway joins DNA ends with short homology (1–3 nt) or no homology, A-EJ shows a preference for joining DNA ends that display microhomology (4–20 nt). This partial bias, it is still unclear how the choice between using A-EJ or C-NHEJ is made and whether A-EJ is in competition with C-NHEJ for repairing the same DSBs.

In this study, we investigate the order and interdependency of association of multiple DNA repair factors with the Igh locus during CSR. Our results reveal that both C-NHEJ and a CtIP/MSH2-dependent A-EJ pathway are simultaneously active during CSR, and, rather than competing, they preferentially repair different types of DSBs as defined by the density of deamination events. Our data support a model in which the spatial distance between AID-initiated breaks on opposite DNA strands determines the type of DSBs requiring repair and that this requirement defines which end-joining pathway is most efficient in repairing the lesion. Hence, the topological nature of the DSB has a critical influence on determining the choice of usage between C-NHEJ and at least a CtIP/MSH2-dependent A-EJ pathway.

Materials and Methods

Mice, cell lines, and CSR analysis

CH12F3-2 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 0.05% 2-ME, and 5% NCTC 109 (Sigma-Aldrich). CH12F3-2 Lig4−/− cells were supplemented with 20% of conditional media from an exponentially growing CH12F3-2 culture. Indeed, knockdown of CtIP produces an important decrease in the level of CSR in C-NHEJ–proficient B cells (27). Although the above-mentioned studies established a role for the A-EJ pathway in normal cells at least during CSR to IgA, it is still unclear how the choice between using A-EJ or C-NHEJ is made and whether A-EJ is in competition with C-NHEJ for repairing the same DSBs.

We generated stable short hairpin RNA (shRNA)-mediated knockdowns in CH12F3-2 cells following protocols from The RNAi Consortium library. We adapted previously published protocols (29) for the ChiP assays with minor modifications, except coimmunoprecipitated DNA was analyzed using real-time PCR with SYBR Green (Applied Biosystems). Briefly, cells were cross-linked with 1% formaldehyde for 20 min at room temperature, and reaction was stopped by addition of glycine to 125 mM final concentration. Cells were washed twice with cold PBS, harvested, and kept at −80°C overnight. Samples were resuspended in RIPA buffer (150 mM NaCl, 1% [v/v] [g/ml] sodium deoxycholate, 0.1% [v/v] SDS, 50 mM Tris-HCl [pH 8], 5 mM EDTA, protease, and phosphatase inhibitors) and sonicated to generate DNA fragments <500 bp using a Bioruptor Next Gen (Diagenode). Samples were then clarified by centrifugation at 20,000 rpm (4°C), and their protein content was measured using the BCA method (Bio-Rad). For immunoprecipitation, 0.5 mg (2 μg/μl) protein extract was pre-cleared for 2 h with 30 μl 50% G protein-Sepharose slurry before addition of Abs. Between 2 and 5 μg each Ab was added to the samples and incubated overnight at 4°C.

Thirty microliters 50% Agarose A/G plus (Santa Cruz Biotechnology) slurry per immunoprecipitation were pre-blocked overnight at 4°C with 1 mg/ml BSA and 0.3 mg/ml tRNA before addition of indicated Abs. Beads were washed twice (15 min at 4°C) with RIPA buffer, three times with ChiP Wash Buffer (100 mM Tris-HCl [pH 8.5], 0.5 M LiCl, 1% [v/v] [g/ml] sodium deoxycholate), and once with 1× TE. Immunocomplexes were eluted for 10 min at 65°C with 100 μl Elution buffer (1% [v/v] SDS), and cross-linking was reversed by adjusting to 200 mM NaCl, 1 mM EDTA, and 1 mM DTT and incubating overnight at 65°C in the presence of 5 μg proteinase K. DNA was purified using QIAquick PCR purification kit (Qiagen), and DNA re-suspended in 60 μl Tris-HCl (pH 8) was used as template in real-time PCR reactions. Primers against multiple regions of the mouse (C57BL/6) Igh locus were designed with Primer Express 2.0 (Applied Biosystems). PCR products spanning between 57 and 123 bp in size were cloned and sequenced to confirm identity. Ten-microliter PCR reactions containing 1× SYBR Green Mix (Applied Biosystems), 1.500 fraction of the ChiP-enriched DNA, and 100 nM primers were set up in 96-well plates. Standard curves using different quantities of the input extracts were run alongside ChiP samples for each individual primer, and plates were read in an Applied Biosystems StepOnePlus Real-time PCR instrument. IgG and input DNA values were used to subtract/normalize the values from ChiP samples. All primer sequences used for the ChiP analyses are available upon request.

Switch junctions analysis

Genomic DNA was purified from CH12F3 cells after switching to IgA with anti-CD40 Ab (BD Biosciences), TG-β (R&D Systems), and IL-4 (PeproTech). Fifty micrograms was used for the PCR amplification step following previously published protocols (30), except Tau Gold polymerase (Applied Biosystems) was used. The PCR products were cloned in a TOPO vector system and sequenced with an automated sequencer at the University of Miami Oncogenomics facility. The primers used for the amplification of the Sμ-Sν loci were: 5′-CCAGACAGAGAAAGCCAGACTCATAAAGCT-3′ and 5′-GATGATGATGGTCCTCACACTGCCAATCC-3′. The junctions were analyzed by aligning the PCR products with the Sμ and Sν sequences. Analysis of mutation pattern at the aligning junctions was performed as previously described (30, 31).

DNA break assay

The DNA break assay was performed as previously described (32) with minor modifications. The HindIII digestion of the genomic DNA was replaced by sonication to DNA fragments of 500–1000 bp using a Bio-ruptor Next Gen (Irvinegen), and the biotin-labeled DNA fragments were purified using Dynabeads-Streptavidin (Invitrogen) following the manufacturer’s instructions. The levels of biotinilated DNA were determined via quantitative PCR using the Sμ primers indicated in Supplemental Table I.
Intra-S deletion analysis

CH12F3 cells expressing shAID-1 or shGFP were stimulated for class switching during 96 h, and clones were isolated by serial dilution. After identifying IgM-secreting clones via dot blot/Western blot analysis and validating by flow cytometry analysis, the selected IgM−/IgA+ clones were expanded in 24-well plates. The extracted genomic DNA from the selected clones was used as template for PCR amplification of the Sμ region using these primers: forward, 5'-CAGTCATTGCTTTAGGGGGAGAAAGAG-ACA-3' and reverse, 5'-GCCACTGCACACTGATGTCT-3'. The PCR products were analyzed by Southern blot using a 32P-labeled Cμ probe as described previously (33). The primer set used for the Cμ probe is: OJ417 5'-AGC-CCCTCCCCACCTCCACCTACCTATTAC-3' and OJ418 5'-CACGAACT-TCACTCCAAAACCGATCACAG-3'.

Statistical analysis

A two-tailed paired Student t test was used for the analyses of CSR efficiency, and a two-tailed Fisher exact test was used for analyses of microhomology length at switch junctions in the different mutants.

Results

Ku70 and CtIP bind independently to the Igh locus during CSR

To determine whether C-NHEJ and A-EJ compete for the repair of DSBs during CSR, we used ChIPs to study the chronological order underlying the recruitment of key components from each end-joining pathway to the Igh locus. We set up a system using the mouse lymphoma cell line CH12F3-2, which is capable of efficient cytokine-induced AID expression and CSR to IgA in vitro through the same molecular mechanisms used by primary lymphocytes (5, 18, 27, 34). Time-course experiments showed that AID and CSR are rapidly induced after cytokine stimulation, as detected by immunoblotting, and by the presence of IgA at the cell surface, as observed by flow cytometry (Fig. 1A, 1B). Accordingly, we found detectable AID at the Sμ as early as 8–10 h poststimulation, reaching the maximum level at ~16 h postinduction (Fig. 1C).

The highest AID levels were observed at the S-regions, more specifically in the region immediately upstream to the Sμ repeat (Pre-Sμ region) and the 3' region within the So repeat (Fig. 1D), consistent with previous reports that analyzed the AID footprint at the Igh (35, 36). For the negative control, we used cytokine-stimulated cells with stable knockdown of AID via shRNA (Fig. 1C, 1D; denoted as shAID-3, see below). The timing of AID occupancy correlated with the activity of AID at the pre-Sμ region [m.2 (36)] (Fig. 1E), as revealed by the presence of G-to-A or C-to-T transitions in CH12F3-2 cells expressing the potent Ugi, which inhibits base excision repair (35–38). These experiments showed that AID was not only rapidly induced in stimulated CH12F3-2 but
also immediately recruited to the S-regions, where it deaminated dC and triggered CSR.

We then analyzed the recruitment of C-NHEJ and A-EJ in this system. Although there are no known factors that are exclusive for A-EJ to use as markers, CtIP has been clearly implicated in a microhomology-mediated pathway for CSR to IgA in CH12F3-2 cells (27), so we analyzed it as a possible marker or at least this particular case of A-EJ. To determine whether there was a chronological order of recruitment of DNA repair proteins to the Igh locus during CSR, we performed kinetic analyses for Ku70, a core component of the C-NHEJ pathway (39), and CtIP. We found that CtIP and Ku70 were both present in high levels at the Sμ region when AID recruitment was maximal (14–16 h; Fig. 2C, 2E). However, CtIP association with chromatin preceded that of Ku70 and was found even when AID was at low concentration in the cells and showed lower Igh occupancy (8–12 h; Fig. 2C, 2E). Control experiments with CH12F3-2 cells expressing shAID-3 demonstrated that the recruitment patterns we observed were AID dependent at all time points (Fig. 2C, 2E).

The different timing observed for the recruitment of Ku70 and CtIP to the Igh locus suggested that these factors do not compete for binding to the S-regions during CSR. Therefore, we tested whether respective knockdown of either CtIP or Ku70 would modify the magnitude of recruitment for the other factor. In agreement with previous reports (17, 27), knockdown of Ku70 and CtIP using specific shRNAs reduced CSR to different extents (~60% reduction for shKu70 and ~25% for shCtIP), and simultaneous knockdown of both factors abolished almost completely CSR (Fig. 2A, 2B). This reduction was not due to nonspecific effects on cell proliferation, AID protein expression, or germline transcript levels (Supplemental Fig. 1). These experiments validated the choice of CtIP as a component of an A-EJ pathway for CSR in CH12F3-2 cells because the other pathway in which CtIP has been implicated, HR, would not produce CSR. Importantly, CtIP knockdown reduced its concentration at the S-regions to background levels during CSR (Fig. 2C) without affecting the presence or distribution of Ku70 at the S-regions (Fig. 2E, 2F). Similar conclusions were obtained regarding the presence and distribution of CtIP at the S-regions in cells depleted of Ku70 (Fig. 2C, 2D).

Recruitment of Ku70 to the Igh locus presumably occurs during G1 phase of the cell cycle during the C-NHEJ repair of the AID-induced DSBs (40), whereas a recent report showed that CtIP is involved in microhomology-mediated end joining of DSBs during S and G2 phases (41). Therefore, to address whether CtIP and Ku70 could be both present at the Igh in the same cell-cycle stage, we performed ChIP assays for Ku70 and CtIP in G1-enriched CH12F3 cells after class-switching stimulation to IgA. We found that CtIP was present at the Sμ in G1-enriched CH12F3 cells and that its enrichment and distribution were not altered by the knockdown of Ku70 (Fig. 2G). Analogous results were obtained for Ku70 in G1-enriched CH12F3 cells (Fig. 2H), thus indicating that the presence of CtIP and Ku70 were not marking cells in different stages of the cell cycle.

These results strongly suggest that these components of the C-NHEJ and microhomology-mediated A-EJ pathways do not compete for association to the Igh locus and suggest that both pathways are independently recruited to the Igh S-regions during CSR, although CtIP was preferentially recruited at early time points (Fig. 2C), suggesting it might be present when AID levels and activity at the Igh are lower (Fig. 1).

The use of C-NHEJ during CSR requires high deamination density of the S-regions

Previous reports have shown that Ku70 knockdown increases the use of microhomologies at the switch junctions during CSR, whereas inhibition of CtIP decreases the use of microhomologies to favor direct joins at the switch junctions (17, 27). We have obtained the same results in this study (Fig. 3A–C). However, when we mapped the location of the junctions within the Sμ genomic region, we noticed a striking difference in the distribution of junctions between Ku70- and CtIP-depleted cells. Indeed, most junctions in the Ku70-depleted cells were found in the regions further away from the Sμ, where the preferred AID substrate motifs (WRCY) are sparser (area a in Fig. 3D). Thus, compared with control cells, Ku70-depleted cells preferentially lost junctions nearby or inside the Sμ repeats, which are rich in AID hot spots (area b in Fig. 3D). Conversely, junctions from CtIP-depleted cells were clustered adjacent to the Sμ repeat and very few closer to Iμ, where AID hot spots are less abundant (Fig. 3D). These results suggest that DSBs in different areas of the Sμ region can be preferentially processed by the CtIP-dependent A-EJ or C-NHEJ pathway and that this correlates with the distribution of WRCY motifs and AID deamination density found in this region.

We therefore asked whether AID levels affect the likelihood of CSR proceeding through the C-NHEJ or CtIP-dependent pathways. To answer this question, we established stable CH12F3-2 cell lines expressing different shRNAs that decreased AID to various levels (Fig. 4A). For each shRNA, the decrease in AID levels observed by Western blot was proportional to the reduction in IgA switching in each cell line (Fig. 4C). Germline transcripts, cell division, and Ku70 or CtIP expression were not affected by shRNA knockdown of AID (Fig. 4A, 4B, Supplemental Fig. 1). Furthermore, we analyzed the AID footprint in CH12F3-2 cells with low levels of AID (i.e., with shAID-1) and control cells (shGFP) expressing Ugi. This analysis confirmed that in cells with low levels of AID, there was a ~60% reduction in the total number of mutations in the Pre-Sμ region and also an increase in the distance between mutations located in opposite DNA strands (Fig. 4E, Supplemental Fig. 2; similar results were observed for shAID-2, not shown). Next, we analyzed the sequence of the switch junctions after inducing CSR in two independent shAID-1– or shAID-2–expressing CH12F3-2 cell lines (referred to as AIDlow). Both lines showed a clear decrease in direct junctions and an increase in the use of microhomologies at the switch-junctions (Fig. 4F, shAID-2 not shown). These results are in agreement with a previous report showing that human patients heterozygous for AID show increased usage of microhomologies at the switch-junctions (30). Our results suggested that partial AID knockdown might promote the use of microhomologies and possibly A-EJ over C-NHEJ in the repair of DSBs during the CSR. In support of these conclusions, knockdown of CtIP but not Ku70 significantly reduced CSR in AIDlow cells, confirming that most CSR was proceeding via a CtIP-dependent pathway in these cells (Fig. 4D). Furthermore, we found a significant decrease in intra-S recombination at the Sμ in AIDlow cells when compared with control cells (shGFP) (Supplemental Fig. 2B), further indicating that the level of AID influences the choice of joining mechanism.

We next analyzed the presence of CtIP as well as of C-NHEJ core elements in the Sμ during CSR in both CH12F3-2 cells and primary mouse B cells. As expected, a partial knockdown of AID (with shAID-1 or shAID-2) yielded a partial reduction (~40–50%) in its occupancy of the Sμ (Fig. 5A, 5B, shAID-2 not shown). Notably, ChIP analysis showed that the concentration of Ku70 and CtIP at the S-regions showed opposite trends under partial AID knockdown. In AIDlow cells, the presence of Ku70 was drastically decreased, whereas presence of CtIP was significantly increased at the Sμ, relative to control cells (Fig. 5A, 5B). Interestingly, we also observed at the Sμ of AIDlow cells an increase of DNA ligase III, which has also been implicated in the microhomology-mediated A-EJ pathway in the absence of C-NHEJ (42, 43). Importantly, overexpression of human AID protein reverted the phenotype,
confirming that the behavior of Ku70, CtIP, and DNA ligase III in AID\textsubscript{low} cells was not due to unspecific effects of shAID1 or shAID-2 (Fig. 5B). Similar results were obtained when we compared the presence of Ku70 and CtIP at the S\textsubscript{m} in stimulated primary splenic B cells from Aicda\textsuperscript{+\textordmasculine} versus Aicda\textsuperscript{+/-} mice (Fig. 5C), showing that this is not a CH12F3-2–specific phenomenon.

Taken together, these data indicate that the distribution of AID deaminations in the S-regions (Fig. 4E), and therefore the density of single-strand breaks (SSBs) in each strand, can influence the choice of end-joining pathway for CSR by determining a different quality of DSBs (i.e., blunt versus staggered).

The mismatch repair pathway is essential for CSR in AID\textsubscript{low} cells

Our results in this study show that a lower density of AID-initiated lesions correlates with an elevated dependence on a CtIP-dependent,
A-EJ pathway that preferentially used microhomologies for CSR. The mismatch repair (MMR) pathway can trigger CSR (38), possibly by processing distal SSBs to generate DSBs (44), thus indicating MMR might be more important in this case than for C-NHEJ (45). We confirmed the importance of MMR for CSR in CH12F3-2 cells by reducing MSH2 expression by shRNA, which significantly decreased the switching from IgM to IgA by \( \sim 45\% \) (Fig. 6A). However, MSH2 knockdown in the AIDlow cells reduced CSR more severely, by \( \sim 80\% \), without affecting cell division, germline transcripts, or AID protein levels (Fig. 6A and Supplemental Fig. 1). To characterize the role of MMR in the shift from C-NHEJ to A-EJ usage in AIDlow cells, we examined the recruitment of the MMR factors MSH2 and EXO1 to S-regions during CSR. In control cells, both proteins were recruited to the S\(_m\) in an AID-dependent fashion (Fig. 6B). Notably, we found EXO1 and MSH2 at the \( Igh \) locus from the same early time points at which AID and CtIP were first detected through later times when Ku70 was also present (compare Fig. 6B to Fig. 2C, 2E). This pattern did not change for MSH2 in AIDlow cells. However, AIDlow cells showed a significant increase in the magnitude of EXO1 recruitment to the S\(_m\) at later time points (Fig. 6B), consistent with the need for DNA resection for the microhomology-mediated joins. Accordingly, we also found an increase in the accumulation of RPA at the \( Igh \) in AID\(_{low}\) cells when compared with control cells (Fig. 6C, 6D). As RPA accumulates in regions where ssDNA is exposed, these results suggest increased DNA resection in AID\(_{low}\) cells compared with the wild-type (WT) cells, subsequent to the processing of AID-initiated lesions by EXO1.

Collectively, these results further support the notion that a CtIP-dependent microhomology-mediated A-EJ, in which MMR plays a major role, predominates for CSR to IgA in AID\(_{low}\) cells.

Reducing AID levels does not affect the DNA damage signaling at the \( Igh \) locus

The DNA damage signaling triggered after detection of a DSB is well characterized, with 53BP1 and the phosphorylated form of histone H2AX (ser129-H2AX or \( gH2AX \)) being two of the main players (46). Both factors are important for CSR, especially 53BP1, which is absolutely required for CSR (4). We showed that lower AID levels lead to more frequent usage of microhomology-mediated A-EJ to resolve CSR, but AID\(_{low}\) cells display fewer mutations in the S-regions, which may translate into fewer DSBs that could affect the strength of DNA damage signaling during CSR. However, the results obtained via ChIP experiments did not show significant differences in the concentration or the distribution of \( gH2AX \) or 53BP1 at the \( Igh \) locus between control and AID\(_{low}\) cells during CSR (Fig. 7A–D and not shown). In addition, direct quantitative comparison of DNA breaks by end-labeling followed by immunoprecipitation and real-time PCR (7) showed that there was no significant difference in the level of breaks at the S\(_m\) between AID\(_{low}\) and control cells (shGFP) before or after class-switching stimulation (Fig. 7C). Furthermore, CSR was ataxia telangiectasia mutated dependent in control as well as AID\(_{low}\) cells, as demonstrated by incubating the cells with the
inhibitor KU-55933 (Fig. 7D). Similarly, γH2AX levels were not affected in AIDlow cells during switching (Fig. 7E). Thus, these results indicate that the DSBs induced during CSR in AIDlow cells trigger a DNA damage response similar to that found in the control cells.

To further analyze the influence of γH2AX over the A-EJ during CSR, we decreased the levels of H2AX in control and AIDlow cells via shRNA. Although partial knockdown of H2AX (not shown) led to a decrease of switching in normal cells, it did not further decrease CSR in AIDlow cells (Fig. 7F, Supplemental Fig. 1E–G), in stark contrast to what we observed for MSH2 or CtIP (Figs. 4D, 6A). Hence, the AIDlow cells still show γH2AX and 53BP1 in the S-regions during CSR, but this does not prevent the activity of the CtIP/MSH2-mediated A-EJ pathway.
Reducing AID levels improve CSR efficiency in C-NHEJ-deficient cells

Based on the results described above, we envisage a model in which DSBs generated during CSR in regions with lower density of AID-mediated deamination would require nucleolytic processing and, at least for CSR to IgA, be preferentially repaired by a CtIP/MSH2-dependent A-EJ pathway. By contrast, C-NHEJ would take care of blunt DSBs that would more often arise in regions with higher density of deamination (Fig. 8A).

A testable prediction of our model is that if the nature of the DSB determines the choice of end-joining pathway, then lowering the density of deamination should increase the activity of this A-EJ by providing more suitable DSBs arising from distal nicks in opposite strands. For this critical test, we measured CSR in AIDlow cells lacking an active C-NHEJ pathway due to Ku70 or Ku80 knockdown (Fig. 8B, 8D). As we predicted, there was ~50% increase in CSR in both the Ku70 AIDlow and Ku80 AIDlow cells compared with the Ku70 and Ku80 expressing WT AID levels (Fig. 8D). Next, we tested DNA Lig4 knockout CH12F3-2 cells, which undergo CSR to IgA using exclusively A-EJ (18). Lig4-null CH12F3-2 cells showed impaired proliferation when compared with control cells as previously reported (Supplemental Fig. 1I) (18). We addressed this problem by using conditional media and evaluating class switching 48 h poststimulation. CSR was increased by 2- to 3-fold in Lig4−/− cells simply by expressing shRNAs that reduced AID levels to ~50% (Fig. 8C, 8E) without affecting the proliferation of these cell lines (Supplemental Fig. 1I). As would be expected from an increase in microhomology-mediated A-EJ activity, AIDlow Ku70 knockdown cells showed increased association of CtIP to the Sμ during CSR when compared with CH12F3-2 expressing WT levels.
of AID (not shown). In any case, our results indicate that WT levels of AID activity favor C-NHEJ by creating suitable DSBs and thereby reducing the usage of the CtIP/MSH2-dependent A-EJ.

Discussion

In this study, we examined whether the C-NHEJ and a microhomology-mediated A-EJ pathway process the same DSBs during CSR. Our results are consistent with a scenario in which C-NHEJ and at least this particular CtIP/MSH2-dependent microhomology-mediated A-EJ pathway do not compete for the repair of DSBs but act in parallel to repair different types of breaks initiated by AID during CSR to IgA in CH12F3-2 cells. Because A-EJ is undefined and probably includes more than one pathway, we can only conclude about the particular pathway that we show in this study is dependent on CtIP and MSH2 and is active even in C-NHEJ–competent cells. We demonstrate that the density of lesions in the S-regions can determine the usage of C-NHEJ or this A-EJ to resolve CSR (Figs. 2–5). Accordingly, diminishing the density of deamination events in the S-regions by controlling the levels of AID leads to an increase in CSR in C-NHEJ–defective cells, entirely catalyzed by the CtIP/MSH2-dependent A-EJ (Fig. 8). Hence, we propose a model for resolving CSR in which the nature of the lesion determines the choice of end-joining pathway: C-NHEJ preferentially repairs DSBs formed from nearby SSBs in opposite DNA strands, whereas A-EJ pathways would repair more staggered DSBs generated by the processing of distant SSBs (Fig. 8A).

Several studies have shown how the initial processing of DSBs influences the occurrence of an end-resection step at the break, which seems to control the decision of whether the DSB will be repaired by homology-dependent pathways (HR or microhomology-mediated A-EJ) or by C-NHEJ (47, 48). Recent reports have shown that 53BP1 and γH2AX inhibit the resection of DNA ends, which favors the C-NHEJ pathway over the A-EJ pathway for the repair of blunt DSBs in G1 cells (47, 49, 50). Our findings demonstrate that at least the CtIP-mediated A-EJ pathway is active even though γH2AX and 53BP1 are present at high concentration in the S-regions, suggesting this pathway is not significantly affected by these DNA damage factors during CSR, at least when the breaks are staggered (see Figs. 2, 7). Moreover, partial knockdown of H2AX did not affect the level of CSR in AIDlow cells, which rely mainly on this A-EJ for CSR (Fig. 7). Our conclusions do not contradict previous reports indicating that γH2AX and 53BP1 prevent end resection. The observed differences can be readily explained due to the different nature of the systems used in each study. Most previous studies on the influence of 53BP1 or γH2AX over the A-EJ pathway used systems in which DSBs were generated by either RAG1/2 during V(D)J recombination or by I-SceI meganuclease in modified S-regions (47, 49–51). In both cases, the DSBs generated are either blunt or have very short overhangs (4 nt) by the nature of the nicking endonucleases. However, AID initiates the formation of SSBs for CSR through DNA processing by multiple enzymes and in which components of the BER pathway (UNG and APE1) play a central role (4). Only those SSBs that are sufficiently near to each other in opposite DNA strands can form a spontaneous DSB and are likely protected from resection by 53BP1 and γH2AX. In contrast, SSBs in opposite strands resulting from deamination of two relatively distant dC need processing by the MMR pathway to be converted into DSBs, which might only then trigger the accumulation of γH2AX and 53BP1 at the damage site (44, 45, 52). Accordingly, MMR is particularly important to allow CSR using the pre-Sμ when the Sμ tandem repeats are deleted (53), because the lower AID hot spot frequency in the pre-Sμ will most preferentially accommodate staggered breaks. The proposed model for processing SSBs by MMR during CSR predicts that the U:G mismatch is recognized by the MSH2/MSH6 complex. After this, EXO1 together with the MLH1-PMS2 heterodimer are recruited to the damage site and induce the excision of the mutated strand from a nearby SSB toward the mismatch. EXO1 requires a nick and would continue until it finds an SSB in the opposite strand (44, 54), but recent evidence indicates that EXO1 functions as a scaffold in

FIGURE 6. The MMR pathway is essential for CSR in AIDlow cells. (A) Analysis of CSR at the indicated time points for CH12F3-2 cells expressing the indicated shRNAs. Student t test; *p = 0.008, **p = 0.001. (B) AID, EXO1, and MSH2 association to the Sμ during CSR analyzed by ChIPs in CH12F3-2 cells expressing the indicated shRNAs. The average values obtained at the different time points are shown. (C) As in (B) for RPA. (D) ChIP experiments showing RPA distribution in Sμ and Sα 20 h after cytokine stimulation of CH12F3-2 cells. Error bars represent SD from at least three independent experiments.
CSR for an unknown exonuclease that would then generate a DSB with a single-strand end or overhang. Mammalian EXO1 encodes both structural and catalytic functions that play distinct roles in essential biological processes (55). In support of the proposed role for the MMR pathway during CSR, the increase in EXO1 and RPA recruitment in the S-regions observed in AIDlow cells occurs at later times and correlates with the decrease in the density of mutations in the S-regions found in these cells (Figs. 4, 6), which suggests that exonucleolytic processing needs to be recruited to produce the DSBs in these cells. MMR-generated overhangs can therefore expose homologous sequences in both DNA ends, helping the A-EJ pathway to bypass the need for CtIP-dependent resection activity that is inhibited by gH2AX and 53BP1 as previously shown in other systems (47, 49–51). Although the use of EXO1 could bypass the need of end-resection by CtIP, AIDlow cells still depend on CtIP for CSR (Fig. 4). One potential explanation for this is that during CSR, CtIP is required to maintain the resected ends generated by the MMR pathway, as was recently suggested in another system (56). Alternatively, this could be explained by the scaffold role of EXO1 in CSR (55). Our results in AIDlow cells agree with previous evidence showing decreased microhomology in switch junctions from MSH2- and EXO1-deficient mice (57, 58) as well as with more recent evidence that MMR functions upstream of the DSB in C-NHEJ mutant cells (45) (Fig. 6). Thus, we propose a model in which the spacing of the DNA lesions dictates an MMR-dependent processing that fails to be inhibited by 53BP1 or γH2AX and facilitates repair by a microhomology-mediated A-EJ. Nevertheless, it is important to point out that the microhomology-mediated A-EJ pathway we analyzed still depends on UNG activity because UNG inhibition almost completely abolishes CSR (Supplemental Fig. 3). This is in agreement with a model in which the main role of MMR is to convert distant SSBs in opposite strands into DSBs as previously suggested (44, 52).

We find less intra-S recombination at the Sμ in cells expressing lower levels of AID. Very limited analysis in WT B cells suggests that both C-NHEJ and A-EJ can mediate intra-S deletions but the situation is complex (59). A limitation of CH12F3 cells is that they may not undergo intra-S deletions through C-NHEJ and A-EJ in the same proportion than WT B cells; hence, we acknowledge that the physiological relevance (or mechanistic insight) of our result is limited. However, it does suggest that lowering the levels of AID changes the pathway of DNA repair used at the Sμ, as our other data indicate.

There are surely other factors that contribute to the choice of end-joining pathway. As expected for a mechanism that relies on DNA annealing, the level of sequence homology present near the breaks is most likely to influence how effective the microhomology-mediated A-EJ can be and how frequently it can be used. In this respect, we would expect the A-EJ to have a different efficiency in compensating for the absence of C-NHEJ depending on the isotype and accordingly to the microhomology that each S-region shares.

FIGURE 7. Reducing AID levels does not change the level of DNA damage factors at the Igh locus. (A) ChIPs showing γH2AX presence at the Igh locus during CSR in CH12F3-2 cells expressing the indicated shRNAs. Average values obtained at each time point are represented in the graph. (B) ChIPs showing the distribution of γH2AX in Sμ and Sα after 16 h of cytokine stimulation. (C) DSB assay in CH12F3-2 cells stimulated for class switching and expressing the indicated shRNAs. DSBs were labeled with TdT and biotin-deoxyuridine triphosphate as previously described (32). Biotin-labeled fragments were purified, and its distribution at the Sμ was analyzed via PCR using the primer sets indicated. (D) Representative flow cytometry analysis of CSR to IgA 24 h postcytokine stimulation in CH12F3-2 cells expressing the indicated shRNAs. DMSO or 2 μM ataxia telangiectasia mutated inhibitor KU55933 (ATMi) was added at time 0. Percentage of IgA+ cells is indicated at top right of boxed areas. (E) Western blot of total H2AX and γH2AX in CH12F3-2 cells expressing the indicated shRNAs, stimulated or not for CSR. The bars represent the average percentage for each group in the indicated cell lines. (F) As in (D) for CH12F3-2 cells expressing the indicated shRNAs. CSR efficiency relative to control cells (shGFP) after 48 h is indicated. Error bars represent SD from at least three independent experiments. *p = 0.02.
with the Sm. In the mouse, this would be maximal for IgA and minimal for IgG1, with 14% identity between Sm and Sg1 and 33% identity between Sm and Sa (19, 60). Indeed, when we analyzed Sm-Sg1 switch joins from Aicda+/−/− mice, the increase in microhomology use was less evident (data not shown), in line with previous reports in human B cells (61–63). A recent report showing that CtIP does not affect class switching to IgG1 in mouse splenic B cells is also compatible with these conclusions (64). Interestingly, some MMR mutants show a similar behavior, with the observed CSR deficiency varying with the isotype (45, 65, 66), which is consistent with our finding of A-EJ more critically relying on MMR (Fig. 6).

Although it is conceivable that reduced levels of AID in AIDlow cells can reduce the possibility of generating simultaneous DSBs in both Sm and Sa, we would expect this to prevent CSR altogether rather than choice between C-NHEJ and the microhomology-mediated A-EJ pathways. The usage of microhomology used as one of our readouts to infer the use of C-NHEJ versus microhomology-mediated A-EJ pathways can be assumed that in those cells in which CSR took place, the simultaneous DSBs did occur. Those data also support a model of differential use according to the level of deamination (Fig. 1F) (30).

In conclusion, our data demonstrate that a microhomology-biased A-EJ pathway functions during CSR to IgA in C-NHEJ–competent cells and that it processes DSBs that cannot be joined by the C-NHEJ pathway. We suggest that by determining the type of DNA breaks through the density of DNA deamination, AID activity influences the end-joining pathway that will be used for CSR. However, further work is needed to determine if A-EJ and C-NHEJ factors can bind to the same S-regions and whether they do so simultaneously. AID seems to indirectly influence the recruitment UNG and MSH2 (67), which could also contribute to determine the quality of DSBs at low concentrations of AID. We cannot rule out that AIDlow cells may attract a lower density of C-NHEJ components to the Sm if AID were to directly recruit Ku70/80 or other critical C-NHEJ factor, although to date there is no solid evidence for this. Despite these unresolved issues, our results support a molecular mechanism that could explain the increased usage of microhomology in the Sm-Sa joins that is characteristic in human AID haploinsufficient individuals (30). It is likely that CSR still preferentially proceeds by C-NHEJ in normal B cells for most isotypes (11–15), but it would be interesting to determine whether there is some type of intrinsic or extrinsic DNA lesion for which a CtIP-dependent microhomology-mediated A-EJ might be the preferred choice. For instance, the preferential use of A-EJ in processes such as chromosome translocations (25) may reflect the complex nature of the DSBs used for the translocations. It is tempting to speculate that by producing a deamination density at the Sm that favors C-NHEJ over A-EJ, the physiological levels of

FIGURE 8. Reducing AID levels improves CSR in C-NHEJ mutant cells. (A) Model for C-NHEJ and A-EJ activity during CSR in cells with WT levels (AID) or reduced levels of AID (AID+/-). Dotted areas mark regions with low or AID-induced high density of DNA SSBs. (B) Western blot analysis of AID knockdown in CH12F3-2 cells expressing the indicated shRNAs. (C) As in (B) for Lig4−/− CH12F3-2 cells. (D) Representative flow cytometry analysis of CSR to IgA 24 h post cytokine stimulation in CH12F3-2 cells expressing the indicated shRNAs. Percentage of IgA+ cells is indicated at top right of boxed areas. Relative CSR efficiency to control cells (shGFP) after 48 h is indicated. Two-tailed Student t test was used for p value test. *p = 0.003. (E) As in (D), but with Lig4−/− CH12F3-2 cells or control cells (Lig4+/+) and after 48 h of class-switching stimulation. t test, *p = 0.005, **p = 0.004. Error bars represent SD from at least three independent experiments.
AID have been optimized during evolution to reduce the risk of chromosomal translocations during CSR.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

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Supplementary Figure 1. Analysis of cell propagation, germline transcripts and AID levels in the different cell lines used in this study. (A) and (E) Propagation of CH12F3-2 cells expressing the indicated shRNAs. (B), (G), and (H) Western blot analysis of AID, Ku70 and CtIP knockdown in CH12F3-2 cells expressing shRNAs against GFP or AID (shAID-1). (C), (D) and (F) Analysis of germline transcripts by RT-PCR followed by Q-PCR in CH12F3-2 cells expressing the indicated shRNAs. (I) as in (A) but for Ligase4 mutants. (J) as in (C) for the indicated cell lines. Error bars represent standard deviation from at least three independent experiments.

Supplementary Figure 2. Distribution of Sμ-associated mutations and Sμ intra-S recombination in AIDlow cells. (A) Plot showing the position and frequency of transitions in the indicated region of pre-Sμ m.2 region in CH12F3-2 cells expressing Ugi and the indicated shRNAs 20 hours post-stimulation. (B) Representative Southern blots showing intra-Sμ recombination in IgM positive CH12F3 clones isolated after 96 hours of class switching stimulation. The Sμ in the different clones was amplified by PCR and the internal Sμ recombination was detected via Southern analysis with a probe specific for the Cμ. The Cμ probe and the primer set used are indicated in the graph. Δ indicates CH12F3 clones with intra-S recombination. The total number of clones analyzed and the percentage of clones with intra-S recombination are indicated at the right.
Supplementary Figure 3. UNG activity is essential for CSR in CtIP or Ku70-depleted cells. CSR efficiency 48 hours post-cytokine stimulation in CH12F3-2 cells expressing the indicated shRNAs. Cells expressing the UNG inhibitors (Ugi) are indicated. Error bars represent standard deviation from at least three independent experiments.
Supplemental Figure 2
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A

Location of switch junctions
CH12F3-2 cells

IgH locus

shGFP

shAID-1

nucleotides

B

Sμ deletions

Sμ deletions

CH12F3-2 clones

shGFP

shAID-1

s = 10/28 (35.7%)
s = 2/27 (7.4%)
Supplemental Figure 3
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### Supplemental Table 1

**shRNAs used in this study.**

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