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Activation-Induced Cytidine Deaminase-Initiated Off-Target DNA Breaks Are Detected and Resolved during S Phase

Muneer G. Hasham,* Kathy J. Snow,* Nina M. Donghia,* Jane A. Branca,* Mark D. Lessard,* Janet Stavnezer,† Lindsay S. Shopland,* and Kevin D. Mills*

Activation-induced cytidine deaminase (AID) initiates DNA double-strand breaks (DSBs) in the IgH gene (Igh) to stimulate isotype class switch recombination (CSR), and widespread breaks in non-Igh (off-target) loci throughout the genome. Because the DSBs that initiate class switching occur during the G1 phase of the cell cycle, and are repaired via end joining, CSR is considered a predominantly G1 reaction. By contrast, AID-induced non-Igh DSBs are repaired by homologous recombination. Although little is known about the connection between the cell cycle and either induction or resolution of AID-mediated non-Igh DSBs, their repair by homologous recombination implicates post-G1 phases. Coordination of DNA breakage and repair during the cell cycle is critical to promote normal class switching and prevent genomic instability. To understand how AID-mediated events are regulated through the cell cycle, we have investigated G1-to-S control in AID-dependent genome-wide DSBs. We find that AID-mediated off-target DSBs, like those induced in the Igh locus, are generated during G1. These data suggest that AID-mediated DSBs can evade G1/S checkpoint activation and persist beyond G1, becoming resolved during S phase. Interestingly, DSB resolution during S phase can promote not only non-Igh break repair, but also Ig CSR. Our results reveal novel cell cycle dynamics in response to AID-initiated DSBs, and suggest that the regulation of the repair of these DSBs through the cell cycle may ensure proper class switching while preventing AID-induced genomic instability. The Journal of Immunology, 2012, 189: 2374–2382.

Unlike site-specific endonucleases such as the RAG1/2 complex, AID lacks known target site specificity (12). In this context, several recent studies have shown that AID can generate point mutations at multiple non-Ig genes (22, 23). Moreover, it has been demonstrated that AID generates widespread DSBs at non-Igh locations (24–26). Although the mechanisms that target AID to Igh are at least partly understood, little is known about the mechanisms or the cell-cycle dynamics governing AID non-Igh (so-called off-target) DNA damage and repair. One model suggests that non-Igh DSBs occur at sites of DNA replication, either by passage of a fork over an AID-initiated single-strand break (SSB), or by direct attack on the fork by AID itself (22, 27). In either case, off-target DSBs would mostly arise at DNA replication sites during S phase. Consistent with this model, we found recently that homologous recombination (HR) is crucial for the repair of AID off-target DSBs. Activated B cells deficient in XRCC2, a member of the RAD51 family of HR factors, are acutely sensitive to AID activity, accumulate numerous off-target DSBs per cell, can incur massive chromosomal instability, and ultimately die (25).

HR is a high-fidelity post-G1 DSB repair pathway with roles in general genome stability maintenance in a range of organisms and cell types (28, 29). Our previous findings highlighted HR as an important genome-protective pathway in B cells, required specifically to survive the promiscuous genome-damaging activity of AID. There is growing evidence that AID-mediated genomic DSBs, if not rapidly and correctly resolved, are substrates for potentially oncogenic chromosomal translocations in stimulated B cells (13, 14, 17, 26, 30–32). Thus, the mechanisms that regulate AID activity at Igh versus off-target locations, as well as the processes that govern DNA repair at on- versus off-target locations, are of considerable importance to understanding normal immune development and the etiology of B cell malignancies.

In this study, we investigate the relationship between AID-mediated Igh (on-target) and non-Igh (off-target) DSBs, in the context of the cell cycle. We find that off-target AID-mediated
DSBs arise during G1, prior to the onset of S phase, similar to on-target CSR-initiating DSBs in the Igh locus. Importantly, we demonstrate that AID-mediated off-target DSBs can arise independently of DNA replication. We also provide evidence that repair of these off-target DSBs, which arise during G1, is largely delayed until S phase. Interestingly, we find that CSR itself can also take place during S phase. Taken together, we propose that AID generates DSBs broadly throughout the genome during G1 and that, when this occurs, delayed repair provides an opportunity to repair off-target DSBs by homologous recombination, while concomitantly ensuring CSR by end joining. We suggest that this phenomenon represents a unique cellular mechanism that allows B cells to generate isotype diversity, while simultaneously protecting the genome from chromosomal instability.

Materials and Methods

Mice

C57BL/6j (stock 000664; The Jackson Laboratory), Atm−/−, Trp53−/− (33), and Aid−/− (11) mouse colonies were maintained in pressurized, individually ventilated caging and fed standard laboratory diet. All mouse work was carried out according to Institutional Animal Care and Use Committee–approved protocols. B cells were isolated from spleens of 4- to 8-wk-old mice following euthanasia.

B cell isolation and culture

Splenic B cells were isolated by magnetic bead-based cell sorting using anti-CD43 (Ly-48) microbeads (catalog 130-049-801; Miltenyi Biotec), according to the manufacturer’s protocol. Isolated cells were tested for purity by flow cytometry analysis, staining for B220, CD43, and IgM (see flow cytometry method). Purified B cells were cultured with RPMI 1640 with l-glutamine (catalog SH30034.01; Life Technologies) supplemented with 10% (v/v) heat-inactivated FBS (catalog S11150; Atlanta Biologicals). Purified B cells at a concentration of 1 × 10^6 cells/ml were stimulated with 1 µg/ml anti-CD40 Ab (clone HM40, catalog 3553721; BD Pharmingen) and 25 ng/ml IL-4 (catalog 214-14; Peprotech) for 2 d, then readjusted to 1 × 10^6 cells/ml and restimulated with an additional 1 µg/ml anti-CD40 and 25 ng/ml IL-4. In some assays, the DNA polymerase inhibitor aphidicolin (APH; catalog A0781; Sigma-Aldrich), dissolved in DMSO and stored as 10 mM aliquots, was used. APH was added to a final concentration of 0.4 or 1.2 µM at the same time anti-CD40 and IL-4 were administered. For cell proliferation assays, B cells were incubated for 10 min with 5 µM CFSE, then transferred to fresh media and activated with or without APH (as described). Lenti-viral transduction, of total splenocytes or purified B cells, with Xvec2 short hairpin RNA or control constructs was performed, as previously described (25).

The 5-ethyl-2′-deoxyuridine incorporation assays

To determine the extent of colocalization of 5-ethyl-2′-deoxyuridine (EdU) and γ-H2AX foci, activated (22-h) Aid−/− and Aid−/− B cells were pulse labeled with 10 µM EdU for 15 min, incubated for 1 h on coverslips, fixed, and stained for γ-H2AX, as described below, for 1 h. The cells were stained for EdU, according to manufacturer’s protocol (Click-it EdU Alexa Fluor 488 Imaging Kit, catalog C-10337; Life Technologies). Samples were imaged using a Leica SP-2 spectral confocal microscope with a ×63 1.4 NA oil objective. Images were manually segmented using Imaris software (Imaris 7.2.0; Bitplane) and were scored for coassociation or purified B cells, with Xvec2 short hairpin RNA or control constructs was performed, as previously described (25).

Immunofluorescence

Cells were adhered to poly-L-lysine (catalog P8920-100ML; Sigma-Aldrich) cover slips, fixed with 3% neutral buffered formalin with 2% sucrose in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 3% FBS for 1 h. Fixed cells were incubated with primary Ab (anti-phosphorylated γ-H2AX, 1:400 dilution, catalog A300-081A; Bethyl; anti-phosphorylated S1981 ATM, 1:500 dilution, catalog 600-401-398; Rockland) overnight at 4°C, followed by secondary Ab conjugated with either Alexa 488 secondary (1:1000 dilution, catalog A11-008; Invitrogen) or tetramethylrhodamine isothiocyanate secondary (1:1000 dilution, catalog 111-26-003; Jackson ImmunoResearch Laboratories) Abs for 30 min at room temperature. Samples were mounted with Vectashield containing DAPI counterstain (catalog H-1200; Vector Laboratories) imaged by epifluorescence wide-field microscopy using a Nikon 90i upright microscope, and analyzed with NIS Elements software.

Flow cytometry

For flow cytometry, 1 × 10^6 to 1 × 10^7 cells were stained for 1 h at 4°C with an Ab-fluorophore mixture containing PE-iodotricarbocyanine anti-B220 (1:100 dilution, catalog 15-0452-82; BioLegend), PE anti-IgG1 (1:100 dilution, catalog 1010-09; Southern Biotechnology Associates), and FITC-IgM (1:100 dilution, catalog 553408; BD Pharmingen) for analysis of class switch recombination. To measure for B cell purity after magnetic bead isolation, cells were stained with PE anti-CD43 (1:400 dilution, catalog 553271; BD Biosciences), FITC anti-CD19 (1:800 dilution, catalog 5539785; BD Biosciences), PE-indodicarbocyanine anti-B220 (1:100 dilution, catalog 561879; BD Biosciences), and allophycocyanin anti-IgM (1:240 dilution, catalog 17-5790-82; eBioscience). The cells were washed with 2% FBS (in PBS) before analysis. Cell-cycle analysis was performed, as previously described, after staining genomic DNA with 20 µg/ml propidium iodide (catalog P4170; Sigma-Aldrich) (34). For flow cytometric analysis of intracellular ATM phosphoseroine 1981, cells were fixed, permeabilized, and blocked, in suspension, as described for immunofluorescence above. The cells were then incubated with anti-phosphorylated S1981 ATM (1:500 dilution, catalog 600-401-398; Rockland) overnight at 4°C, followed by secondary Abs conjugated with either Alexa 488 secondary (1:1000 dilution, catalog A11-008; Invitrogen) for 1 h at room temperature. The cells were washed with 1% FBS in PBS and analyzed. All flow cytometry analysis was performed with FACSCalibur using CellQuest Pro acquisition software (BD Biosciences) and was analyzed with Flowjo software (V8.4.6, Tree Star).

Neutral comet assay

B cells were activated with anti-CD40 plus IL-4 and treated with APH for 2 d, or were exposed to either 0 or 0.5 Gy irradiation (controls). Cell cultures were then washed with PBS, mixed with 1.5 ml 1% low melting point agarose (40°C), and spread on frosted microscope slides (catalog 12-544-5C; Fisher). After agarose polymerization, slides were immersed in 0.5% SDS with 30 mM EDTA (pH 8) for 4 h at 50°C, stored in 1× TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA [pH 8.5]) overnight, then electrophoresed for 25 min in TBE at 7 mA. Slides stained with 2.5 µg/ml propidium iodide for 20 min and imaged by epifluorescence microscopy, as described above. Exhibiting comet tails were quantified as a fraction of the total cells imaged.

Immunoblot analysis

Whole-cell extracts were prepared from total splenocytes or purified splenic B cells, as described (25). A total of 20 µg whole-cell extracts, quantified by Bio-Rad DC (catalog 500-0114; Bio-Rad), was electrophoresed in 10% SDS polyacrylamide Tris-glycine gels (SDS-PAGE, catalog S5850; Lonza), and the proteins were blotted onto a polyvinylidene difluoride membrane (Immobilon-P, catalog IPVHH00010; Millipore) for Western analysis, according to ECL Plus Western blotting detection protocol (catalog RPN2132; GE Healthcare). The following primary Abs were used: anti-p53, R-19 p53 (1:200 dilution, catalog sc1313; Santa Cruz Biotechnology); anti–phospho-p53, Ser15 (catalog 9284, 1:1,000; Cell Signaling); anti–anti-ATM, D222 (dilution 1:1,000, catalog 2873; Cell Signaling); anti–phospho-ATM, Ser1981 (1:1,000 dilution, catalog 10H11.E12 [1:2,000 dilution, catalog 05-740; Millipore]; anti–heat shock protein 90 (1:1,000 dilution, catalog ADA-SPA-835; Enzo Life Sciences); and anti-actin (1:5,000 dilution, catalog #8227-50; Abcam). HRP-labeled secondary Ab was used to detect the primary Abs (anti-rabbit, 1:10,000 dilution, catalog NA934V; GE Healthcare; anti-rat, dilution 1:5,000, catalog 112-035-003; Jackson ImmunoResearch Laboratories; and anti-mouse, dilution 1:15,000, catalog 32320, Thermo Scientific). Detection of the immunoblots was performed using ECL Plus system (catalog RPN2132; GE Healthcare). The blot was exposed to X-OMAT Blue XB film (catalog NEF596; Kodak) to detect the protein bands.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was carried out as previously described (25, 34). After fixation and permeabilization, samples were alkali denatured and stored in 70% ethanol. Samples were blockaded with...
salmon sperm DNA-tRNA-formamide, and then hybridized with iodocarbocyanine-labeled bacterial artificial chromosome (BAC) probes. Cotterisks were washed with 50% (w/v) formamide in 2 × SSC and were then stained for γ-H2AX and imaged, as described above.

**PCR analysis of CSR products**

Genomic DNA was prepared by standard protocols. A total of 200–500 ng input DNA was used per reaction. PCR was performed using GoTaqGreen (M172A; Promega), with PCR parameters, as follows: 94˚C, 2 min, followed by 35 cycles of 94˚C for 15 s, 64˚C for 30 s, 72˚C for 2 min, and a final incubation at 72˚C for 10 min. The primers used were as follows: SuF primer (primer 1), 5’-CCGACTCACTTGGCTTGATCCGATC-3’; SuR primer (primer 2), 5’-ATGTTGAATGCTGGCTGTCGGGCTGGG-3’ (35); and Syl primer (primer 3), 5’-GTATAAGTACCCAGGCTGACGAC-3’. xrc2 PCR was done as previously described (36).

**Statistics**

Unless otherwise indicated, quantitative data are represented as means derived from at least three independent biological samples. Confidence intervals (95% CI) are indicated as error bars, and p values were calculated by the Student t test.

**Results**

**HR repairs recurrent, AID-initiated DSBs at both IgH and non-IgH target sites**

Several recent studies have identified a number of non-Ig (non-Igh) genes that are recurrently mutated by AID in activated B cells (5, 6, 22–24). Although we demonstrated that AID generates multiple, non-Igh (off-target) DSBs, it was not initially known whether off-target DSBs arise at specific, recurrent locations (25). To answer these questions, we identified Bcl6, Myc, Bcl11a, and CD93 as a set of candidate genes, previously published as frequent targets for AID-dependent hypermutation, to test for recurrent AID-dependent DSBs (23, 37). Gene-specific FISH plus immunofluorescence for phospho (γ)H2AX foci was used to measure the fraction of AID-initiated DSBs at each of these loci in Aid+/− (wild-type) versus Aid−/−-activated B cells (Fig. 1). These assays were performed in B cells with or without an XRCC2-specific short hairpin RNA knockdown construct (XKD) that we have previously validated for inhibition of XRCC2-mediated HR in primary B cells (25). IgH, as the major known physiological target for AID, was used as a positive control; and Ltb and Mef2b, which are rarely targeted for point mutations, were used as negative controls. Cells were evaluated for colocalization of γ-H2AX and FISH signals, and the percentage of cells showing at least one colocalization event was determined for each gene (Fig. 1B–D). We observed the highest rate of AID-dependent DSBs at the IgH locus, as expected, and recurrent DSBs, at varying frequencies, at Bcl6, Myc, Bcl11a, and CD93 (Fig. 1B, 1C). The frequency of γ-H2AX foci at all locations was at background level in Aid−/− B cells, confirming AID dependency for both on- and off-target breaks. Interestingly, the frequency of γ-H2AX foci localizing to the IgH locus was significantly elevated in cells with Xrc2 knockdown, suggesting a role for HR in the repair of IgH, as well as non-IgH, DSBs. Because HR is dispensable for Ig class switch recombination, this result suggests that HR can catalyze error-free repair of DSB within IgH, as it does for DSBs at other genomic locations. These data, together with our previously published results, clearly establish that AID-dependent supernumerary γ-H2AX foci (those numbering >2 per cell) included recurrent, non-IgH DSBs at multiple specific locations. This corroborates our earlier findings of a key role for HR in the repair of off-target DSBs, and now suggests that HR is able to access DSBs in the IgH locus as well (25).

**AID-dependent genomic breaks occur, but are not resolved, before the G1/S transition**

It has been shown definitively that AID-mediated IgH DSBs are generated during the G1 phase of the cell cycle (15, 38). Although there has been no previous analysis of cell-cycle dependency for repair of off-target DSBs, the requirement for HR suggests that off-target repair occurs, at least in part, in the S or G2 phase. To determine the cell-cycle dependency of AID-induced DSBs, we developed an assay system to distinguish G1 from S phase chromosomal damage in mouse B cells (Fig. 2A, 2B). Either cell cycle arrest at the G1/S boundary or S-phase enrichment without complete arrest was experimentally induced by exposure to different doses of the DNA polymerase inhibitor APH. Exposure to 1.2 μM APH induced abrupt arrest at the G1/S transition point and led to accumulation of cells with a G1 DNA content, whereas 0.4 μM APH induced an initial accumulation of cells in G1 phase, and slowed but did not halt S-phase progression (Fig. 2C–F; Supplemental Fig. 1A, 1B). Exposure to either 0.4 or 1.2 μM APH inhibited overall B cell expansion (Fig. 2C). However, both CFSE dilution and EdU incorporation analyses confirmed that 0.4 μM-treated B cells continued to cycle but to slowly transit S phase, eventually completing replication, whereas 1.2 μM-treated B cells arrested with minimal DNA replication or cellular expansion (Fig. 2D–F). B cells arrested at the G1/S boundary (1.2 μM) showed a 10-fold increase in comet tails relative to untreated controls, reaching levels comparable to 5 Gy irradiation (Fig. 2A, 2B). This effect was not due to general inhibition of DSB repair by APH, as exposure to neither 0.4 nor 1.2 μM APH impaired overall cell viability, or altered DSB repair kinetics in irradiated B cells (Supplemental Fig. 1B–F). Importantly, after treatment with 1.2 μM APH, Aid+/− B cells showed ∼2-fold higher frequency of comet tails than Aid−/− B cells (0.71 versus 0.34, respectively). This demonstrates AID dependency for the genomic damage, and

![FIGURE 1. Homologous recombination repairs AID-mediated DSBs at IgH and non-IgH sites.](http://www.jimmunol.org/DownloadedForm/332x650 to 406x717)
FIGURE 2. AID-initiated DSBs occur in G1 phase of the cell cycle. (A) Representative wide-field images from neutral comet assays showing comet-negative (top panels) or comet-positive (bottom panels) cells. Comet tails are segmented with dashed lines (original magnification ×60). (B) Bar chart showing quantification of comet-positive Aid+/+ versus Aid−/− purified B cells, 2 d after activation. As a control, purified Aid+/+ or Aid−/− B cells were exposed to 5 Gy ionizing irradiation and subjected to comet analysis. (C) Average number of cells in culture after 4 d of activation; data represent five independent experiments, with three to five mice per experiment. (D) Activated, splenic B cells were cultured with 0, 0.4, or 1.2 μM APH and loaded with 5 μM CFSE. Dilution of CFSE after 5 d was measured by flow cytometry. Boxes show, from right to left, undivided, CFSE-diluted, and unlabeled cells, respectively. The percentage of the population within each gate is shown. Data shown represent B cells pooled from three independent mice. (E) Activated, splenic B cells were incubated with 10 μM EdU and assayed for incorporation via flow cytometry every 0.5 d. The percentage of the population within each gate is shown. Data shown represent B cells (Fig. 3B) (25). This is consistent with prior reports that AID is physiologically active during G1, supports a model for off-target DSBs in which AID initiates bona fide DSBs at multiple non-Igh loci during the G1 phase of the cell cycle, analogous to CSR-initiating DSBs (15, 24, 38).

Unrepaired off-target DSBs are detectable during S phase but are independent of DNA replication sites

One model for the generation of off-target DSBs by AID suggests that they are DNA replication dependent, arising either by passage of a replication fork over an AID-generated SSB or by AID attack of the replication fork itself (23, 27). Either mechanism predicts that AID-dependent supernumerary (>2) DSBs should occur largely during S phase, and coincide with sites of active DNA replication. To directly test these predictions, we evaluated colocalization of AID-mediated DSB foci with regions of nucleotide incorporation (replicating DNA). XKD B cells from either Aid+/+ or Aid−/− mice were activated, pulse labeled with EdU, and costained for γ-H2AX foci and EdU incorporation (Fig. 3A) (39). Consistent with our published results, XRC2-deficient Aid−/− B cells showed a significant increase in supernumerary (>2) γ-H2AX foci relative to Aid+/+ B cells (Fig. 3B) (25). This is consistent with our previously published results, showing that AID induces widespread off-target genomic DSBs. Costained cells were imaged by three-dimensional confocal microscopy to quantify coassociation of γ-H2AX foci with EdU-labeled DNA (Fig. 3C). We observed a higher fraction of AID-mediated DSBs in EdU-positive (replicating) cells than in EdU-negative cells, indicating that supernumerary DSBs accumulate in the S or G2 phase (Fig. 3C). Surprisingly, however, a significant fraction of the AID-initiated γ-H2AX foci present in the EdU+ cells was not colocalized with the EdU labeling (Fig. 3C, 3D). This indicates that, although off-target DSBs accumulate during the S phase of the cell cycle, they are frequently unassociated with sites of active nucleotide incorporation. Although we do not rule out that some AID-dependent DSBs may coincide with DNA replication sites, these results demonstrate that AID-induced genomic breaks often occur independent of DNA replication forks, and thus form γ-H2AX foci that are not coincident with replication sites. This, together with prior reports that AID is physiologically active during G1, supports a model for off-target DSBs in which AID initiates bona fide DSBs at multiple non-Igh loci during the G1 phase of the cell cycle, analogous to CSR-initiating DSBs (15, 24, 38).

AID-initiated DSBs do not strongly activate the G1/S checkpoint

Our data suggest that AID-initiated DSBs, which arise in G1, can persist until S phase. This implies that these DSBs do not activate conventional G1/S DNA damage checkpoints, and thus avoid repair until traversal into S phase. To directly test this, we measured activation of p53 and ATM, both key components of the G1/S DNA damage checkpoint, in activated B cells (Fig. 4). As a positive control, splenic B cells were exposed to ionizing radiation (IR) at doses ranging from 0.1 to 3.0 Gy, confirming that even at the lowest tested doses, nonspecific DNA DSBs could induce checkpoint activation (Fig. 4A, 4B). Western blot analysis of either total or phosphorylated p53 confirmed an increase in both total protein levels and the activated phospho-p53 even at the lowest IR level tested (0.1 Gy), with stronger activation at higher IR doses (Fig. 4B). These data show that primary splenic B cells are sensitive to exogenous DSBs and are able to activate p53 in response to total levels of DNA breakage. By contrast, activation
FIGURE 3.  AID-dependent DSB foci are present in S phase, but are independent of replication forks. (A) Aid+/+ or Aid−/−-activated XKD B cells were cultured for 1 d and pulse labeled with EdU prior to fixation. EdU was detected following conjugation to Alexa Fluor 488 (green), and γ-H2AX was detected with tetramethylrhodamine isothiocyanate (red)-conjugated Ab and imaged by confocal immunofluorescence microscopy. Three-dimensional image reconstruction was used to analyze cells for colocalization of EdU with γ-H2AX foci. Examples of γ-H2AX foci localized (arrows) and not colocalized (arrowheads) with EdU are highlighted in single optical sections. Pie charts indicate the percentage of cells with EdU-positive (green) versus EdU-negative cells (original magnification ×60). (B) Aid+/+ and Aid−/− XKD B cells were scored for the number of γ-H2AX foci and grouped into bins of no foci (none), 1−2 foci, or >2 foci (supernumerary). Bar chart shows the fraction of cells within each bin for Aid+/+ (gray bars) versus Aid−/− (white bars) B cells. (C) Bar chart showing the fraction of activated Aid−/− B cells that were either EdU+ or EdU−. (D) Bar chart showing the fraction of γ-H2AX foci per Aid+/+ or Aid−/− cell that was unassociated with EdU-labeled DNA. For each condition, 20 individual cells were imaged (22−51 optical sections per cell); error bars represent 95% CI of the mean, and the p values were calculated using t-tests.

Discussion

In this study, we have shown that AID initiates non-Igh (off-target) DSBs during the G1 phase of the cell cycle, and these breaks can persist until S phase for repair. We further demonstrate that AID-initiated DSBs arise independently of DNA replication. We provide evidence that AID-mediated DNA breaks are generated during G1, but initially avoid activating the G1/S checkpoint, and then become sensed and repaired during S phase. Interestingly, we find that CSR-initiating DSBs within Igh may, at least under some circumstances, undergo bona fide class switch recombination.

of AID in wild-type splenic B cells by anti-CD40 plus IL-4, which induced γ-H2AX foci approximately equivalent to 0.5 Gy IR, did not strongly induce p53 or phospho-ATM (Fig. 4C, 4D). Whereas we did observe slightly higher levels of phospho-p53 in Aid+/+ than Aid−/− B cells, this occurred in both anti-CD40 and anti-CD40 plus IL-4−treated cells, suggesting that it was not directly related to CSR or AID (Fig. 4D). We did not observe differences in phospho-ATM expression between Aid+/+ and Aid−/− B cells by immunofluorescence, intracellular flow cytometry, or Western blot assays, irrespective of activation (Fig. 4E–G, Supplemental Fig. 2). This suggests that the observed p53 activation was non-specific, and most likely associated with general proliferation induced by anti-CD40 rather than AID-induced DSBs. These results, together with recent studies that have investigated ATM and p53 in germinal center B cells, imply that AID-initiated off-target DSBs, induced after anti-CD40 exposure, do not strongly activate the G1/S checkpoint, at least immediately following their induction (40–44).

G1/S transition and class switch recombination

Previously published data have shown that CSR-initiating DSBs within Igh are generated during G1 and rapidly dissipate as cells traverse into and through S phase (15, 24, 38). Because G1/S arrest appears to forestall resolution of AID-dependent off-target DSBs, the effect of G1/S arrest on resolution of AID-dependent CSR-initiating DSBs was also evaluated (Fig. 5, Supplemental Fig. 3). Activated B cells were treated with 0, 0.4, or 1.2 μM APH, and class switch recombination was quantified by a PCR assay to directly measure recombination products between the Sp and Sy1 elements of the Igh locus (Fig. 5A). As negative controls for Sp-Sy1 recombination, PCR analysis was also carried out for either unstimulated B cells or nonlymphoid DNA isolated from mouse tail. As a CSR specificity control, all assays were carried out in parallel in Aid+/+ and Aid−/− samples. Recombination products were readily detectable in both untreated and 0.4 μM APH-treated B cells isolated from Aid+/+ mice (Fig. 5A). By contrast, recombination efficiency in 1.2 μM APH-treated B cells was reduced ∼30-fold relative to untreated controls, showing background levels similar to Aid−/− B cells (Fig. 5A, Supplemental Fig. 3). Consistent with this finding, we found that exposure to 1.2 μM APH completely abrogated both Ig class switching (Fig. 5B, 5C). This effect was not due to impaired proliferation, as the B cells treated with 0.4 μM APH showed dramatically reduced cellular proliferation, but underwent CSR as efficiently as untreated samples (Fig. 5B). Furthermore, the absence of recombination products or switching in 1.2 μM APH-treated B cells was not due to AID inhibition (Supplemental Fig. 4). These results suggest that, at least under some conditions, CSR-initiating DSBs, although clearly generated by AID during G1, can be recombined in cells that traverse the G1/S boundary and enter into S phase (3, 5, 15, 24, 38) (Fig. 6).
Aid analysis of total ATM and phospho-ATM (pATM S1981) from with 0 or 3 Gy ionizing irradiation were used as controls. Representative images from untreated or activated stained for ATM phosphorylated at serine 1981 (green). DAPI counterstained (blue). (stained for point factors. (Aid+/+, middle panel ionizing irradiation at doses from 0 to 3.0 Gy. Average percentage of phospho-ATM–positive cells, determined from three independent experiments, is indicated. (B) Western blot analysis of total p53 (encoded by Trp53) in extracts from Aid+/+ and Aid−/− B cell nuclei stained for γ-H2AX (green) after 0 or 5 Gy ionizing irradiation. DNA was DAPI counterstained (blue). Bar chart (below) shows average number of γ-H2AX foci per cell after 0, 0.1, or 0.5 Gy ionizing irradiation. Also shown are the average numbers of γ-H2AX foci per cell in activated XKD Aid+/+ versus Aid−/− B cells. Error bars represent 95% CI; the p value was calculated by t tests. For each assay, 20 individual wells were used. (C) Western blot analysis of total p53 (encoded by Trp53) and p53 phosphorylated at serine 15 (phospho-p53) in extracts from Trp53+/+ and Trp53−/− B cells after ionizing irradiation at doses from 0 to 3.0 Gy. Top panel. Shows phospho-p53; middle panel, shows total p53; and lower panel, shows β-actin (loading control). (D) Western blot analysis of phospho-p53 and total p53 from Aid+/+ and Aid−/− B cells cultured with anti-CD40 plus IL-4 (activated) or anti-CD40 alone (control). (E) Representative images from untreated or activated Aid+/+ and Aid−/− B cells stained for ATM phosphorylated at serine 1981 (green). ATM+/+ and ATM−/− B cells treated with 0 or 3 Gy ionizing irradiation were used as controls. DNA was counterstained with DAPI (blue). (F) Histograms of untreated or activated Aid+/+ and Aid−/− B cells intracellularly stained for phospho-ATM serine 1981 and detected by Alexa 488-conjugated secondary Ab. Average percentage of phospho-ATM–positive cells, determined from three independent experiments, is indicated. ATM+/+ and ATM−/− B cells treated with 0 or 3 Gy ionizing irradiation were used as controls. (G) Western blot analysis of total ATM and phospho-ATM (pATM S1981) from Aid+/+ and Aid−/− B cells cultured with anti-CD40 plus IL-4 (activated) or anti-CD40 alone (control). Heat shock protein 90 was used as loading control. Original magnification ×60 (A and B), ×63 (E).

FIGURE 6. Model for cell-cycle modulation and coordinated repair of AID-initiated DSBs. AID initiates DSBs in G1 phase of the cell cycle. DSBs that fail to resolve can bypass the G1/S checkpoint and proceed into S phase for resolution.

recent studies have suggested that other, non-Igh genes may also be targeted by AID for point mutations, albeit at significantly lower frequencies (23, 26, 37, 45). AID has also been implicated in DSBs at non-Igh genes, although the precise mechanisms, frequency, and relationship to off-target point mutations have been unresolved (24, 25). We now show that, in addition to the Igh locus, AID initiates recurrent DSBs at multiple other sites throughout the genome. We further demonstrate that homologous recombination-defective cells accumulate un repaired DNA breaks in Igh and in various non-Igh sites, demonstrating a role for HR in the resolution of both on- and off-target damage. Because productive class switch recombination is known to require DNA end joining, we suggest that HR mediates high-

FIGURE 5. CSR can occur during S phase. (A) The presence of Sµ to Sγ recombination products was detected by PCR from genomic DNA isolated from activated Aid+/+ or Aid−/− B cells treated with 0, 0.4, or 1.2 μM APH. Internal Sµ and Xrcc2 (loading control) products were amplified as controls. Negative controls included B cells treated with anti-CD40 alone and genomic DNA prepared from mouse tails. Band intensity of the recombination products was determined by line scans (Supplemental Fig. 3), and the sum of intensities for the recombination products was quantified relative to loading control. Ratios are shown below the gel images. (B) Representative FACs plots showing CD45R/B220 and IgG1 expression of activated Aid+/+ and Aid−/− B cells, treated with 0, 0.4, or 1.2 μM APH after 4 d in culture. (C) Bar chart showing the percentage of IgG1+ B cells from activated Aid+/+ (gray bars) and Aid−/− (white bars) cultures treated with 0.4, or 1.2 μM APH after 4 d in culture. The error bars are the 95% CI from five independent experiments (3–5 mice per experiment); p values determined by t test.

during S phase. We propose that these cell-cycle dynamics allow for coordination of repair at both Igh and non-Igh locations in cells harboring widespread damage from AID (Fig. 6). This would promote Ig class switching while simultaneously reducing risk of deleterious chromosomal rearrangements.

There is a growing body of evidence that shows AID induces both point mutations and DSBs at numerous non-Igh (off-target) locations throughout the genome (23–25). Whereas the Igh locus is recognized as the preferred, physiological target for AID,
fidelity repair of AID-induced Igh DSBs leading to restoration of the original template without class switching. Although XRCC2-mediated HR is known to promote avian Ig pseudogene conversions, we show a role for HR within the Igh locus in mammalian cells (46).

Two possible models for AID-initiated off-target DSBs can be envisaged. First, AID may generate base-pair mismatches that are directly converted into DSBs via processing of the mutated bases. Excision of two closely apposed bases, leading to juxtaposed single-strand nicks, would generate a DSB. Alternatively, induction of single-strand nicks could produce fragile sites that are converted to frank DSBs by mechanical stress. In either case, AID-mediated DSBs could arise independent of cell-cycle stage. The second model, by contrast, suggests that off-target DSBs are replication dependent, arising either by passage of a replication fork over an AID-initiated SSB, or by direct attack on the fork itself by AID (22, 27). By this model, AID-initiated off-target DSBs should largely coincide with sites of active nucleotide incorporation during S phase. We now demonstrate that AID-dependent off-target DSBs, like those occurring in Igh, are generated during the G1 phase of the cell cycle and occur largely independently of DNA replication sites. Our findings indicate that off-target DSBs arise by mechanisms analogous to those that induce CSR within the Igh locus. In this context, non-Igh DSBs could be considered the products of an ectopic CSR-like process.

This poses an interesting dichotomy. CSR-initiating DSBs within Igh resolve predominantly in G1 via classical nonhomologous end joining or microhomology-mediated end joining. By contrast, off-target DSBs are preferentially repaired by HR, which is repressed during G1 but active in post-G1 cells (25). We now find that both off-target DSBs and Igh breaks are enhanced by HR deficiency (Fig. 1B, 1C), suggesting a multiphasic response to AID-initiated DSBs. We speculate that, under normal circumstances, the majority of Igh S region DSBs are resolved via end joining during G1 (15). However, we show that S region DSBs can be resolved during S phase. Together, these findings suggest a cellular fail-safe mechanism—S region DSBs that fail to recombine in G1 can then be recombined later in S phase. Moreover, we now demonstrate that HR can function in the Igh locus. We propose that S region DSBs that fail to undergo a switch recombination (in either G1 or S) can be repaired by high-fidelity HR, ensuring cell survival and providing another chance to switch in the next cell cycle.

How some AID-mediated DSBs that arise during G1 evade canonical checkpoint activation and persist until S phase for repair is not understood. One possibility is that there are too few AID-dependent DSBs to reach a threshold level for checkpoint activation. Although we cannot definitively rule out this possibility, previous reports have documented p53 activation in B cells stimulated by LPS plus anti–5-dextran, demonstrating checkpont competence in mature splenic B cells (40). Consistent with these, we show that splenic B cells are acutely sensitive to checkpoint activation by IR-induced DSBs (Fig. 4). Thus, another possibility is that checkpoint initiation by AID-mediated DSBs is context dependent, such that some stimuli (e.g., TLR signaling after LPS exposure) initiate checkpoints, whereas others (e.g., CD40 signaling) do not. This may be physiologically important, as CD40 signaling is key in germinai center formation, whereas TLR signaling is not.

Given that we find neither ATM nor p53 to be initially activated by AID-induced damage, Atm or Trp53 mutant B cells might be expected to class switch at least as efficiently as wild type. This seems to be partly true in the case of p53, although the effect of p53 on class switching appears to be mediated by its antioxidant role, rather than its checkpoint function (3). However, defects in Atm generally impinge upon CSR, suggesting that, although ATM initially escapes activation by AID-mediated DSB activation, there are subsequently direct roles for ATM in promoting class switching. The partial inhibition of CSR seen in ataxia telangiectasia patients further suggests a direct role for ATM in promoting class switching (47). Taken together, these diverse observations support the conclusion that class switching B cells mount a multifactorial response to AID-induced DNA damage, which includes checkpoint modulation and dynamic cell-cycle regulation.

Why, and under what circumstances, should activated B cells delay the repair of AID-initiated DSBs until S phase? One possibility is that, in cells incurring numerous AID-initiated breaks, it is advantageous to delay repair until S phase to ensure that both homologous and nonhomologous modes of repair are available to properly repair appropriate targets. By this model, delayed repair would prevent resolution of off-target, non-Igh DSBs by end-joining mechanisms, which could increase the likelihood of deleterious translocations in cells harboring numerous AID-induced DSBs (Fig. 6). Delaying repair until S phase would allow homology-mediated repair of off-target DSBs, but preserve the opportunity for CSR by end-joining processes. Another intriguing possibility is that some CSR events may involve S phase, even in the absence of supernumerary DSBs at non-Igh locations. Previous studies have shown that physiological class switching is cell-division linked, in which increased efficiency of class switching correlates with increased number of cellular divisions. Our data now show that proliferation per se is not strictly necessary for B cells to class switch. Because cells treated with 0.4 µM APH, and therefore enriched in S phase and prevented from undergoing multiple rounds of cell division, are able to switch as efficiently as untreated wild-type B cells, we suggest that S-phase transit is sufficient to promote class switch recombination, but that proliferation enhances CSR and amplifies switched subclones within a B cell population. In this context, both S-phase progression and overall proliferation combine to magnify physiological class switching.

Mechanistically, S phase may promote long-range recombination, which occurs at an unexpectedly high rate in class switching B cells (35, 48). Finally, it is clear that both end-joining and homology-mediated recombination modes of DSB repair are used for different purposes in activated B cells. A major question is how repair pathway choice is regulated and how specific modes of repair might be spatially directed to particular target sites. Although this question is not yet resolved, it is likely that S-phase progression influences the balance between end-joining and homology-mediated modes of repair. Repair choice may be particularly relevant in B cells undergoing CSR. In support of this notion, 53BP1, a key regulator of DNA end resection, modulates both the type of end-joining repair within S regions and the likelihood of ATM activation by persistent S region breaks (47, 49–51).

In summary, we have shown in this study that AID initiates recurrent DNA DSBs at numerous locations throughout the genome, that these DSBs can arise during G1 independently of DNA replication, and that HR preferentially influences off-target DSB repair. This highlights a dichotomous response to Igh versus non-Igh DSBs in activated B cells. Overall, our findings indicate a tightly regulated and highly orchestrated response to AID-initiated genomic damage that involves both cell-cycle regulation and DNA repair pathway choice. Elucidating the detailed mechanisms that link AID, cell-cycle dynamics, and DSB repair.
pathways provides insight into normal adaptive immune development and the etiology of B cell neoplasms associated with genomic instability.

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References

