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Mismatch Repair Proteins MSH2, MLH1, and EXO1 Are Important for Class-Switch Recombination Events Occurring in B Cells That Lack Nonhomologous End Joining

Jennifer Eccleston,* Catherine Yan, † Karen Yuan,* Frederick W. Alt, ‡,§,¶,‖ and Erik Selsing*

In the absence of core nonhomologous end-joining (NHEJ) factors, Ab gene class-switch recombination (CSR) uses an alternative end-joining (A-EJ) pathway to recombine switch (S) region DNA breaks. Previous reports showing decreased S-junction microhomologies in MSH2-deficient mice and an exonuclease 1 (EXO1) role in yeast microhomology-mediated end joining suggest that mismatch repair (MMR) proteins might influence A-EJ–mediated CSR. We have directly investigated whether MMR proteins collectively or differentially influence the A-EJ mechanism of CSR by analyzing CSR in mice deficient in both XRCC4 and individual MMR proteins. We find CSR is reduced and that Igh locus chromosome breaks are reduced in the MMR/XRCC4 double-deficient B cells compared with B cells deficient in XRCC4 alone, suggesting MMR proteins function upstream of double-strand break formation to influence CSR efficiency in these cells. Our results show that MLH1, EXO1, and MSH2 are all important for efficient A-EJ–mediated CSR, and we propose that MMR proteins convert DNA nicks and point mutations into dsDNA breaks for both C-NHEJ and A-EJ pathways of CSR. We also find Mlh1-XRCC4− B cells have an increased frequency of direct S junctions, suggesting that MLH1 proteins may have additional functions that influence A-EJ–mediated CSR. The Journal of Immunology, 2011, 186: 2336–2343.

B cells switch from the production of IgM Abs to other classes of Abs (IgG, IgE, or IgA) by the process of class-switch recombination (CSR); CSR alters the Ab effector function without changing the Ag specificity. Ab effector functions are encoded in the constant gene segments located within the Igh (Igh) locus. DNA breaks are generated in switch (S) regions located upstream of constant gene segments and then joined through long-range recombinational events, bringing a downstream constant gene segment into close proximity with the Ag-specific V region gene segments, to generate different Ab isotypes (1).

One factor required for the generation of DNA breaks in S regions is activation-induced cytidine deaminase (AID) (2). AID deaminates cytidine residues to uracil residues generating U-G mispairings in S-region sequences (2–7). Current models suggest that AID U-G mispairings are recognized by DNA repair factors such as mismatch repair (MMR) and base excision repair (BER) proteins, which, in turn, are important for generating dsDNA breaks in S regions (8–14).

In the absence of each of the individual MMR proteins, MSH2, MSH6, MLH1, PMS2, or exonuclease 1 (EXO1), CSR is reduced ~2- to 5-fold compared with wild-type (15–19). Previous studies have shown that U-G mispairings are targets of the MMR pathway (20–22), which suggests that MMR proteins recognize U-G mispairings in S regions, and that MMR protein activity at S-region U-G mispairings may be similar to the activity of MMR proteins during DNA repair. Evidence suggests that MMR protein activity occurring in S regions increases the frequency of blunt or nearly blunt dsDNA breaks that can be joined by nonhomologous end-joining (NHEJ) factors (11–14).

Factors involved in the NHEJ pathway play a major role in joining S-region DNA breaks during the CSR process (1). Characteristic of NHEJ, S junction structures in wild-type mice are direct (defined as no homology present at the junction site) or display short stretches of microhomology (23). An alternative end-joining (A-EJ) pathway can join S-region DNA breaks in the absence of the core NHEJ factors Ku70, Ku80, XRCC4, or DNA ligase IV, but at a cost of increased Igh-associated chromosomal translocations and reduced CSR efficiency (24–29). The more error-prone, and less efficient, A-EJ pathway used in the absence of XRCC4 results in S-region DNA junctions that exhibit microhomologies mostly ranging in length from 1–7 nucleotides but extending up to 20 nucleotides (24). The pattern of junction structures during A-EJ is different from the pattern observed in wild-type mice; wild-type mice S junctions display an ~40% frequency of direct junctions and few S junctions with greater than four nucleotides of microhomology (23).

During CSR, MMR protein activity has been proposed to lead to the generation of blunt or nearly blunt dsDNA breaks for NHEJ (11–14). To investigate whether MMR proteins have an effect on

*Immunology Program, Department of Pathology, Tufts University School of Medicine, Boston, MA 02111; †Division of Experimental Pathology, Pathology Department, Beth Israel Deaconess Medical Center, Boston, MA 02115; 2Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115; 3The Children’s Hospital, Harvard Medical School, Boston, MA 02115; 4Immune Disease Institute, Harvard Medical School, Boston, MA 02115; and 5Department of Genetics, Harvard Medical School, Boston, MA 02115

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Address correspondence and reprint requests to Dr. Erik Selsing, Tufts University School of Medicine, 150 Harrison Avenue, Boston, MA 02111. E-mail address: erik.selsing@tufts.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: A-EJ, alternative end-joining; AID, activation-induced cytidine deaminase; CSR, class-switch recombination; EXO1, exonuclease 1; FISH, fluorescence in situ hybridization; MMR, mismatch repair; NHEJ, nonhomologous end-joining; S, switch.

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A-EJ, we crossed mice deficient in Msh2, Mlh1, or Exo1 with mice that have a conditional deletion of the Xrc4 gene in mature B cells. We found that MMR proteins are important for CSR in the absence of NHEJ and suggest that MMR protein activity increases the frequency of DNA breaks that can be used for CSR. Our results demonstrate that MMR proteins are important for A-EJ-mediated CSR, and we propose that MMR proteins function collectively to increase the frequency of DNA substrates for CSR end-joining pathways.

Materials and Methods

Mouse strains

Exo1−/−, Mlh1−/−, and Msh2−/− mice (30-32) were crossed with conditional CD21 cre Xc/− (cXc−) mice described previously (24) to generate Exo1−/− cXc−, Mlh1−/− cXc−, and Msh2−/− cXc− mice. These mice are referred to as Exo1-XRC4−, Mlh1-XRC4−, and Msh2-XRC4− in this text. All animal studies have been reviewed and approved by the Tufts Medical Center Division of Laboratory Animal Medicine.

In vitro B cell cultures for flow cytometry

Splenic B cells were purified by negative selection (Easy Sep Mouse B cell Enrichment kit; StemCell Technologies) (11). Exo1-XRC4− and Mlh1-XRC4− sets of experiments used B cells that were cultured at 0.5 × 10^6/ml. Msh2-XRC4− experiments used B cells that were cultured at 0.7 × 10^6/ml. B cell cultures were stimulated to induce IgG3 (20 μg/ml LPS), IgG1 (0.5 μg/ml anti-CD40 + 20 ng/ml IL-4), or IgG2a (20 μg/ml LPS + 100 ng/ml IFN-γ) CSR.

Fluorescence in situ hybridization

Metaphases were prepared from B cells stimulated in culture with either LPS (20 ng/ml from Sigma) or anti-CD40 (0.5 μg/ml from BD Pharmingen or ebioscience) plus IL-4 (20 ng/ml from PeproTech or ebioscience) to induce CSR. After 4 d of culture, cells were fixed in metaphase by incubating with Cocolend (Life Technologies), then swollen in 70 mM KCl and fixed in 3:1 methanol/acetic acid. Metaphases were then dropped on glass slides to be used for fluorescence in situ hybridization (FISH) analysis (24). To determine general genomic instability, we hybridized slides with a Cy3-labeled telomeric probe (Cy3-3’[TTAGGG]n; Applied Biosystems). For IgH locus instability, the 3’ region of the IgH locus is detected using BAC199, and the 5’ region of the IgH locus is detected using BAC207. BACs are labeled with either biotin or digoxigenin by nick translation and then hybridized to slides overnight. All three probes used for FISH have been described previously (24). Fifty metaphases are analyzed for each sample. Statistical significance was determined using a Student two-tailed t test.

CSR junction amplification and analysis

Splenic B cells were stimulated in vitro as described earlier. DNA was isolated from 4-d cultures by phenol/chloroform separation and ethanol precipitation.

Syb-Syl junctions were amplified using nested PCR primers, Sαα.8 (5′-GATGCTGCTCTTATCAGTATAC-3′) and Syl10.8 (5′-TGGATGTAG-GACTCCACAC-3′) for the first round of PCR. The second round of PCR used primer set Sαα.8 (5′-GAATTCTACTTGTGCTCCTTAAACGCT-3′) and Syl118 (5′-CTGTGATTACTCCCAACTGACT-3′). Both rounds of Sp-Syl PCR amplification performed were 35 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 4 min. All PCRs were performed using Expand Long Template Taq and Pfu polymerase mix (Roche). PCR products were cloned and sequenced as previously described (11). Sequenced switch junctions were aligned to published sequences MUSIGC07 and D78344, and analyzed using the EMBOSS program.

Hybridoma Southern blot analysis

Splenic B cells were cultured for 4 d with 0.5 mg/ml anti-CD40 plus 20 ng/ml IL-4, then fused with the myeloma NS-1 cell line and plated at a concentration of 1 cell/well in 96-well plates. IgM* hybridomas were identified by ELISA. DNA was prepared from individual IgM* hybridomas and digested overnight with restriction enzymes EcoRI or EcoRI plus BamHI. Digested hybridoma DNA was run on a 1% agarose gel, then transferred to a nylon membrane (Bio-Rad Zeta Probe) and hybridized with a genomic DNA probe specific to the Cμ region to identify intraswarm region recombinations, as described previously (33).

Results

To determine whether the MMR proteins MSH2, MLH1, and EXO1 are important for CSR events mediated by the A-EJ pathway, Msh2−/−, Mlh1−/−, and Exo1−/− mice were crossed to conditional XRC4−/− mice to condition XRC4−/− mice that have been described previously (24). These crosses generated mice with mature B cells that are deficient in the core NHEJ protein, XRCC4, as well as MSH2, MLH1, or EXO1. Mature B cells from these mice were used to determine whether MMR proteins are important for A-EJ-mediated CSR.

To determine whether MMR proteins are important for CSR in the absence of XRCC4, we activated B cells from Msh2-XRCC4−/−, Mlh1-XRCC4−/−, and Exo1-XRCC4−/− mice in vitro for 4 d to induce CSR to multiple isotypes and then assayed B cells by flow cytometry to determine the percentage of cells that class switched. Msh2-XRCC4−/−, Mlh1-XRCC4−/−, and Exo1-XRCC4−/− B cells were each analyzed in separate sets of experiments and compared with wild-type or single protein (Msh2, MLH1, or EXO1)-deficient B cells from littermate animals. Table I shows the percentage of B cells that had undergone CSR during each set of experiments for multiple isotypes, and Fig. 1 shows representative dot plots depicting the percentage of IgG1 B cells after 4 d of αCD40 plus IL-4 stimulation. Similar to results previously reported (15, 17, 18, 24), EXO1−/−, MLH1−/−, MSH2−/−, and XRCC4−/− deficient B cells have a reduction in CSR compared with wild-type B cells (Fig. 1, Table I). About 50% of wild-type IgG1 CSR occurs in EXO1−/− deficient B cells, whereas 20–40% of wild-type CSR occurs for IgG3 and IgG2a. Similar results were observed for XRCC4−/−, MSH2−/−, and MLH1−/− deficient B cells (Fig. 1, Table I).

In comparison with B cells with single-protein deficiencies, we found that B cells deficient in both EXO1 and XRCC4 have a further reduction in CSR. Exo1-XRCC4−/− double-deficient B cells have an ~2-fold reduction in IgG1 CSR efficiency compared with Exo1−/− or XRCC4−/− deficient B cells (Fig. 1A). Analyses of Msh2-XRCC4−/− and Mlh1-XRCC4−/− double-deficient B cells revealed similar results to those observed for Exo1-XRCC4−/− (Fig. 1B, 1C).

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The number of independent experiments performed for each data set: n = 6 for the Exo1 and Mlh1 data sets; n = 7 for the Msh2 data set.

aFor the Exo1 data set, seven Exo1-XRCC4−/− and six WT, Exo1−/−, and XRCC4−/− mice were included in these experiments. The p value is <0.05 when compared with Exo1−/− and p < 0.05 when compared with XRCC4−/− as calculated by a two-tailed t test.

bFor the Mlh1 data set, nine Mlh1-XRCC4−/− mice and six WT, MLH1−/−, and XRCC4−/− mice were included in these experiments. The p value is <0.05 when compared with Mlh1−/− and p < 0.05 when compared with XRCC4−/− as calculated by a two-tailed t test.

cFor the Msh2 data set, analyses of IgG3 and IgG2a included 13 Msh2-XRCC4−/− and 9 WT, Msh2−/−, and XRCC4−/− mice. For IgG1, 10 Msh2-XRCC4−/−, 8 WT and Msh2−/−, and 7 XRCC4−/− mice were included in these experiments. The p value is <0.05 when compared with Msh2−/− and p < 0.05 when compared with Xrc4−/− as calculated by a two-tailed t test.

WT, wild-type.
observed for wild-type (24). S-junction microhomology lengths are also increased in the absence of MLH1 and EXO1 but decreased in the absence of MSH2 (11, 34). To determine whether individual MMR protein deficiencies influence S-junction structures during A-EJ, we analyzed S junctions from Exo1-<sup>-</sup>XRCC4<sup>-</sup>, Mlh1<sup>-</sup> XRCC4<sup>-</sup>, and Msh2-XRCC4<sup>-</sup>, and compared the joins with those obtained from their XRCC4<sup>+</sup> littermates. Sµ-Sy1 switch junctions were cloned and sequenced from B cells activated in vitro from each mouse strain.

Fig. 2 shows the frequency of Sµ-Sy1 junction structures that we find in each mouse strain analyzed. All S-junction sequences are listed in Supplemental Fig. 1. Similar to previous reports, the XRCC4-deficient B cells contained S junctions with microhomology or inserts (sequences at the junction that did not align to either Sµ or Sγ1). Analyses of Msh2-XRCC4<sup>-</sup> and Exo1-<sup>-</sup>XRCC4-deficient B cells revealed a similar pattern of Sµ-Sy1 junction structures as those amplified from their XRCC4<sup>+</sup> littermates. Exo1-<sup>-</sup>XRCC4<sup>-</sup> and Msh2-XRCC4<sup>-</sup>-deficient B cells contained both short and long stretches of microhomology, as well as inserts at Sµ-Sy1 junction sites (Fig. 2). Thus, the lack of either EXO1 or MSH2 in B cells deficient for XRCC4 does not seem to impact the CSR junction structures.

Similar to Sµ-Sy1 junctions from Exo1-<sup>-</sup>XRCC4<sup>-</sup> and Msh2-<sup>-</sup>XRCC4-deficient B cells, Sµ-Sy1 junctions from Mlh1<sup>-</sup>-XRCC4-deficient B cells display both short and long stretches of microhomology, as well as inserts (Fig. 2). However, unlike Exo1-<sup>-</sup>XRCC4<sup>-</sup> and Msh2-<sup>-</sup>XRCC4-deficient B cells, there is substantial increase in the level of direct join S junctions that do not contain microhomology in Mlh1<sup>-</sup>-XRCC4-deficient B cells compared with XRCC4-deficient B cells alone (Fig. 2). This result suggests that the absence of the MLH1 protein alters the pattern of S-junction structures that occur during CSR in XRCC4-deficient B cells. We propose that this altered S junction pattern may result from a change in the types of DNA ends available for CSR in the absence of MLH1 (see Discussion).

In the absence of XRCC4, in vitro CSR induction results in the presence of Igh locus DNA breaks that are not repaired (24). We therefore investigated whether MMR protein deficiency affects the frequency of Igh locus chromosomal breaks that occur in the absence of XRCC4. To determine the frequency of Igh locus

All three mouse strains, Exo1-<sup>-</sup>XRCC4<sup>-</sup>, Msh2-<sup>-</sup>XRCC4<sup>-</sup>, and Mlh1<sup>-</sup> XRCC4<sup>-</sup>, have a significant reduction in CSR efficiency to all Igh isotypes analyzed when compared with single-protein-deficient B cells (Table I). These results demonstrate that EXO1, MLH1, and MSH2 are important for the A-EJ-mediated CSR events that occur in XRCC4-deficient B cells. In addition, in the absence of XRCC4, each MMR protein analyzed appears to affect CSR to a similar degree.

Previous studies have shown that, in the absence of XRCC4, most CSR junctions display microhomology, and that for some junctions, the length of microhomology is longer than what is observed for wild-type (24). S-junction microhomology lengths are also increased in the absence of MLH1 and EXO1 but decreased in the absence of MSH2 (11, 34). To determine whether individual MMR proteins influence S-junction structures during A-EJ, we analyzed S junctions from Exo1-<sup>-</sup>XRCC4<sup>-</sup>, Mlh1<sup>-</sup> XRCC4<sup>-</sup>, and Msh2-XRCC4<sup>-</sup>, and compared the joins with those obtained from their XRCC4<sup>+</sup> littermates. Sµ-Sy1 switch junctions were cloned and sequenced from B cells activated in vitro from each mouse strain.

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In the absence of XRCC4, in vitro CSR induction results in the presence of Igh locus DNA breaks that are not repaired (24). We therefore investigated whether MMR protein deficiency affects the frequency of Igh locus chromosomal breaks that occur in the absence of XRCC4. To determine the frequency of Igh locus

FIGURE 1. Reduced IgG1 CSR efficiency in Exo1-XRCC4<sup>-</sup>, Msh2-<sup>-</sup>XRCC4<sup>-</sup>, and Mlh1-XRCC4<sup>-</sup> B cells compared with Exo1, Msh2, Mlh1, and XRCC4 deficiency alone. B cells from Exo1-XRCC4<sup>-</sup>, Msh2-<sup>-</sup>XRCC4<sup>-</sup>, Mlh1-XRCC4<sup>-</sup>, and various control mice were stimulated in vitro for 4 d with αCD40 plus IL-4 to induce IgG1 CSR. After 4 d of culture, B cells were analyzed by flow cytometry to determine percentage of B cells that had undergone CSR to IgG1. A, Representative FACs dot plots of Exo1-XRCC4<sup>-</sup> and control B cells from one experiment. Gated population represents percentage of IgG1<sup>+</sup> cells. B, Same as in A with Mlh1-XRCC4<sup>-</sup> B cells. C, Same as in A for Msh2-XRCC4<sup>-</sup> B cells.

FIGURE 2. MLH1 deficiency alters CSR junctions in XRCC4-deficient B cells, whereas Msh2-XRCC4<sup>-</sup> and Exo1-XRCC4<sup>-</sup> switch junctions resemble XRCC4<sup>+</sup> mice. Sµ-Sy1 junctions from XRCC4<sup>-</sup>, Exo1-<sup>-</sup>XRCC4<sup>-</sup>, Mlh1-XRCC4<sup>-</sup>, and Msh2-XRCC4<sup>-</sup> B cells were amplified by PCR and aligned to μ and γ germline sequences. Amount of microhomology at switch junctions was determined by counting the number of uninterrupted shared nucleotide (nt) identity with Sµ and Sy sequences at the junction. Cultures from three Exo1-XRCC4<sup>-</sup>, three Msh2-XRCC4<sup>-</sup>, and two Mlh1-XRCC4<sup>-</sup> mice were used to generate the Sµ-Sy1 junctions. The slight difference in the percentage of junctions with five or more nts of microhomology observed for Msh2-XRCC4<sup>-</sup> and other XRCC4-deficient strains is not statistically different.
chromosomal breaks, we performed FISH assays using B cells that were fixed in metaphase after 4 d of in vitro culture with αCD40 plus IL-4. The DNA breaks are detected as chromosomal aberrations visualized by FISH using probes specific for the 3′ and 5′ regions of the Igh locus (Fig. 3A–C).

FISH analysis was used to investigate whether the decreased CSR in double-deficient B cells is the result of an increase in S-region DNA breaks that either cannot be joined or are joined aberrantly in a chromosome translocation event. We first determined the frequency of Igh locus breaks detectable by FISH in the context of MMR protein deficiency alone. Wild-type B cells were included in each experiment as a control. Similar to previously published results, B cells from wild-type mice had few detectable Igh locus chromosome aberrations (Fig. 3D–F). Furthermore, Igh locus breaks were not detected at levels greater than wild-type in EXO1−, MLH1−, and MSH2-deficient B cells (Fig. 3B).

We next performed the same FISH assay using B cells from Exo1−XRCC4−, Mlh1−XRCC4−, and Msh2−XRCC4−, as well as wild-type and XRCC4− littermate controls. Fig. 3 displays the percentage of cells with chromosomal aberrations, as well as the types of aberrations observed for the various mouse strains. Wild-type B cells had few detectable Igh locus chromosome aberrations (1.4%), whereas aberrations were present in ~15% of XRCC4−deficient B cells. We found that B cells from each of the Exo1−XRCC4−, Mlh1−XRCC4−, and Msh2−XRCC4− double-deficient strains have a lower frequency of Igh locus chromosomal aberrations than B cells deficient in XRCC4 alone. Exo1−XRCC4−, Mlh1−XRCC4−, and Msh2−XRCC4− mouse strains have 40–50% fewer B cells with Igh locus chromosomal aberrations after 4 d of in vitro αCD40 plus IL-4 stimulation compared with XRCC4 deficiency alone (Fig. 3D–F; Supplemental Tables I–III). In addition, no difference in cell survival during CSR activation was observed between XRCC4-deficient B cells compared with double-deficient B cells, demonstrating the reduction in Igh breaks in these cells is not due to an increase in the death of cells that contain breaks (Supplemental Fig. 2). These results suggest that MMR protein activity may be involved in the generation of dsDNA breaks that persist in the absence of XRCC4. MMR protein activity may lead to the generation of dsDNA breaks that can be used for CSR by converting widely spaced DNA nicks into dsDNA breaks (11–14). Therefore, the reduced CSR efficiency of MMR/XRCC4 double-deficient B cells compared with XRCC4 deficiency alone may be caused by a decrease in the overall number of S-region DNA breaks/substrates that can be used for CSR during MMR protein deficiency.

**FIGURE 3.** Igh locus chromosome aberrations are decreased in Exo1−XRCC4−, Msh2−XRCC4−, and Mlh1−XRCC4− double-deficient B cells compared with XRCC4−deficient B cells. A, Metaphases were prepared from B cells cultured with anti-CD40 plus IL-4 for 4 d. Metaphases were hybridized with fluorescently labeled BAC199 and BAC207, which are specific for regions 3′ and 5′ of the Igh locus. Chromosomes from an individual metaphase with an intact Igh locus (normal) are shown. B, As in A, where pictures show chromosomes from individual metaphases with an Igh locus-associated break. C, As in A, where pictures show chromosomes from individual metaphases with Igh locus-associated translocations. Cartoons depicting the status of each Igh locus shown in the metaphase pictures are displayed with the pictures. Pictures of Igh locus breaks and translocations are from the XRCC4−deficient strains analyzed. D, Bar graphs represent percentage of metaphases with Igh locus-specific chromosomal aberrations from Exo1−XRCC4− and littermate controls. Total number of metaphases analyzed is in parentheses below each bar graph. Seven Exo1−XRCC4− mice, six wild-type (WT), six XRCC4−, and three Exo1− mice were used. E, As in D, results are from five Mlh1−XRCC4− mice, four XRCC4−, four Mlh1−, and three WT. F, As in D, results are from nine Msh2−XRCC4− mice, five XRCC4−, five WT, and four Msh2− mice. Statistical significance was determined using a two-tailed t test. G, Bar graphs represent the percentage of DNA breaks observed that were chromosomal (white bars) versus chromatid (black bars) breaks. H, Bar graphs represent the percentage of abnormal chromosomes that had free (white bars) versus translocated (black bars) DNA ends.
Although MMR protein deficiency decreased the frequency of IgH locus chromosome aberrations, the types of aberrations observed were similar to XRCC4 deficiency alone. A majority of the chromosomal aberrations found in XRCC4-deficient B cells are chromosome breaks characterized by a loss of the 5' signal (BAC207) on both sister chromatids, indicative of breaks that occur during the G1 phase of the cell cycle when CSR is ongoing (12, 35). Similar to XRCC4-deficient B cells, we find that the IgH locus chromosome aberrations observed in Exo1-XRCC4−, Mlh1-XRCC4−, and Msh2-XRCC4−-deficient B cells are mainly chromosome breaks. For all mouse strains analyzed, few chromatid breaks characterized by a loss of the 5' signal on one sister chromatid were observed (Fig. 3G; Supplemental Tables I–III).

In XRCC4-deficient B cells, on average, 16% of the IgH locus aberrations are translocations (number of translocations/number of aberrations) (24) (Supplemental Tables I–III). The proportion of the total aberrations that are characterized as chromosome translocations is ~15% for both Mlh1-XRCC4− and Msh2-XRCC4−-deficient B cells, similar to the proportion observed for XRCC4-deficient B cells (Fig. 3H; Supplemental Tables II–III). Chromosome translocations account for <5% of the IgH locus aberrations in Exo1-XRCC4−-deficient B cells (Fig. 3H; Supplemental Table I), an ~67% reduction compared with XRCC4 deficiency alone. The decrease in translocations in Exo1-XRCC4−-deficient B cells compared with XRCC4-deficient B cells could suggest a possible role for EXO1 in the error-prone pathway of A-EJ that is responsible for the translocations.

To determine whether the reduction in chromosome aberrations in the absence of MMR is an IgH-specific effect or an overall general effect, we performed a second FISH assay using a telomere-specific probe (Fig. 4A). This assay allowed us to determine genome-wide instability by identifying chromosome aberrations positioned on any of the 40 mouse chromosomes. First, genome-wide genomic instability was determined during MMR protein deficiency alone. Using a telomere-specific probe in FISH analyses, we found that after IgG1 CSR induction, MSH2−, MLH1−, and EXO1-deficient B cells display a slightly greater level of genome-wide chromosome aberrations than wild-type B cells (Fig. 4; Supplemental Table IV). Approximately 8–10% of MMR-deficient B cells contained chromosome aberrations, most of which were characterized as chromosome breaks (Fig. 4; Supplemental Table IV). A slight increase in genome-wide genomic instability was also observed after 4 d of LPS activation (Supplemental Table V). This result suggests that MMR proteins are important for repair of some genome-wide damage that occurs after in vitro CSR induction.

We next assayed Exo1-XRCC4−, Mlh1-XRCC4−, and Msh2-XRCC4−-deficient B cells for genome-wide chromosome aberrations using the telomere-specific probe. For comparison, wild-type and XRCC4-deficient B cells were included in each experiment. The frequencies of genome-wide chromosomal aberrations observed for each mouse analyzed are shown in Fig. 4 and Supplemental Tables VI–VIII. As previously reported, chromosomal aberrations are observed at a high frequency in XRCC4-deficient B cells after CSR induction. We also find high frequencies of chromosomal aberrations in Exo1-XRCC4−, Mlh1-XRCC4−, and Msh2-XRCC4−-deficient B cells after CSR induction. We find that the frequency of genome-wide chromosome aberrations in Exo1-XRCC4−, Mlh1-XRCC4−, and Msh2-XRCC4− mouse strains is similar to the frequency in XRCC4− alone (Fig. 4). A slight reduction in the percentage of cells with genome-wide chromosome aberrations is observed, but the reduction is equivalent to the reduction in the percentage of cells with IgH locus chromosome aberrations, suggesting that most of the decrease found using the telomere-specific probe is due to the decrease in the IgH locus. Therefore, MMR deficiency does not appear to result in a decreased frequency of chromosome aberrations outside the IgH locus. These results may suggest the MMR protein effect on chromosome aberrations is an IgH locus-specific effect.
CSR efficiency in Exo1−/−XRCC4−/−, Mlh1−/−XRCC4−/−, and Msh2−/−XRCC4−/− deficient B cells is decreased compared with XRCC4-deficient B cells; however, the decrease in CSR does not lead to increased IgH locus breaks or translocations. This suggests that either fewer S-region DNA breaks occur or the DNA breaks are repaired by another mechanism. One such mechanism possible is intraswitch region recombination, where CSR-induced DNA breaks within the same S region are joined to another (36, 37). The frequency of intraswitch region recombination events can be determined using genomic DNA from IgM+ B cell hybridomas generated after in vitro activation of CSR. Sµ internal recombination can be detected by Southern blotting as a change in the size of a restriction enzyme digest fragment detected with a Cµ probe (Fig. 5).

To investigate whether EXO1, MLH1, or MSH2 deficiency leads to an increased frequency of intraswitch region recombination events, we performed Southern blotting analysis using a Cµ probe on IgM+ hybridomas generated from Exo1−/−, Mlh1−/−, and Msh2−/−, as well as wild-type B cells activated in vitro for 4 d with anti-CD40 plus IL-4. For wild-type controls, we found 27% of IgM+ hybridomas had undergone an Sµ internal recombination event (Fig. 5; Supplemental Table IX), similar to previously published wild-type results (26). We found MSH2-deficient IgM+ hybridomas have a similar frequency of Sµ intraswitch region recombination events as wild-type (Fig. 5; Supplemental Table IX). About 45% of IgM+ B cell hybridomas deficient in MLH1 or EXO1 have Sµ internal recombinations. EXO1 and MLH1 deficiencies result in a slightly higher, but nonstatistically significant, increase in intraswitch region recombination events compared with wild-type. Because intraswitch region recombination events are not increased, and no detectable increase in chromosome breaks are observed by FISH in MMR-deficient B cells, these results suggest that the defect in CSR of MMR-deficient B cells could be caused by a decrease in the overall number of S-region dsDNA breaks.

**Discussion**

During CSR, S-region DNA breaks can be joined by NHEJ factors. In the absence of classical NHEJ, an A-EJ pathway is used to join S-region DNA breaks during CSR. Previous studies investigating CSR during MMR protein deficiency showed that MMR proteins are critical for CSR in the absence of the Sµ tandem repeat region, and that they are important for generating a high frequency of blunt dsDNA breaks in S regions, suggesting that MMR proteins are important for NHEJ-mediated CSR (11–13). We have crossed mice deficient in the individual MMR proteins, MSH2, MLH1, and EXO1 with mice that have a conditional deletion of XRCC4 in mature B cells to investigate whether MMR proteins have additional individual or collective roles in other end-joining pathways of CSR. In our studies, we have determined that MSH2, MLH1, and EXO1 each influence CSR events that occur in the absence of the core NHEJ factor XRCC4. We find that Msh2−/−XRCC4−/−, Mlh1−/−XRCC4−/−, and Exo1−/−XRCC4−/− deficient B cells have further reductions in CSR when compared with the corresponding single-protein-deficient B cells. These findings demonstrate that MSH2, MLH1, and EXO1 each influence the efficiency of A-EJ–mediated CSR and show that the MMR proteins are important for CSR events mediated by A-EJ.

In the absence of the core NHEJ factors Ku70, XRCC4, or DNA ligase IV, B cells that are activated to undergo CSR contain IgH locus-associated chromosome breaks and translocations (24, 26). The presence of these IgH-associated chromosome breaks and translocations suggests that the A-EJ pathway, operating in the absence of NHEJ, is more error prone than NHEJ and is not capable of repairing all of the S-region DNA breaks that are generated in response to CSR induction. The inability to join some S-region DNA breaks in the absence of NHEJ factors results in significantly decreased CSR efficiency.

The reduced CSR efficiency that we observe in MMR/XRCC4 double-deficient B cells (compared with XRCC4-deficient B cells) could result from a further decrease in the fraction of IgH breaks that are able to be joined during CSR. However, we also find that B cells deficient in both MMR and XRCC4 exhibit fewer unrepaired CSR-induced IgH locus chromosome breaks when compared with XRCC4-deficient B cells. This finding indicates that the decreased CSR in MMR/XRCC4-deficient B cells is not caused by an increased fraction of unreparable IgH locus DNA breaks. Instead, our observations suggest that, when both MMR and XRCC4 proteins are absent, less AID DNA damage is converted into IgH locus dsDNA breaks that can be used by end-joining factors for CSR. Therefore, we conclude that the major role of MMR proteins during CSR is to convert AID DNA damage into suitable broken DNA substrates for both C-NHEJ and A-EJ pathways. This conclusion is in accordance with previous studies showing that MMR proteins are important for the generation of S-region dsDNA breaks for CSR in wild-type B cells (12).

During DNA replication, MMR proteins repair base–base mispairings, as well as short nucleotide insertions and deletions. DNA repair initiates when MSH2-MSH6 (or MSH2-MSH3) heterodimers recognize DNA damage. After recognition, MLH1-PMS2 heterodimers and EXO1 are recruited to the sites of DNA damage, resulting in excision of the nucleotide strand that contains the damage. DNA repair is complete once DNA polymerases correctly fill in the gaps left by EXO1 excision (38). Previous reports have proposed a model that describes the activity of MMR proteins during CSR as similar to their activity during DNA repair (11–14).
In our model, MSH2-MSH6 heterodimers recognize U-G mismatches generated by AID in S-region sequences and recruit MLH1-PMS2 and EXO1 to these sites. MLH1-PMS2 and EXO1 then process the DNA surrounding the U-G mismatches, possibly initiating excision at nicks or base excision repair intermediates, as previously suggested by Schanz et al. (22). This activity could convert widely spaced DNA nicks into double-stranded breaks in IgH S regions that can be used for CSR (11–14). In this model, MMR proteins are critical for optimal CSR efficiency because they generate DNA ends that can be recognized by NHEJ factors during CSR. In a similar manner, CSR efficiency could be reduced in B cells double deficient in MMR and XRCC4 because the collective activity of MMR proteins may also generate DNA ends that can be recognized by A-EJ factors during CSR. Therefore, MMR proteins are critical for efficient CSR mediated by NHEJ and A-EJ because their activity would increase the frequency of DNA ends that can be joined by either of these pathways.

MMR protein activity could generate DNA ends suitable for A-EJ-mediated CSR by EXO1 excising DNA past the initial U-G mismatch, leading to DNA ends with short overhangs. Studies investigating EXO1-mediated excision tracts during the MMR repair pathway suggest that EXO1 excision likely proceeds slightly past the mismatch before it is inactivated (39). Alternatively, the endonucleolytic activity of the MLH1-PMS2 heterodimer may be important for A-EJ-mediated CSR. An endonucleolytic function of the MLH1-PMS2 heterodimer that is dependent on MSH2 mismatch recognition has been identified in human MMR (40). The endonucleolytic activity of MLH1-PMS2 could generate nicks in S-region DNA sequences, and this activity may lead to increased A-EJ efficiency.

Our results show that Mlh1-XRCC4-deficient B cells have an increase in the frequency of S junctions that are joined without microhomology compared with XRCC4 deficiency alone. No increase in direct S junctions is observed in Msh2-XRCC4 or Exo1-XRCC4 B cells, suggesting that MLH1 may have an additional functional independent of other MMR proteins during CSR. A function for Mlh1 independent of other MMR proteins was also previously suggested when switch junctions from Msh2-Mlh1 double-deficient B cells were found to more closely resemble Mlh1 single-deficient B cell junctions than Msh2-deficient B cell junctions (41).

During CSR, the A-EJ pathway almost exclusively uses microhomology to join S-region DNA breaks in the absence of XRCC4 or DNA ligase IV (24, 25). These findings suggest that the A-EJ pathway of CSR uses microhomology to join S-region DNA breaks. However, studies investigating DNA repair in XRCC4- or Ku80-deficient cell lines have demonstrated that although microhomology is used more often, it is not used exclusively for DNA break repair in NHEJ-deficient cells (42–44). In addition, ~10% of switch-junction sequences from Ku70-deficient B cells are joined without the use of microhomology (25). Furthermore, ~10–20% of switch junctions from B cells that are deficient in both Ku70 and DNA ligase IV are joined without the use of microhomology (25). Therefore, A-EJ is capable of joining S-region DNA breaks without using microhomology and suggests that S-junction structures may depend partially on the DNA ends available for ligation.

An increased frequency of direct S junctions in Mlh1-XRCC4-deficient B cells suggests that the absence of the MLH1 protein alters the structures of S junctions in XRCC4-deficient B cells. One possible explanation for this result is that MLH1 protein presence may influence which DNA ends are available for CSR. One function of MLH1 independent of other MMR proteins is to inhibit recombination between similar but nonidentical sequences, particularly when short lengths of homology are present; this role is suggested to possibly prevent recombination events that are damaging to the cell (45). MLH1 may play an active role in inhibiting CSR breaks that lack microhomology (blunt breaks) from being used by the A-EJ pathway.

Based on our results, we propose that a major collective function of MSH2, MLH1, and EXO1 is to increase the frequency of substrates for the end-joining pathways used for CSR. MMR proteins could recognize U-G mismatches in S-region sequences leading to an MMR-like activity that results in more DNA ends that can be used for CSR. This MMR-like activity may be similar to the activity described previously for MMR proteins during NHEJ-mediated CSR (11, 12, 14). It is possible that MMR proteins generate DNA nicks in S regions and convert widely spaced DNA nicks into near-blunt DNA ends and DNA ends with short overhangs. This activity would make MMR proteins important for efficient CSR, independent of the dsDNA end-joining repair pathway used.

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Disclosures
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