Reassessment of the Role of Mut S Homolog 5 in Ig Class Switch Recombination Shows Lack of Involvement in *cis*- and *trans*-Switching[1]

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Abstract

When B cells are activated after immunization or infection, they exchange the gene encoding the Ig H chain C region by class switch recombination (CSR). CSR generally occurs by an intrachromosomal deletional recombination within switch (S) region sequences. However, ~10% of CSR events occur between chromosome homologs (*trans*- or interallele CSR), suggesting that the homologous chromosomes are aligned during CSR. Because the Mut S homolog 4 (Msh4) and Msh5 bind to Holliday junctions and are required for homologous recombination during meiosis in germ cells, we hypothesized these proteins might be involved in *trans*-chromosomal CSR (*trans*-CSR). Indeed, Msh4-Msh5 has recently been suggested to have a role in CSR. However, we find a large variety of alternative splice variants of Msh5 mRNA in splenic B cells rather than the full-length form found in testis. Most of these mRNAs are unlikely to be stable, suggesting that Msh5 might not be functional. Furthermore, we find that *msh5* nullizygous B cells undergo CSR normally, have unaltered levels of *trans*-CSR, normal levels of DNA breaks in the Sμ region, and normal S-S junctions. We also show that the S-S junctions from *cis*- and *trans*-CSR events have similar lengths of junctional microhomology, suggesting *trans*-CSR occurs by nonhomologous end joining as does intrachromosome (*cis*)-CSR. From these data, we conclude that Msh5 does not participate in CSR.

Class switch recombination (CSR)[4] is a unique type of recombination that occurs in B cells upon appropriate activation. It results in a change from IgM and IgD expression by naive B cells to expression of IgG, IgE, or IgA. This switch changes C_H, and improves the ability of the Ab to eliminate the pathogen. It generally occurs by an intrachromosomal deletional recombination between switch (S) region sequences located upstream of each C_H gene, except Cδ. The C_H genes are arranged in the following order: Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, and Cα, with the Cμ gene being closest to the recombined VDJ gene segment. CSR is initiated by activation-induced cytidine deaminase (AID), which converts dC bases in S regions to dU bases, some of which are subsequently excised by the base excision repair enzyme uracil DNA

glycosylase, and others are recognized by the mismatch repair (MMR) proteins Mut S homolog (Msh)2-Msh6 (1-7). Additional base excision repair enzymes, apurinic/apyrimidinic endonucleases 1 and 2, and several MMR enzymes are required to generate the dsDNA breaks (DSBs) in the S regions that are required for CSR (8,9). Deficiencies in the MMR proteins Msh2, Msh6, Mlh1, Pms2, and Exo1 reduce CSR by 2- to 5-fold (10-14).

Msh5 is one of the Msh that are specialized for recognizing aberrant DNA duplexes. However, Msh5 and its partner Msh4 do not perform mismatch repair, but instead are essential for meiosis, and mice deficient in either protein are sterile (15,16). Msh5 does not homodimerize, and its only known partner is Msh4 (17). Msh4-Msh5 forms a sliding clamp that binds to Holliday junctions in vitro (18), and during meiosis Msh4 is distributed along the synaptonemal complex early in prophase I, from the leptonema stage until pachynema (16). Although meiotic chromosomes in $msh4^{-/-}$ or $msh5^{-/-}$ mice begin to form synaptonemal complexes, they do not condense correctly, and show abnormally high levels of association with Rad51. Rad51 association suggests that meiosis is initiated and that DSBs and ssDNA breaks (SSB) are generated as normal, but recombination between homologs fails to proceed normally. Deletion of either the $msh4$ or $msh5$ gene results in increased aberrant pairing between nonhomologous chromosomes and greatly decreased pairing between homologs during meiosis in both spermatocytes and oocytes (15,16). Taken together, the data suggest that during meiosis Msh4-Msh5 binds Holliday junctions and monitors DNA sequences to ensure that recombination occurs between homologs. An additional heterodimer, Mlh1-Mlh3, is also required for fertility and is thought to bind and stabilize Msh4-Msh5 bound at Holliday structures (19-22).

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[4]Abbreviations used in this paper:

CSR
    class switch recombination

AID
    activation-induced cytidine deaminase

cis-**CSR**
    intrachromosome-CSR

DSB
    dsDNA break

KO
    knockout

LM-PCR
    ligation-mediated PCR

MMR
    mismatch repair

Msh
    Mut S Homolog

S
    switch

SSB
    ssDNA break

TR
    tandem repeat

trans-**CSR**
    trans-chromosomal CSR

WT
    wild type

Msh4 and Msh5 mRNAs have been detected in several tissues that do not contain cells undergoing meiosis, but whether they have a role in these tissues is unknown. Recently, Sekine et al. (23) reported that Msh5 contributes to CSR. They showed that MRL/*lpr* mice congenic for a 13-megabase segment from C57BL/6 (H-2^{b/b}) that includes the *msh5* gene and the entire MHC locus have 5% of the level of Msh5 mRNA in their spleens as do spleens from MRL/*lpr* H-2^{k/k} mice. They found that these congenic mice have low levels of serum IgG3. They also found that splenic B cells from *msh4* or *msh5* knockout (KO) mice had altered $S\mu$-$S\gamma3$ and $S\mu$-$S\alpha$ junctions relative to wild-type (WT) littermates. From additional analyses of patients with various single-nucleotide mutations within the *msh5* gene, they concluded that Msh5 has a role in CSR, although the role was not defined.

Most switch recombination occurs by an intrachromosomal deletion event, although recently it was demonstrated that 7-17% of the CSR events occur between the two homologous chromosomes (24-26). Because *trans*-chromosomal CSR would require alignment of the two chromosome homologs, we hypothesized that Msh5 might regulate this *trans*-chromosomal switch. To investigate this possibility, we performed extensive analyses of CSR in splenic B cells from mice with a null mutation in the *msh5* gene due to targeted gene deletion (15). We find that Msh5 deficiency has no effect on CSR. Consistent with these findings, all the Msh5 RNAs in splenic B cells that we sequenced have mutations leading to amino acid replacements and/or alternative splicing relative to the functional Msh5 mRNA in testis. Most of these splice variants would result in premature translation termination and mRNA degradation. Furthermore, the Msh5 proteins that might be produced would be unable to interact with Msh4.

Materials and Methods

Mice

All mouse strains were backcrossed to C75BL/6 for at least four generations. WT^{+/+} littermates (of the *msh5*^{-/-} mice) were used as controls for all experiments reported in this study. The Msh5 null mice have been described previously (15). Similar to the *msh5* targeted strain of mice used by Sekine et al. (23) and De Vries et al. (27), the mice are nulls for Msh5, and have a phosphoglycerate kinase-hygromycin cassette remaining within the targeted locus. Mice were housed in the Institutional Animal Care and Use Committee-approved specific pathogen-free facility at the University of Massachusetts Medical School. The mice were bred and used according to the guidelines from University of Massachusetts Animal Care and Use Committee.

B cell isolation and culture

Spleen cells were dispersed and RBCs were lysed in Gey's solution, followed by T cell depletion with a mixture of anti-T cell Abs, as described previously (11). To track cell division, in some experiments cells were stained with $1 \mu M$ CFSE for 15 min at 37°C, and quenched with FCS to remove unbound CFSE. Cells were cultured at 1×10^5/ml in 24-well plates and activated to induce CSR for 4 days (or 3 days if stained with CFSE). All cultures contained LPS ($50 \mu g$/ml; Sigma-Aldrich), and most contained human BLyS (100 ng/ml; Human Genome Sciences). IL-4 (800 U/ml) was added to induce switching to IgG1; IFN-γ (10 U/ml) for IgG2a; anti-δ-dextran (0.3 ng/ml) for IgG3; and TGF-β (2 ng/ml) for IgG2b; and to induce IgA switching, we added TGF-β (2 ng/ml), IL-4 (800 U/ml), IL-5 (1.5 ng/ml; BD Biosciences), and anti-δ-dextran (0.3 ng/ml).

Flow cytometry

For FACS analysis, cells were washed twice with PBS, 1% FCS, and 0.2% NaN_3, and incubated for 30 min on ice with PE goat F(ab′)_2 anti-mouse IgG1, IgG2a, IgG2b, and IgG3, or PE goat anti-mouse IgA (Southern Biotechnology Associates). In some experiments, FITC goat F(ab

′)₂ anti-mouse IgM (Southern Biotechnology Associates) was used to stain un-switched cells. For splenic B cell subset analysis, cells were stained with anti-B220 allophycocyanin (RA3-6B2; Caltag Laboratories; Invitrogen), anti-CD23 PE (2G8; Southern Biotechnology Associates), anti-CD21 FITC (7G6; BD Pharmingen), and biotinylated anti-CD24 (Caltag Laboratories), followed by streptavidin-PerCP (BD Pharmingen). Expression of IgM[a] and IgM[b] allotypes was analyzed by staining peripheral blood cells with anti-mouse IgM[a] PE (DS1; BD Pharmingen) and anti-mouse IgM[b] FITC (AF6-78; BD Pharmingen). CFSE fluorescence and Ab staining were acquired on a LSR flow cytometer (BD Biosciences) and analyzed using the FlowJo software package (Tree Star).

Western blotting

Cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1.0% Nonidet P-40, 50 mM Tris-Cl (pH 7.5), Complete protease, and phosphatase inhibitor mixture; Roche) and subjected to three freeze-thaw cycles to obtain whole-cell protein extracts. Protein content was determined using Bradford assay (Bio-Rad). Equal amounts (25 μg) of proteins were electrophoresed on 8% SDS-polyacrylamide gels, and blotted onto Immobilon-P polyvinylidene fluoride membranes (Millipore). Immunoblotting was performed using rabbit anti-Msh5, followed by goat anti-rabbit HRP (Santa Cruz Biotechnology) and enhanced chemiluminescent substrate (Pierce).

Genomic DNA preparation and linker ligation-mediated PCR (LM-PCR)

After culture for 2 days, viable cells were isolated by Ficoll/Hypaque gradients ($r = 1.09$) or Lympholyte (Cedarlane Laboratories) centrifugation. A total of 1×10^6 cells was imbedded in low melt agarose, and DNA was isolated by proteinase K treatment and used for LM-PCR, as described (28). Briefly, DNA was ligated overnight to double-stranded linker, which was prepared by annealing 5 nmol each of LMPCR.1 (5′-GCGGTGACCCGGGAGATCTGAATTC-3′) and LMPCR.2 (5′-GAATTCAGATC-3′) in 300 μl of 1× ligase buffer, resulting in a double-stranded oligonucleotide with a 14-nt single-stranded overhang that ligates unidirectionally. Ligated DNA samples were assayed for GAPDH by PCR to adjust DNA input before LM-PCR. The primer 5′Sμ (5′-GCAGAAAATTTAGATAAAATGGATACCTCAGTGG-3′) was then used in conjunction with linker primer (LMPCR.1) to amplify linker-ligated DNA breaks. Three-fold dilutions of input DNA were amplified by HotStar *Taq* (Qiagen). PCR products were electrophoresed on 1.25% agarose gels and vacuum blotted (VacuGene XL; Pharmacia) onto nylon membranes (GeneScreen Plus; PerkinElmer). Blots were hybridized at 42°C overnight with a Sμ-specific oligonucleotide probe (μ probe 5′: 5′-AGGGACCCAGGCTAAGAAGGCAAT-3′) end labeled with [γ-^{32}P]ATP and washed three times at 42°C and three times at 55°C with 2× SSC/0.1% SDS.

Amplification, cloning, and sequencing germline Sμ segments

Genomic DNA was isolated from purified B cells, either ex vivo or cultured with LPS plus anti-δ-dextran for 4 days. Cell pellets were incubated with proteinase K (0.5 mg/ml), RNase A (100 μg/ml), and SDS (0.5%) in STE (0.1M NaCl, 20 mM Tris, 1 mM EDTA) for 2 h at 37°C, followed by three to four extractions with phenol/chloroform (1:1) and precipitation with 0.3 M sodium acetate (pH 7) and ethanol. DNA was wound out on glass rods and resuspended in Tris-EDTA buffer (pH 8). Germline Sμ was amplified using the following primers: 5μ3 (5′-AATGGATACCTCAGTGGTTTTTAATGGTGGGTTA-3′) and 3μ2 (5′-AGAGGCCTAGATCCTGGCTTCTCAAGTAG-3′). The amplified fragments were examined by agarose gel electrophoresis. Germline Sμ segments that migrated at ~3 kb were excised from the gel and cloned into the pCR4-TOPO vector. S-S junctions were cloned directly

into the pCR4-TOPO vector and sequenced by Macrogen. Sequences were analyzed by alignment to germline sequences, as described earlier (29,30).

PCR to detect trans-*chromosomal CSR (*trans-*CSR)*

Genomic DNA was isolated from splenic B cells from 129 (IgH[a]) × B6 (IgH[b]) *msh5*^{-/-} and 129 (IgH[a]) × B6 (IgH[b])WT F$_1$ mice after culturing for 4 days with LPS plus anti-δ-dextran. Sμ-Sγ3 junctions were PCR amplified using Expand Long Template *Taq* polymerase (Roche) and the primers Sμ-nest2F (5′-CCAGCCACAGTAATGACCCAG-3′) and Sγ3-nest2R (5′-CTGACCCAGGAGCTGCATAAC-3′), as previously described (26). PCR products were cloned into the pCR4-TOPO vector and sequenced by Macrogen. Sequences were analyzed by comparison with germline Sμ and Sγ3 regions from IgH[a] and IgH[b] allotypes (26). *Trans*-CSR and intrachromosome-CSR (*cis*-CSR) could be distinguished on the basis of single-nucleotide polymorphisms between a and b allotypes. Only sequences that had at least two polymorphisms on either the Sμ and Sγ3 side of the junction were included in the analysis.

Amplification of Sμ-Sγ3 junctions from ex vivo spleen cells

Genomic DNA was isolated from total spleen cells and amplified by a nested PCR using the identical primers described by Sekine et al. (23). Junctions from two *msh5*^{-/-} and two WT littermates were amplified in 12 independent PCR.

Results

Alternatively spliced Msh5 RNA is expressed in mouse splenic B cells

In order for Msh5 to have a role in CSR, it must be present in B cells. We performed quantitative RT-PCR to attempt to detect Msh5 mRNA. Using five different sets of primers for various regions of this large gene, we detected Msh5 RNA in WT C57BL/6 (B6) B cells, both ex vivo and under conditions that induce CSR (LPS and IL-4) (Fig. 1). Recently, it was reported that the amount of mRNA for Msh5 is 100 times lower in ex vivo B cells from B6 than from BALB/c mice and 10 times lower in activated B cells (23). Because we use B6 mice in our experiments, it was important to examine this result further. We have similar, but not identical results; when averaging the results from all five sets of primers, we find that B6 B cells express ~12 times less Msh5 mRNA than do BALB/c B cells, and after activation express ~10 times less than similarly activated BALB/c cells.

We cloned and sequenced the Msh5 PCR products from B6 and BALB/c splenic B cells, both resting and activated to switch. All 10 *msh5* cDNAs sequenced from B6 cells either have replacement mutations or a premature termination codon due to either a 4-bp deletion at the exon 18-19 junction, or inclusion of the intron after exon 18 or 20 (Table I). These splice variants would result in deletion of at least 124 aa from the C terminus of the 833-residue protein, which are required for binding to Msh4 (31,32). However, it is highly likely these mRNAs would be degraded during attempted translation due to nonsense-mediated mRNA decay, because the premature stop codon is located more than 50-55 nt upstream of the last coding exon (33). This is consistent with the very low levels of the Msh5 mRNA detected. Furthermore, although present at higher levels, none of the nine BALB/c cDNAs sequenced could encode normal Msh5 protein (Fig. 2 and Table I). They were all splice variants relative to full-length testis Msh5 mRNA, and sometimes also had single amino acid mutations. Most of the variants would result in premature termination of translation. The apparent heterogeneity in Msh5 mRNA levels using the different primer sets shown in Fig. 1*A* can be partly explained by alternative splicing events. The highest levels of Msh5 mRNA are found using primers for exons 5-6 and exons 22-24. Sequence analysis shows that exons 5-6 are retained in all of the splice variants. By contrast, all 10 of the testes Msh5 cDNAs that we cloned encoded unmutated full-length mRNA.

These results are reminiscent of previous findings showing that all of the Msh4 mRNAs cloned from liver and placenta are splice variants compared with the full-length form of Msh4 mRNA found in testis (34). However, it is unknown whether these Msh4 RNA variants encode proteins, or whether B cells contain these splice variants.

Consistent with the structure of the Msh5 mRNA in B cells, we could not detect Msh5 protein in ex vivo B6 splenic B cells by Western blotting. Nor could we detect it in B cells activated to undergo CSR, although we detected a strong signal in testis (Fig. 1*B*). Because the Msh5 Ab is specific for the C terminus, this result is in agreement with our finding that splenic Msh5 mRNAs have aberrant splicing that prevents them from encoding the C terminus. In conclusion, although we find Msh5 mRNA in splenic B cells, the RNA is spliced differently from the full-length form of mRNA found in testis, where Msh5 is known to function. It is likely that the observed mutations and splicing events perturb normal protein expression, resulting in either no Msh5 protein or an aberrant form of Msh5 that is unable to interact with Msh4. However, due to the previous report that Msh5 functions in CSR (23), we decided to analyze CSR in the $msh5^{-/-}$ mice.

B cell subsets and CSR are normal in msh5$^{-/-}$ splenic B cells

To determine whether splenic B cell subsets are altered in Msh5-deficient mice, we characterized splenocytes by flow cytometry after staining with anti-B220, anti-CD24 (Fig. 3, *upper panels*), anti-CD21, and anti-CD23 Abs (*lower panels*). The proportions of cells in mature splenic B cell subsets from $msh5^{-/-}$ mice are similar to those in WT mice.

To determine whether Msh5 functions in CSR, splenic B cells were isolated by T cell depletion and induced to undergo CSR to IgG and IgA isotypes by treatment with LPS, various cytokines, and/or anti-δ-dextran, after staining the cells with CFSE to track cell division. On day 3 or 4 of culture, cells were stained with isotype-specific Abs and analyzed by flow cytometry. Fig. 4 shows that $msh5^{-/-}$ and WT B cells switched equally well to all isotypes tested. There was no difference in DNA synthesis in the cultures as determined by [^{3}H]thymidine incorporation (not depicted), nor any difference in proliferation as determined by dilution of CFSE during cell division.

We considered the possibility that Msh5 might have a role in CSR only in the absence of Msh2, i.e., these two MMR proteins might be partially redundant. We mated $msh2^{+/-}$ and $msh5^{+/-}$ mice to obtain $msh2^{-/-}msh5^{-/-}$ double-KO mice. Splenic B cells were isolated and CSR was assayed in comparison with B cells from both single-KO and WT mice. As shown in Fig. 4, CSR in the double-KO B cells is not further reduced relative to $msh2^{-/-}$ B cells, suggesting that Msh2 deficiency does not increase the dependence of CSR on Msh5.

In B cells from mice in which all of the Sμ tandem repeats (TR) have been deleted (SμTR$^{-/-}$), switching is ~50% reduced compared with WT cells (35). However, CSR is nearly ablated in SμTR$^{-/-}$ B cells that are also deficient in Msh2 (36). This is most likely because mismatch repair appears to function to convert SSBs on opposite strands to DSBs, if the SSBs are too far apart to spontaneously form a DSB (7,9). As there are relatively few AID hotspot targets (AGCT) in the remnants of the Sμ region present in these mice, there are likely to be fewer SSBs. We asked whether Msh5 might also be essential for CSR in the SμTR$^{-/-}$ B cells by breeding SμTR$^{-/-}msh5^{-/-}$ mice. However, CSR is no further reduced in B cells from these mice than from SμTR$^{-/-}$ mice (Fig.4), further indicating that Msh5 does not function in the same pathway as Msh2 during CSR.

Sμ DNA DSBs are not altered in Msh5-deficient mice

In cells undergoing CSR, DSBs can be detected by LM-PCR in both donor (Sμ) and acceptor S regions, and these DSBs are dependent upon AID, uracil DNA glycosylase, and apurinic/apyrimidinic endonuclease (8,28,37,38). The Sμ DSBs are also reduced in mismatch repair-deficient B cells and in B cells lacking the SμTR (9). We asked whether Sμ DSBs are reduced in *msh5*^{-/-} B cells and found they are not (Fig. 5).

Sμ-Sγ3 junctions and Sμ mutations are unaltered in Msh5-deficient mice

CSR normally occurs by nonhomologous end-joining recombination, usually resulting in 0-2 bp of microhomology at the S-S junctions, consistent with the requirement for Ku70-Ku80 for CSR (39-41). Msh5 deficiency does not alter the length of junctional microhomology at Sμ-Sγ3 junctions in cells induced to switch in culture, suggesting it does not alter the end-joining reaction (Fig. 6*A*). However, the study by Sekine et al. (23) reported a significant increase in the Sμ-Sγ3 junctional microhomology in ex vivo spleen cells from *msh5*^{-/-} mice (1.4 bp in WT vs 2.5 bp in *msh5*^{-/-}, *p* = 0.023). The FvB strain of mice was used in that study, which they reported express low levels of Msh5 mRNA. Using the identical nested primers they used, we have cloned and sequenced Sμ-Sγ3 junctions from ex vivo spleen cells from two *msh5*^{-/-} mice and two WT littermates (B6 strain). We find no difference in the distribution of microhomology lengths between WT and *msh5*^{-/-} mice (Fig. 6*B*). The average junctional microhomology in WT mice was 1.9 bp, and in *msh5*^{-/-} cells was 1.8 bp.

During induction of CSR, mutations are introduced in the un-rearranged (germline) Sμ region and into the recombining S regions due to the activity of AID and the repair activities that follow (30,42-44). There was no difference in mutation frequency in the 5′ portion of the germline Sμ segments in WT and *msh5*^{-/-} B cells induced to switch to IgG3 with LPS plus anti-δ-dextran, WT: 30.9×10^{-4} mutations per nt (19,417 nt sequenced) and *msh5*^{-/-}: 28.3×10^{-4} (42,160 nt sequenced).

Msh5 does not regulate trans-chromosomal (interallele) CSR

CSR occurs between the two chromosome homologs at a surprisingly high frequency in rabbits and mice. For example, in rabbits the frequency of interallelic CSR to IgA is ~7% of the total CSR(24). Interallelic CSR to both IgA and IgG3 has also been observed in mice, occurring in 7 or 17% of the cells that have switched to IgA or IgG3, respectively (25,26). In these experiments, F_1 animals were used, in which the two alleles can be distinguished. The investigators either examined allelic markers in both the C_H and V_H genes (rabbits) or used a nonfunctional V_H gene knockin allele, and determined whether after CSR, the functional allele became associated with the C_H allotype from the nonfunctional knockin allele (mice). The high frequency of *trans*-CSR indicates that during CSR the two homologous chromosomes are closely positioned in nuclei, and also suggests that *trans*-CSR might be useful for rescuing cells with nonfunctional alleles (26).

Because the Msh4-Msh5 heterodimer binds to Holliday junctions and regulates crossing-over events between two homologous chromosomes during meiosis, it seemed possible that Msh5 in B cells might be involved in regulating *trans*-CSR. To assay *trans*-CSR, we bred 129 (IgH[a]) × B6 (IgH[b]) *msh5*^{-/-} F_1 mice and compared *trans*-CSR in B cells from these mice with B cells from WT 129 × B6 F_1 mice. Ex vivo B cells from both types of mice expressed either IgM[a] or IgM[b] in equal proportions (Fig. **7**). B cells were induced to switch to IgG3 with LPS plus anti-δ-dextran and on day 4, DNA was isolated from the cells, and Sμ-Sγ3 junctions were amplified by PCR and cloned. *Trans*- and *cis*-CSR were determined by analyzing single-nucleotide differences in the amplified Sμ-Sγ3 sequences. There are five and seven single-nucleotide polymorphisms between the regions amplified in the Sμ and Sγ3 sequences, respectively (see *Materials and Methods*). However, if S-S recombination results in deletion

of the segments of the S regions containing these polymorphic nucleotides, we only include sequences that had at least two of the polymorphisms, to prevent misidentification due to possible mutations in the S regions. The frequency of *trans*-CSR to IgG3 was 8.3% in 108 junctions from WT and 12.7% in 110 junctions from *msh5*^{-/-} B cells. This small difference is not significant ($p = 0.40$). The finding that Msh5 does not regulate *trans*-CSR is consistent with the fact that CSR does not occur by homologous recombination, and suggests that *trans*-CSR also does not involve homologous recombination, but most likely involves an end-joining reaction, as does normal *cis*-CSR.

It has been proposed that chromosomal translocations such as those that recombine the IgH locus with the c-*myc* oncogene preferentially occur using an alternative end-joining pathway rather than classical nonhomologous end joining (45,46). Recombination occurring by the alternative pathway is characterized by having longer microhomologies at the junctions and the absence of blunt-end junctions. To examine whether the *trans*-CSR junctions might occur by the alternative pathway, we compared the lengths of the microhomologies occurring at the Sμ-Sγ3 junctions in the *trans*-CSR junctions with those occurring by *cis*-CSR. However, we found no difference between the lengths of junctional microhomology in *trans*- and *cis*-CSR junctions, nor between *trans*- and *cis*-junctions from WT and *msh5*^{-/-} B cells (Fig. 6C). These results suggest that *trans*-CSR does not occur by a different type of end joining pathway from *cis*-CSR, and due to the fact that the average junctions have only ~2 bp of microhomology, most likely the recombination occurs predominantly by classical nonhomologous end-joining.

Discussion

The results reported in this study indicate that Msh5 does not have a role in CSR. Furthermore, we were unable to detect in splenic B cells (ex vivo or activated) any cDNAs that encode the form of Msh5 found in testis, because all the cDNAs sequenced encoded splice variants or had amino acid substitutions. This is reminiscent of the report that somatic tissues express only splice variants of Msh4 mRNA and not the full-length form predominant in testis (34). It is possible that spleen B cells contain a short form of Msh5 protein that does not interact with Msh4, but because our Ab is specific for the C terminus, we could not address this possibility. The only well-established role of Msh5 is as a partner with Msh4, where it is known to be essential for meiotic recombination (47-49). Also, dimerization of Msh4 and Msh5 is important for nuclear localization (50), suggesting that the C-terminal truncation of Msh5 may prevent it from acting in the nucleus. We hypothesize that alternative splicing that results in nonfunctional mRNAs is used to suppress inappropriate functionality of the *msh4* and *msh5* genes, whose activity in B cells might result in erroneous recombinations, given the involvement of these proteins in meiotic recombination during gametogenesis, and the presence of induced DSBs in the Ig genes during development and during B cell activation.

Recently, Sekine et al. (23) reported that MRL/*lpr* mice congenic for a 13-megabase segment from B6 (H-2[b/b]), which includes the entire MHC locus in addition to the Msh5 gene, have 5% of the level of Msh5 mRNA in their spleens relative to spleens from MRL/*lpr* H-2[k/k] mice. Some of these MRL/*lpr* H-2[b/b] mice expressed very low levels of serum IgG3, but because there was incomplete penetrance of this phenotype and the 13-megabase region encodes numerous genes, it is unknown whether the phenotypes they observed in these mice are due to Msh5 levels. The investigators also studied CSR in cultured B cells from *msh5*^{-/-} (FvB background) and *msh4*^{-/-} (B6 background) mice. Similar to our results, they found no significant reduction in CSR to IgG1, IgG3, or IgA in B cells deficient for Msh5, nor in B cells deficient for Msh4. However, in the one other analysis they performed with the KO mice, their results appear to differ from ours. They found significant increases in the lengths of junctional microhomologies at Sμ-Sγ3 and Sμ-Sα junctions in ex vivo FvB *msh5*^{-/-} spleen cells relative to FvB WT cells, although the average increase was modest (1.4 bp in WT; 2.5 in *msh5*^{-/-}).

However, because we have not examined *msh5*$^{-/-}$ mice in the FvB background, it is possible the background causes the difference. The difference is unlikely to be due to the levels of Msh5, because they found that B cells from FvB mice express low levels of Msh5 mRNA, as do B6 B cells.

Msh5 deficiency results in sterility and diminution in testicular size and a complete loss of ovarian structures (15). Surgical castration causes a loss of estrogen production in both females and males (51,52). Estrogen has been shown to inhibit B cell development at the pro- and pre-B cell stages (53), and surgical removal of the ovaries or testicles increases the numbers of B220^{+} cells in the bone marrow (54). In agreement with these data, we have found that Msh5 deficiency results in increased numbers and proportion of B220^{+} cells in the bone marrow (our unpublished data). In apparent contrast with the effect on B cell development, estrogen treatment of mice greatly increases the numbers of IgM-, IgG-, and IgA-secreting B cells in both bone marrow and spleen (52), but the effect of castration on Ab-secreting cells has not been reported. These results illustrate the complexity inherent in analyzing the effects of Msh5 deficiency on Ig class switching in vivo, and support the use of splenic B cell cultures as a model for analyses of the effects of DNA repair genes on CSR.

Our results suggest that Msh5 is not involved in CSR, consistent with the known role of Msh5 in monitoring homologous recombination during meiosis and its ability to bind Holliday junctions, which do not form during end-joining recombination. Our results also suggest that *trans*-CSR, which must involve close alignment of homologous chromosomes at the Ig S regions, does not require Msh5. Furthermore, our finding that *trans* S*μ*-S*γ*3 junctions do not show increased lengths of microhomology relative to intrachromosomal CSR junctions, nor any differences between WT and *msh5*$^{-/-}$ B cells, suggests that *trans*-CSR in both WT and *msh5*$^{-/-}$ cells occurs by a normal end-joining recombination. However, because we failed to detect any normally spliced Msh5 mRNA in splenic B cells, and were unable to detect Msh5 protein, it is possible that splenic B cells do not express normal Msh5, and that this explains the absence of a CSR phenotype when the *msh5* gene is deleted.

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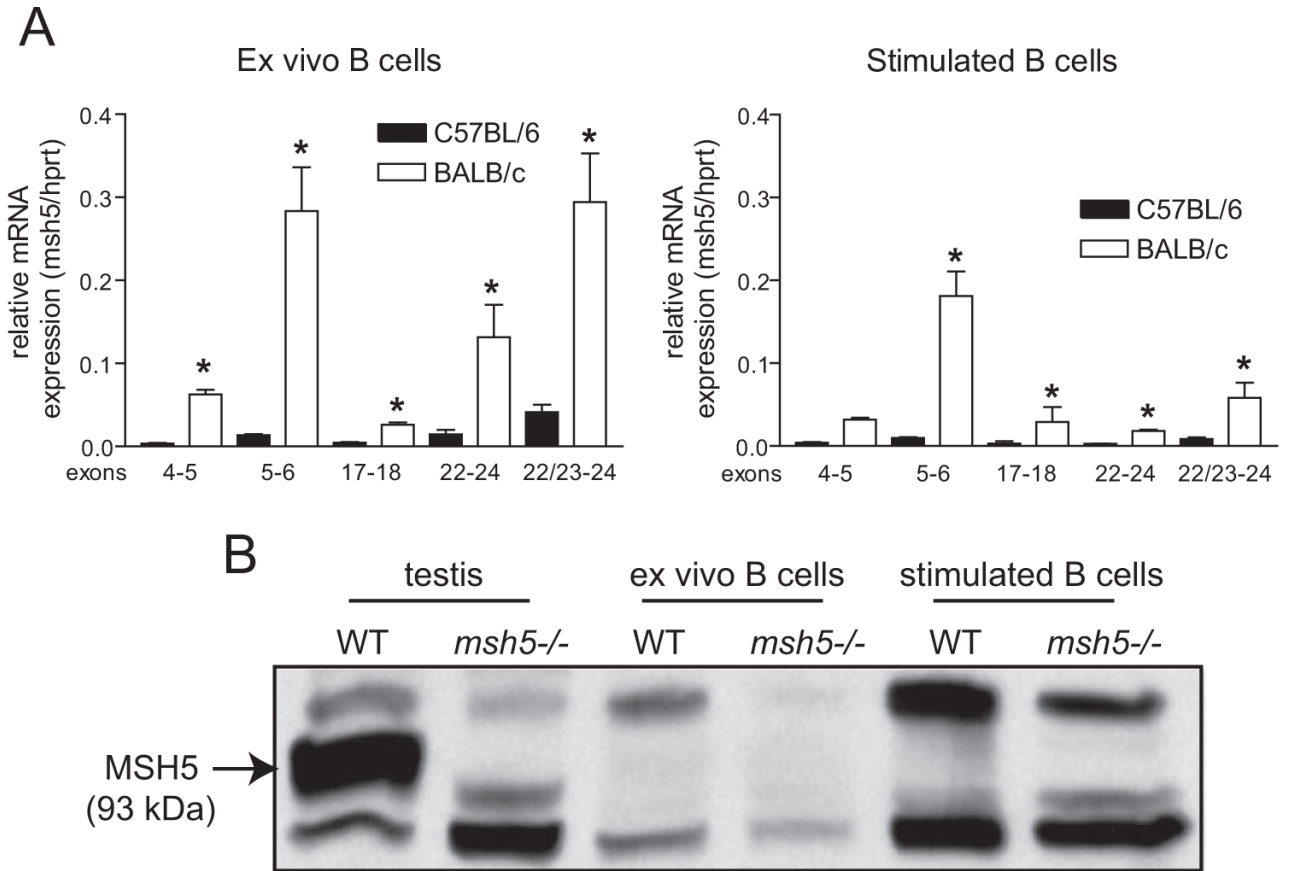
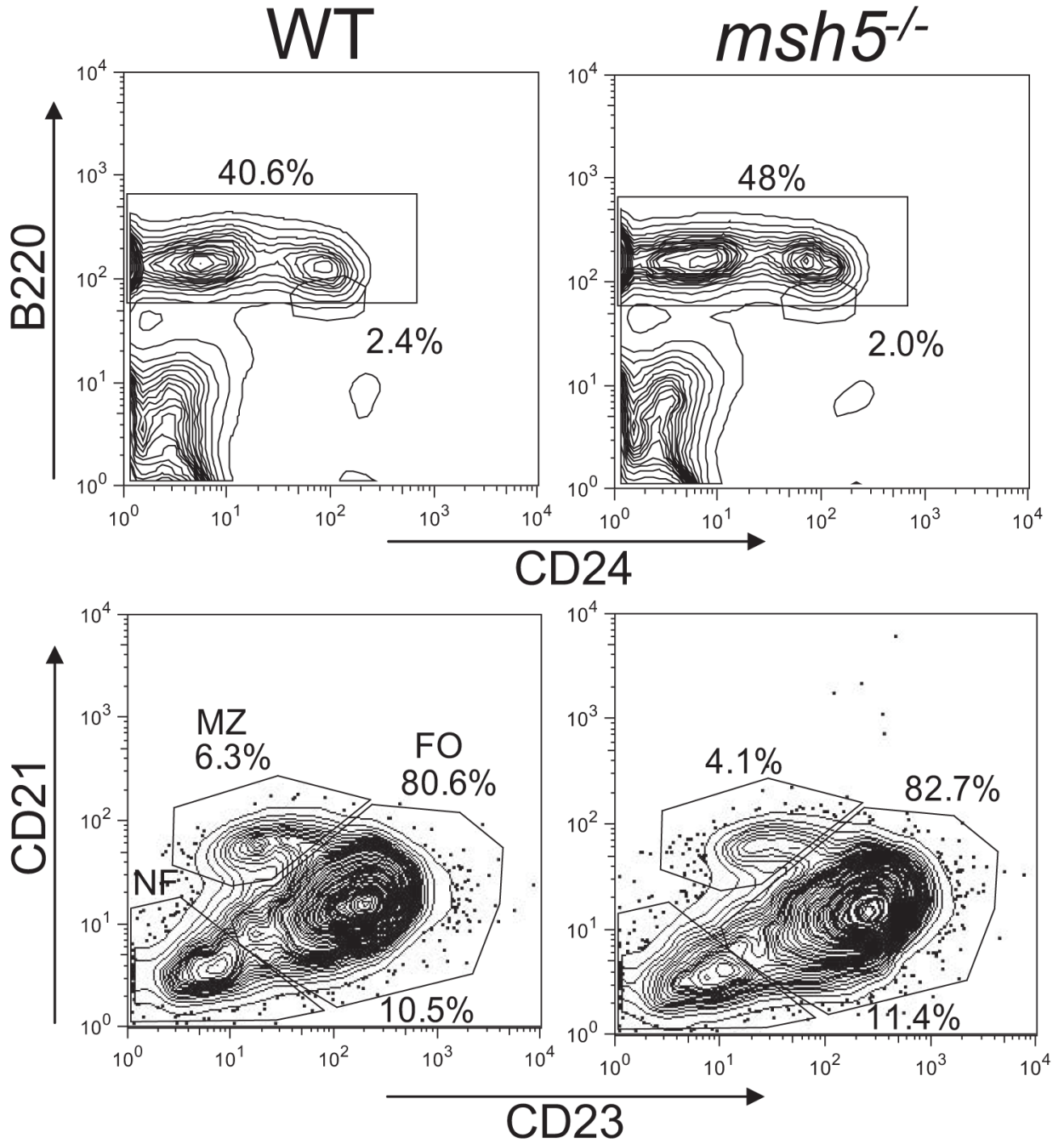**FIGURE 1.**
Msh5 expression in B cells from C57BL/6 and BALB/c mice. *A, Msh5* mRNA expression relative to steady-state control gene (*Hprt*) was measured by quantitative RT-PCR on cDNA from ex vivo and LPS plus IL-4-stimulated B cells from C57BL/6 and BALB/c mice. Primers for designated exons are given below the *x*-axis of the graph. Means + SEM are shown ($n \geq$ 4); *, $p < 0.05$, calculated by two-tailed Student's *t* test. *B*, A total of 25 μg of whole-cell extracts obtained from testis, ex vivo B cells, and LPS plus IL-4-stimulated B cells from WT and *msh5*^{-/-} mice was electrophoresed and immunoblotted with rabbit anti-Msh5.
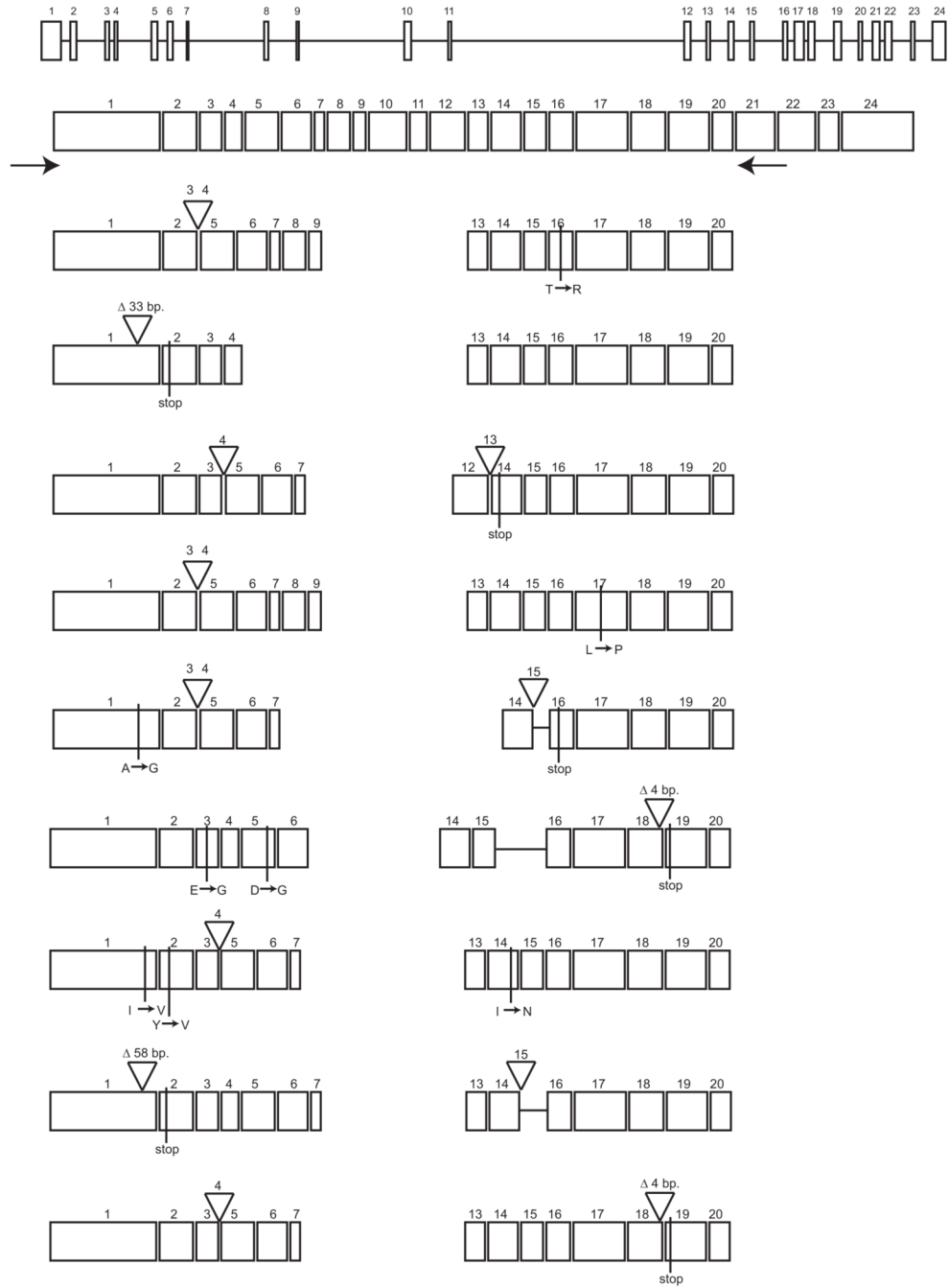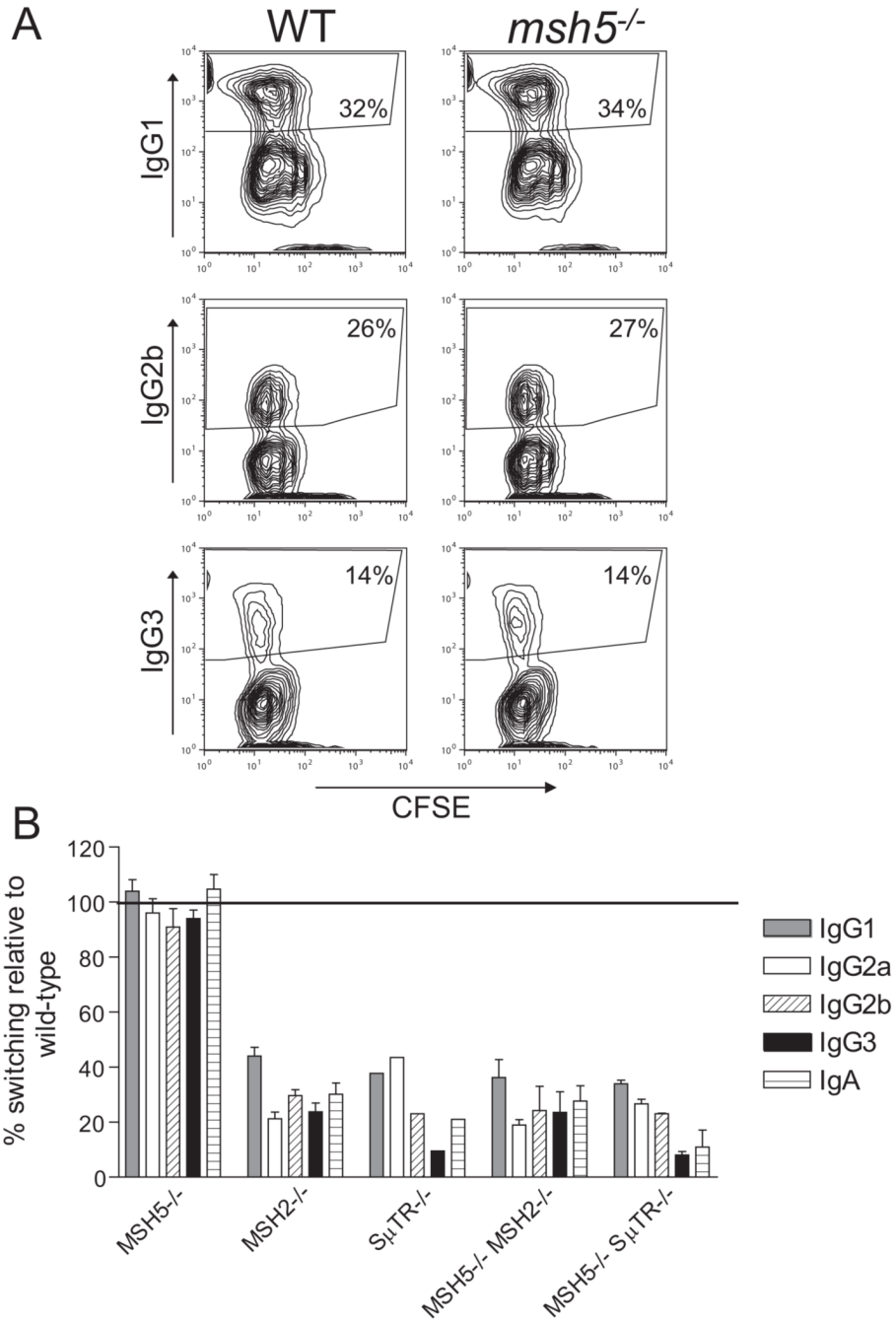
FIGURE 2.
Msh5 splice variants in ex vivo splenic B cells from BALB/c mice. *Upper panel*, Depicts a map of the mouse *msh5* gene, encompassing 24 exons. *Second panel*, Shows the mRNA; primers that were used to identify splice variants are shown as arrows. *Lower panels*, Show different splice variants found in ex vivo splenic B cells from BALB/c mice. Spliced out sequences are depicted as triangles. Amino acid replacement mutations and stop codons are shown as vertical lines; retained introns/sequences are shown as horizontal lines. No normal Msh5 mRNA was found in nine clones that aligned to full-length Msh5. The obtained sequences do not fully cover the region amplified by the indicated primers as shown by the gap between the two fragments shown for each cDNA.
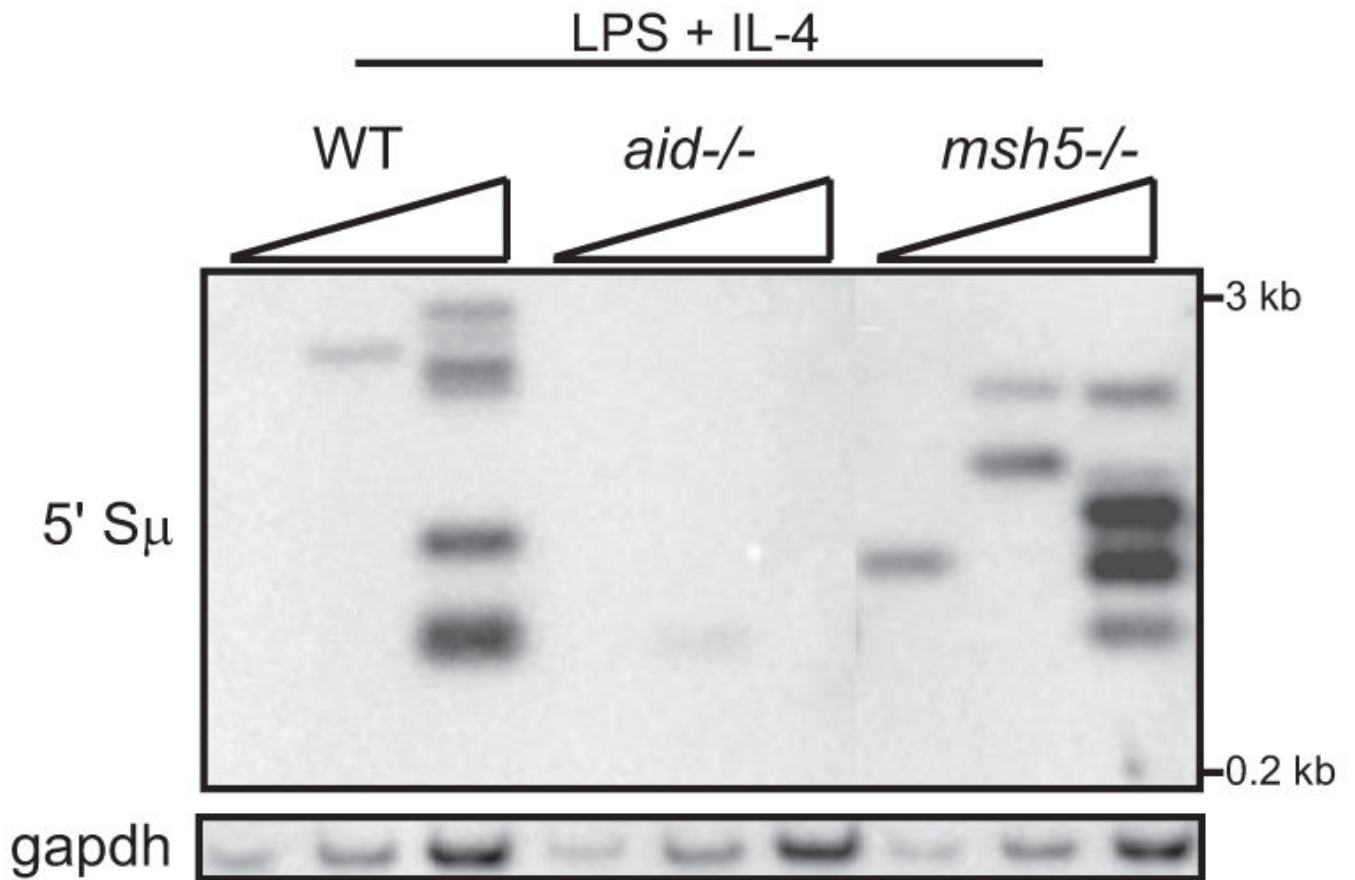
FIGURE 3.
Mature splenic B cell subsets are similar in *msh5*^{-/-} and WT littermate mice. FACS analysis of total ex vivo splenocytes after staining with anti-B220, anti-CD24, anti-CD21, and anti-CD23. *Upper panels*, Show B220+ (total B cells) and B220^{low} CD24^{high} (immature B cells). *Lower panels*, Show CD21 and CD23 staining within the B220+ gate: CD21^{high}CD23^{low} (marginal zone B cells), CD21^{low}CD23^{high} (follicular B cells), and CD21^{low}CD23^{low} (newly formed B cells).

FIGURE 4.
Msh5 deficiency does not alter CSR. Splenic B cells were induced to undergo CSR to IgG1 (LPS plus IL-4), IgG2a (LPS plus IFN-γ), IgG2b (LPS plus TGF-β), IgG3 (LPS plus anti-δ-dextran), and IgA (LPS plus IL-4 plus IL-5 plus TGF-β plus anti-δ-dextran). Ig class switching was assayed by flow cytometry using Abs specific for Ig isotypes. Splenic B cells from WT littermate controls were used in each experiment. Seven independent experiments were performed for msh5^{-/-}; three for msh2^{-/-}, msh5^{-/-}msh2^{-/-}, and msh5^{-/-}SμTR^{-/-}; and one for SμTR^{-/-}. *A*, Representative examples of FACS analyses for IgG1, IgG2b, and IgG3 switching in CFSE-stained B cells. *B*, Mean percentages plus SEM of switching relative to WT (=100%)

are shown. One-sample Student's *t* test showed no significant difference between *msh5*^{-/-} vs WT, or *msh5*^{-/-}*msh2*^{-/-}, *msh5*^{-/-}*SµTR*^{-/-} double KO vs single KO (*msh2*^{-/-} or *SµTR*^{-/-}).
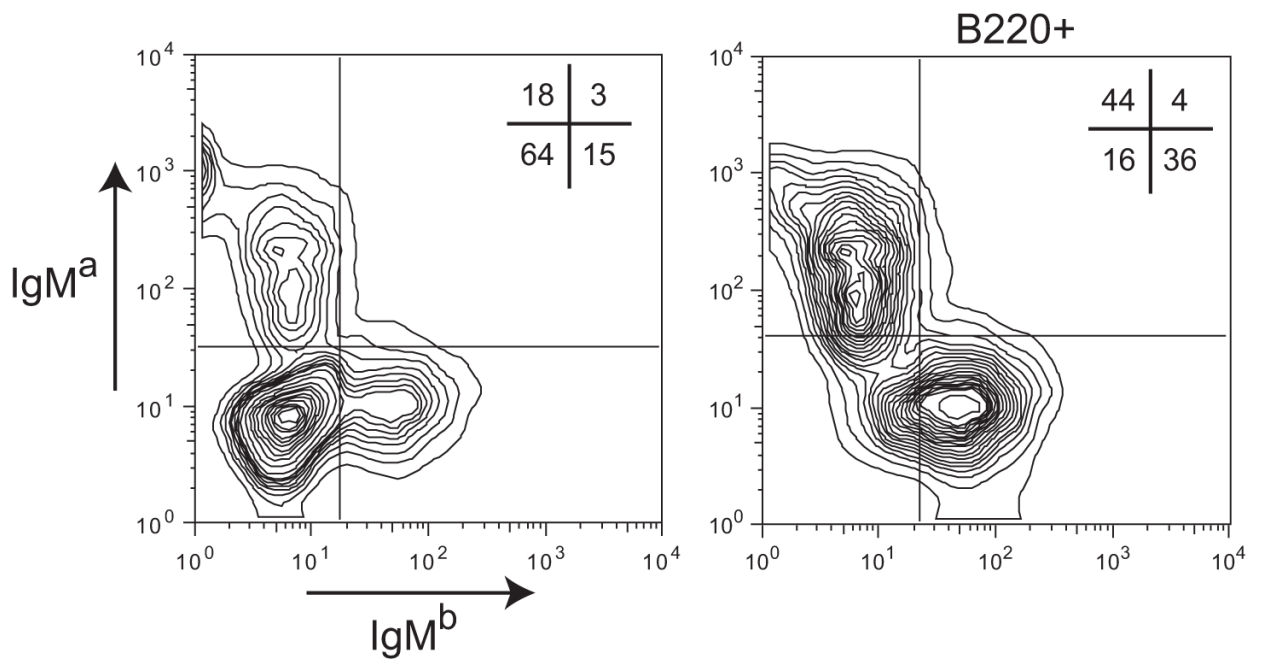
FIGURE 5.
Sμ DSBs are normal in Msh5-deficient switching B cells. Sμ-blunt DSBs were assessed by LM-PCR on 3-fold dilutions of DNA from splenic B cells stimulated with LPS plus IL-4 for 2 days. Input of DNA was normalized by PCR for the *gapdh* gene. LM-PCR was performed for the 5′ portion of Sμ, detecting blunt DSBs downstream of this primer. Genomic DNA from *aid*^{-/-} splenic B cells stimulated with LPS plus IL-4 is shown as a negative control.
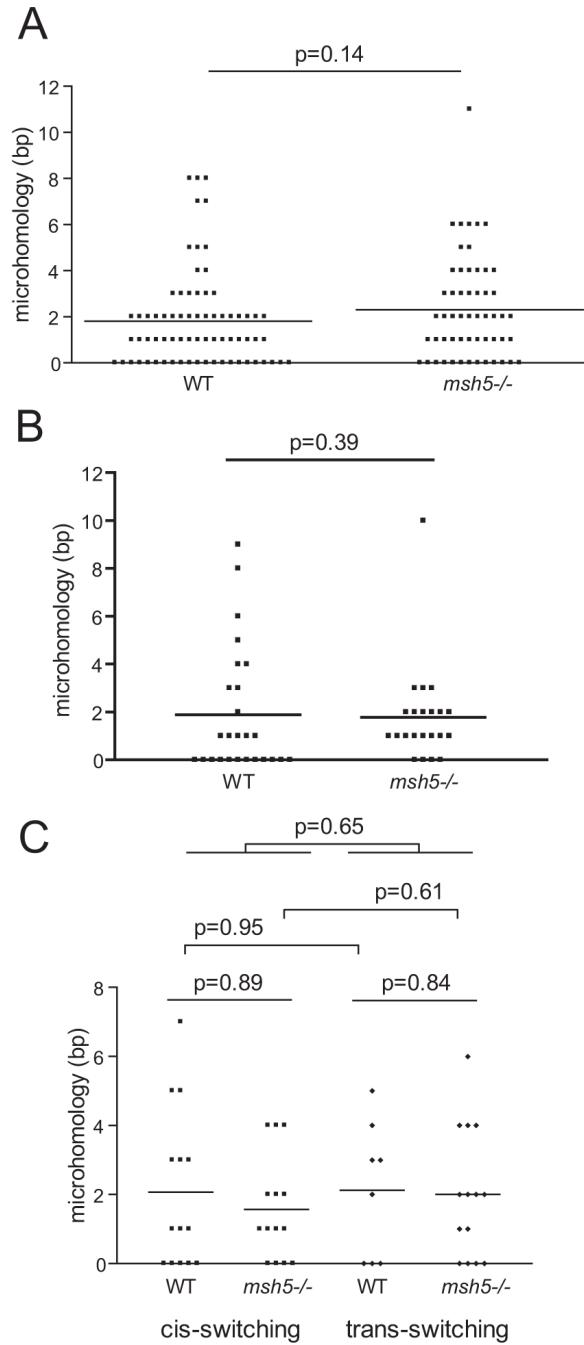
FIGURE 6.
Unaltered microhomology at Sμ-Sγ3 junctions in *msh5*^{-/-} B cells and in *trans*-chromosomal switching. S-S junctions were amplified from genomic DNA isolated from splenic B cells stimulated for 4 days with LPS plus anti-δ-dextran (*A* and *C*), or from ex vivo spleen cells (*B*). Squares/diamonds represent microhomology between donor/acceptor sequences at the junction. The horizontal lines indicate the means. *A*, Microhomology at Sμ-Sγ3 junctions is not significantly different in WT and *msh5*^{-/-} cells from B cells induced to switch in culture. *B*, Microhomology is not significantly different in Sμ-Sγ3 junctions from ex vivo WT and *msh5*^{-/-} spleen cells. *C*, Microhomology is not significantly different in *trans*-chromosomal

switching in WT or in *trans*-chromosomal switching in *msh5*$^{-/-}$ or WT vs *msh5*$^{-/-}$ (Mann-Whitney U *t* test used for all comparisons).

FIGURE 7.
IgM^a and IgM^b are equally expressed in 129 (IgH^a) $\times$ B6 (IgH^b) F$_1$ mice. Representative FACS analysis of peripheral blood from 129 (IgH^a) $\times$ B6 (IgH^b) F$_1$ WT mouse. Percentages of cells in each quadrant are given in *upper right corner* of FACS plots. *Left FACS plot*, Shows IgM^a/IgM^b staining within forward light scatter/side light scatter gate; *right FACS plot*, shows IgM^a/IgM^b staining within B220$^+$ gate.

Table I

Msh5 cDNA sequences from mouse B cells

	cDNAs Sequenced	cDNAs Aligning to Full-Length *msh5*	cDNAs Encoding Normal *msh5* Protein	Mutations in Msh5 cDNAs
C57BL/6	10	2	0	Q603W
				Q88R, S169P, I402M
C57BL/6	17	8	0	S24C, A480V, Y505H, S598G
Activated B cells				4-bp deletion at exon 18-19 junction: STOP (4 clones)
				Intron inclusion after exon 18: STOP
				Intron inclusion after exon 20: STOP (2 clones)
BALB/c	10	9	0	Splice variants; Fig. 2
Ex vivo B cells				
BALB/c	10	0	0	
Activated B cells				