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CUTTING EDGE

Cutting Edge: The G-U Mismatch Glycosylase Methyl-CpG Binding Domain 4 Is Dispensable for Somatic Hypermutation and Class Switch Recombination[1]

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Affinity maturation of the humoral response is accomplished by somatic hypermutation and class switch recombination (CSR) of Ig genes. Activation-induced cytidine deaminase likely initiates these processes by deamination of cytidines in the V and switch regions of Ig genes. This activity is expected to produce G-U mismatches that can be substrates for MutS homolog 2/MutS homolog 6 heterodimers and for uracil DNA glycosylase. However, G-T and G-U mismatches are also substrates of the methyl-CpG binding domain 4 (Mbd4) glycosylase. To determine whether Mbd4 functions downstream of activation-induced cytidine deaminase activity, we examined somatic hypermutation and CSR in Mbd4$^{-/-}$ mice. In this study, we report that CSR, as analyzed by an in vitro switch assay and by in vivo immunizations, is unaffected in Mbd4$^{-/-}$ mice. In addition, the hypermutated JH2 to JH4 region in Peyer's patch B cells showed no effects as a result of Mbd4 deficiency. These data indicate that the Mbd4 glycosylase does not significantly contribute to mechanisms of Ab diversification. The Journal of Immunology,* 2003, 170: 1620–1624.

Somatic hypermutation (SHM)[4] and class switch recombination (CSR) of Ig genes leads to the affinity maturation of the Ab response (1). Both of these processes occur at the same stage of B cell differentiation and both require activation-induced cytidine deaminase (AID) (2). Although the substrate for AID has yet to be defined biochemically, a previously proposed model for SHM and CSR in which cytidines in DNA are deaminated (3, 4) gained further support when AID was found to produce many transition mutations at G-C base pairs when expressed in hybridomas and fibroblasts (5, 6). Indeed, recent findings show that uracil, a product of cytidine deamination, is an intermediate in SHM and CSR (7).

If AID is indeed a DNA-specific cytidine deaminase, different DNA repair pathways could act on its product, a G-U mismatch. First, MutS homolog (MSH)2 and MSH6 heterodimers together with MutL homolog (MLH)1 and postmeiotic segregation 2 could bind to and initiate mismatch repair. In fact, mice deficient in MSH2 or MSH6 (8–14) or in MLH1 or postmeiotic segregation 2 (11, 15–17) are defective in SHM and CSR. Second, uracil DNA glycosylase (UNG) could compete with MSH2/6 dimers to excise uracil from DNA. Recent evidence shows that B cells from UNG-deficient mice have reduced CSR activity and an altered spectrum of SHM. Mutations occurring at G-C base pairs were mostly transition mutations, suggesting that, in UNG$^{-/-}$ mice, adenines are placed opposite to uracils during replication of the Ig V region (7).

Methyl-CpG binding domain 4 (Mbd4) is a novel DNA repair enzyme that has been implicated in the repair of G-T and G-U mismatches, especially in CpG sites (18, 19). Mbd4, which is ubiquitously expressed (18), belongs to a family of genes that encode for proteins that bind preferentially to methyl-CpG dinucleotides (18). In addition to an N-terminal region that recognizes methyl-C, this enzyme contains a C-terminal catalytic domain that shares amino acid identity with many known bacterial DNA glycosylases and has DNA glycosylase activity on G-T or G-U mismatches at CpG sites (20, 21). In support of these findings, mice deficient in Mbd4 have a 2- to 3-fold increase in C to T transition mutations at CpG motifs (22, 23). Because the human homolog of Mbd4, also called methyl-CpG binding endonuclease 1, interacts with MLH1, it was suggested that it might also be a component of or recruit the mismatch repair system (24). And because AID creates G-U mismatches, Mbd4 could affect the rate or outcome of SHM and CSR through the mismatch repair pathway. Therefore, in

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[4] Abbreviations used in this paper: SHM, somatic hypermutation; AID, activation-induced cytidine deaminase; CGG, chicken γ globulin; CSR, class switch recombination; Mbd4, methyl-CpG binding domain 4; MSH, MutS homolog; MLH, MutL homolog; NP, nitrophenyl; UNG, uracil DNA glycosylase.

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this study, we have examined whether mice deficient in Mbd4 have defects in mechanisms of Ab diversification.

Materials and Methods

Mice and immunizations

The Mbd4$^{-/-}$ mice have been previously described (22). The mice used in this study were backcrossed to C57BL/6 mice for four generations and were housed in a pathogen-free facility. Throughout this article, Mbd4$^{+/-}$ and Mbd4$^{+/+}$ mice will be referred to as Mbd4$^{+/-}$ because no differences were observed between them. Two-mo-old Mbd4$^{-/-}$ and Mbd4$^{+/-}$ mice were immunized i.p. with nitrophenyl (NP) coupled to chicken γ globulin (CGG) (NP_{23}-CGG), and sera was monitored for Abs specific to NP by ELISA, as previously described (25). High and low affinity anti-NP Abs were measured on NP_2-BSA- and NP_{17}-BSA-coated plates (Biosearch Technologies, Novato, CA), respectively. Titers were assigned to the serial dilution corresponding to half maximum absorbance.

In vitro switching assay

Switching was induced essentially as described (11). Briefly, nonimmunized mice were sacrificed and spleens were harvested. Splenocytes were isolated and depleted of T cells by complement-mediated lysis using rat anti-mouse CD4 (ATCC GK1.5; American Type Culture Collection, Manassas, VA), CD8 (ATCC 2.43), and CD90.2 (Southern Biotechnology Associates, Birmingham, AL) Abs followed by addition of an anti-rat κ L chain Ab and guinea pig complement (Life Technologies, Rockville, MD). Splenocytes were plated in RPMI 1640 medium containing 10% FCS and 50 μg/ml LPS (Sigma-Aldrich, St. Louis, MO) for 4 days, and cells were harvested and stained with anti-IgG1 or anti-IgG3 FITC (BD PharMingen, San Diego, CA) and anti-IgM CY5 (Jackson Immunochemicals, West Grove, PA) with the appropriate unstained and single-color controls. Cells were washed with PBS and fixed with 1% paraformaldehyde in PBS. A FACSCalibur (BD Biosciences, Mountain View, CA) apparatus was used to acquire the data. FACS analysis was performed using the FloJo software package (Tree Star, San Carlos, CA). Cells were gated for viability based on the forward- and side-scatter profiles, which was confirmed by propidium iodide staining. The IgG-positive population was scored according to the gating profile shown in Fig. 1.

PCR amplification and sequence analysis

A nested PCR strategy was used to amplify V186.2 IgG1 transcripts from NP-immunized mice as described previously (25). To sequence the JH2 to JH4 region, Peyer's patches were harvested from two Mbd4$^{-/-}$ and two Mbd4$^{+/+}$ 1-year-old mice, and peanut lectin agglutinin-high B cells were FACS sorted as previously described (14). The JH2 through JH4 regions were amplified from Peyer's patch B cells using Pfu turbo (Stratagene, La Jolla, CA) and the following primers: 5′ primer, 5′-GGC ACC ACT CTC ACA GTC TCC TCA GG-3′, and 3′ primer, 5′-TGA GAC CGA GGC TAG ATG CC-3′, using the following conditions: 95° for 30 s, 61° for 30 s, and 72° for 1.5 min for 35 cycles. PCR products were cloned and sequenced as previously described (5). Statistics for sequencing data in Table I were measured by the independent-sample *t* test with equal variances assumed (Excel; Microsoft, Redmond, WA).

Results

In vitro switching analysis

To determine whether CSR activity was compromised in Mbd4$^{-/-}$ mice, B cell splenocytes were isolated from naive 2-mo-old Mbd4$^{-/-}$ and Mbd4$^{+/-}$ mice and then stimulated with LPS to induce switching. LPS stimulation in vitro induces mouse B cells to switch from IgM to IgG3 (11). Cells were cultured for 4 days, and LPS blasts were stained with anti-IgM and anti-IgG1 or anti-IgG3, and two-color FACS was used to determine the percentage of IgG1- or IgG3-positive cells within the live populations (Fig. 1*A*). Switching to IgG1 served as control for spontaneous switching in short-term cultures. This assay has been successfully used in a number of studies to show that mice with genetic defects in mismatch repair have defects in CSR activity (10, 11). Two independent experiments were performed, each using two male or female Mbd4$^{-/-}$ mice with two age-matched heterozygous or wild-type littermates. Approximately 10% of Mbd4$^{-/-}$ and control B cells switched from IgM to IgG3 with LPS stimulation (Fig. 1), while only

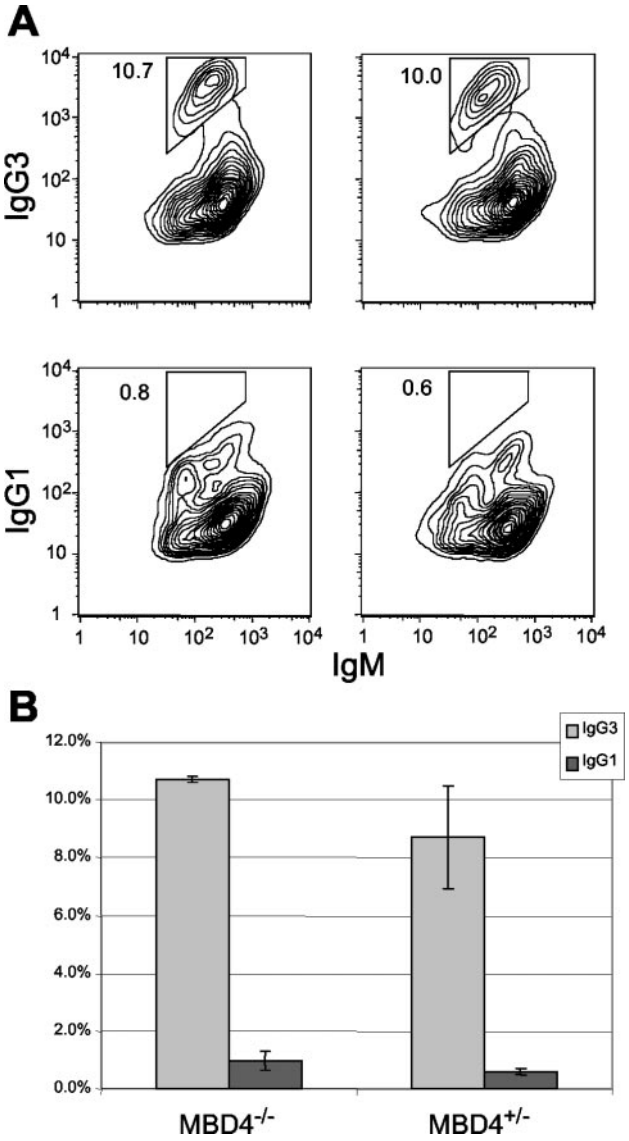

FIGURE 1. In vitro CSR of splenic B cells. *A*, CSR in LPS-stimulated splenic B cells. Splenic B cells from Mbd4$^{+/-}$ and Mbd4$^{-/-}$ mice were purified (see *Materials and Methods*) and stimulated with LPS for 4 days. Two-color FACS for representative Mbd4$^{-/-}$ (*left two panels*) and Mbd4$^{+/-}$ mice (*right two panels*) are displayed. Percentages of IgG3- and IgG1-switched B cells (gated) are shown for each panel (*inset*). *B*, Bar graph depicts summary of two independent experiments using B cells from two Mbd4$^{+/-}$ and two Mbd4$^{-/-}$ mice.

~0.5% switched to IgG1. These data show that Mbd4 deficiency does not result in an appreciable loss of in vitro CSR activity in B cells.

Normal in vivo humoral response in Mbd4$^{-/-}$ mice

Although the in vitro switching assay did not reveal any defects in CSR to IgG3, we sought to confirm this result in vivo. Mbd4$^{-/-}$ and littermate control mice were immunized by i.p. injection with the hapten NP coupled to CGG (NP_{23}-CGG) in alum. This protocol elicits a T cell-dependent Ab response that was assayed by ELISA on BSA highly substituted with NP (i.e., NP_{17}-BSA; Fig. 2*A*). Sera were harvested from wild-type, heterozygous, and Mbd4$^{-/-}$ mice after primary immunizations and analyzed by ELISA for an IgG1 response specific for NP. As shown in Fig. 2*A*, control and Mbd4$^{-/-}$ mice produced similar

Table I. *SHM of Ig sequences in splenic and Peyer's patch B cells*[a]

	Spleen B cell V 186.2[b]		Peyer's Patch B Cell JH2 to JH4 Region[c]	
	Mbd4$^{-/-}$	Mbd4$^{+/-}$	Mbd4$^{-/-}$	Mbd4$^{+/-}$
Mutation frequency[d] ($\times 10^{-3}$)	1.5	2.3	4.2	2.1
Mutated sequences/total (%)	25/90 (28)	32/80 (40)	14/33 (42)	26/62 (42)
G-C mutations/total (%)	25/39 (64)	27/54 (50)	71/176 (40)	71/172 (41)
Transition/total (%)	21/39 (54)	27/54 (50)	84/176 (48)	83/172 (48)
Hot spot/total (%)	13/39 (33)	12/54 (22)	19/176 (11)	23/172 (13)

[a] PCR products amplified by PFU polymerase were cloned and plasmids were sequenced. Identical mutations that were observed in more than one plasmid for each mouse were scored only once.
[b] The V 186.2 region is 294 bp long. These data were obtained from three Mbd4$^{-/-}$ and three littermate controls. Codon 33 mutations were excluded from this analysis because it affects Ab affinity (26).
[c] The JH2 to JH4 region is 1460 bp long, of which 1300 bp was sequenced. These data were obtained from two Mbd4$^{-/-}$ and two littermate controls. Three and six insertion/deletions were found in Mbd4$^{-/-}$ and Mbd4$^{+/-}$ mice, respectively.
[d] Frequency is defined as mutations per base pair sequenced (mut/bp).

total titers of IgG1 that bind to high substituted NP_{17}-BSA in the primary response. In addition, when the sera were examined on ELISA plates that were coated with NP_2-BSA, which captures only high affinity Abs (26), control and Mbd4$^{-/-}$ mice produce similar levels of high affinity IgG1 to NP in both the primary and secondary response (Fig. 2*B*). Because IgG1 Abs were assayed in vivo and IgG3 in vitro, these data show that Mbd4 deficiency does not have a detectable effect on CSR. In addition, similar high affinity anti-NP Ab titers were observed in both groups of mice suggesting that SHM is occurring normally in the Mbd4-deficient mice.

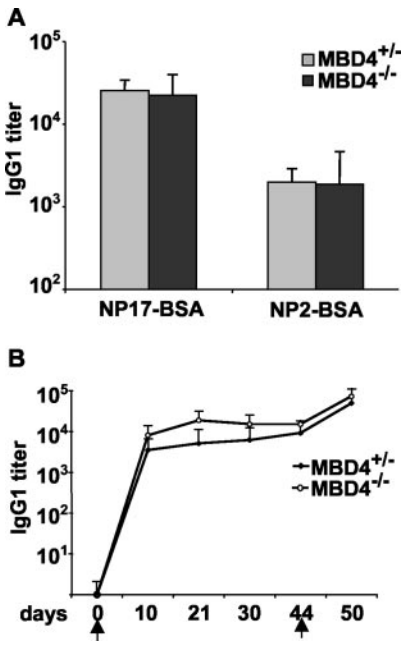

FIGURE 2. In vivo serum response to the NP hapten. *A*, Primary response to NP immunizations. IgG1 titers were determined in Mbd4$^{-/-}$ and littermate controls 10 days after immunizations. ELISAs were performed using NP_{17}-BSA and NP_2-BSA that capture total NP-specific IgG and high affinity NP-specific Ig, respectively. Data summarize results from five Mbd4$^{-/-}$ mice and five littermate controls. *B*, High affinity NP-specific IgG1 response. Two Mbd4$^{-/-}$ mice and two littermate controls were immunized with NP_{23}-CGG on days 0 and 44 (▲), and sera were withdrawn in 10-day intervals. ELISAs were performed using NP_2-BSA.

SHM is unaffected in Mbd4$^{-/-}$ mice

The V 186.2 H chain V region dominates the immune response to NP in C57BL/6 mice, making it possible to study SHM in

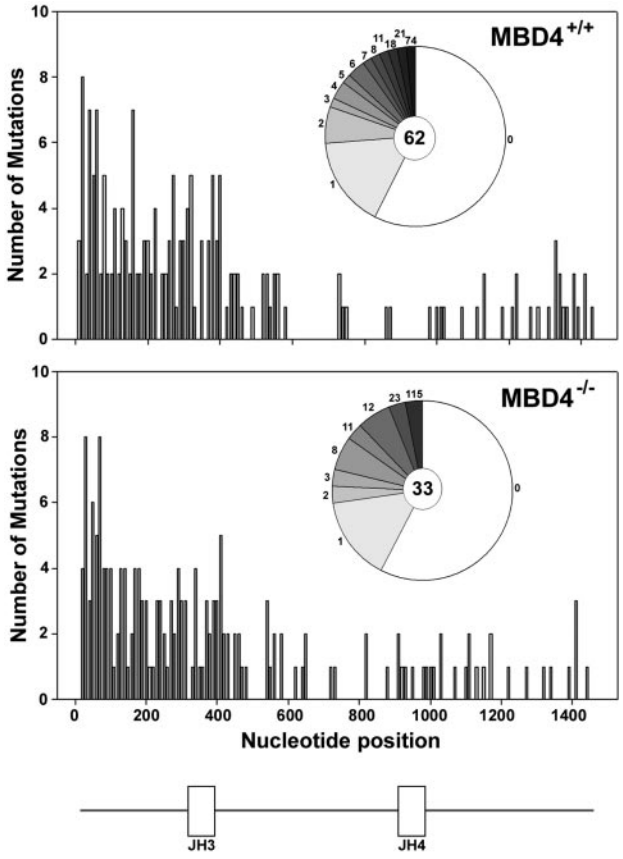

FIGURE 3. Somatic hypermutation of the JH2 to JH4 region in Peyer's patch B cells. The JH2 to JH4 region was sequenced in 1-year-old mice. Two wild-type (*upper panel*) and two Mbd4$^{-/-}$ mice (*lower panel*) were used for this analysis, and the data for each were grouped for display purposes, although no significant differences were seen for each individual mouse. The line graph depicts the location of mutations (in groups of 10 bp) identified in Mbd4$^{-/-}$ and littermate controls. Pie charts depict the distribution of mutation frequencies for the plasmid clones that were sequenced (center of pie) and the proportion of sequences with 0, 1, 2, and greater mutations (numbers of mutations outside the pie slices). The JH3 and JH4 exons displayed below the graph correspond to the nucleotide position of the mutations.

vivo (27). The V 186.2 region was amplified from Mbd4$^{-/-}$ and Mbd4$^{+/-}$ mice 10 days after NP-CGG immunization, and ~40–50 mutations/group were analyzed (Table I). The frequency of mutation was slightly lower in the Mbd4$^{-/-}$ mice, but this was not statistically significant. There was also a modest, but statistically insignificant, increase in the frequency of G/C mutations (64 vs 50% for Mbd4$^{-/-}$ and Mbd4$^{+/-}$) and in the targeting of mutation to RGYW hot spots (33 vs 22%) in the Mbd4$^{-/-}$ mice compared with their littermates (Table I).

Because mutations identified in the above analysis might have been influenced by selection for base pair changes that increase Ab affinity to NP, we sequenced the JH2 to JH4 region in germinal center B cells from Peyer's patches. This region is 3′ to the V region and mutates at high levels but does not encode protein and should not be subjected to selection (28, 29). In addition, because it comes from Peyer's patch B cells that have repeatedly recycled through germinal centers, some of the V region genes will have undergone multiple rounds of mutation and accumulated many base changes (29). Because the Mbd4$^{-/-}$ mice do not have microsatellite instability (22), there is no reason to believe that there will be any defects in B cell differentiation (12, 13, 29). In this case, ~170 mutations were analyzed in Mbd4$^{-/-}$ mice and in littermate controls (Fig. 3 and Table I). A comparison of the mutations found in this region showed no difference in the patterns of mutation between Mbd4$^{-/-}$ and littermate control mice (Table I). In addition, no difference was observed in the targeting of specific dinucleotide motifs identified by Shapiro et al. (30) (data not shown). A small increase in the mutation frequency of the JH2 to JH4 region was observed in Mbd4$^{-/-}$ mice (Table I); however, this difference was attributed to one heavily mutated sequence in one Mbd4$^{-/-}$ mouse (Fig. 3). Removal of this one sequence from the analysis did not significantly alter the data or the conclusions.

Discussion

The processes of SHM and CSR result in single base changes and in recombination at rates that far exceed those that are normally observed in somatic cells (4). It is unclear how B cells that are undergoing these processes avoid the many mechanisms that cells have evolved for sensing and repairing DNA damage. It appears that at least some of these mechanisms are actually involved in creating and fixing the mutations that are required for the production of Abs. This is true of mismatch repair, because mice that lack MSH2 or MSH6 have lower rates of SHM and CSR than wild-type mice and have changes in the patterns of the mutations that do occur (4, 31). Although some pathways of base and nucleotide excision repair do not seem to contribute to SHM or CSR (4), it is still important to examine other mechanisms of repair to determine whether they play a role in either of these processes.

Mbd4 has glycosylase activity and is involved in the repair of G-T and G-U mismatches, especially if they arise from 5-methyl cystosines that were part of CpG dinucleotides. This is an important activity because the deamination of 5-methylcytosine in CpG sites causes >20% of the mutations that are responsible for human diseases (19). Nevertheless, weak Mbd4 glycosylase activity has also been observed in vitro for G-T mismatches that are not in CpG dinucleotides (21). Because the product of AID seems to be a G-U mismatch (7), and because Mbd4 associates with components of the mismatch repair system (24) which is involved in SHM and CSR (4), it was important to determine whether Mbd4 might contribute to these processes. Our results with Mbd4$^{-/-}$ mice indicate that Mbd4 does not contribute significantly to Ab diversification.

The lack of a detectable role for Mbd4 in SHM and CSR could be due to a number of reasons. First, thymidine DNA glycosylase also has an activity similar to that of Mbd4, so redundancy could explain the lack of effect of the loss of Mbd4 (32). Second, Mbd4 preferentially binds to methylated CpG; however, the highly transcribed Ig genes are expected to be unmethylated (33). Third, the CpG dinucleotide, which is the primary substrate for Mbd4, is infrequent in the coding region of V186.2 (two CpG in 294 bp) and JH2 to JH4 regions (nine CpG in 1460 bp), and is also not part of the RGYW/WRCY motif, which is a sequence that is most frequently targeted by AID and mutated during SHM in vivo and in vitro (5, 34). Thus, mutations at CpG dinucleotides due to SHM are probably infrequent. In fact, none of the 348 mutations identified in the JH2 to JH4 region in Peyer's patch B cells from both Mbd4$^{-/-}$ mice and littermates (Table I) were in a CpG dinucleotide (data not shown). Thus, the lack of Mbd4 activity on G-U mismatches caused by SHM supports the notion that Mbd4 has primary activity on mismatches that result from deaminations of cytidines within CpG motifs.

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