

Severe Attenuation of the B Cell Immune Response in Msh2-deficient Mice

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Summary

Recently, results obtained from mice with targeted inactivations of postreplication DNA mismatch repair (MMR) genes have been interpreted to demonstrate a direct role for MMR in antibody variable (V) gene hypermutation. Here we show that mice that do not express the MMR factor Msh2 have wide-ranging defects in antigen-driven B cell responses. These include lack of progression of the germinal center (GC) reaction associated with increased intra-GC apoptosis, severely diminished antigen-specific immunoglobulin G responses, and near absence of anamnestic responses. Mice heterozygous for the Msh2 deficiency display an "intermediate" phenotype in these regards, suggesting that normal levels of Msh2 expression are critical for the B cell response. Interpretation of the impact of an MMR deficiency on the mechanism of V gene somatic hypermutation could be easily confounded by these perturbations.

Key words: mismatch repair • germinal center • B cell response • somatic hypermutation

Postreplication mismatch repair (MMR)¹ functions to identify and correct nucleotide misincorporations in the nascent DNA strand during chromosomal replication in prokaryotes and eukaryotes (1–3). The genes encoding the components of this system were initially identified in *Escherichia coli* as mutations that resulted in increased genome-wide mutation rates, and were designated Mut genes. A central component of the postreplication MMR system in *E. coli* is MutS, the protein involved in the recognition of mispaired nucleotides (1, 4). Homologues of MutS have been shown to function in a similar manner in *Saccharomyces cerevisiae* and humans (4). Both humans and mice have at least five homologues of MutS, termed Msh2–6 (4, 5). Recent interest has focused on the human homologues of bacterial MMR genes, since mutations in these genes have been associated with a large fraction of hereditary nonpolyposis colorectal cancer kindreds (2, 3). Strains of mice deficient in several of the MSH proteins have now been generated via gene targeting technology, and most display expected defects in MMR and a propensity to develop certain types of cancer. For example, mice deficient in Msh2 are predisposed to the development of a form of pre-T cell

leukemia with onset at 2–3 mo of age, with older mice developing intestinal and skin neoplasias (6–9). Nevertheless, Msh2-deficient mice develop normally and are fertile (6, 7, 9).

Recently, several groups have presented evidence obtained from the analysis of antibody V gene somatic hypermutation in mice deficient in MMR proteins. Three of these groups have concluded that their data support the idea that MMR proteins are involved in the incorporation or fixation of mutations in V genes during this process. Cascalho et al. have proposed that the mammalian MutL homologue Pms2 is directly involved in the introduction of mutations into V genes (10), whereas Gearhart and colleagues have argued that both Msh2 (11) and Pms2 (12) alter the spectrum of mutations resulting from the action of the V gene "mutator," by preferentially repairing certain types of lesions. Rada et al. have observed that the V genes in antigen-activated B cells of Msh2-deficient mice have a reduced V gene hypermutation frequency, but an increased frequency of mutations at sites previously identified as "hot spots" in normal mice. Therefore, they conclude that hypermutation takes place in sequential Msh2-independent and -dependent phases during immune responses in normal mice (13).

It is well documented that the somatic hypermutation of V genes is intimately associated with, and may be a prerequisite for, memory B cell genesis in mice (14–17). In most of the studies on a possible role of MMR in V gene somatic hypermutation, the potential for an MMR deficiency resulting in pleiotropic defects in the memory B cell response

¹Abbreviations used in this paper: AFC, antibody-forming cell; GC, germinal center; HSA, heat stable antigen; MMR, mismatch repair; NP-CGG, (4-hydroxy-3-nitrophenyl)acetyl chicken gamma globulin; PALS, periarteriolar lymphoid sheath; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

(18, 19) was acknowledged but not directly or extensively examined. However, preliminary investigation of this issue has provided support for this idea. Rada et al. observed a decreased IgG1 response in *Msh2*^{-/-} compared with +/- mice (13). Moreover, Frey et al. observed that B cells in the Peyer's patches of *Msh2*-deficient mice that are subjected to chronic antigenic stimulation and proliferation displayed high levels of length variation at the D6Mit59 microsatellite locus (20). Instability at several such microsatellite loci is a signature of elevated chromosome-wide mutation rate (21).

In this report, we present data demonstrating that an *Msh2* deficiency in mice indeed results in wide-ranging defects in the B cell immune response. Among these are an attenuated progression of the germinal center (GC) reaction, dramatically reduced levels of antigen-induced IgG isotypes, and a greatly reduced anamnestic response. Significantly, *Msh2*^{+/-} mice display an "intermediate" phenotype in these regards, suggesting that normal levels of expression of *Msh2* are crucial to antigen-driven B cell proliferation and development. Despite the previously recognized propensity of *Msh2*-deficient mice to develop T cell leukemia (6, 8), T cell numbers and proliferative function appear essentially normal in young *Msh2*-deficient mice. In addition, stimulation of B cells from *Msh2*-deficient mice in vitro revealed only subtle differences in proliferation, apoptosis, or isotype class switching compared with wild-type B cells. We discuss how an *Msh2* deficiency might result in these phenotypic outcomes, and how such pleiotropic effects on the B cell response in vivo could confound evaluation of a potential role of the MMR system in V gene somatic hypermutation.

Materials and Methods

***Msh2*-deficient Mice.** The line of *Msh2*-deficient mice used in this study was created by targeted inactivation of exon 11 of the *Msh2* gene, and has been described previously (8). Mice used in our studies were maintained by brother-sister mating and were of a mixed C57BL/6 × 129/o/a background. Homozygous knockout, heterozygous knockout, and *Msh2* wild-type offspring were identified using DNA derived from ear-clip tissue, and a previously described PCR assay (9). Age-matched mice of 7–12 wk of age were used in all experiments, and littermates were used in individual experiments when possible.

Immunizations and Serology. Preparation of and immunization with (4-hydroxy-3-nitrophenyl)acetyl chicken gamma globulin (NP-CGG) were performed as described previously (22–24). Mice received 100 µg i.p. of antigen in alum for primary immunization, and the same amount of antigen in PBS i.p. for secondary immunization. TNP-Ficoll was injected in PBS at a dose of 50 µg i.p. per mouse. Mice were bled via the retroorbital sinus at various times after immunization, and the levels of anti-NP, anti-CGG, or anti-TNP antibodies of various isotypes were assayed in sera obtained from these samples by previously described ELISA assays (23, 25). These same assays were also used to evaluate antibody levels in supernatants obtained from in vitro stimulation of B cells, with the exception that anti-Ig reagents were used to capture secreted antibody.

Immunohistochemistry and Flow Cytometry Analysis. Spleen isola-

tion, flash freezing, sectioning, and immunohistochemistry were all conducted essentially as described previously (23, 24). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed on spleen sections to evaluate numbers of apoptotic nuclei using the ApopTag™ kit obtained from Oncor, according to the manufacturer's instructions. Numbers of apoptotic nuclei in GCs were counted at 400× using a compound microscope. GC sizes were determined by counting the number of PNA⁺ cell diameters at 100× magnification in the largest GC dimension. Size categories were as follows: small, 10–29 diameters; medium, 30–39 diameters; and large, 40 or more diameters. GC microdissection, Vλ gene PCR amplification, and nucleotide sequencing were performed using techniques (26) and primers (27) described previously.

Flow cytometric analysis of spleen and bone marrow cells was performed essentially as described previously (28) using PE-labeled anti-B220 (RA3-6B2; PharMingen), FITC-labeled anti-IgM (donkey anti-mouse; Jackson ImmunoResearch Labs), biotin-labeled anti-IgD (SBA-1; Southern Biotechnology Associates), and FITC-labeled anti-CD24 (heat stable antigen [HSA], M1/69; PharMingen). Stained cells were analyzed on a Coulter Epics Profile II or an Epics Elite using the Coulter Elite software.

Isolation and In Vitro Stimulation and Analysis of T and B Cells. In some experiments (see legends for details), total lymph node cells were used as an enriched source of T cells for in vitro analysis. In others, splenic T cells were enriched by treating total spleen cells with anti-class II (MKD6) and anti-HSA (J11d) mAbs and guinea pig complement (Rockland), followed by Percoll gradient purification of small dense lymphocytes. The enriched T cells were incubated with various concentrations of mitogenic reagents, antigen, or with 4 × 10⁵ allogeneic, irradiated (2,000 rads) spleen cells, at 2 × 10⁵ cells per well in 100 µl RPMI plus 10% FCS. For plate bound anti-CD3 stimulation, 96-well tissue culture plates were coated with 25 µg/ml 2C11 mAb, followed by extensive washing with sterile PBS and blocking with media containing 10% FCS. Proliferation was evaluated 45–50 h after T cell plating on anti-CD3-coated wells via a pulse of [³H]thymidine for 6–18 h, followed by cell harvesting on glass fiber filters and scintillation counting.

Small dense splenic B cells were isolated from spleens via T cell depletion using a cocktail of anti-CD4 (172), anti-CD8 (31M), and anti-Thy1 (polyclonal rabbit anti-mouse; Sigma) antibodies and guinea pig complement, followed by purification of high-density cells on Percoll gradients. The resulting cells were incubated in vitro in RPMI plus 10% FCS including various concentrations of LPS (Difco), goat anti-mouse IgM F(ab')₂S (Pierce) or an anti-murine CD40 mAb (FGK45; reference 29). In some experiments, recombinant murine IL-4 (PeproTech) was included in the cultures at 50 ng/ml. To evaluate proliferation, such cultures were pulsed after 45–50 h with [³H]thymidine, cells were harvested onto glass fiber filters, and ³H incorporated into DNA was evaluated by scintillation counting.

To evaluate cell cycle progression and apoptosis, cells were harvested at various times after initiation of culture, fixed and permeabilized in 70% EtOH, treated with RNase (Fisher Scientific), and stained with propidium iodide (PI; Sigma) followed by flow cytometric analysis. Numbers of G₁, S, G₂, and apoptotic cells were evaluated using a Coulter Epics Profile II and the Elite software. Supernatants from such cultures were also harvested at various times and evaluated for levels of antibodies of various isotypes, as described above.

Analysis of Microsatellite Instability. DNA was isolated as described (30) from in vitro cultures of B cells, and instability at the

D6Mit59 locus was evaluated using a PCR-based assay essentially as described previously (9). One of the primers used for this analysis was end-labeled with ^{32}P using T4 polynucleotide kinase; the PCR reaction products were then separated on acrylamide/urea DNA sequencing gels, and the gels were exposed to x-ray film. Microsatellite primer sequences were obtained from the Whitehead Institute for Genome Research (<http://www.genome.wi.mit.edu>).

Results

The GC Reaction Does Not Progress in MSH2-deficient Mice. Our studies were initiated, as were those of others (10–13, 20), to test the idea that a deficiency in MMR might directly alter the V gene hypermutation process. Since hypermutation takes place predominantly in GCs in normal mice, we used an immunohistochemical GC microdissection PCR approach to evaluate levels of V gene mutation during the immune response to NP-CGG (16, 31). We observed a relatively normal frequency and chemical spectrum of V λ somatic mutation among PCR DNA clones isolated from λ^+ splenic GCs at days 12 and 15 of the primary NP-CGG response in a pilot study (data not shown). However, during the course of this study, we found that the GC reaction in Msh2 $-/-$ mice was abnormal. The results of an extensive analysis of this phenomenon are summarized in Table I. In Msh2 $-/-$ mice immunized with 100 μg of NP-CGG in alum, antigen-specific splenic GCs were present at day 8 after immunization at a normal

frequency compared with wild-type mice, but their average size appeared somewhat smaller, and large GCs (≥ 40 cell diameters) were absent. At 12 d after immunization of Msh2 $-/-$ mice, the average size of antigen-specific GCs had not increased, resulting in continued absence of the large GCs that make up a sizable percentage of all GCs observed in $+/+$ mice at this time. In addition, at this later time point the average number of antigen-specific GCs per unit area of spleen in Msh2 $-/-$ mice had decreased twofold compared with day 8, whereas Msh2 $+/+$ mice showed no significant difference in this frequency at the two time points. Analysis of spleens from NP-CGG-immunized Msh2 $+/-$ mice revealed an intermediate phenotype with respect to these alterations, and the intermediate level of total GCs at day 12 in such mice was statistically significant compared with the values obtained from either $+/+$ or $-/-$ mice.

Evaluation of levels of apoptosis in the GCs of Msh2 $+/+$, $+/-$, and $-/-$ mice 8 d after immunization via the TUNEL assay showed significantly higher levels of apoptotic nuclei in the small and medium size antigen-specific GCs of Msh2 $-/-$ mice compared with $+/+$ mice, with $+/-$ mice displaying intermediate levels of such nuclei (Fig. 1). Interestingly, the variation in number of apoptotic nuclei per GC was much larger in Msh2 $-/-$ compared with $+/-$ and $+/+$ mice. The reduced number of antigen-specific GCs at day 12 in Msh2 $-/-$ mice precluded obtaining a statistically significant comparison of levels of apoptotic nuclei at this time point.

Staining of spleen sections from mice immunized 8 and 12 d earlier with NP-CGG with an IgG-specific reagent showed a reduction in the number of IgG $^+$ B cells in

Table I. Antigen-specific GC Numbers and Sizes in Msh2-deficient Mice

	Total NP $^+$ GCs per 10 \times field*		
	Small	Medium	Large
Day 8			
$+/+$	2.2 \pm 1.6	3.5 \pm 2.17	1.2 \pm 0.98 ‡
Total	6.83 \pm 2.56		
$+/-$	3.83 \pm 1.17	2.33 \pm 1.03	0.5 \pm 0.84
Total	6.67 \pm 2.25		
$-/-$	3.33 \pm 2.07	3.33 \pm 2.58	0 ‡
Total	6.67 \pm 1.37		
Day 12			
$+/+$	2.17 \pm 1.17	1.83 \pm 0.75	1.67 \pm 1.33 ‡
Total	5.67 \pm 1.5§**		
$+/-$	3.25 \pm 1.89	1.25 \pm 1.26	0.25 \pm 0.5 ‡
Total	4.75 \pm 1.70$^{\parallel**}$		
$-/-$	1.67 \pm 1.5	2.0 \pm 1.41	0 $^+$
Total	3.67 \pm 1.37$^{\S\parallel}$		

*Values were obtained from three mice of each genotype. Two random fields per section and two sections per mouse from different areas of the single spleen were analyzed.

‡ , § , $^\parallel$, ‡ , ** , $^+$ Value pairs that pass the Student's t test at the 90% confidence level.

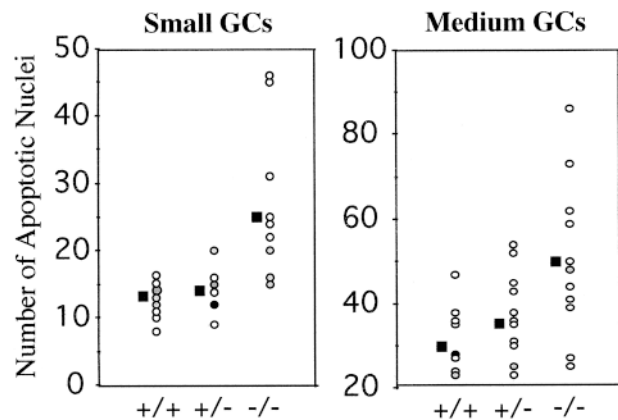


Figure 1. Levels of apoptotic nuclei in antigen-specific GCs of Msh2-deficient mice. Mice were immunized with NP-CGG, and spleens were taken at day 8 after immunization, sectioned, and stained with PNA and NP, all as described in Materials and Methods. Parallel sections were stained with PNA, and apoptotic nuclei were elaborated by the TUNEL assay. Apoptotic nuclei were counted in eight randomly chosen NP $^+$ GCs of small and medium size (see Table I). Each value obtained is indicated by a circle in the scatter plots, with values obtained two, three, or four times indicated by light gray, dark gray, and black filled circles, respectively. Filled squares indicate mean value of apoptotic nuclei for each experimental category. A Student's t test analysis revealed that the differences in levels of apoptotic nuclei observed between $+/+$ and $-/-$ mice in both GC size categories were statistically significant at the 90% confidence level.

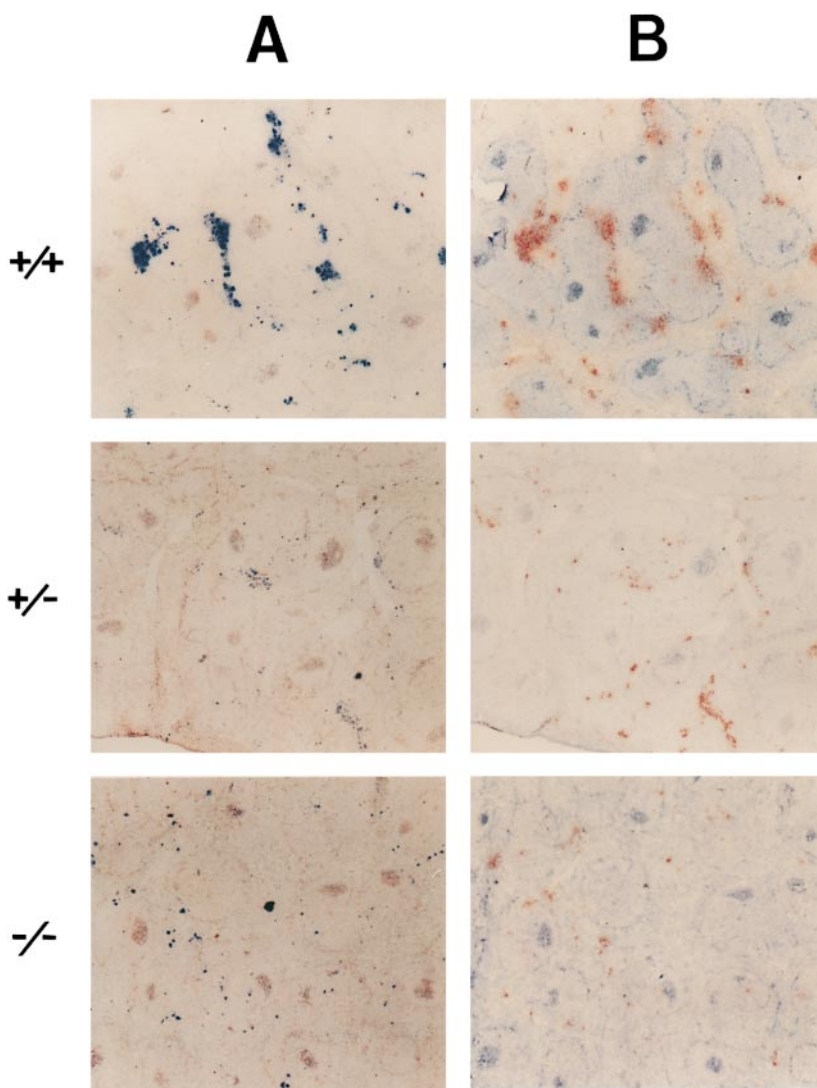


Figure 2. GC and PALS focus responses in the spleens of Msh2-deficient mice at day 8 after immunization. Mice were immunized with NP-CGG, and spleens were taken at 8 d after immunization and sectioned for immunohistochemistry, all as described in Materials and Methods. Parallel sections were stained either with (A) PNA (red) and NP (blue), or with (B) PNA (blue) and a polyclonal anti-IgG reagent (red). Notice the intense antigen- and IgG-specific staining at the edges of the white pulp in the $+/+$ spleen sections, characteristic of the PALS AFC focus response. This reaction is obviously reduced in $+/-$ spleens and nearly absent in $-/-$ spleens. At day 8 of the response, reduction in the number of GCs in Msh2 $-/-$ mice has not occurred, but at day 12, when the PALS focus response has waned, there are twofold fewer GCs in $-/-$ compared with $+/+$ spleens, and the average size of these GCs is much smaller (see Table I).

the GCs of Msh2 $-/-$ mice relative to $+/+$ mice (data not shown). Moreover, the periarteriolar lymphoid sheath (PALS)-associated antibody-forming cell (AFC) focus reaction, a predominant component of the early primary anti-NP-CGG response (22, 32), was also substantially reduced in Msh2 $-/-$ spleens (Fig. 2). This was observed using both antigen- and IgG-specific staining. Again, Msh2 $+/-$ mice displayed an intermediate phenotype with respect to these alterations (Fig. 2). Despite these differences, no obvious abnormalities in splenic architecture, the size of other splenic B and T cell microenvironments, size and location of follicular dendritic cell networks, or locales of antigen-specific B cell proliferation and differentiation were observed among Msh2 $+/+$, $+/-$, and $-/-$ mice during these studies.

Severely Reduced Mature Serum Antibody Responses in Msh2-deficient Mice. These observations led us to conduct detailed analysis of the anti-NP-CGG serum antibody responses of Msh2-deficient mice. As shown in Fig. 3 A, at early stages of this response, antigen-specific IgM levels were only slightly

lower in Msh2 $+/-$ and $-/-$ mice compared with $+/+$ mice. However, dramatic differences in the levels of NP-specific κ , λ , and IgG isotypes were apparent between Msh2 $+/+$ and $-/-$ mice at all times in the primary response, with Msh2 $+/-$ mice displaying an intermediate phenotype in most cases (Fig. 3, A and B). Msh2 $+/-$ mice also showed a delayed serum IgG1 response (peaking at day 21 instead of 14), and expressed increased levels of IgG2a, although this isotype was a minor component of the total response in all mice. Also readily apparent was the severely diminished or reduced secondary serum antibody response in Msh2 $-/-$ and $+/-$ mice, respectively. In $-/-$ mice, this secondary response was of a magnitude not noticeably different from peak levels in the primary response in most assays. This result was not idiosyncratic to the anti-NP response, as the secondary response to the carrier, CGG, was also severely blunted in $-/-$ mice and reduced in $+/-$ mice (Fig. 3 C). Analysis of the serum antibody response to TNP-Ficoll, a T cell-independent antigen, revealed analogous IgM responses in $+/+$ and $-/-$ mice, but the total (kappa) anti-TNP re-

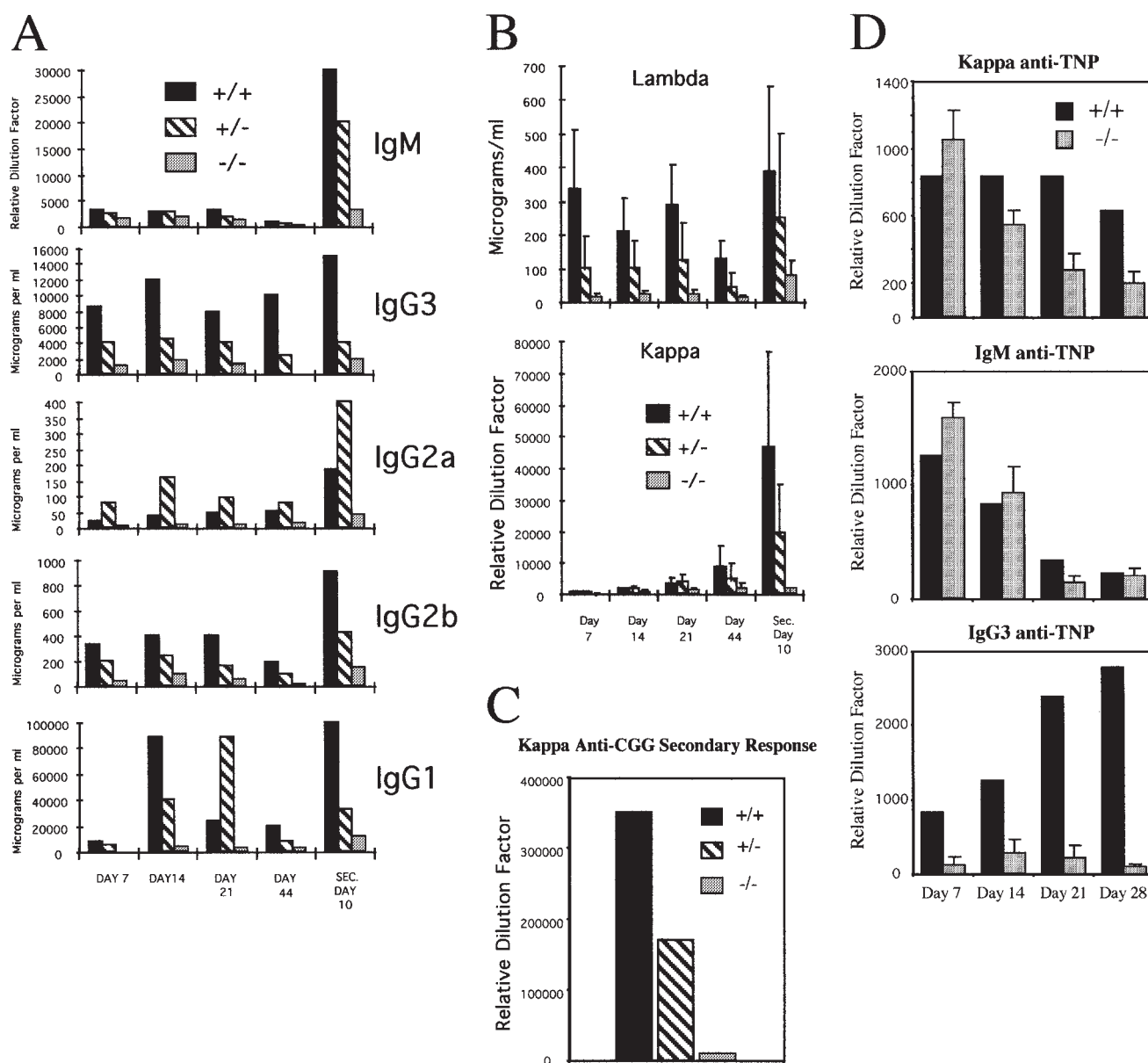


Figure 3. Serum antibody responses of Msh2-deficient mice. Immunization was performed with either NP-CGG (A, B, and C) or TNP-Ficoll (D), as described in Materials and Methods, and mice were bled at various times thereafter. Levels of antigen-specific antibody of a particular isotype were determined by ELISA. In many of these assays, anti-NP mAbs with similar affinities for NP were used as standards, allowing the results to be presented as micrograms per milliliter of these standards. In other assays, relative levels of antigen-binding antibodies in different sera were determined using serial dilutions. The points at which the resulting OD curves were 50% maximal were then used to calculate the relative dilution factor giving an equivalent OD for each serum sample. The data illustrated in A and C were obtained using sera pooled from at least three different animals of the each genotype. The data illustrated in B and D were obtained from at least three individual sera per time point, and error bars are shown (except for the +/+ anti-TNP-Ficoll data, which were obtained from one animal).

response decayed more rapidly in Msh2 $-/-$ mice. This more rapid decay appeared to be accounted for by a severe deficiency in the IgG3 response, particularly at late times after immunization (Fig. 3 D). Interestingly, total serum IgG levels were found not to differ significantly in Msh2 +/+, +/-, and -/- mice (data not shown), suggesting that long-term homeostatic regulation of these levels is not perturbed by an Msh2 deficiency.

Peripheral B Cell, T Cell, and Bone Marrow B Cell Compartments in Msh2-deficient Mice. Alteration of the T cell-

dependent B cell response in Msh2-deficient mice could result from preexisting abnormalities in the T cell compartment, the B cell compartment, or both. Flow cytometric analysis of splenic B lymphocytes revealed no apparent abnormalities in numbers of mature and immature subsets in Msh2-deficient mice (Fig. 4). Similar analyses of the T cell compartment revealed no obvious differences between Msh2 +/+ and -/- mice (data not shown). These observations are consistent with previous publications indicating that the splenic lymphoid compartment of

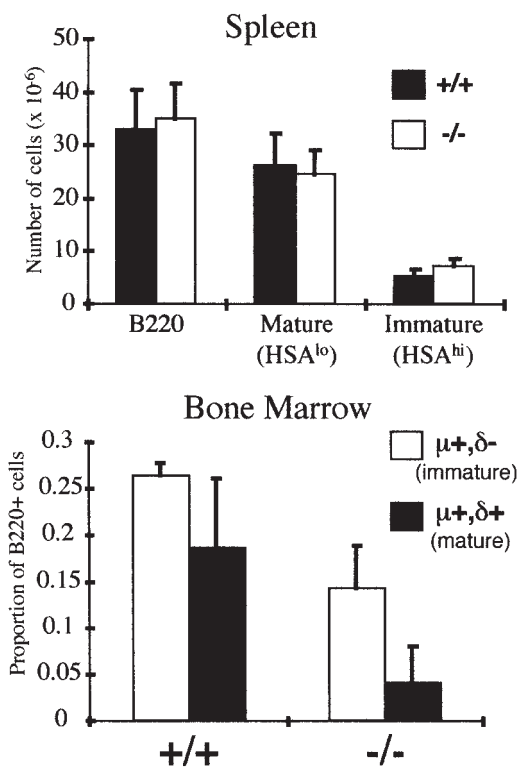


Figure 4. Levels of B cells in bone marrow and spleens of Msh2-deficient mice. Numbers and proportions of B220⁺ cells expressing cell surface antigens diagnostic of mature B cells were measured in spleen and bone marrow cells from at least three animals per genotype via flow cytometry, as described in Materials and Methods. In bone marrow, the proportion of B220⁺ cells that expressed surface IgM but not IgD was measured to evaluate immature B cell levels, and the proportion of B220⁺ cells that expressed both isotypes was used to evaluate levels of mature, recirculating cells. B cell numbers and maturity in the spleen were evaluated using B220 and levels of expression of HSA.

Msh2-deficient mice is overtly normal (6–8). Analysis of B lineage cells in the bone marrow of such mice revealed a slight decrease in the proportion of B220⁺ immature IgM⁺, IgD⁻ B cells in Msh2^{-/-} compared with +/+

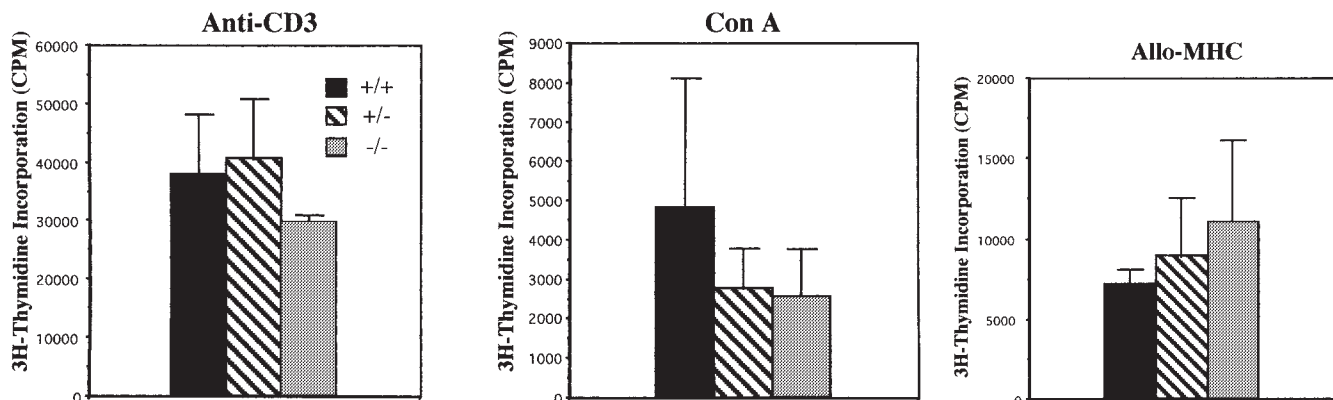


Figure 5. In vitro proliferative responses of Msh2-deficient T cells. Total lymph node cells were stimulated with either 4 μg/ml Con A or irradiated spleen cells from BALB.K mice (allo-MHC) for 48 h, as described in Materials and Methods, and pulsed with [³H]thymidine to measure proliferation. Plate-bound anti-CD3 stimulation was also performed in this fashion (see Materials and Methods), but using splenic T cells enriched as described. The cells used for each experiment were derived from at least two mice of each genotype.

mice, but a more dramatic four- to fivefold reduction in mature “recirculating” B220⁺, IgM⁺, IgD⁺ B cells in -/- mice. The latter result is consistent with the previous results of Rada et al. (13).

In Vitro Proliferative Responses of Msh2-deficient T Cells. To assess generic T cell activation and proliferation function, in vitro stimulations of lymph node and splenic T cells were performed. No significant differences in anti-CD3-, Con A-, or alloantigen-induced proliferative responses were observed among Msh2 +/+, +/-, or -/- T cells (Fig. 5). In addition, KLH-primed lymph node T cells obtained from Msh2-deficient mice proliferated in response to re-stimulation with KLH in vitro to an extent that did not differ reproducibly from wild-type cells (data not shown).

Proliferative and Apoptotic Responses of Msh2-deficient B Cells In Vitro. The lack of evidence for defects in T cell function and mature peripheral B cell numbers and maturity in Msh2 mice prompted a detailed analysis of B cell responses in vitro. Small dense B cells were purified from Msh2 +/+, +/-, and -/- mice and stimulated with anti-IgM F(ab')₂ fragments, LPS, or a mitogenic anti-CD40 mAb in vitro. The cultures were then evaluated for cell cycle progression via [³H]thymidine incorporation. Cell cycle progression and apoptosis were also evaluated using PI-flow cytometric DNA content analysis. Msh2 -/- B cells displayed only slightly reduced proliferation that was not statistically significant compared with +/+ B cells, except at the highest concentrations of stimulant (Fig. 6). PI cell cycle analysis revealed only minor differences in cell cycle progression and apoptosis induced by these same stimuli among the various types of B cells during a 4-d culture period. Msh2 -/- cultures contained only slightly elevated levels of apoptotic cells at early time points in a few experiments (data not shown).

In Vitro Isotype Class Switching by Msh2-deficient B Cells. Since our serological and immunohistochemical analysis of the B cell response of Msh2-deficient mice revealed a severe attenuation of the IgG response, induction of isotype class switching in vitro was examined using small dense

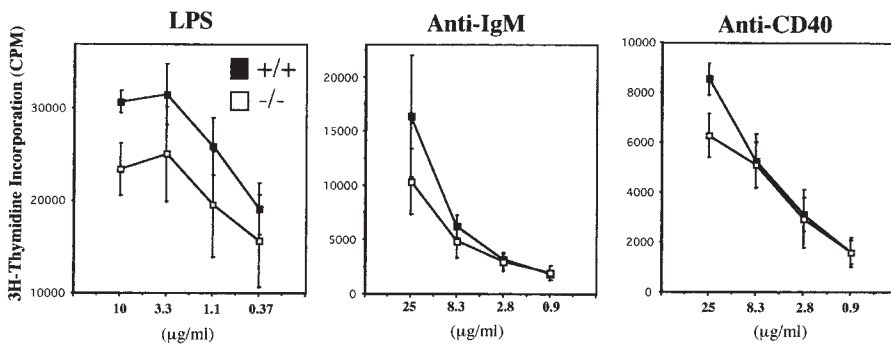


Figure 6. In vitro proliferative responses of *Msh2*-deficient B cells. Small dense splenic B cells were purified from pools of cells derived from three mice of each genotype, as described in Materials and Methods, and cultured for 45–50 h with the indicated concentrations of LPS, a polyclonal goat anti-mouse IgM F(ab)₂ preparation, or the mitogenic anti-mouse CD40 mAb FGK45. Cell proliferation was then evaluated via a 12–16-h pulse of [³H]thymidine, also as described in Materials and Methods. All culture conditions were performed in triplicate, and error bars are shown. The data shown are representative of the results of three independent experiments.

splenic B cells. B cells were cultured with LPS, which results in low levels of switching to IgG3 and IgG2b in normal B cells, or with LPS plus IL-4, which suppresses switching to IgG3 and drives efficient switching to IgG1 and IgE isotypes in normal B cells (33). Levels of class-switched IgG1 and IgG3–secreted antibody were then determined by ELISA. *Msh2* +/+, +/-, and -/- B cells stimulated with LPS gave rise to levels of IgG3 that did not differ greatly, and *Msh2* -/- B cells produced only slightly reduced amounts of this isotype (Fig. 7). Little or no IgG1 was produced by any of these cell types in response to LPS. In response to stimulation with LPS plus IL-4, *Msh2* +/+, +/-, and -/- B cells gave rise to similar levels of IgG1, and little IgG3. These data argue that the regulation and efficiency of isotype class switching in vitro are not greatly perturbed by an *Msh2* deficiency in B cells.

Discussion

Mice lacking *Msh2* display a striking defect in their ability to sustain a B cell response. Moreover, mice containing

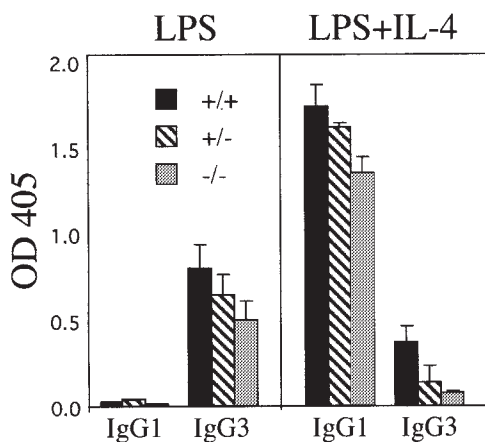


Figure 7. IgG expression by *Msh2*-deficient B cells in vitro. Small dense splenic B cells derived from pools of cells obtained from three mice of each genotype were cultured for 4.5 d with either LPS (25 µg/ml) or LPS (25 µg/ml) and recombinant murine IL-4 (50 ng/ml), and supernatants were harvested. Levels of total IgG3 and IgG1 in these supernatants were then determined by ELISA, as described in Materials and Methods. All assays were performed in triplicate, and error bars are shown.

only one copy of the functional *Msh2* gene (resulting in 50% of the wild-type level of *Msh2* protein expression [7]) display an “intermediate” phenotype in this regard, indicating that even a slight reduction in *Msh2* activity can perturb this response. It should be noted that the intermediate B cell response defect displayed by *Msh2* +/- mice makes them inappropriate controls for studies on the effects of an *Msh2* deficiency in vivo. *Msh2* deficiency results in an inhibition of the progression of the GC reaction, dramatically reduced levels of IgG-producing AFCs and antigen-induced IgG serum antibody, and a blunted anamnestic response. We have also observed that *Msh2* expression is elevated in GCs (Vora, K.A., R. Fishel, and T. Manser, unpublished observations), consistent with the idea that this protein plays a critical role in B cell development in this microenvironment.

Nonetheless, why an *Msh2* deficiency leads to such a severe attenuation of mature B cell responses in vivo remains a subject of speculation. Although we found that T cell numbers and proliferative function in vitro were normal in *Msh2* -/- mice, a more detailed analysis of Th function might have exposed differences between antigen-primed wild-type and *Msh2*-deficient T cells. A complicating factor in this regard is that in an environment where B cell function is deficient, defects in levels of T cell activation or differentiation may arise secondarily (34). Since *Msh2* +/- mice are predisposed to the development of pre-T cell leukemia, it is tempting to speculate that general T cell physiology may be perturbed in these mice. Our experiments were performed in young mice with no evidence of T cell abnormalities. In addition, *Msh2* +/- mice, which do not frequently develop T cell leukemia (8; Cranston, A., and R. Fishel, unpublished observations), displayed obviously altered B cell responses, and the immune response to the T cell-independent antigen TNP-Ficoll was reduced in *Msh2* -/- mice. Finally, initiation of the GC reaction, a T cell-dependent process, appeared normal in *Msh2*-deficient mice. Nevertheless, given the previous conclusions of others that the GC reaction appears to require lower levels of T cell help than does the AFC response (35, 36), and that class switching to many IgG isotypes is driven by Th cells (33), a primary or secondary defect in the development of an efficient Th response in *Msh2*-deficient mice must still

be considered. Resolution of this issue will require the construction of mice with selective Msh2 deficiencies in the B and T cell compartments.

In principle, an intrinsic B cell defect due to Msh2 deficiency might result from perturbations in any of the somatic DNA alteration pathways essential to normal B cell development. Taken together, however, our data argue against this possibility. Analysis of B lineage cells in the bone marrow of Msh2 $-/-$ mice revealed a major deficiency in the size of the mature, IgM⁺, IgD⁺ “recirculating” subpopulation, but immature IgM⁺, IgD⁻ B cell numbers were only slightly reduced and peripheral B cell numbers and maturity levels appeared normal, consistent with the idea that primary B cell development is not greatly altered due to an Msh2 deficiency. As suggested previously (18), an increased rate of V gene hypermutation due to the absence of a “counteracting” MMR system might dramatically increase the frequency of generation of nonfunctional antigen receptors on GC B cells, resulting in substantial increases in GC cell death rate. However, this would not easily explain the severely attenuated PALS AFC focus and T cell-independent B cell responses we observed, since V gene mutation does not take place at a high rate during these responses (26, 37). Finally, the possibility that Msh2 might be involved in class switch recombination, while intriguing due to its documented role in suppression of homeologous recombination (38), is not supported by our finding that Msh2-deficient B cells can efficiently and accurately class switch *in vitro*.

On the other hand, our data strongly suggest that the effects of an Msh2 deficiency on B cell function do not become manifest until the stages of antigen-driven responses characterized by high rates of proliferation. The early stages of the primary T cell-dependent antigen-driven response, including IgM production and initiation of the GC reaction, were not greatly affected by an Msh2 deficiency. We also observed a normal early IgM response to TNP-Ficoll. In contrast, GC expansion in Msh2 $-/-$ mice appears blocked, and this alteration is associated with a substantially higher frequency of apoptotic cells in GCs. The observation that the PALS AFC focus response is severely attenuated in Msh2-deficient mice could also be explained by the rapid clonal expansion necessary for this response (22, 32), or by the derivation of precursors of this response from the GC reaction (39). The reason for the deficiency in the mature IgM⁺, IgD⁺ recirculating bone marrow B cell population in Msh2 $-/-$ mice is more difficult to explain, but might indicate a reduced peripheral B cell life span, or derivation of a subset of this population from the memory B cell pathway.

Interestingly, we observed more variability in the number of apoptotic nuclei per GC in Msh2 $-/-$ mice compared with controls (Fig. 1). In normal mice, intra-GC B cell apoptosis is thought to result from negative selection of autoreactive B cells and a lack of positive selection of B cells with low affinities for antigen (40–44). The increased and greater variability in numbers of GC apoptotic nuclei characteristic of Msh2-deficient mice is consistent with the

superimposition of another, highly stochastic process leading to intra-GC apoptosis. A generic defect in high rate B cell proliferation due to increased genome-wide mutation rate (the “mutator” phenotype) is a reasonable candidate for this process. The perturbations we observed in IgG expression in Msh2-deficient B cell responses could well result secondarily from such a proliferative defect, as class switching requires cell division and usually takes place after a period of clonal expansion (22, 33).

These considerations raise the question of why we observed only subtle changes in Msh2 $-/-$ B cell proliferation, apoptosis, and class switching during our *in vitro* studies. The answer may simply relate to the fact that rates and extents of B cell proliferation approaching those characteristic of certain stages of antigen-driven B cell development are not attained by most B cells subjected to *in vitro* stimulation (45). Moreover, B cells undergoing the initial stages of apoptotic death are probably rapidly engulfed by phagocytic cells *in vivo*, but may remain intact for extended periods *in vitro*. Under conditions of low to moderate rates of clonal expansion of Msh2-deficient B cells, DNA lesions normally recognized by Msh2-containing complexes may be recognized and repaired, albeit less efficiently, by other factions of the DNA repair system. However, as cell cycle time decreases, a less efficient MMR system might well become limiting for clonal expansion. Accumulation of point mutations in genes pivotal in regulating cell viability, or a sudden block to cell cycle progression due to more severe lesions whose repair, suppression, or detection is normally in part mediated by Msh2 (such as double strand breaks [46] or homeologous recombination products [38]) would likely culminate in induction of apoptotic pathways and cell death. Indeed, analysis of MSH2-deficient Peyer’s patch GC B cells, a population undergoing chronic, high rate proliferation, revealed high levels of instability at the D6Mit59 microsatellite locus (20). In contrast, when we performed a similar analysis of this locus in Msh2 $+/+$, $+/-$, and $-/-$ B cells stimulated for 5 d *in vitro* with anti-IgM, LPS, or anti-CD40, no instability at this locus could be detected (data not shown). Clearly, these observations warrant a more detailed analysis of Msh2-deficient B cell cycle progression, life span, and genome stability *in vivo*.

Previous conclusions regarding the effect of MMR deficiencies on V gene hypermutation have been garnered largely from the analysis of the V gene products of antigen-driven B cell responses taking place *in vivo* (11–13, 20). As our data show that the T cell-dependent B cell response, during which the hypermutation process takes place, is grossly perturbed by such a deficiency, these data mandate a reevaluation of these conclusions. Memory B cell development takes place simultaneously in a single animal in many distinct lymphoid microenvironments, the most important of which are probably the GCs (40, 47, 48). Because the B cell clonal composition of a given GC is limited (31, 49), the nature of antigen selection forces and clonal proliferation will vary in different GCs. Such differences are amplified by the rather random nature of hypermutation, resulting in very different V region mutant rep-

ertoires in different GCs. In addition, levels of V gene hypermutation have been observed to vary in GCs at the same time during an immune response (50). Thus, since GCs are "independent islands of antibody V region somatic evolution" (22, 51), the impact of an MMR deficiency on the progression of the GC reaction may vary dramatically from GC to GC, depending on the variables discussed above.

These observations make it clear that sampling biases are likely to be problematic in the analysis of the effects of MMR deficiencies on V gene somatic hypermutation. Such sampling biases could result from substantial GC to GC, and even mouse to mouse, variation in the effects of an MMR deficiency on memory B cell genesis. For example, in our analysis of V λ mutation in Msh2 $-/-$ mice, we chose to microdissect only the GCs that stained strongly with NP for PCR recovery of V λ genes. This limited analysis revealed approximately normal frequencies of GC V gene mutation, supporting the idea that Msh2 is not involved in the hypermutation process (20, 27). However, such NP $^{++}$ GCs become progressively rare in Msh2 $-/-$ compared with $+/+$ mice, and those we picked might only have been representative of a small clonal subpopulation that had not succumbed to the stochastic effects of absence of Msh2, perhaps due to a lower rate of proliferation.

Indeed, if high proliferation rate predisposes a B cell clone to death in an Msh2-deficient situation, the outcome of antigen-driven clonal selection during B cell responses may often be reversed in Msh2-deficient mice compared with normal mice, to favor those antigen-stimulated clones that exit the cell cycle earliest during the response. Somewhat analogous arguments have been presented by Frey et al. (20). Such biases might be further exacerbated when the memory B cell compartment is sampled, as even in normal mice this population is derived from a minor fraction of all clones that participate in the primary GC reaction (15, 31,

52, 53). Overall, an Msh2 deficiency may result in majority representation of responding B cells that have failed to complete or activate normal steps in memory B cell genesis, including steps in clonal expansion, selection, and V gene hypermutation. For example, Kuo and Sklar (54) have observed that the mammalian homologue of the MutM gene (8-oxyguanine DNA glycosylase) is highly induced in the rapidly proliferating B cells of GCs. This enzyme is involved in the pathway leading to repair of oxidatively damaged G nucleotides in DNA (55). Since an Msh2 deficiency attenuates progression of the GC reaction, perhaps via promoting the death of cells undergoing rapid proliferation, such a deficiency could result, secondarily, in overrepresentation of GC B cells that have not induced MutM. This could lead to a preferential sampling of mutations at germline G residues, if oxidative damage to these residues was elevated during the GC reaction. Such a scenario could explain the observations of Jacobs et al. (27), Phung et al. (11), and Rada et al. (13) that V gene mutations sampled on one strand in Msh2-deficient mice are observed predominantly at positions in which a germline G or C nucleotide was present.

Following from these arguments, the differences in hypermutation frequency and pattern observed in previous studies of MMR-deficient mice (10–13, 20) could well have resulted from biases in sampling of the V gene products of memory B cell responses (18, 19). Thus, while these differences are intriguing, whether MMR proteins play any direct role in the generation, fixation, or repair of DNA lesions during the hypermutation process remains an unresolved question. Clearly, if such a role is to be accurately evaluated in the future, experiments will need to be conducted under conditions where the effects of MMR deficiencies or defects on other aspects of B cell differentiation are minimized.

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