The translesion DNA polymerase θ plays a dominant role in immunoglobulin gene somatic hypermutation

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Immunoglobulin (Ig) somatic hypermutation (SHM) critically underlies the generation of high-affinity antibodies. Mutations can be introduced by error-prone polymerases such as polymerase ζ (Rev3), a mispair extender, and polymerase η , a mispair inserter with a preference for dA/dT, while repairing DNA lesions initiated by AID-mediated deamination of dC to yield dU:dG mismatches. The partial impairment of SHM observed in the absence of these polymerases led us to hypothesize a main role for another translesion DNA polymerase. Here, we show that deletion in C57BL/6J mice of the translesion polymerase θ , which possesses a dual nucleotide mispair inserter–extender function, results in greater than 60% decrease of mutations in antigen-selected V186.2DJ_H transcripts and greater than 80% decrease in mutations in the Ig H chain intronic J_H4-iE μ sequence, together with significant alterations in the spectrum of the residual mutations. Thus, polymerase θ plays a dominant role in SHM, possibly by introducing mismatches while bypassing abasic sites generated by UDG-mediated deglycosylation of AID-effected dU, by extending DNA past such abasic sites and by synthesizing DNA during dU:dG mismatch repair.

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Introduction

Somatic hypermutation (SHM) targets immunoglobulin (Ig) V(D)J region DNA, thereby providing the structural substrate for selection of higher affinity antibody mutants. It emerged before class switch DNA recombination (CSR) in phylogeny, first appearing in sharks (Flajnik and Du Pasquier, 2004). It requires T-cell help and effects mainly single nucleotide

changes with rare insertions or deletions (Diaz and Casali, 2002; Papavasiliou and Schatz, 2002; Neuberger *et al*, 2003; Wu *et al*, 2003; Chaudhuri and Alt, 2004; Honjo *et al*, 2004). It introduces point mutations at a rate of 10^{-3} – 10^{-4} per base per cell generation, while virtually sparing the Ig C region, and targets preferentially the mutational RGYW/WRCY hotspot (R for purine, Y for pyrimidine and W for A or T). Like CSR, SHM requires activation-induced cytidine deaminase (AID), which is expressed by activated B cells in germinal centers of peripheral lymphoid organs (Honjo *et al*, 2002, 2004).

The RGYW/WRCY hotspot includes WRC/GYW, which is preferentially deaminated by AID, as suggested by *in vitro* experiments involving single-stranded DNA (Pham *et al*, 2003; Yu *et al*, 2004). Within WRC/GYW, AID deaminates dC to yield dU:dG mispairs (Neuberger *et al*, 2003, 2005; Chaudhuri and Alt, 2004). dU:dG can be ‘replicated over’ resulting in dC→dT or dG→dA transitions (Phase 1a) or processed by the mismatch repair (MMR) pathway, possibly involving patch DNA synthesis by error-prone (translesion) DNA polymerases (Phase 2) (Neuberger *et al*, 2003, 2005). dU can also be deglycosylated by uracil DNA glycosylase (UDG), thereby yielding an abasic site. This can be bypassed by translesion DNA polymerases leading to insertion of transitions and transversion mutations (Phase 1b) or they could be processed by the base excision repair (BER) pathway, which entails base excision by apyrimidinic endonuclease (APE), followed by short-patch repair. This would unlikely yield a mismatch because of the high fidelity of the DNA polymerase involved in the process, polymerase (pol) β . Finally, transition and transversion mutations can be introduced by translesion DNA polymerases (Diaz and Casali, 2002; Wu *et al*, 2003; Diaz and Lawrence, 2005; Neuberger *et al*, 2005) while repairing DNA breaks, including double-stranded DNA breaks (DSBs) involving resected ends, as generated by AID (Papavasiliou and Schatz, 2002; Zan *et al*, 2003).

There are more than 15 distinct eukaryotic DNA polymerases, not all with a well-defined function (Hubscher *et al*, 2002). Some of these DNA polymerases can efficiently bypass DNA lesions, such as abasic sites arising through hydrolytic loss of purines or as intermediates during BER removal of dUTP, thereby minimizing chromosomal breakage and helping cells tolerate endogenous DNA damage, which can block progression of replication forks and dampen cell survival. These ‘translesion’ polymerases bypass DNA lesions effectively at the replication fork, but are highly error-prone when copying undamaged DNA. Bypass of abasic sites mostly requires the intervention of two distinct DNA polymerases: one, such as pol η or pol ι , that incorporates a nucleotide across from the lesion (mismatch inserter), and another that elongates past the mispair from the newly created primer end (mismatch extender), as pol ζ (Rev3) does

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(Prakash *et al*, 2005). The damage repair DNA pol β , pol μ , pol λ and pol κ do not seem to be involved in SHM (Diaz and Lawrence, 2005). In contrast, a role has been identified for DNA pol ζ and pol η , and, perhaps, pol ι (Diaz *et al*, 2001b; Zan *et al*, 2001; Zeng *et al*, 2001; Diaz and Casali, 2002; Faili *et al*, 2002; Wu *et al*, 2003; Faili *et al*, 2004; Delbos *et al*, 2005; Martomo *et al*, 2005). However, inhibition of pol ζ expression or deficiency in pol η has resulted in only partial reduction of SHM (Diaz *et al*, 2001b; Zan *et al*, 2001; Delbos *et al*, 2005; Martomo *et al*, 2005), prompting us to hypothesize that another translesion polymerase plays a dominant role in the SHM DNA repair process (Diaz and Casali, 2002; Wu *et al*, 2003; Xu *et al*, 2005).

Pol θ is the first DNA polymerase known to efficiently bypass an abasic site by functioning both as a mispair inserter and as a mispair extender, it synthesizes through a damaged DNA template including randomly inserted abasic sites with an efficiency comparable to that displayed on the corresponding undamaged template (Seki *et al*, 2004). Although residues in the polymerase catalytic site are highly homologous to high-fidelity A family DNA polymerases, pol θ displays an extremely low fidelity (Seki *et al*, 2004). Because of its dual nucleotide inserter-extender ability and its upregulation in the germinal center (Kawamura *et al*, 2004), we addressed the role of pol θ in SHM using C57BL/6J pol $\theta^{-/-}$ mice (Shima *et al*, 2004). pol $\theta^{-/-}$ mice were immunized with the alum-precipitated hapten (4-hydroxy-3-nitrophenyl) acetyl coupled to chicken gamma globulin (NP-CGG). The cDNA sequence of the rearranged V186.2 gene, which is used dominantly in response to NP, and the noncoding Ig H chain J_H4-iE μ intronic DNA were then analyzed for mutations. Here, we show that deletion of pol θ results in a greater than 80% decrease in mutations in the intronic Ig H chain

J_H4-iE μ DNA, thereby defining a significant role for this translesion polymerase in the DNA repair process, which is central to SHM.

Results

Pol θ is upregulated in hypermutating B cells

DNA pol θ expression has been reported to be upregulated in mouse germinal center B cells (Kawamura *et al*, 2004), in which SHM and CSR occur. To understand the involvement of pol θ in SHM, we sorted human tonsil B lymphocytes into IgD⁺ CD38⁻ (naïve B cells), IgD⁺ CD38⁺ (early centroblasts), IgD⁻ CD38⁺ (germinal center centroblasts and centrocytes) and IgD⁻ CD38⁻ (memory B cells) fractions, representing four sequential stages of B-cell differentiation (Zan *et al*, 2003). SHM is silent in naïve B cells, is activated in early centroblasts and centroblasts, abates in centrocytes and is quiescent in memory B cells. We analyzed the expression of pol θ and compared to that of pol ζ and pol ι , which are involved in SHM (Zan *et al*, 2001; Faili *et al*, 2002). We found that like pol ζ , pol θ was upregulated in hypermutating IgD⁺ CD38⁺ and IgD⁻ CD38⁺ B cells, but not in nonhypermutating IgD⁺ CD38⁻ or IgD⁻ CD38⁻ B cells, while pol ι was expressed at comparable levels at all four stages of B-cell differentiation (Figure 1A). We further analyzed the expression of pol θ , pol ζ , pol η and pol ι in the spleen, Peyer's patches, thymus, liver and heart of C57BL/6J mice. In contrast with pol ζ , pol η and pol ι , which were expressed in all different tissues analyzed, albeit at different degrees, pol θ was preferentially expressed in the spleen, Peyer's patches and thymus (Figure 1B). In the spleen, in which germinal center B cells are admixed with other lymphoid and non-lymphoid cells, pol θ transcripts were detected at a low level.

Figure 1 DNA pol θ is upregulated in hypermutating B cells. (A) Expression of pol θ is upregulated in human CD38⁺ germinal center B cells. IgD⁺ CD38⁻, IgD⁺ CD38⁺, IgD⁻ CD38⁺ and IgD⁻ CD38⁻ B cells were isolated from human tonsils. Transcripts of pol θ , pol ζ (Rev3), pol ι and β -actin were analyzed by specific RT-PCR using serially two-fold diluted cDNA as a template (lanes 1–5 in each column). (B) Preferential expression of pol θ in lymphoid tissues. Transcripts of pol θ , pol ζ , pol η , pol ι and GAPDH in mouse spleen, Peyer's patches, thymus, liver and heart were analyzed by specific RT-PCR using serially two-fold diluted cDNA as a template (lanes 1–5 in each column). (C) Upregulation of pol θ in stimulated IgD⁺ B cells. Purified human IgD⁺ B cells were cultured in the presence or absence of anti-CD40 mAb and IL-4 with or without pretreatment with anti-BCR Ab (Zan *et al*, 2001, 2003) and harvested after 2 days. RNA was purified to analyze the expression level of pol θ , pol ζ , pol ι and β -actin by RT-PCR using serially two-fold diluted cDNA as a template (lanes 1–5 in each column). (D) Upregulation of pol θ in stimulated mouse B cells. B220⁺ B cells from the spleen of WT C57BL/6J mice were cultured in the presence or absence of LPS and IL-4 with or without treatment with anti-mouse BCR Ab. After 2 days, B cells were harvested and used to analyze the expression level of pol θ , pol ζ , pol η , pol ι and GAPDH by RT-PCR using serially two-fold diluted cDNA as a template (lanes 1–5 in each column).

In the thymus, which contains a high proportion of proliferating T cells, the level of *pol* θ transcripts was also low. In contrast, like *pol* ζ , *pol* θ was significantly upregulated in Peyer's patches, which contain a large proportion of hypermutating germinal center B cells.

We then verified whether *pol* θ expression could be induced by the stimuli that are critical to induce germinal center B-cell differentiation, SHM and CSR. To this end, human peripheral blood B cells were BCR crosslinked and then stimulated *in vitro* with anti-CD40 monoclonal antibodies (mAb) and interleukin-4 (IL-4); accordingly, mouse spleen B cells were BCR crosslinked and then stimulated with bacterial lipopolysaccharide (LPS) and IL-4. BCR crosslinking is necessary together with CD40 engagement and IL-4 for the induction of SHM in human B cells (Zan et al, 1999, 2000), and upregulates DNA *pol* ζ in a dose-dependent fashion (Zan et al, 2001); LPS can substitute CD40 engagement in mouse B cells. These stimuli upregulated *pol* θ expression in both human and mouse B cells (Figure 1C and D), while they left *pol* η and *pol* ι expression unchanged. Thus, *pol* θ is upregulated by germinal center B-cell differentiation-inducing stimuli *in vitro* and is expressed in hypermutating B cells *in vivo*.

***Pol* θ deficiency does not affect B- and T-cell number or *in vitro* B-cell responses**

In *pol* $\theta^{-/-}$ mice, the size of the spleen, and the number and the size of the Peyer's patches were comparable to those in

pol $\theta^{+/+}$ mice (not shown). The number of B cells and T cells, the proportion of CD4⁺ T cells and the level of B- and T-cell death in the spleen and the Peyer's patches, as analyzed by staining with 7-amino-actinomycin (7-AAD), of *pol* $\theta^{-/-}$ mice were also comparable to those of *pol* $\theta^{+/+}$ mice (Figure 2A–D). In addition, *pol* $\theta^{-/-}$ B cells upregulated AID in response to LPS and IL-4 at a level comparable to *pol* $\theta^{+/+}$ B cells, which also upregulated *pol* θ expression (Figure 2E). Finally, after stimulation, *pol* $\theta^{-/-}$ B lymphocytes were not significantly different in cell cycle, as analyzed by propidium iodide (PI) staining, and division, as shown by carboxyfluorescein diacetate succinimidyl ester (CFSE) vital dye incorporation, from *pol* $\theta^{+/+}$ cells (Figure 2F and G).

***Pol* θ deficiency impairs SHM in the V186.2DJ_H transcripts encoding antibodies induced by NP-CGG**

To understand the role of *pol* θ in SHM, we examined cDNA sequences of the rearranged Ig V186.2 segment in *pol* $\theta^{-/-}$ C57BL/6J mice immunized with NP-CGG, as this V_H gene dominates the response to NP (Takahashi et al, 1998). Four *pol* $\theta^{-/-}$ mice as well as two heterozygote *pol* $\theta^{+/-}$ and two wild-type (WT) *pol* $\theta^{+/+}$ controls were injected intraperitoneally with NP-CGG twice within 4 weeks. At 4 days after the booster injection, spleen (B220⁺) B cells were isolated for mutation analysis. In such B cells, the proportion of PNA^{hi} cells was comparable in *pol* $\theta^{-/-}$ and *pol* $\theta^{+/+}$ mice (Figure 3A and B). V186.2DJ_H-C γ 1 cDNAs were amplified by an RT-PCR involving two nested forward V186.2 leader

Figure 2 *Pol* θ deficiency does not affect the B- and T-cell numbers, the proportion of CD4⁺ T cells and levels of B- and T-cell death in spleen and Peyer's patches, nor does it impair B-cell cycle or division. Data are representative of three experiments. (A–D) Flow cytometric profiles of cells from spleen or Peyer's patches stained with (A) PE-conjugated anti-B220 mAb and FITC-conjugated anti-CD3 mAb (17A2), (B) FITC-conjugated anti-CD3 mAb and PerCP-conjugated anti-CD4 mAb (GK1.5), (C) 7-AAD and PE-conjugated anti-B220 mAb and (D) 7-AAD and FITC-conjugated anti-CD3 mAb. (E) *Pol* θ and AID expression in induced *pol* $\theta^{-/-}$ or *pol* $\theta^{+/+}$ B cells. Transcripts from LPS- and IL-4-stimulated *pol* $\theta^{-/-}$ or *pol* $\theta^{+/+}$ spleen B cells were analyzed by RT-PCR specific for the cDNA sequences encoding exons 2 and 3 of the *pol* θ gene, or AID using serially two-fold diluted cDNA as a template (lanes 1–5 in each column). (F) Cell cycle analysis of *pol* $\theta^{-/-}$ and *pol* $\theta^{+/+}$ B cells induced *in vitro* by LPS and IL-4. After 1 and 3 days, cells were harvested for PI staining and flow cytometry analysis. Cells in G0/G1, S and G2/M phases identified by DNA content as measured by PI staining are shown. (G) Cell division in *pol* $\theta^{-/-}$ or *pol* $\theta^{+/+}$ B cells. *pol* $\theta^{-/-}$ or *pol* $\theta^{+/+}$ spleen B cells were labeled with CFSE and cultured in the presence of LPS and IL-4. After 3 days, the cells were harvested for flow cytometry analysis. Cell division is indicated by a progressive left shift in fluorescence histograms. Dark line filled with gray depicts the fluorescence tracing; gray vertical lines within the fluorescence tracing are integrations representing individual cell generations that are enumerated above the graph.

Figure 3 Pol θ deficiency does not result in alteration of germinal centers and *in vivo* B-cell proliferation. **(A)** Detection of germinal centers in *pol θ*^{-/-} and *pol θ*^{+/+} mice spleen sections prepared 10 days after immunization with NP-CGG. Germinal centers were revealed by staining with PE-labeled anti-B220 mAb and FITC-labeled PNA. The original magnifications are indicated at the bottom. **(B)** Flow cytometric profiles of cells from spleen or Peyer's patches of NP-CGG-immunized *pol θ*^{-/-} and *pol θ*^{+/+} mice stained with PE-labeled anti-B220 mAb and FITC-labeled PNA (dot plots were gated on B220⁺ cells). **(C, D)** *In vivo* B-cell proliferation indicated by the frequency of BrdU⁺ cells among B220⁺ or B220⁺ PNA^{hi} cells from spleen or Peyer's patches. *Pol θ*^{-/-} and *pol θ*^{+/+} mice were immunized with SRBC. After 6 days, the mice were injected twice within 16 h with BrdU (1 mg) and killed 4 h after the last injection. Cells from spleen or Peyer's patches were stained with PE-labeled anti-mouse B220 mAb or this mAb together with FITC-labeled PNA. The incorporated BrdU was stained with APC-conjugated anti-BrdU antibody and analyzed using FACSCaliburTM. Dot plots were gated on (C) B220⁺ or (D) B220⁺ PNA^{hi} cells, respectively. Data are representative of three experiments.

DNA-specific primers and two nested C γ 1-specific reverse primers. Preliminary experiments have suggested that *pol θ*^{-/-} B cells display normal or even increased levels of V_HDJ_H-C γ 1 transcripts and assured that 'comparable' populations of B cells were analyzed (not shown). The amplified V186.2DJ_H-C γ 1 cDNA was cloned and sequenced, and unique mutations in V186.2 and a 135 bp C γ 1 segment were compiled from a total of 106, 111 and 107 sequences from *pol θ*^{+/+}, *pol θ*^{+/-} and *pol θ*^{-/-} mice, respectively. Only two mutations were found in a total of 217 C γ 1 sequences from *pol θ*^{+/+} and *pol θ*^{+/-} mice, yielding a mutation frequency (6.83×10^{-5} change/base), which is within the range of PCR error using Platinum Pfx DNA polymerase (6.0×10^{-5} change/base after two PCR rounds of 30 cycles each). In *pol θ*^{-/-} mice, the mutation frequency of V186.2 (0.87×10^{-2} change/base) was reduced by more than 60% as compared to that in *pol θ*^{+/+} (2.25×10^{-2} change/base) or *pol θ*^{+/-} mice (2.34×10^{-2} change/base) ($P < 0.001$) (Figure 4A).

Impaired SHM in *pol θ*^{-/-} mice is not due to alteration of germinal centers, B-cell cycle or proliferation

The impairment of SHM observed in *pol θ*^{-/-} mice was not due to obvious defects in B-cell number or reactivity. In these mice, the number and architecture of the germinal centers in

the spleen were comparable to those of *pol θ*^{+/+} mice (Figure 3A). The proportion of PNA^{hi} B cells in both spleens and Peyer's patches was also comparable to that in *pol θ*^{+/+} mice (Figure 3B). Finally, as shown by *in vivo* bromodeoxyuridine (BrdU) incorporation, the proportions of proliferating B220⁺ B cells and B220⁺ PNA^{hi} cells in the spleen or Peyer's patches of *pol θ*^{-/-} mice were also comparable to those of the *pol θ*^{+/+} littermate controls (Figure 3C and D).

Pol θ deficiency alters the spectrum mutations in the rearranged V186.2 gene

Survey of the V186.2DJ_H-C γ 1 transcript sequences in response to NP-CGG revealed that *pol θ* deficiency altered the spectrum of the residual mutations, although the changes were relatively small. While the ratio of mutations at dC/dG to dA/dT was not significantly different in *pol θ*^{-/-} mice compared to their *pol θ*^{+/-} or *pol θ*^{+/+} counterparts, overall transition mutations increased by 21.2 and 23.6% compared to *pol θ*^{+/-} or *pol θ*^{+/+} mice, respectively ($P < 0.001$) (Figure 4B and C). Such increased transition mutations reflected a dominance of dG $\rightarrow$ dA transitions (33.3% of the total mutations), which increased by 48.7 and 29.6%, as compared to *pol θ*^{+/+} (22.4%) and *pol θ*^{+/-} (25.7%) mice, respectively ($P < 0.001$). WRC/GYW accounts for 102 (34.7%) of the

Figure 4 Mutations in the V186.2 region of V186.2DJ_H-C γ 1 transcripts from spleen B cells of NP-CGG-immunized $pol \theta^{-/-}$, $pol \theta^{+/+}$ or $pol \theta^{+/-}$ mice (secondary response). Data are pools from four $pol \theta^{+/+}$, two $pol \theta^{+/-}$ and six $pol \theta^{-/-}$ mice. (A) Pie charts depict the proportions of sequences that carry one, two, three, etc. mutations over the 294 bp region analyzed. The numbers of the sequences analyzed are at the center of the pies. (B) Numbers and nature of independent mutational events scored. (C) Compilations, with the numbers indicating percentages of all mutations scored in the pool of the target sequences of panels A and B. Below the compilations, the ratio of mutations at dC/dG to those at dA/dT is indicated, as is the ratio of transition:transversion substitutions at both dC/dG and dA/dT. P -values are from comparison of $pol \theta^{-/-}$ with $pol \theta^{+/-}$ and $pol \theta^{+/+}$ mice.

294 bp germline V186.2 gene sequence. In the secondary response to NP in $pol \theta^{-/-}$ mice, the proportion of the residual mutations that segregated within WRC/GYW was comparable to that in $pol \theta^{+/+}$ and $pol \theta^{+/-}$ mice, 52.2 versus 53.1 and 47.6% of total mutations, respectively (Figure 5A and B). In $pol \theta^{-/-}$ mice, 44.7% of the mutations within WRC/GYW were dG $\rightarrow$ dA transitions, which accounted for 23.6% of total residual mutations, while only 34.7 and 34.2% of WRC/GYW mutations in $pol \theta^{+/+}$ and $pol \theta^{+/-}$ mice were dG $\rightarrow$ dA transitions, which accounted for about 18.5 and 15.4% of total mutations, respectively. Thus, the residual mutations in $pol \theta^{-/-}$ mice show an increased frequency of transitions at both dC/dG and dA/dT with a preference for the WRC/GYW motif.

Transgenic $pol \theta$ expression does not affect SHM

Because of the significant contribution of $pol \theta$ to SHM, we wanted to determine whether further upregulation of this DNA polymerase would boost SHM above the level of WT C57BL/6J mice. We immunized $pol \theta$ transgenic (Tg) C57BL/6J mice with NP-CGG once and analyzed V186.2DJ_H-C γ 1 transcripts after 21 days. We chose to perform sequence analysis during the primary response, as we reasoned that any moderate change in SHM would be better appreciated

before any NP-CGG boost (Takahashi *et al*, 1998). As compared to their WT counterparts, $pol \theta$ expression was further upregulated in the spleen, Peyer's patches, liver and heart of $pol \theta$ Tg mice (Supplementary Figure 1). In WT mice, mutations in the primary response were approximately 70% of those in the secondary response (not shown); during such a primary response, the mutations in $pol \theta$ Tg mice were comparable to the control WT mice (Supplementary Figure 2A) in overall frequency and spectrum (Supplementary Figure 2B and C), indicating that transgenic $pol \theta$ expression does not enhance SHM.

$Pol \theta$ deficiency impairs SHM in the unselected H chain intronic J_H4-iE μ DNA

The greater than 60% decrease in mutation frequency in the V186.2 DNA coding for the NP-binding V_H site in $pol \theta^{-/-}$ mice did not likely reflect the full impact of the lack of $pol \theta$ deficiency, as mutations that increased antibody affinity for NP were under positive selection pressure. We analyzed the J_H4-iE μ intronic sequence downstream of rearranged V_H J558DJ_H4 DNA, which is targeted by SHM but is not subjected to positive or negative selection pressure, in PNA^{hi} B220⁺ cells from Peyer's patches of three pairs of $pol \theta^{-/-}$ and $pol \theta^{+/+}$ mice. In these mice, the proportions of Peyer's

Figure 5 Distribution of mutations in the V186.2 region of V186.2DJH-C γ 1 transcripts from spleen B cells of NP-CGG-immunized *pol* $\theta^{-/-}$ mice as compared to *pol* $\theta^{+/+}$ or *pol* $\theta^{+/-}$ mice (secondary response). Mutations from *pol* $\theta^{+/+}$ or *pol* $\theta^{+/-}$ mice are depicted above the germline V186.2 sequence and mutations from *pol* $\theta^{-/-}$ mice below. CDR1 and CDR2 regions are underlined. WRC, GYW and the imbricated WRC/GYW motifs are highlighted with yellow, blue and green, respectively. (A) Sequences derived from two pairs of *pol* $\theta^{-/-}$ and *pol* $\theta^{+/+}$ mice. (B) Sequences derived from two pairs of *pol* $\theta^{-/-}$ and *pol* $\theta^{+/-}$ mice.

patch PNA^{hi} B cells were comparable (19.7–20.3% of total B220⁺ cells). V_HJ558DJ_H4-iE μ DNA was amplified using a seminested PCR involving one J558 FR3 forward primer and two different J_H4 intronic reverse primers. We chose to analyze only the sequences downstream of V_HJ558DJ_H4 rearrangements, as V_HDJ_H rearrangements involving different J_H genes place the J_H-iE μ intron at different distances from the V_H promoter and may result in different mutation rates. Analysis of 231 and 235 J_H4-iE μ intronic DNA sequences (578 bp) from *pol* $\theta^{+/+}$ mice and *pol* $\theta^{-/-}$ mice showed that mutations in *pol* $\theta^{-/-}$ mice were decreased by 80.1%: 4.18×10^{-3} versus 0.83×10^{-3} change/base, respectively (Figure 6A). J_H4-iE μ intronic sequences from Peyer's patches PNA^{low} B220⁺ cells were also analyzed. Five mutations were found in 18 sequences from *pol* $\theta^{-/-}$ PNA^{low} B220⁺ cells, resulting in a frequency of 0.48×10^{-3} change/base, and six mutations were found in 19 sequences from *pol* $\theta^{+/+}$ PNA^{low} B220⁺ cells, resulting in a frequency of 0.55×10^{-3} change/base. This mutation range was considerably lower than that of PNA^{hi} B220⁺ cells, and is consistent with the notion that the PNA^{low} B220⁺ cells are not absolutely 'negative' (Gonzalez-Fernandez and Milstein, 1993), possibly because they include newly generated IgM memory B cells that underwent only a first round of mutations. Thus, *pol* θ deficiency effectively results in a major impairment of SHM.

Pol θ deficiency alters the spectrum of the residual somatic mutations

The residual mutations in *pol* $\theta^{-/-}$ mice reflected a decrease in absolute numbers of all mutations. These included a comparable reduction in mutations at dC/dG and dA/dT, with an increase in transitions, by 68.7 and 43.8%, respectively ($P < 0.001$) (Figure 6B and C). While the increase in transition mutations at dA/dT resulted from increased transitions at both dA and dT, by 32.2 and 61.8%, respectively ($P < 0.001$), the increase in transition mutations at dC/dG was exclusively due to an increase of dG $\rightarrow$ dA transitions by 101.9% ($P < 0.001$), in front of an actual decrease in dC $\rightarrow$ dT transitions. Absence of *pol* θ also resulted in segregation of the residual mutations within WRC/GYW. There are total 12 WRC, 22 GYW and seven imbricated WRC/GYW motifs within J_H4-iE μ , together accounting for 22.4% of the whole 578 bp J_H4-iE μ sequence. More than 45.1% of the residual mutations in *pol* $\theta^{-/-}$ mice were within WRC/GYW, as compared to 27.2% in *pol* $\theta^{+/+}$ mice. *Pol* θ deficiency ablated two-thirds of the mutations in WRC/GYW with dG $\rightarrow$ dA and dT $\rightarrow$ dC transitions accounting for more than 68% of the residual mutations (Figure 7 and Table I); in fact, dG $\rightarrow$ dA transitions were increased (19 versus 23) and dT $\rightarrow$ dC transitions were unchanged (12 versus 12) in absolute numbers (Table I). Thus, most mutations inserted in the absence of *pol* θ consist of dG $\rightarrow$ dA and dT $\rightarrow$ dC transitions, and these segregate within the WRC/GYW AID hotspot.

Discussion

Understanding of SHM has been furthered by the identification of AID as a central factor in this process. AID can directly deaminate dC in DNA, yielding dU:dG mispairs. The generation of this and other lesions, such as DNA cleavage (Papavasiliou and Schatz, 2002; Wu *et al*, 2003; Zan *et al*, 2003; Casali and Zan, 2004; Xu *et al*, 2005), would constitute

the initial step in SHM and CSR. The second step, DNA repair, is critical for the insertion of mismatches (mutations), mainly through the intervention of error-prone DNA polymerases. Our findings show that the translesion *pol* θ plays a significant role in the DNA repair process associated with SHM in both overall effectiveness of the process and the spectrum of the mutations. Accordingly, they also show that *pol* θ is selectively expressed in hypermutating B cells and is upregulated by stimuli that are essential for the induction of SHM and CSR.

Like mice homozygous for *chaos 1* (*chaos-1*, chromosome aberration occurring spontaneously 1) recessive dT $\rightarrow$ dC missense mutation (Shima *et al*, 2003, 2004), which results in a nonfunctional *pol* θ gene product, *pol* $\theta^{-/-}$ mice exhibit significant spontaneous and DSB-induced genome instability in the form of elevated micronuclei in reticulocytes ('micronucleus' phenotype), in response to radiation or mitomycin C treatment, indicating that *pol* θ deficiency causes a defect in homologous recombination (HR) and/or interstrand cross-link DNA repair (Shima *et al*, 2003, 2004). The genetic instability generated by *pol* θ deficiency did not result in a deficiency or alteration of B-cell number or response. The number and morphology of germinal centers and PNA^{hi} (B220⁺) B cells in the spleen and Peyer's patches in *pol* $\theta^{-/-}$ mice were comparable to those in *pol* $\theta^{+/+}$ mice, and so were B-cell cycle and proliferation as well as the level of B- and T-cell death. Therefore, the profound SHM defect in *pol* $\theta^{-/-}$ B cells is specific and points at a specific and physiologic role of *pol* θ in this process, as further emphasized by the preferential expression of this DNA polymerase in Peyer's patches, which contain a high proportion of hypermutating B cells, but not in the liver or the heart. In contrast, *pol* ζ , *pol* η and *pol* ι were expressed in all the lymphoid and nonlymphoid tissues analyzed. The meaning of *pol* θ expression in the thymus is unclear but support the involvement of *pol* θ in the DSB repair process possibly associated with T-cell receptor V(D)J gene rearrangement and T-cell division. Accordingly, *pol* θ is expressed in the bone marrow (Kawamura *et al*, 2004), where B cells differentiate by undergoing Ig V(D)J gene rearrangement and divide.

Following direct DNA deamination by AID, mutations can stem from 'replicating over' dU:dG lesions, yielding transitions at dC:dG (Phase 1a), or can be inserted by translesion DNA polymerases while bypassing abasic sites generated by deglycosylation of dU (Phase 1b) or while carrying out a patch repair as part of MMR or BER (Phase 2) (Neuberger *et al*, 2003, 2005). In *ung*^{-/-} *msh2*^{-/-} mice, in which Phases 1b and 2 are 'blocked' and no resolution to the dU:dG mispair exists other than 'replicating over', the overall frequency of mutations accumulated in the noncoding unselected Ig H chain intronic area is normal, but 99% of the mutations occur at dC/dG and all of them are transitions (Rada *et al*, 2004). Such double knockout mice, however, provide an artificial situation, which is unlike the physiological SHM process. Under physiological conditions, transitions at dC/dG account for 22–28% of the total unselected mutations (Rada *et al*, 2004). Thus, even assuming that 'replicating over' dU:dG accounts for all dC/dG transitions, this mechanism would be involved in the generation of approximately one out of four mutations. At least three out of four mutations could be inserted through DNA repair processes that involve error-prone DNA synthesis by translesion DNA polymerases in

Figure 6 Mutations in the Ig H chain intronic J_H4-iE μ DNA in PNA^{hi} Peyer's patches (B220⁺) B cells from *pol θ*^{-/-} mice and *pol θ*^{+/+} littermates. Data are from three *pol θ*^{-/-} and three *pol θ*^{+/+} mouse pairs. (A) Pie charts depict the proportions of sequences that carry one, two, three, etc. mutations over the 578 bp J_H4-iE μ region analyzed. The numbers of the sequences analyzed are at the center of the pies. (B) Numbers and nature of independent mutational events scored. (C) Compilations, with the numbers indicating percentages of all mutations scored in the pool of the target sequences of panels A and B. Below the compilations, the ratio of mutations at dC/dG to those at dA/dT is indicated, as is the ratio of transition:transversion substitutions at both dC/dG and dA/dT.

Phases 1b and 2. In fact, Phase 2 is the major pathway of SHM, as shown by the greater than 75% decrease in the frequency of somatic mutations in *msh2*^{-/-} mice and the normal mutation frequency in *ung*^{-/-} mice (Rada *et al*, 1998, 2004; Neuberger *et al*, 2005).

The greater than 80% decrease in the mutational load of the Ig H chain locus intronic sequence in *pol θ*^{-/-} mice

indicates that *pol θ* is dominant in the overall SHM process. *Pol θ* can bypass abasic sites generated by UDG-mediated deglycosylation of dU (Phase 1b), thereby introducing mutations with a 1:2 ratio of transitions:transversions, if all four bases are inserted with equal efficiency across from an abasic site by this polymerase. It can introduce mutations at dA/dT and dC/dG while copying undamaged DNA template past

J_H4-IE μ intronic sequences

Figure 7 Distribution of mutations in the intronic J_H4-iE μ sequence. Unique mutations from *pol* $\theta^{+/+}$ mice are depicted above the unmutated J_H4-iE μ sequence and unique mutations from *pol* $\theta^{-/-}$ mice below. WRC, GYW and imbricated WRC/GYW motifs are highlighted with yellow, blue and green, respectively. Sequences were derived from three pairs of *pol* $\theta^{-/-}$ and *pol* $\theta^{+/+}$ mice.

abasic sites or as part of the DNA patch repair, which is central to Phase 2. Pol θ bypass of abasic sites would be consistent with the increased transitions at dC:dG among residual mutations in *ung*^{-/-} mice (Rada *et al*, 2002), in which the failure to generate abasic adducts by UDG from dU residues prevents pol θ from inserting dC, dT or dG across from the adduct, while boosting the replicating over of dU:dG, which yields dC→dT and dG→dA transitions (Phase 1a). Likewise, the absence of pol θ would abort abasic site bypass and perhaps enhance the role of BER. In Phase 2, pol θ would introduce both transitions and transversions at dC/dG and dA/dT, consistent with the properties displayed by human recombinant pol θ *in vitro* (Seki *et al*, 2004).

Pol θ effectively bypasses lesions by inserting mispairs and extending past mispairs; it also copies undamaged DNA template effectively but in an error-prone fashion. The mispair extender function is shared by another translesion

polymerase, pol ζ (Diaz *et al*, 2001a; Zan *et al*, 2001), which can extend DNA past mispairs inserted by other translesion polymerases, such as pol η (Delbos *et al*, 2005; Martomo *et al*, 2005) or pol ι (Faili *et al*, 2002; Prakash *et al*, 2005). The significant residual mutations (40–57%) in human and mouse B cells, in which the expression of the pol ζ rev3 catalytic subunit was inhibited by *rev3* antisense DNA (Diaz *et al*, 2001b; Zan *et al*, 2001), suggest that pol θ compensates for pol ζ deficiency in mispair extension. Other translesion polymerases including pol η or pol ι can incorporate a base opposite an abasic site or undamaged DNA, but none can efficiently extend past the incorporated nucleotide (Prakash *et al*, 2005), supporting the prevailing contention that shift to a second DNA polymerase is required to extend the primer after insertion, as exemplified by the effective synergy between pol ι and pol ζ , as sequential mispair inserter and extender, respectively (Prakash *et al*, 2005). However, while

Table 1 Segregation of mutations within WRC/GYW in the J_H4-iE μ DNA

Mutation	<i>pol</i> $\theta^{+/+}$		<i>pol</i> $\theta^{-/-}$	
	Number	% Total	Number	% Total
dA $\rightarrow$ dC	11	1.97	4	3.54
dA $\rightarrow$ dG	30	5.38	7	5.31
dA $\rightarrow$ dT	7	1.25	1	0.88
dG $\rightarrow$ dA	19	3.41	23	20.35
dG $\rightarrow$ dC	7	1.25	0	0.00
dG $\rightarrow$ dT	19	3.41	1	0.88
dC $\rightarrow$ dA	7	1.25	0	0.00
dC $\rightarrow$ dG	12	2.15	0	0.00
dC $\rightarrow$ dT	17	3.05	2	1.77
dT $\rightarrow$ dA	0	0.00	0	0.00
dT $\rightarrow$ dC	12	2.15	12	10.62
dT $\rightarrow$ dG	10	1.79	1	0.88
Total	151	27.06	51	45.13

Total mutations in *pol* $\theta^{+/+}$ mice = 558; mutations in WRC = 82, mutations in GYW = 89 and mutations in WRC/GYW = 151. Total mutations in *pol* $\theta^{-/-}$ mice = 113; mutations in WRC = 32, mutations in GYW = 37 and mutations in WRC/GYW = 51. J_H4-iE μ DNA consisted of 578 nucleotides.

pol ζ is devoid of any mispair inserting activity, *pol* θ can effectively extend past mispairs in addition to inserting them (Seki *et al*, 2004). When synthesizing a DNA strand across an abasic site (Phase 1b) or as part of a patch repair process (Phase 2), *pol* θ could extend past the mispair inserted by *pol* η , *pol* ι or itself. Mispairs inserted by *pol* η , *pol* ι or *pol* θ can also be extended by *pol* ζ . The interchangeability of *pol* ζ and *pol* θ as mispair extenders would explain the presence of residual mutations in the absence of the former or the latter.

The relative overall increase in both dA $\rightarrow$ dG and dT $\rightarrow$ dC transitions in *pol* $\theta^{-/-}$ mice likely resulted from the activity of *pol* η in Phase 2. Indeed, this DNA polymerase displays a preference for inserting transitions at dA/dT, as indicated by the decreased transition mutations at dA/dT in *pol* $\eta^{-/-}$ mice (Delbos *et al*, 2005; Martomo *et al*, 2005) and in patients with the variant form of *Xeroderma pigmentosum* (XP-V) (Zeng *et al*, 2001; Yavuz *et al*, 2002; Faili *et al*, 2004), in whom *pol* η is reduced or absent, as well as experiments involving mouse DNA *pol* η *in vitro* (Pavlov *et al*, 2002). In *pol* $\theta^{-/-}$ mice, transitions incorporated at dA/dT by *pol* η or at dC/dG by *pol* ι (Zhang *et al*, 2001) would have been extended by *pol* ζ (Diaz and Lawrence, 2005; Prakash *et al*, 2005). In the absence of *pol* η , the MMR complex may recruit a DNA polymerase that is more accurate at copying dA/dT bases; the inserted nucleotides would then be extended by *pol* θ . The significant role of *pol* η as a mispair inserter in Phase 2, possibly working in concert with *pol* θ as a mispair extender, would explain the decreased mutation frequency in *pol* η -deficient mice (Delbos *et al*, 2005), although such a decrease has not been confirmed in different *pol* $\eta^{-/-}$ mice (Martomo *et al*, 2005). The relative increase in dC/dG transitions in the residual *pol* $\theta^{-/-}$ mice mutations resulted solely from an increase in dG $\rightarrow$ dA transitions. dC $\rightarrow$ dT transitions were actually decreased in these mice, suggesting an asymmetrical deamination of dC residues by AID.

A possible asymmetry, that is, strand polarity, in AID-mediated dC deamination is further supported by the segregations of dG $\rightarrow$ dA transitions but not dC $\rightarrow$ dT transitions within WRC/GYW in *pol* $\theta^{-/-}$ mice (Table 1), suggesting that

under physiological conditions, AID preferentially deaminates dC in the bottom DNA strand. The concentration of mutations within WRC/GYW in the absence of *pol* θ would further indicate that this polymerase plays a major role in Phase 2. The deamination of dC in the bottom strand yields a dG:dU mispair. In the absence of *pol* θ , the dG:dU mispair can be replicated over, yielding a dG $\rightarrow$ dA transition in the top strand, or can be processed by UDG yielding an abasic site that can be bypassed by other translesion DNA polymerases, repaired through BER or dealt with by MMR. The MMR complex promotes excision of a stretch of nucleotides containing the dU. The subsequent patch DNA synthesis would involve *pol* η and result in dT $\rightarrow$ dC transitions on the top strand. Indeed, *in vitro* analysis have shown that *pol* η preferentially introduce dG across from dT, yielding dT $\rightarrow$ dC transitions (Matsuda *et al*, 2000).

The increase in dG $\rightarrow$ dA transitions within WRC/GYW in *pol* $\theta^{-/-}$ mice would be inconsistent with the putatively preferential incorporation by human recombinant *pol* θ of dA across from an abasic adduct emerging from a dC, and can be explained in different ways. First, the biological activity of mouse *pol* θ *in vivo* may be different from that of human recombinant *pol* θ *in vitro* (Seki *et al*, 2004). Second, *pol* θ can also incorporate dT across from abasic sites resulting in dG $\rightarrow$ dT or dC $\rightarrow$ dA transversions, although with lower efficiency (Seki *et al*, 2004). Third, the absence of *pol* θ would diminish Phases 1b and 2 and may enhance replication over dU:dG in Phase 1a, which yields exclusively dC $\rightarrow$ dT and dG $\rightarrow$ dA transitions. Finally, the effective dual mispair inserter/extender function of *pol* θ would allow this DNA polymerase to introduce mismatches not only while bypassing abasic sites (Phase 1b), but also while extending DNA past such abasic sites (Phase 1b) or while synthesizing DNA during the dU:dG MMR/DNA patch repair of Phase 2.

Interestingly, no significant increase in SHM of the rearranged V186.2 gene segment in the primary immune response to NP-CGG was brought about by *pol* θ expression above its physiological level, as achieved in *pol* θ Tg mice, suggesting that physiological upregulation of this DNA polymerase is sufficient to ensure an optimal function of the SHM machinery. Rather, a highly effective mechanism to enrich for mutations was provided by the application of a positive antigen (NP-CCG) pressure to the V_HDJ_H gene product, as emphasized by the significant higher mutational load in the V186.2DJ_H region compared to the intronic J_H4-iE μ sequence in *pol* $\theta^{+/+}$ mice (2.25×10^{-2} versus 4.18×10^{-3} change/bp). Current experiments are aimed at further defining the *in vivo* activities of *pol* θ , the only known DNA polymerase with a helicase domain, in B-cell DNA repair. The role of *pol* θ in DSB repair and HR is consistent with a function of this DNA polymerase in CSR. Indeed, our preliminary experiments suggest that *pol* θ plays a significant and complex role in CSR. This will be investigated using newly generated double knockout mice, including *pol* $\theta^{-/-}$ *msh2* $^{-/-}$ and *pol* $\theta^{-/-}$ *ung* $^{-/-}$ mice.

Materials and methods

Mice and immunization

The *pol* $\theta^{-/-}$ (*pol* θ^{mt1Jcs}/pol θ^{mt1Jcs}) mice used in this study were at the N4 backcross generation into strain C57BL/6J (93.75% contribution of this background, on average, the remainder coming

from strain 129) and *pol* θ Tg mice were generated on the C57BL/6J background and maintained as coisogenics (Shima *et al*, 2004). The *pol* $\theta^{-/-}$ mice were generated by placing an in-frame stop codon into exon 1 and replacing exons 2–5 with a neomycin resistance (*neo*) gene. Although there have been reports that on very rare occasions the *neo* gene in the targeted allele can affect expression of neighboring genes, and thus gives unexpected phenotypes, the phenotypes we observed in the *pol* $\theta^{-/-}$ mice were solely due to DNA *pol* θ inactivation as (1) the phenotype of our *pol* $\theta^{-/-}$ mice is congruous with the biochemical and functional nature of DNA *pol* θ , (2) the genetic instability phenotype of the *pol* $\theta^{-/-}$ mice is identical to that of the mice carrying the *chaos1* point-mutant allele (Shima *et al*, 2003, 2004), suggesting that there is no additional mutant phenotype in the knockout and (3) *pol* $\theta^{-/-}$ mice heterozygotes for the knockout allele have no phenotype, indicating that the *neo* gene does not create a dominant effect by activating inappropriate expression of a neighboring gene. Neither the *pol* $\theta^{-/-}$ nor the *pol* θ Tg mice could be homozygous for the 129 mutant allele of *pol* ι gene. In fact, most of them do not carry that allele at all, as revealed by amplification and sequencing of the *pol* ι gene (not shown). C57BL/6J *pol* $\theta^{+/+}$ mice were from the Jackson Laboratory (Bar Harbor, ME).

Mice (8- to 10-week-old) that were free of obvious disease were given a first intraperitoneal injection of 100 μ g of NP-CGG at a ratio of 16:1 (NP₁₆-CGG) (Biosearch Technologies, Novato, CA) in alum (Pierce, Rockford, IL). After 4 weeks, they were boosted intraperitoneally with another 100 μ g NP₁₆-CGG. B cells from spleens were obtained 4 days after the boost to analyze the sequences of V186.2DJ_H-C γ 1 cDNA. Peyer's patches B cells were obtained from 15- to 18-week-old *pol* $\theta^{-/-}$ and *pol* $\theta^{+/+}$ C57BL/6J mice and used to analyze the intronic DNA downstream of rearranged V_H J558 genes. *Pol* θ Tg mice were immunized once with NP-CGG and after 21 days spleen B cells were isolated for analysis. All animal protocols were approved by the Institutional Animal Care and Use Committee of University of California, Irvine, CA.

Human B cells

IgD⁺ CD38⁺, IgD⁺ CD38⁺, IgD⁺ CD38⁺ and IgD⁺ CD38⁺ B cells were purified from human tonsils. After T-cell depletion by sheep red blood cell (SRBC) rosetting and fractionation through Histo-paque 1077[®] (Sigma-Aldrich Co., Saint Louis, MO), IgD⁺ CD38⁺, IgD⁺ CD38⁺, IgD⁺ CD38⁺ and IgD⁺ CD38⁺ B cells were purified through solid-phase selection using specific mouse mAbs (Zan *et al*, 2003). In other experiments, human IgD⁺ B cells were isolated from normal peripheral blood using similar procedures (Zan *et al*, 2001). Purified B cells were analyzed for purity using FACSCalibur[™] (BD Biosciences, San Jose, CA) and appropriate labeled mAbs (Zan *et al*, 2003).

Mouse B cells

Single B220⁺ cell suspensions were prepared from the spleen or Peyer's patches using a B-cell enrichment kit (BD Biosciences). B cells were then sequentially incubated with phycoerythrin (PE)-labeled anti-mouse CD45R (B220) mAb (RA3-6B2) (BD Biosciences) and fluorescein isothiocyanate (FITC)-labeled peanut agglutinin (PNA) (E-Y Laboratories, San Mateo, CA). Labeled lymphocytes were then sorted using a MoFlow[™] cell sorter (Dako-Cytomation, Fort Collins, CO), yielding 95% pure B220⁺ PNA^{hi} cells.

In vitro B-cell activation

Human IgD⁺ B cells were reacted for 1 h at 4°C with Sepharose[®]-conjugated rabbit Ab to human Ig μ chain and rabbit Ab to human Ig (H + L chain) (Irvine Scientific, Santa Ana, CA), mixed 1:1 at 2 μ g/ml (anti-huBCR Ab), washed with cold PBS and then cultured in the presence or absence of anti-human CD40 mAb (IgG1 mAb G28-5, ATCC HB 9110, Manassas, VA) (2 μ g/ml) and human IL-4 (100 U/ml) (Genzyme Co., Cambridge, MA) in flat-bottomed six-well plates for 2 days. B220⁺ lymphocytes were purified from splenocytes of nonintentionally immunized C57BL/6J mice. These B cells were reacted for 1 h at 4°C with goat F(ab')₂ anti-mouse Ig (10 μ g/ml) (SouthernBiotech, Birmingham, AL), washed with cold PBS and then cultured in the presence or absence of *Escherichia coli* LPS (50 μ g/ml) (Sigma-Aldrich Co.) and rmIL-4 (2 ng/ml) (R&D Systems, Minneapolis, MN) in flat-bottomed six-well plates for 2 days. All cultures were performed in RPMI-1640 medium supplemented with 10% heat-inactivated FBS (Sigma-Aldrich Co.), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

FACS analysis

The number of (B220⁺) B cells and (CD3⁺) T cells, the proportions CD4⁺ T cells, the level of B- and T-cell death and the proportions of PNA^{hi} B cells were determined by FACS analysis. Single cell suspensions were prepared from the spleen or Peyer's patches of *pol* $\theta^{-/-}$ and *pol* $\theta^{+/+}$ mice and stained with PE-conjugated anti-mouse B220, FITC-conjugated anti-mouse CD3 mAb (17A2) (BioLegend, San Diego, CA), PerCP-conjugated anti-mouse CD4 mAb (GK1.5) (BioLegend, San Diego, CA), 7-AAD (BD Biosciences) or FITC-conjugated PNA. Cells were fixed with 1% paraformaldehyde in PBS and analyzed using a FACSCalibur[™] flow cytometer (BD Biosciences).

In vitro B-cell cycle and proliferation

Cell cycle was analyzed by PI staining (Zan *et al*, 2003). Proliferation was analyzed using the CellTrace CFSE cell proliferation kit (Molecular Probes, Eugene, OR). Cells were washed in serum-free HBSS (Invitrogen Corp., Carlsbad, CA) and resuspended at 40 $\times$ 10⁶/ml. After adding an equal volume of 2.4 μ M CFSE, the cell suspension was incubated at 37°C for 12 min and then washed with medium containing 10% FCS. Cells were then diluted and cultured in the presence of nil or LPS from *E. coli* (Sigma-Aldrich Co.) and rmIL-4 (R&D Systems), harvested at various time points after activation and analyzed using a FACSCalibur[™] flow cytometer.

In vivo B-cell proliferation

Mice were injected intraperitoneally with SRBCs (Colorado Serum Company). After 6 days, they were injected intraperitoneally twice within 16 h with BrdU (1 mg) and killed 4 h after the last injection. Cells from the spleen or Peyer's patches were stained with PE-labeled anti-mouse B220 mAb (BD Biosciences) or this mAb together with FITC-labeled PNA (E-Y Laboratories). Incorporated BrdU was stained with APC-conjugated anti-BrdU mAb using the BrdU Flow kit (BD Biosciences) and analyzed using a FACSCalibur[™] flow cytometer.

Histology

Spleens from immunized mice were embedded in OCT compound, snap-frozen and stored at -80°C. Cryostat sections (7 μ m) were fixed using cold acetone and stored at -80°C for 25 min. Acetone-fixed sections were air dried for 30 min at room temperature. PE-conjugated B220 (1:200 dilution) and FITC-conjugated PNA (1:100 dilution) were applied to the sections and kept in a dark box at room temperature for 1 h. The sections were washed using PBS and mounted using anti-fade reagent (Invitrogen Corp.) for examination.

DNA *pol* θ , *pol* ζ , *pol* η , *pol* ι , β -actin, AID and GAPDH expression

RNA was extracted from 2 $\times$ 10⁶ cells using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The first strand cDNAs were synthesized from equal amounts of total RNAs (4 μ g) using the SuperScript[™] Preamplification System (Invitrogen Corp.). The expression of the human or mouse DNA *pol* θ , *pol* ζ and *pol* ι , β -actin and GAPDH was analyzed by specific RT-PCR. This was made semiquantitative by performing dilution analysis so that there was a virtually linear relationship between the amount of cDNA used and the intensity of the PCR product. PCR consisted of 25 cycles, each of 45 s denaturation at 94°C, 45 s annealing at 58°C and 45 s extension at 72°C. Each amplification cycle was completed by an additional 10 min extension at 72°C. *Pol* θ , *pol* ζ and *pol* ι cDNAs were amplified using specific primers (Supplementary Table I) and equal amounts of first strand cDNA template.

PCR amplification of Ig V186.2DJ_H cDNA and intronic V_HJ558DJ_H-iE μ DNA

Platinum Pfx DNA polymerase (Invitrogen Corp.) was utilized for all cDNA and DNA amplifications. Rearranged V186.2DJ_H-C γ 1 DNA coding for the anti-NP V region was amplified from spleen cell cDNA using a nested PCR involving two V186.2 leader-specific primers, 5'-CATGCTCTTCTTGGCAGCAACAGC-3' and 5'-CAGGTCC AACTGCAGCAG-3' (forward), and two C γ 1 primers, 5'-GTGCACA CCGCTGGACAGGGATCC-3' and 5'-CCATGGAGTTAGTTTGGGCAG-3' (reverse). Parameters were 30 cycles of 45 s at 94°C, 45 s at 58°C and 1 min at 72°C for the first round. In the second round, 1/20 of the first-round PCR product was amplified for another 30 cycles of 45 s

at 94°C, 45 s at 58°C and 45 s at 72°C. The intronic H chain region downstream of rearranged V_HDJ_H was amplified using a seminested PCR involving one V_HJ558 framework region (FR)3-specific primer, 5'-GCCTGACATCTGAGGACTCTGC-3' (forward), and two primers specific for sequences 1184 and 580 nucleotides downstream of J_H4, 5'-CCTCTCCAGTTTCGGCTGAATCC-3' and 5'-TGAGACCGAGGCTAGATGCC-3' (reverse), yielding an amplified DNA of ~658 bp if rearrangement involves J_H4, ~1278 bp if rearrangement involves J_H3, ~1658 bp if rearrangement involves J_H2 and ~1977 bp if rearrangement involves J_H1. Reaction conditions for the first round were 94°C for 1 min, 58°C for 1.5 min, 72°C for 2 min for 30 cycles, and for the second-round PCR were 94°C for 45 s, 58°C for 1 min and 72°C for 1 min for 30 cycles. PCR products were cloned into the pCR-TOPO™ vector (Invitrogen Corp.) and sequenced.

Sequence analysis

Sequences were analyzed using the MacVector version 7.2 software (Accelrys Inc., San Diego, CA). Mutations targeting the Ser33 codon were excluded from analysis, as such mutations are known to

undergo a strong positive selection in the V186.2DJ_H gene product in NP-CGG-immunized mice and would bias the sequence analysis (Takahashi *et al*, 1998; Bardwell *et al*, 2004). In the case of the J_HiE μ intronic DNA, only J_H4-iE μ sequences were analyzed. WRC/GYW motifs include WRC, GYW and imbricated/adjacent WRC/GYW motifs, when these hotspots were analyzed for the target of mutations. The differences in frequency and spectrum of mutations in *pol* $\theta^{+/+}$, *pol* $\theta^{+/-}$ and *pol* $\theta^{-/-}$ mice were analyzed using χ^2 tests.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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