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ARTICLE *in* EUROPEAN JOURNAL OF IMMUNOLOGY · OCTOBER 2008

Impact Factor: 4.03 · DOI: 10.1002/eji.200838502 · Source: PubMed

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 DOI 10.1002/eji.200838502 Eur. J. Immunol. 2008. 38: 2796–2805

European Journal of Immunology

DNA polymerase η is a limiting factor for A:T mutations in Ig genes and contributes to antibody affinity maturation

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DNA polymerase η (POLH) is required for the generation of A:T mutations during the somatic hypermutation of Ig genes in germinal center B cells. It remains unclear, however, whether POLH is a limiting factor for A:T mutations and how the absence of POLH might affect antibody affinity maturation. We found that the heterozygous *Polh*$^{+/-}$ mice exhibited a significant reduction in the frequency of A:T mutations in Ig genes, with each type of base substitutions at a level intermediate between the *Polh*$^{+/+}$ and *Polh*$^{-/-}$ mice. These observations suggest that *Polh* is haplo-insufficient for the induction of A:T mutations in Ig genes. Intriguingly, there was also a reduction of C to T and G to A transitions in *Polh*$^{+/-}$ mice as compared with WT mice. *Polh*$^{-/-}$ mice produced decreased serum titers of high-affinity antibodies against a T-dependent antigen, which was associated with a significant reduction in the number of plasma cells secreting high-affinity antibodies. Analysis of the V region revealed that aa substitutions caused by A:T mutations were greatly reduced in *Polh*$^{-/-}$ mice. These results demonstrate that POLH is a limiting factor for A:T mutations and contributes to the efficient diversification of Ig genes and affinity maturation of antibodies.

Key words: Activation-induced cytidine deaminase · Affinity maturation · DNA polymerase η · Immunoglobulin gene · Somatic hypermutation

Supporting Information available online

Introduction

High-affinity antibodies are critical effectors against pathogens and are generated in the germinal center (GC) B cells by the active introduction of mutations at both C:G and A:T pairs in the Ig genes [1]. This hypermutation process is initiated by the activation-induced cytidine deaminase (AID) [2], which is thought to catalyze the deamination of cytosine (C) to uracil (U) on DNA in a transcription-dependent manner [3]. Available genetic evidence suggests that mutations are introduced during replication and the error-prone repair of the AID-triggered U:G mismatch [4–6]. Direct replication of the U:G mismatch, or the abasic site formed after excision of U via the uracil DNA glycosylase, could result in mutations at C:G pairs. A number of

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DNA polymerases, including POLQ and the deoxycytidyl transferase (REV1), have been implicated in generating C:G mutations by replicating over the abasic site [7–11]. The mechanism leading to the mutations at non-damaged A:T pairs still remains largely elusive. The components of the mismatch repair (MMR), including MSH2 and MSH6, and DNA polymerase η (POLH) are required for the induction of A:T mutations [12–19]. Absence of POLH or MSH2/MSH6 results in >80% reduction of A:T mutations in Ig genes. POLH is a translesion DNA polymerase capable of correctly bypassing UV-induced cyclobutane thymine dimmers and is involved in the suppression of sunlight-induced skin cancers [20, 21]. However, POLH is highly inaccurate when replicating undamaged DNA and has an average base substitution error rate that is ~1000-fold higher than that for replicative polymerases including Polδ and Polε [22, 23]. It has been suggested that the AID-mediated U:G lesion is recognized by the components of MMR, which then recruit POLH to catalyze a short patch DNA synthesis. Mutations are thought to be induced during the short patch DNA synthesis since POLH frequently incorporates wrong nucleotide opposite template A or T. Consistent with this possibility, the patterns of A:T mutations observed in Ig genes were found to correlate with the base substitution specificity of POLH [24, 25]. These observations suggest that POLH is the primary enzyme that introduces A:T mutations during Ig gene somatic hypermutation (SHM). Other polymerases may cooperate with POLH and contribute to the generation of some A:T mutations [26].

POLH is widely expressed in many tissues and cell types [27]. Although it is a low fidelity enzyme for undamaged DNA, there is no evidence that it participates in MMR and generates mutations in normal tissues. It is thus unclear why GC B cells are able to utilize POLH and generate A:T mutations in Ig genes during the repair of the AID-triggered U:G lesion. *Polh* transcript levels were increased in GC B cells [28], raising the possibility that elevated *Polh* expression may be important for the induction of a high frequency of A:T mutations. In the present study, we have investigated whether the levels of POLH affect the frequency of A:T mutations in Ig genes *in vivo* and how the absence of POLH might affect the affinity maturation of antibodies. Our results demonstrate that POLH is a limiting factor for A:T mutations in Ig genes. Moreover, we found that absence of POLH results in reduced frequency and altered patterns of aa substitutions in Ig genes and decreased production of high-affinity antibodies.

Results

Mutation frequency and patterns in Ig genes in WT, *Polh*$^{+/-}$ and *Polh*$^{-/-}$ mice

We analyzed GC B cells isolated from the spleen of mice immunized with a T-dependent antigen. These GC B cells are induced by acute antigen stimulation and are suitable sources for the analysis of both mutation frequency and mutation patterns [9, 29]. We analyzed J_H4 intronic sequences from three WT, four *Polh*$^{+/-}$ and two *Polh*$^{-/-}$ mice that were derived from breeding of *Polh*$^{+/-}$ mice. This region was selected since the mutations are not under antigenic selection and therefore reflect the unbiased mutation patterns. For WT and *Polh*$^{-/-}$ mice, we also included data from our previous study [26], allowing the analysis of a large number of mutations of each type of base substitution. The detailed results of individual mice in each group are shown in Supporting Information 1. The pooled data in each group are summarized in Table 1. Consistent with our previous studies [7, 9], the overall mutation frequency in the J_H4 intronic region was 1.049% in WT mice with approximately half of the mutations occurring at C:G (0.518%) and A:T (0.531%). In agreement with earlier observations, *Polh*$^{-/-}$ mice had dramatically reduced mutation frequency at A:T (0.083%), confirming that POLH is a critical enzyme for the generation of A:T mutations. Remarkably, we found a significant reduction in the frequency of A:T mutations in *Polh*$^{+/-}$ heterozygous mice (0.317 *versus* 0.531% in WT mice, $p<0.001$, unpaired *t*-test). In fact, the frequency of each type of base substitutions at A:T in *Polh*$^{+/-}$ mice was intermediate between that found in WT and *Polh*$^{-/-}$ mice (Fig. 1). These results demonstrate that *Polh* is haplo-insufficient for the induction of A:T mutations and that its function cannot be compensated for by other enzymes. In addition, there was a significant reduction of C to T and G to A transitions in *Polh*$^{+/-}$ mice, which was not observed in WT or *Polh*$^{-/-}$ mice (Fig. 1). Although the reduction of C:G mutations was more restricted to transitions, the overall mutation frequency at C:G was significantly decreased in *Polh*$^{+/-}$ mice (Table 1). This was largely due to the fact that C:G transitions represented ~60% of the total C:G mutations (Fig. 1) and their reduction greatly affected the overall frequency at C:G.

Decreased serum titers of high-affinity antibodies in *Polh*$^{-/-}$ mice

To investigate the role of A:T mutations in antibody affinity maturation, we next examined the immune responses against a T-dependent antigen, 4-hydroxy-3-nitrophenyl-acetyl coupled to chicken γ-globulin (NP-CGG). *Polh*$^{-/-}$ mice produced significantly reduced amounts of NP-specific high-affinity antibodies during the primary response ($p<0.05$, unpaired *t*-test), as measured by ELISA with NP3 (Fig. 2A). The production of total (high- and low-affinity) antibodies was also reduced in *Polh*$^{-/-}$ mice as compared with WT mice (Fig. 2B), but this was largely attributable to the reduction of the high-affinity antibodies. The reduced affinity maturation in *Polh*$^{-/-}$ mice was illustrated by comparing the ratios of NP3- and NP30-binding antibody titers, which revealed a significant reduction in serum titers of the high-affinity antibodies at weeks 2 and 3 after immunization (Fig. 2C; $p<0.01$, unpaired *t*-test). The majority of the antibodies were high-affinity during the secondary response and the ratio of NP3- and NP30-binding antibodies was close to 1 in both WT and *Polh*$^{-/-}$ mice (Fig. 2C). This probably is a result of selection that obscures the primary defect in antibody affinity maturation in

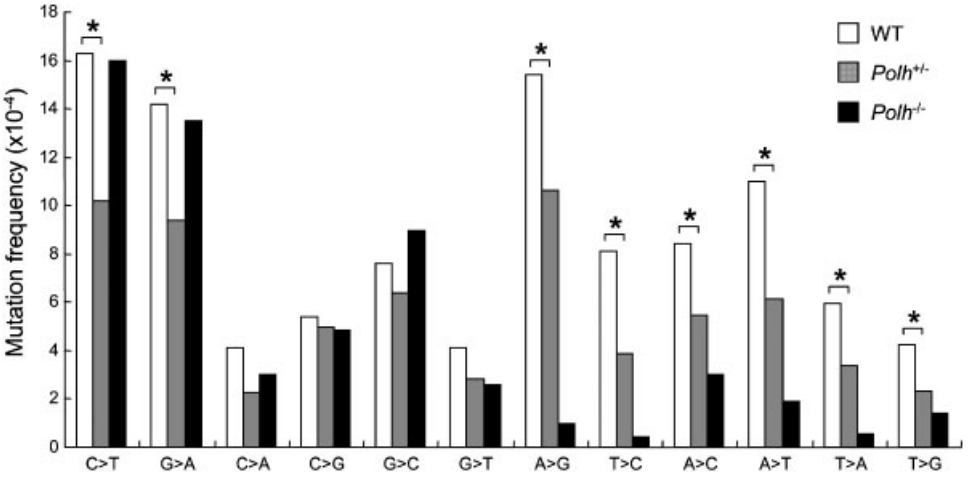

Figure 1. Mutation frequency in the J_H4 intronic region of GC B cells isolated from the spleen of WT, *Polh*$^{+/-}$ and *Polh*$^{-/-}$ mice immunized with NP-CGG. Absolute frequency was obtained by dividing the number of each type of nucleotide substitutions by the length of the mutated sequences. The results of eight WT, four *Polh*$^{+/-}$ and six *Polh*$^{-/-}$ mice were included in the analysis (see Supporting Information Table 1 for detailed results of individual mice). The data are corrected for base composition. Each type of base substitutions at A:T, as well as C to T and G to A transitions, was significantly decreased in *Polh*$^{+/-}$ mice as compared with WT mice (*p<0.01, unpaired t-test).

Table 1. Mutation frequency in WT, *Polh*$^{+/-}$, and *Polh*$^{-/-}$ mice

J_H4 intron (509 bases)	WT (sum of eight mice)	*Polh*$^{+/-}$ (sum of four mice)	*Polh*$^{-/-}$ (sum of six mice)
Number of clones	1070	553	826
Mutated clones (%)	821 (76.7%)	441 (79.7%)	532 (64.4%)
Total length of mutated sequences	417 889	224 469	270 788
Total number of mutations	4384	1523	1548
Overall mutation frequency (%)	1.049	**0.678**[a)]	**0.572**
Mutation frequency at C:G (%)	0.518	**0.361**	0.489
Mutation frequency at A:T (%)	0.531	**0.317**	**0.083**
%mutation at C:G : A:T	49.3:50.7	53.2:46.8	85.5:14.5

[a)] The values in bold type indicate significant differences from WT mice (p<0.001, unpaired t-test). In addition, the overall mutation frequency (0.572%) and the frequency at A:T (0.083%) in *Polh*$^{-/-}$ mice are significantly lower compared to those in *Polh*$^{+/-}$ mice, respectively (p<0.05, unpaired t-test).

Polh$^{-/-}$ mice. These results demonstrate that *Polh*$^{-/-}$ mice have impaired affinity maturation against a T-dependent antigen.

Reduced number of plasma cells secreting high-affinity antibodies in *Polh*$^{-/-}$ mice

The observed reduction in the serum titers of high-affinity antibodies in *Polh*$^{-/-}$ mice could be either due to a reduction in the number of plasma cells producing high-affinity antibodies or due to decreased secretion of antibodies by individual plasma cells. To distinguish these two possibilities, we next performed ELISPOT assay to determine the frequency of the cells secreting NP-specific high- and low-affinity antibodies. Mice were immunized with NP-CGG and 2 wk after the immunization single cell suspension of spleen cells were analyzed for NP-specific antibody-forming cells (AFC). Consistent with the reduction in the production of high-affinity antibodies, *Polh*$^{-/-}$ splenocytes contained a decreased frequency of AFC producing NP3-binding antibodies (Fig. 3A, $p = 0.087$). In contrast, the frequency of NP30-binding AFC was similar between WT and *Polh*$^{-/-}$ mice (Fig. 3B, $p = 0.55$). The reduction in the number of AFC producing high-affinity anti-NP antibodies was further illustrated by calculating the ratio of NP3- and NP30-binding AFC in individual mice (Fig. 3C, $p = 0.015$). These results demonstrate that the reduction in the serum titers of high-affinity antibodies was due to a reduction in the number of plasma cells that produce high-affinity antibodies.

Reduced frequency and altered patterns of V_H aa replacements in *Polh*$^{-/-}$ mice

The reduced antibody affinity maturation in *Polh*$^{-/-}$ mice suggests that A:T mutations are important for the diversification of Ig genes. To directly validate this possibility, we sequenced the V_H186.2 gene (see Supporting Information Table 2 for mutation frequency in individual mice), which is the primary V gene

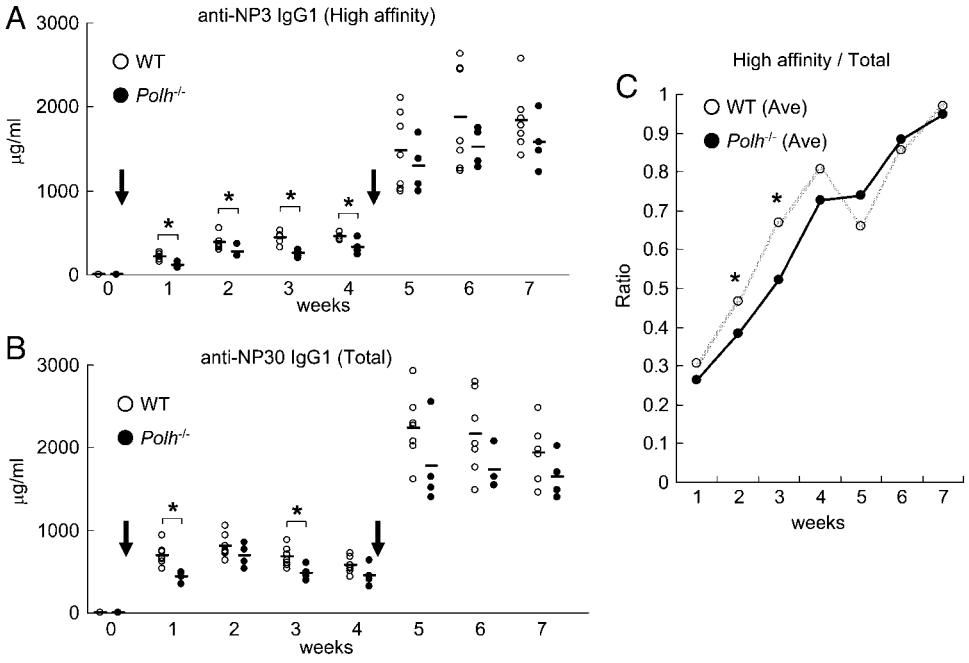

Figure 2. Immune responses and affinity maturation in WT and *Polh*$^{-/-}$ mice. Mice (seven WT and four *Polh*$^{-/-}$) were immunized with 100 μg of NP-CGG in alum and boost 4 wk later. Arrows indicate immunization times. NP-specific IgG1 antibodies in the serum were measured by ELISA. (A) Titers of high-affinity NP-specific antibodies (*$p<0.05$, unpaired *t*-test). (B) Titers of total (high and low-affinity) NP-specific antibodies (*$p<0.05$, unpaired *t*-test). (C) Average ratio of high-/low-affinity antibodies (*$p<0.01$, unpaired *t*-test).

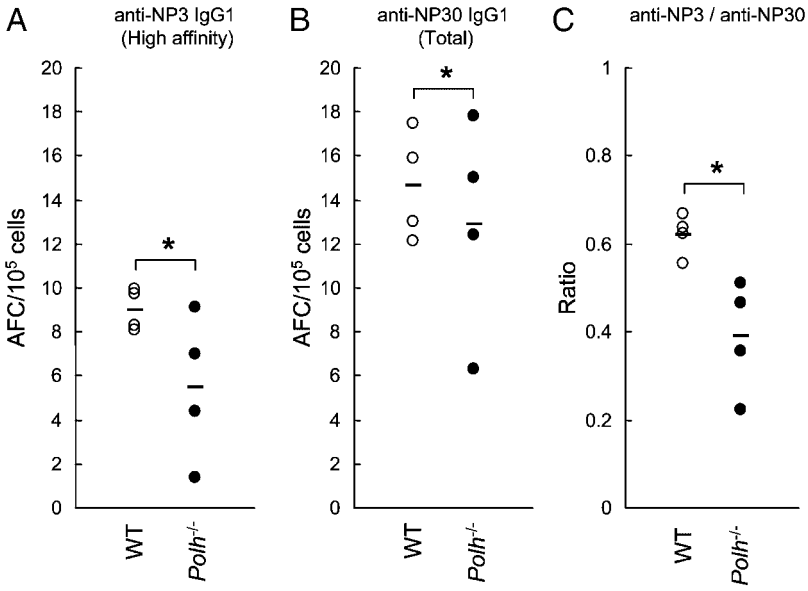

Figure 3. Reduced number of plasma cells producing high-affinity NP-specific antibodies in *Polh*$^{-/-}$ mice. Mice (four WT and four *Polh*$^{-/-}$) were immunized with NP-CGG in alum and 2 wk later splenocytes were analyzed for NP-specific AFC by ELISPOT assay as described in *Materials and methods*. (A) Frequency of NP-specific high-affinity AFC (*$p = 0.087$, unpaired *t*-test). (B) Frequency of NP-specific total (high and low-affinity) AFC (*$p = 0.549$, unpaired *t*-test). (C) The ratio of high-/low-affinity AFC was significantly reduced in *Polh*$^{-/-}$ mice compared with WT mice (*$p<0.05$, unpaired *t*-test).

responding to NP in mice immunized with NP-CGG. We analyzed 99 WT and 99 *Polh*$^{-/-}$ unique sequences (Tables 2 and 3). It is well known that the antibody affinity against NP increases ~10-fold by a single aa substitution (tryptophan to leucine, W to L) at position 33 of CDR1, which is almost exclusively due to a G to T transversion mutation. Consistent with the normal frequency of C:G mutations in *Polh*$^{-/-}$ mice, the W33L occurred at a similar frequency in WT (78 of 99 clones, 79%) and *Polh*$^{-/-}$ (71 of 99 clones, 72%) sequences.

We first compared aa substitutions in sequences that contained W33L (Table 2, clones with W33L). The W33L was strictly associated with the presence of a tyrosine (Y) at position 99 as previously reported [30] both in WT and in *Polh*$^{-/-}$ mice. Among these, 51 WT and 54 *Polh*$^{-/-}$ clones contained aa

Table 2. Amino acid substitutions in CDR1 and CDR2 of V_H186.2 gene in WT and *Polh*$^{-/-}$ mice[a)]

Clones with W33L			CDR1					CDR2																	
	Position		31	32	33	34	35	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	99
	Germline sequence		S	Y	W	M	H	R	I	D	P	N	S	G	G	T	K	Y	N	E	K	F	K	S	
WT	51	37	–	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		6	–	–	*L*	*I*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		3	*N*	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		2	–	–	*L*	**L**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	*I*	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	**Y**	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	*T*	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
	15	2	–	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**R**	–	–	–	Y
		2	*T*	–	*L*	–	–	–	–	–	–	**D**	–	–	–	–	–	–	–	–	–	–	–	–	Y
		2	*T*	–	*L*	–	–	–	–	–	–	–	*T*	–	–	–	–	–	–	–	**M**	–	–	–	Y
		1	–	–	*L*	–	–	–	–	–	–	–	**G**	–	–	–	–	–	–	–	–	–	–	–	Y
		1	*N*	–	*L*	–	–	–	–	–	–	**Y**	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	–	–	*L*	*I*	–	–	–	–	–	–	**R**	–	–	–	–	–	–	–	–	–	–	–	Y
		1	*T*	–	*L*	–	–	–	–	–	–	–	–	–	–	**S**	–	–	–	–	–	–	–	–	Y
		1	*N*	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**R**	–	Y
		1	*N*	–	*L*	–	–	–	–	–	–	**S**	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	*N*	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	**S**	–	**M**	–	–	–	Y
		1	*N*	–	*L*	–	–	–	–	**A**	–	**D**	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	*N*	–	*L*	–	–	–	–	–	–	–	–	–	S	–	–	–	**S**	–	–	–	–	N	Y
	12	2	–	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*T*	Y
		2	–	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	N	Y
		2	*I*	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	N	Y
		2	–	–	*L*	*I*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	N	–	–	N	Y
		1	–	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	N	–	Y
		1	*N*	–	*L*	–	–	–	–	–	–	–	–	–	Y	–	–	–	–	–	–	–	–	–	Y
		1	*I*	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*T*	Y
		1	*T*	–	*L*	–	–	–	–	–	–	–	–	–	R	–	–	–	–	–	–	–	–	–	Y
Polh$^{-/-}$	54	37	–	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		6	*N*	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		4	*I*	–	*L*	*I*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		3	*T*	–	*L*		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		3		–	*L*	*I*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	*I*	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
	2	1	–	–	*L*	–	–	–	–	–	–	**K**	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	–	–	*L*	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**Q**	–	–	–	Y
	15	2	–	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*T*	Y
		2	–	–	*L*	–	–	–	–	–	–	–	–	–	–	S	–	–	–	–	–	–	–	–	Y
		2	–	–	*L*	–	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–	–	Y
		1	–	–	*L*	–	–	–	–	–	–	–	–	–	–	–	N	–	–	–	–	–	–	–	Y
		1	–	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	R	Y
		1	–	–	*L*	–	–	–	–	–	–	–	–	–	S	–	–	–	–	–	–	–	–	–	Y
		1	–	–	*L*	–	–	–	–	–	–	–	–	–	N	–	–	–	–	–	–	–	–	–	Y
		1	–	–	*L*	–	–	–	–	–	–	–	N	–	–	*I*	–	–	–	–	–	–	–	–	Y
		1	–	–	*L*	–	–	–	–	–	–	–	–	–	–	*I*	–	–	–	–	–	–	–	*T*	Y
		1	*K*	–	*L*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	N	Y
		1	*T*	–	*L*	–	–	–	–	–	–	–	–	–	–	*I*	–	–	–	–	–	–	–	–	Y
		1	–	–	*L*	*I*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	N	–	Y

[a)] 78 WT and 71 *Polh*$^{-/-}$ unique clones were analyzed. aa substitutions induced by A:T and C:G mutations are shown in bold and italic, respectively.

Table 3. Amino acid substitutions in CDR1 and CDR2 of V_H186.2 gene in WT and *Polh*$^{-/-}$ mice[a)]

Clones with W33			CDR1					CDR2																		
	Position		31	32	33	34	35	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	99
	Germline sequence		S	Y	W	M	H	R	I	D	P	N	S	G	G	T	K	Y	N	E	K	F	K	S	
WT	15	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*T*	Y
		1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**R**	–	–	–	–	–	–	–	Y
		1	*T*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**E**	–	Y
		1	*N*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	–	–	–	–	–	–	–	–	–	–	**G**	–	*D*	–	–	–	–	–	–	–	–	–	Y
		1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**G**	–	–	–	–	Y
		1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**R**	–	–	–	–	–	–	–	*G*
		1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**M**	–	–	–	*G*
		1	*N*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*G*
		1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**E**	–	–	–	–	–	–	*N*	*F*
		1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*T*	*A*
		1	–	–	–	–	–	–	–	–	–	–	*T*	–	*V*	–	–	–	–	–	–	–	–	–	*D*
		1	–	–	–	*I*	–	–	**F**	–	–	–	–	–	*D*	–	**H**	–	–	**G**	–	–	–	*N*	W
	6	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*G*
Polh$^{-/-}$	20	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*N*	–	–	–	–	–	–	–	Y
		2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*N*	Y
		1	–	–	–	–	–	–	–	–	–	–	–	*D*	–	–	–	–	–	–	–	–	–	–	Y
		1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*N*	–	–	–	Y
		1	–	–	–	–	–	–	–	–	–	–	–	–	*S*	–	*N*	–	–	–	–	–	–	–	Y
		1	*N*	–	–	*I*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	–	–	–	–	–	–	–	–	–	–	*N*	–	–	*I*	–	–	–	–	–	–	–	–	Y
		1	*N*	–	–	–	–	*S*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*T*	Y
		1	*N*	–	–	–	–	–	–	–	–	–	–	–	*D*	–	–	–	–	–	–	–	–	*N*	Y
		1	–	–	–	*I*	Q	–	–	–	–	–	–	*D*	*V*	–	–	–	–	*D*	*N*	–	–	–	Y
		1	–	–	*Y*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	–	–	–	–	–	–	–	–	–	–	–	*D*	*I*	–	–	–	–	–	–	–	–	*R*	*G*
		1	–	–	–	–	*N*	–	–	–	–	–	–	–	*D*	–	*N*	–	–	–	–	–	–	*T*	*G*
		1	–	–	*C*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*G*
		1	–	–	–	–	–	–	–	–	–	–	–	–	*D*	–	–	–	–	**A**	–	–	–	–	*F*
		1	*T*	–	–	–	–	–	–	–	–	–	–	–	–	–	**R**	–	–	–	–	–	–	*T*	*F*
		1	–	–	–	–	–	–	–	–	–	–	–	*D*	–	–	–	–	–	–	–	–	–	–	*S*
		1	–	–	–	*I*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*N*	–	–	–	*H*
	8	6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y
		1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*G*
		1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	W

[a)] 21 WT and 28 *Polh*$^{-/-}$ unique clones were analyzed. aa substitutions induced by A:T and C:G mutations are shown in bold and italic, respectively.

substitutions only within CDR1, with 14 WT and 17 *Polh*$^{-/-}$ clones also had aa changes at positions 31 and/or 34. The remaining W33L sequences had additional aa replacement in CDR2. Among these, 15 WT but only 2 *Polh*$^{-/-}$ clones contained aa replacement caused by A:T mutations (shown in bold), consistent with the dramatic decrease of A:T mutations in *Polh*$^{-/-}$ mice. In contrast, a similar number of WT (12 clones) and *Polh*$^{-/-}$ (15 clones) sequences contained aa replacement in CDR2 caused by C:G mutations. In WT mice, C:G mutations frequently targeted position 66 and to a less extent position 57 whereas A:T mutations frequently targeted positions 54 and 63, indicating that mutations at C:G and A:T preferentially targeted aa at different positions. In *Polh*$^{-/-}$ mice, C:G mutations also targeted positions 66 and 57 as was the case for WT mice. However, positions 54 and 63, the target of A:T mutations, were rarely mutated due to the great reduction of A:T mutations.

Instead, position 58 was frequently targeted. In fact, there were a total of 17 aa substitutions caused by A:T mutations in CDR2 of WT W33L clones but only two were observed in *Polh*$^{-/-}$ W33L clones (Table 2). Consequently, the patterns of aa substitutions in CDR2 were significantly different between WT and *Polh*$^{-/-}$ W33L sequences.

We next compared the aa substitutions in sequences that did not contain the W to L substitution (Table 3, clones with W33). In these clones, position 99 contained glycine (G) and other aa, as well as Y, in agreement with earlier observations [29]. In the 15 WT clones that contained aa substitution in CDR1 and/or CDR2, a total of 23 aa replacements was observed, of which 10 were due to A:T mutations. In contrast, among the 41 aa replacement observed in the 20 *Polh*$^{-/-}$ clones, only two were caused by A:T mutations. As was the case for the W33L clones, the patterns of aa substitution within CDR2 were also quite different between WT and *Polh*$^{-/-}$ clones due to the great reduction of A:T mutations in *Polh*$^{-/-}$ mice. When the total numbers of aa changes in CDR2 of both W33L and W33 clones were counted, WT had 53 aa replacements, of which 28 were induced by A:T mutations. In contrast, *Polh*$^{-/-}$ had 49 aa substitutions and only four were derived from A:T mutations. Collectively, these results demonstrate that the absence of POLH resulted in altered patterns of aa substitutions in CDR2 of V_H186.2 genes in mice immunized with NP-CGG.

Discussion

Multiple DNA polymerases are involved in the introduction of point mutations during Ig gene SHM. Among these, POLH plays a major role in the generation of A:T mutations. In the present study, we have carefully analyzed a large number of point mutations in Ig genes of *Polh*$^{+/+}$, *Polh*$^{+/-}$ and *Polh*$^{-/-}$ mice. We found that the frequency of A:T mutations in Ig genes was strictly dependent on the gene dosage of *Polh*, indicating that POLH is a limiting factor for A:T mutations. The upregulation of *Polh* expression in GC B cells therefore represents an important mechanism to achieve a high frequency of A:T mutations in Ig genes. The results of *Polh*$^{+/-}$ mice are in contrast to that of *Msh2*$^{+/-}$ mice. Although *Msh2*$^{-/-}$ mice exhibit an $>80\%$ reduction of A:T mutations as is the case for *Polh*$^{-/-}$ mice, *Msh2*$^{+/-}$ heterozygous mice have relatively normal A:T mutations [12]. These observations suggest that the amount of MSH2 required for the induction of A:T mutations is in excess in GC B cells when compared with that of POLH. Notably, each type of nucleotide substitutions at A:T pairs was similarly decreased in *Polh*$^{+/-}$ mice as compared with WT mice, indicating that the reduction in POLH expression did not result in altered patterns of A:T mutations. This is consistent with the idea that the patterns of A:T mutations reflect the base substitution specificity of POLH [24, 25]. Humans with xeroderma pigmentosum variant (XP-V) disease are deficient in POLH and have greatly reduced A:T mutations in the Ig genes [28]. It remains to be determined whether human carriers of *POLH* mutations also have reduced A:T mutations relative to control individuals.

Unexpectedly, we also observed a significant reduction of C to T and G to A transitions in *Polh*$^{+/-}$ mice. Since transversions at C:G were only marginally decreased, it is unlikely that *Polh*$^{+/-}$ GC B cells had a general depression of SHM in which case each type of C:G mutations would have been uniformly reduced as was the case for A:T mutations. In supporting this conclusion, B cells from *Polh*$^{+/-}$ mice exhibited normal proliferative responses to anti-IgM stimulation and CD40 ligation *in vitro*. In addition, the development of the GC B cells *in vivo*, as measured by the size of the B220$^+$PNA$^+$ population in the spleen after immunization with a T-dependent antigen, appeared normal in *Polh*$^{+/-}$ mice (data not shown). The reduction of C to T and G to A transitions in *Polh*$^{+/-}$ GC B cells was not observed in WT or *Polh*$^{-/-}$ mice and therefore cannot be simply explained as a gene dosage effect. POLH has recently been shown to interact with RAD18, RAD6 and REV1 [31]. RAD 18 is an E3 ubiquitin ligase which, together with RAD6, monoubiquitinates PCNA on stalled replication forks and has been implicated in SHM of Ig genes in chicken DT40 cells [32, 33]. REV1 has been shown to interact with multiple Y-family DNA polymerases [34] and contribute to the generation of C to G and G to C transversions in Ig genes in mice [10, 11]. The reduction in POLH protein levels in *Polh*$^{+/-}$ GC B cells might have resulted in altered protein–protein interactions and indirectly affected the function of other low fidelity polymerases involved in the generation of C:G mutations. The lack of effect on C:G mutations in the complete absence of POLH could be due to functional compensation by other polymerases. In any case, the reduction of transitional mutations at C:G in *Polh*$^{+/-}$ mice is difficult to explain at this point but might suggest unidentified mechanisms that control the interactions and the recruitment of different polymerases during Ig gene SHM. Further studies are required to understand why C:G transitions were reduced in *Polh*$^{+/-}$ but not *Polh*$^{-/-}$ mice.

Ig genes undergo SHM at C:G and A:T base pairs at roughly the same frequency. Although it seems obvious that both types of mutations are important for the diversification of Ig genes, it has not been formerly proven whether a great reduction of either C:G or A:T mutations would really affect the affinity maturation of antibodies. We utilized a well-established T-dependent antigen, NP-CGG, to address this question. The affinity of antibodies against NP is largely dependent on a single aa substitution (W to L) in CDR1 of the V_H186.2 gene, which is predominantly caused by a G to T mutation. Since *Polh*$^{-/-}$ mice had normal C:G mutations, we anticipated that the great reduction of A:T mutations in *Polh*$^{-/-}$ mice would have minimal effect on the affinity maturation of antibodies against NP. The results of the present study, however, demonstrate that the reduced A:T mutations have substantial effect on the affinity maturation of NP-specific antibodies. Both the serum titers of high-affinity anti-NP antibodies and the frequency of plasma cells producing high-affinity antibodies were significantly reduced in the absence of POLH. These observations suggest that while the W to L substitution is

important for increased antibody affinity against NP, other aa changes, particularly those occurring within the CDR2, also likely contribute to the affinity maturation of NP-specific antibodies. It is tempting to speculate that affinity maturation in *Polh*$^{-/-}$ mice would be severely compromised if the affinity is more dependent on aa substitutions caused by A:T mutations.

The results of the present study demonstrate that the frequency of A:T mutations in Ig genes is strictly dependent on the gene dosage of *Polh*. Paradoxically, in a separate study we found that the ectopic overexpression of POLH in fibroblasts failed to induce A:T mutations in an AID-mediated mutagenesis system. These observations collectively suggest that POLH is essential but not sufficient for the induction of A:T mutations and that additional factors present in GC B but not fibroblasts are likely required. One hypothesis is that GC B cells express a factor that functions to recruit POLH instead of high fidelity DNA polymerases during the repair of the U:G mismatch. POLH is a Y-family low fidelity polymerase, which also includes POLK and POLI [35]. However, neither POLK nor POLI appears to be involved in Ig gene SHM [36–39]. It remains to be investigated how POLH participates in the SHM of Ig genes and introduces mutations at A:T pairs.

Materials and methods

Establishment of *Polh*$^{-/-}$ mice

Polh$^{-/-}$ mice were generated in a 129/C57BL/6 mixed background [40] and have been backcrossed with C57BL/6 mice for more than ten generations. Mice were maintained under specific pathogen-free conditions and all animal experiments have been approved by the RIKEN Yokohama Institute (permission number 20-025).

Somatic hypermutation assays

Mice were immunized i.p. with 100 μg of NP-CGG precipitated with alum and 2 wk later B220^{+}PNA^{high} GC B cells were sorted from spleen. Genomic DNA extraction and analysis of somatic mutations in the J_H4 intronic region were performed as described previously [7, 9]. For the analysis of the $V_H186.2$ gene, mice were similarly immunized with NP-CGG with alum and 2 wk later splenocytes were isolated. Total RNA was extracted with Trizol reagent and the first strand cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen). $V_H186.2$ gene was amplified with a forward primer V_H (5′-CAGCCTGACATCTGAGGACTCTGC-3′) and a reverse primer Cγ1 (5′-CTCCACCAGACCTCTCTAGACAGC-3′) as described previously [2]. PCR was carried out with the high fidelity KOD plus polymerase (TOYOBO, Japan) under the following conditions: 94°C for 5 min and then 94°C for 20 s, 65°C for 30 s and 68°C for 30 s for 30 cycles. The PCR products were cloned into the pCR2.1 vector for sequencing. Only clones with unique sequences were analyzed.

ELISPOT assay

ELISPOT assay was performed using a Multiscreen HTS filter plate (Millipore). The HTS plate was coated with 50 μg/mL of NP3-BSA or NP30-BSA at 4°C overnight. The coated plate was then washed with PBS-T (PBS containing 0.1% Tween 20) three times and blocked with PBS containing 1% BSA for 1 h at RT. Splenocytes (5×10^5, 2.5×10^5 and 1.25×10^5) were then seeded and incubated at 37°C for 100 min in a CO_2 incubator. The plate was then washed twice with PBS-T containing 50 mM EDTA, three times with PBS-T and blocked again with PBS containing 1% BSA for 1 h at RT. The plate was further incubated with alkaline phosphatase-conjugated goat anti-mouse IgG1 antibodies (1 μg/mL in PBS containing 1% BSA) at 37°C for 60 min in a CO_2 incubator, washed four times with PBS-T and developed with BCIP/NBT reagent (MOSS INC) for 2–3 min. The plate was then washed four times with H_2O, air dried and colonies were counted using an IMMUNOSPOT Analyzer (CTL Analyzers LLC, Cleveland, OH). The number of colonies obtained with different numbers of splenocytes was converted to number of colonies/10^5 cells and the average numbers are shown.

Acknowledgements: The authors thank Drs. Tomohiro Kurosaki and Masaki Hikida for their helpful discussions, and Akiko Ukai and Hiromi Mori for their excellent technical assistance. The authors also thank the RCAI Animal Facility for breeding and maintaining the mice, the FACS Facility for cell sorting, and the Immunogenomics group for sequencing.

Conflict of interest: The authors declare no commercial or financial conflict of interest.

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Abbreviations: **AFC:** antibody-forming cells · **AID:** activation-induced cytidine deaminase · **MMR:** mismatch repair · **NP-CGG:** 4-hydroxy-3-nitrophenyl-acetyl coupled to chicken γ-globulin · **POLH:** DNA polymerase η · **SHM:** somatic hypermutation

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Supporting Information for this article is available at www.wiley-vch.de/contents/jc_2040/2008/38502_s.pdf

Received: 11/5/2008
Revised: 10/7/2008
Accepted: 24/7/2008

