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Allelic 'choice' governs somatic hypermutation *in vivo* at the immunoglobulin κ-chain locus

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Monoallelic demethylation and rearrangement control allelic exclusion of the immunoglobulin κ-chain locus (*Igk* locus) in B cells. Here, through the introduction of pre-rearranged *Igk* genes into their physiological position, the critical rearrangement step was bypassed, thereby generating mice producing B cells simultaneously expressing two different immunoglobulin-κ light chains. Such 'double-expressing' B cells still underwent monoallelic demethylation at the *Igk* locus, and the demethylated allele was the 'preferred' substrate for somatic hypermutation in each cell. However, methylation itself did not directly inhibit the activation-induced cytidine-deaminase reaction *in vitro*. Thus, it seems that the epigenetic mechanisms that initially bring about monoallelic variable-(diversity)-joining rearrangement continue to be involved in the control of antibody diversity at later stages of B cell development.

The main mechanism for preimmune antigen receptor diversity is variable-(diversity)-joining (V(D)J) recombination mediated by the recombination-activating proteins RAG-1 and RAG-2. To ensure that each B cell or T cell expresses a single product, however, this process is subject to allelic exclusion. Experiments have indicated that the mechanisms that bring about allelic exclusion actually begin early during development when all of the immune receptor loci become asynchronously replicating, thereby generating a clonally inherited allele-specific 'mark'[1]. This structure is apparently 'interpreted' in the immune system, causing the 'preferential' initiation of nuclear relocalization[2], chromatin opening[3] in the form of histone modification, demethylation[4] and rearrangement on the early replicating allele[1]. After the actual recombination event, allelic exclusion is then maintained through an additional mechanism involving feedback inhibition, whereby the product of successful rearrangement on one allele causes a general inhibition of the recombination machinery in *trans*[5].

After rearrangement in B cells, additional antibody diversity can be generated by receptor editing[6,7] in the bone marrow and somatic hypermutation (SHM), which occurs in the germinal centers of secondary lymphoid tissues[8]. SHM is a carefully controlled process involving the introduction of DNA strand breaks in a reaction mediated by activation-induced cytidine deaminase (AID)[9], which probably functions as a DNA-editing enzyme[8,10–12]. It seems that the main targets of SHM are productively and nonproductively rearranged immunoglobulin alleles[13–17], whereas the precise positioning of mutations is dependent on the transcription machinery, which apparently operates by recruiting the enzymes required for mediating mutagenesis[8]. Like V(D)J recombination, SHM can occur only in regions with increased transcription[18,19], demethylation[20] and chromatin accessibility[21,22], and this is directed by local enhancer elements[23,24]. Such observations collectively show that the allele-specific developmentally regulated opening of B cell receptor loci is key in targeting DNA-alteration events, thus suggesting that SHM, like V(D)J rearrangement, may be subject to allelic exclusion.

To test that hypothesis, we generated a targeted mouse carrying two nearly identical pre-rearranged immunoglobulin κ-chain alleles (*Igk* alleles) that were potentially equal candidates for SHM. We constructed one of the alleles to encode a human κ-constant region (C_κ), which allowed us to distinguish the products of each allele by simple flow cytometry. By bypassing rearrangement, single B cells then were able to express both *Igk* alleles simultaneously. Despite that equivalence, the main molecular mechanisms responsible for the establishment of allelic exclusion were still operative, as indicated by the finding that only one allele was 'preferred' for demethylation as well as SHM. Our results suggest that the same epigenetic factors that initially 'mark' one allele for V(D)J rearrangement continue to be involved in directing allele specificity at later stages of B cell development.

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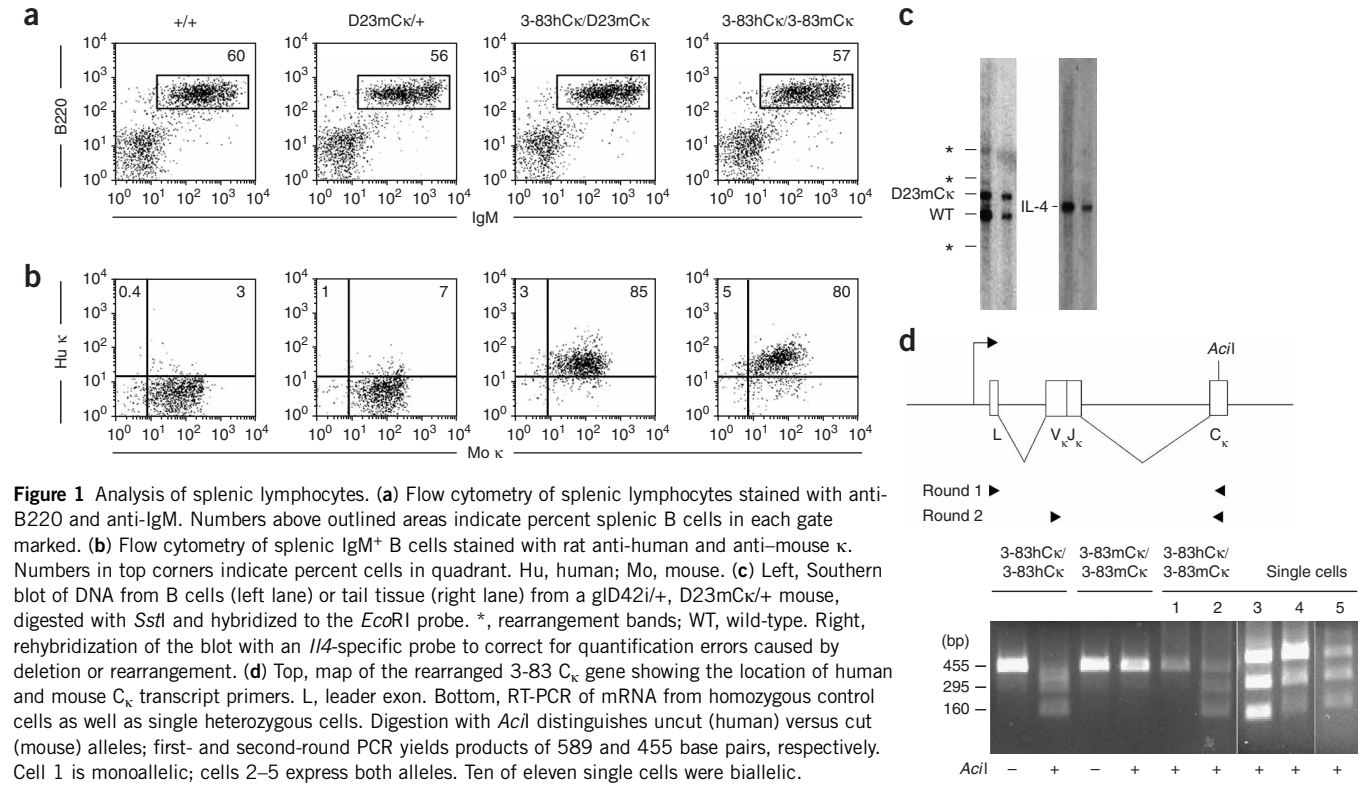

Figure 1 Analysis of splenic lymphocytes. (**a**) Flow cytometry of splenic lymphocytes stained with anti-B220 and anti-IgM. Numbers above outlined areas indicate percent splenic B cells in each gate marked. (**b**) Flow cytometry of splenic IgM$^+$ B cells stained with rat anti-human and anti–mouse κ. Numbers in top corners indicate percent cells in quadrant. Hu, human; Mo, mouse. (**c**) Left, Southern blot of DNA from B cells (left lane) or tail tissue (right lane) from a gID42i/+, D23mCκ/+ mouse, digested with *Sst*I and hybridized to the *Eco*RI probe. *, rearrangement bands; WT, wild-type. Right, rehybridization of the blot with an *Il4*-specific probe to correct for quantification errors caused by deletion or rearrangement. (**d**) Top, map of the rearranged 3-83 C_κ gene showing the location of human and mouse C_κ transcript primers. L, leader exon. Bottom, RT-PCR of mRNA from homozygous control cells as well as single heterozygous cells. Digestion with *Aci*I distinguishes uncut (human) versus cut (mouse) alleles; first- and second-round PCR yields products of 589 and 455 base pairs, respectively. Cell 1 is monoallelic; cells 2–5 express both alleles. Ten of eleven single cells were biallelic.

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RESULTS

Introduction of human C_κ into a pre-rearranged $V_\kappa J_\kappa$ allele

Our strategy required a mouse with two pre-rearranged *Igk* alleles encoding κ–light chains that could be distinguished by antibodies. To accomplish this, we inserted a human C_κ exon into a previously designed *Igk* locus that already had a pre-rearranged 3-83 $V_\kappa J_\kappa$ gene segment, producing the '3-83hCκ' vector. We transfected embryonic stem cells containing the 3-83 $V_\kappa J_\kappa$ joint[25] on one allele with the newly constructed 3-83hCκ targeting vector (**Supplementary Fig. 1** online). Only the rearranged allele had long stretches of homology to the 3-83hCκ vector and could therefore undergo recombination. We screened 1,400 potentially recombinant colonies that were resistant to the aminoglycoside G418 and the antiviral nucleoside analog ganciclovir and then transiently transfected clones containing the correctly integrated sequence with a plasmid expressing Cre recombinase to produce deletion of the neomycin-resistance gene (**Supplementary Fig. 1**). We injected the successfully targeted embryonic stem cell clones into blastocysts to generate chimeras that produced heterozygous progeny (called '3-83hCκ/+ mice' here) that we subsequently interbred to obtain 3-83hCκ-homozygous mice (called '3-83hCκ/3-83hCκ mice' here). As ascertained by immunoblot (data not shown) and flow cytometry (reported below), B cells from these mice were capable of expressing functional antibodies, and by enzyme-linked immunosorbent assay we also confirmed that these antibodies were secreted normally (**Supplementary Fig. 2** online).

Allelic inclusion in a 'double-targeted' mouse

Because studies have shown that 'double-targeted' heavy-chain loci can express two different μ–heavy chains in the same cell[26], we used a similar strategy to test whether the same is true at the *Igk* locus. As a first step we generated mice with two *Igk* alleles that could be distinguished from each other, and then we crossed 3-83hCκ-homozygous mice with mice homozygous for the corresponding mouse vector (3-83mCκ)[25]. We obtained B cells from the resulting heterozygous '3-83hCκ/3-83mCκ' mice, stained the cells with specific antibodies to human or mouse κ–light chains and analyzed them by flow cytometry. Almost all cells expressed both κ-chains (**Fig. 1**); we confirmed this result with single-cell RT-PCR (**Fig. 1d**).

It could be argued that the double-expression pattern in these 3-83hCκ/3-83mCκ mice represented a special case, in that both alleles encode the same κ-chain specificity. To rule out that possibility, we constructed a second targeted mouse containing a pre-rearranged *Igk* gene with a different specificity (D23)[27,28] (**Supplementary Fig. 1**). We targeted embryonic stem cells with a vector carrying the D23κ V region (**Supplementary Fig. 1**). Of 152 colonies resistant to G418 and ganciclovir, three had undergone homologous recombination. We used two embryonic stem clones to generate mice carrying the targeted allele. We then crossed those mice to the *deleter* strain[29] to excise the neomycin-resistance gene and then mated the mice with 3-83hCκ/3-83hCκ mice to obtain '3-83hCκ/D23mCκ' heterozygous mice. By flow cytometry we found that despite the different specificities, both alleles were still expressed in almost all B cells (**Fig. 1a**,**b**), as shown before[30].

One simple explanation for the lack of allelic exclusion could be that the targeted mice lacked the ability to eliminate *Igk* alleles through rearrangement involving a recombination sequence located about 25 kilobases (kb) 3′ of the C_κ exon. To test whether that was the case, we generated mice expressing a pre-rearranged 3-83 IgH chain in combination with 3-83hCκ (together constituting an antibody recognizing H-2K^k and H-2K^b) on an autoreactive genetic background[31]. As expected[31], almost 25% of B cells from these mice expressed

Table 1 Cellularity and proportion of bone marrow and splenic compartments

	Bone marrow					Spleen			Peritoneal cavity			
	Ly ($\times10^7$)	IgM$^+$ ($\times10^6$)	preB ($\times10^6$)	proB ($\times10^6$)	hκ$^+$mκ$^+$ (%)	Ly ($\times10^7$)	IgM$^+$B220$^+$ ($\times10^7$)	hκ$^+$mκ$^+$ (%)	Ly ($\times10^6$)	hκ$^+$mκ$^+$ (%)	CD5$^+$IgM$^+$ ($\times10^6$)	CD5$^-$IgM$^+$ ($\times10^6$)
+/+	1.1	3.1	2.9	0.4	12	10.3	5.8	7.2	5.3	6.5	1.7	2.0
3-83hCκ/+	0.9	3.5	0.8	0.5	49	5.8	2.5	36	3.5	25	1.0	1.4
D23mCκ/+	0.8	2.9	0.9	0.6	12	7.5	3.6	9	3	11	0.8	1.2
3-83hCκ/3-83hCκ	1.4	5.2	0.75	0.8	97	5.4	2.6	1.2	0.3	ND	0.1	0.1
3-83hCκ/3-83mCκ	0.8	1.8	0.48	4.1	83	11.5	6.3	84	2.5	90	1.0	1.2
3-83hCκ/D23mCκ	1.4	5.3	1.4	1.0	71	6.5	3.0	77	4.5	54	0.4	2.0
B1-8i/+, 3-83hCκ/+	1.2	3.6	0.04	0.4	8	5	1.5	18	3	18	0.15	0.6
B1-8i/+, 3-83hCκ/D23mCκ	4	11	0.1	1.6	97	13	5.7	91	0.6	84	0.2	1.2

Bone marrow was isolated from two femurs; cells were counted and were stained for CD43, B220 and IgM. Numbers are based on the total number of lymphocytes (Ly). Percentages of 'double-producer' cells were calculated from IgM$^+$ cells in the bone marrow and from B220$^+$ cells in the spleen. The B1-8 heavy chain (B1-8i) represents an 'innocuous' specificity in combination with both the 3-83 (ref. 31) and the D23 (ref. 28) Igκ light chains. preB, pre–B cell; proB, pro–B cell; hκ, human κ-chain; mκ, mouse κ-chain; ND, not done. At least four mice were analyzed in each group.

λ-chains, and essentially all cells were negative for the 3-83 idiotype (data not shown). As a further test, we also bred mice of the D23 line with mice expressing a pre-rearranged heavy-chain gene (glD42) encoding an antibody to DNA[32]. Once again, λ-chains were produced by about 30% of the cells (data not shown), and Southern blot analysis confirmed that the initial D23 rearrangement was destroyed by RAG-mediated recombination (**Fig. 1c**).

We next sought to determine whether the absence of κ-chain allelic exclusion had an effect on B cell development. For this, we analyzed by flow cytometry bone marrow and splenic cells from the 'double-knock-in' mice. These mice had a normal B cell subset distribution (**Table 1**). The only deviations from that pattern were that they had 25% more immature cells in the spleen and a smaller pre–B cell fraction in bone marrow; those characteristics were further exaggerated in mice with a pre-rearranged B1-8 heavy chain (**Table 1** and **Supplementary Fig. 3** online). The last change was indicative of accelerated B cell maturation, probably due to the presence of functional pre-rearranged light chain genes at the pre–B cell stage of development[25].

Methylation analysis of mice with pre-rearranged *Igk*

During B cell development, κ-chain genes undergo monoallelic demethylation before $V_\kappa J_\kappa$ rearrangement. Germline *Igk* alleles in B cells retain their methylated state[4]. With that in mind, we analyzed the DNA-methylation patterns of the two *Igk* alleles in 3-83hCκ/3-83mCκ mice. We obtained DNA from B cells expressing both human and mouse κ-light chains and first digested the DNA with *Hin*dIII to visualize the 3-83hCκ (2.0-kb) and 3-83mCκ (3.0-kb) alleles and then tested for methylation in the J_κ region with the methylation-sensitive restriction enzyme *Hha*I[4]. Each allele was about 50% methylated, and this was true for B cells expressing 3-83mC_κ and 3-83hC_κ from 3-83hCκ/D23mCκ mice as well (**Fig. 2a**,**b**). We obtained similar results with bisulfite analysis of the V_κ region (**Fig. 2c**). These experiments demonstrate that as in normal B cells, demethylation in these cells takes place monoallelically and independently of rearrangement[3,4].

Notably, almost all allelically included cells expressed the mouse and human alleles in similar amounts, as demonstrated both by flow cytometry and single-cell RT-PCR (**Fig. 1**). It thus seemed that the presence of DNA methylation in the $J_\kappa V_\kappa$ region did not substantially

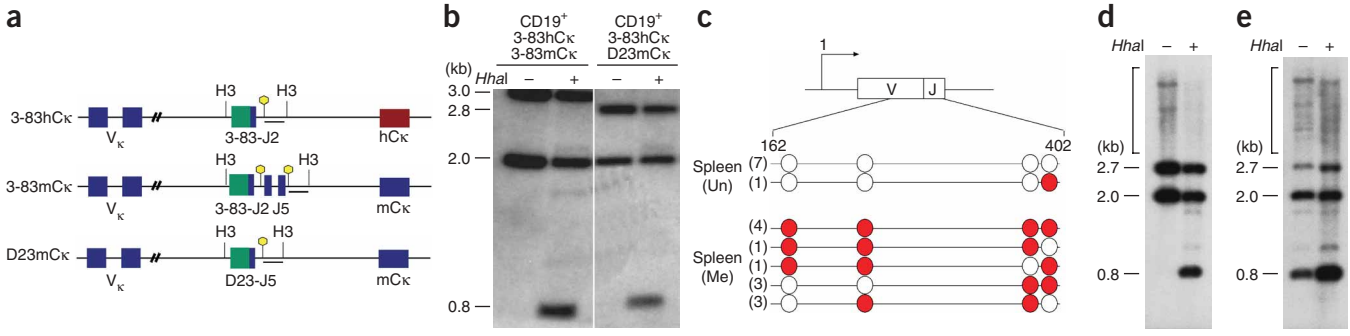

Figure 2 Methylation analyses of 'double-targeted' mice. (**a**) Partial restriction map of the targeted alleles showing probes used for Southern blot (underlined). H3, *Hin*dIII site; open hexagons, *Hha*I sites. (**b**) Methylation analysis of DNA from CD19$^+$ splenic cells derived from 3-83mCκ/3-83hCκ and D23mCκ/3-83hCκ mice, digested with *Hin*dIII in the presence (+) or absence (–) of *Hha*I (to assess methylation). (**c**) Sequencing of bisulfite-treated molecules from the 3-83 V region containing four CpG residues. Filled circles indicate methylation; numbers in parentheses (left margin) indicate number of each molecule type. Un, unmethylated; Me, methylated; 162 and 402, location of product relative to transcription start site (1). (**d**) Methylation analysis as described in **b** of DNA from splenic B cells expressing the 3-83hCκ allele, sorted from 3-83hCκ/+ mice. Left margin: 2.7 kb, mouse germline allele; 2.0 kb, 'knocked-in' 3-83hCκ allele. Both lanes stained similarly with ethidium bromide. (**e**) Methylation analysis as described in **b** of DNA from splenic B cells expressing both 3-83hCκ and 3-83mCκ, sorted by flow cytometry from 3-83hCκ/+ mice. The 0.8-kb band in the '+' lane is derived from unmethylated rearranged mouse molecules; the 0.8-kb band in the '–' lane is nonspecific. Smears (brackets, **d**,**e**) indicate endogenous $V_\kappa J_\kappa$ rearrangements.

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Table 2 Analysis of SHM in rearranged V3-83Jκ2 genes

Cell	Segment	Clones[a]	Mutation frequency[b]	Mutations per 10^3 bases
GC	3-83-J_2	27	0.64 (70/10,989)	6.4
GC-C	3-83-J_2	28	0.17 (19/11,396)	1.7
T	3-83-J_2	35	0.03 (4/14,245)	0.3
T-C	3-83-J_2	20	0.08 (7/8,140)	0.8

Germinal center B cells from Peyer's patches of 3-83mCκ 'knock-in' mice were isolated by flow cytometry sorting for $B220^+PNA^{hi}$ cells; genomic DNA from the germinal center B cells and from tail tissue was cut with *Hha*I (GC-C and T-C, respectively) or was left uncut (GC and T, respectively) and then was amplified with $V_κ$ 5′ primers and 3′ primers located downstream of $J_κ4$.
[a]Total number of PCR clones sequenced (each clone contains 407 base pairs). [b]Percent mutation frequency (with number of mutations/total number of base pairs sequenced in parentheses).

affect transcription. To confirm that idea, we sorted B cells from 3-83hCκ/+ heterozygous mice and selected those that stained positive only for the 'humanized' κ-chain. At least half of the 3-83hCκ alleles were methylated (**Fig. 2d**), even though flow cytometry showed a single uniform population of cells expressing human κ-chain. Had the presence of methylated DNA inhibited transcription, we would have expected a bimodal staining pattern. These experiments thus suggest that in cells containing two rearranged *Igk* genes, both alleles were transcribed to a similar degree. They also demonstrate that DNA methylation does not affect transcription of the rearranged *Igk* alleles to an appreciable extent, consistent with published observations showing that transcription in the immune system can also take place on methylated DNA[33–35].

The existence of a prearranged 3-83Cκ allele 'marked' with a human C region provided a unique opportunity to also test the hypothesis that rearrangement is initially targeted to only one allele in each cell. We sorted B cells from 3-83hCκ/+ mice to select those expressing both the mouse and the 'humanized' *Igk* alleles (about 30–35% of the total, as the 3-83 allele does not cause complete allelic exclusion)[25] (**Table 1**). We assumed that in those selected cells, the endogenous mouse germline *Igk* allele must have undergone demethylation and subsequent rearrangement. In keeping with that idea,

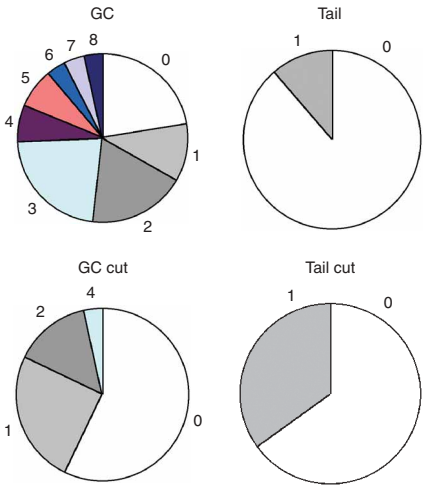

Figure 3 Mutation analysis. Analysis of somatic mutations in DNA from germinal center cells before (GC) and after (GC cut) digestion with *Hha*I. Right, DNA obtained from tail tissue before (Tail) and after (Tail cut) digestion with *Hha*I serves as a control. Numbers outside wedges indicate the number of mutations in the $V_κJ_κ$ segment for that fraction of clones.

Southern blot analysis indeed showed an absence of mouse germline *Igk* alleles in these cells, whereas the 3-83hCκ pre-rearranged allele (1.9 kb) was intact and was completely methylated (**Fig. 2e**). Thus, despite active demethylation in the cells, the 3-83hCκ allele remained fully methylated. This result demonstrates that demethylation is a controlled process initiated on only a single allele in each cell. As noted above, this demethylation was required only for rearrangement (not expression), as the modified human allele was still efficiently expressed in the same cells.

Somatic hypermutation

Because both the 3-83hCκ and 3-83mCκ alleles were transcribed to about the same extent in splenic B cells of double-targeted mice, we were able to assess the relationship between DNA methylation and SHM. We dissected Peyer's patches from mice with pre-rearranged Igκ genes on both alleles and isolated germinal center B cells by flow cytometry on the basis of their $B220^+PNA^{hi}FAS^{hi}$ phenotype. Undigested or *Hha*I-restricted DNA from these cells served as substrates for amplification of the 3-83 $V_κ$ region with one $V_κ$-specific primer together with a J-$C_κ$ intron primer located 3′ of $J_κ4$. These primers specifically amplify the 3-83mCκ allele; thus, in this way we avoided comparing the mutation load in two different alleles (human and mouse). We then cloned and sequenced the amplified DNA. PCR products from the *Hha*I-digested DNA were derived exclusively from methylated molecules, whereas undigested DNA yielded products from both methylated and unmethylated copies of this region. DNA obtained from tail tissue of these same mice served as a control for the background error rate inherent in the PCR and cloning procedures (**Table 2**).

As expected, we readily detected mutations in PCR products from uncut DNA from Peyer's patch germinal center B cells. In contrast,

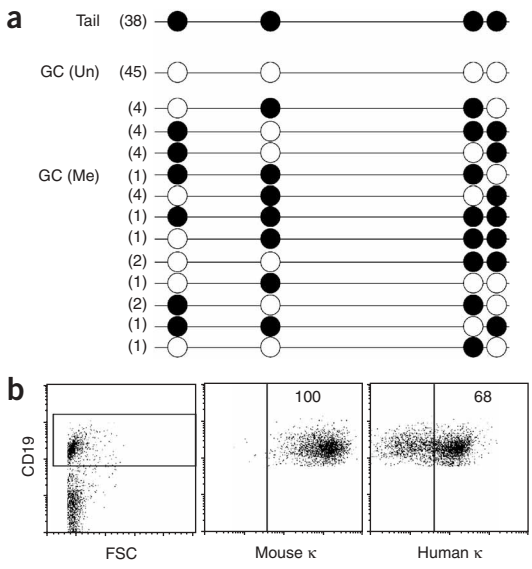

Figure 4 DNA-methylation and expression analyses of Peyer's patch B cells. (**a**) Sequencing of bisulfite-treated molecules from the 3-83 V region containing four CpG residues. Filled circles indicate methylation; numbers in parentheses (left margin) indicate the number of each type of molecule from germinal centers (GC) or DNA obtained from tail tissue (Tail). (**b**) Flow cytometry of Peyer's patch cells from 3-83hCκ/3-83mCκ mice. $CD19^+PNA^{hi}Fas^{hi}$ germinal center cells were stained with rat antibody to human or mouse κ-chain. Numbers in plots indicate percent positively stained cells in the outlined area. FSC, forward scatter.

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Table 3 Single-cell RT-PCR analysis

B cell	Spleen	Germinal center
Monoalllelic	1 (1 mouse)	3 (2 human, 1 mouse)
Biallelic	10	10
Total cells analyzed	11	13

Single-cell RT-PCR analysis of 3-83 $V_\kappa J_\kappa$ rearranged κ-chain transcripts in Peyer's patch and splenic B cells isolated from 3-83hCκ/3-83mCκ mice; PCR products were digested with *Aci*I and were separated by 2% agarose gel electrophoresis.

Table 4 Single-cell mutation analysis

Cell	Allele 1	Allele 2	*P* value
D1	10	5	1.5×10^{-1}
E1	8	1	2.0×10^{-2}
E7	15	1	2.6×10^{-4}
F6	14	0	6.1×10^{-5}
G7	23	0	1.2×10^{-7}
I2	3	3	–
L2	3	2	–
L8	13	0	1.2×10^{-4}
M2	12	0	2.4×10^{-4}
M5	21	0	4.8×10^{-7}
M6	27	1	1.1×10^{-7}
N5	17	0	7.6×10^{-6}
N8	10	0	9.8×10^{-4}
K1	4	1	1.9×10^{-1}
R1	36	4	9.3×10^{-8}

Mutation analysis of germinal center cells obtained from three different 3-83hCκ/3-83mCκ or 3-83mCκ/3-83mCκ mice by sorting and assessed by single-cell RT-PCR (453 base pairs) and mutation analysis in the VJ region (240 base pairs). Sequencing results from both alleles occurred with 32 cells, and those with the most total mutations are presented. Cells ($n = 17$) with less than a total of 5 mutations are not shown, as such a relatively low mutation count is not sufficient for statistical analysis comparing the two alleles. For each cell, hypergeometric analysis was used to calculate the probability (*P* value) of randomly obtaining this degree of skewing (or more) between the two alleles in single cells; the probability of randomly picking this combination of 13 of 15 cells with a *P* value of less than 0.02 is low ($P < 10^{-7}$), and the probability of finding 9 of 15 cells with a *P* value of less than 10^{-3} is much lower ($P < 10^{-30}$).

however, the frequency of somatic mutations was much lower for methylated molecules (**Fig. 3** and **Supplementary Table 1** online). Sequence analysis of products originating from undigested DNA, representing both methylated and unmethylated alleles, showed that undigested DNA had a high frequency of mutations per base (6.4×10^{-3}), whereas methylated DNA underwent many fewer mutations per base (1.7×10^{-3}). After taking into consideration the average background frequency of mutations in PCR products of DNA obtained from tail tissue, we calculated that unmethylated DNA was tenfold more likely to undergo somatic mutations than methylated copies in germinal center cells.

To confirm those results and to obtain a better idea of the V_κ DNA-methylation pattern in germinal centers themselves, we treated DNA with bisulfite and then cloned individual molecules for sequencing analysis. About 40% of the *Igk* alleles were methylated in germinal centers, slightly less than in splenic B cells (**Fig. 4a**). Once again, we noted far fewer mutations on methylated molecules (**Supplementary Fig. 4** online). This may not have been an exclusive effect of DNA methylation but instead may have reflected overall epigenetic differences between the two alleles (**Supplementary Fig. 5** online).

As local RNA synthesis is a prerequisite of SHM, we wanted to be sure that both 3-83κ alleles indeed remained transcriptionally active in germinal centers as they were in splenic B cells. For this, we purified germinal center cells with specific antibodies to human or mouse κ–light chains and analyzed the cells by flow cytometry. Over 60% of the cells expressed both κ-chains (**Fig. 4b**); we confirmed this result by single-cell RT-PCR of a small sample of individual germinal center cells (**Table 3**). These data were consistent with the observation that many germinal center cells undergo random inactivation of one of the two alleles because of active mutagenesis that takes place during the affinity maturation process. Thus, both flow cytometry and single-cell RT-PCR showed that most Peyer's patches B cells expressed both rearranged 3-83 $V_\kappa J_\kappa$ alleles.

Although those findings indicated that methylated *Igk* genes are a poor substrate for SHM, it was still possible that some of the methylated molecules were derived from germinal center cells that had not yet initiated the mutation process. To demonstrate definitively that SHM actually occurs 'preferentially' on only one allele in actively mutating cells, we isolated single cells from germinal centers and determined the number of mutations on each individual allele by amplifying and sequencing κ-chain RNA products. Many individual cells had highly skewed patterns with an excessive number of mutations present on a single allele (**Table 4**). As the probability of so many cells having this degree of asymmetry simply by chance was extremely low ($P < 10^{-30}$), these data indicated that one allele in each cell was indeed protected from SHM even when that same cell accumulated a relatively large number of total mutations. In these experiments, single allele sequences were always derived from the RNA products of expressed *Igk* genes, thus providing additional support for the idea that this *cis* effect was not due to differences in transcription. The results reported above collectively showed that the process of SHM at the *Igk* locus was inhibited on the methylated allele even though it seemed to be expressed in a way similar to the unmethylated allele. Such a result is consistent with studies showing that whereas an unrearranged methylated allele is not subject to SHM *in vivo*, nonproductive rearranged alleles that have undergone demethylation undergo SHM to a similar degree as a productive allele in the same cell[15].

DISCUSSION

Allelic exclusion in the lymphoid system is achieved through a series of epigenetic events that ensure that only one allele in each B cell or T cell undergoes productive rearrangement. Here we sought to determine whether such a process of allelic 'choice' represents a more general mechanism for distinguishing the two alleles and thus 'preferentially' limiting molecular changes at the DNA level to one of the alleles. In particular, our experiments were aimed at determining if SHM is also subject to this type of regulation. To test that hypothesis, we created a mouse targeted with two nearly identical pre-rearranged *Igk* alleles. In such mice, allelic exclusion is bypassed, thus allowing the examination of 'downstream' alterations, such as SHM, in the DNA coding sequence.

Our studies have definitively demonstrated that in mice carrying two pre-rearranged *Igk* loci, almost all B cells were able to express both alleles simultaneously. That finding is consistent with published experiments showing that two pre-rearranged alleles can also be expressed in a single B cell[26,30]. Those observations collectively provide evidence that allelic exclusion of immune receptor molecules is mainly controlled at the level of rearrangement. Furthermore, this regulatory process must operate at high efficiency, as 'double-expressing' cells are rare in normal mice[36]. Our studies have also demonstrated that cells expressing two different *Igk* alleles developed and matured normally, with only slightly accelerated differentiation in the bone marrow. Thus, although allelic exclusion may be important in optimizing

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antibody selection, the exclusive expression of a single allele per cell is not a prerequisite for B cell development.

The productive rearrangement and expression of a single allele in each cell (allelic exclusion) is preceded by other monoallelic changes at the molecular level. Before lymphoid development in the early embryo, loci encoding immune receptors become asynchronously replicating in each cell and in this way acquire an allele-specific 'mark'[1]. That different structure is then used in lymphoid cells to direct epigenetic changes that cause one allele to undergo nuclear repositioning[2], demethylation[4] and chromatin opening[3], thus making it a 'preferred' substrate for the initial rearrangement event. Our experiments have shown that even in the absence of active rearrangement, endogenous *Igk* alleles in the 3-83hCκ/3-83mCκ targeted mouse still replicated asynchronously[1] and underwent monoallelic demethylation, indicating that they were still subject to this epigenetic 'marking' process.

Despite a distinct difference in V_κ-J_κ DNA methylation for the two *Igk* alleles in each B cell, both alleles seem to express the 3-83hCκ and 3-83mCκ products with equal efficiency. Indeed, flow cytometry and single-cell RT-PCR indicated that each protein was uniformly expressed to a similar extent in all B cells. That finding, along with published reports[34], suggests that methylation does not greatly influence the amount of rearranged gene transcription. Such a result may be due to the fact that the promoter of the 3-83 V region has very few CpG residues, with only one present over a 500–base pair stretch of upstream sequence. Overall, our data strengthen the idea that DNA-methylation changes in the V_κ-J_κ region are 'programmed' to control recombination rather than transcription.

Our studies have indicated that like rearrangement in normal B lymphocytes, SHM occurs 'preferentially' (with a tenfold higher frequency) on the unmethylated allele. As both alleles are essentially equivalent in terms of DNA sequence, this effect cannot be due to differences in *cis*-acting elements or *trans*-acting factors. Furthermore, expression analysis demonstrated that this skewing was probably not a result of allele-specific transcription, a finding consistent with a study showing that high rates of transcription are not sufficient for robust SHM[37]. These studies collectively suggest that allele-specific epigenetic differences generated during B cell development influence SHM; this hypothesis is consistent with the observation that both demethylation and SHM are regulated by the same combination of enhancers[4,23,24]. It is likely that this mechanism also contributes to the inhibition of SHM on nonrearranged methylated *Igk* alleles in normal B cells[17,38].

A series of studies has demonstrated that SHM is accomplished by AID, which apparently acts as a DNA-deaminase enzyme[10–12]. Thus, one possibility to explain monoallelic SHM is that DNA methylation itself directly inhibits the action of AID. To test that hypothesis, we developed an *in vitro* AID assay with a DNA fragment containing a single methylated CpG and two flanking cytosine residues. This experiment demonstrated that although methylated CpG itself is not a good substrate for AID-mediated deamination[12], it does not inhibit this reaction on local cytosine residues; similar results have been reported in other studies[39]. It thus seems that whereas DNA methylation is involved in preventing SHM, it probably works indirectly by altering histone modification or other aspects of chromatin structure, thereby restricting accessibility to the SHM machinery. These pre-SHM events should be distinguished from alterations in histone modification that occur as part of the hypermutation reaction and may actually be dependent on AID itself[21,22].

DNA methylation is involved in the maintenance of genome integrity by inhibiting DNA damage[40,41], and such modification seems to be especially critical in the immune system, in which sequence alteration represents an inherent aspect of differentiated cell function. In keeping with that idea, it seems that demethylation of the *Igk* locus is tightly regulated in a way that is not only spatially localized to a specific gene region but is also restricted to a single allele. Such a mechanism could be part of a more general strategy aimed at limiting somatic mutations to select target sequences, thereby preventing unnecessary genomic damage.

In deciphering the mechanisms involved in SHM restriction, the idea that undermethylation is only one aspect of the monoallelic marking system must be taken into consideration. Before SHM in activated B cells, the *Igk* locus has already undergone monoallelic changes in nuclear localization and DNase I sensitivity in addition to DNA methylation[42]. It is likely that this selection process is actually directed by recognition of allele replication asynchrony, a key epigenetic 'mark' that is established in each cell early in development. Any or all of these mechanisms could be responsible for 'marking' one allele in each cell to be the 'preferential' target for SHM. It thus seems that epigenetic factors not only direct allele-specific V(D)J rearrangement but also can persist to influence later events (such as SHM) that ultimately affect gene diversity.

METHODS

Target vectors and the generation of 'knock-in' mice. For the generation of mice expressing human C_κ, the 3-83hCκ targeting vector was constructed. It contains the pre-rearranged 3-83V_κ J_κ2 (ref. 25) and human C_κ sequences. Embryonic stem cells containing the pre-rearranged 3-83$V_\kappa J_\kappa$ genes were transfected with the 3-83hCκ targeting vector to obtain a 'humanized' pre-rearranged 3-83 $V_\kappa J_\kappa$ allele. For the generation of mice expressing the D23κ light chain (D23κi mice; called 'D23mCκ' here), the κ23T vector, based on the shuttle vector pVKR2neo[25], was used. The κ23T vector contains the D23V_κ-J_κ5 rearranged sequence[27] driven by its endogenous promoter[28]. Embryonic stem cells were cultured, were transfected with the targeting vectors and were analyzed, and the appropriate clones were injected into blastocysts as described[43]. Expression of the two 'knock-in' alleles was assessed by immunoblot, enzyme-linked immunosorbent assay (data not shown) and flow cytometry. For analysis of expression of the κ–light chain from the D23mCκ allele, a D23mCκ/D23mCκ mouse was also crossed with a mouse from which the mC_κ sequence was deleted[44]. Analysis of the resultant D23mCκ/CκT mice, which produced κ-chains only from the D23mCκ allele, showed that D23κ was expressed efficiently, similar to expression of κ-chains in wild-type mice (**Supplementary Fig. 6** online).

Mice. All animals were kept in a conventional animal facility at the University of Cologne (Germany) or at the Hadassah Medical School (Jerusalem, Israel). Unless specified otherwise, all experiments used mice 8–12 weeks old. The genotype of transgenic mice was determined by PCR and by Southern blot. For amplification of the D23mCκ (D23κi) allele, primers Jκ5A and D23κL were used. The expected product was 0.7 kb. The 3-83hCκ mice were genotyped by Southern blot. All mice were maintained in the specific pathogen–free unit of Hadassah Medical School according to guidelines of the ethics committee and in keeping with National Institutes of Health guidelines.

Antibodies. Fluorescence staining was done as described[45]. Antibodies were prepared and conjugated to fluorescein isothiocyanate, phycoerythrin, allophycocyanin or biotin in the laboratory in Cologne except where a manufacturer is specified. Biotinylated antibodies were developed with streptavidin conjugated to CyChrome (BD PharMingen). Stained cells were analyzed on a FACScan (Becton Dickinson) or on a FACSCalibur (Becton Dickinson). Cells were sorted on a FACStar (Becton Dickinson). Antibodies used in this study included antibody to B220 (anti-B220; RA3-6B2), anti-IgM (R33-24.12), anti-CD43 (S7; BD PharMingen), anti–mouse κ (R33-18.10), anti-CD21/35 (7G6; BD PharMingen), anti-CD23 (B3B4; BD PharMingen), anti-493 (ref. 46), anti-IgD (1.3-5), anti-CD24 (heat-stable antigen; 30-F1; BD PharMingen), anti–human κ (LO-hK-3; Biosource), anti–human κ (goat serum; Southern Biotechnology Associates) and biotinylated anti-CD19 (ID3; PharMingen).

Cell sorting and purification. Cell suspensions from the spleen were prepared by disruption in PBS, followed by gentle pipetting and centrifugation through PBS. Samples were enriched for $CD19^+$ cells by staining with biotinylated anti-CD19 followed by incubation with streptavidin-coated magnetic beads or by anti-CD19 directly coupled to magnetic beads (Miltenyi Biotec). Cells were sorted by magnetic cell sorting with the MACS system[47] (Miltenyi Biotec). Cells bound to the column in the magnetic field were eluted and were recovered for subsequent DNA isolation and analysis. Enrichment (over 90%) was assessed by flow cytometry.

Methylation assays. DNA-methylation patterns were assessed as described before[4]. Genomic DNA (5–10 μg) was left undigested or was digested with *Hha*I, a methyl-sensitive enzyme (NEB). Digested DNA was separated by electrophoresis through agarose gels, was transferred to nitrocellulose filters, was hybridized for 16 h at 65 °C to specific radioactive probes and was analyzed by autoradiography. The degree of undermethylation was assessed with a PhosphorImager (Fujifilm BAS-1800).

Bisulfite analysis. DNA isolated from Peyer's patches germinal centers, spleen or tail tissue was treated with bisulfite[48], and the 3-83 V region was then amplified specifically with nested primers and purified by gel extraction (Qiagen), and individual molecules were isolated by TA cloning (Invitrogen) and were sequenced.

SHM analysis. SHM was analyzed as described[23]. Peyer's patches were dissected from the small intestines of 5- to 6-month-old mice and single-cell suspensions were prepared. The cells were double-stained with fluorescein isothiocyanate–conjugated peanut agglutinin (PNA; Sigma) and phycoerythrin-conjugated anti-B220. $B220^+PNA^{hi}$ cells were fractionated by flow cytometry and DNA was prepared as described[23]. Genomic DNA (50–100 ng) was left undigested or was digested for 16 h at 37 °C with *Hha*I (20 U). The Expanded High Fidelity PCR system (Roche) and standard conditions (50 mM KCl, 0.1% (vol/vol) Triton X-100, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, 200 mM of each of four dNTPs and 300 ng of each primer) were used for PCR. First-round PCR (50 μl total volume) used primers P5′SM, in the 3-83 V region, and P170-2, 3′ to $J_κ4$. Amplification was done as follows: 3 min at 94 °C; 35 cycles of 20 s at 94 °C, 20 s at 60 °C and 30 s at 72 °C; and 3 min at 72 °C. Second-round PCR conditions were identical to those of the first round except that 2 μl of the first-round amplification product was added to the PCR mixture containing primers PSM-5 and PSM-3M. The second-round products were purified by gel extraction (Qiagen) and were cloned with the TA Cloning System (TOPO; Invitrogen), followed by sequencing with reverse and forward primers (Applied Biosystems). Sequence alignment with the CLUSTAL_X program[49] allowed the identification of mutations from the consensus 3-83 V region of each clone. The mutation frequency was measured for total versus methylated DNA from Peyer's patches (6.4×10^{-3} versus 1.7×10^{-3} mutations per base, respectively). For calculation of the difference between methylated and unmethylated molecules, the average background mutation for DNA obtained from tail tissue (0.55×10^{-3} mutations per base) was first subtracted, and then the frequency of mutations in unmethylated molecules was calculated based on the assumption that 50% of the κ-alleles were methylated and resistant to digestion by restriction enzyme. This method showed that mutations were nine times more frequent on unmethylated molecules than on methylated molecules (5.85 – 0.575 / 0.575). Bisulfite analysis showed a mutation frequency of 3.9×10^{-3} mutations per base for methylated molecules and 0.8×10^{-3} mutations per base for unmethylated molecules from germinal centers, whereas methylated DNA obtained from tail tissue had a background of 0.6×10^{-3} mutations per base.

Single cell RT-PCR for 3-83hCκ/3-83mCκ mice. Splenic and Peyer's patches germinal center B cells were stained with fluorescein isothiocyanate–conjugated rat monoclonal anti–mouse B220 (RA3–6B2; BD Biosciences) or with a combination of allophycocyanin-conjugated anti-mouse CD19 (BD Pharmingen), fluorescein isothiocyanate–conjugated anti-PNA (Vector Laboratories) and phycoerythrin-conjugated anti-Fas (BD Pharmingen). Individual splenic $B220^+$ cells and germinal center $CD19^+PNA^{hi}Fas^{hi}$ cells were sorted by flow cytometry directly into 12.5 μl reverse-transcription buffer (1× RT buffer; Promega), 20 U Rnasin (Promega) and 100 pmol pd(N)6 Random Hexamer (Pharmacia Biotech) in individual PCR tubes. The tubes were incubated for 1 min at 65 °C, then for 3 min at 22 °C, and then were placed on ice. AMV reverse transcriptase (150 U; Promega) was added. The tubes were incubated for 3 min at 22 °C, followed by the reverse-transcription reaction for 50 min at 42 °C. Standard conditions and ABgene Red Hot (1× PCR buffer from ABgene, 2.5 mM $MgCl_2$, 1 mM of each of all four dNTPs and 0.2 μg each of each primer) were used for nested PCR.

For each round of PCR, one V 3-83–specific primer (first PCR, L2; second PCR, L133) and two complementary human-mouse C-region primers (mixed at a ratio of 1:1; first PCR, human hR1 and mouse mR1; second PCR, human hR2 and mouse mR2) were used. The total volume of 30 μl included 3 μl cDNA or 3 μl of the first-round PCR product as the template for the first or second round of PCR, respectively. Amplification conditions were as follows: 5 min at 94 °C; 45 cycles of 35 s at 94 °C, 40 s at 58 °C and 1 min at 72 °C; and 5 min at 72 °C. The second-round PCR product was digested with *Aci*I (R0551S; New England Biolabs) and was analyzed by agarose gel electrophoresis.

Single-cell mRNA mutation analysis. RT-PCR products of single germinal center cells derived from three 3-83hCκ/3-83mCκ or 3-83mCκ/3-83mCκ adult mice (over 1 year of age) were obtained as described. PCR products were analyzed by agarose gel electrophoresis followed by gel extraction (Qiagen). For 3-83hCκ/3-83mCκ cells, allele-specific primers were used to amplify the human and mouse products separately, which were directly sequenced. In 3-83mCκ/3-83mCκ cells, five individual molecules were isolated by TA cloning (Promega) and were sequenced. The two alleles were distinguished by their mutation profiles. Statistical significance was determined by hypergeometric analysis.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/natureimmunology/.

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