

Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation

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Background: Individuals with genetic defects in CD40 ligand (CD40L) or B-cell antigen receptor coreceptor molecules CD19 and CD81 suffer from an antibody deficiency. Still, these patients carry low levels of memory B cells and serum antibodies.

Objective: We sought to assess why the remaining memory B cells and antibodies in the blood of these patients do not provide functional immunity.

Methods: We included CD19-deficient patients (n = 8), CD40L-deficient patients (n = 8), and healthy controls (n = 50) to perform detailed flow cytometry on blood B cells, molecular analysis of IgA and IgG transcripts, as well as functional analysis of B-cell activation.

Results: CD19-deficient and CD40L-deficient patients carried reduced numbers of all memory B-cell subsets except CD27⁺ IgA⁺ B cells. Their immunoglobulin heavy chain class-switched transcripts contained less somatic mutations and reduced usage of IgM-distal IgG2 and IgA2 subclasses. The selection strength of mutations for antigen binding was significantly lower than in controls, whereas selection to maintain superantigen binding was normal. Furthermore, the patients showed impaired selection against inherently autoreactive properties of their immunoglobulins. Somatic hypermutation analysis revealed decreased activation-induced

cytidine deaminase and uracil-DNA glycosylase 2 activity in CD40L deficiency and increased uracil-DNA glycosylase 2 but decreased mismatch repair in CD19 deficiency. B-cell activation studies revealed that this was at least in part due to transcriptional regulation of DNA repair genes.

Conclusions: This study on CD19 and CD40L deficiencies illustrates that both the B-cell antigen receptor and CD40 signaling pathways are required for the selection of immunoglobulin reactivity. Still, they differentially mediate DNA repair pathways during somatic hypermutation, thereby together shaping the human *in vivo* antigen-experienced B-cell repertoire. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: CD19, CD40L, somatic hypermutation, selection, immunoglobulin, autoreactivity

Circulating B cells each express a unique B-cell antigen receptor (BCR), which has been generated through genomic recombination of their immunoglobulin loci.¹ On recognition of the antigen with their BCR, B cells will initiate an immune response that is characterized by clonal proliferation, and affinity maturation and class switch recombination (CSR) of their immunoglobulin loci. Affinity maturation is accomplished through somatic hypermutation (SHM) in the variable domains of rearranged immunoglobulin heavy (IgH) and immunoglobulin light (Igk or Igl) chains, and selection of individual cells for high-affinity antigen binding.² CSR changes the effector function of the immunoglobulin molecule without affecting the antigen specificity by replacing the IgH chain constant region.³

Both SHM and CSR are genomic processes that are mediated by the activation-induced cytidine deaminase (AID) enzyme (reviewed in Liu and Schatz,⁴ Saribasak and Gearhart,⁵ and Xu et al⁶). The AID enzyme is upregulated in activated B cells and deaminates cytosines, especially in WRCY/RGYW motifs, in highly transcribed genes. The introduced uracil can be replicated over, giving rise to transition mutations: C → T; or G → A. Alternatively, the uracil can be excised by uracil-DNA glycosylase (UNG), an enzyme involved in the base excision repair (BER) pathway, followed by error-prone repair allowing transversion mutations (C → A/G, G → C/T) of the AID-targeted C·G pair. Finally, the U·G mismatch can be processed by mismatch repair (MMR), which is prone to introduce mutations in A and T residues, preferentially in WA/TW motifs. High-density lesions in immunoglobulin switch regions upstream of the constant genes can result in double-stranded DNA breaks, thereby mediating CSR.

In a classical germinal center (GC) response, the AID enzyme is upregulated through signals from the CD40 molecule following binding with its ligand (CD40L) on activated T follicular helper cells.⁷ This interaction is critical for T-cell-dependent immunoglobulin responses: individuals with genetic defects in the

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Abbreviations used

AID:	Activation-induced cytidine deaminase
BCR:	B-cell antigen receptor
BER:	Base excision repair
CDR:	Complementarity determining region
CD40L:	CD40 ligand
CSR:	Class switch recombination
EXO1:	Exonuclease 1
FR:	Framework region
GC:	Germinal center
IgH:	Immunoglobulin heavy chain
MMR:	Mismatch repair
MSH2:	MutS homolog 2
MSH6:	MutS homolog 6
SHM:	Somatic hypermutation
SpA:	<i>Staphylococcus aureus</i> virulence factor protein A
UNG:	Uracil-DNA glycosylase

CD40 or CD40L genes lack GC, have strongly reduced serum IgA and IgG levels, and are susceptible to infections with encapsulated bacteria (reviewed in Durandy et al⁸). Still, these patients have IgA⁺ plasma cells in gut and IgM⁺ and IgA⁺ memory B cells in blood carrying SHM.^{9–11} Likely candidates for the upregulation of the AID enzyme in the absence of CD40 signaling are TACI and Toll-like receptors that can activate nuclear factor- κ B through signaling via MyD88.^{12–14}

Developing and mature B cells depend on tonic BCR signaling through PI3K for their survival.^{15,16} Antigen-induced BCR signaling depends on CD19, which is complexed with CD21, CD81, and CD225 on the membrane of B cells. Genetic defects in *CD19* or *CD81* that impair CD19 protein expression result in an antibody deficiency with strongly reduced serum immunoglobulin levels.^{17–19} Still, naive B-cell survival appears largely normal,^{17,20,21} and CD19-deficient patients have normal appearing GC in lymphoid tissue and their BCR-mediated signaling is not completely abolished.¹⁷

Despite the defects in B-cell activation and the antibody deficiency, patients with mutations resulting in a CD19 deficiency or a CD40L deficiency have memory B cells with molecular signs of immunoglobulin maturation.^{9,17–19,22} We here studied memory B cells from these patients as a human *in vivo* model to dissect the roles for BCR and CD40 signaling in immunoglobulin maturation and selection. We found that defective BCR and CD40 signaling both resulted in impaired SHM, CSR, and immunoglobulin repertoire selection, but differentially affected SHM targeting. Thus, these 2 signals are required for optimal antibody responses and selection against inherent autoreactivity.

METHODS**Patients**

Eight CD19-deficient and 8 CD40L-deficient patients suffering from an antibody deficiency were included in this study (see Table E1 in this article's Online Repository at www.jacionline.org). Seven CD19-deficient patients had biallelic genetic defects in the *CD19* gene, and 1 patient had a homozygous mutation in the *CD81* gene.^{17–19,23,24} Eight male patients suffered from a hyper-IgM syndrome because of the absence of CD40L expression on activated T cells and carried hemizygous mutations in their *CD40L* genes.⁹

Diagnostic workup of blood samples was carried out after oral informed consent and according to the guidelines of the local medical ethics committees in Belgium, Colombia, Japan, Turkey, Poland, and The Netherlands. Controls provided written consent.

Flow cytometry and Ca²⁺ flux analysis

6-Color flowcytometric immunophenotyping of blood B cells was performed for 4 CD19-deficient patients, 5 CD40L-deficient patients, and 50 age-matched healthy controls to quantify 6 memory B cells on a fluorescence-activated cell sorting LSRII.⁹

Ca²⁺ influxes on BCR stimulation were measured in CD20⁺ B cells by using flow cytometry as described before, following stimulation with *Staphylococcus aureus* Cowan I (Calbiochem, La Jolla, Calif) diluted 1:500, or with 20 μ g/mL goat antihuman IgM-F(ab')₂ (Jackson Immunoresearch Laboratories, Inc, West Grove, Pa).¹⁷

Amplification and sequence analysis of rearranged IgH transcripts

IgA and IgG transcripts were amplified from the cDNA of thawed mononuclear cells by using subgroup-specific forward primers in framework region (FR) 1 or the leader sequence of IGHV3 and IGHV4 in combination with a C α or C γ consensus reverse primer.⁹ The *IGHV1-69* genes that were amplified with the IGHV4 primer were included in all analyses, except for IGHV4 subgroup use. All PCR products were cloned into the pGEM-T easy vector (Promega, Madison, Wis) and prepared for sequencing on an ABI PRISM 3130XL (Applied Biosystems, Foster City, Calif). Obtained sequences were analyzed with the IMGT database (<http://www.imgt.org/>).²⁵

Targeting of SHM in the FR and complementarity determining regions (CDRs) was analyzed with the JoinSolver program.²² The selection strength for mutations in the FR and the CDR was determined with the Bayesian estimation of Antigen-driven SElectIoN program (<http://selection.med.yale.edu/baseline/>).²⁶ This program allows assessment of positive selection of mutations in CDRs as well as negative selection of FR mutations. IgG subclasses and IgA were identified by using the germline sequence of the IGH locus (NG_001019).

In vitro activation of purified B cells from human tonsils

Human tonsils were obtained from otherwise healthy children who underwent routine tonsillectomy. Resting B cells were purified by negative selection with CD43 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and subsequently cultured at a density of 1 million cells/mL in 3 mL culture medium with combinations of IL-4 (10 ng/ μ L; R&D Systems, Abingdon, United Kingdom), anti-IgM F(ab')₂, anti-CD40 (Bioceros B.V., Utrecht, The Netherlands), CpG ODN2006 (1 μ g/mL; Miltenyi Biotec), and IL-21 (50 ng/mL; PeproTech, London, United Kingdom). After 72-hour culture, cells were harvested for TaqMan-based quantitative RT-PCR on a StepOnePlus (Applied Biosystems). Intron-spanning primers were designed for each gene by using the universal probe library Assay Design Center (Roche, Basel, Switzerland), and fluorogenic probes were obtained from Roche (see Table E2 in this article's Online Repository at www.jacionline.org). Target gene expression levels were corrected for *ABL* expression levels and expressed as fold difference from uncultured cells.¹⁸ All quantitative RT-PCR reactions were performed in duplicate.

Statistical analyses

Statistical analyses were performed with the Mann-Whitney *U* test, the student paired *t* test, or the χ^2 test as indicated in detail in figure legends. *P* values of less than .05 were considered statistically significant.

RESULTS**Memory B-cell subsets in CD19 and CD40L deficiencies**

To study the extent of impaired humoral immunity in CD19-deficient and CD40L-deficient patients (Table E1), we performed extensive flow cytometric immunophenotyping of their blood B-cell compartments. CD40L-deficient patients showed a nearly complete lack of primary (CD27⁺IgM-only and CD27⁺IgG⁺) and secondary GC-derived (CD27⁺IgG⁺ and CD27⁺IgA⁺) memory B cells, but had clearly detectable CD27⁺IgM⁺IgD⁺ “natural effector” B cells and normal numbers of CD27⁺IgA⁺ B cells (see Fig E1 in this article's Online Repository at

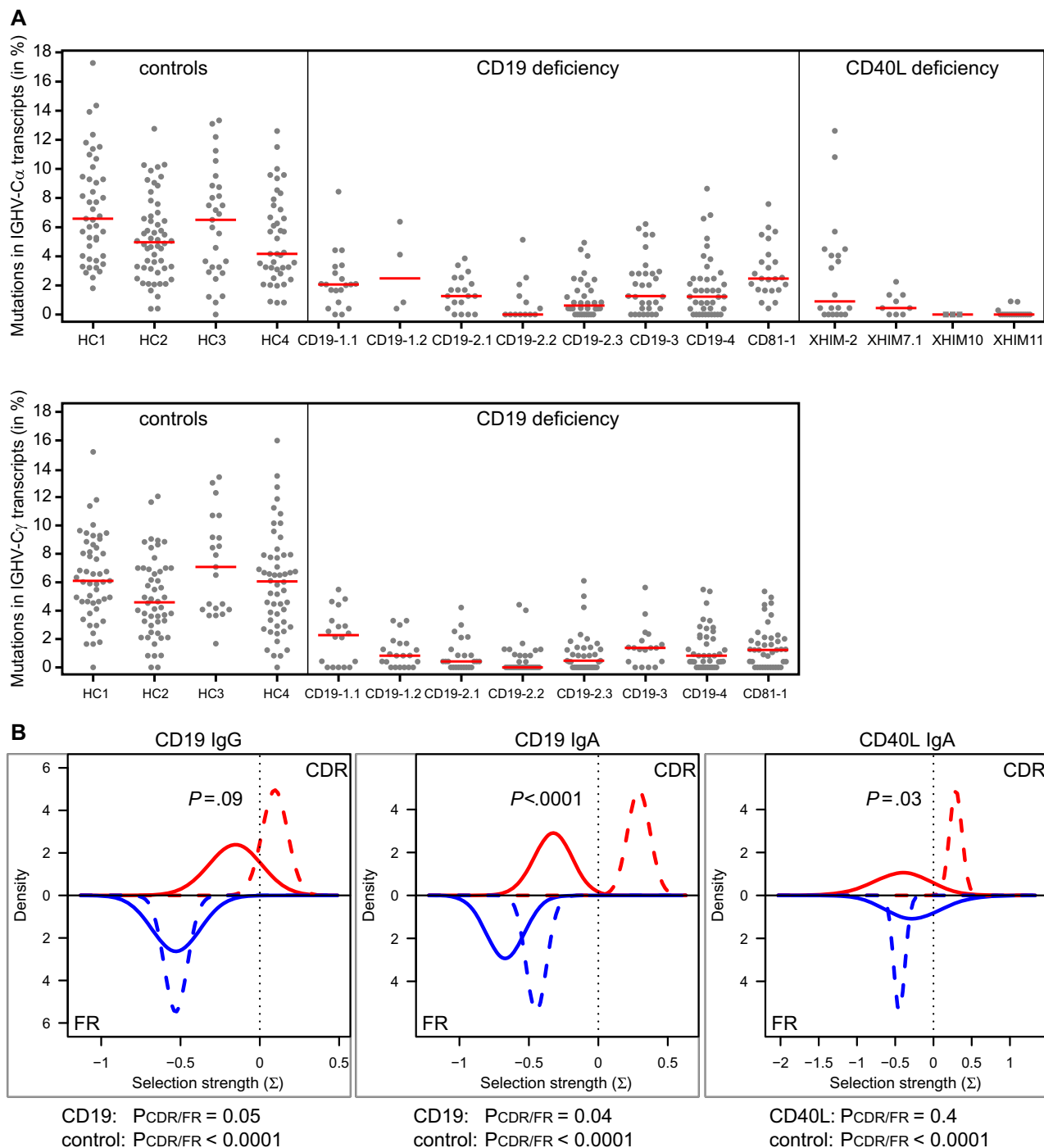


FIG 1. SHM analysis. **A**, Mutation frequencies in *IGHV* genes of $C\alpha$ and $C\gamma$ transcripts of healthy controls (HC) and patients. Gray dots represent unique sequences; red lines represent median values. Ages: HC1 and HC2, 7 years; HC3 and HC4, 22 years. **B**, Selection for replacement mutations in *IGHV*-CDRs (red lines) and *IGHV*-FRs (blue lines) as determined with the Bayesian estimation of Antigen-driven SElectIoN program.¹⁶ Solid lines represent patients; dotted lines represent healthy controls. A selection strength of more than 0 is indicative of positive selection.

www.jacionline.org.⁹ CD19-deficient patients also showed a marked reduction in all memory B cells, except for the $CD27^-IgA^+$ subset (Fig E1, B). Their natural effector B-cell numbers were more severely decreased than in CD40L-deficient patients, whereas low numbers of circulating $CD27^-IgG^+$ and $CD27^+IgA^+$ memory B cells were detectable (Fig E1).

SHM frequencies and patterns

IgA and IgG transcripts using the large *IGHV3* and *IGHV4* subgroups^{27,28} were amplified to study molecular signs of antibody selection. IgG transcripts could not be detected in CD40L-deficient patients. SHM levels in *IGHV* genes of IgA and IgG transcripts were similar between childhood and adult

TABLE I. Targeting and selection of individual mutations in rearranged *IGHV*

	IgA			IgG	
	Control (n = 153)	CD19-deficient (n = 202)	CD40L-deficient (n = 50)	Control (n = 163)	CD19 (n = 221)
Transitions (%)	1394/2725 (51.2)	672/1532 (43.9)*	103/177 (58.2)†	1516/2990 (50.7)	641/1493 (42.9)*
Transversions (%)	1331/2725 (48.8)	860/1532 (56.1)*	74/177 (41.8)†	1474/2990 (49.3)	852/1493 (57.1)*
Transitions at C·G (%)	809/1612 (50.2)	459/1005 (45.7)*	66/99 (66.7)*†	897/1790 (50.1)	384/938 (40.9)*
Targeting of C·G (%)	1612/2725 (59.2)	1005/1532 (65.6)*	99/177 (55.9)†	1790/2990 (59.9)	938/1493 (62.8)
RGYW (%)	644.8/2725 (23.7)	284.3/1532 (18.6)*	40.6/177 (22.9)	745.6/2990 (24.9)	256.5/1493 (17.2)*
WRCY (%)	393.7/2725 (14.4)	287.1/1532 (18.7)*‡	24.6/177 (13.9)	436.4/2990 (14.6)	235.0/1493 (15.7)
WA (%)	309.9/2725 (11.4)	116.5/1532 (7.6)*	21.6/177 (12.2)†	337.8/2990 (11.3)	104.5/1493 (7.0)*
TW (%)	197.6/2725 (7.3)	78.9/1532 (5.2)*	18/177 (10.2)†	183.3/2990 (6.1)	76.9/1493 (5.2)
FR (R/S)	1203/631 (1.9)	817/330 (2.5)*	78/49 (1.6)†	1334/743 (1.8)	822/360 (2.3)*
CDR (R/S)	693/173 (4.0)	274/80 (3.4)	39/11 (3.5)	713/179 (4.0)	235/52 (4.5)

The number of analyzed sequences is indicated in parentheses next to the population name.

All analyses were performed with the JoinSolver program, and the differences between controls and patients were analyzed with the χ^2 test. R/S, Ratio between replacement (R) and silent mutations (S).

*Significant differences ($P < .05$) between patient and control cells.

†Significant differences ($P < .05$) between IgA of CD40L and CD19 deficiency.

‡Significant differences ($P < .05$) between IgA and IgG of CD19 deficiency.

controls (median, 4%-7%; Fig 1, A). SHM frequencies in CD19-deficient patients were reduced to less than 3% and in CD40L-deficient patients to less than 1%.

CD19-deficient and CD40L-deficient patients showed an accumulation of replacement mutations in CDR1 and CDR2 of rearranged *IGHV* genes, similar to controls (see Fig E2 in this article's Online Repository at www.jacionline.org). Furthermore, *IGHV*-CDR R/S ratios of controls, CD19-deficient patients, and CD40L-deficient patients were similarly increased as compared to *IGHV*-FRs and ranged between 3.4 and 4.5 (Table I). However, the increased R/S ratios in CDRs versus FRs do not necessarily reflect selection processes because CDR codons are more susceptible than FR codons to replacement mutations.²⁹ Therefore, we analyzed the *IGH* sequences with the Bayesian estimation of Antigen-driven SElectIoN program, which determines whether the mutation patterns differed from what can be expected from random targeting.²⁶ Similar to previous observations,³⁰ we found positive selection for replacement mutations in the CDR and negative selection in the FR of IgA and IgG transcripts of healthy controls (Fig 1, B; dotted lines). In contrast, CD19-deficient and CD40L-deficient patients lacked positive selection of replacement mutations in the CDR. Thus, IgH-switched transcripts in CD19-deficient and CD40L-deficient patients showed impaired SHM levels with reduced selection for replacement mutations in the CDR.

Impaired immunoglobulin class switching to downstream constant regions

The human *IGH* locus contains 2 IgA and 4 IgG constant regions (Fig 2, A). IgA2 was almost as frequently used as IgA1 in healthy controls. This was significantly reduced in CD19-deficient (23%) and CD40L-deficient patients (4%; Fig 2, B). Furthermore, the SHM frequencies did not differ between IgA1 and IgA2 transcripts and were strongly reduced for both isotypes in CD19-deficient and CD40L-deficient patients (Fig 2, C).

The IgG subclass distribution in CD19-deficient patients was also skewed toward the usage of IgM-proximal IgG subclasses (Fig 2, B). While in controls, IgG1 (56%) and IgG2 (35%) were mostly used, CD19-deficient patients mostly used IgG1 (73%) and IgG3 (12%). The SHM frequencies in all subclasses were significantly lowered in CD19-deficient patients than in controls,

but these increased with more downstream IgG subclass usage (Fig 2, C). A similar trend was seen for controls, but this was not significant. We therefore conclude that the IgG subclass distribution molecularly reflects the defective secondary immune responses of CD19-deficient patients.¹⁷

Immunoglobulin repertoire selection for superantigen binding

Similar to healthy controls, CD19-deficient and CD40L-deficient patients showed diverse use of *IGHV3* subgroup genes with *IGHV3-23*, *IGHV3-30*, and *IGHV3-48* predominating (not shown). The *Staphylococcus aureus* virulence factor protein A (SpA) triggers supraclonal B-cell responses through interactions with several contact residues in the FR1 and FR3 of *IGHV3*-encoded BCRs.³¹ To study the selection for SpA binding, we analyzed the frequencies of IgG and IgA rearrangements with mutations in 1 or more of the 7 critical residues: ARG/LYS-20, GLY-74, ARG-75, THR-77, SER-79, GLN-90, and ASN-92 (Fig 3, A).³¹ The mutation frequencies in *IGHV3* transcripts from controls and CD19-deficient patients were compared with *IGHV1/4* transcripts that cannot bind SpA. The number of transcripts of CD40L-deficient patients was too low for this analysis. The frequencies of mutated *IGHV1/4* genes were similar to the theoretical chance, that is, 7 times the overall replacement mutation frequency (Fig 3, B). These mutation rates were much higher in controls (63% and 76%) than in CD19-deficient patients (28% and 24%), due to the lower replacement mutation rates in the patients. Importantly, in both controls and CD19-deficient patients, the frequencies of *IGHV3* rearrangements with mutated core residues were significantly lower than those of *IGHV1/4* rearrangements, despite comparable overall mutation frequencies. Thus, *IGHV3* rearrangements are selected for maintaining SpA superantigen binding, a process that is not dependent on CD19.

Because SpA superantigen binding was normally retained in CD19-deficient patients, whereas other immunoglobulin selection processes were abnormal, we addressed whether SpA is able to activate CD19-deficient B cells. In line with previous observations, CD19-deficient patients showed impaired Ca^{2+} influx following anti-IgM stimulation (Fig 3, C).¹⁷⁻¹⁹ In contrast, *S aureus* Cowan I lysate, in which SpA is the dominant B-cell

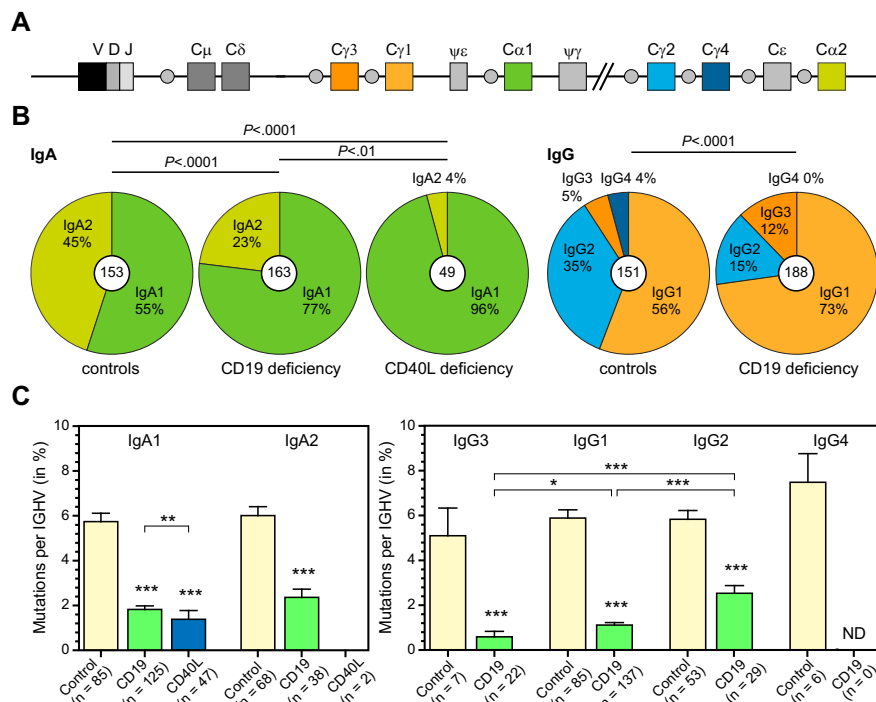


FIG 2. IgA and IgG subclass analysis. **A**, Schematic representation of the constant region of the human *IGH* locus. **B**, Distribution of IgA and IgG subclass use in switched transcripts of healthy controls, CD19-deficient patients, and CD40L-deficient patients. Total numbers of analyzed sequences are indicated in the center of each plot. Differences in the distributions were statistically analyzed with the χ^2 test. **C**, *IGHV* mutation frequencies in distinct IgA and IgG subclasses (mean $\pm$ SEM). ND, Not detected. Statistical significance was calculated with the Mann-Whitney test. * $P < .05$; ** $P < .01$; and *** $P < .001$.

stimulant,³² induced a normal initial Ca^{2+} influx followed by an increased baseline level that is reminiscent of BCR signaling.³³ Thus, CD19-deficient B cells display normal signaling and immunoglobulin selection for SpA superantigen binding.

Immunoglobulin repertoire selection against inherently autoreactive properties

Within the *IGHV4* subgroup, the *IGHV4-34* and *IGHV4-59* genes were most frequently used in naive mature B cells of healthy controls, likely because of their highly efficient recombination signal sequences.^{34,35} *IGHV4-34* genes were hardly used in IgA and IgG transcripts of controls (Fig 4, A and B) because they display inherent autoreactivity in their FR1 that binds the I antigen on red blood cells.³⁶ Moreover, 3 of 5 *IGHV4-34* transcripts in control IgG and IgA transcripts were mutated in 1 or more of the I antigen-binding residues—TRP-7, ALA-23, TYR-25³⁶—thus likely removing autoreactivity. The selection against *IGHV4-34* use was impaired in CD19-deficient and CD40L-deficient patients (Fig 4, A and B). Furthermore, nearly all these transcripts (24 of 25 of CD19 and 2 of 2 of CD40L) retained all 3 interacting residues. Thus, CD19-deficient and CD40L-deficient patients lack selection against I antigen binding in their immunoglobulin class-switched B cells.

Memory B cells carry on average smaller IgH-CDR3 regions than do naive B cells.^{9,37} Indeed, the IgG and IgA transcripts from controls showed smaller IgH-CDR3 sizes (14 aa) than did naive B-cell subsets (17 aa; Fig 4, C). The median sizes of IgH-CDR3 in IgA and IgG transcripts in CD19-deficient patients were

smaller than in naive B cells, but significantly higher (16 and 15 residues, respectively) than switched transcripts in controls. CD40L-deficient patients showed no reduction in IgH-CDR3 of IgA transcripts.

Together, these findings indicate that in contrast to normal selection for superantigen binding, inherent autoreactivity is not counterselected in CD19-deficient and CD40L-deficient patients.

Targeting and repair of SHMs

To study whether the reduced SHM levels in CD19 and CD40L deficiencies were associated with abnormal targeting and repair, we analyzed mutations in hypermutable motifs and the transition to transversion ratios. In line with previous findings,²² CD40L-deficient patients showed an increased overall transition over transversion mutation ratio as compared with controls (58/42) (Table I). This was mostly due to transition mutations at C·G (67/33) and significantly increased G→A transitions (see Fig E3 in this article's Online Repository at www.jacionline.org), reminiscent of reduced UNG2 and BER function. Overall targeting of C·G as well as targeting of RGYW/WRCY motifs in CD40L patients seemed unaffected. Targeting of WA/TW motifs was slightly, but not significantly, increased over controls. Together, CD40L-deficient patients show decreased SHM induction by the AID enzyme and decreased UNG2 function in C·G repair, but normal MMR.

Interestingly, CD19-deficient patients showed a decreased transition over the transversion ratio than did controls for both

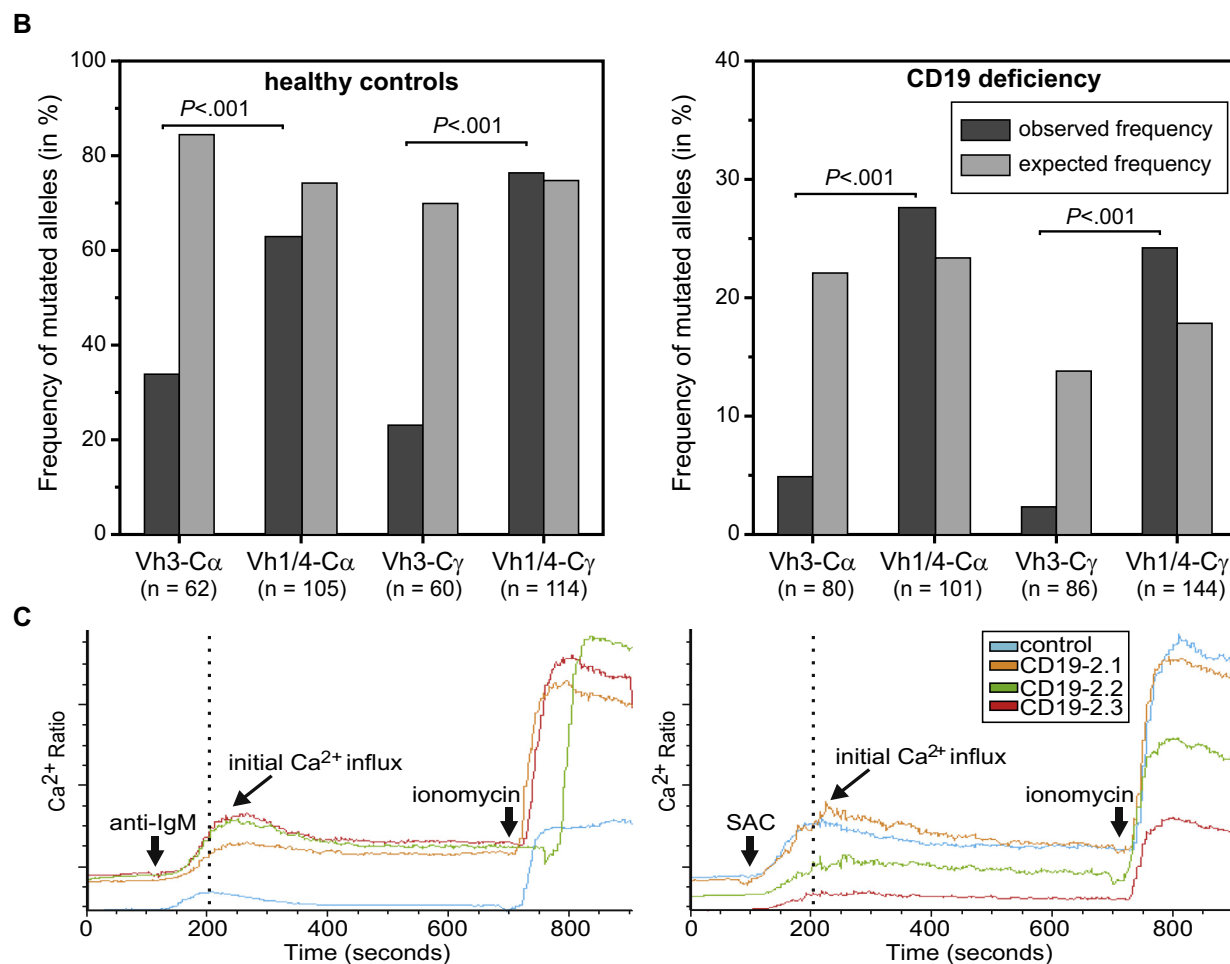
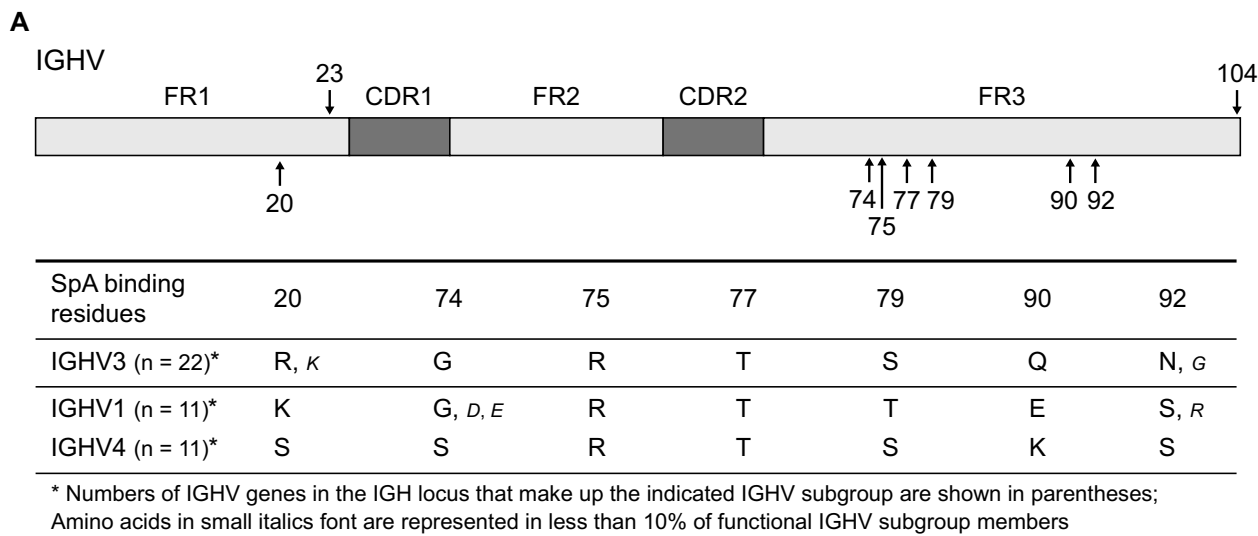


FIG 3. Selection against mutations in SpA-binding residues in *IGHV3* genes of healthy controls and CD19-deficient patients. **A**, Schematic representation of germline-encoded IGHV genes and 7 core residues for SpA binding.³¹ **B**, Frequencies of *IGHV* genes with mutations in 1 or more of the 7 core residues for SpA binding. The observed mutation frequencies are shown in *black bars*. The *gray bars* depict the expected mutation frequencies, that is, 7 times the overall mutation frequency of that subset of sequences. Differences in frequencies of mutated *IGHV3* and *IGHV1/4* genes were analyzed with the χ^2 test. **C**, Ca^{2+} influx on B-cell stimulation with anti-IgM or SAC. SAC, *Staphylococcus aureus* Cowan.

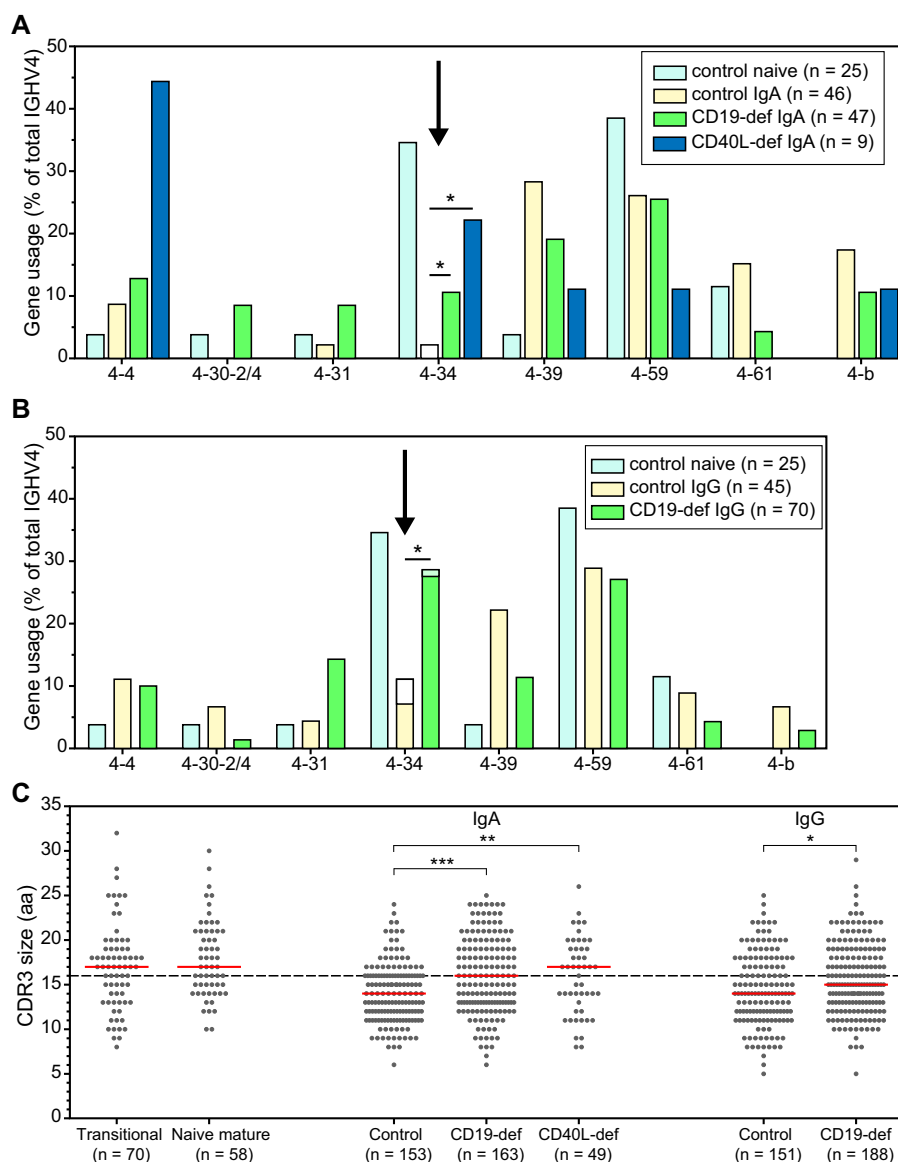


FIG 4. Impaired selection against inherently autoreactive *IGHV4-34* genes and long IgH-CDR3 regions. **A** and **B**, *IGHV4* gene usage in IgA and IgG transcripts. *IGHV4-34* genes with mutations in their I-antigen-binding domain are shown in a lighter shade. **C**, IgH-CDR3 sizes of unique transcripts. All individual data points are shown as gray dots, with red lines indicating the median value. The number of analyzed sequences is indicated in parentheses for each subset. Statistical significance was calculated with the Mann-Whitney test. * $P < .05$; ** $P < .01$; and *** $P < .001$.

IgG and IgA (Table I). These were partly the result of decreased transitions at C·G, due to decreased G→A transitions and increased C→A transversions (Fig E3), indicative of increased UNG2 and BER activity. Overall targeting was more skewed toward C·G than in healthy controls or CD40L-deficient patients, with normal targeting of WRCY and slightly decreased targeting of RGYW motifs (Table I; also see Table E3 in this article's Online Repository at www.jacionline.org). Furthermore, mutations in WA/TW motifs were reduced, indicating reduced MMR of SHM in CD19-deficiency. Thus, the repair of AID-induced lesions seems differentially affected by the 2 signaling pathways: CD40 signaling is required for UNG2 function, but not for MMR, whereas IgM signaling seems to inhibit BER and favors MMR.

Regulation of AID and error-prone repair genes by IgM and CD40 signaling

To dissect how IgM and CD40 signaling affect SHM induction and repair, we stimulated control naive B cells with anti-IgM, anti-CD40, and IL-4, which supports B-cell survival.³⁸ Of the previously described genes,²² *AID*, *UNG2*, MutS-homologues 2 and 6 (*MSH2* and *MSH6*), exonuclease 1 (*EXO1*), and *PCNA* were upregulated after 3 days of culture (Fig 5, A and B).

Stimulation with anti-IgM in complete absence of anti-CD40 resulted in hardly any upregulation of the studied genes (Fig 5, C). Moreover, *RPA1* and *APEX1* were downregulated as compared with unstimulated cells. These effects could not be restored by the addition of B-cell activation stimuli IL-21 or CpG (see Fig E4 in this article's Online Repository at www.jacionline.org).

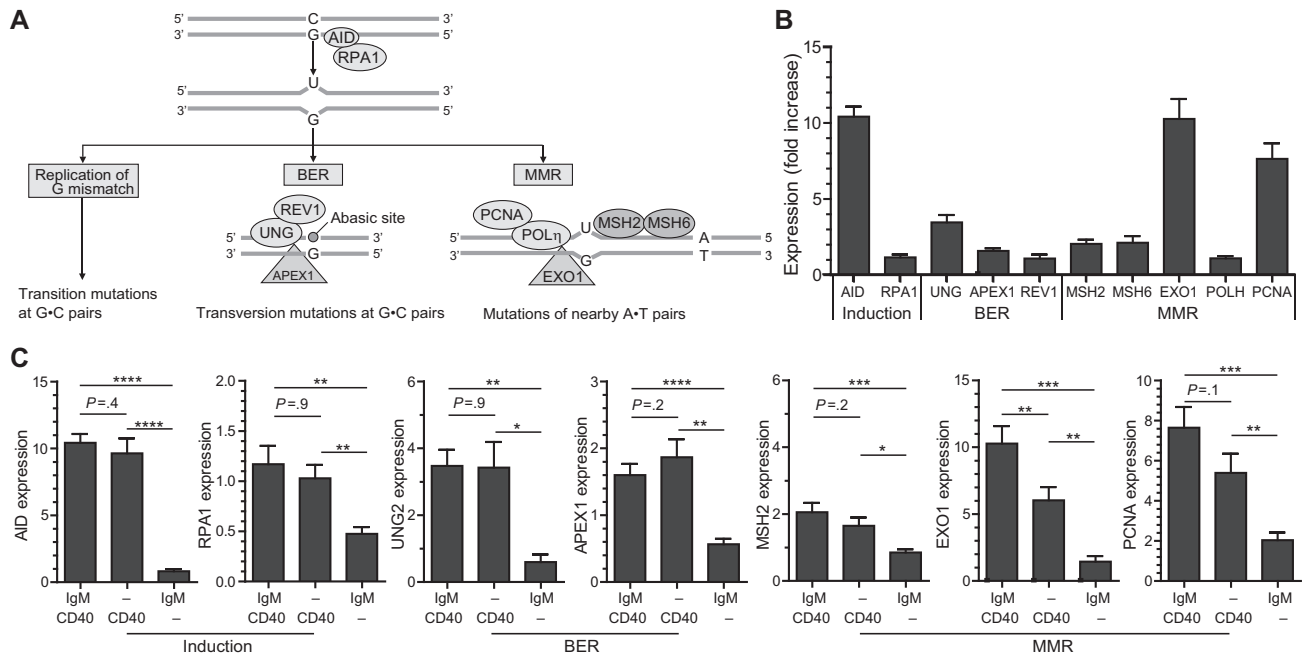


FIG 5. Gene expression levels of SHM targeting and repair genes in activated B cells. **A**, Schematic representation of error-prone repair of AID-induced lesions by replication, BER, or MMR. **B**, Gene expression levels of *AID*, *RPA1*, *UNG2*, *MSH2*, *MSH6*, *EXO1*, *POLH*, and *PCNA* in purified naive B cells from childhood tonsil following stimulation with 10 ng/mL anti-IgM, anti-CD40, and IL-4. **C**, Gene expression levels in the absence of either anti-IgM or anti-CD40 stimulation. The bars in (B) and (C) represent the average fold upregulation of 10 independent donors as compared with unstimulated B cells with SEM error bars. Statistical significance was calculated with the student paired *t* test; **P* < .05; ***P* < .01; ****P* < .001; and *****P* < .0001. *PCNA*, Proliferating cell nuclear antigen; *POLH*, polymerase η .

The effects on gene expression levels were less severe in the absence of IgM signaling. *AID* was only mildly decreased, and no effects were seen for *RPA1*, *UNG2*, or *APEX1*. Of the MMR genes, *MSH2*, *EXO1*, and *PCNA* expression levels were reduced, but not to the extent seen in the absence of CD40. These results confirm the *ex vivo* observations that CD40L-deficient patients, who completely lack CD40 signaling, show strongly decreased *AID* and *UNG2* function. The slight upregulation of *EXO1*, *MSH6*, and *PCNA* is indicative for residual MMR function of the few lesions created by *AID* in these patients. IgM signaling was redundant for *UNG2* upregulation but impaired MMR genes. This could explain the observed skewing toward BER over MMR in CD19 deficiency.

DISCUSSION

Here, we analyzed antibody maturation in patients with CD19 and CD40L deficiencies to understand the absence of functional B-cell immunity in these patients. Although selection for superantigen binding was normal in CD19 deficiency, both patient groups showed decreased SHM and reduced selection strengths for antigen binding. Furthermore, selection against inherently autoreactive *IGHV* genes and *IGH*-CDR3 properties were impaired. Interestingly, SHM targeting and repair were differentially impaired: CD40L-deficient patients displayed reduced *UNG2*-dependent BER activity, whereas CD19-deficient patients showed increased BER and reduced MMR activity. Thus, the BCR and CD40 signaling pathways together control antibody maturation and selection against autoreactivity.

CD19-deficient and CD40L-deficient patients showed similarly reduced numbers of CD27⁺IgA⁺ and both IgG⁺ memory B-cell subsets. This contrasts the much higher serum IgG and IgA levels that can be found in CD19-deficient patients as compared with CD40L-deficient patients. Apparently, the CD19 deficiency drastically impairs circulating B-cell numbers. Although this could result from sequestration in tissue compartments, this likely underlies the lack of a memory response to booster vaccinations seen in CD19-deficient patients.¹⁷ In line with defective secondary responses, IgG2/4 transcript frequencies were strongly reduced in CD19-deficient patients. Immunoglobulin CSR to IgG2/4 frequently occurs via a first switch to IgG1/3. Considering the significantly higher SHM frequencies in IgG2 than in IgG1/3 transcripts, indirect switching to IgG2 does take place in CD19-deficient patients, but to a much smaller extent than in healthy individuals.

CD19-deficient patients had more severely reduced CD27⁺IgM⁺IgD⁺ natural effector B-cell numbers than did CD40L-deficient patients. In the absence of CD40 signaling and functional GCs, these cells are thought to be derived from the splenic marginal zone, while in healthy controls these might be a mixture of GC and marginal zone-derived B cells. The low numbers of circulating natural effector cells in CD19-deficient patients indicate that both differentiation pathways are affected, suggesting a crucial role for IgM signaling in both GC and marginal zone responses. Both CD19-deficient and CD40L-deficient patients showed normal numbers of CD27⁺IgA⁺ B cells, indicating normal formation of memory from local mucosal tissue. Still, their SHM levels and selection for replacement

mutations in IGHV-CDR were impaired, and the patients displayed reduced use of the IgA2 subclass, which is resistant to bacterial proteases. Thus, despite seemingly normal numbers, local mucosal IgA responses are qualitatively impaired in the absence of CD40 or CD19-dependent BCR signaling.

The strongly reduced SHM frequencies in CD19-deficient and CD40L-deficient patients provide little sequence variation for the subsequent selection of high-affinity receptors. This could be the basis for the reduced selection strength in immunoglobulin-switched transcripts of the patients. Moreover, the selection processes against inherently autoreactive features of IgH, long CDR3 and IGHV4-34 were absent. The defects seemed more severe in CD40L-deficient patients than in CD19-deficient patients. This could be due to the impaired upregulation of AID, which is required for human B-cell tolerance.³⁹ Alternatively, it could reflect either the importance of CD40 signaling over BCR signaling, or, more likely, residual BCR signaling in the absence of CD19.

CD19-deficient patients showed normal Ca^{2+} responses to *S aureus* Cowan and normal selection against mutations in SpA-binding residues in the FR. Thus, CD19 seems redundant in the response to this superantigen. It has been disputed whether the binding of SpA to IGHV3 induces immune responses or B-cell death.⁴⁰ This is most likely dependent on the local environment, because IL-4 and CD40L protect B cells from SpA-induced apoptosis.⁴⁰ Our findings that in healthy individuals SpA-binding residues are selectively unmutated indicate that there are selection processes *in vivo* to retain SpA superantigen binding. It appears that the maintenance of a large population of antigen-experienced B cells capable of SpA binding is favorable for the host.

CD19 deficiency showed a unique SHM targeting profile with decreased transition mutations and increased MMR, which, to our knowledge, has not been described before. Rather, deficiencies in DNA repair genes result in increased transition mutations (UNG, MSH2, MSH6, and EXO1 deficiencies),^{41–46} or in increased G·C targeting (polymerase η and proliferating cell nuclear antigen deficiencies).^{47–50} Our *in vitro* studies indicated that BCR signaling was needed to upregulate MMR genes, but not UNG2 and APEX1. This contrasts CD40L deficiency that impaired both BER and MMR. Thus, CD19-dependent BCR signaling seems to specifically affect MMR at the transcriptional level, although the exact mechanism behind this remains to be elucidated.

IgA transcripts of CD40L-deficient patients had few mutations, which showed signs of reduced UNG2 activity at G·C, but normal MMR as evidenced by the normal A·T targeting. These observations were mostly in line with published data on SHM targeting in $\text{CD27}^+\text{IgM}^+\text{IgD}^+$ B cells of CD40L-deficient patients.²² In contrast to this report, we observed normal targeting of G residues in RGYW motifs, and higher SHM levels in IgA transcripts, suggestive of more AID activity. This could be inherent to IgA responses, because in healthy controls also, IgA^+ memory B cells show higher levels of SHM than do IgM^+ or IgG^+ memory B-cell subsets.⁹ Our results support other observations of CD40-independent SHM and CSR in intestinal mucosa through TACI and Toll-like receptor signaling.^{12–14} However, these processes are unable to upregulate AID and UNG2 levels to the same extent as CD40. Moreover, GC-independent $\text{CD27}^-\text{IgA}^+$ memory B cells in healthy controls show increased SHM, transversion mutations, and IgA2 CSR,⁹ indicative of higher AID and UNG2 activity than in CD40L deficiency.

Thus, it remains possible that in healthy controls CD40 signaling is directly or indirectly involved in generating their antigen-experienced repertoire.

In conclusion, BCR and CD40 signals are required for AID-induced DNA breaks, affinity maturation, and selection against inherently autoreactive features, whereas these signals skew DNA break repair: CD40 signaling promotes BER, and BCR-CD19 signaling promotes MMR. Thus, the human antigen-experienced B-cell repertoire is shaped *in vivo* through balanced signaling via the BCR and CD40 to ensure optimal affinity maturation and selection against autoreactivity. These new insights from well-defined human inherited disorders provide means to study antibody maturation and selection defects in patients with immune-mediated diseases, including primary immunodeficiencies and autoimmune disorders.

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Key messages

- Memory B cells in patients with CD19 or CD40L deficiency are impaired not only in numbers but also in the quality of their antibodies.
- B-cell antigen receptor and CD40 signaling balance the 2 DNA repair mechanisms involved in affinity maturation of antibodies.
- Impaired antibody selection in patients with B-cell antigen receptor signaling defects might underlie their autoimmunity.

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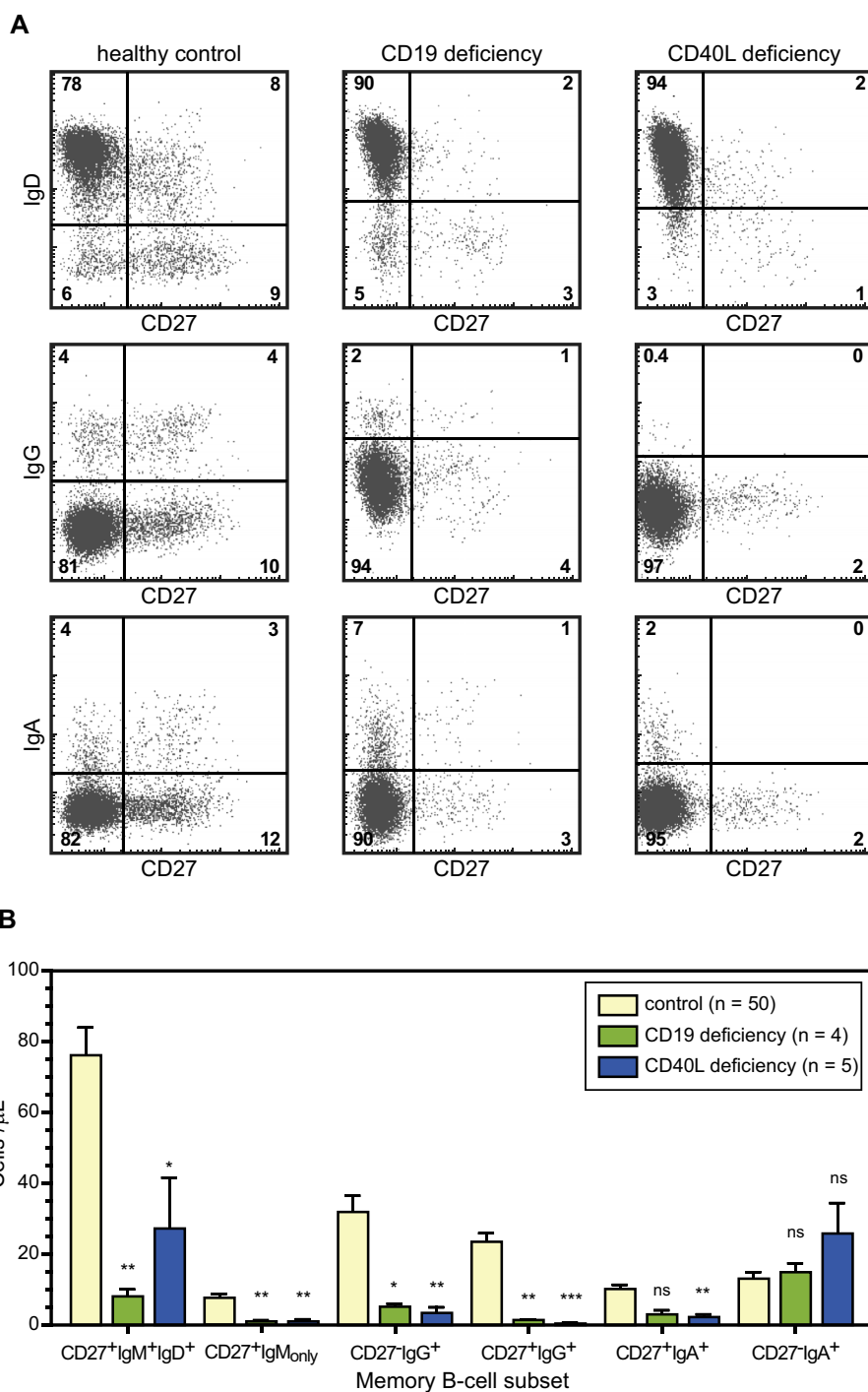


FIG E1. Memory B cells in CD19-deficient and CD40L-deficient patients. **A**, Representative FACS plots. **B**, Absolute cell numbers of 6 memory B-cell subsets (mean $\pm$ SEM). Data included from patients XHIM-1.2, XHIM-1.3, XHIM-9, XHIM-11, and XHIM-12, CD19-1.1, CD19-1.2, and CD19-4, and CD81-1. Statistical significance was calculated with the Mann-Whitney test: ns, Not significant; * $P < .05$; ** $P < .01$; and *** $P < .001$. FACS, Fluorescence-activated cell sorting.

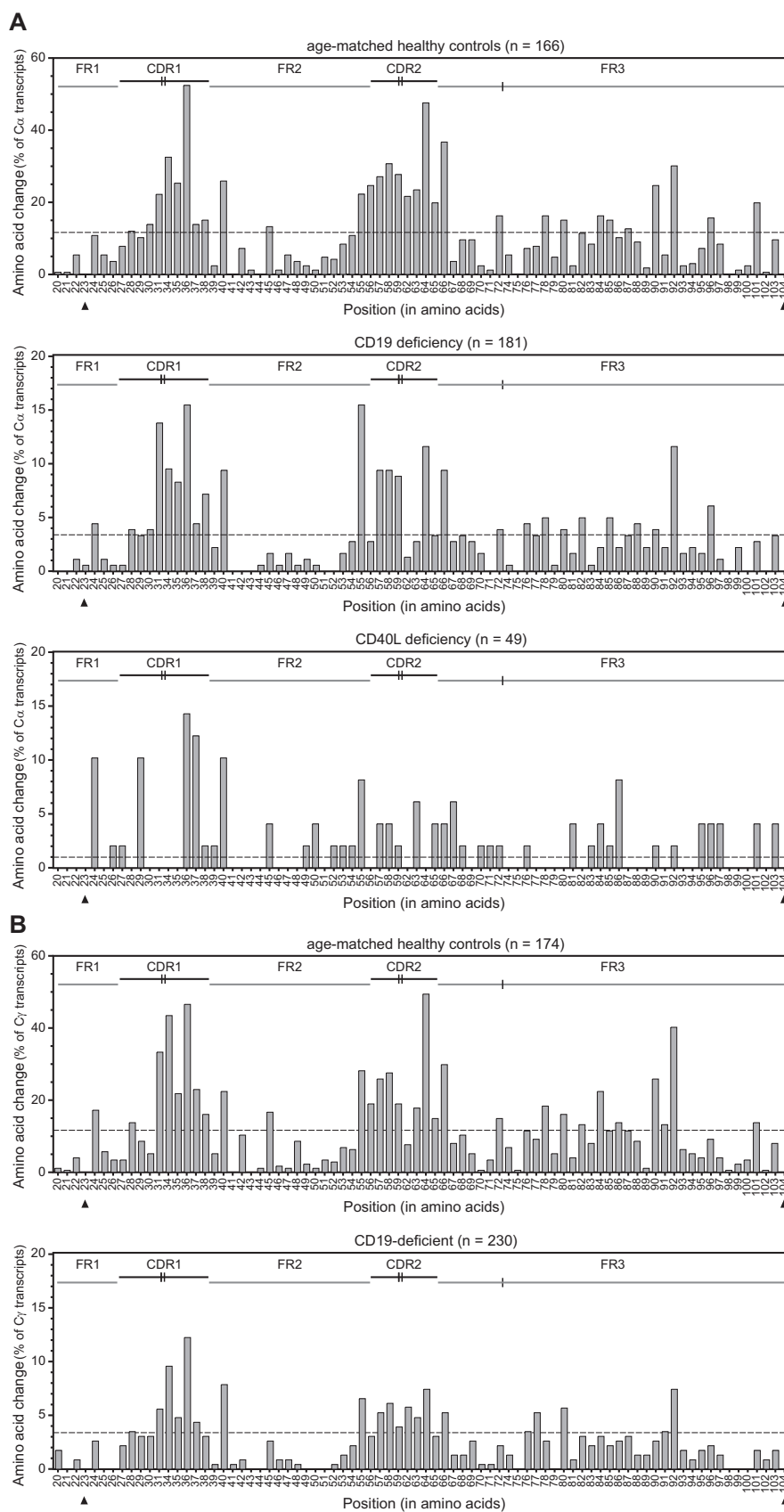


FIG E2. Distribution of amino acid replacements in rearranged *IGHV* genes. Distribution of amino acid replacements in rearranged *IGHV* genes derived from sequence analysis of (A) C α transcripts and (B) C γ

Control IgG					
Absolute numbers					
To From	A	C	G	T	
A		196	398	173	767
C	119		241	410	770
G	487	329		204	1020
T	110	221	102		433
					2990
Frequencies					
To From	A	C	G	T	
A		6.6	13.3	5.8	25.7
C	4.0		8.1	13.7	25.8
G	16.3	11.0		6.8	34.1
T	3.7	7.4	3.4		14.5
					100

CD19 IgG					
Absolute numbers					
To From	A	C	G	T	
A		82	158	88	328
C	109 *		124	167	400
G	217 *	155		166 *	538
T	55	99	73 *		227
					1493
Frequencies					
To From	A	C	G	T	
A		5.5	10.6	5.9	22.0
C	7.3		8.3	11.2	26.8
G	14.5	10.4		11.1	36.0
T	3.7	6.6	4.9		15.2
					100

Control IgA					
Absolute numbers					
To From	A	C	G	T	
A		164	361	174	699
C	97		203	378	678
G	431	306		197	934
T	85	224	105		414
					2725
Frequencies					
To From	A	C	G	T	
A		6.0	13.2	6.4	25.7
C	3.6		7.4	13.9	24.9
G	15.8	11.2		7.2	34.3
T	3.1	8.2	3.9		15.2
					100

CD19 IgA					
Absolute numbers					
To From	A	C	G	T	
A		65	126 *	120 * [†]	311
C	92 * [†]		121	246 [†]	459
G	213 *	171		162 *	546
T	64 *	87 *	65		216
					1532
Frequencies					
To From	A	C	G	T	
A		4.2	8.2	7.8	20.3
C	6.0		7.9	16.1	30.0
G	13.9	11.2		10.6	35.6
T	4.2	5.7	4.2		14.1
					100

CD40L IgA					
Absolute numbers					
To From	A	C	G	T	
A		10	21 *	8 [‡]	39
C	4		13	22	39
G	40 * [‡]	14		6 * [‡]	60
T	12	20	7		39
					177
Frequencies					
To From	A	C	G	T	
A		5.6	11.9	4.5	22.0
C	2.3		7.3	12.4	22.0
G	22.6	7.9		3.4	33.9
T	6.8	11.3	4.0		22.0
					100

FIG E3. Substitution of individual nucleotides in rearranged *IGHV*. Individual nucleotide substitutions for IgG rearrangements of controls and CD19-deficient patients and IgA rearrangements cells of controls, CD19-deficient patients, and CD40L-deficient patients are given both in absolute number (*left panels*) and in relative frequencies (*right panels*). The number of analyzed sequences is similar as in Table I and Table E2. All analyses were performed with the JoinSolver program and statistically analyzed with the χ^2 test. Significant differences are indicated with an *asterisk* between patient and control cells, with a hash between IgG and IgA of controls, with a single dagger between IgG and IgA of CD19 deficiency, and with a *double dagger* between IgA of CD19 and CD40L deficiency.

transcripts from healthy controls, CD19-deficient patients, and CD40L-deficient patients. The number of analyzed sequences is indicated in parentheses. Each *bar* represents the frequency of substitutions at each amino acid position starting from 20 (first codon following primer sequence) to 104 (last codon of the FR3). *Horizontal dotted lines* indicate average replacement mutation frequencies; *triangles* indicate conserved cysteines: first CYS-23 and second CYS-104.

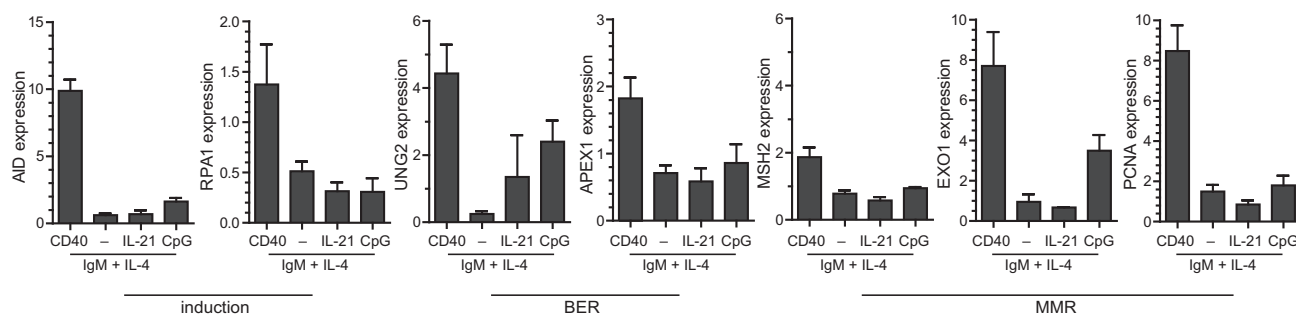


FIG E4. Gene expression levels of SHM targeting and repair genes in IgM + IL-4 activated B cells with CD40, IL-21, or CpG costimulation. The bars represent the average fold upregulation of 4 independent donors as compared with unstimulated B cells with SEM error bars.

TABLE E1. Patients with absence of CD19 or CD40L protein expression

Patient	Age (y); sex	Gene	Mutation		Membrane expression			Serum immunoglobulin (mg/dL)			Reference
			cDNA	Protein	CD19	CD81	CD40L	IgG	IgA	IgM	
CD19-1.1	10; F	<i>CD19</i>	c.972insA	p.Arg325AlafsX4	—	+	ND	325	292	25	17
CD19-1.2	12; M	<i>CD19</i>	c.972insA	p.Arg325AlafsX4	—	+	ND	91	1	59	23
CD19-2.1	35; M	<i>CD19</i>	c.1384delGA	p.Asn463ArgfsX3	—	+	ND	204	18	47	17
CD19-2.2	33; F	<i>CD19</i>	c.1384delGA	p.Asn463ArgfsX3	—	+	ND	198	7	30	17
CD19-2.3	49; F	<i>CD19</i>	c.1384delGA	p.Asn463ArgfsX3	—	+	ND	256	19	63	17
CD19-3	8; M	<i>CD19</i>	c.947-1G>T; gene deletion	p.Ala316Asp fsX5; p.X	—	+	ND	249	10	18	24
CD19-4	6; F	<i>CD19</i>	c.G156C	p.Trp52Cys	—	+	ND	300	50	40	19
CD81-1	4; F	<i>CD81</i>	c.561+1G>A	p.Glu188MetfsX13	—	—	ND	240	71	35	18
XHIM-1.2	13; M	<i>CD40L</i>	c.761C>T	p.Thr254Met	+	ND	—	115	<20	124	9
XHIM-1.3	1; M	<i>CD40L</i>	c.761C>T	p.Thr254Met	+	ND	—	80	<1	<30	9
XHIM-2	3; M	<i>CD40L</i>	c.617T>C	p.Leu206Pro	+	ND	—	23	35	139	
XHIM-7.1	3; M	<i>CD40L</i>	c.662A>C	p.Gln221Pro	+	ND	—	<20	<7	306	
XHIM-9	2; M	<i>CD40L</i>	c.474delG	p.Gly158fsX4	+	ND	—	80	47	154	9
XHIM-10	4; M	<i>CD40L</i>	c.435delC	p.Tyr146ThrfsX3	+	ND	—	<134	<6	71	
XHIM-11	1; M	<i>CD40L</i>	c.154A>T	p.Lys52X	+	ND	—	152	6	178	9
XHIM-12	1; M	<i>CD40L</i>	c.521A>C	p.Gln174Pro	+	ND	—	92	<6	121	9

Subnormal values as compared with age-matched controls are shown in boldface. F, Female; M, male.

TABLE E2. PCR primers and UPL FAM-labeled hydrolysis probes for gene expression analysis

Gene	Forward primer (5'-3')	Position (exon)	Reverse primer (5'-3')	Position (exon)	UPL probe no.
AID	ACTCTGGACACCACTATGGACA	1	GCGGACATTTTGAATTGGT	2	69
APEX1	GCTTCGAGCCTGGATTAAGA	3	TTGGTCTCTTGAAGGCACAGT	4	45
EXO1	TGAGCTCTGGAAAACTTTGG	15	CTGGGGACAGGGGTTTCT	16	69
MSH2	CAAGGAGAATGATTGGTATTTGG	2	CCAAAGAGAATGTCTTCAAAGTGAAG	3	2
MSH6	AAGGCGAAGAACCTCAACG	1	CTGGTGAGAAGTCACAAGTGGT	2	40
PCNA	TGGAGAACTTGGAAATGGAAA	4	GAAGTGGTTCATTCATCTCTATGG	5	69
POLH	TGAATCCCAGCTCCAGAGTC	7	GCCTGGGTTTAACTGGATCA	8	7
REV1	CAATTTTATAGTGGAGTTGCCATC	3	CCTCCATGCAACATCATTAGTTT	4	73
RPA1	TGCCCTATAATGAAGGACTCG	5	GAACCCATTCCCAGACTT	7	3
UNG2	TGAGGAAAGCGGAGATGC	1	GATCCGGTCCAAGTCTC	2	51

FAM, Fluorescein; PCNA, proliferating cell nuclear antigen; POLH, polymerase η ; UPL, universal probe library.

TABLE E3. Targeting of individual nucleotides in hypermutable motifs

	IgA						IgG			
	Control (n = 153)		CD19-deficient (n = 202)		CD40L-deficient (n = 50)		Control (n = 163)		CD19-deficient (n = 221)	
G										
Inside <u>RGYW</u>	364.5/1439 (25.3)	4.1	157.5/1936 (8.1)	2.6	25/437.5 (5.7)	5.2	434/1518.5 (28.6)	4.8	132/2105.7 (6.3)	2.2
All other G	569.5/9241 (6.2)		388.5/12543 (3.1)		35/3072.5 (1.1)		586/9758.5 (6.0)		406/14002.3 (2.9)	
C										
Inside <u>WRCY</u>	229.8/1267.7 (18.1)	3.8	135.5/1693 (8.0)	3.1	11/397.5 (2.7)	2.5	259.1/1301.7 (19.9)	3.9	98/1965.5 (5.0)	2.3
All other C	448.2/9397.3 (4.8)		323.5/12457 (2.6)		28/2657.5 (1.1)		510.9/9919.3 (5.2)		302/14024.4 (2.2)	
A										
Inside <u>WA</u>	332/2249.5 (14.8)	2.6	126/3032 (4.2)	2.0	23/798.5 (2.9)	3.6	367.5/2391.5 (15.4)	2.6	115.1/3349.7 (3.4)	1.6
All other A	367/6452.5 (5.7)		185/8740 (2.1)		16/2116.5 (0.8)		399.5/6773.5 (5.9)		212.8/9826.3 (2.2)	
T										
Inside <u>TW</u>	202.5/1724 (11.7)	3.6	92/2505 (3.7)	2.6	23/604.5 (3.8)	4.7	201.8/1820.8 (11.1)	3.3	78.5/2721.7 (2.9)	1.9
All other T	211.5/6483 (3.3)		124/8709 (1.4)		16/1987.5 (0.8)		231.3/6889.3 (3.4)		148.5/9761.3 (1.5)	
A										
Inside <u>RGYW</u>	127.5/649 (19.6)	2.8	48/1006.5 (4.8)	2.0	6.5/249 (2.6)	2.1	159/735 (21.6)	3.0	53/1061 (5.0)	2.2
All other A	571.5/8053 (7.1)		263/10765.5 (2.4)		32.5/2666 (1.2)		608/8430 (7.2)		275/12115 (2.3)	
A										
Inside <u>WRCY</u>	94.5/1174 (8.0)	1.0	33/1430.5 (2.3)	0.9	3.5/266 (1.3)	1.0	105/1209 (8.7)	1.0	48/1797 (2.7)	1.1
All other A	604.5/7528 (8.0)		278/10341.5 (2.7)		35.5/2649 (1.3)		662/7956 (8.3)		280/11379 (2.5)	

The number of underlined (commonly mutated) nucleotides inside and outside the motifs was calculated with the JoinSolver software (frequency in parentheses). The ratio between the frequency of mutations inside and outside the motifs is depicted in boldface and used as a measurement of targeting. The number of analyzed sequences is indicated in parentheses next to the name of each population.