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The origin of *CD95*-gene mutations in B-cell lymphoma

Markus Müschen, Klaus Rajewsky, Martin Krönke and Ralf Küppers

CD95 (Apo-1/Fas) is crucial for the negative selection of B cells within the germinal center (GC). Impairment of CD95-mediated apoptosis results in defective affinity maturation and the persistence of autoreactive B-cell clones. *CD95* was defined recently as a tumor-suppressor gene and is silenced in many tumor entities. In contrast to other malignancies, in GC-derived B-cell lymphomas, inactivation of the *CD95* gene is often a result of deleterious mutations. Such mutations occur also at a low frequency in normal GC, but not naive, B cells. We propose that *CD95* mutations in B-cell lymphomas originate from the GC reaction and are introduced most probably as targeting errors of the somatic hypermutation machinery, which bears – besides its physiological role – an inherent risk of malignant transformation and the persistence of autoreactive B-cell specificities.

The CD95 (Apo-1/Fas) molecule belongs to the tumor necrosis factor receptor (TNFR) family [1] and is an almost ubiquitously expressed transmembrane death receptor [2]. Usually, the induction of apoptosis by CD95 requires crosslinking of CD95 by CD95 ligand (CD95L), which is expressed only in a few anatomically well-defined structures, including germinal centers (GCs) [3]. Crosslinking of CD95 by CD95L leads to the assembly of a death-inducing signaling complex (DISC), which includes trimerized CD95, CD95/Fas-associated death-domain-containing protein (FADD) and procaspase-8 (Fig. 1). The DISC is assembled around the cytoplasmic death domain (DD) of CD95, which thus, plays a pivotal role in the transduction of the death signal [2].

'…the somatic-hypermutation machinery can act occasionally outside of the Ig loci.'

CD95 mediates negative selection of B cells within the germinal center

In the B-cell lineage, expression levels of CD95 peak at the GC stage of differentiation [4], which contributes to the susceptibility of GC B cells to apoptosis [3,4] (Fig. 1). Indeed, human GC B cells carry a preformed DISC that is maintained in an inactive configuration by FADD-like interleukin-1β-converting-enzyme-inhibitory protein (c-FLIP) (Fig. 1) [5]. CD40 stimulation and Ig crosslinking – mimicking the T-cell–B-cell interaction – prevent the degradation of c-FLIP, suggesting that inhibition of the CD95 pathway is involved in positive selection and affinity maturation within the GC [3,5]. This idea is supported by the analysis of

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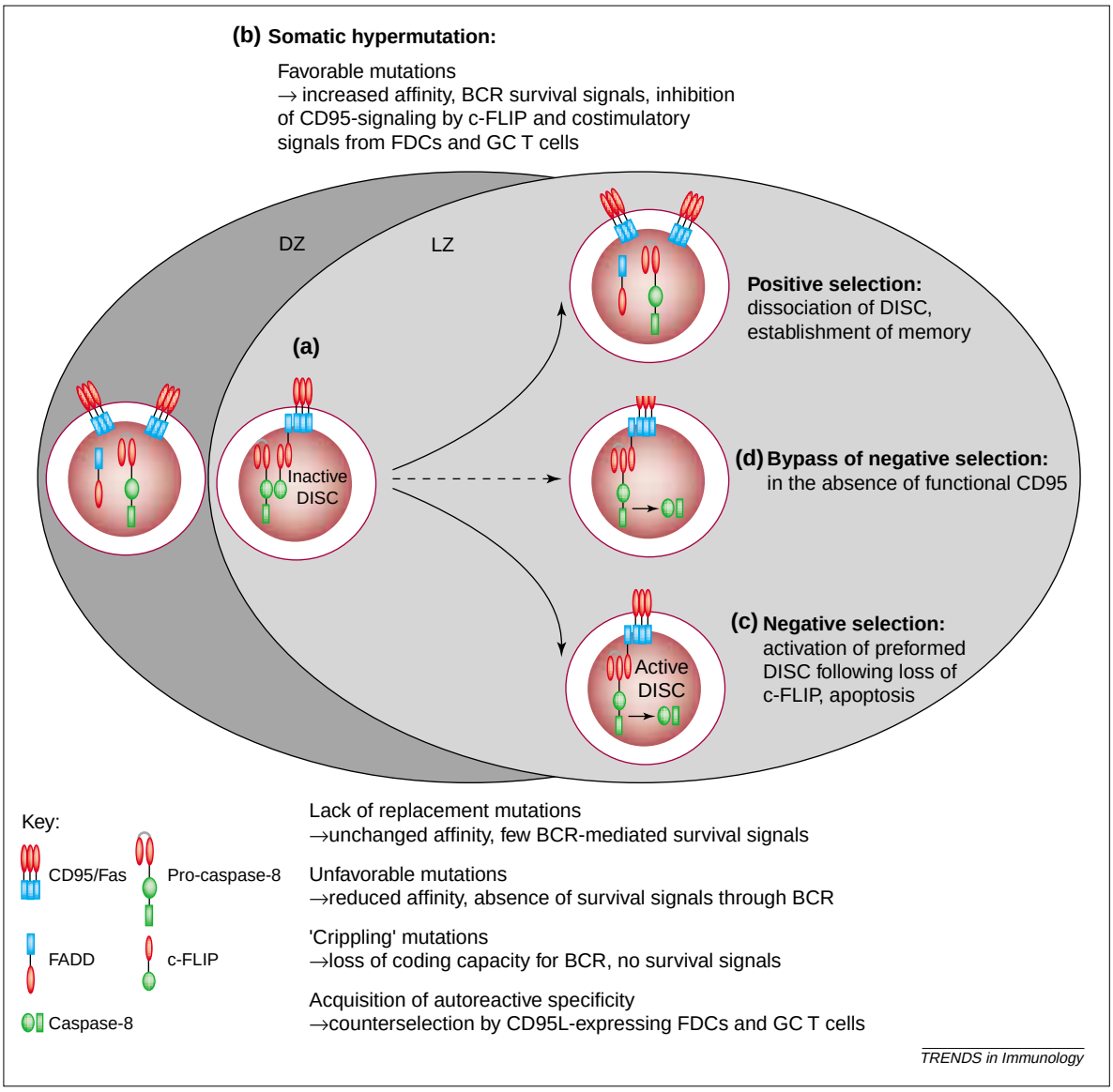

Fig. 1. B-cell receptor (BCR) and CD95 mediate clonal selection within the germinal center (GC). (a) B cells that enter the GC acquire susceptibility to apoptosis, resulting from the assembly of a CD95 death-inducing signaling complex (DISC), which includes trimerized CD95, Fas-associated death-domain-containing protein (FADD) and procaspase-8, held in an inactive form owing to association with FADD-like interleukin-1β-converting-enzyme-inhibitory protein (c-FLIP). (b) The process of somatic hypermutation either increases the affinity of a BCR for its cognate antigen, introduces silent or irrelevant mutations in the BCR, decreases BCR affinity for antigen, abolishes BCR coding capacity ('crippling' mutations) or generates an autoreactive specificity. GC B cells are subject to removal by apoptosis within the GC unless they have improved sufficiently the affinity of their BCR for antigen. (c) For those B cells with reduced or unchanged BCR affinity, c-FLIP is lost from the DISC in the absence of BCR-mediated survival signals and the constitutively initiated apoptosis program is executed. (d) In the absence of functional CD95 (e.g. owing to somatic mutations), B cells that carry a BCR with low affinity for antigen, an autoreactive BCR or no BCR at all might, eventually, bypass negative selection. Abbreviations: DZ, dark zone; FDC, follicular dendritic cell; LZ, light zone.

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CD95-deficient mice carrying the germline *lpr* mutation. In these animals, affinity maturation in response to immunization with the 4-hydroxy-3-nitrophenyl-acetyl (NP) antigen is disturbed, and the mutant mice are unable to establish an NP-specific memory B-cell pool in the absence of CD95 [6]. Moreover, CD95 signaling has been implicated also in the negative selection of autoreactive GC B cells (Fig. 1) [7]. The CD95 signaling pathway in GC B cells is, presumably, regulated also by other factors, such as the specific inhibition of CD95 signaling by CD95/Fas apoptosis inhibitory molecule (FAIM) upon B-cell receptor (BCR) crosslinking [8].

CD95 as a tumor-suppressor gene in malignancies of B-cell and other lineages

The *CD95* gene was proposed recently to act as a tumor-suppressor gene [9] and it is silenced in many tumor entities. Frequently, malignant cells lose their susceptibility to CD95-mediated apoptosis during tumor progression [9]. Loss of CD95 function has a dual effect on the tumor cell and its environment. CD95-deficient tumor cells can escape immunosurveillance and rejection (e.g. by CD95L-expressing cytotoxic T cells) [10]. In addition, the absence of functional CD95 is a prerequisite for

Table 1. Frequency of somatic *CD95* mutations in hematological and solid malignancies[a]

Malignancy	Number of cases with *CD95*-gene mutations	%	Refs
Solid tumors			
Bladder cancer	3/43	–	[32]
Colon carcinoma	0/12	–	[33]
Malignant melanoma	3/44	–	[34]
Non-small-cell lung carcinoma	5/65	–	[35]
Squamous-cell carcinoma	3/71	–	[36]
Ovarian cancer	0/8	–	[37]
Primary breast cancer	0/48	–	[38]
Breast cancer cell lines	0/10	–	[39]
Prostatic carcinoma	4/166	–	[40]
Subtotal	**18/467**	**3.8**	–
Leukemia			
Acute myelogenous leukemia	0/6	–	[41]
Acute lymphoblastic leukemia:	0/6	–	[41]
i. B-lineage acute lymphoblastic leukemia	0/32	–	[42]
ii. T-lineage acute lymphoblastic leukemia	1/35	–	[43]
	0/81	–	[44]
Chronic myelogenous leukemia	0/30	–	[41]
Subtotal	**1/190**	**0.6**	–
T-cell lymphoma			
Peripheral T-cell lymphoma	0/35	–	[14]
T-cell Hodgkin's disease	0/1	–	[45]
Subtotal	**0/36**	**0**	–
Pre-GC B-cell lymphoma			
Mantle-cell lymphoma	0/9	–	[14]
B-CLL[b]	1/17	–	[14]
	0/31	–	[41]
Subtotal	**1/57**	**1.8**	–
Non-B-lineage tumors and pre-GC B-cell lymphoma total	**20/750**	**2.7**	–
(Post-) GC B-cell lymphoma			
Nodal B-cell lymphoma:			
i. Burkitt's lymphoma	0/5	–	[14]
	0/3	–	[41]
ii. Diffuse large-cell lymphoma	9/43	–	[14]
	1/3	–	[16]
	1/17[c]	–	[31]
iii. Hairy cell leukemia	0/3	–	[41]
iv. Follicle center cell lymphoma	2/33	–	[14]
v. Multiple myeloma	7/54	–	[13]
vi. Hodgkin's disease	2/9[c]	–	[28]
Subtotal	**22/170**	**12.9**	–
Extranodal B-cell lymphoma:			
i. Diffuse large-cell lymphoma	5/10	–	[15]
ii. Follicle center cell lymphoma	6/8	–	[15]
iii. MALT-lymphoma	3/5	–	[14]
	0/18[d]	–	[46]
	6/8	–	[15]
	4/5	–	[16]
Subtotal	**24/54**	**44.4**	–
(Post-) GC B-cell lymphoma total	**46/224**	**20.5**	–

[a]Abbreviations: B-CLL, B-cell chronic lymphocytic leukemia; GC, germinal center; MALT, mucosa-associated lymphoid tissue.
[b]A fraction of B-CLL cases is derived from post-GC B cells. Information about whether the B-CLL case harboring a mutated *CD95* gene is actually derived from a post-GC B cell was not available [14].
[c]The analysis [28,31] also includes mutations within 5′ intronic regions and the 5′ untranslated region (UTR) of the *CD95* gene.
[d]This study [46] contrasts with the findings of three other groups, the reason for which is unclear.

aberrant expression of CD95L, which is seen frequently in malignant cells [11]. Transcriptional down-regulation of expression of CD95 is a common feature of malignancies of epithelial origin [10,11] and is a result of aberrant DNA methylation induced by oncogenic Ras [12]. In other cases, downstream elements of the CD95 signaling pathway might be deranged [2].

Whereas transcriptional silencing of *CD95* is common in epithelial malignancies, somatic mutations of the *CD95* gene are rare in these tumors (Table 1) [9]. Also, hematopoietic malignancies derived from the myeloid or T-cell lineages, or immature or pre-GC B cells are virtually devoid of mutations in the *CD95* gene (Table 1). This is, however, not the case for tumors derived from GC or post-GC B cells. Approximately 20% of B-cell lymphomas derived from (post-)GC B cells carry mutations of the *CD95* gene, the large majority of which are deleterious (Table 1) [13–16]. Such mutations are found mainly within the last exon of the *CD95* gene, which encodes the DD. These mutations act in a dominant-negative manner, probably because there is cooperativity of the receptor subunits in the trimeric receptor such that mixed complexes of wild-type and mutant CD95 are inefficient in forming a stable signaling complex [2].

'…20% of B-cell lymphomas derived from (post-)GC B cells carry mutations of the *CD95* gene …mainly within the last exon…, which encodes the death domain.'

The role of *CD95* as a tumor-suppressor gene in B-cell lymphomas is supported also by the fact that germline mutations of the *CD95* gene are associated with B-cell lymphomas in both humans and mice. *lpr* mice lacking functional CD95 develop lymphadenopathy and are prone to autoimmunity and the development of B-cell lymphomas [17]. Germline mutations of the *CD95* gene, leading to autoimmune lymphoproliferative syndrome (ALPS) and predisposing to B-cell lymphoma, have been observed in humans as well [2,18]. Straus *et al.* [18] found that germline *CD95* mutations are associated with a tenfold increased risk of B-cell non-Hodgkin's lymphoma and a 51-fold higher risk of Hodgkin's lymphoma. The incidence of other tumors was not increased significantly in this study.

Intriguingly, somatic mutations of the *CD95* gene are particularly frequent in extranodal B-cell lymphomas (Table 1). Whereas nodal, diffuse large-cell and follicular lymphomas harbor somatic *CD95*-gene mutations in 17% and 6% of cases, respectively, the percentages for extranodal disease are 50% and 75%, respectively (Table 1). In addition,

Table 2. Mutation frequencies of Ig and non-Ig genes in various B-cell subsets[a]

Gene	Mutation frequency ($\times 10^{-4}$ base pairs^{-1})			
	Naive B cells ($CD27^-IgM^+IgD^+$)	GC B cells ($CD19^+CD38^+$)	Memory B cells ($CD19^+CD27^+$)	Refs
IgV_H	<0.5	650	750	[21]
IgV_k	<0.5	450	400	[21]
$IgV\lambda$	<0.5	500	550	[21]
$IgC\gamma$	<0.5	<0.5	<0.5	[22]
TBP	<0.5	ND	<0.5	[22]
c-Myc	<0.5	ND	0.7	[22]
Survivin	<0.5	ND	0.6	[22]
Bcl-6	<0.5	7.5	12.2	[23]
CD95	<0.5	2.5	3.1	[24]
$IgC\kappa$	<0.5	<0.5	<0.5	[25]

[a]Abbreviations: GC, germinal center; ND, not determined.

the presence of *CD95*-gene mutations in a B-cell lymphoma is often linked to pre-existing chronic autoimmune disease (e.g. chronic lymphocytic thyroiditis) [14,15]. The findings of these studies might indicate that deleterious mutations of the *CD95* gene can enable a pre-malignant autoreactive B cell to survive in extranodal GC-like structures until further transforming events take place. Such ectopical GCs are found frequently in chronic lymphocytic thyroiditis [15]. However, it is conceivable that B-cell lymphomas that arise classically within GCs from a nonautoreactive precursor might develop independently from the presence or absence of functional CD95.

Mutations of the *CD95* gene might reflect increased mutability owing to the malignant transformation and defective DNA-repair pathways of neoplastic cells. DNA-repair deficiency, however, is a frequent feature also in many non-B-lineage malignancies [19]. That *CD95*-gene mutations are approximately tenfold more frequent in (post-)GC B-cell-derived tumors than in other malignancies [46 of 224 cases (20.6%) compared with 20 of 750 cases (2.7%)] is suggestive of a B-cell-specific mutation mechanism within the GC (Table 1). *CD95* mutations might have been acquired by the normal B-cell precursor of the tumor clone during the GC reaction.

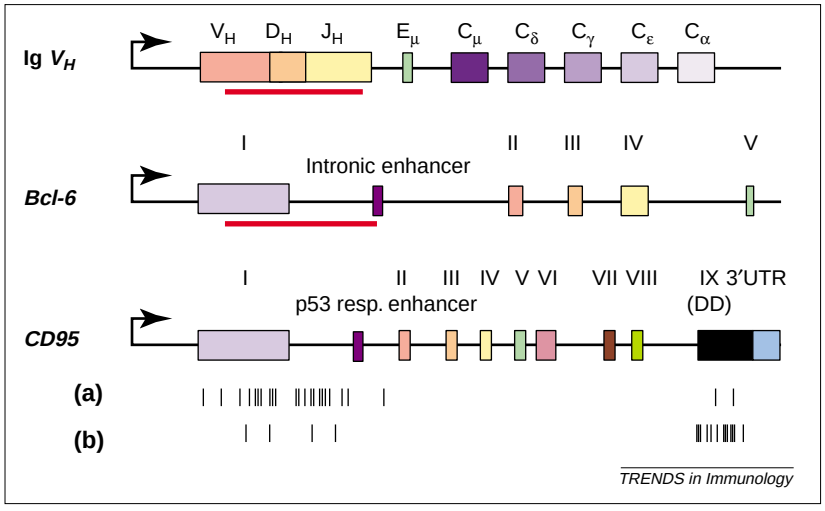

Fig. 2. Targeting of somatic mutations within Ig and non-Ig genes. The organization of the Ig heavy-chain variable-region (V_H) [after variable–diversity–joining (*VDJ*)-gene rearrangement], *bcl-6* and *CD95* loci is depicted. For the V_H and *bcl-6* loci, the window in which somatic mutations occur is indicated with a red bar. The clustering of somatic mutations within the *CD95* gene is shown for (a) single sorted germinal-center (GC) B cells and memory B cells and (b) single sorted GC B cells that have been selected *in vitro* for resistance against an agonistic anti-CD95 antibody. Abbreviations: resp., response; UTR, untranslated region.

The somatic-hypermutation machinery acting inside and outside the Ig loci

When antigen-activated B cells establish GCs and proliferate vigorously, the somatic-hypermutation process introducing somatic mutations into rearranged Ig genes is activated [20]. The mutation process targets specifically a region of approximately 1–2 kb downstream of the Ig *V*-gene promoter, including the rearranged variable (V)-region genes. Then, the GC B cells are selected for expression of a BCR with improved affinity for the respective antigen. Cells that fail to express a receptor with increased affinity undergo apoptosis. GC B cells undergo repeated rounds of proliferation, mutation and selection, acquiring an average mutation load of ~6% in the heavy-chain genes and slightly lower in the light-chain genes (Table 2) [21]. Although highly specific for Ig V-region genes, the somatic-hypermutation machinery can act occasionally outside of the Ig loci. Indeed, the *bcl-6* gene was identified as the first non-Ig gene that can become a target of somatic hypermutation (Table 1) [22,23]. Somatic mutations within the *bcl-6* gene are, however, 50–100 times less frequent than within rearranged Ig V-region heavy-chain genes (Table 2).

Mutation mechanism of the *CD95* gene

Based on the association of somatic *CD95* mutations with (post-)GC B-cell lymphomas, we wondered whether *CD95* might be another example of a non-Ig gene that undergoes somatic hypermutation in the GC. Therefore, we purified naive, GC and memory B cells of healthy donors and analyzed them separately for mutations within two distinct regions of the *CD95* gene at the single-cell level. The analysis was focused on the 5′ region of the gene (i.e. within the typical window of somatic hypermutation) (Fig. 2) and the last exon encoding the DD. In naive B cells, there was no indication of any mutation of the *CD95* gene. However, a small, but significant, fraction (one in six) of GC and memory B cells carried somatic mutations in the 5′ region of the *CD95* gene [24]. The pattern of *CD95* mutations within 5′ untranslated and intronic regions is compatible with somatic hypermutation because the mutations cluster within the typical window (Fig. 2a) and occur predominantly as transitions rather than transversions [21]. There was no significant preference for the somatic hypermutation

hotspot-motif RGYW [21]. However, the number of mutations in the analysis might have been too small to identify mutational hotspots. We conclude that mutation in the 5′ region of the *CD95* gene in B cells can occur independently from malignant transformation and is due probably to targeting errors of somatic hypermutation.

From two of 45 GC B cells (and none of 33 memory B cells), somatic mutations within the DD were amplified that were not compatible with the normal function of CD95 (one nonsense and one frame-shift mutation) owing to the dominant-negative effect of destructive monoallelic mutations within the DD [24]. To test whether these rare DD mutations in normal GC B cells can, indeed, result in resistance to CD95 signaling, viable GC B cells were challenged *in vitro* with an apoptosis-inducing anti-CD95 antibody [24]. Surviving GC B cells were sorted and analyzed for *CD95* mutations at the single-cell level. In these cells, the frequency of mutations within the *CD95* DD was increased by more than tenfold compared with the nonselected GC B cells (seven of 22 cells carried 12 DD mutations compared with two mutations in 45 cells, respectively) (Fig. 2b). All mutated anti-CD95-resistant GC B cells harbored inactivating mutations of the CD95 gene, seven of which have been identified previously in the germline of ALPS patients [18] or as somatic mutations in B-cell lymphomas [15]. Among the remaining five mutations, for which a deleterious effect has not been established so far, two deletions resulted in a frame-shift within the DD. Thus, loss-of-function mutations of the *CD95* gene can occur in normal GC B cells (Fig. 2a) and can be positively selected *in vitro* (Fig. 2b). Whereas <5% of normal GC B cells harbor deleterious DD mutations, the percentage of loss-of-function DD mutants is considerably higher in B-cell lymphomas (>20%) (Table 2).

Mutations within the DD lie outside of the somatic-hypermutation window (i.e. >15 kb downstream of the transcription initiation site). The constant regions within the Ig heavy-chain locus that are located <5 kb distant from the site of transcription initiation (e.g. Cγ) in, for example, IgG-expressing B cells are usually devoid of somatic mutations (Table 2). The observation that DD mutations are found nonetheless in (post-)GC B cells seemingly contradicts the view that these mutations were introduced by somatic hypermutation. One might, however, envision that mutations owing to the somatic-hypermutation machinery are introduced also into genomic regions beyond the typical window in close proximity to the site of transcription initiation. If such mutations occur, they are usually below the detection limit, unless they confer a survival advantage and are positively selected – as deleterious DD mutations in GC B cells. Also, a hypothetical site of transcription initiation in the proximity of the DD could explain the clustering of mutations within this region. According to a transgenic mouse model developed by Peters and Storb [25], the *Cκ*-gene segment, which is otherwise not subject to somatic hypermutation (Table 2), can acquire somatic mutations upon insertion of a promoter 5′ of this gene segment.

As an alternative, it cannot be ruled out presently that DD mutations are rare mutations due to errors of replication. These mutations might be acquired primarily by B cells in GCs because these cells proliferate extensively and also, express CD95 at high levels; there could be strong selection pressure for *CD95*-gene mutations to escape apoptosis. This situation would be reminiscent of the *p53* gene, which is mutated in a large percentage of human cancers [19]. It will be interesting to see whether B cells harboring DD mutations can be detected in humans affected by activation-induced cytidine-deaminase (AID) deficiency, which is characterized by large GCs but the absence of somatic hypermutation in GC B cells [26,27].

'…*CD95* might be another example of a non-Ig gene that undergoes somatic hypermutation in the GC.'

In contrast to normal B cells, evidence for selection in favor of loss-of-function mutants is difficult to obtain in B-cell lymphomas. In a study of the clonal evolution of nine cases of B-cell-derived Hodgkin's lymphoma, in terms of *CD95* mutation at the single-cell level, there was indication of clonal selection in favor of *CD95*-loss mutants in one case [28]. All of the Hodgkin's lymphoma cells analyzed shared clonal, somatically mutated Ig-gene rearrangements and clonal mutations of the *IκBα* gene, but were distinguished by two distinct mutations within the DD. The presence of clonal mutations of the *IκBα* gene together with two 'subclonal' mutations within the DD of the *CD95* gene suggests that inactivation of *IκBα* occurred earlier than loss of CD95 function, but *CD95* mutations were, indeed, positively selected and conferred a survival advantage [28].

Concluding remarks

We propose that *CD95* mutations in B-cell lymphomas are targeting errors of the somatic-hypermutation machinery during the GC reaction. CD95 plays a crucial role in negative selection during the GC reaction; therefore, occasional targeting errors of somatic hypermutation might interfere with the affinity maturation and counterselection of autoreactive B cells. B cells expressing a BCR with low affinity for antigen, an autoreactive BCR or no BCR at all (Fig. 1) might escape negative selection owing to inactivating mutations of the *CD95* gene. The finding that GC B cells can acquire *CD95*-gene mutations that might be involved in malignant transformation adds to the emerging picture of an important role for the GC reaction – and, in

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particular, the Ig-remodeling processes taking place in GC B cells – in lymphomagenesis [29]. Now, there is increasing evidence that not only class-switch recombination (and perhaps BCR revision) but also, somatic hypermutation, owing to its association with DNA double-strand breaks, might give rise occasionally to reciprocal chromosomal translocations that are a hallmark of many B-cell non-Hodgkin's lymphomas [30]. Moreover, a specific aberrant targeting of multiple proto-oncogenes has been reported recently for diffuse, large B-cell lymphomas, indicating a specific role for targeting errors of hypermutation in the pathogenesis of this type of B-cell lymphoma [31].

Given the role of CD95 signaling in the negative selection of autoreactive GC B cells and the frequent association of B-cell lymphomas carrying *CD95*-gene mutations with autoimmune diseases, it is intriguing to speculate that *CD95*-gene mutations might play a role not only in the pathogenesis of (primarily extranodal) B-cell lymphomas but also, in the generation of B cells producing autoantibodies that might be involved in autoimmune diseases. This idea should be tested experimentally.

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