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Chronic lymphocytic leukaemia: could immunological tolerance mechanisms be the origin of lymphoid neoplasms?

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Summary

Immunological tolerance theory in chronic lymphocytic leukaemia (CLL): we suggest that B cells that express B-cell receptors (BCR) that recognize their own BCR epitopes are viewed by immune system as 'dangerous cells'. BCR autonomous signalling may induce constant receptor editing and mistakes in allelic exclusion. The fact that whole BCR recognizes a self-antigen or foreing antigen may be irrelevant in early B cell development. In early B cells, autonomous signalling induced by recognition of the BCR's own epitopes simulates an antigen-antibody engagement. In the bone marrow this interaction is viewed as recognition of self-molecules and induces receptor editing. In mature B cells autonomous signalling by the BCR may promote 'reversible anergy' and also may correct self-reactivity induced by the somatic hypermutation mechanisms in mutated CLL B cells. However, in unmutated CLL B cells, BCR autonomous signalling in addition to self-antigen recognition augments B cell activation, proliferation and genomic instability. We suggest that CLL originates from a coordinated normal immunologic tolerance mechanism to destroy self-reactive B cells. Additional genetic damage induced by tolerance mechanisms may immortalize self-reactive B cells and transform them into a leukemia.

Keywords: anergy; chronic lymphocytic leukaemia; clonal selection theory; immunological tolerance; receptor editing.

Introduction

Much evidence has accumulated supporting the idea that neoplasms, including those of a lymphoproliferative nature, are influenced by their environment.[1] This evidence has come from the study of a range of conditions such as inflammation, bacterial and viral infections, endemic diseases and autoantigen stimulation of the immune system.[1] Immunological tolerance mechanisms, in a way similar to other stimuli, also induce the persistence of B-cell receptor changes that induce genetic instability and finally result in molecular aberrations that promote the development of a neoplasm.[2–8]

Here, we review tolerance mechanisms to prevent self-reactivity in chronic lymphocytic leukaemia (CLL) B lymphocytes and propose a hypothesis in which tolerance mechanisms play a key role in CLL development.

The objective in writing this review was to present a hypothesis about the generation of CLL in terms of the clonal selection approach. Using the same method as Burnet,[5] we try to express CLL development as a logical and linear development of the forbidden clone concept formed within the framework of clonal selection theory.

B-cell development occurs initially in the bone marrow

Stem cells differentiate into pro-B lymphocytes. These pro-B lymphocytes undergo a rearrangement of their immunoglobulin heavy chain genes and are known as pre-B lymphocytes. Subsequent rearrangement of the light chain enables the cells to express surface IgM and they becomes immature transitional B lymphocytes. When these lymphocytes leave the bone marrow they are called naive B lymphocytes. During this process several tolerance mechanisms control the survival of self-reactive B-lymphocytes.

It is interesting to note that the B-lymphocyte differentiation from pro-B to pre-B to early B to mature B lymphocyte is marked by the expression of the B-cell

receptor (BCR) on the cell surface and by constant 'education' to avoid autoimmunity. Despite the fact that unmutated CLL (U-CLL) and mutated CLL (M-CLL) lymphocytes receive the same strategies (receptor editing and anergy) to correct their self-reactive BCR, it appears that the U-CLL subset is resistant to immunological tolerance, but the M-CLL subset is not.[9–11] Finally, germinal centre exclusion[12–15] may be the censor checkpoint which divides CLL cells into M-CLL and U-CLL (germinal centre excluded B cells).

The essence of the 'forbidden clone' concept

On differentiation, a stem cell becomes a lymphocyte carrying a specific pattern of BCR. If accessible, antigenic determinants are present soon after differentiation. Any normal lymphocyte capable of reacting with them will be eliminated. If, however, the stem line has at some point undergone a somatic mutation increasing resistance, the differentiated lymphocyte can, on specific stimulation, initiate a forbidden clone.[5]

Hypothesis

CLL B lymphocytes fulfil the immunological tolerance rules proposed by Burnet and can be classified as 'malignant' forbidden clones, which suggests that CLL B lymphocytes may be generated by immunological tolerance mechanisms.

CLL B lymphocytes qualify as a malignant forbidden clone

The cause of CLL is unknown. Several cellular origins of CLL have been proposed, supporting the idea that CLL may be generated at different levels of maturation and activation. In particular, marginal zone B cells,[16] human B1 cells,[17] $CD5^+$ B cells,[18] regulatory B cells[19,20] and memory B cells[21] have been suggested as the cellular origin of CLL. Despite extensive searching it is not yet known whether there is an equivalent normal cell from which the CLL lymphocyte arises,[22–26] and it is not certain at what stage of lymphocyte maturation the cell arises, because roughly equal numbers seem to come from pre- and post-germinal centre B lymphocytes.[27] However, BCR of CLL lymphocytes demonstrate highly selected immunoglobulin heavy chain variable (IGHV) gene usage, or even very similar entire antigen-binding sites, coded by both heavy-chain[28–35] and light-chain genes.[36,37] These findings argue in favour of a limited set of autoantigens promoting the division of precursor cells and clonal evolution.[38–42] Immunoglobulin genes are rearranged with 40–50% of cases unmutated (> 98% homology with germline) and 50–60% showing somatic hypermutation (SHM). IGHV gene usage in CLL is highly selective and often associated with autoantibody reactivity.[43–45] Importantly, U-CLL and M-CLL lymphocytes derive from self-reactive B lymphocytes, despite expressing different antibody reactivity.[9]

Burnet's rules about immunological tolerance and their application in CLL B-cell development

Taking advantage of the comparison of CLL development data with a linear normal B-lymphocyte differentiation we can observe that at all stages of B-cell development there is evidence that CLL cells may be induced or promoted by the persistent action of a tolerance mechanism.

Burnet's immunological tolerance rules about generation of self BCR and autoantigens indicate that:

(1) Since an immune pattern is generated by a random process, a mechanism must exist whereby the 'self-reactive' cells that may emerge can be eliminated or functionally inhibited.[5] More than one mechanism may be needed to establish and maintain this intrinsic immunological tolerance toward self components.[5,46–58]
(2) The accessibility, amount and physiological character of the [autoantigen] plays an essential part in determining both to what extent a potentially pathogenic clone is stimulated to proliferate and what opportunity it has to damage cells and tissues in the body.[5]

These rules are reflected in CLL development because on differentiation the CLL stem cell becomes a lymphocyte carrying a specific pattern of BCR.[59] CLL haemopoietic stem cells generate aberrantly increased amounts of pro-B lymphocytes that have an intrinsic propensity to generate clonal CLL-like B lymphocytes with normal karyotypes.[59] In pro-B lymphocytes, DH-JH joining precedes VH-DJH rearrangement, followed by VL-JL joining in the late-stage pre-B lymphocytes. Pro-B lymphocytes derived from patients with CLL are also altered because they continue to rearrange heavy chains and some of them fail to induce allelic exclusion.[60] Intriguingly, CLL B lymphocytes express BCR that recognize their own BCR and also have an autonomous signalling capacity,[61] in a way similar to the pre-B-cell receptor expressed in pre-B lymphocytes.[62,63] These BCR are continuously accessible to antigenic determinants present in their own BCR.[61] This persistent stimulus induces an early positive selection and promotes their differentiation.[62] We suggest that this early B-cell stage BCR (whether self-reactive or not) can be viewed as 'dangerous BCR' because CLL B cells try to delete or correct it. These new B lymphocytes, capable of reacting with their own BCR (internal self antigen), will be eliminated or silenced.[63,64] However, this forbidden B lymphocyte is resistant to tolerance mechanisms such as receptor editing because its heavy and light chains,[64] frequently used by its

BCR, are similar to heavy chains that cannot be silenced by receptor editing.[31,36,37,50] For this reason, CLL B lymphocytes are in constant receptor editing[65] and some of them have a lack of allelic exclusion.[60] This suggests that CLL B lymphocytes retain the BCR autonomous signalling capacity acquired during the pre-B-cell development in both U-CLL and M-CLL.[61] This is also supported by the fact that CLL B lymphocytes recognize galectin-1, a ligand of pre-B BCR in pre-B cells.[66,67] CLL B lymphocytes also fail to inhibit the autonomous signalling capacity of the BCR induced by the production of conventional light chain.[61,64]

Burnet's immunological tolerance rule about 'points of decision' such as anergy indicates that:

> There are many genetically determined anomalies in the functioning of the immune system. It is reasonable to suggest that it functions by shifting the 'point of decision', determining whether a lymphocyte reacting with its corresponding determinant will be stimulated to proliferation and functional activity or will be destroyed or functionally inhibited.[5]

Chronic lymphocytic leukaemia was considered to be a tumour of circulating B lymphocytes, variably stimulated and anergized following exposure to antigen in lymphoid tissue.[68] This is in concordance with our hypothesis that CLL B lymphocytes are persistently under check by a tolerance mechanism because they are chronically exposed to self antigens (or their own BCR).

The low expression of BCR is the hallmark of CLL and anergic B lymphocytes. Despite their initial resistance to receptor editing, these CLL B lymphocytes are forced to engage with the environment as a test of

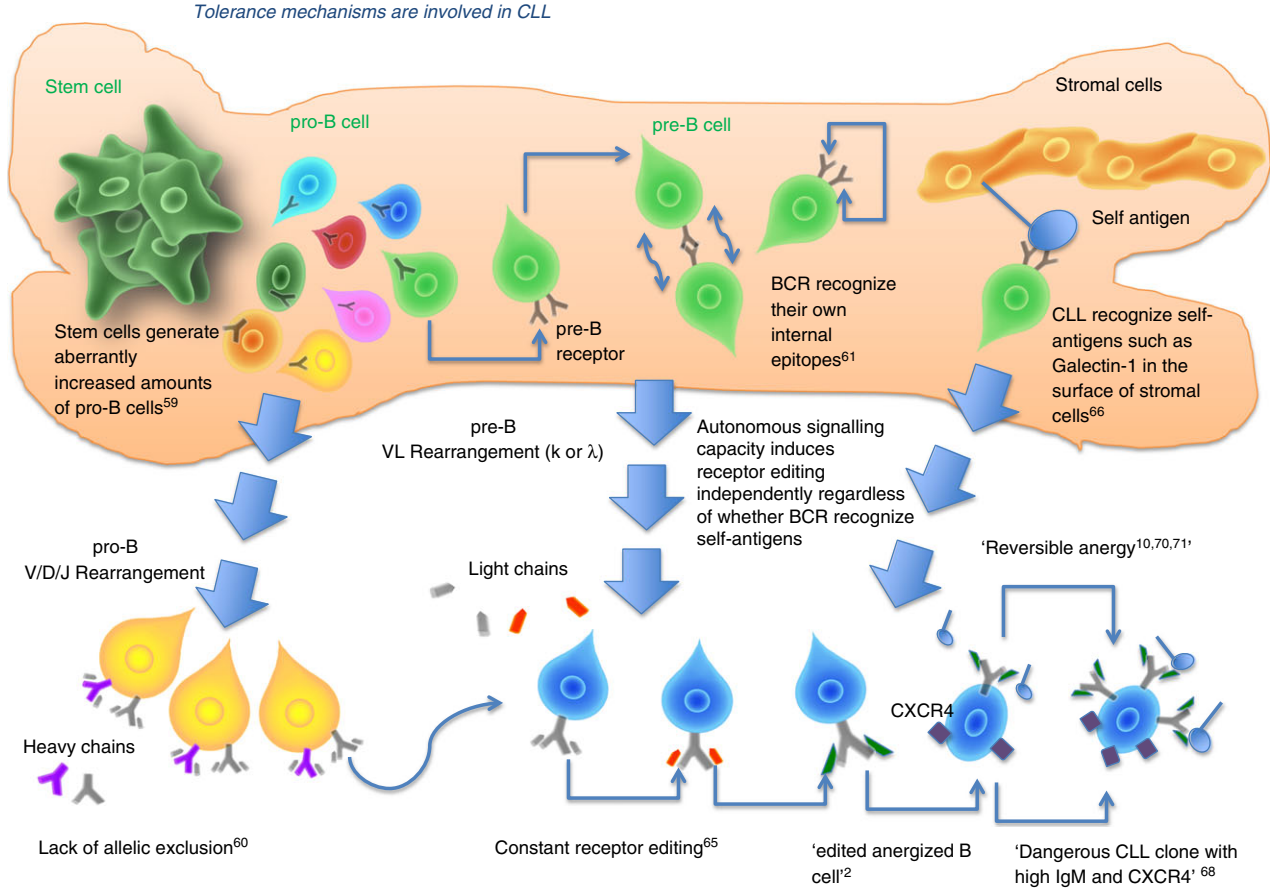

Figure 1. Tolerance mechanisms are involved in chronic lymphocytic leukaemia (CLL). Stem cells from patients with CLL produce an increased amount of pro-B cells. These pro-B cells undergo tolerance in bone marrow. Pro-B lymphocytes derived from patients with CLL continue to rearrange heavy chains and some of them fail to induce allelic exclusion. Some pro-B cells mature into pre-B cells that express B-cell receptors (BCR) that recognize their own BCR and also have an autonomous signalling capacity. These BCR (self-reactive or not) with autonomous signalling capacity induce constant receptor editing, a mechanism involved in inducing immunological tolerance. Despite active receptor editing, B cells that express BCR with autonomous signalling capacity are viewed by the immune system as 'dangerous cells' and suffer different degrees of 'reversible anergy'. These mechanisms are common in monoclonal B-cell lymphocytosis, unmutated CLL (U-CLL) B cells and mutated CLL (M-CLL) B cells. However, M-CLL B cells may be expressing a profound level of anergy compared with U-CLL B cells. Importantly, M-CLL can correct their initial self-reactive BCR during central tolerance mechanisms in bone marrow. U-CLL B cells remain self-reactive B cells.

self-reactivity (negative selection),[67,69] becoming 'edited, anergized CLL B lymphocytes'.[10,70,71] However, there is a difference between BCR signalling in U-CLL and M-CLL.[10] The molecular signature of anergy has been detected in both U-CLL and M-CLL.[10,70,71] In Fig. 1 we provide a schematic representation that summarizes several central tolerance mechanisms involved in the development of CLL B cells and monoclonal B-cell lymphocytosis.

Burnet's immunological tolerance rules about 'resistance to elimination' indicate that:

(1) Changes due to somatic mutation or to physiological factors may render a newly differentiated lymphocyte (the antigen-reactive cell) non-susceptible to elimination or inhibition by the 'censorship' mechanisms concerned. As in virtually every statement one can make in an immunological context, the word 'non-susceptible' is not an absolute but simply refers to a range of susceptibility below the level, determined by genetic factors, of the normal newly differentiated lymphocyte.[5]
(2) When, through somatic mutation, a lymphocyte line develops that has an undue resistance to elimination by antigenic contact, and reacts specifically with an accessible body component functioning as antigenic determinant, it is potentially capable of initiating a forbidden clone of directly or indirectly pathogenic cells. As such, they will have all the essential characteristics of a conditioned malignancy.[5]

Self-reactive B lymphocytes decrease from 75% in immature B lymphocytes in the bone marrow to 20% in the mature naive B-lymphocyte population in healthy individuals.[47] Lymphocytes require very low affinity binding to self antigens to remain viable in the periphery. Interestingly, aberrations in PTPN22 over-expression in CLL represent a protective mechanism that allows autoantigen-activated CLL cells to escape from negative selection and tolerance mechanisms.[72] Importantly, decreased BCR signalling is sufficient to alter the removal of developing self-reactive B cells,[56,73] on the one hand, and PTPN22 alterations might induce a failure in central tolerance in healthy donors,[73] on the other. One of the most intriguing phenomena related to immunological tolerance is that sometimes a mechanism created to protect against self-reactivity leads to damage and promotes malignancy ('horror autotoxicus').[3,6–8]

Several lines of evidence suggest that normal cells that become CLL lymphocytes progress through various stages before becoming overtly leukaemic. Healthy individuals may show monoclonal or oligoclonal B-cell expansions with the characteristic phenotype of CLL in about 3·5% of tested subjects > 40 years old, and the estimated rate of progression of monoclonal B-cell lymphocytosis to CLL requiring treatment is 1·1% per year.[74–76] This implies that B CLL lymphocytes (forbidden clone) undergo at some point somatic mutations or molecular aberrations as microRNA disturbances,[11,77,78] mutations in MyD88,[79] NOTCH-1[80–82] and SF3B1[83,84] or trisomy 12,[85,86] 13q deletion,[87] 11q deletion,[87] and 17p deletion,[87] increasing their resistance to being eliminated. Interestingly, karyotype evolution has been reported during the long-term follow up of patients with untreated early-stage CLL.[76,88] We suggest that genetic programmes are progressively disrupted during B-cell development and tolerance checkpoints. Combinations of disrupted genetic programmes originate different ways to make a CLL-like clone. These new 'edited anergized' B CLL lymphocytes continue with their differentiation and can, on specific stimulation, initiate a process of forbidden clone proliferation.

Burnet's immunological tolerance rule about 'autoantibodies' indicates that:

> It is necessary for survival that neither lymphocytes nor antibodies which are reactive to more than a minimal degree with any accessible body component should exist in the body.[5]

Within germinal centres, naive B lymphocytes undergo activation, proliferation, SHM, isotype switching and subsequent positive and negative selection by antigen. Somatic mutations are introduced at a high rate in the germinal centres, and are typically involved in single nucleotide exchanges.

An important peripheral checkpoint is entry into the T-lymphocyte-dependent, long-term memory compartment. Lymphocytes that express antibodies encoded by the VH4-34 gene are self-reactive B cells.[12,13] B lymphocytes that express self-reactive BCR encoded by the IGHV4-34 are excluded from T-lymphocyte-dependent IgG memory and plasma cell populations, suggesting that these autoreactive lymphocytes fail to cross a developmental checkpoint following activation in normal individuals.[12,13] This implies that negative selection of autoreactive cells occurs at the transition of naive to germinal centre cells. The comparison of the methylation patterns between B-cell populations and CLL B cells leads to the conclusion that malignant U-CLL cells are related to pre-germinal (germinal-centre-excluded?) B cells, whereas M-CLL lymphocytes probably stem from germinal-centre-experienced B cells [24] (memory B cells?).

Remarkably, 79·3% of U-CLL antibodies are polyreactive and some of them recognize apoptotic cells, IgG, thyroglobulin, DNA, actin, cardiolipin and other nuclear antigens.[9,38–41] These features of U-CLL are in concordance with the negative selection at the transition from naive to germinal centre cells and suggest that U-CLL lymphocytes are excluded from germinal centres. In Fig. 2 we provide a schematic overview of

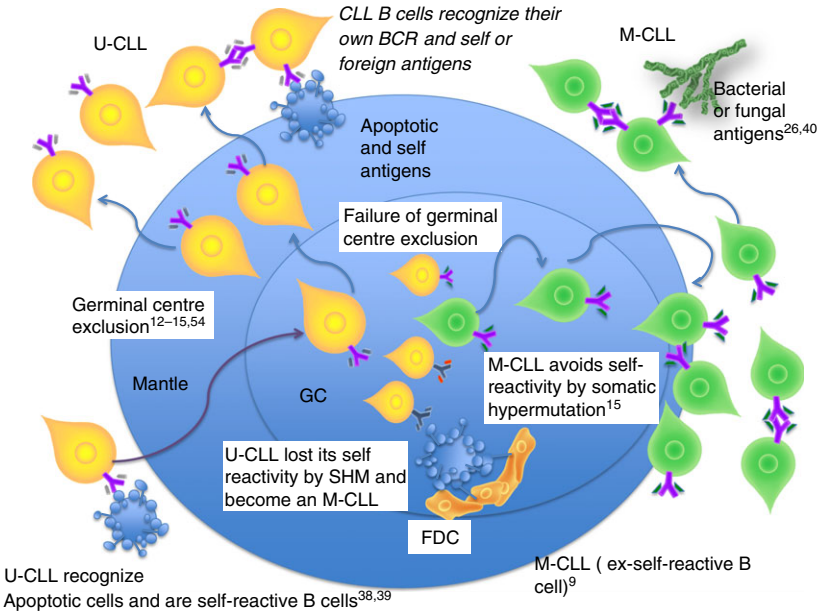

Figure 2. Tolerance mechanisms in the germinal centre are involved in chronic lymphocytic leukaemia (CLL). Germinal centre exclusion of self-reactive B cells maintains the unmutated CLL (U-CLL) subset without somatic mutations in their heavy and light chains. However, if a failure of germinal centre exclusion occurs, somatic hypermutation may correct the initial self-reactive B-cell receptor (BCR). Newly generated ex-self-mutated CLL may recognize their own BCR to avoid apoptosis and/or recognize new antigens (self or foreign).

the genesis of the U-CLL B-cell subset and M-CLL subset.

Mutated CLL lymphocytes appear to have undergone the normal process of SHM and antigen selection in the germinal centres before transformation and might be considered ex-self-reactive B cells.[9] Although receptor editing[65–67] and anergy[10,70,71] may play a role in the tolerance of M-CLL cases, the current hypothesis is that M-CLL avoids self reactivity by SHM.[78] This implies a failure of germinal centre exclusion of self-reactive B cells,[12–15] or the generation of new self-reactive B cells during SHM.[52,53] In the first scenario a self-reactive B cell (U-CLL) loses its self-reactivity when passing through the germinal centre reaction by SHM.[89] This is important because point mutations can both correct self-reactive BCR and abolish autonomous signalling of CLL BCR. In the second scenario, new self-reactive B cells could avoid self recognition by anergy and heavy-chain or light-chain editing. Both contexts result in an ex-self-reactive M-CLL cell. Figure 3 summarizes the different processes of M-CLL subset genesis taking into consideration the origin of B cells (non-self B cells, ex-self B cells or self B cells that produce natural autoantibodies).

Tolerance mechanisms involved in the development of memory B cells

B cells expressing self-reactive antibodies and antibodies with broad specificity for bacteria are removed from the repertoire in the transition from naive to IgM^+ memory B cells.[15] However, rare IgM^+ memory B cells that produce antibodies with low levels of self reactivity acquire this reactivity by SHM.[15] In a similar way, autoreactivity in human IgG^+ memory B cells is mostly created by SHM.[54] The fact that CLL B cells undergo isotype switching with and without acquiring somatic mutations suggests that germinal centre chekpoints may be disrupted in CLL patients.[30–35,40] Some BCR of M-CLL B cells could bind carbohydrate determinants of bacterial capsules, viral coats and certain autoantigens.[25,32–34,42,54] These facts indirectly support a failure in tolerance mechanisms within germinal centre checkpoints.[32–34,40,42,54]

M-CLL B lymphocytes might also be generated during a normal immune response against some pathogens without any stigma of self reactivity. Recently, a new subset of M-CLL lymphocytes expressing BCR highly specific for a major antigenic determinant of yeasts and filamentous fungi has been reported.[26] Importantly, this subset represents > 0·3% of CLL and is not associated with germline self reactivity.[26]

Burnet's immunological tolerance rule about 'self-reactive T cells and autoantibodies' indicates that:

> In all probability, the clinical manifestations of autoimmune disease are due to aggressive T lymphocytes producing damage to target cells; to the deposition of antigen–antibody complexes elsewhere; and to a directly damaging action of an antibody, but probably only when the autoantigen is present in the circulating blood either in cells or as a soluble component.[5]

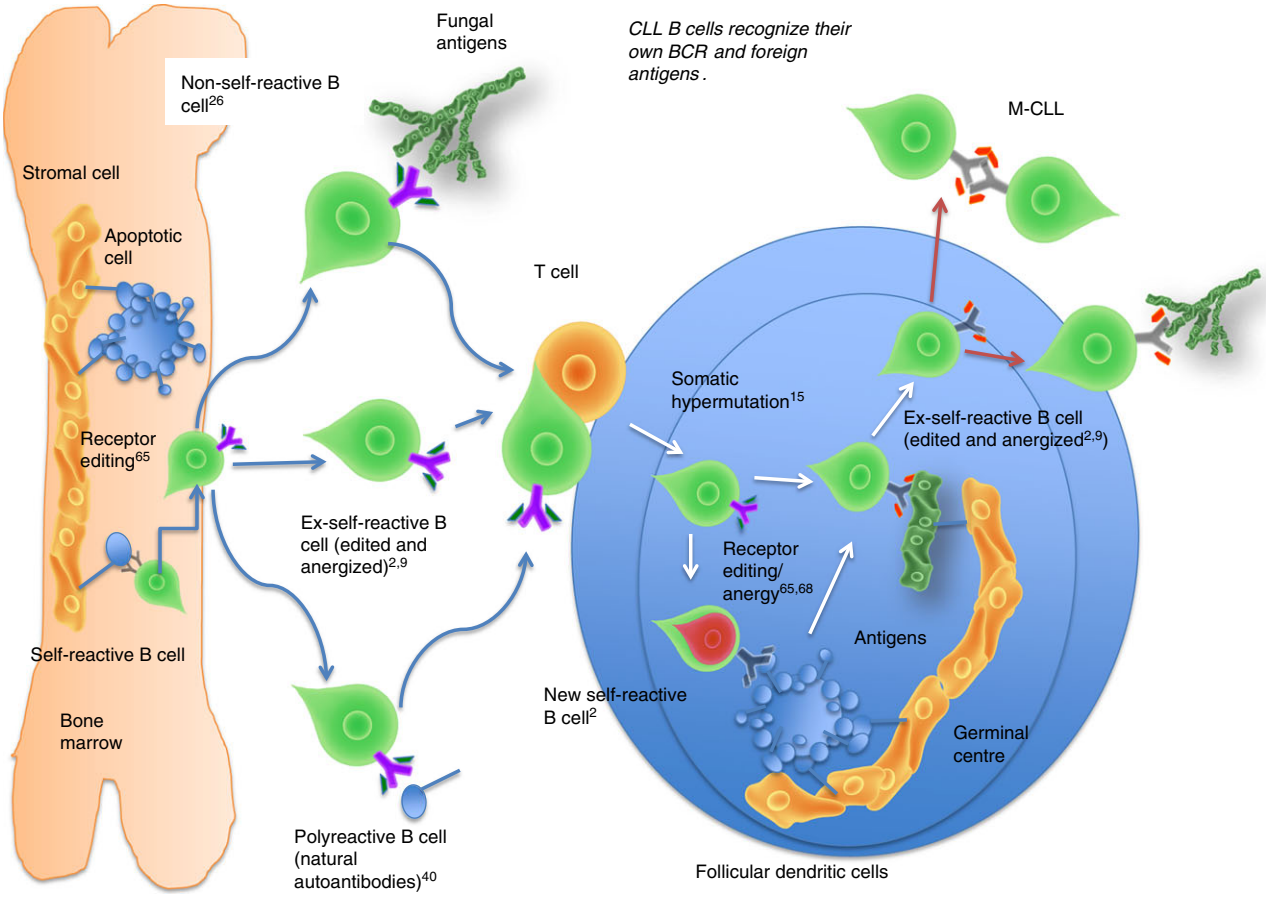

Figure 3. Immunological consequences of developing B-cell receptors (BCR) that recognize their own BCR epitopes. Non-self-reactive B cells, ex-self-reactive B cells and B cells that encode natural autoantibodies may also be an origin of mutated chronic lymphocytic leukaemia (M-CLL) cells if these cells previously acquire BCRs that recognize their own BCR in the bone marrow. When they enter a normal immune response they may acquire self reactivity during the somatic hypermutation process. However, persistent receptor editing and anergy induced by their 'special BCR' with autonomous signalling capacity may correct their recently acquired self-reactive BCR during somatic hypermutation within the germinal centre.

Indeed, although the BCR of CLL B cells may be non-self-reactive, BCR of CLL B lymphocytes may be viewed for the immune system as a self antigen in an early developmental stage because these 'autonomous signalling BCR' recognize their own BCR internal epitopes.[61] It is improbable that an immune attack against this epitope would induce an autoimmune disease.[5] In theory, however, an attack against their self BCR may increase their own BCR stimulation; induce chronic activation of mechanisms of tolerance as receptor editing; and promote BCR surface elimination with the consequent low surface IgM detected in CLL B lymphocytes.

The immunodeficiency of CLL

All patients with CLL have a degree of immunodeficiency,[90,91] which may be a direct consequence of leukaemic cell action. The CLL lymphocytes themselves can induce profound immunosuppression by direct contact or through cytokines such as transforming growth factor-β and interleukin-10 (IL-10).[20,92] Direct contact with CLL lymphocytes can induce synapse inhibition in previously healthy T lymphocytes,[93,94] and probably may also promote the induction of regulatory T cells.[95–97] Remarkably, CLL B lymphocytes may kill plasma cells during cell–cell contact.[98] Over-expression of CAV1 in CLL B lymphocytes from lymph nodes increases the capacity to interact with T lymphocytes and promotes an immunosuppressive environment.[99] Once T-cell functions are blocked or disturbed[100,101] the rest of the immune system also fails.[102] Decreased immunoglobulin synthesis develops in about 85% of patients and > 50% suffer recurrent infections. Surprisingly, the innate immune system also has defects in natural killer cells,[103] phagocytic cells[104] and complement system.[105] Finally, low levels of immunoglobulin and complement may decrease the clearance of self antigens (apoptotic blebs), with the subsequently increased risk of autoimmunity and CLL disease progression.[2,106,107]

The role of transcription factors in CLL development

B-cell ontogeny-determining transcription factors may also support the hypothesis about several cellular origins of CLL B cells. As mentioned before, B-cell differentiation is typically viewed as a linear process as defined by regulated expression of specific sets of transcription factors and BCR expression.

PAX5

Haemopoietic stem cells (HSC) are capable of developing into all the blood cell types. B cells are continually generated from HSC in the bone marrow. Initial commitment to the B-cell lineage requires activation of a series of transcriptional factors. At the nuclear level, the transcription factors PU.1, Ikaros, E2A, early B-cell factor (EBF) and PAX-5 play major roles in committing progenitor cells to the B-cell lineage.[108] However, after lineage commitment has been established, it is the composition of the BCR that controls further development, as mentioned above. Throughout B-cell development, precursor B cells produce $CD19^+$ pro-B cells that are irreversibly committed to becoming B cells, due to expression of PAX-5. PAX-5 is a paired-box transcription factor which, among the progeny of HSC, is expressed exclusively in cells of the B-cell lineage. CD79A and PAX-5 appear at the time of heavy chain gene rearrangement. Importantly, PAX-5 is expressed at higher levels in U-CLL B cells, when it is compared with normal B cells and M-CLL B cells.[109] Interestingly, PAX-5 mRNA decreases in plasma B cells, suggesting that the lower level of PAX-5 in M-CLL cells may be a sign of developmental block before M-CLL cells become plasma B cells.

Helix-loop-helix transcription factors E2A and EBF

E2A codes two transcription factors E12 and E47, members of the basic helix-loop-helix family, and its induction is crucial from the earliest stages of B-cell lineage development. E12 is a better activator of EBF and PAX-5 and E47 play a greater role in driving TdT and RAG, implicating this transcription factor in the process of chromatin remodelling of the immunoglobulin heavy chain locus that permits accessibility by the recombinase machinery.[108,110] Interestingly, in CLL B cells E2A is elevated at the mRNA and protein levels compared with normal B-cell subsets.[111] This finding is consistent with the circumstance that CLL B cells have constant BCR rearrangement and (auto)antigen-driven receptor editing[65] to avoid self reactivity or autonomous BCR signalling.[61] Moreover, receptor editing in marginal zone B-cell development is regulated by E2A.[112] E2A proteins are required to regulate secondary gene rearrangement in B cells that express an autoreactive BCR, due in part to activated RAG expression and also because E2A proteins are required to promote developmental progression of autoreactive B cells.[112] Moreover, this circumstance may explain the increased ability to activate secondary immunoglobulin light chain gene rearrangement[65] in CLL. However, this increased receptor editing is not enough to induce IgL gene rearrangement that will permit surface immunoglobulin expression without autonomous signalling.[61] Importantly, E2A binds to a large subset of genes involved in pre-BCR signalling,[113] supporting the notion that links autonomous BCR signalling[61] in CLL B cells with normal pre-B cells.[62–64]

Early B-cell factor-1 (EBF1) is a helix-loop-helix like transcription factor. EBF1 is expressed at all stages of differentiation except in plasma cells and may be critical in the progression of B cells past the early pro-B cell stage. Significantly low levels of EBF1 in both M-CLL and U-CLL cells compared with $CD5^+$ or conventional B cells have been reported.[18] EBF1 is also a repressor of the transcription factor TCF7.[113] TCF7 is known to be a T-cell-specific transcription factor required for T-cell development. Importantly, high expression of TCF7 is associated with prolonged survival times and M-CLL.[114] Importantly, the low expression of EBF1 may lead to reduced levels of numerous B-cell signalling factors, contributing to an anergy signature of CLL cases.[18] Taken together it is possible that the low levels of EBF1 and high expression of TCF7 contribute to the diminished response of BCR to stimulus and are associated with prolonged survival of patients with M-CLL.

Transcription factors control pro-B and pre-B cell development

PU.1 is an ETS family loop-helix-loop (winged helix) transcription factor. PU.1 regulates a number of B-cell-specific genes including CD79a, joining chain, μ chain, κ chain, λ chain, RAG and TdT.[115,116] Significantly, PU.1 is poorly expressed in CLL B cells compared with $CD5^+$ B cells from normal blood, control tonsils and non-CLL B-cell lymphomas.[117] Interestingly, in pro-B cells specifically deleted for PU.1 these genes continue to be expressed.[118,119] We suggest that low levels of PU.1 may contribute to the increased BCR rearrangements[65] and lack of allelic exclusion[60] detected in CLL B cells. PU.1 directly activates the expression of EBF1.[120] Importantly, low levels of EBF1 detected in CLL B cells are in concordance with the low levels of PU.1 observed in CLL B cells. Such low levels of both PU.1 and EBF1 in B cells do not compromise their ability to establish an immune response upon BCR stimulation.[119] The recent finding that PU.1 is a potent tumour suppressor in classical Hodgkin's lymphoma cells[121] suggests that low expression of PU.1 in CLL B cells might also play a role in the progression of disease.

LEF-1 is specifically expressed in pro-B and pre-B cells.[122,123] Surprisingly, monoclonal B-cell lymphocytosis and both M-CLL and U-CLL B cells express LEF-1.[124,125] Interestingly, pro-B, pre-B and CLL B cells share the expression of LEF-1 as a pro-survival factor. We speculate that some CLL B cells derive from pro-B cells taking into consideration that some CLL B cells lack allelic exclusion[60] and express LEF-1.[124,125] In the same line of thinking some CLL B cells derive from pre-B cells because they share with pre-B cells the expression of LEF-1[124,125] and an autonomous BCR signalling similar to pre-BCR.[62,64]

ID-1 and ID-2 have a helix-loop-helix domain, but lack a DNA binding domain. Hence, they can function as a dominant negative factor, inhibiting the function of helix-loop-helix transcription factors such E2A. ID-1 is expressed only in pro-B cells. However, pre-B cells in adults, showed highly up-regulated expression of ID-2, in the absence of changes in expression of E2A[126] in bone marrow. Over-expression of ID-2 in follicular lymphoma cells may regulate proliferation or contribute to the maturation arrest in these tumour cells.[127] In M-CLL B cells ID-2 expression is high but may be down-regulated after stimulation in a similar manner to normal B cells.[109] However, in U-CLL B cells, high expression of PAX-5 and lower levels of ID2[109] are associated with constitutive expression of activation-induced cytidine deaminase (AID). Importantly, U-CLL B cells are frequently self-reactive and more prone to acquire more genetic lesions than M-CLL B cells. Interestingly, AID is required for the establishment of both central and peripheral B-cell tolerance.[128,129] Taken together it is possible that low expression of ID-2 contributes to constitutive expression of AID[130,131] and reflects the self-reactive nature of U-CLL B cells.

FOXP1 is a forkhead transcription factor involved in the control of V/D/J recombination of the genes encoding immunoglobulin heavy chain.

FOXP1 deficiency is associated with a block in the transition from pro-B to pre-B cells.[132] FOXP1 also regulates recombination-activating genes (RAG1 and RAG2) in B cells and in cancer cells.[133] In human B cells, FOXP1 shows the opposite expression pattern to BCL-6, suggesting that FOXP1 regulates the transition from resting follicular B cell to activated germinal centre B cell.[134] Intriguingly, FOXP1 is down-regulated in some autoreactive human naive B cells refractory to antigenic stimulation (anergic B cells).[58] However, FOXP1 expression is higher in some high-risk U-CLL B cells, especially in those which also have SF3B1 mutations[83] and deletions in chromosome 11q.[84] We speculate that this deregulation in some transcription factors such as FOXP1 may induce some resistance to U-CLL B cells to becoming anergic B cells. High expression of FOXP1 may also explain germinal centre exclusion and the lack of SHM in U-CLL B cells.[58,134]

The chromatin regulator Aiolos and the transcriptional co-activator OBF-1 have been implicated in the control of genes involved in pre-BCR function.[135] They also play a key role in the transition from pre-B to immature B cells.[136] OBF-1 has comparable levels of mRNA and protein in CLL B cells and normal blood $CD5^+$ B cells.[117] However, Aiolos is over-expressed in CLL B cells in comparison with healthy donors. Remarkably, Aiolos is also involved in cell survival.[137] In addition, OCT-2 and OBF1 transcription factors are expressed in CLL B cells at comparable levels to $CD5^+$ B cells.[117] SPI-B is also expressed in both CLL B cells and normal B cells.[138]

Transcription factors associated with marginal zone, follicular B cells and human B1 B cells

NOTCH-2 is preferentially expressed in mature B cells and supports marginal zone B-cell development and type 2 transitional B cells.[139]

Although the precursor of CLL remains elusive, marginal zone B cells have been suggested to have one normal counterpart,[16] because they display an activated B-cell phenotype, and express either mutated or unmutated IGHV genes that can be biased in usage and produce poly/autoreactive antibodies. Interestingly, the transcription factor NOTCH-2 is constitutively expressed in both U-CLL and M-CLL B cells but not in normal B cells.[80] The expression of NOTCH-2 in CLL is in agreement with marginal zone B cells as CLL B-cell precursors.

Nonetheless, marginal zone B cells may in several cases be the precursors of CLL B cells; some CLL also may derive from follicular B cells. c-Myb is critical for B-cell development and maintenance of follicular B cells [140] and is expressed also in normal pre-B cells. Interestingly, only a few CLL B cells express the c-Myb transcription factor.[141] This finding argues in favour of multiple origins for CLL B-cell development theory. The expression pattern of Myb in B cells inversely correlates with that of miR-150.[142] Interestingly, miR-150 is highly expressed in the majority of CLL tumour cells, but not in the proliferation centres.[143] M-CLL (clinically indolent) has significantly higher levels of miR-150 than U-CLL (clinically aggressive).[11,77] The mantle zone is rich in VH4-34 self-reactive B cells excluded from germinal centres.[3,12,13] Remarkably, in the mantle zone, the expression of miR-150 is high and of c-Myb is weak[144] in a similar way to stereotypical subset 4 (IGHV4-34/IGKV2-30 mutated, good prognosis).[11] However, subset 1 (IGHV1/5/7-IGKV1 (D)39, unmutated, bad prognosis) down-regulates miR-150 in a similar manner to germinal centres and proliferation centres. Notably, miR-150 null mice possess a higher proportion of B1 cells and show a significant increase in the production of 'natural' antibodies.[142] This is in keeping with the lower levels of miR-150, increased expression of ZAP-70, increased proliferative response to BCR stimulation and the suggestion that human B1 B

cells or marginal zone B cells[16,17] may be a possible origin of the U-CLL subsets.

Transcription factors control receptor editing, germinal centre formation and exclusion, class switch recombination and SHM

The transcription factor IRF4 regulates immunoglobulin class switch recombination and plasma cell differentiation. Transient expression of IRF4 induces the expression of germinal centre genes including BCL-6 and AID.[145] However, sustained and higher concentration of IRF4 promotes the generation of plasma cells.[145] IRF4 mutations in CLL induce higher IRF4 mRNA expression.[146] Surprisingly, patients with IRF4 mutations have a paradoxically good prognosis because they express high risk factors such as U-CLL B cells and trisomy 12.[146] We speculate that sustained IRF4 in self-reactive B cells may induce germinal centre exclusion and block the differentiation to plasma cells in U-CLL B cells. Importantly, increased IRF4 transcripts are associated with increased receptor editing in self-reactive pre-B cells.[55] Along the same lines, expression of MUM1/IRF4 correlates with M-CLL, post-germinal centre origin and a more favourable clinical course.[147] This suggests that only ex-self-reactive or re-educated M-CLL B cells may form germinal centres and suffer SHM.[2,13,15,54]

The differentiation of mature B cells into antibody-secreting plasma cells and memory B cells is controlled by the presence or absence of transcription factors, including BLIMP-1, BCL-6 and IRF-4.[148,149]

Chronic lymphocytic leukaemia cells are fixed in a transition stage between immature and mature resting naive B cells, which continue to express PAX-5 and do not up-regulate BLIMP-1.[138] BCL-6 is a central transcription factor that promotes the germinal centre phenotype, and while doing so, prevents plasmablast differentiation. However, in CLL B cells BCL-6 is expressed at very low levels compared with germinal centre B cells although CLL B cells have higher BCL-6 levels than memory B cells.[150] Taken together, this suggests that some tolerance mechanism prevents the transition of self-reactive B cells into autoantibody-producing plasma B cells.

The increased expression of PAX-5 in U-CLL[130] supports the notion that U-CLL cells are immature self-reactive B cells excluded from germinal centres. In M-CLL a low level of PAX-5[130] may represent the induction of the plasma cell/memory B-cell transcription programme, which leads to the down-regulation of BCL-6, but this process is not complete. We speculate that interruption of this transition from activated-IgH mutated B cells to plasma B cells represents a mechanism of control for potentially dangerous autoantibody-secreting B cells or the transition of an ex-self-reactive B cell to a 'fixed' memory B cell. Importantly, BCL-6 mutations are associated with M-CLL.

Chromosomal abnormalities in M-CLL B cells and their relationship with tolerance

Deletion at 13q

The commonest abnormalities include del 13q in > 50% of patients and are usually associated with good prognosis.[87] Deletion of 13q14.3 contains two micro-RNAs, miRs15a and 16-1.[78] Interestingly the miR15/16 cluster controls the production of BCL-2, an anti-apoptotic factor produced at high levels in CLL B cells. This suggests that miRNAs can act as tumour suppressors. Deletions at 13q often have leukaemic CLL clones with mutated IGHV genes.

MyD88

Whole genome sequencing demonstrated that recurrent mutations in some genes are more frequent in patients with M-CLL. Two types of mutations in MyD88 have been described in CLL; an activating mutation (MyD88 L265P) and an inactivating mutation (MyD88 E52DEL).[88]

Interestingly, IRAK.4/MyD88 complexes play a major role in the establishment of central B-cell tolerance in the bone marrow by counterselecting developing autoreactive B cells.[57] Defects in receptor editing contribute to persistence of self-reactive B cells in patients with MyD88 deficiency.[57] We speculate that patients with CLL clones with an inactivating mutation (MyD88 E52DEL) have self-reactive B cells that are inactivated by anergy or SHM and not by receptor editing. This is in accordance with the fact that M-CLL are ex-self-reactive B cells. However, in M-CLL with the activating mutation (MyD88 L265P), receptor editing may be increased with the subsequent abolition of self reactivity.

Another important consequence in M-CLL with activating mutation (MyD88 L265P) is the function of Toll-like receptors (TLR). Most self-reactive B cells are normally counterselected during early B-cell development and TLR7, TLR8 and TLR9 may prevent the recruitment of developing self-reactive B cells in healthy individuals. TLR9 promotes tolerance by restricting the survival of anergic B cells with specificity for DNA.[151] Activating MyD88 mutation enhances the response of TLR to their ligands.[88] Even more, IL-10 production by B cells stimulated by contact with apoptotic cells results from the engagement with TLR9.[152] Interestingly human circulating $CD27^+$ B cells respond to DNA-bearing apoptotic cells secreting IL-10.[84]

These facts suggest a role for MyD88 mutations in the development of M-CLL and their similarities with IL-10 regulatory B cells. Interestingly, TLR9 stimulation can also induce apoptosis in M-CLL and proliferation in U-CLL.[153] This may be explained by the fact that TLR9 stimulation after BCR triggering can also increase BCR-initiated activation. The synergic stimulation of TLR9 and BCR might be

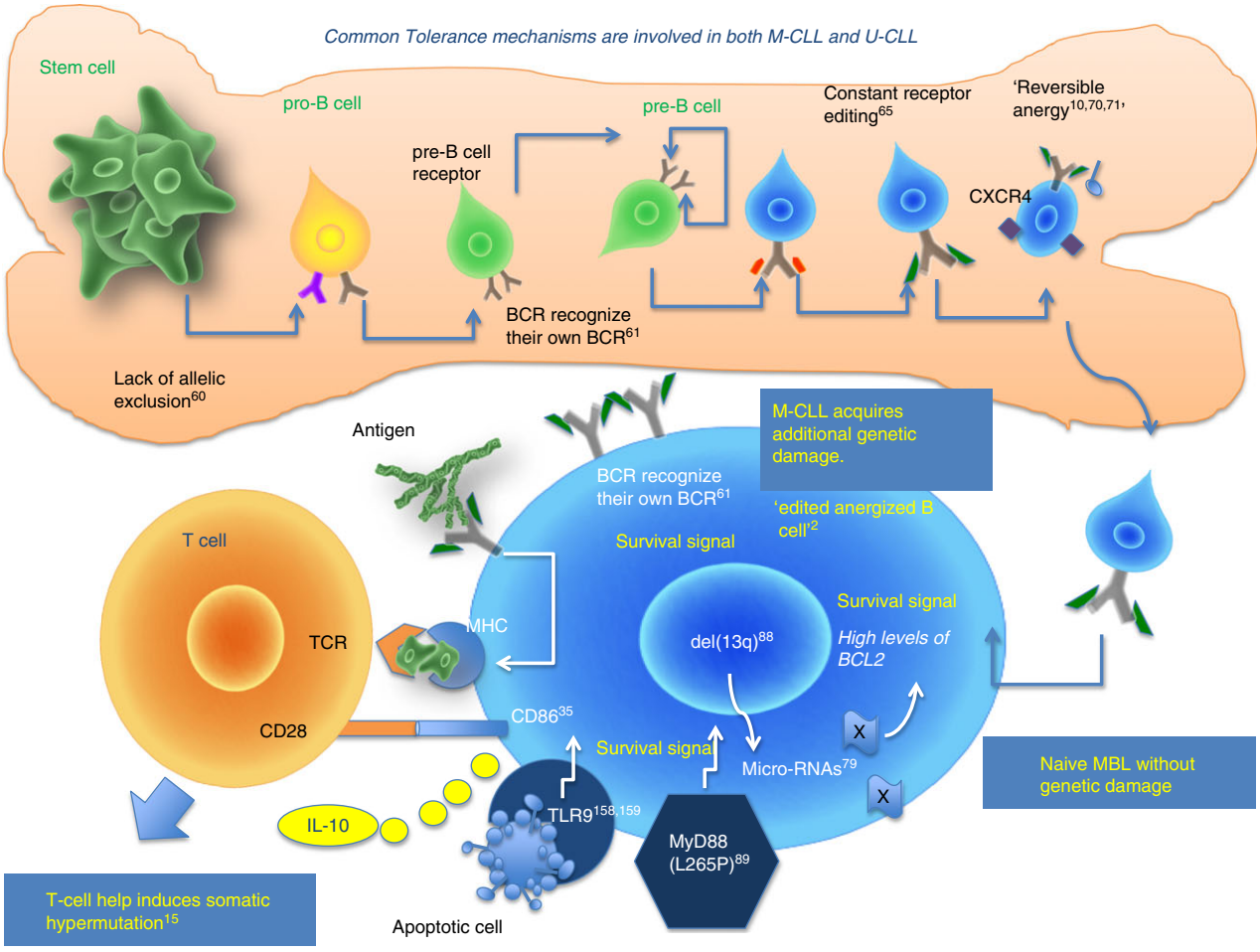

Figure 4. Mutated chronic lymphocytic leukaemia (M-CLL) lymphocytes have molecular and genetic disturbances that promote survival and tolerance. Deletion of 13q14.3 contains two micro-RNAs, miRs15a and 16-1, involved in the control and the production of BCL-2, an anti-apoptotic factor produced at high levels in CLL B cells. Activating MyD88 mutation enhances the response of Toll-like receptor (TLR) to their ligands. Immunosuppressive cytokines such as interleukin-10 (IL-10) may be induced in M-CLL B cells by the engagement of TLR9 with apoptotic cells. TLR9 stimulation induces CD86 expression in M-CLL. The interaction of CD86 with CD28 provides co-stimulatory signals for T cells activated through the T-cell receptor. These interactions may promote survival of M-CLL B cells but also promote tolerance and B-cell maturation arrest. Interactions of B-cell receptors (BCR) that recognize their own BCR epitopes may promote survival and prevent apoptosis.

present only in U-CLL because M-CLL cases involve mainly anergic B cells. It is possible that signalling through mitogen-activated protein kinase and nuclear factor-κB, which are downstream of both TLR9 and the BCR, promote proliferation in U-CLL B cells; however, in M-CLL B cells promote apoptosis and IL-10 secretion.

Importantly, TLR9 stimulation induces CD86 expression preferentially in M-CLL.[35] The interaction of CD86 with CD28 provides important co-stimulatory signals for T cells activated through CD3/T-cell receptor. Furthermore, stimulation of TLR9 in concert with the secretion of various chemokines and IL-10 from M-CLL cells contribute to the impaired synapses, T-cell exhaustion and generation of an increased amount of regulatory T cells in patients with CLL. In Fig. 4 we provide a schematic model of M-CLL subset genesis and the role of TLR, MyD88 mutations, del13q, microRNAs and immunological tolerance.

Chromosomal abnormalities in U-CLL B cells and their relationship with tolerance

Trisomy 12 and NOTCH-1 mutations

Trisomy 12 is found in about 15% of CLL patients. Interestingly, the NOTCH-1 mutations are associated with trisomy 12 and U-CLL cases.[80–82,88] These mutations in NOTCH-1 result in the accumulation of an active protein isoform in CLL B cells, and these patients appear to have a worse clinical course.

NOTCH-1 is expressed throughout normal B-cell development and in leukaemic B-cell lineage cells and

may regulate human B-cell development. The NOTCH ligand Delta is expressed in the bone marrow B lineage cells and the NOTCH ligand Jagged-1 is expressed in bone marrow stromal cells.[154] The NOTCH-1–Delta interaction promotes the differentiation of B lymphocytes and co-engagement of NOTCH and the BCR results in increased activation of the mitogen-activated protein kinase pathway.[155] It is possible that NOTCH-1 ligands also contribute to the development of CLL.

Deletion at 11q and SF3B1 mutations

The approximately 20% of patients who exhibit this deletion often present beyond Binet A stage and follow an aggressive disease course with bulky adenopathy and diminished survival.[87] These patients have an increased proportion of U-CLL clones. Del 11q22-q23 in most cases affects the ataxia telangiectasia mutated (ATM) gene, the deficiency of which causes genomic instability. Interestingly, mutations in the SF3B1 gene are associated with del(11).[83,84] The SF3B1 gene regulates the alternative splicing programme of genes controlling cell cycle progression and apoptosis.[83,84]

We suggest that once an autoreactive lymphocyte acquires a chromosomal abnormality induced by the tolerance mechanisms, it may be considered a malignancy of self-reactive B cells. It is possible that the criteria to differentiate a benign proliferation of self-reactive B cells as monoclonal B-cell lymphocytosis[76] from leukaemia may be the presence of chromosomal or genetic abnormalities. In Fig. 5 we provide a schematic model of U-CLL subset genesis and the role of TLR, NOTCH and FOXP1 mutations, del11q, and immunological tolerance mechanisms that promote chromosomal instability and progression.

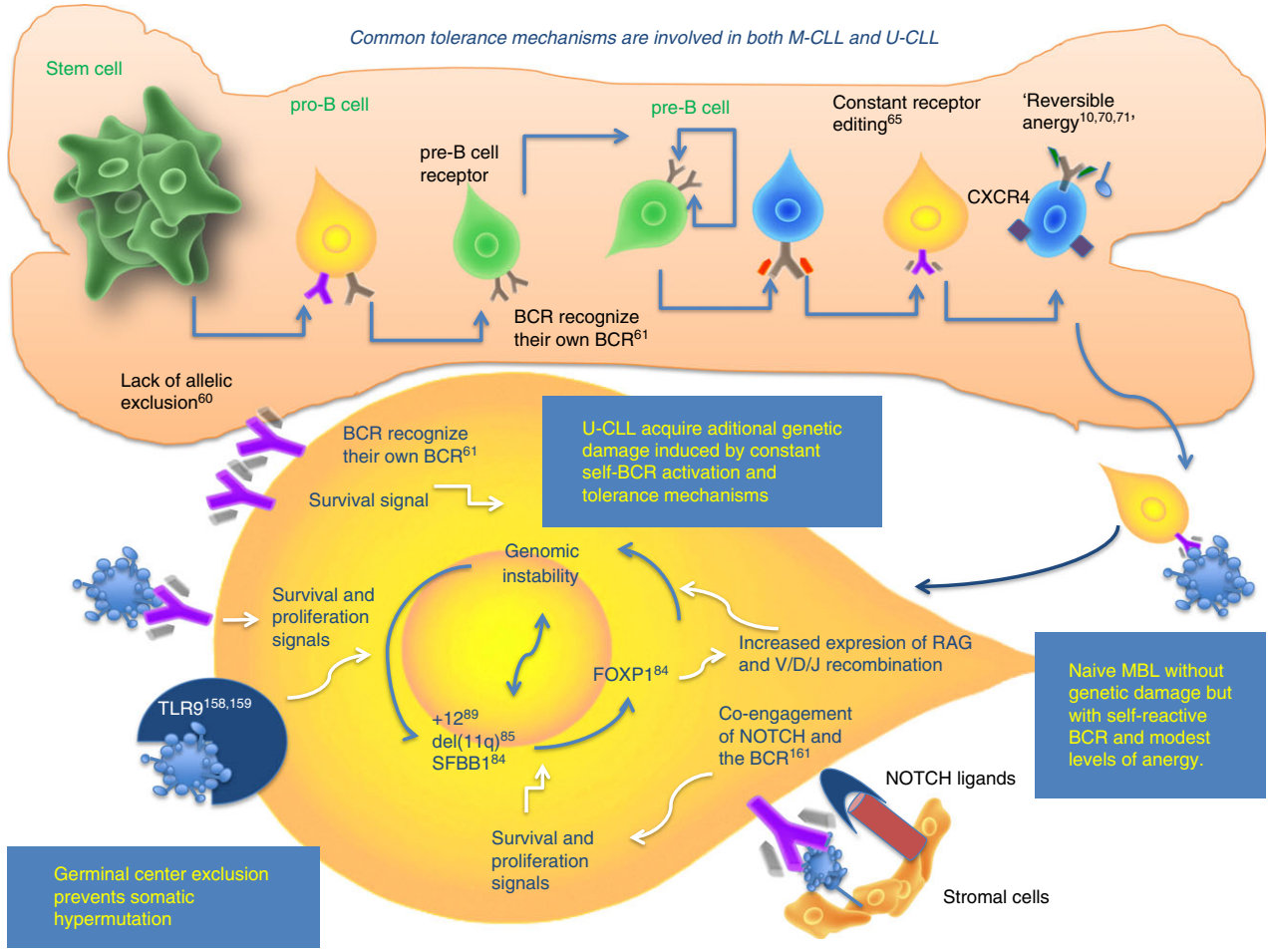

Figure 5. Unmutated chronic lymphocytic leukaemia (U-CLL) lymphocytes have molecular and genetic disturbances that promote survival, proliferation and genomic instability. Deletion of 11q and acquisition of aberrations in SF3B1, Notch-1 and FOXP1 causes genomic instability with the risk of acquiring further genetic damage and progression. Co-engagement of Notch ligands and self-B-cell receptor (BCR) activation results in increased proliferation and survival in U-CLL. These interactions may overcome the low level of anergy that the U-CLL subset undergoes. Signalling through self-reactive BCR, Toll-like receptor 9 (TLR9) with apoptotic cells and interactions of BCR with their own BCR epitopes promote proliferation of U-CLL B cells. In cells with increased levels of FOXP1, augmented expression of RAG enzymes may also promote new genetic damage by persistent V/D/J recombination or receptor editing.

In summary, we suggest that B cells that express BCR that recognize their own BCR epitopes are viewed by the immune system as 'dangerous cells'. We speculate that BCR autonomous signalling in early B-cell development may induce constant receptor editing (in heavy and light chains) a mechanism involved in immunological tolerance. This constant activation of receptor editing may increase risks of mistakes in allelic exclusion. It may also induce genomic instability and increase the risk of other molecular and genetic disturbances. We advocate that autonomous signalling by the BCR is the clue to understanding the concept of receptor editing and allelic exclusion in CLL B cells. The fact that the whole BCR recognizes a self antigen or foreign antigen may be irrelevant in early B-cell development. In early B cells, autonomous signalling induced by recognition of the BCR's own epitopes simulates an antigen–antibody engagement. In the bone marrow this interaction is viewed as recognition of self molecules and induces receptor editing. This receptor editing may correct germline autoreactivity in the M-CLL subset but fails to induce tolerance in the U-CLL subset. In mature B cells, autonomous signalling by the BCR may promote 'reversible anergy' and also may correct self reactivity induced by the somatic hypermutation mechanism in M-CLL. However, in U-CLL, BCR autonomous signalling in addition to self antigen recognition augments B-cell activation, proliferation and genomic instability.

Therapy approaches at the immunological level

B-cell receptor inhibitors, such as ibrutinib, a Bruton′s tyrosine kinase (BTK) inhibitor, have demonstrated significant activity against CLL.[156–158] Recently, the effect of ibrutinib in potentially dangerous CLL clones with high sIgM and CXCR4 expression levels was proposed as a critical factor in therapeutic success.[68] CLL was considered to be a tumour of circulating B lymphocytes, variably stimulated and anergized following exposure to antigen in lymphoid tissue.[68] This is in concordance with our hypothesis that CLL B lymphocytes are persistently under check by a tolerance mechanism because they are chronically exposed to self antigens. In this context, inhibition of BCR signalling is very important because it can control autonomous CLL lymphocyte BCR signalling capacity and self antigen BCR activation. A consequence of this BCR inhibition may be the inhibition of tolerance mechanisms such as receptor editing and reduction of the risk of acquiring of new molecular aberrations and clonal evolution.

Conclusion

We suggest that CLL originates from a coordinated normal immunological tolerance mechanism to destroy self-reactive B cells. CLL is a malignancy of lymphocytes that acquire a self BCR with autonomous BCR signalling that induces them to proliferate and mature independently of their maturation stage. Additional genetic damage induced by tolerance mechanisms may immortalize them and transform a self-reactive B cell into a leukaemia. The result of tolerogenic mechanisms and genetic aberrations is the survival of CLL B-cell clones with similar markers and homogeneous gene expression signatures despite the different stages of maturation at which the initial damage occurs. BCR signalling inhibitors may correct the immunological disturbances blocking the stimuli that induce chronic activation or tolerance mechanisms in CLL B lymphocytes.

Disclosures

The authors declare no conflict of interest.

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