Signal-dependent and signal-independent functions of the B-cell receptor in chronic lymphocytic leukemia

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Signals generated by the B-cell receptor (BCR) are considered to play a key role in the pathogenesis of several B-cell malignancies. This especially refers to chronic lymphocytic leukemia (CLL), where the malignant B cells frequently express BCRs with nearly-identical "stereotyped" antigen-recognition units, encoded by a restricted set of immunoglobulin heavy-chain variable (IGHV) region gene combinations.[1-3] This feature, along with the shared reactivity toward common antigenic determinants,[4-8] strongly suggests that the initial expansions of the malignant clones are antigen-driven. In addition, freshly isolated CLL cells frequently express high levels of BCR-target genes[9] and display increased basal activity of signaling molecules along the BCR pathway,[10] suggesting that they continue to be stimulated by antigen even after the initial transforming event. Continued antigen stimulation is believed to promote the growth and survival of the leukemic cells, and this possibility is further supported by recent clinical trials demonstrating remarkable activity of drugs that inhibit BCR signaling in patients with CLL.[11,12]

In this issue of Haematologica, Bergh *et al.* investigated the consequences of cognate antigen binding to CLL cells that express BCRs belonging to the so-called "stereotyped subset 1".[13] This large stereotyped subset is characterized by the expression of IGHV-unmutated BCRs encoded by the IGHV1-5-7/IGKV1-39 combination.[1] In a previous study by the same group, subset #1 CLL BCRs were shown to bind to oxidized phospholipids found on low-density lipoproteins (LDL), apoptotic blebs and certain microbes.[4] The specificity of these interactions was further confirmed in the current study by competition ELISA assays, showing binding of CLL subset #1 antibodies to oxidized but not to native LDL. In another set of experiments performed with primary subset #1 CLL cells and biotin-labeled oxidized LDL (oxLDL), antigen binding was shown to induce BCR clustering and subsequent internalization. Surprisingly, analysis of downstream signaling events, such as intracellular Ca^{2+} flux and ERK phosphorylation, failed to detect any response upon ligation of the BCR with cognate antigen. Responsiveness to BCR ligation was recovered is some cases after 48 h in culture, but this change was primarily observed in cells stimulated with anti-IgM and was less frequently seen in cells stimulated with cognate antigen.

The unexpected finding that BCR ligation with cognate antigen does not generate an activatory signal in this stereotyped CLL subset raises several interesting questions. The first question is why are these BCRs positively selected during leukemia development. One possible explanation could be that they are selected because of functions that are not necessarily related to BCR signaling. It is well known that signals generated by the BCR are necessary but not sufficient for cellular activation and that co-stimulatory signals, such as those provided by T-helper cells (via CD40L engagement of CD40 on the B cells) or Toll-like receptor 9 (TLR9), are also required. T-helper cells and TLR9 can productively acquire their ligands only after they have been internalized and delivered to the endosomes, which is a process that requires BCR-mediated endocytosis. Thus, the primary role of the stereotyped subset #1 CLL BCRs could be to capture, internalize and deliver such ligands. Importantly, oxidized phospholipids and the TLR9 ligand CpG-DNA co-localize in apoptotic blebs, suggesting that stereotyped subset #1 CLL cells could acquire proliferative signals by binding to such complexes. This possibility is further supported by experiments performed in the study of Bergh *et al.*, showing that CLL subset #1 cells efficiently internalize oxLDL and proliferate following TLR9 stimulation. Internalized oxLDL could also provide peptides for T-cell activation, as has previously been reported for T cells isolated from human atherosclerotic plaques.[14] Moreover, recent studies have shown that CLL cells can efficiently activate autologous T cells in an antigen-specific manner, and such activated T cells can drive leukemic cell proliferation both *in vitro* and in a murine xenograft model *in vivo*.[15,16]

The second question that arises from this study is: what is the reason for the silenced BCR signaling response in CLL cells from subset #1? One possibility is that this unresponsiveness results from chronic repetitive engagement of subset #1 BCRs by oxLDL molecules present in the patients' sera. Chronic BCR occupancy by autoantigen in the absence of co-stimulatory signals induces anergy, which is a state of cellular lethargy characterized by attenuated BCR signaling responses and elevated basal activity of certain downstream signaling molecules. The experiments performed by Bergh *et al.* show that CLL cells from subset #1 display many features of anergic cells, including elevated basal phospho-ERK levels and inability to proliferate, secrete IgM and induce CD86 expression upon antigen binding. Thus, subset #1 CLL cells isolated from the peripheral blood may have been rendered anergic because of chronic occupancy of their BCRs with circulating oxLDL molecules. It is worth noting, however, that anergy is a reversible state, so these cells could still be capable of mounting a proliferative response in the tissues, where they would encounter the same antigen in a different form (i.e. presented by antigen-presenting cells) and associated with the necessary co-stimulatory ligands.[17] In addition, considering the frequent polyreactivity of CLL BCRs, including subset #1 BCRs, it is possible that the antigens encountered in the tissues will be different from those predominantly encountered in the circulation.

An alternative, not mutually exclusive possibility, is that this state of hypo-/non-responsiveness is induced by the recently discovered cell-autonomous BCR-BCR interaction, which is caused by the apparently unique capacity of CLL CDR3 regions to bind to internal immunoglobulin epitopes.[18] These BCR-BCR interactions have been shown to generate a persistent low-intensity signal that is manifested by increased intracellular Ca^{2+} levels[18] and which appears to lead to increased apoptosis resistance of the leukemic cells.[19] However, these cell-autonomous BCR-BCR interactions would also be expected to compete with the BCR/external-antigen interactions, thus reducing the number of BCRs available for acute antigen

engagement below the threshold required for cellular activation. This possibility may also explain why the BCR signaling response to oxLDL did not recover in 3 of the 5 subset #1 CLL cases after prolonged *in vitro* culture.

Finally, the most important question from a clinical perspective is whether cases with a silenced BCR response to cognate antigen would be expected to respond to treatment with BCR pathway inhibitors. As mentioned earlier, the lack of a signaling response of circulating CLL cells does not necessarily preclude that such an event could occur in the tissues, where the malignant cells could regain responsiveness and be driven to proliferate by immune complexes containing the antigen and co-stimulatory ligands. In addition, BCR pathway inhibitors could also function by targeting the cell-autonomous BCR signal, which appears to be a common feature of CLL cells and a therapeutic target in itself, as evidenced by the increased apoptosis of CLL cells treated *in vitro* with kinase inhibitors or siRNA molecules that target BCR pathway components.[10,20] Thus, until proven otherwise, BCR pathway inhibitors should be considered a rational therapeutic option also in this large and typically aggressive CLL subset characterized by a silenced BCR response to cognate autoantigen.

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