The role of antigen in the development of B-cell chronic lymphocytic leukemia
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Chapter 7

General discussion
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B-cell receptor (BCR)-mediated signaling plays an important role in the development of B-cells\(^1\). Numerous studies report that BCR signaling may also have a pivotal role in the development and expansion of malignant B-cells\(^2\). BCR-signaling in lymphomas is either ligand independent, such as in diffuse large B-cell lymphomas harbouring activating mutations in CD79a and CD79b\(^3\), or ligand dependent, as proposed for most low-grade B-cell non Hodgkin’s lymphomas (NHL)\(^2\). Most of the evidence for ligand-dependent BCR-signaling in NHL is indirect, i.e. by studies on the BCR repertoire, revealing restricted usage of immunoglobulin heavy chain variable region (\(\text{IGHV}\)) genes in MALT lymphoma\(^4\), mantle cell lymphoma\(^5\) and in B-cell chronic lymphocytic leukemia (CLL). In CLL, \(\text{IGHV1-69}\), \(\text{IGHV3-7}\) and \(\text{IGHV4-34}\) are overrepresented\(^6\). Moreover, over 30% of CLL can be grouped into subsets based on similarities of the amino acid sequences in the highly variable complementary determining region 3 (CDR3), strongly suggesting that distinctive antigens are involved in the development of CLL\(^4,8,11\). In recent years, an effort has been made to elucidate what these cognate antigens are by producing CLL BCRs as recombinant immunoglobulins.

Cognate antigens identified for U-CLL

On average, CLL with unmutated \(\text{IGHV}\) (U-CLL) express longer \(\text{IGHV}\)-CDR3s, as compared to healthy donor-derived B-cells and CLL with mutated \(\text{IGHV}\) (M-CLL)\(^9,12\). Long CDR3s are predisposed for polyreactivity based on increased conformational diversity\(^13,14\). Indeed, the majority of U-CLL express polyreactive BCRs that bind with low-affinity to a variety of exo- and self-antigens, such as ssDNA, dsDNA, LPS, insulin, IgG, cardiolipin, oxidized LDL and cytoskeletal components (Chapter 2)\(^12,15-20\). Polyreactive binding patterns have been identified for both U-CLL that express stereotypic BCRs as well as for non-subset U-CLL\(^12\). Self-reactivity of U-CLL BCRs was confirmed by stainings of Hep2 cells, which were mostly directed at cytoplasmic structures\(^12\). In agreement, we found that U-CLL-derived sIgM frequently bound to cytoplasmic structures in tissue-microarrays (Chapter 2 and 6). Putative cognate antigens have been identified for two U-CLL subsets. Subset #1 (IGHV1-U) CLL recognize vimentin and subset #6 (IGHV1-69-U) CLL bind non-muscle myosin IIA (MYHIIA)\(^21,22\). Many cytoplasmic antigens, including vimentin and MYHIIA, move to the outside of the cell during apoptosis, suggesting that U-CLL may recognize apoptotic cells\(^17,22\). In support, the vast majority of U-CLL indeed bound apoptotic cells by flow cytometry, including representatives of subset #1 (IGHV1-U), subset #6 (IGHV1-69-U), subset #8 (IGHV4-39-U), subset #9 (IGHV1-69-U) and subset #28 (IGHV1-2-U) and binding to apoptotic cells is associated with worse clinical outcome\(^23\).
Although the assumption of a correlation between apoptotic cell binding and disease aggressiveness is tantalizing, pivotal evidence that apoptotic cells are the cognate antigens for U-CLL is missing. Currently, it is not known if apoptotic cell binding is mediated by the stereotypic features of the IGHV-CDR3 and if it depends at all on subset-shared IGLV. Moreover, it has not been shown that interaction with apoptotic cells drives proliferation of primary U-CLL cells. Apoptotic cell binding is common also among non-stereotypic CLL and it can be readily detected in the serum of healthy donors, suggesting that this specificity is common for many antibodies. Therefore, it cannot be ruled out that binding to apoptotic cells is a reflection of germline-encoded polyreactivity rather than selection by distinctive antigens.

Cognate antigens identified for M-CLL

In contrast to U-CLL, M-CLL are generally not polyreactive (Chapter 2) and M-CLL BCRs do not bind apoptotic cells. Intriguingly, reversion of somatic mutations results in polyreactivity in the majority of M-CLL, demonstrating that M-CLL have derived from polyreactive precursors. Moreover, this suggests that polyreactivity is not important in sustaining M-CLL growth. In this thesis, we have uncovered the specificity of three stereotypic M-CLL subsets. First, a subset of IGHV3-7-encoded BCRs was found to be highly specific for the Fc-tail of IgG, so called rheumatoid factors (RFs) and this specificity was dependent on both the stereotypic IGHV as well as the IGLV (Chapter 3). Second, we identified a subset of M-CLL that express unusually short IGHV-CDR3s, which we designated V3-7Short (IGHV3-7-M). V3-7Sh BCRs bind with high-affinity to β-(1,6)-glucan, a major antigenic determinant of fungi, which is not expressed by human cells (Chapter 4). Binding to β-(1,6)-glucan depends on both the IGHV and the IGLV, as well as distinct amino acids in the IGHV-CDR3. Moreover, reversion of somatic mutations showed that V3-7Sh are affinity selected for β-(1,6)-glucan (Chapter 4). Importantly, primary CLL cells of both the IGHV3-7-RF and IGHV3-7Sh subsets were induced to proliferate when cultured in the presence of their cognate antigen (Chapters 3 and 4), implying that cognate antigens may also drive CLL expansion after transformation. Finally, we show in Chapter 5 that subset #4 CLL (IGHV4-34-M) are affinity-selected for distinct poly-N-acetyllactosamine (NAL) epitopes expressed by a subset of B-lymphocytes. Virtually all IGHV4-34-encoded antibodies bind NAL epitopes by a hydrophobic patch in the IGHV-FR1. However, subset #4 CLL (IGHV4-34-M) specifically bound a distinct NAL epitope as expressed by the DLBCL cell line Ly-3 and this specificity clearly depended on both the stereotypic IGHV and the IGLV. Recently, the specificity of a fourth M-CLL subset was elucidated. BCRs of subset #13 (IGHV4-59-M) were found to also be RFs. Collectively, these studies strongly suggest that M-CLL in majority...
are highly selected for single extrinsic antigens and that these antigens can be both self-antigens and exo-antigens. In contrast, it has been claimed that CLL growth is driven by antigen-independent, cell-autonomous Ca\textsuperscript{2+} signaling due to self-recognition of an intrinsic IGHV amino acid motif, based on signaling studies of retrovirally expressed CLL BCRs in mouse cells and increased basal Ca\textsuperscript{2+} levels in primary CLL cells\textsuperscript{27}. Autonomous signaling was independent of IGHV-mutation status, IGHV-restriction or IGHV-stereotypy. It is therefore unlikely that these observations relate to the occurrence of subsets of CLL expressing stereotypic BCRs with shared somatic mutations. Moreover, primary CLL cells do not proliferate \textit{in vitro} in the absence of extrinsic BCR crosslinking (Chapters 3 and 4), implying that extrinsic cognate antigens drive CLL expansion. The role of antigen-independent BCR signaling and whether it is a requirement for CLL development remains to be determined.

**Future directions**
Our finding that primary V3-7Sh and V3-7RF CLL cells are induced to proliferate by their cognate antigens may reflect a general principle for CLL and possibly more low-grade NHL entities. The challenge is now to identify the true cognate antigens for U-CLL and the remaining M-CLL subsets. Convenient methods to produce CLL-derived immunoglobulins, as described in Chapter 6, may accelerate this field of research. After identification of putative cognate antigens it is of importance to provide evidence that interaction with these antigens accounts for the observed stereotypy by exchanging the stereotypic IGHV and IGLV and by substitution of stereotypic IGHV-CDR3 amino acids. Moreover, the functional consequence of interaction with the cognate antigens should be studied in primary CLL cells, focusing on proliferation. CLL cells proliferate in lymph nodes, where they have gene expression profiles indicative of ongoing BCR signaling\textsuperscript{28}. This suggests that CLL cells encounter their cognate antigen in lymph nodes. It is noteworthy that the cognate antigens thus far identified (IgG, β-(1,6)-glucan NAL-epitopes and apoptotic cells) may all be continuously present in lymph nodes. Along these lines, it may be hypothesized that CLL require continuous or intermittent stimulation by cognate antigens for expansion. In support, interference with the BCR signaling pathway \textit{in vivo} results in migration of CLL cells from lymph nodes to the periphery and disease remission\textsuperscript{29,30}. The recent success of these agents interfering with BCR signaling pathway components, such as Syk and Btk\textsuperscript{29-31}, might be exemplary and paves the way for personalized targeted therapies based on BCR specificity, e.g. anti-fungal therapy for V3-7Sh. It is therefore a necessity to unveil the specificity of more CLL subsets. Alternatively, it may be possible to shield cognate antigens from interacting with CLL cells by treating patients with their own CLL-derived soluble immunoglobulins.
Reference List


