Microenvironment and anti-CD20 based therapies in CLL
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Chapter 1

Background and scope of thesis
Characteristics and cellular origin of CLL

Chronic lymphocytic leukemia (CLL) is a chronic lymphoproliferative disorder characterized by the progressive accumulation of CD5⁺CD19⁺CD23⁺ mature B cells in peripheral blood and secondary lymphoid organs (lymph nodes (LNs) and spleen) ¹. Classically, CLL cells were thought to be long-lived tumor cells that accumulate as a result of defects in intrinsic apoptosis. However, growing evidence suggests that CLL is a dynamic disease with substantial proliferation rates and increased death rates in vivo ²,³. The normal counterpart of CLL is still a matter of debate (reviewed in ⁴).

Normal B cell maturation and differentiation

Most important for the maturation and differentiation of B cells is the formation of a B cell receptor (BCR). The BCR consists of a membrane bound immunoglobulin (Ig) molecule composed of two Ig heavy (H) chains and two light (L) chains, encoded by variable, diversity and joining (VDJ) region gene segments that have gone through a process of RAG-mediated V(D)J-rearrangement. The RAG-mediated V(D)J rearrangement is responsible for the initial antibody repertoire of B cells (at least 10¹¹ specificities) developing in the bone marrow. First the pre-BCR is formed, consisting of a H chain and a surrogate L chain. BCR mediated signalling (constitutively or delivered by autoantigens from the microenvironment) then becomes important. It induces positive selection, clonal expansion, heavy chain allelic exclusion and formation of the L chain (Igkappa or Iglambda) resulting in a complete BCR expressed on immature B cells. These cells are IgM⁺IgD⁺, IGHV/D/J, and IGK/J or Igl/J are paired, and cells can be autoreactive. Now negative selection for higher affinity autoreactive BCRs takes place by a process of clonal deletion and receptor editing or receptor revision of the IgHV gene. B cell development in the bone marrow is foreign antigen independent. Cells with no or low affinity for autoantigen move to the periphery as naive B cells. Foreign antigen-binding B cells meet helper T cells at the border between T- and B cell zones in secondary lymphoid tissue. This interaction gives rise to an initial proliferation of B cells in a primary focus. Some of these proliferating B cells differentiate into plasmablasts that secrete antibody, others migrate together with their associated T cells into a primary lymphoid follicle and ultimately form a germinal center (GC). In the GC reaction a stepwise differentiation occurs during processes of somatic hypermutation (SHM) under the influence of activation-induced cytidine deaminase (AID), positive selection of high affinity BCRs and class switch recombination (CSR). After the GC reaction, cells are isotype switched and affinity matured, and differentiate into a) plasma cells that produce oligo/monoreactive Igs or b) memory cells. Whereas T cell-dependent BCR stimulation occurs in GC reactions, T-cell independent B cell responses take place in the marginal zone (MZ) of the spleen. Here, MZ B cells encounter T-cell independent antigens and can respond in 2 ways: 1. without undergoing isotype switching or IGV gene mutations they give rise to either plasmacells secreting polyreactive, unmutated IgMs or to non-switched memory cells 2. after isotype switching and IGV gene
mutation they give rise to plasma cells producing oligo/monoreactive mutated IgMs, IgGs or IgAs or to switched memory cells.

**Origin of CLL cells**
Importantly, most of our knowledge about CLL cells derives from studying circulating cells. CLL cells residing in lymph nodes and other secondary lymphoid organs proliferate and expand as result of survival signals from their microenvironment. Once they leave their ‘sanctuary sites’ these cells become prone to apoptosis. There is strong evidence that signaling via the BCR plays a major role in the development of CLL and that it determines its clinical behaviour (reviewed in 6). Most cases of CLL express IgM and IgD and use either mutated (M-CLL) or unmutated (UM-CLL) immunoglobulin variable heavy chain (IGHV) genes. This feature distinguishes two patient subgroups with distinct clinical courses 7,8. UM-CLL can be considered to be derived from a pre-GC cell that has not undergone SHM, whereas M-CLL is the malignant counterpart of a post-GC cell that has undergone SHM in a germinal center reaction. The IGHV gene and immunoglobulin light chain variable (IGHV) gene usage differs markedly between the 2 major subsets. The third (minor) subset of CLL, expressing isotype-switched lgs usually derived from mutated V genes, displays IGHV gene patterns distinct from M-CLL 9. This suggests a separate origin of the 3 subsets and it appears that each subset has arisen independently during B cell differentiation. The routes leading to the 3 subsets of CLL are depicted in Figure 1, where antigen stimulation (auto- or foreign antigen) is the likely initial drive for all.

CLL cells continuously need support from the surrounding microenvironment for their survival in vivo. One key component in the microenvironment is antigen. Both UM-CLL and M-CLL undergo engagement of surface IgM (sIgM) in vivo. Interestingly, UM-CLL express higher levels of IgM than M-CLL. This is probably the result of diminished down-modulation after antigen encounter, possibly caused by a lower affinity for antigen of UM-CLL compared to affinity matured M-CLL. Furthermore, in both UM-CLL and M-CLL endocytosis of sIgM takes place after antigen engagement. Endocytic events lead to modulation of glycans of the μ-chains with relative higher surface retention of a mannosylated immature form, which binds to mannose binding lectins in the microenvironment. This surface retention is more evident in UM-CLL where it could mediate new microenvironmental interactions. Finally, less dramatic down-modulation of sIgM in UM-CLL results in stronger signaling capacity as compared to M-CLL, characterized by partial activation of downstream pathways (reviewed in 6).

Other factors correlating with sIgM signaling capacity (e.g. CD38 expression, ZAP70 expression) are of prognostic significance (reviewed in 6), indicating the important role of BCR signaling in the pathophysiology and behaviour of the disease.

In conclusion, UM-CLL and M-CLL cells can be considered as antigen-experienced pre- and post-GC cells respectively and UM-CLL cells show more aggressive behaviour in vivo, possibly as a result of stronger IgM signaling capacity and easier access to other survival factors from the microenvironment.
Bidirectional interaction of T cells and CLL cells

The cellular crosstalk between T- and B cells belongs to the major microenvironmental stimuli regulating B cell survival, proliferation and differentiation. The interaction between CLL cells and T cells is a dynamic process with reciprocal effects on both cell types, which will be discussed below.
Effects of CLL cells on T cells

1. CLL cells exert different effects on basal T-cell numbers. CLL patients have an increased absolute number of CD4+ and CD8+ T cells. Furthermore, in CLL an inversed CD4/CD8 ratio is observed. Interestingly, an increase in total CD4+ and CD8+ T cell numbers is found only in CLL and not in other B cell malignancies including monoclonal B cell lymphocytosis (unpublished data). This suggests CLL cell specific induction of T cell proliferation. The presence of an oligoclonal or even monoclonal T cell receptor (TCR) repertoire in CD8+ and (more frequently) in CD4+ T cells suggests (tumor) antigen-selected memory T cells. There is growing evidence however that these T cells are not tumor-specific. The increased pool of cytotoxic CD8+ T cells in CMV seropositive CLL patients consists largely of CMV-specific cytotoxic CD8+ T cells. The fact that CMV seronegative CLL patients do not show an increase in total number of cytotoxic CD8+ T cells, supports the hypothesis that it’s not a tumor-specific T cell pool that accounts for the T cell expansion in CLL.

There is evidence for CLL-dependent differentiation of certain T cell subsets. A significant increase in relative numbers of central and effector memory T cells is observed in the CD4+ T cell pool from CLL patients with unmutated IGHV genes as compared with patients with mutated IGHV genes. This is associated with a high Rai stage, progressive disease and shorter treatment-free survival. In a recent study, also the numbers of CMV-specific CD4+ T cells exhibiting the late differentiated CD45RO+CD27-CD28-CCR7- phenotype are found to be increased in CLL as compared to normal controls. Furthermore, there is evidence for CLL cell-induced Treg formation.

2. CLL cells also have an effect on T cell function. Although total CD4+ and CD8+ T cell numbers are increased in CLL, the frequent occurrence of secondary cancers and increased incidence of autoimmune diseases suggest T cell dysfunction. Aberrant T-cell physiology in CLL is characterized by reduced expression of numerous genes involved in the TCR signaling cascade upon TCR and CD28 costimulation. At the protein level, this pattern results in a reduced secretion of cytokines. T cells from CLL patients are impaired in their capacity to differentiate towards a Th1 phenotype. Also, adequate cytotoxic anti-CLL immune responses are hampered by reduced expression of costimulatory molecules on CLL cells. On the other hand, the cytotoxic capacity of CMV specific T-cells in CLL and healthy donors is comparable. Finally, by expressing programmed death-1 (PD-1) and CD200, CLL cells might contribute to the impairment of the immunologic synapse formation, resulting in the inhibition of T cells. Interestingly, lenalidomide can reverse cellular immune dysfunction in CLL by enhancing T cell immunological synapse formation and functional activity, reviewed in.

Recently, T-cell function in CLL Eμ-TCL1 transgenic mouse model was studied. Eμ-TCL1 transgenic mice develop functional T-cell defects and alterations of gene and protein expression closely resembling changes seen in CLL patients. Infusion of ‘CLL cells’ from Eμ-TCL1 mice into young syngeneic mice induces comparable defects, suggesting a causal...
relationship between CLL and the T cell defects. Lenalidomide also reverses dysfunctional immunological synapse formation and T-cell signaling in vivo in TCL1 mice.

**Effect of T cells on CLL cells**

There is growing evidence for a key role of T cells in the pathogenesis of CLL.

1. Very recently, in a mouse model Bagnara et al. elegantly show in vivo proliferation of CLL cells under the influence of activated autologous CLL-derived T lymphocytes.
2. Increased numbers of regulatory T cells in CLL could interfere with adequate tumor surveillance by putative tumor-specific T cells, thereby contributing to diminished T cell function in CLL and eventually disease progression. Indeed, the increased numbers of Treg in CLL patients is associated with unmutated IGHV genes, high Rai stage and poor clinical outcome.
3. In this thesis we show the effect of activated T cells on important characteristics of CLL cells in vivo: activation, proliferation and drug resistance of CLL cells. Altogether, these studies suggest that T cells favour disease progression in CLL patients. The exact underlying mechanisms however have to be determined.

**Treatment of CLL**

At present a curative treatment for CLL is not available, although treatment results have improved considerably over the last decade. A watch-and-wait approach is applied in asymptomatic patients. Until recently, in case of symptomatic disease and/or disease progression patients were treated with alkylating agents (chlorambucil) or purine analogue containing regimens (fludarabine). Remission rates are variable but cures are never obtained. However, recently it was shown in a large randomized phase III trial that addition of the chimeric IgG1 anti-CD20 monoclonal antibody (mAb) Rituximab (R) to FC (Fludarabine, Cyclophosphamide) improves both progression free survival (PFS) and overall survival (OS) in p53 functional, previously untreated CLL patients. Combination treatment with fludarabine, cyclophosphamide, and rituximab (FCR) has now emerged as the standard of care in the treatment of CLL in fit young patients (reviewed in). Although this approach seems encouraging, patients with CLL eventually all relapse and require additional therapies. Also, this treatment is too toxic for many patients since CLL mainly affects elderly people.

Many of the current therapeutic regimens for CLL are myelotoxic, immunosuppressive, and associated with infectious complications. Finally, upon sequential treatments in up to 50% of the patients selection of p53 dysfuctional clones occurs, resulting in chemoresistance. It is now unclear how to treat patients that do not respond to or cannot tolerate the current agents. Therefore, there is an urgent need for targeted novel therapeutic approaches that are effective, associated with low toxicity, circumvent microenvironmental chemoresistance and act independently of p53. Examples of targeted therapies are small molecules targeting the PI3K/Akt/mTor pathway (e.g. everolimus, temsirolimus or CAL-101 a selective inhibitor of PI3K.
6), or inhibiting BCR signaling targets SYK (fostamatinib) and BTK ((PCI-32765) 6, Antibody-drug conjugates and targeted monoclonal antibodies (mAbs) to B-cell lineage antigens or to TNF-receptor family members like TRAIL-death receptor or CD40 (reviewed in 36). Alemtuzumab (anti-CD52 mAb) has been shown to be effective in the treatment of p53 dysfunctional patients 37, possibly by inducing p53-independent non-classical apoptosis 38;39. However, T cell depletion following treatment with Alemtuzumab can result in serious opportunistic infections and CMV reactivation. Also immunotherapy, targeting CD23 40 in vitro and in vivo, or CD47 mAbs 41 in vitro, has been shown to be effective. At present, anti-CD20 therapy is the most widely used targeted treatment in CLL and will be discussed below.

**Anti-CD20mAbs: types and mechanisms of cell death induction**

The CD20 molecule, a transmembrane tetra-span, is a non-glycosolated 30-to 35-kDa phosphoprotein (Figure 2) expressed by B lymphocytes already at early stages of differentiation and by most B cell malignancies 42-44. Although CD20 is the most frequently antibody-targeted antigen in general, its exact function is still unknown. Until now there is no known natural ligand. Tedder et al 45;46 and Golay et al 47 were among the first to show that different anti-CD20 antibodies were able to induce changes in growth patterns of B cells. Moreover,
it has been shown that CD20 can function as a calcium channel, as transfection of CD20 into cell lines results in increase of Ca\(^{2+}\) conductance, and initiate intracellular signals \(^{42,48,49}\). Also, intracellular calcium responses are significantly reduced in CD20\(^{-/-}\) mouse B cells \(^{50}\). In CD20\(^{-/-}\) mice, B cell development, tissue localization, signal transduction, proliferation, T cell-dependent antibody responses and affinity maturation are normal \(^{50}\). However T cell independent B cell responses are severely impeded in CD20-deficient mice \(^{51}\). Interestingly, in humans CD20 deficiency also results in impaired T cell-independent antibody responses, although the response of antigen-independent B cells is normal \(^{51}\).

The chimeric anti-CD20 mAb rituximab (Figure 2) was first approved in 1997 for the treatment of relapsed or refractory low-grade or follicular B-cell NHL. Since then it has become a major element in the treatment of many B-cell malignancies including B-CLL \(^{52,53}\) and auto-immune diseases such as rheumatoid arthritis \(^{54}\).

Important mechanisms of anti-CD20 mAbs action in vivo are antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC), mediated by cells expressing Fc\(\gamma\) receptors (Fc\(\gamma\)Rs) such as Fc\(\gamma\)RIIIa- expressing NK cells and macrophages (reviewed in \(^{55}\)). Furthermore, intracellular signals initiated upon CD20 binding potentially result in growth inhibition and cell death ("direct cell death") (reviewed in \(^{55}\)). The last mechanism is much less clear, remains controversial and its role in vivo is still a matter of debate. There is evidence that CD20 crosslinking can lead to cell death of certain B-cell lines \(^{39,56,57}\), however many other B-cell lines appear to be insensitive to such death \(^{58,59}\).

There is further controversy concerning the possible mechanisms of anti-CD20 induced cell death. Many have shown that cell death following CD20 crosslinking has features of classical apoptosis (including caspase activation and DNA fragmentation) \(^{57,58,60,61}\). Others could not confirm these findings \(^{39,57,62,63}\). Regarding this issue, it is important to note that there are 2 types of anti-CD20 mAbs: Type I (rituximab-like) and type II (tositumomab-like), based on their ability to redistribute CD20 molecules in the plasma membrane (lipid rafts) and activate various effector functions (Table 1). Although both types bind bivalently to CD20, distinct complexes are formed with CD20. The B cell surface can accommodate approximately twice as many of type I antibodies as type II antibodies. Type I antibodies stabilize CD20 into

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<th>Table 1. Differences between type I and type II anti-CD20 mAbs.</th>
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<td><strong>Type I mAbs</strong></td>
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<tr>
<td>Localize CD20 to lipid rafts</td>
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<tr>
<td>High CDC</td>
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<tr>
<td>ADCC activity</td>
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<td>Ca(^{2+})-flux +</td>
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<td>Weak homotypic aggregation (HA)</td>
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<td>Weak direct cell death induction</td>
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<td>Rituximab, Ocrelizumab, Ofatumumab</td>
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lipid rafts leading to stronger C1q binding and potent induction of CDC. However, type I antibodies trigger only low levels of direct cell death. In contrast, type II antibodies do not stabilize CD20 into lipid rafts and thus have reduced CDC capacity, but they potently induce direct cell death 55;64;65. Recent studies showed that type II anti-CD20 mAbs induce homotypic adhesion-related cell death through a lysosome-dependent pathway 64;66. Figure 3 summarizes the mechanisms of cell death induction by anti-CD20 mAbs.

**Anti-CD20 therapy in CLL and new anti-CD20 mAbs**

Rituximab monotherapy is effective in the treatment of CLL, but the efficacy is relatively low at dosages used in the treatment of non-hodgkin lymphoma (NHL). Therefore, higher doses and more intensified regimens must be used 67. Unfortunately, a considerable fraction of patients are resistant or become resistant to therapy with rituximab. Possible underlying mechanisms are not well understood. Several possible explanations might exist: first, CLL cells express low levels of CD20 68;69 and CD55 and CD59 expressed on CLL cells inhibit complement activation70. Second, freshly isolated CLL cells are resistant to ADCC by NK cells in vitro 71. In general, CLL cells seem resistant to apoptosis-inducing processes 72, possibly caused by an intrinsic defect in apoptosis. Third, circulating CD20 has been shown to interfere
with the binding of rituximab. Finally, after rituximab treatment transient downmodulation of CD20 expression in CLL patients has been described, which could explain the lack of response of CLL patients to rituximab. Several mechanisms can be responsible for CD20 loss. After rituximab binding CD20 can be removed from CLL cells by internalization and degradation of CD20/mAb complexes in CLL cells themselves. Another mechanism is loss via an endocytic process called shaving/trogocytosis, mediated by FcγR on acceptor cells including monocytes/macrophages, which remove and internalize rituximab-CD20 immune complexes from B cells. A very recent study shows that shaving occurs more rapidly and induces considerably greater loss of CD20 and bound mAb from opsonized B cells than in the process of internalization.

At the moment, various new anti-CD20 mAbs with superior efficacy are being developed. An example of a newly developed type I anti-CD20 mAb is Ofatumumab: a fully human IgG1 anti-CD20 mAb that binds to the small loop of CD20 and displaying enhanced CDC inducing capacity. Recently it was shown that ofatumumab is active in fludarabin-refractory CLL patients irrespective of prior rituximab treatment. The first glyco-engineered type II humanized IgG1 anti-CD20 mAb is GA101, that shows increased direct and immune effector cell-mediated cytotoxicity when compared to rituximab. Also, GA101 is more potent than rituximab at equivalent concentration in depleting CLL cells in vitro. Finally, a phase I/II study with GA101 in heavily pre-treated relapsed or refractory CLL patients shows promising activity when given as single agent.

Scope of the thesis

The aim of this thesis is two fold:
1. to study the bidirectional interaction between CLL cells and T cells.
2. to analyze mechanisms and modulation of direct cell death induced by type I- and type II anti-CD20 mAbs in CLL.

Chapter 2 describes the effect of activated T cells on activation, proliferation and drug resistance of CLL cells. These effects are compared with other microenvironmental stimuli. In chapter 3 the mechanism of Treg formation in CLL patients is investigated. In chapters 4 and 5 the effect of the microenvironment on the sensitivity of CLL cells to type I anti-CD20 mAb (rituximab) and type II anti-CD20 mAb (GA101) induced cell death is investigated and the underlying cell death mechanisms are explored. In chapter 6 the effect of CD40 stimulation on sensitivity of B cells to rituximab and GA101 is investigated in vivo in a CD20 transgenic (CD20TG) mouse model. Finally, in chapter 7 an integrated summary and discussion is presented and future approaches towards the treatment of CLL are suggested.
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