Allogeneic cellular immunotherapy for chronic B-cell leukemia

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Chapter 1

Introduction

CLL and MCL

B-cell chronic lymphocytic leukemia

B-cell chronic lymphocytic leukemia (CLL) is characterized by the clonal proliferation and accumulation of neoplastic B lymphocytes in peripheral blood, bone marrow and lymphoid tissue. The malignant B cells show the typical morphology of small lymphocytes with a narrow border of cytoplasm and a dense nucleus with partially aggregated chromatin and the immunophenotype of CD5, CD19, and CD23 in combination with low expression levels of surface immunoglobulin (Ig). The pathologic features of the lymph nodes are those of a small lymphocytic lymphoma. CLL is the most frequent type of leukemia in the western hemisphere with an incidence of 3 in 100,000. While the disease classically occurs in elderly people with a median age range at diagnosis of 65-68 years, an increasing number of patients are diagnosed at a younger age with now one-third of the patients under the age of 60 [1,2]. Most patients present themselves with peripheral lymphocytosis and are asymptomatic at presentation. In symptomatic patients, the most common features are generalized lymphadenopathy, splenomegaly and cytopenias. The cytopenias can be due to progressive bone marrow infiltration or to the development of autoantibodies against self-antigens present on erythrocytes and/or platelets. Furthermore patients with CLL generally acquire an immunodeficiency, characterized by hypogammaglobulinemia, impaired production of secondary Ig isotypes and T-cell defects, resulting in an enhanced susceptibility for bacterial infections [3,4]. It is now recognized that the clinical behaviour of patients with CLL is heterogeneous [5]. A subset of patients with early stage CLL have a disease that will rapidly evolve to a more advanced, ultimately fatal disease whereas other patients remain with early stage for decades.

Mantle cell lymphoma

Mantle cell lymphoma (MCL) is a non-Hodgkin lymphoma (NHL), characterized by the t(11;14)(q13;q32) translocation involving the PRAD-1/bcl 1 gene resulting in overexpression of cyclin D_1 [6]. Immunophenotyping shows expression of CD5, CD19, and surface Ig but lacks expression of CD23. Although longer recognized as a distinct clinicopathologic entity not until 1992 the consensus terminology of *mantle cell lymphoma* was used [7]. MCL is an uncommon disease, accounting for only approximately 5% of NHL, occurring in patients with a median age of approximately 65 years [8,9]. Clinically, patients with MCL present with advanced stage disease with frequent involvement of bone marrow, peripheral blood and extranodal sites. The clinical course of MCL is characterized by a high overall response rate to induction chemotherapy with a relatively short time to progression and a poor overall survival of approximately 3 to 4 years [8,10]. Many chemotherapeutic regimens are highly active against the disease but relapses typically occur and patients usually die of their disease. Therefore, MCL is regarded incurable with the conventional cytotoxic therapies.

Biology of CLL

Historically CLL cells were believed to represent a leukemic transformation of naïve B lymphocytes that had not undergone germinal center antigen exposure and subsequent somatic mutation of their Ig genes. Recent studies demonstrating that approximately 50% of CLL clones exhibit somatic mutation of their Ig chains, have altered the biological insights of CLL [11-13]. Some CLL clones, expressing unmutated variable heavy chain Ig (IgV_H) genes may originate from naïve B cells whereas other CLL clones with mutated IgV_H genes may be derived from post-germinal center memory B cells. More importantly the presence or absence of somatic mutations of IgV_H genes has been shown to distinguish between two disease subsets providing important prognostic information [11,12]. Patients whose CLL cells express mutated IgV_H genes, defined as > 2% difference from the corresponding germ-line gene, have a distinctly longer median survival than CLL patients with unmutated IgV_H genes [11,12,14]. Median survival in CLL patients with unmutated IgV_H genes ranges between 79 and 119 months whereas patients whose CLL cells express mutated IgV_H genes have a significant longer median survival, reaching 293 months in one study [11,12,14,15]. These survival differences between IgV_H gene mutation status were even observed in patients with early stage disease, indicating that the mutation status is a powerful predictor of clinical outcome in CLL. The proportion of CLL cells with unmutated IgV_H genes is close to 30-40%.

Gene expression profile studies identified a small number of genes that were differently expressed by mutated and unmutated CLL subtypes [16]. One of these genes encodes 70-kD zeta-associated protein (ZAP-70), an intracellular tyrosine kinase required for the TCR signaling normally expressed by T cells and natural killer (NK) cells and not by normal B cells. Several studies have shown that ZAP-70 is preferentially expressed in CLL cells with unmutated IgV_H genes and that its expression is associated with aggressive disease [17-19]. In patients showing discordance between ZAP-70 level and the IgV_H mutational status, ZAP-70 was a better predictor of clinical course than the mutation status [17]. Studies focusing on the role of ZAP-70 in the B-cell receptor (BCR) signaling demonstrated that CLL cells with ZAP-70 expression renders IgM signaling more effective than CLL cells lacking ZAP-70, thereby potentially enhancing survival and/of proliferation of ZAP-70 positive CLL cells [20]. There is also increasing evidence for the role of antigen in the pathogenesis of CLL. The structurally similarity of the B-cell receptors and the observed limited diversity of antigen-binding pockets of CLL cells from various patients suggest a common promoting antigen relevant for the pathogenesis of CLL [21,22]. Ongoing antigen-mediated activation of the leukemic cells through the BCR is suggested by the presence of cellular activation markers on the cell surface [23]. Conceivably, it can be hypothesized that CLL cells that express unmutated IgV_H genes, which typically also express ZAP-70 are susceptible to repetitive stimulation by a distinct yet unknown antigen resulting in proliferation of CLL cells and allowing them to avoid apoptosis [5,20]. This could account in part for the more aggressive clinical behavior. Cellular kinetics studies using deuterated water illustrated the correlation between division rates of CLL cells and progressive disease [24].

Genomic aberrations are often encountered in CLL. Interphase fluorescence in situ hybridization (FISH) enables to identify genetic abnormalities in 82% of the patients with CLL [24-26]. 17p deletion (17p-) and 11q deletion (11q-) and dysregulation of the p53 gene, located on the short arm of chromosome 17, are independent prognostic factors identifying subgroups of patients with rapid disease progression and short survival times whereas 13q deletion (13q-) or a normal karyotype are associated with favorable outcomes [24-27]. The high-risk genomic aberrations (11q-, 17p- and p53 dysfunction) were strongly associated with the presence of unmutated IgV_H genes [15,27]. As hypothesized above CLL cells with unmutated IgV_H genes may have increased proliferative capacity and ability to survive and may be selectively prone to the acquisition of genetic changes, which may further impair the prognosis in these patients. Finally, in CLL upregulation of anti-apoptotic genes such as Bcl-2, MCL1 and surviving is observed, resulting in further accumulation of leukemic cells and enhanced survival [28,29]. Other potential factors influencing apoptosis in CLL are interactions with stromal cells, “nurse-like” cells or activated $CD4^+$ T cells, expressing CD40L [28,30]. Cytokines produced by the micro-environment, such as interleukin (IL)-4 or vascular endothelial growth factor may further enhance expansion of CLL clones [31,32].

Biology of MCL

The genetic hallmark of MCL is the chromosomal translocation t(11;14)(q13;q32) leading to deregulation and overexpression of cyclin D_1. Cyclin D_1 , one of the key regulators of the cell cycle, controls after encountering cyclin-dependent kinase (CDK) the G1 phase and the G1/S-phase transition of the cell cycle. Hence, overexpression of cyclin D_1 in MCL cells may accelerate G1/S-phase transition and therefore tumor cell proliferation [33]. The increase of intracellular cyclin D_1/CDK leads to phosporylation of the retinoblastoma protein (Rb), that, in turn, loses its suppressive effect on cell cycle progression [34]. The level of cyclin D_1 expression appears to be directly correlated with the tumor cell proliferation rate in MCL [35]. Several additional molecular alterations that target predominantly cell cycle regulatory elements have been described [33].
Another important pathogenetic mechanism in MCL is dysregulation of the DNA damage response pathway. 40-75% of MCL patients carry mutations of the ataxia-telangietasia mutated (ATM) gene, located on chromosome 11, resulting in inactivation of the gene [36]. ATM plays a central role in the cellular response to DNA damage by activating p53 after DNA damage and by controlling phosphorylation of effector genes [33]. ATM inactivation in MCL is associated with a high number of chromosomal alterations suggesting that it may be partly responsible for the chromosomal instability in these tumors. P53 is also frequently directly targeted by genetic alterations and as observed in CLL patients, these dysregulations are associated with a poor prognosis [37,38]. A recent gene expression profiling study has defined a subset of proliferation-associated genes that may predict the length of survival in different subgroups of MCL patients [35].

Therapy

Treatment of CLL

CLL still has been considered an incurable disease despite the availability of highly active chemotherapeutic agents and monoclonal antibodies [39,40]. The decision to treat is guided by the stage of the disease, the presence of symptoms, disease activity as expressed by lymphocyte doubling time (LDT) and probably in the near future the presence of unfavourable prognostic factors, such as ZAP-70 expression, unmutated IgV_H genes and genetic aberrations, involving chromosome 11 and 17 as described in the biology of CLL paragraph. The staging classification of Rai and Binet are used to estimate the prognosis based on the extent of lymphadenopathy, hepatosplenomegaly measured by palpation and anemia and thrombocytopenia measured by blood counts. The clinical stages according to RAI and Binet are shown in table 1. These well validated clinical staging systems describe early (Rai 0, Binet A), intermediate (RAI I/II, Binet B) and advanced (RAI III/IV, Binet C) stages with estimated median survival times of >10, 5-7 and 1-3 years, respectively [41,42].

Table 1. Clinical staging system according to Rai and Binet [41,42].

Binet classification			Rai classification				Median survival (years)
Stage	Definition	% of patients	Risk group	Stage	Definition	% of patients	
A	< 3 lymphoid areas	60	low	0	lymphocytosis only	30	>10
B	> 3 lymphoid areas	30	intermediate	I II	lymfadenopathy Hepato or splenomegalie lymfadenopathy	25 25	5-7
C	anemia and/or thrombocytopenia	10	high	III IV	lymfocytosis with anemia lymfocytosis with anemia and thrombocytopenia	10 10	1-3

Since the use of immunophenotyping and automated differential blood counts, more early stage asymptomatic individuals with CLL are being diagnosed. For this group of patients the Rai and Binet staging systems lack the ability to prospectively identify those patients that will rapidly evolve to more aggressive disease. Importantly, new clinical and biological markers are determined which could be considered as additional prognostic factors. LDT, as indicator for disease kinetics, is the time required for doubling of the peripheral blood lymphocytes. Although a short LDT has prognostic value in patients with early stage disease, host factors can interfere with the LDT thereby influencing treatment decisions [43]. Other interesting potential prognostic factors are ß-2 microglobulin or solubable CD23 but all these parameters needs to be validated in prospective trials [44]. Importantly, treatment of unselected patients in early stage increased toxicity without improving overall survival, therefore these patients may be better off by monitoring with a watch and wait strategy [45]. Consensus guidelines for treatment of patients with CLL and response criteria have been proposed by the National Cancer Institute (NCI) [46]. However, with the introduction of new more effective therapeutic strategies such as chemoimmunotherapy and allogeneic stem cell transplantion (SCT) following reduced-intensity conditioning (RIC) resulting in long duration of responses, revision of these NCI response criteria is

needed. Using 4-color flow cytometry and real-time quantitative polymerase chain reaction (PCR), assessment of minimal residual disease (MRD) after effective therapy is available. Elimination of MRD after therapy may become the therapeutic goal in the future [47].

Chemotherapy

Until recently the alkylating agent chloorambucil has been the cornerstone of treatment in patients with CLL. Three randomized trials have demonstrated that the nucleoside purine analog fludarabine yielded superior overall and complete response rates and longer progression-free survival (PFS) as compared with alkylator-based therapy. Unfortunately, all patients treated with fludarabine ultimately relapsed and no survival benefit was observed. [39,48-50]. Based on these encouraging results, fludarabine was combined with cyclophosphamide as first-line therapy showing better overall response rates and PFS compare to fludarabine alone [51]. Again after a follow-up period of 22 months, no difference in overall survival was noticed. To further enhance therapeutic efficacy and eradicate residual malignancy, fludarabine or fludarabine-based regimens were combined with monoclonal antibodies (MoAb).

Chemoimmunotherapy

Rituximab (anti-CD20 MoAb), a humanized MoAb, has single-agent activity in CLL [52,53]. Probably due to low expression levels of CD20 on the CLL cells, its single agent efficacy is limited but it may be synergistic in combination with fludarabine or fludarabine and cyclophosphamide [54,55]. The combination fludarabine and rituximab did not protect high-risk CLL patients with unmutated IgV_H genes and unfavourable cytogenetics from disease progression [56]. Alemtuzumab (anti-CD52 MoAb), currently approved for the treatment of refractory CLL, is highly active in both blood and bone marrow but its efficacy is limited in the presence of bulky lymfadenopathy [47,57,58]. In patients with refractory CLL (66% fludarabine-refractory) impressive response rates were reported with the combination fludarabine and alemtuzumab emphazing the complementary modes of actions and illustrating the synergistic effects the combination [59]. Randomized controlled trials are now pivotal to define the optimal combination, dose and schedules for chemoimmunotherapy.

High dose chemotherapy followed by rescue autologous stem cell transplantation

Patients with chemotherapy-refractory CLL and/or patients with adverse prognostic markers might benefit from more intensive approaches aiming to prolong survival. The efficacy of autologous SCT relies exclusively on the cytotoxic therapy administered. In accordance with this concept outcomes were better for patients transplanted in earlier stage with chemotherapy sensitive CLL [60]. Autologous SCT up front after initial treatment with fludarabine in previously untreated CLL patients was shown to be feasible with low treatment-related mortality (TRM). However, despite complete molecular remissions in a large cohort of patients shortly after autoSCT, ultimately all patients relapsed, indicating that this treatment modality is not curative for CLL [61]. In high-risk CLL patients (90% unmutated IgV_H genes) treated with an autologous SCT after myeloablative-conditioning regimen

including total body irradiation (TBI) no evidence of a plateau of DFS or survival was observed [62]. In both studies, late TRM due to secondary malignancies such as myelodysplastic syndromes (MDS) negatively effected outcome [61,62]. In conclusion, combinations of chemotherapy and MoAbs and/or dose intensification of chemotherapy in combination with autologous SCT do not have the capacity to definitely cure CLL.

Treatment of MCL

At diagnosis most patients have advanced disease, necessitating the administration of chemotherapy. Several chemotherapeutic regimens have proven efficacy as induction treatment with response rates of 80-95% [63,64]. Although rituximab as a single agent has limited activity against MCL [65], in combination with anthracycline-based regimens such as CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone) it significantly increased overall and complete response rates and time to treatment failure compared to CHOP but not progression-free and overall survival [66]. A recent randomized study observed an improved overall survival in patients with relapsed or refractory MCL who were treated with a combination of rituximab and the purine nucleoside analog-based regimen fludarabine, cyclophosphamide and mitoxantrone (FCM) in comparision with FCM alone [63,67]. Intensification of the chemotherapy by combining an anthracycline/based regimen with alternating cycles of rituximab and high dose methotrexate and cytarabine compared favourably with other studies at the cost of significant toxicity with 8% toxic deaths [68]. Unfortunately, in none of the studies a plateau in the failure-free survival and overall survival curves was observed. Since these regimens do not offer cure, high-dose chemotherapy followed by autologous SCT has been studied in order to improve survival rates [40,69]. High-dose chemotherapy followed by autologous SCT as consolidation therapy after successful induction therapy prolonged progression-free survival in comparison to interferon maintenance but not overall survival [70]. Approaches to improve the preparative regimen for autologous SCT by adding rituximab or a radioimmunoconjugate or by applying rituximab maintenance therapy after autologous SCT may prolong survival but will probably not cure the disease [64,71].

Cellular immunotherapy against CLL and MCL

Cognate interactions between antigen-presenting B and T cells are pivotal for immunologic responses. For an efficient immune response the antigen-presenting cell (APC) must present the peptide antigen in the context of the major histocompatibility complex (MHC) to deliver a signal to the T-cell receptor (TCR) of the antigen-specific T cells. Next, an antigen-nonspecific, MHC-nonrestricted costimulatory signal must be delivered to the T cell. CD40, a molecule of the family of tumor necrosis factor (TNF) receptors expressed throughout B-cell development, plays a critical role. Its ligand CD40L (CD154) is induced on activated CD4+ T cells upon antigen recognition by the TCR. CD40/CD40L interactions significantly enhances the antigen-presenting capacity of B cells by upregulating the adhesion molecules ICAM-1 (CD54) and LFA-3 (CD58) and the costimulatory molecules B7-1 (CD80), B7-2 (CD86) and CD83. Binding of the costimulatory molecules by CD28 on the T cell surface induces T-cell proliferation, activation and increase the production of IL-2 whereas binding by CTLA-4 on the T

cell results in T-cell anergy [72,73]. Hence, professional APC like dendritic cells (DC) or activated monocytes or B cells, expressing high levels of adhesion and costimulatory molecules are capable of provoking strong antigen specific T-cell responses, whereas immature APCs lacking costimulation may lead to the induction of anergy or suppression.

Malignancies generally fail to induce a clinically significant antigen-specific antitumor response in the autologous host. The inability of tumor cells to induce autologous T-cell proliferation and cytotoxicity may be caused by insufficient numbers of functional antigen-specific T cells in the repertoire or a suppression of antigen-specific immune responses. Other explanations might be the absence or low expression levels of unique tumor-associated antigens or the low immunogenicity of these antigens. Finally, neoplastic cells may not be capable of functioning efficiently as APC. Ideally, tumor-associated antigens should be overexpressed or de novo expressed in tumor cells compared to normal cells, they should be bind to the MHC molecules and they should be recognized by the T-cell repertoire in MHC-restricted fashion.

In B-cell malignancies the Ig is an unique clonal marker containing tumor-specific epitopes or idiotypes that can function as target for T-cell-mediated immune responses [74-76]. In addition several other tumor-associated antigens highly overexpressed in CLL and/or MCL have been identified. Autologous T-cell responses against bcl-2, survivin, fibromodulin and oncofetal antigen-immature laminin receptor have been reported [77-80]. In these studies it was shown that the neoplastic B cells were not capable to adequately present the tumor antigen to the T cell due to lack of costimulatory molecules. Modification of the B-cell tumor into an APC phenotype or ex-vivo pulsing of professional DC with the tumor-associated antigen was necessary for the recognition of the tumor antigen by autologous T cells [78-81]. Since both normal B cells and malignant B cells highly express CD40, CD40 stimulation is an effective tool to activate tumor B cells and modify them into professional malignant APC with high expression levels of CD80 and CD86. Transformation of B-cell malignancies, including CLL, follicular lymphoma and hairy cell leukemia into APC has been studied previously, but the modification of MCL cells into APCs have not been reported [82-86]. Using these malignant APC as stimulator cells, autologous T-cell responses *in vitro* could be induced [82,83]. For CD40 stimulation, coculture of neoplastic B cells in the presence of CD40L-transfected feeder cells have been used [82,83,85-87]. Another approach is the direct gene transfer of CD40L into CLL cells via adenovirus vectors or the *ex vivo* infection with vectors encoding for multiple costimulatory molecules [84,88,89]. To translate these results into clinical practice vaccination strategies with tumor-specific idiotype vaccines [75,90,91], CD40L-transduced CLL cells [88] and tumor antigen-pulsed DC [78] were developed. Clinical trials have illustrated the feasibility of this immunotherapeutic strategies and showed clinical responses after the vaccinations [88,90,91]. However, these clinical responses were transient and did not lead to the induction of sustained durable anti-tumor T-cell response. More importantly, best responses were seen in patients with relative indolent disease whereas patients with aggressive and/or bulky disease minimal response rates were observed. In conclusion, although autologous T cells capable of reacting against tumor-associated

peptides may exist in the T-cell repertoire of the patient, tolerance for tumor antigens or suppression of tumor-specific T cells might be induced.

In patients with CLL several abnormalities involving the T cell as well as composition of T-cell populations, possibly impairing tumor-specific immune responses, have been reported. Recently, significantly increased amounts of regulatory T cells (T_{reg}) were found in CLL patients compared to healthy individuals and the highest frequencies were observed in untreated patients and progressing patients presenting with extended disease [92]. T_{reg} are $CD4^+CD25^+$ T cells that can suppress antigen-specific T-cell immune responses and may be partly responsible for the lack of antitumor immune responses [93]. Additional immune suppressive factors including cytokines TGF-ß, interleukin (IL)-10 and IL-4 may further suppress T-cell activation, expansion and T-cell effector functions. High expression levels of these immuno-modulatory cytokines in CLL cells and autocrine IL-10 production by the MCL cells have been described [94-96]. Finally, $CD4^+$ and $CD8^+$ T cells from CLL patients showed reduced expression of CD40L and CD28 respectively, thereby possibly interfering with T cell-APC interaction [3,97]. The usage of a limited oligoclonal TCR repertoire by $CD4^+$ T cells in patients with CLL further suggest that these cells might be involved in the disease process [98]. The T-cell compartment of the patient is further impaired by the administration of chemotherapy and MoAbs. The introduction of a whole new T-cell repertoire derived from a healthy donor may be an attractive approach to reconstitute T-cell reactivity against tumor antigens. More importantly, after an allogeneic SCT a variety of alloantigens, expressed by the malignant cells, may serve as potential targets for alloreactive donor T-cells thus definitely eradicating persistent disease.

Allogeneic SCT

Following myeloablative conditioning or reduced-intensity conditioning (RIC) allogeneic SCT leads to fully or partially replacement of recipient-derived hematopoiesis by donor hematopoiesis. Once durable donor engraftment is established the administration of non-tolerized donor T cells is possible. The transfer of non-selected donor T cells in the stem cell graft or in the DLI have beneficial effects through reactivity against (allo)antigens on leukemic cells, the graft-versus-leukemia (GvL) effect but may also have detrimental alloreactive activity against non-hematopoietic tissue resulting in graft-versus-host disease (GvHD). After allogeneic HLA partially mismatched SCT T-cell responses may be directed against the differentially expressed HLA complex from the patient. Transplantation over HLA barriers will thus lead to alloreactivity against the polymorphic part of the HLA molecules. Following HLA-matched allogeneic SCT, T-cell responses can be directed against minor histocompatibility antigens (mHag). MHag are immunogenic peptides encoded by polymorphic genes that can be recognized by alloreactive T cells in the context of “self” HLA-molecules [99,100]. In donor/recipient pairs of allogeneic SCT mHag can be differently expressed in various tissues and can induce T-cell responses that contribute to GvHD and GvL activity [99,100]. Ubiquitously expressed mHag, such as the male-specific mHag [101] and the HA-8 mHag [102] may serve as target for T-cell responses associated with GvHD. In contrast, T-cell reactivity against mHags such as HA-1, HA-2 or BCL2A1 that are selectively expressed in hematopoietic cells including leukemic cells, but not widely expressed in non-

hematopoietic tissue may be preferentially associated with GvL activity [103-106]. The B-cell lineage restricted mHag HB-1 was capable of eliciting donor-derived cytotoxic T lymphocyte (CTL) reactivity against B-cell acute lymphoblastic leukemia [107]. Recently, a novel B-cell restricted mHag, encoded by an alternative transcript of the proliferation associated nuclear element 1 (PANE1) was identified that is selectively expressed in B-lymphoid cells with the highest levels of expression in resting B cells and CLL cells [108]. Because of the B-cell and CLL-restriction of this mHag, it might be a potential therapeutic target for mHag-specific adoptive cellular immunotherapy in patients with persistent CLL after allogeneic SCT.

Allogeneic SCT in patients with CLL and MCL

Allogeneic SCT provides new therapeutic opportunities for cure of advanced chronic B-cell malignancies. New prognostic markers (see “the biology of CLL” paragraph) enable to identify high-risk CLL patients with a poor prognosis who will merit this experimental treatment modality with curative intent. As illustrated in figure 1, the much lower incidence of relapse after allogeneic SCT compared to autologous SCT as well as the elimination of post-transplant residual disease by adoptive cellular therapy such as donor lymphocyte infusions (DLI) suggests susceptibility of CLL cells and MCL cells to a GvL effect [60,109-112] . However, overall survival rates after allogeneic SCT reported to be only 40-60% at 4-5 years follow-up due to substantial toxicity associated with allografting using standard myeloablative regimens [60,110,113-115]. High TRM up to 40% was observed caused by organ toxicity, infectious complications related to the impaired immune system following the conditioning regimen and graft-versus-host disease (GvHD) in an extensively pretreated older patient population.

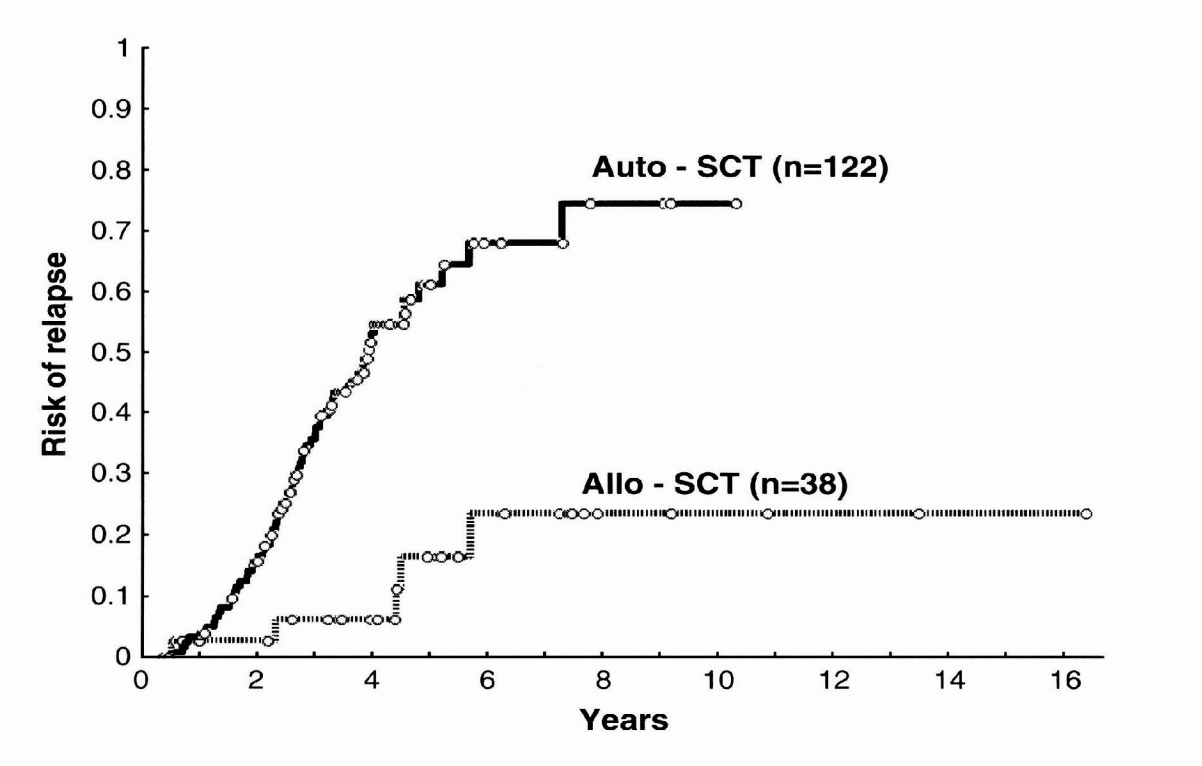

Figure 1. Relapse rate in patients with CLL entering complete remission after SCT. Series from the International Group on CLL/Transplants.

Various strategies of reducing the preparative regimen's toxicity while preserving its immunosuppressive effect necessary to prevent graft rejection and to establish donor chimerism have been developed. Using RIC, short-term TRM was significantly reduced while maintaining the GvL-mediated curative potential of allogeneic SCT [116-119]. Since incidences of acute and chronic GvHD were not substantial altered following RIC, the reduced mortality was specifically related to a decrease of infectious complications [117,119]. However, considerable toxicity due to a high incidence of acute GvHD (>40%) and extensive chronic GvHD (30-60%), caused by alloreactive T cells, was observed [117,119,120]. The use of alemtuzumab to deplete for donor and recipient T cells as part of the RIC strategy effectively reduces GvHD [111,118,121]. A beneficial effect of using alemtuzumab for T-cell depletion is its intrinsic anti-CLL activity thus offering a time frame after allogeneic SCT to optimize the potential of adoptive immunotherapy. Due to profound T-cell depletion *in vitro* and/or *in vivo* using this approach no GvL effect without additional treatment with donor T cells is anticipated [117,118]. The two-step approach of allogeneic SCT following RIC by first the use of T-cell depleted grafts avoiding the risk of GvHD, followed by the postponed administration of DLI was demonstrated to be feasible even in older patients [118,121].

Donor lymphocyte infusion

Incorporation of alemtuzumab in the RIC to deplete recipient and incoming donor T cells has resulted in durable engraftment in sibling and unrelated donor transplantations while significantly reducing the risk for GvHD. The reduced antitumor activity of such protocols necessitates the use of adjuvant DLI infusions to promote GvL activity [109,111,118,121]. The efficacy and curative effect of DLI has been demonstrated in a variety of hematopoietic malignancies and was dependent on the sensitivity of the malignancy to the effects of DLI [122]. The remarkable sensitivity of chronic myeloid leukemia (CML) to DLI had led to the successful use of T-cell depleted protocols with salvage DLI early in the course of the disease relapse [123]. Several studies have shown that CLL and MCL are susceptible to DLI [111,118,124]. However, continuous relapses after allogeneic SCT and the application of DLI were frequently observed with relapse rates 3 years after transplantation of 50% for the MCL patients and 44% for CLL patients in one study [111] and 27% of CLL patients, relapsing after 2 years in another recent study [120]. In these patients escalating doses of DLI were necessary to achieve disease control, resulting in considerable toxicity due to GvHD. Performing the transplantation procedure while patients were in CR resulted in significantly better DFS. These data indicate that in some patients with CLL or MCL, treated with an allogeneic SCT and DLI alloreactive donor T cells are not capable of eliciting a GvL effect against persisting leukemic cells. Therefore both the specificity of the alloimmune response as well as the magnitude of the T-cell response has to be improved to achieve long-term disease control and ultimately cure. Strategies for adoptive immunotherapy augmenting the GvL reactivity and reducing the GvHD are urgently needed.

Adoptive cellular immunotherapy with *in vitro* generated tumor-reactive CTL

The frequently observed relapses after allogeneic SCT and DLI reflecting the incapacity of donor T cells to definitely eradicate resistant CLL or MCL after allogeneic SCT may likely be caused by the inappropriate APC function of the malignant cells. B-cell malignancies can be modified into malignant

professional APCs [82,83,85,125]. Tumor-reactive T cells can be generated by repetitive stimulation of donor-derived T cells with the transformed malignant B cell as stimulator cell. These *in vitro* generated and expanded CTLs can then be infused in the patient. The feasibility and success of this approach has been demonstrated by the induction of CR in a patient with resistant leukemia after allogeneic SCT [126]. Alternatively, mHags with a relatively hematopoiesis-restricted or even more preferable B-cell lineage-restricted expression may serve as tumor-specific antigen. The *in vitro* generation of mHag-specific CTLs by stimulating donor T cells with professional APC from the donor loaded with mHag peptide may also be an approach to enhance GvL reactivity without causing GvHD. Whether transformed CLL and MCL cells as stimulator cells has the capacity to initiate a primary T-cell response in a HLA-matched setting and what the optimal conditions are to generate such a immune response needs to be determined.

Aim of the study

With the introduction of less toxic preparative conditioning regimens, resulting in reduced TRM, allogeneic SCT is now considered a potential curative treatment modality for patients suffering from chronic B-cell malignancies such as CLL and MCL. However, the observation that after allogeneic SCT and the administration of DLI relapses frequently occur, suggests that these neoplastic B cells are capable of escaping T-cell mediated immunity. Furthermore, considerable morbidity due to GvHD caused by alloreactive T cells was observed. In this thesis we investigated the APC capacity of primary CLL and MCL cells and analyzed opportunities to effectively transform them into professional malignant APC. To mimic the clinical transplantation setting, the induction of primary T-cell responses against the malignant APCs were performed using T cells from HLA-matched donors. To elucidate the nature of graft-versus-CLL responses and to gain more insight into the success and failure of cellular adoptive immunotherapy, T-cell responses involved in the GvL and GvHD in patients treated with DLI were studied.

In **chapter 2** we analyzed the expression levels of adhesion and costimulatory molecules on CLL cells. The most optimal method to transform CLL into efficient APC cells using activating cytokines, by triggering toll-like receptors (TLR) using microbial pathogens and by CD40 stimulation with CD40L-transfected fibroblast was determined. The production of immunostimulatory cytokines by these modified malignant cells was established. Finally, the allostimulatory capacity of the obtained CLL APCs and the primary CLL was examined in a HLA class I-matched setting.

To further translate the results from chapter 2 into a clinically applicable transplantation model, and to investigate whether modified CLL cells are sufficiently immunogenic to initiate an adequate alloimmune response in a complete HLA-matched sibling setting, primary T-cell responses against CLL-APCs were generated. In **chapter 3** we described the results of these experiments and we further analyzed the specificity of the obtained CLL-reactive CTL clones.

In **chapter 4** we described the phenotypic characteristics of MCL cells from six different patients. Pro-inflammatory, MCL- and B-cell activating cytokines were tested for their costimulatory upregulating capacity. Futhermore, CD40 activation was performed to modify MCL cells into an antigen-presenting phenotype. The generation of MCL-reactive CTL lines and clones using MCL APC was investigated.

In **chapter 5** we report the clinical outcomes and response rates in patients with advanced CLL, treated with allogeneic SCT following RIC with *in vitro* T-cell depletion using alemtuzumab. To improve our understanding of the graft-versus-CLL activity we characterized allogeneic immune responses in a patient, successfully treated with DLI and in a patient suffering from progressive disease despite the administration of escalating doses of DLI. To investigate the CLL-reactive T-cell repertoire in the unprimed donor, primary T-cell responses against the CLL and the transformed CLL were assessed.

In **chapter 6** the results of these studies are summarized. Alternative strategies in cellular immunotherapy in the context of RIC allogeneic SCT to improve the specificity and efficacy of GvL responses are discussed. Finally, suggestions to apply these results into a clinical protocol are provided.

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