Allogeneic cellular immunotherapy for chronic B-cell leukemia
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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	lymfadenopathy	25	5-7
				II	Hepato or splenomegalie lymfadenopathy	25	
C	anemia and/or thrombocytopenia	10	high	III	lymfocytosis with anemia	10	1-3
				IV	lymfocytosis with anemia and thrombocytopenia	10	

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.

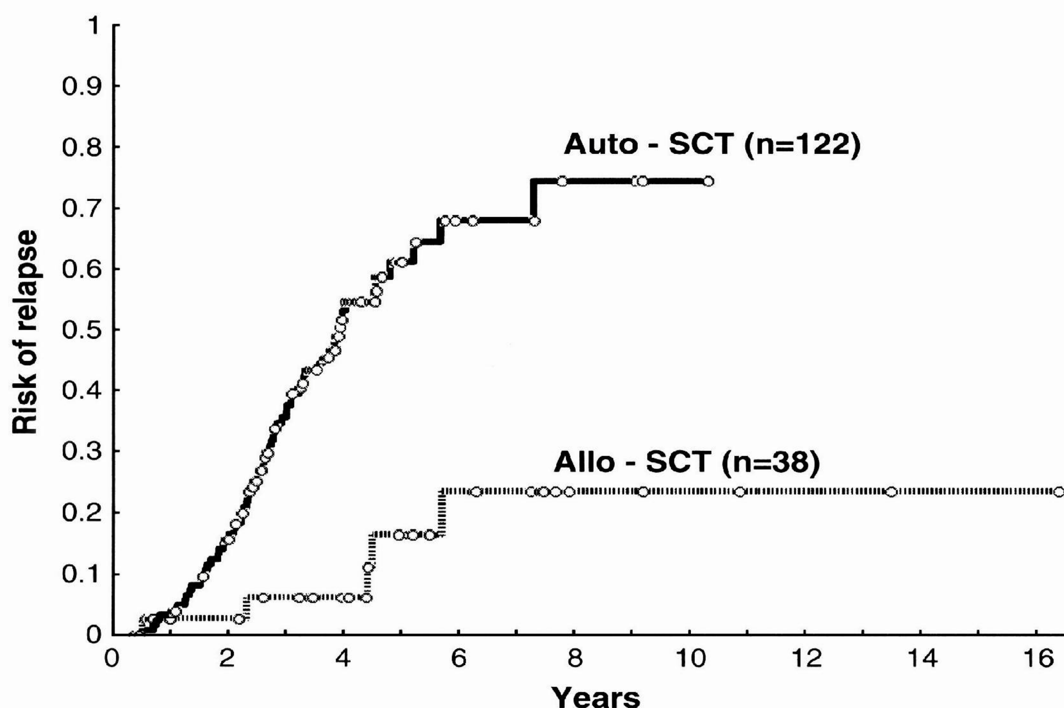

Figure 1. Relapse rate in patients with CLL entering complete remission after SCT. Series from the International Group on CLL/Transplants.

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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Chapter 2

Generation of B-cell chronic lymphocytic leukemia (CLL)-reactive T-cell lines and clones from HLA class I-matched donors using modified CLL cells as stimulators: implications for adoptive immunotherapy

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Abstract

Allogeneic stem cell transplantation following reduced-intensity conditioning is being evaluated in patients with advanced B-cell chronic lymphocytic leukemia (CLL). The curative potential of this procedure is mediated by donor-derived alloreactive T cells, resulting in a graft-versus-leukemia effect. However, CLL cells may escape T cell-mediated immune reactivity since these cells lack expression of costimulatory molecules. We examined the most optimal method to transform CLL cells into efficient antigen presenting cells (APC) using activating cytokines, by triggering toll-like receptors (TLRs) using microbial pathogens and by CD40 stimulation with CD40L-transfected fibroblasts. CD40 activation in the presence of IL-4 induced strongest upregulation of costimulatory and adhesion molecules on CLL cells and induced the production of high amounts of IL-12 by the leukemic cells. In contrast to primary CLL cells as stimulator cells, these malignant APCs were capable of inducing the generation of CLL-reactive $CD8^+$ CTL lines and clones from HLA class I-matched donors. These CTL lines and clones recognized and killed primary CLL as well as patient derived lymphoblasts, but not donor cells. These results show the feasibility of *ex-vivo* generation of CLL-reactive $CD8^+$ CTL's. This opens new perspectives for adoptive immunotherapy, following allogeneic stem cell transplantation in patients with advanced CLL.

Introduction

Chronic lymphocytic leukemia of B cell origin (CLL) can not be cured with conventional chemotherapy [1]. Although novel chemotherapeutic agents including purine analogues [2] and targeted therapy with monoclonal antibodies (MoAb) like alemtuzumab or anti-CD20 [3-5] are promising, these treatment modalities rarely lead to cure of the disease. Autologous stem cell transplantation (SCT) did not result in longer relapse-free survival than conventional therapies [6]. Allogeneic SCT is increasingly considered for treatment of patients with advanced CLL. The rationale for allogeneic SCT relies on adoptive transfer of donor-derived alloreactive T cells that may eradicate refractory or recurrent CLL. Complete sustained remissions in CLL patients have been observed after allogeneic SCT suggesting a susceptibility of CLL cells to a graft-versus-leukemia (GvL) effect [6-8]. However, the application of allogeneic SCT in CLL has been hampered by high treatment-related mortality (TRM) in this extensively pretreated older patient population [6-8]. Reduced-intensity conditioning (RIC) may reduce short-term TRM [8-10]. Longterm follow-up of CLL patients treated with allogeneic SCT after dose-reduced conditioning regimens is limited, but short-term follow-up showed reduction of TRM with preservation of GvL reactivity [7-10]. Acute or chronic graft-versus-host disease (GvHD) remains a major cause of morbidity and mortality [7,11]. An approach to reduce GvHD reactivity while conserving GvL activity is to perform allogeneic SCT with *in vitro* T-cell depletion followed by treatment using *in vitro*-selected cytotoxic T lymphocytes (CTLs) with relative specificity for the CLL or using hematopoiesis-restricted minor histocompatibility antigen (mHag)-specific CTLs [12,13].

Although CLL cells show high expression of HLA class I and II molecules necessary for presentation of antigens to T cells, CLL cells are unable to stimulate normal allogeneic T cells in a mixed lymphocyte reaction (MLR) due to inadequate expression of costimulatory and adhesion molecules [14-16]. For the induction of a sustained T-cell response, the expression of the costimulatory molecules CD80 or CD86 on the antigen-presenting cells (APC) is essential [17]. Both normal B cells and CLL cells highly express CD40, and stimulation of these cells via the CD40-CD40L pathway may enhance the immunogenicity of these cells by upregulating costimulatory molecules [14,18-21]. Hence, CD40-activated CLL cells may induce specific T-cell responses capable of reacting with leukemic cells [14,19,22]. Modification of B cells, monocytes or immature DC into efficient APCs can also be initiated by microbial products such as endotoxin (LPS), viral double-stranded RNA, or immunostimulatory bacterial CpG-DNA sequences (CpG) [23-25]. These microbial pathogens can be recognized by distinct toll-like receptors (TLRs) expressed on APC. Signaling through TLRs strongly activates APC to upregulate costimulatory molecules and to synthesize and release inflammatory cytokines [23]. Normal and neoplastic B cells express most TLRs at low or undectable levels except TLR9 and TLR10, which are abundantly expressed in B cells [26,27]. Unmethylated CpG motifs, characteristic of bacterial DNA, are detected by TLR9, and may therefore stimulate normal and malignant B cells [26-28].

In this study, we investigated the most optimal method to modify CLL cells into efficient malignant APCs using several proinflammatory cytokines including interleukin (IL)-1, IFN-α, and TNF-α, and

cytokines IL-2, IL-3 and IL-4, known to upregulate costimulatory molecules in plasmacytoid dendritic cells (DC) and B cells [29,29-31,31]. We further tested whether activation of TLRs by various microbial pathogens or triggering CD40 with CD40L-transfected fibroblasts could induce further upregulation of costimulatory molecules. Moreover, the additional stimulatory effects of TLRs triggering by microbial products on CD40-activated CLL cells were studied. To examine the potential use of *in vitro* manipulated CLL for adoptive immunotherapy after allogeneic SCT, we used the modified CLL cells as APCs to generate allogeneic CLL-reactive $CD8^+$ CTL lines and clones from HLA class I-matched donors. Stimulation of CD40 on CLL cells in the presence of IL-4 induced the strongest upregulation of costimulatory molecules. Using these malignant APCs as stimulator cells, CLL-reactive $CD8^+$ CTL lines and clones were generated from three HLA class I-matched donors. These CTL lines and clones recognized and killed primary CLL as well as PHA blasts or EBV transformed B cells (EBV-LCL) from the patient, but not donor specific cells. Our findings indicate that *ex-vivo* generation of CLL-reactive $CD8^+$ CTL lines and clones from HLA class I-matched donors is feasible.

Material and methods

Cell samples

After informed consent peripheral blood samples were obtained from 14 untreated patients with CLL, and from 3 healthy donors. Peripheral blood mononuclear cells (PBMC) of CLL patients were isolated from blood samples by Ficoll density separation and cryopreserved. As assessed by flow cytometry, more than 90% of the PBMC from CLL patients coexpressed CD19 and CD5 surface molecules. Three HLA class I-matched patient / donor pairs were used to induce a T-cell response against CLL cells. In two of the three patient / donor combinations a one locus HLA class II mismatched donor was available. The HLA types of these patients and donors are shown in table 1.

Table 1. HLA type of donor and patients.

	HLA class I						HLA class II			
CLL 3	A2	A3	B7	B14	Cw7		DR13	DR7	DQ1	DQ2
donor	A2	A3	B7	B14	Cw7		DR13	DR7	DQ1	DQ7*
CLL 4	A3	A31	B7	B27	Cw2	Cw7	DR2		DQ1	
donor	A3	A31	B7	B27	Cw2	Cw7	DR15*	DR4*	DQ1	DQ3
CLL 13	A2	A26	B7	B44	Cw5	Cw7	DR4	DR15	DQ3	DQ6
donor	A2	A26	B7	B44	Cw5	Cw7	DR13*	DR15		DQ6

HLA-A, -B, and -C typing was performed by standard serology methods, and HLA-DR and -DQ typing was done by DNA analysis using sequence specific primers. *donor-patient disparate alleles

Modification of CLL cells into APCs

CLL cells were cultured in IMDM (BioWhittaker, Verviers, Belgium) containing 10 % human serum at 37˚C in a 5% CO_2 humified atmosphere in 24-well plates (Costar, Cambridge, MA, USA) at a concentration of 10^6 cells/well in a total volume of 1 mL per well. Leukemic cells were cultured in the presence or absence of the cytokines IL-1α (10 ng/mL, Hoffmann-La Roche, New Jersey, USA), IL-2 (100 U/mL, Chiron, Amsterdam, Netherlands), IL-3 (50 ng/mL, Novartis, Basel, Switzerland), IL-4 (500 U/mL, Schering-Plough, Amsterdam, the Netherlands), TNF-α (10 ng/mL, Boehringer Ingelheim, Ingelheim am Rhein, Germany) or interferon-α (IFN-α,1000 U/mL, Hoffmann-La Roche), or in the presence or absence of the cytokine combinations IL-3 and IL-4, IL-4 and IFN-α, or IL-4 and TNF-α . For activation of innate immunity, CLL cells were incubated with synthetic CpG oligodeoxynucleotide 2006 (CpG; 5' TCGTCGTTTTGTCGTTTTGTCGTT-3', 10 µg/mL; Eurogentec, Seraing, Belgium), lipopolysaccheride (LPS, 100 ng/mL, Sigma-Aldrich, St.Louis, MO, USA) or polyriboinosinic polyribocytidylic acid (Poly(I:C), 50 µg/mL, Sigma-Aldrich) in the presence or absence of IL-4 (500 U/mL). Finally, to enhance further upregulation of costimulatory molecules, CLL were cocultured on ltk murine fibroblast cells transfected with the human CD40-ligand [32] (tCD40L; kindly provided by Dr.C.van Kooten, Department of Nephrology, Leiden University Medical Center). The fibroblasts were irradiated (70 Gy), and seeded at a concentration of $1x10^5$ cells/well in 24-well plates (Costar). CLL were added at a concentration of $1x10^6$ cells/well in presence or absence of cytokines, cytokine combinations and/or immunostimulators. After 24, 48, 96 or 144 hours of culture, CLL cells were harvested and washed. Morphology of the cells was analyzed, the number of viable cells was counted using eosin exclusion, and the cells were analyzed by flow cytometry.

Immunophenotyping and cytokine measurement

To perform immunophenotyping, mouse MoAbs conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or phycoerythrin cyanine 5 (PE-Cy5) were used. These MoAbs included FITC-conjugated IgG1 antibodies specific for CD5, CD25, CD40, CD54 or CD86, and FITC-conjugated IgG2a antibodies specific for CD58 or HLA-DR. PE-conjugated IgG1 antibodies specific for CD14, CD19, CD23, CD56, CD80 or CD123, PE-conjugated IgG2a antibodies specific for CD8, PE-conjugated IgG2b antibodies specific for CD83, and PE-Cy5-conjugated IgG1 antibodies specific for CD3 were used. Appropriate isotype controls (IgG1, IgG2a and IgG2b) were used. All MoAbs were purchased from Becton Dickinson (BD, San Jose, CA, USA) except for anti-CD40 and anti-CD58 (Serotec, Oxford, England), anti-CD54 (CLB, Amsterdam, the Netherlands), anti-CD8 and anti-CD83 (Caltag, Burlingame, AL, USA) and anti-CD80 (Immunotech, Marseille, France). 10^5 cells were incubated with MoAbs for 30 minutes at 4˚C, washed twice, and analyzed on a FACScan (BD). Results were analyzed using the CellQuest software (BD). The relative expression of surface antigen is described as the mean fluorescence intensity ratio (MFIR). This value is calculated by dividing MFI of cells stained with a specific MoAbs by the MFI of cells stained with an isotype-control MoAbs. If the percentage of positive events was more than 10 % the leukemic sample was considered positive for that surface marker, and then the MFIR was calculated. Cell-free supernatants were harvested after 96 hours of culturing the CLL cells with or without cytokines, microbial pathogens, and tCD40L cells.

Cytokine measurements were performed using commercial IL-10 (CLB) or IL-12 p40/p70 (U-CyTech, Utrecht, the Netherlands), ELISA kits according to the manufacturer's instructions.

Generation of CLL-reactive CTL lines and clones

PBMC from unrelated healthy HLA class I-matched donors (table 1.) at a concentration of 0.5 x 10^6 cell/well in 24-well plates (Costar) were stimulated with irradiated primary CLL, or CLL cells cultured under various conditions at a responder/stimulator (R/S) ratio of 10:1. IL-2 (100 IU/mL) was added at day 6, and 2 days after each (re)stimulation. At day 9, the T cell lines were harvested, and depleted of $CD4^+$ T cells using anti-CD4-conjugated magnetic beads (Milteny Biotec, Bergisch Gladbach, Germany). The T cell lines were restimulated with irradiated stimulator cells at the same R/S ratios at days 9, 16 and 23. T cells were harvested 4-5 days after the third or fourth stimulation for phenotypic analysis, and used as effectors in cytotoxicity assays. T cell clones were generated from CLL-reactive $CD8^+$ CTL lines by cell sorting using a FACS-Vantage flow cytometer (BD). Viable $CD8^+$ cells were sorted into 96-well microtiter plates at a concentration of one cell per well (single cell/well sorting). The T cell clones were expanded in the presence of irradiated CD40-activated CLL cells (5 x 10^3 cells / well), irradiated allogeneic feeder cells (5 x 10^4 cells / well) in medium consisting of IMDM plus 10% human serum, IL-2 (100 U /mL) and phytohemagglutinin (PHA, 800 ngr/mL, Murex Biotech Limited, Dartfort, UK). After 21-24 days, proliferating T cell clones were tested for specific lysis of the primary CLL cells, CD40 activated CLL cells, or PHA blasts and/or EBV-LCL from patient and donor in a ^{51}Cr-release assay.

Cytotoxicity assay

^{51}Cr-release assays were performed as described previously [33]. Briefly, primary CLL cells, or CLL cells cultured under various conditions, EBV-LCL or PHA blasts from patient or donor were used as target cells. Effector cells and 5,000 ^{51}Cr labeled target cells were added to wells of U-bottom microtiter plates at E/T ratios ranging from 30:1 to 1:1. Spontaneous release was measured by addition of 100µl IMDM with 10% human serum, and maximum release by adding 100µl 1% Triton X-solution to target cells. After 4 hours of incubation at 37ºC ^{51}Cr release was measured in a luminescence counter (Topcount-NXT, Packard, Meriden, CT, USA). The percentage lysis was calculated using the following formula: 100x [(experimental release cpm – spontaneous release cpm) / (maximum release cpm – spontaneous release cpm)]. T cell lines and clones showing more than 10% specific lysis of target cells were considered cytotoxic. To determine HLA class I- and class II-restriction of the recognition of the target cells, blocking studies were performed in selected experiments. Target cells were incubated with anti-HLA class I antibodies (W6/32) or anti-HLA class II antibodies (PdV5.2, kindly provided by Dr.A. Mulder, Department of Immunohematology and Bloodtransfusion, Leiden) at final concentrations of 10 µg/mL for 30 minutes before effector cells added. Blocking experiments at effector level were performed by adding anti-CD8 MoAb (FK18, RIVM, Bilthoven, the Netherlands) at a final dilution of 8µg/mL 30 minutes prior addition of target cells.

Statistical analysis

Differences between experimental groups were analyzed using the Student's *t*-test.

Table 2. Immunophenotype of freshly isolated CLL cells.

	Recognition		Adhesion		Costimulation			
Patient	MHC I	MHC II	CD54	CD58	CD86	CD80	CD83	CD40
	MFIR (% positive cells)							
CLL 1	++ (83)	++ (80)	+ (87)	± (27)	0 (3)	0 (7)	0 (0)	++ (99)
CLL 2	+++ (79)	++ (92)	+ (78)	± (22)	0 (4)	0 (0)	0 (9)	++ (94)
CLL 3	++ (68)	++ (21)	0 (9)	± (54)	0 '(4)	0 (0)	0 (0)	++ (96)
CLL 4	ND	++ (92)	+ (86)	± (55)	± (15)	++ (16)	0 (0)	++ (97)
CLL 5	+++ (91)	+++ (85)	+ (16)	± (12)	0 (5)	0 (0)	0 (2)	++ (94)
CLL 6	+++ (93)	++++ (98)	++ (96)	+ (92)	+ (25)	++ (40)	0 (0)	++ (98)
CLL 7	ND	+++ (92)	+ (18)	+ (69)	0 (4)	+ (12)	0 (0)	++ (92)
CLL 8	+++ (85)	++++ (96)	++ (10)	± (33)	0 (2)	0 (3)	0 (0)	++ (89)
CLL 9	+++ (78)	+++ (99)	± (48)	± (24)	0 (5)	0 (2)	0 (0)	++ (94)
CLL 10	++ (66)	+++ (98)	+ (85)	± (50)	± (12)	0 (2)	0 (2)	++ (99)
CLL 11	+++ (93)	+++ (84)	+ (17)	± (10)	0 (5)	0 (0)	0 (0)	++ (84)
CLL 12	++ (83)	++ (76)	0 (3)	± (10)	0 (6)	0 (2)	0 (1)	++ (63)
CLL 13	ND	++ (90)	0 (8)	± (12)	0 (25)	0 (0)	0 (2)	++ (85)
CLL 14	ND	+++ (96)	± (33)	± (10)	± (13)	± (42)	± (20)	++ (96)

Mean fluorescence intensity ratio (MFIR) is mean fluorescence intensity (MFI) of cells stained with a fluorochrome-conjugated antigen-specific MoAb divided by MFI of cells stained with a fluorochrome-conjugated isotype-control MoAbs. If the percentage of positive events was <10 %, MFIR of the leukemic sample was not calculated and is expessed as 0. If the percentage of positive events was ≥ 10 % than the MFIR is calculated and depicted as: ±, < 10; +, 10 – 20; ++, 20 – 50; +++, 50 – 100; ++++, >100. (ND = not done)

Results

Upregulation of costimulatory and adhesion molecules on CLL cells

Cell surface expression of adhesion molecules CD54 and CD58, and costimulatory molecules CD40, CD80, CD86 and CD83 on freshly isolated CLL cells are shown in table 2. All patients showed intermediate (MFIR 20 to 50) or high expression (MFIR >50) of HLA class I and II. CD58 was expressed at low levels (MFIR <20) in all patients. CD54 expression was negative in 3 patients and low or intermediate in the other patients. CD40 was expressed at intermediate levels in all patients. In only 5 patients expression of CD80 and/or CD86 was observed.

To upregulate the expression of adhesion and costimulatory molecules on CLL cells, we first tested several cytokines, including IL-1, IFN-α, TNF-α, IL-2, IL-3, and IL-4. Next, upregulation of the molecules on CLL cells by triggering TLRs using LPS, poly(I;C) or CpG as microbial components in combination with the various cytokines was tested. Finally, upregulation was investigated after CD40 activation of the CLL cells by tCD40L in the presence of cytokines and/or microbial products. Figure 1 summarizes the FACS analysis of the most optimal combinations to upregulate adhesion and costimulatory molecules on CLL cells. The data are presented as percentages positive CLL cells for a specific molecule (figure 1A) and the MFIR (figure 1B), and were obtained after a culture period of 96 hours. No significant upregulation of adhesion and costimulatory molecules with the cytokines or cytokine combinations alone was found (data not shown). Of the tested microbial components, only CpG in combination with IL-4 induced an increased expression of costimulatory and adhesion molecules. As shown in figure 1, this combination increased the percentage of positive CLL cells for CD86 1.8 fold (MFIR 2.4 fold, $p=0.015$), for CD83 3.8 fold (MFIR 4.0 fold, $p<0.01$) and for CD54 1.3 fold (MFIR 2.3 fold, $p=0.015$). CD40 activation significantly increased the percentage positive CLL cells ($p<0,01$) for all adhesion and costimulatory molecules and the expression levels ($p<0,001$) and transformed CLL cells into characteristic APC phenotypes with high expression of CD80, CD86 and CD83. CD40 triggering in the presence of IL-4 further increased the expression levels of CD80 (MFIR 1.5 fold, $p=0.05$) and of CD86 (MFIR 1.3 fold, $p=0.14$). Additional stimulation with the microbial products did not further enhance the upregulation.

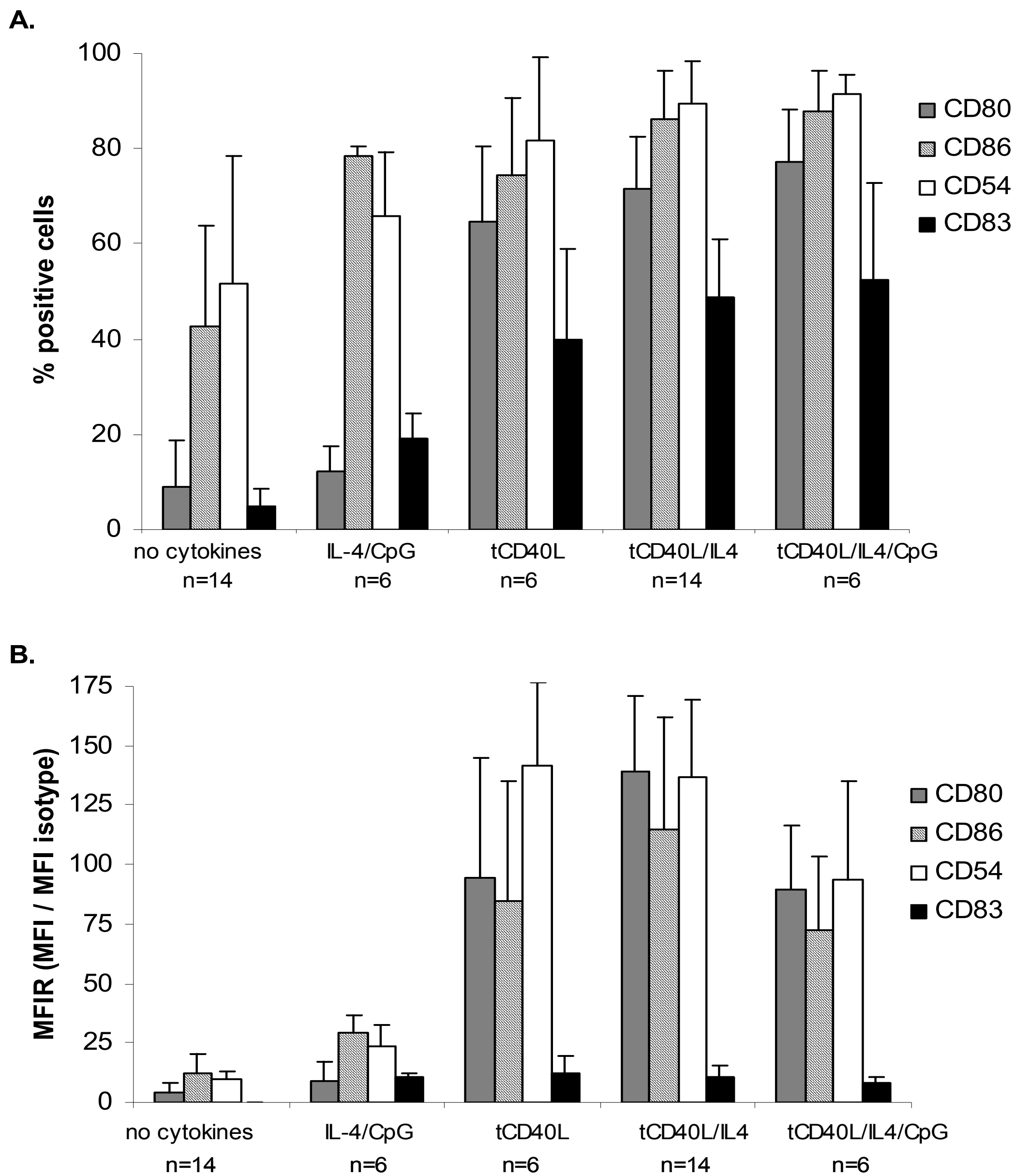

Figure 1. Expression and upregulation of costimulatory and adhesion molecules on CLL cells activated by IL-4, CpG and/or tCD40L. (A) Combinations using CD40 activation give significant higher percentages of positive CLL cells for CD80, CD85, CD54 and CD83 ($p<0.01$) (B) CD40 activation of CLL cells caused significant upregulation of expression levels of all costimulatory and adhesion molecules ($p<0.001$) and the addition of IL-4 further enhanced the expression levels of CD80 and CD86 ($p=0.05$ and $p=0.14$ respectively). CLL cells were cultured for 96 hours in the presence or absence of IL-4 (500 UI/mL) , CpG (10 µg/mL) and/or tCD40L. Expression of CD80, CD86, CD54 and CD83 was analyzed by flow cytometry. Mean fluorescence intensity ratio (MFIR) was calculated as described in Material and Methods section, only if percentage of positive cells was >10%. Results are expressed as mean ± SD of number of CLL patients as shown in figure.

To determine the optimal time period of CD40 and IL-4 stimulation, phenotypic analysis of CLL cells was performed 48, 96 and 144 hours after stimulation. As shown in figure 2, IL-4 and CD40 activation of CLL cells caused strong upregulation of CD80, CD86, CD83 and CD54 within 48 hours. After 96 hours of stimulation a further enhancement of expression levels of CD80 (MFIR 1.2 fold, p= 0.07) and CD86 (MFIR 1.4, p=0.05) was observed. However, after 48 hours a significant downregulation of

percentages and expression levels of CD83 were found ($p<0.05$). Therefore, we considered activation of the CLL cells with tCD40L and IL-4 to be optimal after 96 hours.

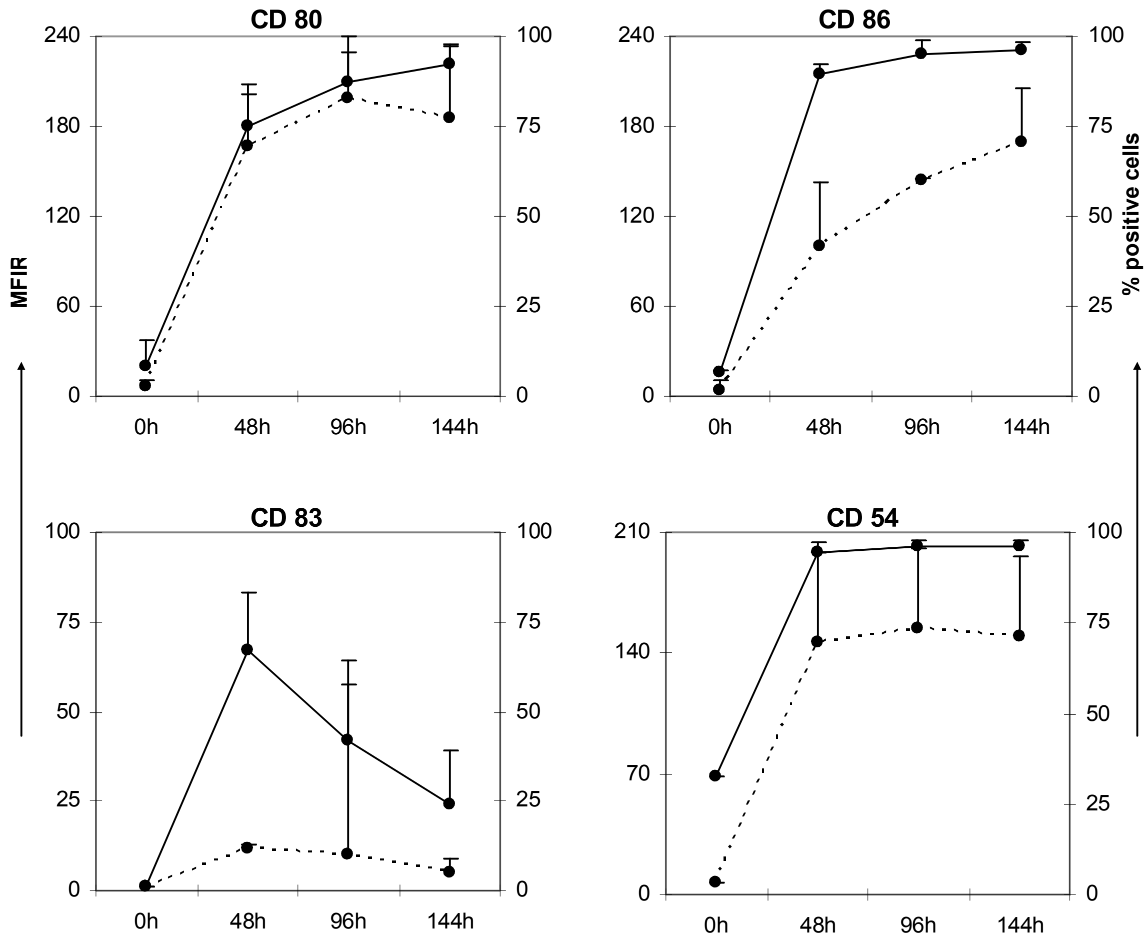

Figure 2. Kinetics of upregulation of costimulatory molecules on CLL cells activated by tCD40L and IL-4. CLL cellls were incubated with tCD40L and IL-4. MFIR and percentage of CLL cells positive for CD80, CD86, CD83 and CD54 were analyzed at different time points by flow cytometry. Data are shown as means ± SD for 4 patients.(straight line: % positive cells, dotted line MFIR).

Stimulation of tCD40L induced a dendritic-like morphology in 60-80% of the CLL cells and as determined by forward /sideward analysis using flowcytometry a 2 to 3 fold increase in size of CD40-activated CLL cells was seen (data not shown). Recovery of viable CLL cells after 96 hours was 29% ± 11% (mean ± SD) for CLL cells cultured in medium alone. Enhanced survival of 45% ± 12 % was found when CLL cells were cultured on tCD40L in medium containing IL-4. In conclusion, stimulation of CLL cells by tCD40L and IL-4 for a period of 96 hours caused the strongest upregulation of costimulatory and adhesion molecules and modified CLL cells into morphologically and phenotypically characteristic APC.

Production of IL-10 and IL-12 by activated CLL cells

As demonstrated in figure 3, after 96 hours of culture primary CLL cells and CpG/IL-4 activated CLL cells produced only minimal amounts of IL-12 (65 ± 22 pg/mL and 50 ± 27 pg/mL, respectively, n=7,

mean ± SD). However, significant higher levels of IL-12 were produced by tCD40L-activated CLL cells (2539 ± 2301 pg/mL, p=0.005, n=6). Additional stimulation with IL-4 or CpG further enhanced the production of IL-12 to 7953 ± 3980 pg/mL (n=7) and 5632 ± 3310 pg/mL (n=5) respectively. In all conditions only low amounts of IL-10 were produced (<20 pg/mL).

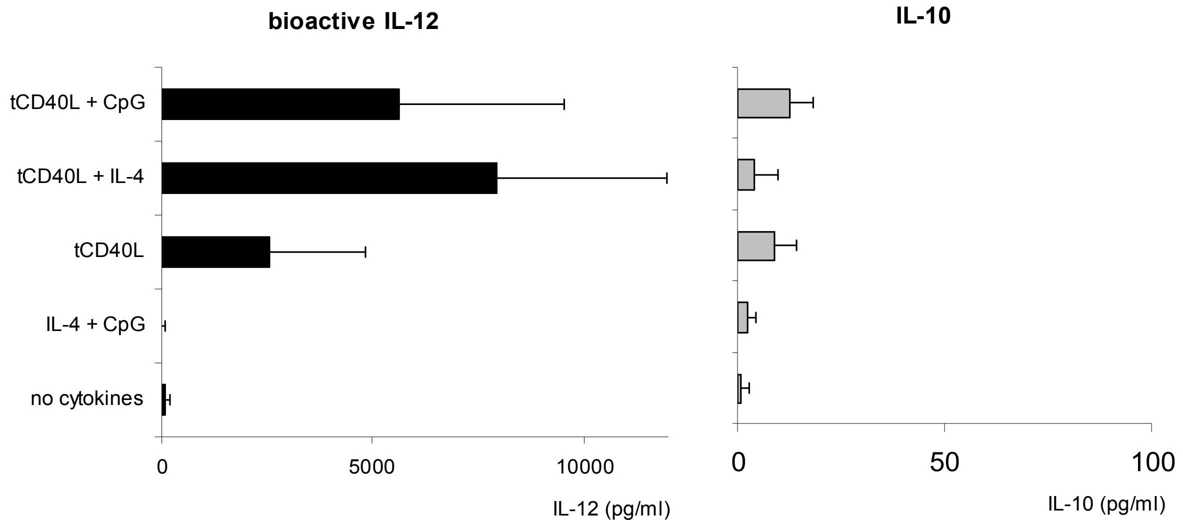

Figure 3. IL-12 and IL-10 production by CLL cells in response to different stimuli. Significant higher levels of IL-12 were produced by tCD40L-activated CLL cells than primary CLL cells (2539 ± 2301 pg/mL and 65 ± 22 pg/mL respectively, p=0.005, n=6). CLL cells were incubated with different stimuli for 96 hours. IL-12 and IL-10 was measured in the supernatant by ELISA. Data are shown as means ± SD of 5-7 experiments.

Proliferation and cytotoxicity of allogeneic CD8$^+$ T cells from HLA-class I-matched donors stimulated with tCD40L/IL-4 activated CLL cells as APCs

Since CD40 activation by xenogeneic fibroblasts is limiting its clinical applicability, we investigated whether CpG/IL-4 activated CLL cells were capable of inducing CTL responses against CLL or that CD40 ligation was essential for the induction of a CLL-reactive T-cell response. For the generation of allogeneic CLL reactive T-cell responses, the stimulatory capacity of primary CLL cells, CpG/IL-4 activated CLL cells and tCD40L/IL-4 activated CLL cells was tested. Figure 4 illustrates the phenotypic characterization of the three different types of CLL stimulator cells tested, obtained after 96 hours of activation.

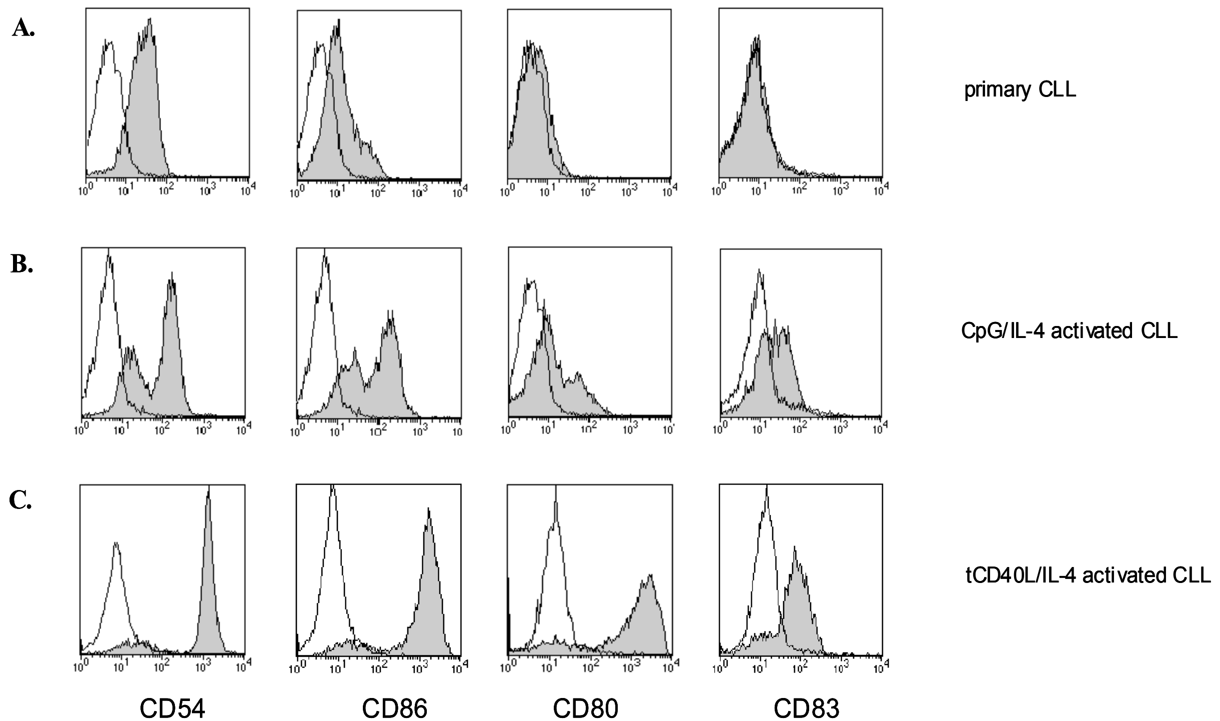

Figure 4. Change of phenotype of CLL cells from one representative patient stimulated with CpG and IL-4, or with tCD40L and IL-4 or unstimulated. (A) Representative examples of CLL cells, obtained from one patient, cultured for 96 hours in medium alone. (B) stimulated with CpG (10µg/mL) and IL-4 (500U/mL) (C) stimulated with tCD40L and IL-4. Unshaded histogram represent staining of CLL cells with the appropriate isotype control MoAb and shaded histogram the staining of CLL cells with specific MoAb

T cells from three unrelated HLA-class I-matched donors (see table 1) were stimulated with the three types of stimulator cells. Since donors were HLA-class II mismatched, CD4 depletion was performed to eliminate undesirable allo-HLA responses. The growth kinetics of the $CD8^+$ T cells in response to the different stimulator cells are shown in figure 5. No proliferation of donor T cells was seen using primary CLL as stimulator cells. Using CpG/IL-4 activated CLL cells as stimulators, in two of the three patient / donor pairs limited proliferation of $CD8^+$ T cells was observed. In contrast, using tCD40L/IL-4 activated CLL cells as APCs in all three patient/ donor combinations tested a 12 to 15-fold increase of $CD8^+$ T cells was observed.

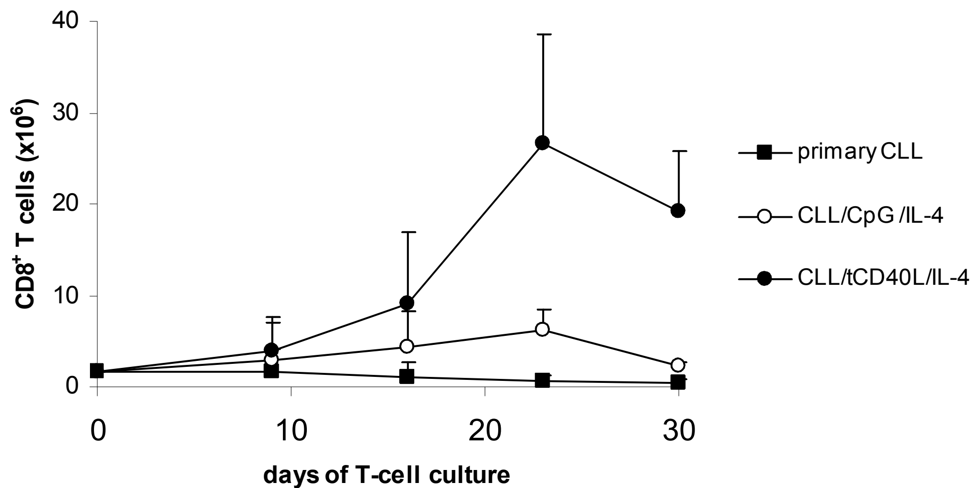

Figure 5. Proliferation of allogeneic $CD8^+$ T cells in response to primary CLL, CpG/IL-4 activated CLL, or tCD40L/IL-4 activated CLL cells. Higher numbers of $CD8^+$T cells at day 23 were obtained using tCD40L/IL-4 activated CLL as stimulators than CpG/IL-4 stimulated CLL cells (26,5 ± 12,01 and 6,18 ± 2,36 $x10^6$ cells respectively, p <0.01, n=3). Proliferation of allogeneic $CD8^+$T cells in response to weekly stimulation. $CD8^+$T cells in PBMC plated on day 0 were 1.5 x 106. The results represent the mean ± SD of experiments performed using 3 donor/patients pairs.

The cytotoxic activity of the CTL lines was tested using primary CLL, tCD40L/IL-4 activated CLL cells, PHA blasts or EBV-LCL from patients and donors as target cells. In all three tested patient / donor pairs, CTL lines generated in response to tCD40L/IL-4 activated CLL cells as stimulators, effectively killed at E/T ratios of 30:1, the primary CLL (41.1 ± 24.7%, mean ± SD, n=3), the tCD40L/IL-4 activated CLL cells (46.3 ± 26.2%), and PHA blasts or EBV-LCL of patients (52.8 ± 10.8%) in a 4-hour ^{51}Cr release assay. In contrast, PHA blasts or EBV-LCL from the donor were not killed (4.3 ± 3.0%, n=3). Cytotoxicity of the generated CTLs is shown in figure 6. In only one of the three donor / patients pairs, cytotoxicity of a CTL line generated using CpG/IL-4 activated CLL cells as APCs could be tested, illustrating cytotoxicity to the CLL-specific targets and PHA blasts from the patient as shown in figure 6A2. In summary, in contrast to primary CLL cells, tCD40L/IL-4 activated CLL cells can be used as stimulator cells to generate CTL lines recognizing and killing CLL-specific targets and PHA blasts or EBV-LCL from the patients. To exclude HLA-non-restricted killing and to exclude that the cytotoxicy of the CTL lines was exerted by allo-HLA-driven contaminating $CD4^+$ T cells, blocking studies were performed using a representative example of a generated CLL-reactive CTL line. Cytoxicity was completely blocked by anti-HLA class I or anti-CD8 antibodies and not by the addition of anti-HLA class II (figure 6D). These results confirmed HLA class I-restricted recognition of the targets by the $CD8^+$ CTL lines.

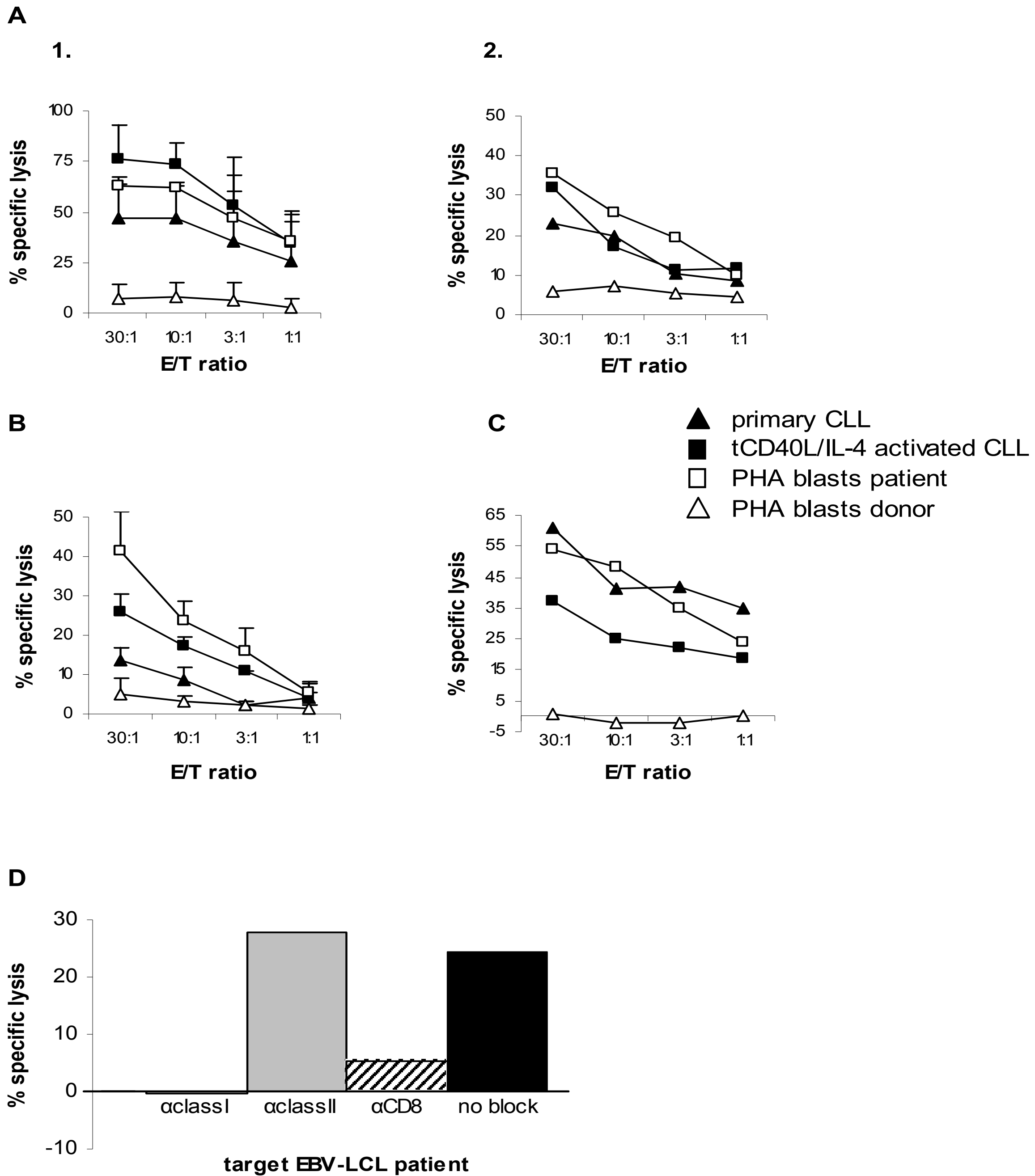

Figure 6. Cytotoxic activity of donor-derived CTL lines from three different HLA class I-matched donors and HLA class I-restriction of cytotoxicity. (A) Cytotoxic activity of CTL lines in donor / CLL 3 combination, generated using tCD40L/IL-4 activated CLL cells as stimulator cells, measured in 4-hour ^{51}Cr release assays (1.) or using CpG/IL-4 activated CLL cells as stimulators (2.) (B) Cytotoxicity of CTL lines in donor / CLL 4 (C) Cytotoxicity of the CTL line in donor / CLL 13 combination. Results are the mean ± SD of two independent experiments (in A1. and B.) or are results of a single experiment (A2. and C.) (D) HLA class I-restriction of cytotoxicty was demonstrated by the following blocking experiments using EBV-LCL of the patient as target at an E/T ratio of 10:1. CTL effectors were preincubated for 30 minutes with anti-CD8 antibodies or the target cells were 30 minutes preincubated with anti-HLA class I (W6/32) or anti-HLA class II (PdV5.2) antibodies prior to the CTL assay.

Generation of CLL-reactive CTL clones

To determine whether the cytotoxicity of the generated CTL lines was exerted by cytotoxic T cells with different specificity, a CLL-reactive CTL line was sorted one cell per well, at day 23. A total of 25 proliferating $CD8^+$ T-cell clones were obtained. Ten of the 25 clones showed sustained proliferation after restimulation, allowing testing for cytotoxicity. Four of these $CD8^+$ clones specifically lysed primary CLL cells (13,9 ± 5,9%, mean ± SD) , tCD40L/IL-4 activating CLL cells (39,4 ± 14,4%) and PHA blasts from the patient (42,3 ± 19,0%) and not PHA blasts from the donor (3,0 ± 1,5%), when tested at an E/T ratio of 10:1 (figure 7). The other 6 clones showed antigen-driven proliferation, but were not cytotoxic to CLL specific targets (data not shown).

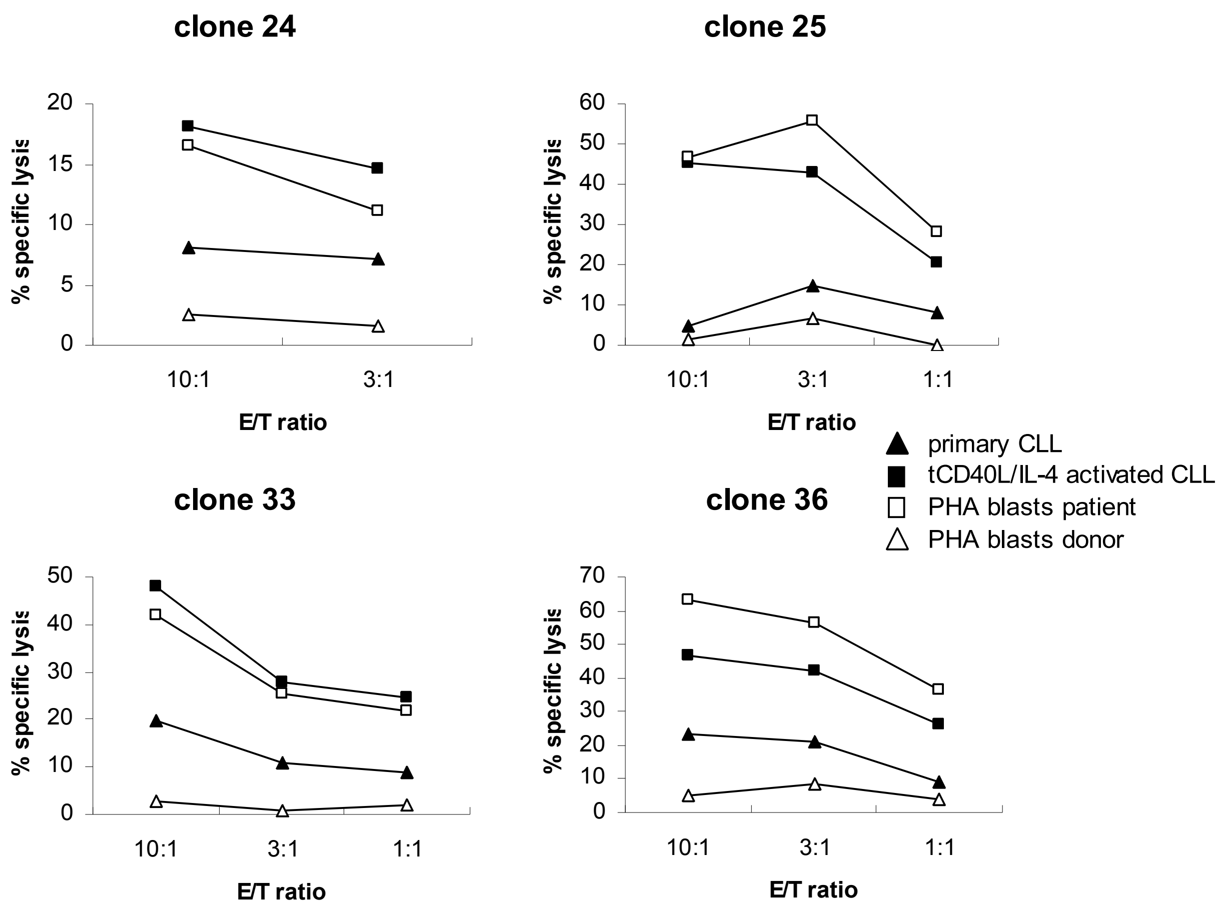

Figure 7. Cytotoxicity of the four donor-derived CD8+ CTL clones. CTL clones were generated in donor / CLL 3 combination by one cell / well sorting of the donor-derived CTL line at day 23. At day 0 and 14, the T cell was stimulated with irradiated tCD40L/IL-4 activated CLL cells and allogeneic feeder cells in medium with IL-2 and PHA. Proliferating clones were tested in a ^{51}Cr release assay against primary CLL, tCD40L/IL-4 activated CLL and PHA blasts from patient and donor at day 21.

Discussion

The aim of this study was to investigate the best method to modify CLL cells into malignant APCs and to test their capacity to activate allogeneic T cells to generate CLL-reactive T cell reponses. CD40

activation in the presence of IL-4, was shown to most effectively transform CLL cells into efficient APCs, capable of producing high amounts of IL-12. In all three HLA class I-identical donor / patient pairs tested, using these malignant APC as stimulator cells, allogeneic $CD8^+$ T cell lines were generated, recognizing CLL as well as patient derived PHA blasts. Cloning of these CLL-reactive T-cell responses revealed that single CTL clones recognized both non malignant patient-derived and CLL-specific targets.

Primary CLL cells have previously been demonstrated to be unable to stimulate donor T-cell proliferation and activation. This has been explained by the inadequate expression of costimulatory and adhesion molecules [14,19,22,34]. CD40 ligation has been shown to be an effective tool to upregulate costimulatory molecules on the surface on CLL cells [14,19,20,22,34]. CD40-triggered CLL cells, as stimulator cells, can activate allogeneic T cells in MLRs and can induce allogeneic immune responses against CLL [14,15,19,22,34,35]. In attemps to improve the immunogenicity of CLL cells without using the CD40 system, we investigated several alternatives. We first explored the ability of several cytokines to upregulate costimulatory molecules. The proinflammatory cytokines, proliferating cytokines, plasmacytoid-DC stimulatory factors (IL-3) or B-cell activating cytokines (IL-4) tested [29-31,36] did not cause significant upregulation of costimulatory molecules. Recent studies have demonstrated that normal, but also malignant B cells, express a distinct TLR expression profile in which TLR9 and TLR10 predominate [26,27,37,38]. We therefore tested triggering of TLRs on CLL cells and showed that CpG, agonist of TLR 9, in combination with IL-4, increased the expression of costimulatory molecules. However, CD40 activation significally further enhanced the expression levels of all costimulatory and adhesion molecules and was superior to all combinations tested without CD40 ligation. The addition of IL-4 further enhanced the expression of CD80 and CD86, thus confirming results from other studies [14,19,20,22,34]. Although CD40 activation can upregulate the expression levels of TLR9 in B cells and can increase the responsiveness to CpG [26,27], no additional upregulating effect but rather a downregulating effect was observed by adding CpG.

Professional APCs can express and secrete IL-12 after challenge with microbial stimuli or after CD40L-CD40 interactions between T cells and the APCs [39]. IL-12 is a pivotal cytokine in the Th1-response to antigen and its effect includes enhanced CTL activity of $CD8^+$ T cells and natural killer cells, and further differentation of antigen activated $CD4^+$ and $CD8^+$ T cells [40]. We demonstrated that CD40 activation is essential for the IL-12 production by CLL cells and showed that additional activation using CpG or IL-4 resulted in a higher IL-12 production. These results are in accordance with studies showing that CD40 activation induced enhanced IL-12 mRNA expression in normal and neoplastic B lymphocytes, including follicular lymphoma and mariginal zone lymphoma cells, resulting in IL-12 secretion by these B cells [41,42]. In summary, the CD40-CD40L pathway is critical to modify CLL cells into phenotypically professional APC, capable of producing significant amounts of IL-12. Cytokines and / or microbial pathogens are insufficient to stimulate CLL cells.

Expression of adhesion and costimulatory molecules on APCs is necessary to induce efficient T-cell responses. We confirmed that T cells demonstrate very low proliferative responses upon restimulation with unmodified CLL cells. Since CpG/ IL-4 activated CLL cells could be generated under good manifacturing practice (GMP) conditions, we analyzed whether the minimal upregulation of costimulatory molecules on CpG/IL-4 acitvated CLL cells was sufficient to overcome T-cell anergy. In two of three HLA class I-matched combinations proliferation and in one of the three couples tested, cytotoxicity against CLL- and patient-derived targets of $CD8^+$ CTL lines using these APCs was obtained. Although our results extend reports from others [35], only very limited proliferation of donor T cells in response to CpG/IL-4 activated CLL cells was observed (figure 5) and the generation of a CLL-reactive CTL line was successful in only one of three donors tested. The importance of high expression of costimulatory molecules on APCs was demonstrated by the capacity of tCD40L/ IL-4-activated CLL cells to induce vigorous expansion of CLL-reactive $CD8^+$ CTL lines. We were able to generate $CD8^+$ CTL lines from HLA class I-matched donors with high cytotoxic activity against primary and modified CLL cells. Moreover, using one cell / well sorting several CLL-reactive proliferating $CD8^+$ CTL clones from a HLA class I-matched donor could be obtained, illustrating that the cytotoxicity could not be due to contaminating allo-HLA class II CD4 responses. Furthermore the cytotoxicity of the generated $CD8^+$ CTL lines was completely abrogated by anti-HLA class I antibodies and not by anti-HLA class II antibodies.

In conclusion, this study not only demonstrates that CD40-activated CLL cells have the stimulatory capacity to induce an alloresponse over a MHC barrier, but more importantly it shows the feasibility to generate $CD8^+$ CTL lines and clones from HLA class I-matched donors, recognizing leukemia-specific as well as non-malignant patient-derived targets. These results suggest that the antigens recognized by the CTL lines and clones are not tumor-specific but rather mHag-specific[19,22,34,35]. Whether the mHags, recognized by the CTL lines and clones are hematopoiesis-restricted and/or B-cell-specific remains to be elucidated.

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Chapter 3

Minor Histocompatibility Antigen (mHag)-specific And B-cell Chronic Lymphocytic Leukemia (CLL)-reactive T Cells Can Be Derived From Matched Sibling Donors Using Transformed CLL Cells As Stimulator Cells.

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Submitted

Abstract

Objective: Allogeneic stem cell transplantation (SCT) following reduced-intensity conditioning provides new therapeutic opportunities for cure of advanced B-cell chronic lymphocytic leukemia (CLL). Although treatment of relapsed or persistent CLL after allogeneic SCT with donor lymphocyte infusions (DLI) can lead to complete sustained remissions, illustrating susceptibility of CLL for the graft-versus-leukemia (GvL) effect, effectiveness of this therapy is limited due to low immunogenicity of CLL cells and the lack of specificity resulting in concurrent development of graft-versus-host-disease (GvHD). To improve the specificity of the immune response, we investigate whether modified CLL cells are sufficiently immunogenic to initiate an adequate alloimmune response *in vitro* in a complete HLA-matched setting representing a clinical applicable transplantation model.

Methods: First, CLL cells were modified into efficient antigen-presenting cells (CLL-APC) by CD40 triggering in the presence of IL-4. Next, T cells derived from three unprimed HLA-matched sibling donors were stimulated with primary CLL or CLL-APCs from the patients.

Results: In contrast to primary CLL, CLL-APC cells as stimulators were capable of inducing cytotoxic $CD8^+$ and $CD4^+$ T-cell (CTL) clones from all three sibling donors. These CTL clones effectively lysed CLL-specific and patient-derived targets but not non-hematopoietic targets of the patient or donor-specific targets. The recognition of all clones was HLA-restricted and most likely mHag-specific.

Conclusion: In all three donor-recipient pairs tested, CLL-APC as stimulators were capable of inducing mHag-specific and CLL-reactive T cells clones. These results allow the development of new cellular immunotherapeutic interventions to further exploit the GvL effect following allogeneic SCT.

Introduction

B-cell chronic lymphocytic leukemia (CLL) is clinically and biologically heterogeneous [1,2]. Some patients may have an indolent course with long-term survival, whereas others have a rapidly fatal disease despite intensive therapy [1,3]. In addition to characteristic cytogenetic abnormalities, new molecular and protein markers such as the mutation status of the immunoglobulin variable heavy (VH) genes, expression of ZAP-70, and profiling of the expression of genes, enables to predict better patients, who will suffer from an aggressive and ultimately fatal course [4-8]. For this group of patients new effective treatment modalities are needed.

Recently, it has been demonstrated that allogeneic stem cell transplantation (SCT) may overcome the adverse prognosis of patients with CLL with unmutated VH genes [9]. Since allogeneic SCT following reduced-intensity conditioning (RIC) has resulted in lower short term treatment-related mortality (TRM) [10-14] this treatment modality is increasingly being explored in patients with advanced CLL [11,15,16]. Donor T cells in the graft, recognizing leukemia-associated antigens or minor histocompatibility antigens (mHag) on CLL cells may initiate a robust alloimmune response and thus eradicate persisting or relapsing CLL in the recipient. Complete remissions have been reported after allogeneic SCT illustrating susceptibility of CLL cells to a graft-versus-leukemia (GvL) effect [11,15,17]. We and others demonstrated the feasibility of a two-step approach of allogeneic transplantation and immunotherapy following RIC by the use of T-cell depleted grafts avoiding the risk of graft-versus-host disease (GvHD), followed by a postponed administration of donor lymphocyte infusion (DLI) [13,18,19]. The approach was feasible even in older patients, but acute or chronic GvHD caused by the administration of donor lymphocytes remained a major cause of morbidity [13,20]. Furthermore, in some patients *in vivo* alloimmune responses sufficient to completely suppress the CLL cells could not be evoked. Therefore, further selection of donor T cells with high avidity for CLL cells, and further enrichment of leukemia-reactive donor T cells may be pivotal to achieve long-term control of the disease after allogeneic SCT.

Enrichment of donor T-cells for leukemia-reactive T-cell specificity may be achieved by *in vitro* stimulation of donor lymphocytes with leukemic cells from the patient [21,22]. This approach appears to select for T-cell responses against mHag that are relatively specifically expressed on hematopoietic cells [22]. However, although CLL cells highly express HLA class I and II molecules, they are inappropriate to function as antigen-presenting cells (APC) due to inadequate expression of costimulatory molecules [23-27]. We and others demonstrated that by CD40 triggering in the presence of IL4, CLL cells can be modified into efficient malignant APC, capable of inducing vigorous allogeneic T-cell responses [23,25-27]. CLL-reactive mHag specific cytotoxic T lymphocytes (CTL) derived from unrelated HLA class I-matched donors could be generated [27].

To further translate these results in 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 setting, T cells derived from three unprimed HLA-matched sibling donors

were stimulated with CLL-APCs from the patients. In all three donor-recipient pairs, CLL-APC as stimulators were capable of inducing mHag-specific and CLL-reactive T cells clones. These results support the potential of this approach to treat disease recurrence after allogeneic SCT.

Material and methods

Cell samples

Three patients treated for advanced CLL and eligible for allogeneic SCT using HLA-identical sibling donors were included in this study. After informed consent peripheral blood (PB) samples were obtained from the patients, and from their HLA-identical sibling donors. The HLA types of patients and donors are shown in Table 1. Mononuclear cells (MNC) were isolated from the samples by Ficoll density separation, and cryopreserved. As assessed by flow cytometry, more than 90% of the MNC from the CLL patients coexpressed CD19 and CD5 surface molecules. Phytohemagglutinin (PHA) activated T cells (PHA blasts) and stable transformed B cell lines (EBV-LCL) were generated as described previously [28]. Mesenchymal stem cells (MSC) were derived from the bone marrow (BM) of one patient by culturing the adherent cells for several weeks on DMEM supplemented with L-alanyl-L-glutamine, sodium pyruvate, 1 mg/mL glucose, and pyridoxine (Gibco BRL), and 10% fetal bovine serum (FBS)

Table 1. HLA types of patient/donor pairs.

	HLA class I						HLA class II					
Pair 1	A3	A31	B8	B35	Cw4	Cw7	DR1	DR3	DQ2	DQ5	DP2	DP3
Pair 2	A2*	A68	B51	B53	Cw4	Cw1	DR4	DR1	DQ3	DQ6	DP2	DP4
Pair 3	A1	A2†	B8	B35	Cw4	Cw7	DR3	4	DQ2	DQ5	DP2	

* patient was negative for HA-1, donor was positive for HA-1. † patient was positive for HA-1, donor was negative for HA-1

Modification of CLL cells into CLL-APC

CLL cells were transformed into CLL-APC as recently described [27]. Briefly, CLL cells were cocultured on Itk murine fibroblast cells transfected with the human CD40-ligand (tCD40L; kindly provided by Dr.C.van Kooten, Department of Nephrology, Leiden University Medical Center). The fibroblasts were irradiated (70 Gy), and seeded at a concentration of $1x10^5$ cells/well in 24-well plates (Costar, Cambridge, MA, USA). CLL cells were added at a concentration of $1x10^6$ cells/well in medium consisting of IMDM (BioWhittaker, Verviers, Belgium) with 10% human serum and IL-4 (500 U/mL, Schering-Plough, Amsterdam, The Netherlands). After 4 days of culture at 37˚C in a 5% CO_2 humified atmosphere, the CLL cells were harvested and washed twice. Viable cells were counted using eosin exclusion, analyzed by flow cytometry, and used as stimulator cells.

Phenotypic analysis of the CLL-APC and the cytotoxic T cell lines and clones

To perform immunophenotyping of the CLL-APC and of the generated T cell lines and clones, mouse MoAbs conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or phycoerythrin cyanine 5 (PE-Cy5) were used. These MoAbs included FITC-conjugated antibodies specific for CD3, CD5, CD40, CD54, CD58, CD86, or HLA-DR, PE-conjugated antibodies specific for CD4, CD19, CD23, CD56, CD80 or CD83, and Cy5-conjugated anti-CD8. All MoAbs were purchased from Becton Dickinson (BD, San Jose, CA, USA) except for anti-CD40 and anti-CD58 (Serotec, Oxford, England), anti-CD54 (CLB, Amsterdam, the Netherlands), anti-CD8 and anti-CD83 (Caltag, Burlingame, AL, USA) and anti-CD80 (Immunotech, Marseille, France). PE-labeled tetrameric complexes of HA-1 peptide bound to HLA-A2 molecules were prepared and used as described previously [22]. After labelling all cell samples were analyzed on a FACScan (BD).

Generation of CLL-reactive CTL lines and clones

MNC from the three healthy HLA matched sibling donors at a concentration of 0.5 x 10^6 cell/well in 24-well plates (Costar) were stimulated with irradiated CLL-APC at a responder/stimulator (R/S) ratio of 10:1. IL-2 (100 IU/mL, Chiron, Amsterdam, Netherlands) was added at day 6, and 2 days after each (re)stimulation. The T cell lines were restimulated with irradiated stimulator cells at the same R/S ratios at days 9, 16 and 23. T cells were harvested 4-5 days after the third or fourth stimulation for phenotypic analysis, and used as effectors in cytotoxicity assays. T cell clones were generated from CLL-APC reactive CTL lines by single cell / single well sorting using a FACS-Vantage flow cytometer (BD). To exclude dead cells the suspensions were counterstained with propidium iodide (PI; Sigma, St.Louis, USA) at a final concentration of 0.5 µg/ml immediately prior to cell sorting. PI negative cells within the lymphocyte gate were sorted, and plated as single cells per well (single cell/well sorting) into 96-well microtiter plates containing 100 µl of feeder mixture consisting of culture medium, IL-2 (100 IU/mL), phytohemagglutinin (PHA, 800 ng/mL, Murex Biotech Limited, Dartfort, UK) and 15 Gy-irradiated allogeneic feeder cells (5 x 10^6 /mL). Proliferating T cell clones were selected, and restimulated with feeder-mixture. From day 21 the clones were functionally analyzed. The nomenclature used for each T cell clone represents the patient number followed by clone number.

Analysis of cytotoxicity and cytokine production

To determine the cytotoxicity of the T cell lines and clones standard 4 hours and 10-16 hours ^{51}Cr-release assays were performed as described previously [27]. Primary CLL cells, CLL-APC cells, EBV-LCL or PHA blasts from patient or donor were used as target cells. T cell lines and clones showing more than 10% specific lysis of target cells were considered cytotoxic. To determine HLA class I- and II-restriction of the recognition of the target cells, blocking studies were performed in selected experiments. Target cells were incubated with α-HLA class I antibodies (W6/32) or α-HLA class II antibodies (PdV5.2) at final concentrations of 10 µg/mL for 30 minutes before effector cells added. A human MoAb specific for HLA-B8 (BVK5B10) was used to determine HLA-B8 restricted recognition of the targets by CTL clones derived from donor 3. For analysis of interferon (IFN) γ production by the T cells against MSC, 5,000 T cells were cocultured on a monolayer of 5,000 plated MSC in 96 wells flat-

bottom plates. After 24 hours, supernatants were harvested and the concentration of IFN-γ was measured by ELISA (CLB, Amsterdam, The Netherlands).

T-cell receptor (TCR) analysis

The T-cell receptor (TCR) α and ß chains of the CTL clones were determined by polymerase chain reaction and sequencing, as previously described [29]. The sequences of the TCRß chains were named according to the nomenclature described by Arden et al [30].

Results

Generation of CLL-reactive CTL lines and clones

Our previous study had illustrated that CD40 activation in the presence of IL-4 is an effective tool to increase APC function of CLL cells [27]. Four days of CD40 and IL-4 stimulation effectively transformed CLL cells, derived from the three patients in this study, into CLL-APC with high expression of the costimulatory molecules CD54, CD58, CD80 and CD86 and enhanced expression of CD83 (figure 1).

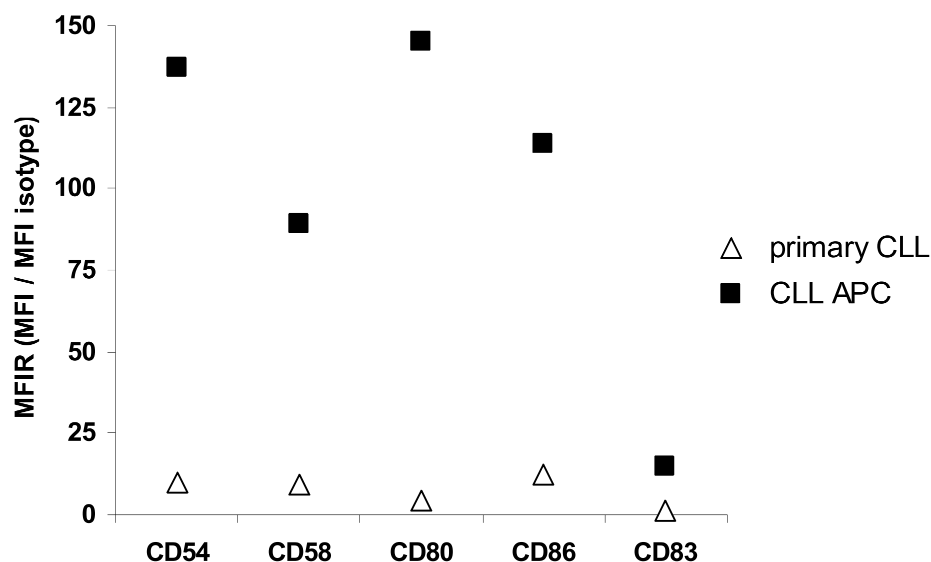

Figure 1. Expression of costimulatory and adhesion molecules on primary CLL and CLL-APC derived from patient 2. All costimulatory and adhesion molecules on the CLL cells were significantly upregulated by CD40 activation in the presence of IL-4. Primary CLL cells from patient 2 were cultured in 24 wells plate (1x106) for 96 hours in the presence or absence of IL-4 and tCD40L. The expression of CD54, CD58, CD80, CD86, and CD83 was analyzed by flow cytometry. Mean fluorescence intensity ratio (MFIR) was calculated as MFI cells stained by a specific MoAb / MFI cells stained by isotype-control MoAb, only if the percentage of positive cells was >10%. Results are representative for all three patients.

These CLL-APC and primary CLL cells were used as stimulator cells for T cells from the HLA-identical sibling donors. In all three donor-patient pairs, no proliferation but a decrease of T cells was observed in response to primary CLL cells as stimulator cells. In contrast, using CLL-APC as stimulators, vigorous expansion of donor T cells was observed, resulting in a 6-10 fold increase of the T cell

population after three weekly stimulations. Experiments to generate CLL-reactive CTL lines were performed twice for each patient-donor combination. The CTL lines derived from donor 1 consisted of 84 ± 4% (mean ± SD) CD4+ T cells and of 12 ± 2% CD8+ T cells, and the T cell line derived from donor 2 consisted of 91 ± 2% CD4+ T cells and 4 ± 2% CD8+ T cells. Flow cytometric analysis of the CTL lines derived from donor 3 at day 21 showed a large population CD3-CD56+ cells (78 ± 4%) and only 11 ± 6% CD4+ T cells and 6 ± 3% CD8+ T cells. All T cell lines generated were examined for their ability to kill primary CLL and CLL-APC as well as patient- and donor-derived other hematopoietic targets (PHA-blasts and/or EBV-LCL). Due to very low (<1%) normal B-cell counts in the MNC of patient 1 and 2, no EBV-LCL from these two patients could be generated. As illustrated in figure 2, CLL-APCs induced T cell lines with cytolytic activity against CLL-APC and patient-derived hematopoietic targets in all donor-patient pairs. No cytotoxicity was observed against primary CLL. In our previous study we had demonstrated that primary CLL could adequately be recognized and killed by alloreactive donor-derived T cells 27. Therefore, to determine whether low precursor frequencies of mHag-specific and/or CLL-specific cytotoxic T cells within the predominantly CD4+ T cell and the CD56+ populations were responsible for the low cytolytic activity against CLL targets, and to select for these cytotoxic T cells, cloning experiments of the α-CLL and the α-CLL-APC CTL lines in the donor-patients pairs were performed.

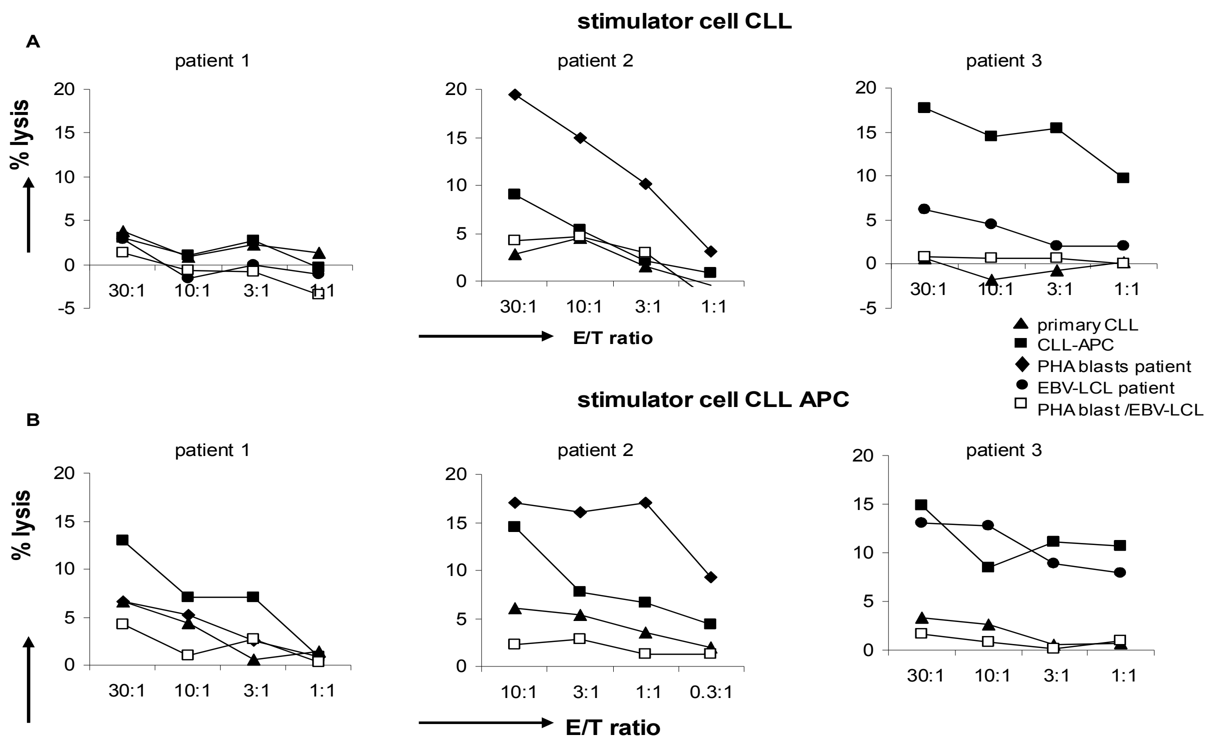

Figure 2. Cytotoxicity of CTL lines, derived from the three HLA-identical sibling donors, generated against primary CLL cells or against CLL-APC cells. (A) Using primary CLL as stimulator cells, no cytotoxicity was observed in the first donor-recipient pair (1), reactivity against patient-derived PHA blasts was observed in the second couple (2), and reactivity against CLL-APC was observed in the third couple (3), as measured in a 4-hours 51Cr-release assay. (B) Stimulation with CLL-APC induced cytolytic ctivity against CLL-APC in all donors and cytotoxicity against patient-derived targets in donor 2 and 3. Results are representative for two experiments for each donor-recipient combination.

In donor-recipient combination 1, from the α-CLL-T cell line, 5% of the 1 cell/well clonal T cells showed proliferation. None of the 11 $CD4^+$ and the 3 $CD8^+$ T cell clones were cytotoxic. The plating efficiency in the donor-recipient pair 1, from the α-CLL-APC T cell line, was 11%, resulted in 31 proliferating clones. As shown in figure 3A 3 out of 8 $CD8^+$ T cell clones and 4 out of 23 $CD4^+$ T cell clones exerted cytotoxic activity against CLL-specific targets and not against other patient- and donor-derived targets. In the donor-recipient pair 2, from the α CLL-T cell line, two proliferating $CD8^+$ clones and 12 $CD4^+$ clones were obtained (plating efficiency 3%) with one $CD8^+$ T cell clone recognizing CLL-APC and PHA blasts from the patient but not primary CLL (data not shown).

A total of 110 proliferating clones were generated from the α-CLL-APC T cell line from donor-recipient pair 2. As illustrated in figure 3B, nine out of 48 $CD8^+$ T cell clones and one out of 62 $CD4^+$ T cell clones recognized CLL- and patient-specific but not donor-derived targets. Only two CTL clones (clone 2.57 and 2.89) show reactivity against primary CLL cells whereas the other clones recognized the CLL-APC and/or PHA blasts from the patient (figure 3B). Because the CTL clones generated were not highly effective in killing primary CLL cells and because we did not succeed to generate EBV-LCL from the patients, these CTL clones were not further analyzed for HLA-restriction.

In the third donor-patient combination 101 proliferating clones (72 $CD4^+$ and 29 $CD8^+$ T cell clones) were obtained from the α-CLL CTL line and none of these clones exhibited patient- or CLL-specific cytotoxicity. Single cell sorting of the α-CLL-APC CTL line resulted in high clonal expansion efficiency of 22% of the cells isolated. Four out of 208 T cell clones (112 $CD4^+$ and 96 $CD8^+$ T cell clones) were reactive against patient-derived and CLL-specific targets. These $CD8^+$ CTL clones failed to stain with the HA-1 specific tetramer. As illustrated in Figure 3C, three clones (clone 3.76, 3.82 and 3.87) were highly cytotoxic against primary CLL (42 ± 11%, mean ± SD, n=3), CLL-APC (82 ± 10%), EBV-LCL of the patient (88 ± 12%) and not against donor-derived EBV-LCL (3 ± 1%) in a 16 hour ^{51}Cr-release assay. Clone 3.62 exhibited reactivity against CLL-APC (lysis 47%) and EBV-LCL of the patient (68%) but not against primary CLL and donor-derived targets. Significant although lower cytotoxicity (38 ± 7%) was observed against PHA blasts (figure 3), indicating that the clones are mHag-specific and that the recognized mHag is not B lineage-restricted.

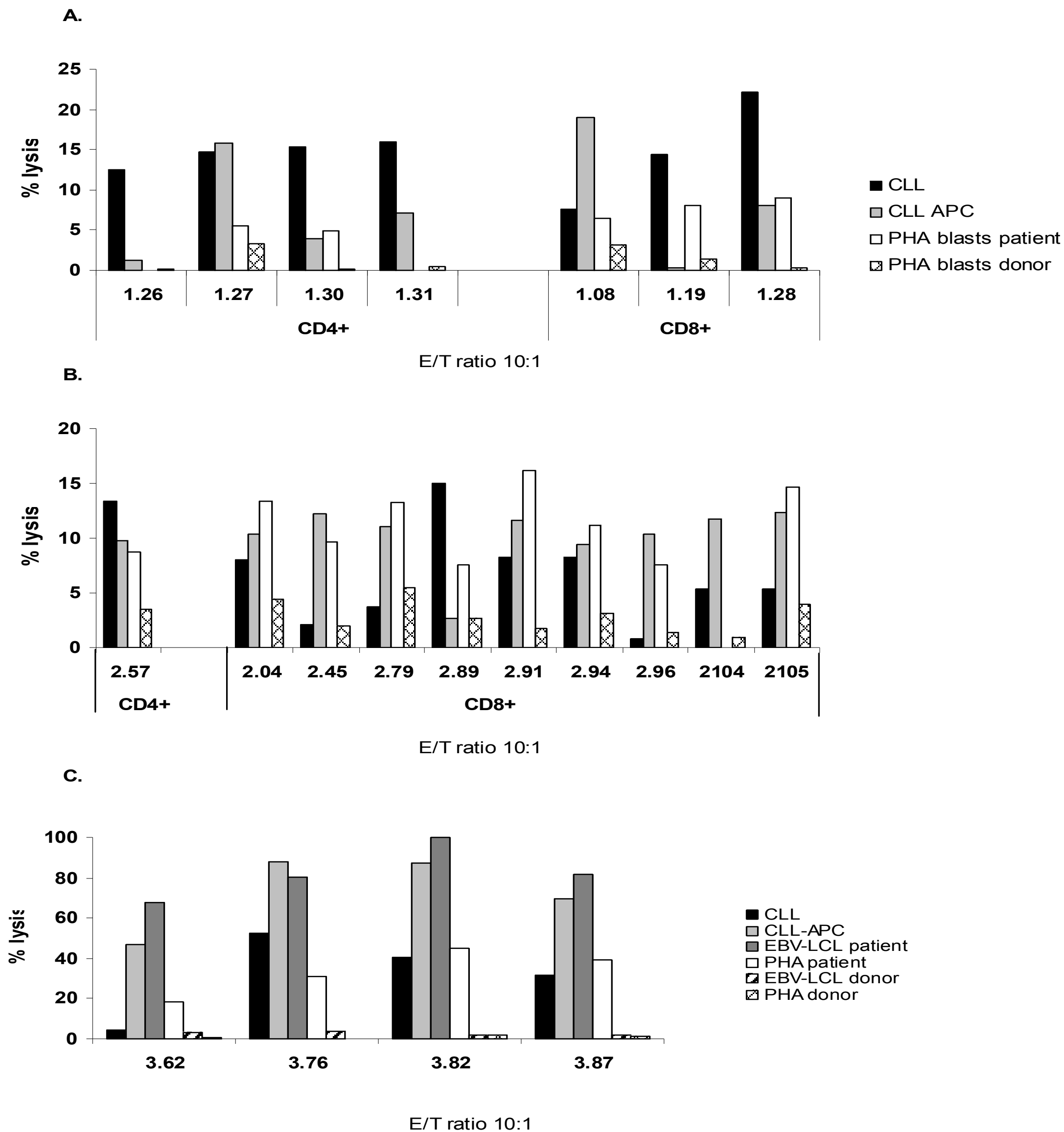

Figure 3. Cytotoxicity and specificity of CTL clones derived from HLA-matched sibling donors, generated against CLL-APC as stimulator cells. (A) In donor-patient pair 1 four CD4+ and three CD8+ CTL clones, recognizing CLL-specific targets but not patient- or donor-derived targets, were generated using CLL-APC as stimulators. (B) Out of 110 proliferating T cell clones, one CD4+ (clone 2.57) and 9 CD8+ CTL clones were obtained. Primary CLL as target was only recognized by clone 2.57 and clone 2.89, whereas CLL-APC and PHA blasts from the patient were recognized by most CTL clones. (C) Four CD8+ T cell clones out of 208 T cell clones showed cytotoxicity against primary CLL, CLL-APC and patient- derived targets and not against donor-derived EBV-LCL and PHA blasts. Clone 3.62 recognized CLL-APC and EBV-LCL and PHA blasts from the patient but not primary CLL cells whereas the other clones efficiently killed all CLL-specific and patient-specific targets and not donor-derived targets. All four clones stained negative with the HA-1 specific tetramer. Cytotoxicity was tested at E/T ratios of 10:1 and measured in a 10-16 hour ^{51}Cr-release assay. The ^{51}Cr-release assay was performed twice for each CTL clone. Results are representative for these experiments.

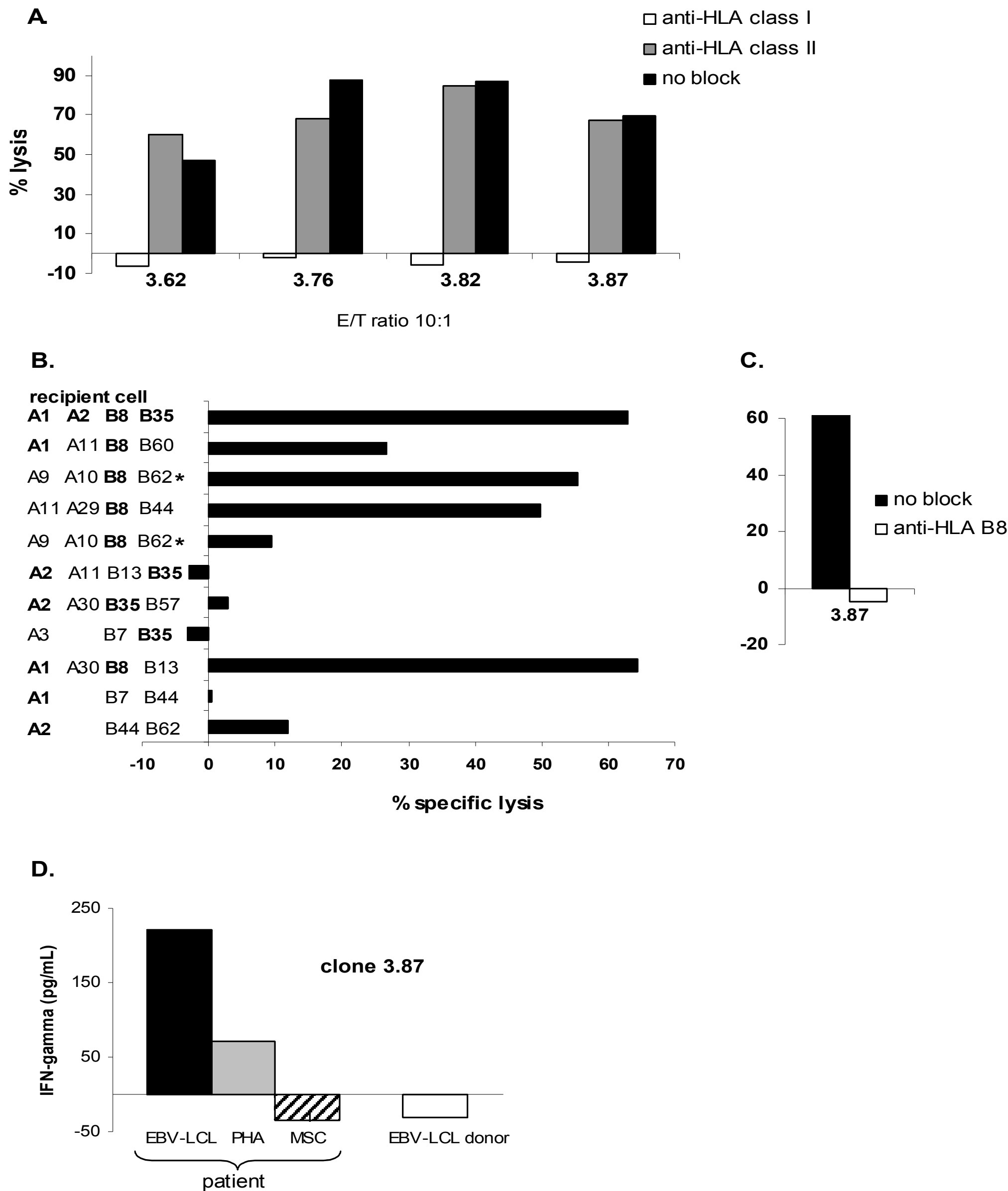

Figure 4. Characterization of the CLL-reactive clones derived from donor 3.(A) HLA class I-restriction of cytotoxicity. EBV -LCL of the patient were used as target cells at an E/T ratio of 10:1 in a 10-hour ^{51}Cr-release assay and anti-HLA class I MoAb (W6/32) and anti-HLA class II MoAb (PdV5.2) were used to block recognition by the CTL clones. (B) Determination of the HLA class I-restricting element. To determine the HLA class I-restricting element, the CD8+ CTL clone 3.87 was tested for reactivity against a panel of EBV-LCL from unrelated individuals. The recognition of EBV-LCL targets by clone 3.87 was shown to be HLA-B8 restricted. mHag specificity was further illustrated by differential recognition of two individuals who were siblings, unrelated to the donor-recipient pair (see *). (C) Confirmation of HLA-B8 restricted recognition of the CTL clone 3.87 by blocking with anti-HLA B8 MoAb. (D) Hematopoietic-restricted recognition by the CLL-reactive CTL clone 3.87. Recognition of hematopoietic cells of the patient (EBV-LCL and PHA blasts), the non-hematopoietic mesenchymal stem cells (MSC) of the patient and EBV-LCL of the donor by the CTL clone 3.87 as measured by IFN-γ ELISA.

Cytolytic activity of the CTL clones could completely be abrogated using the α-HLA class I MoAb and not by α-HLA class II MoAb (figure 4A), confirming HLA class I restricted recognition. The TCRs of the CLL-reactive CTL clones were analyzed by PCR and showed that clone 3.76, 3.82 and 3.87 were identical (AV3 AV21 BV1). To further analyze the HLA class I-restricting element of these identical CTL clones, clone 3.87 was tested for reactivity against a panel of EBV-LCL from unrelated individuals that shared an HLA molecule with the recipient. As shown in figure 4B, the recognition of the targets was HLA-B8 restricted. To confirm HLA-B8 restricted recognition, a blocking study using α-HLA B8 MoAb (BVK5B10) was performed and showed no cytolytic activity of clone 3.87 in the presence of this MoAb (figure 4C). From patient 3, we were capable of culturing MSC from the BM of the patient. These cells were used as targets to determine the possible reactivity of the CLL-reactive T cells against non-hematopoietic tissues of the patient (figure 4D). Whereas the leukemia-reactive T cells produced significant amounts of IFN-γ against EBV-LCL (221 pg/mL) or PHA blasts (72 pg/mL) of the patient, no IFN-γ was produced after coculture on the MSC or against donor-derived EBV-LCL.

In conclusion, in contrast to primary CLL cells, CLL-APCs strongly induced a proliferative response of T cells from HLA-identical donors. In all CTL lines generated low precursor frequencies of CLL-reactive and mHag-specific T cells were present. Repetitive stimulation with CLL-APCs and single cell per well cloning enriched for these CLL-reactive T cells. In the third donor-patient pair the CLL-APCs were characterized to have induced mHag-specific HLA-B8 restricted CTL clones, highly effectively killing CLL-specific targets and not non-hematopoietic cells.

Discussion

Allogeneic SCT following RIC has resulted in lower TRM and may prolong survival in patients with advanced CLL [11,15]. Although several studies have shown the susceptibility of CLL cells to a GvL effect relapses and/or progression of the disease after allogeneic SCT do occur indicating that a more efficient alloimmune anti CLL response is pivotal to achieve long-term remissions [9,11,12,15]. Recently, we demonstrated the feasibility to generate CLL-reactive CTLs in an unrelated HLA class I-matched setting [27]. Following these results this translational study was performed to evaluate whether T cells from HLA-identical sibling donors can be triggered to preferential kill CLL-specific targets, allowing application of these CTLs as adoptive immunotherapy to treat disease recurrence after allogeneic SCT.

It has been demonstrated that activation of the CLL cells by IL-4 and CD40 triggering is essential to overcome allogeneic T cell anergy towards primary CLL cells [23-27]. In this study, no proliferative impulse of donor T cells was observed in response to primary CLL cells and repetitive stimulation with these malignant cells, expressing low levels of costimulatory molecules, failed to induce CLL- or mHag-specific T-cell responses. Only CLL-APC as target was recognized by the CTL line derived from donor 3. In contrast CLL-APC as stimulators induced proliferation of T cells in all three donor-patient combinations. These CTL lines showed enhanced cytotoxicity against patient-derived targets and CLL-APC. As illustrated in figure 2, the CTL lines showed no activity against the primary CLL. We

have demonstrated that primary CLL as target can be efficiently lysed by T cells, derived from HLA class I-matched donors [27]. Therefore, to analyze whether low precursor frequencies of mHag-specific and/or CLL-specific cytotoxic T cells were present within the predominantly $CD4^{+}$ T cell and the $CD3^{-}$ $CD56^{+}$ populations single cell per well cloning was performed of the α-CLL and the α-CLL-APC CTL lines to identify cytotoxic T cells with reactivity against primary CLL and/or patient-derived targets.

Only one cytotoxic CTL clone was obtained from one of the three donor-α CLL T cell lines. In contrast, from each donor-α CLL-APC T cell line several CTL clones, recognizing CLL-specific and patient-derived targets, were generated (see figure 3). In accordance with the observed cytotoxicity of the α-CLL-APC CTL lines, the CTL clones generated, preferentially recognized CLL-APC as target. However, in addition several CTL clones (e.g. clone 1.28, clone 2.57, clone 2.89 and clone 3.76) showed cytotoxicity against primary CLL. Some clones were active against patient-derived hematopoietic targets and CLL-APC (clone 2.45, clone 2.105 and clone 3.62) and not against primary CLL. These CTL clones may be able to recognize the CLL cells, once these leukemic cells become activated or start dividing. One might speculate that for an adequate alloimmune reponse in patients with disease recurrence after allogeneic SCT, CTL clones with different specificities might be necessary to control the disease. Our results indicate that CLL-APC as stimulator cells are sufficient immunogenic to induce T-cell responses in HLA-matched sibling donors and enrich for precursor T cells with reactivity to CLL- and patient-specific targets. Three CTL clones, derived from donor 3 effectively lysed primary CLL as well as EBV-LCL from the patient. Further analysis of the specificity of these CTL clones showed that these clones were mHag-specific, recognized their targets in a HLA-B8 restricted manner and used the same TCR. These clones were not reactive to MSC, cultured from BM of the patient, suggesting that the recognized mHag is not widely expressed in non-hematopoietic tissue. Our study illustrates that CLL-APCs are capable of stimulating mHag-specific CLL-reactive T cells, present at low precursor frequencies in unprimed MNC of the donor. Although other patient-derived APC may also be capable of eliciting mHag-specific T-cell responses, we think that CLL-derived APC will likely skew the immune response toward recognition of CLL-specific antigens of mHags, highly expressed on the CLL cells thus leading to a more specific and efficient T-cell response.

Our two-step approach of T cell-depleted allogeneic SCT following RIC using alemtuzumab with its intrinsic anti-CLL activity followed by the postponed administration of DLI was shown to be feasible in CLL patients [13]. With the data from our study several strategies to limit the occurrence of severe GvHD after adoptive immunotherapy while preserving and enhancing GvL activity can be considered. High doses of CLL-reactive and relatively hematopoiesis-restricted mHag-specific T cells or low dose DLI in combination with these leukemia-reactive T cells can be administered. In addition, $CD8^{+}$ alloreactive T-cells are considered to be main effectors of GvHD and may not be necessary for the GvL reactivity [31].

Since only 5% of all T cell clones tested showed cytotoxicity against CLL-specific targets, our study clearly demonstrates that the use of extensive *in vitro* culture periods is not very efficient in selecting and isolating leukemia-specific T cells, is labor intensive. Early detection and isolation of leukemia-reactive T cells in the immune response using the IFN-γ capture assay may result in higher efficiency rates of CLL-reactive CTL clones [29,32].

In conclusion, this study illustrates that mHag-specific CLL-reactive CTL clones, derived from HLA-identical sibling donors, can be generated using CLL-APCs as stimulator cells. Recently, follow-up of a large cohort of patients with chemo-refractory CLL, treated with an allogeneic SCT following RIC, showed the feasibility of the transplant procedure, but also reported a relapse rate of 26% after 2 years [15]. In this setting adoptive immunotherapy with donor-derived mHag-specific CTLs, which preferently kill CLL cells and not non-hematopoietic targets could be of great value and could treat or even prevent disease recurrence after allogeneic SCT.

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Chapter 4

Primary allogeneic T-cell responses against mantle cell lymphoma antigen presenting cells for adoptive immunotherapy after stem cell transplantation

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Abstract

Purpose: In patients, treated with allogeneic stem cell transplantation (SCT) for advanced mantle cell lymphoma (MCL), complete sustained remissions have been observed illustrating susceptibility of MCL cells to a graft-versus-lymphoma (GvL) effect. To potentiate this GvL effect adoptive transfer of *in vitro*-selected MCL-specific cytotoxic T cells (CTLs) can be an attractive approach. The lack of expression of costimulatory molecules on MCL cells hampers the generation of MCL-reactive T cell-responses. The purpose of this study was to modify MCL cells into antigen-presenting cells (APC) and to use these MCL-APC to induce allogeneic MCL-reactive T-cell responses.

Experimental Design: Interleukin (IL)-4, IL-10, CpG, and CD40 activation were tested for their capacity to upregulate costimulatory molecules on MCL cells. Primary MCL cells or the modified MCL-APC were then used to evaluate the induction of MCL-reactive T-cell responses in HLA matched donors.

Results: Ligation of CD40 on MCL cells was essential to upregulate costimulatory molecules and to induce production of high amounts of IL-12. In contrast to primary MCL cells, MCL-APC cells as stimulators were capable of inducing cytotoxic $CD8^+$ T-cell (CTL) lines from HLA class I-matched donors. High numbers of CTL clones could be generated capable of efficiently killing the primary MCL cells and MCL-APC but not donor-specific targets.

Conclusion: These results demonstrate the feasibility to generate primary allogeneic T-cell responses against MCL-APC, and may provide new immunotherapeutic tools to further exploit the GvL effect following allogeneic SCT in patients with MCL.

Introduction

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 [1]. Patients with MCL frequently present with advanced-stage disease and have a median survival of only 3 to 4 years [2]. Although in general MCL is initially responsive to conventional chemotherapy or to high-dose chemoradiotherapy followed by autologous stem cell transplantation (SCT), persistent remissions are usually not achieved reflected in the absence of a plateau phase in the survival curves [3,4]. The addition of rituximab to autologous SCT as an *in vivo* purging agent or as maintenance therapy appears promising, but has not yet definitively shown to result in a survival benefit [5-7].

Allogeneic SCT is currently being explored as a treatment modality in patients with advanced or relapsed chemosensitive MCL. Complete sustained remissions have been observed after allogeneic SCT illustrating susceptibility of MCL cells to a graft-versus-lymphoma (GvL) effect [5,8]. High treatment related mortality (TRM) has hampered large scale application of allogeneic SCT in this extensively pretreated older patient population. Allogeneic SCT following reduced-intensity conditioning (RIC) has been shown to be feasible and resulted in a lower TRM, allowing the application of non-selected donor lymphocyte infusion (DLI) [9-12]. However, acute and chronic graft-versus-host disease (GvHD) caused by alloreactive T cells present in the graft and/or in the DLI and persistence or recurrence of MCL after allogeneic SCT has limited favourable long-term outcomes [13]. Therefore, both the specificity of the immune reactivity in the context of allogeneic SCT, as well as the magnitude of the immune response specific for MCL cells have to be improved. The use of T-cell depleted grafts avoiding the risk of GvHD, followed by *in vitro* selected alloreactive T cells with specificity for the MCL cells or for hematopoiesis-restricted minor histocompatibility antigens (mHag) could be an attractive approach, and may induce complete remissions with only minor graft-versus-host disease (GvHD) [14].

Malignant B cells inefficiently induce allogeneic T-cell responses despite their strong expression of HLA class I and class II due to inadequate expression of costimulatory and adhesion molecules [15-17]. However, both normal and malignant B cells highly express CD40 molecules. Ligation of these receptors induces expression of the costimulatory molecules CD80 and CD86, adhesion molecules such as CD54 and CD58, and upregulates cytokine production [16-21]. Stimulation of normal B cells can also be initiated by activating toll-like receptor (TLR) 9. TLR 9, expressed on normal and various neoplastic B cells [22-25], detects CpG motifs within bacterial DNA and induces upregulation of costimulatory molecules on these cells. Synthetic CpG oligodeoxynucleotide (ODN) 2006 mimic microbial DNA and has the ability to activate normal and malignant B cells [26]. Transformation of B cell malignancies, including B-cell chronic lymphocytic leukemia, follicular lymphoma and hairy cell leukemia, into antigen-presenting cells (APC) has been studied previously [15,17,20,21,27-29], but the modification of MCL cells into APC and the generation of MCL-reactive T-cell responses have not been reported.

In this study, we hypothesized that by transforming MCL cells into professional APC and using these MCL-APC to stimulate HLA-matched donor T cells, the generation of MCL-reactive cytotoxic T lymphocytes (CTL) lines will be possible. We first examined the stimulatory capacity of cytokines, including the proinflammatory cytokines TNF-α and interferon-α, the B-cell activating cytokine interleukin (IL)-4 [30], and the MCL-stimulating cytokine IL-10 [31] and the additional triggering of the TLR9 by the synthetic agonist CpG ODN 2006, or triggering CD40 with CD40 ligand transfected fibroblasts (tCD40L) to upregulate costimulatory molecules on MCL cells, and to induce the production of IL-12 by these cells. Using CD40-activated MCL cells as stimulator cells, we could demonstrate that MCL-reactive mHag-specific CTL lines and clones from HLA class I-matched donors could be generated. These anti-MCL T-cell responses may be used to treat patients, suffering from relapse of MCL after allogeneic SCT.

Material and methods

Cell samples

After informed consent peripheral blood samples were obtained from 6 patients with MCL in the leukemic phase. The diagnosis of MCL was confirmed by detection of cyclin D_1 overexpression using cytohistochemical staining and verification of the t(11;14) translocation by karyotype or interphase fluorescence in situ hybridization (FISH) [32]. The patient characteristics are shown in table 1.

Table 1. Patient characteristics.

Patient	sex/age/stage	phenotype	cyclin D1	t (11;14)
MCL 1	M / 68 / IV	CD5+CD23-	+	+
MCL 2	M / 44 / IV	CD5+CD23-	+	+
MCL 3	M / 56 / IV	CD5+CD23-	+	+
MCL 4	M / 88 / IV	CD5+CD23±	+	+
MCL 5	V / 52 / IV	CD5+CD23-	+	+
MCL 6	M / 64/ IV	CD5+CD23-	+	+

M, male; F, female. Cyclin D1 overexpression was detected using cytohistochemical staining. The t(11;14) translocation was verified by karyotype or FISH analysis.

Mononuclear cells (MNC) were isolated from peripheral blood samples by Ficoll density separation, and cryopreserved. The percentage of CD5+/CD19+ MCL cells in the MNC fraction was always higher than 90%. HLA-A, -B, and -C typing of patients was performed by standard serology methods, and HLA-DR and –DQ typing was done by DNA analysis using sequence specific primers. To induce T-cell responses, MNC from a fully HLA class I-matched URD and an HLA-A- and HLA-B-matched unrelated donor (URD) were tested against MCL 3 and MCL 4, respectively (table 2).

Table 2. HLA type of patients and unrelated donors (URD).

Donor/patient	HLA class I						HLA class II			
MCL 3	A2	A33	B15	B58	Cw3		DR13*	DR15	DQ6*	
URD	A2	A33	B15	B58	Cw3		DR17	DR15	DQ1	DQ3
MCL 4	A1	A11	B37	B44	Cw6	Cw16*	DR7*	DR15	DQ2*	DQ6*
URD	A1	A11	B37	B44	Cw6	Cw5	DR3	DR4	DQ1	DQ7

HLA-A, -B, and -C typing was performed by standard serology methods. HLA-DR and -DQ typing was done by DNA analysis using sequence specific primers. *donor-patient disparate alleles

Transformation of MCL cells into APCs

MCL cells were cultured in medium consisting of IMDM (BioWhittaker, Verviers, Belgium) with 10 % human serum in 6-well plates (Costar, Cambridge, MA, USA) at a concentration of 10^6 cells/mL in a total volume of 5 mL per well. MCL cells were cultured in the presence or absence of the cytokines IL-4 (500 U/mL, Schering-Plough, Amsterdam, the Netherlands), IL-10 (10 ng/mL, Sanquin, Amsterdam, The Netherlands), TNF-α (10 ng/mL, Boehringer Ingelheim, Ingelheim am Rhein, Germany) or interferon-α (IFN-α 1000 U/mL, Hoffmann-La Roche, Basel, Switzerland), or in the presence or absence of the cytokine combinations IL-4 and IL-10. For triggering TLR 9, MCL cells were incubated with its ligand synthetic CpG ODN 2006 (CpG; 5' TCGTCGTTTTGTCGTTTTGTCGTT-3', 10 µg/mL; Eurogentec, Seraing, Belgium) in the presence or absence of the cytokine combination IL-4 and IL-10. Finally, to further enhance upregulation of costimulatory molecules MCL cells were cocultured on ltk murine fibroblast cells transfected with the human CD40-ligand [33] (tCD40L; kindly provided by Dr. C. van Kooten, Department of Nephrology, Leiden University Medical Center). The fibroblasts were irradiated (70 Gy), and seeded as a feeder layer at a concentration of $5x10^5$ cells/well in 6-well plates (Costar). MCL cells were added at a concentration of $5x10^6$ cells/well in presence or absence of the cytokines, cytokine combinations and/or immunostimulators as described above. After 1, 2, 4 or 6 days of culture, MCL cells were harvested and washed. Cytospin preparations were made on day 4 for morphological assessment by May-Grunwald-Giemsa (MGG) staining. The number of viable cells was counted using eosin death cell exclusion, and the cells were analyzed by flow cytometry.

Immunophenotyping and cytokine analysis

To perform immunophenotyping, a panel of mouse MoAbs conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) was used. These MoAbs included FITC-conjugated IgG1 antibodies specific for CD3, CD4, CD5, CD40, CD54 or HLA-DR, and FITC-conjugated IgG2a antibodies specific for CD58. PE-conjugated IgG1 antibodies specific for CD14, CD19, CD23, CD56 or CD80, PE-conjugated IgG2a antibodies specific for CD8, and PE-conjugated IgG2b antibodies specific for CD83 and CD86 were used. Appropriate isotype controls (IgG1, IgG2a and IgG2b) were used. All MoAbs were purchased from Coulter (Coulter Corporation, Miami, Florida, USA) except anti-CD40 (Serotec, Oxford, England). Cytoplasmatic TLR9 was measured using PE-conjugated IgG2a antibodies

(eBioscience, Anaheim, CA, USA). A quantity of 25 μL of each MoAb in the appropriate dilution was added to 10^5 cells in 50 μL PBS containing 0.8 g/L albumin (Sanquin). After 30 minutes of incubation at 4˚C, the cells were washed twice, and analyzed on a Coulter FACScan (Coulter). Results were analyzed using the Coulter Expo 32 software (Coulter). The relative expression of surface antigens is described as the mean fluorescence intensity ratio (MFIR). This value is calculated by dividing the MFI of cells stained with fluorochrome-conjugated antigen-specific MoAb by the MFI of cells stained with fluorochrome-conjugated isotype-control MoAb. If the percentage of positive events was more than 10% the leukemic sample was considered positive for that surface marker, and then the MFIR was calculated. Cell-free supernatants were harvested after 96 hours of culturing the MCL cells with or without cytokines, with or without CpG, and in the presence or absence of irradiated tCD40L cells. Cytokine measurements were performed using commercial IL-10 (Sanquin), IL-12 p40/70 (U-CyTech, Utrecht, the Netherlands) and IL-12 p70 (U-Cy Tech) ELISA kits according to the manufacturer's instructions.

Mixed lymphocyte reaction

A modification of the allogeneic mixed lymphocyte reaction (MLR)[34] was used to analyze the functional significance of IL-10 and IL-12 to the APC capacity of the MCL cells. Freshly thawed or CD40-activated MCL cells from patients 3, 4 and 5 were used as stimulator cells. MNC from a healthy donor was used as responder cells. In a 96-well flat-bottom culture plates (Costar) responder cells at concentration of 1.5×10^4 were stimulated with 1×10^5 irradiated (15Gy) stimulator cells in the presence of IL-10 (10ng/mL), IL-12 (5 U/mL) or without cytokines. After 4 days of culture in IMDM with 10% human serum, 1 μCi of [^{3}H]thymidine (Amersham, Roosendaal, The Netherlands) was added and after additional 18 hours of culture [^{3}H]thymidine incorporation was measured. As a control, [^{3}H]thymidine incorporation in wells containing responder or stimulator cells only was used. All counts were corrected by subtracting the mean stimulator control counts (18-fold) and the mean of responder control counts (6-fold). A Stimulation Index (SI) was calculated by dividing the counts, measured in wells with different stimulators and culture conditions, by the counts produced by unstimulated responder cells, cultured in medium alone.

Generation of MCL-reactive CTL lines and clones

MNC from two unrelated healthy HLA class I-matched donors at a concentration of 10^6 cells / well in 6-well plates (Costar) were stimulated with irradiated (30 Gy) primary MCL cells, or CD40-activated MCL cells at responder/stimulator (R/S) ratios of 10:1 in medium consisting of IMDM (BioWittaker) and 10 % human serum. IL-2 (Chirion, Amsterdam, the Netherlands) at a final concentration of 100 U/mL was added at day 6. Twice weekly, half of the medium was refreshed with IL-2 containing medium. At day 9 the T cells lines were harvested, and depleted of $CD4^+$ T cells using anti-CD4-conjugated magnetic beads (Milteny Biotec, Bergisch Gladbach, Germany) since the donors were HLA class II-mismatched. The T cell lines were restimulated with irradiated stimulator cells at the same R/S ratios at days 9, 16 and 23. T cells were harvested 5-7 days after the third or fourth stimulation for phenotypic analysis, and used as effectors in cytotoxicity assays. To generate MCL-reactive CTL clones, the donor-derived leukemia-reactive CTL lines were plated at frequencies of 3, 1 and 0.3 cells/well in 96-well microtiter

plates, and expanded in the presence of irradiated allogeneic feeder cells (5 x 10^4 cells / well) in medium consisting of IMDM plus 10% human serum, IL-2 (100 U /mL) and phytohemagglutinin (PHA, 800 ng/mL, Murex Biotech Limited, Dartfort, UK). When sufficient cell numbers were reached T cell clones were harvested, and tested for specific cytolytic activity against the primary MCL cells, CD40 activated MCL cells, and PHA blasts from patient and donor. As read-out system for T-cell-mediated cytotoxicity, our recently developed carboxyfluorescein diacetate succinimidyl ester (CFSE)-based cytotoxicity assay was used [35].

CFSE-based cytotoxicity assay

The CFSE-based cytotoxicity assay facilitates the quantitative analysis of susceptibility to T-cell-mediated lysis of malignant cell types within a heterogeneous target cell population. Briefly, after washing with phosphate-buffered saline (PBS) the target cells were resuspended at 20 x 10^6 cells/mL and labeled with 10 µM CFSE (Molecular Probes Europe, Leiden, the Netherlands) for 10 minutes at 37°C. The reaction was stopped by the addition of an equal volume of fetal calf serum (FCS), followed by a 2-minute incubation at room temperature. After 2 washes the CFSE-labeled target cells were resuspended in culture medium at a cell concentration of 5 x 10^4 cells/mL, and 100 µL/well was plated in 96-well microtiter plates. CTLs were added at different effector/target (E/T) ratios ranging from 0.3:1 to 10:1. After 5 hours and 12 hours of incubation in a humidified atmosphere of 5% CO_2 and 37°C the wells were harvested, and to allow quantitative analysis of the cell populations 10,000 Flow-Count Fluorospheres (Coulter Corporation, Miami, FL) were added. To stain for dead cells, propidium iodide (0.5 µg/mL) was added, samples were mixed properly, and directly analyzed by flow cytometry. For each sample 5,000 microbeads were acquired and the absolute number of surviving cells was determined at each time point by calculation of the ratio between the number of viable cells and the number of beads. The percentage of survival was calculated as follows: % survival = [absolute # viable $CFSE^+$ target cells (t = x)]/[absolute # viable $CFSE^+$ target cells (t = 0)] x 100. Percentage of specific lysis was defined as: 100% - % survival. To analyze the HLA class-I or -II restriction of the target cell lysis blocking studies were performed. Target cells were incubated with saturating concentrations of anti-HLA class I or anti-HLA class II MoAb (W6/32 and PdV5.2, respectively) for 30 minutes before effector and target cells were cocultured. Blocking experiments at effector level were performed by adding anti-CD8 MoAb (FK18, RIVM, Bilthoven, the Netherlands) at a final dilution of 8µg/mL 30 minutes prior to the addition of target cells.

Statistical analysis

Statistical significance of differences was determined by the paired two-tailed Student's *t*-test or by the Wilcoxon test using the absolute values.

Results

Expression and upregulation of adhesion and costimulatory molecules on MCL cells.

To determine cell surface expression of the adhesion molecules CD54 and CD58, and the costimulatory molecules CD40, CD80, CD86 and CD83, immunophenotyping of freshly isolated MCL cells from 6 patients was performed.

Table 3. Phenotype of isolated MCL cells.

	Recognition	Adhesion		Costimulation			
Patient	MHC II	CD54	CD58	CD80	CD86	CD83	CD40
	MFIR (% positive cells)						
MCL 1	+++ (97)	+ (42)	++ (26)	± (13)	0 (4)	0 (9)	++ (95)
MCL 2	+++ (97)	++ (37)	0 (6)	± (11)	± (10)	0 (2)	+ (91)
MCL 3	+++ (94)	+ (83)	+ (93)	± (52)	0 (3)	0 (2)	++ (88)
MCL 4	+++ (99)	+ (13)	+ (29)	+ (84)	0 (3)	+ (20)	++ (88)
MCL 5	+ (72)	+ (14)	+ (15)	0 (2)	0 (5)	0 (2)	++ (83)
MCL 6	+++ (96)	+ (17)	++ (28)	0 (7)	0 (1)	0 (7)	++ (96)

Mean fluorescence intensity ratio (MFIR) is mean fluorescence intensity (MFI) of cells stained with a fluorochrome-conjugated antigen-specific MoAb divided by MFI of cells stained with a fluorochrome-conjugated isotype-control MoAb. If the percentage of positive events was <10 %, MFIR of the leukemic sample was not calculated and is expressed as 0. If the percentage of positive events was ≥ 10 % then the MFIR is calculated and depicted as: ± < 10; + 10 – 20; ++ 20 – 50; +++ 50 – 100

As shown in table 3, the MCL cells of all patients strongly expressed HLA class II (MFIR >50). In most patients (n=5), a minority of the MCL cells (<45%) expressed CD54 at low levels (MFIR <20). One patient (MCL 2) had intermediate levels (MFIR 20-50) of CD54 expression, but lacked CD58 expression. In MLC 3, almost the entire population (>80%) expressed the adhesion molecules but expression levels were low. Two out of six patients showed intermediate levels of CD58. In only one patient (MCL 4) some expression of CD80 and CD83 was observed, all other MCL cases expressed no or neglectable levels of CD80, CD86 and CD83. Only the costimulatory molecule CD40 was expressed by all MCL cells at intermediate levels. In summary, in accordance with other B cell malignancies, inadequate expression of costimulatory molecules on MCL cells was observed.

To upregulate the expression of adhesion and costimulatory molecules on MCL cells, we first examined several cytokines, including IFN-α, TNF-α, IL-4 and IL-10. Next, activation through TLR 9 by its synthetic ligand CpG ODN 2006 in combination with the various cytokines was tested. Finally, CD40 engagement as stimulus to modify MCL cells into APCs was analyzed in the presence of cytokines and/or CpG. Figure 1 summarizes the FACS analysis data of the most optimal combinations

to upregulate adhesion and costimulatory molecules on MCL cells. The data are presented as percentages of positive MCL cells (figure 1A) and the MFIR (figure 1B), and were obtained after a culture period of 96 hours.

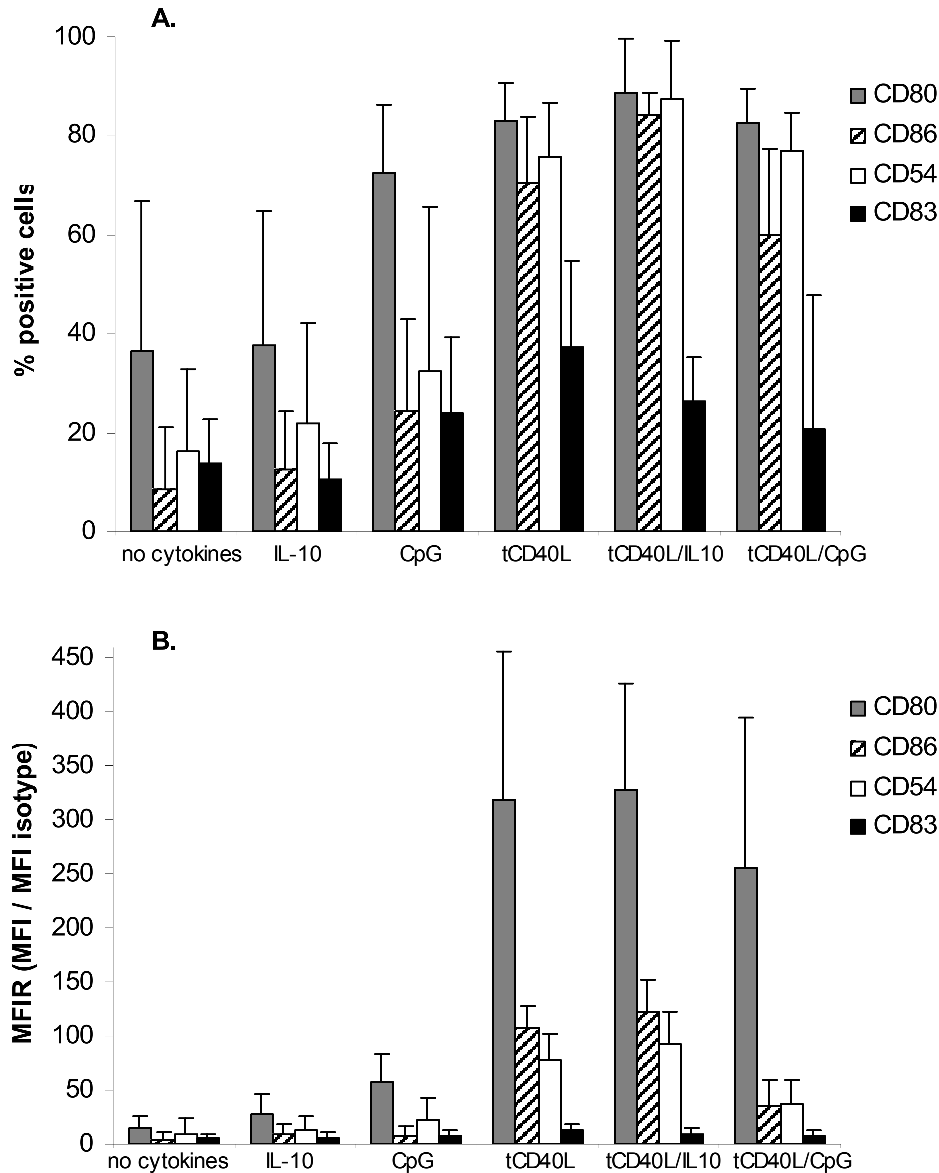

Figure 1: Expression and upregulation of costimulatory and adhesion molecules on MCL cells activated by IL-10, CpG and/or tCD40L. (A) Combinations using CD40 activation give significant higher percentages of positive MCL cells for CD80 and CD86 ($p<0.01$) (B) Stimulation of MCL cells by tCD40L gives maximal upregulation of expression levels of CD80 and CD86. MCL cells were cultured for 96 hours in the presence or absence of IL-10 (10 ng/mL), CpG (10 µg/mL) and/or tCD40L. Expression of CD80, CD86, CD54 and CD83 was analyzed by flow cytometry. Mean fluorescence intensity ratio (MFIR) was calculated as described in Material and Methods section, only if percentage of positive cells was >10%. Results are expressed as mean ± SD of 6 patients with MCL.

None of the tested cytokines caused any upregulation. CpG increased the percentage of MCL cells positive for costimulatory molecules tested, but only the expression level of CD80 was enhanced. Using flowcytometry, only weak expression of TLR9 was observed (data not shown). Accordingly, only

minor changes were observed after stimulation of MCL cells with CpG. CD40 activation by tCD40L significantly increased the percentage of positive MCL cells for all adhesion and costimulatory molecules ($p<0.01$). As demonstrated in figure 1B, the expression levels of all these molecules, especially CD80 (MFIR 21.3 fold, $p=0.001$) on MCL cells were strongly upregulated, and transformed MCL cells into characteristic APC phenotypes with high expression of CD80, CD86 and CD54. Additional stimulation with CpG or IL-10 did not further enhance the upregulation.

To investigate the optimal time period of stimulation for full activation of MCL, phenotypic analysis of MCL cells of all six patients was performed 2, 4 and 6 days after stimulation with tCD40L. As shown in figure 2, CD40 activation of MCL cells by tCD40L caused strong upregulation of CD80, CD86, and CD54 within 2 days. After 4 days of stimulation a further enhancement of expression levels of CD80 (MFIR 2.9 fold, $p= 0.01$) and CD86 (MFIR 3.5, $p=0.02$) was observed. Although a significantly higher percentage of leukemic cells expressed CD83 after CD40 stimulation, the expression levels of CD83 were only minimally upregulated.

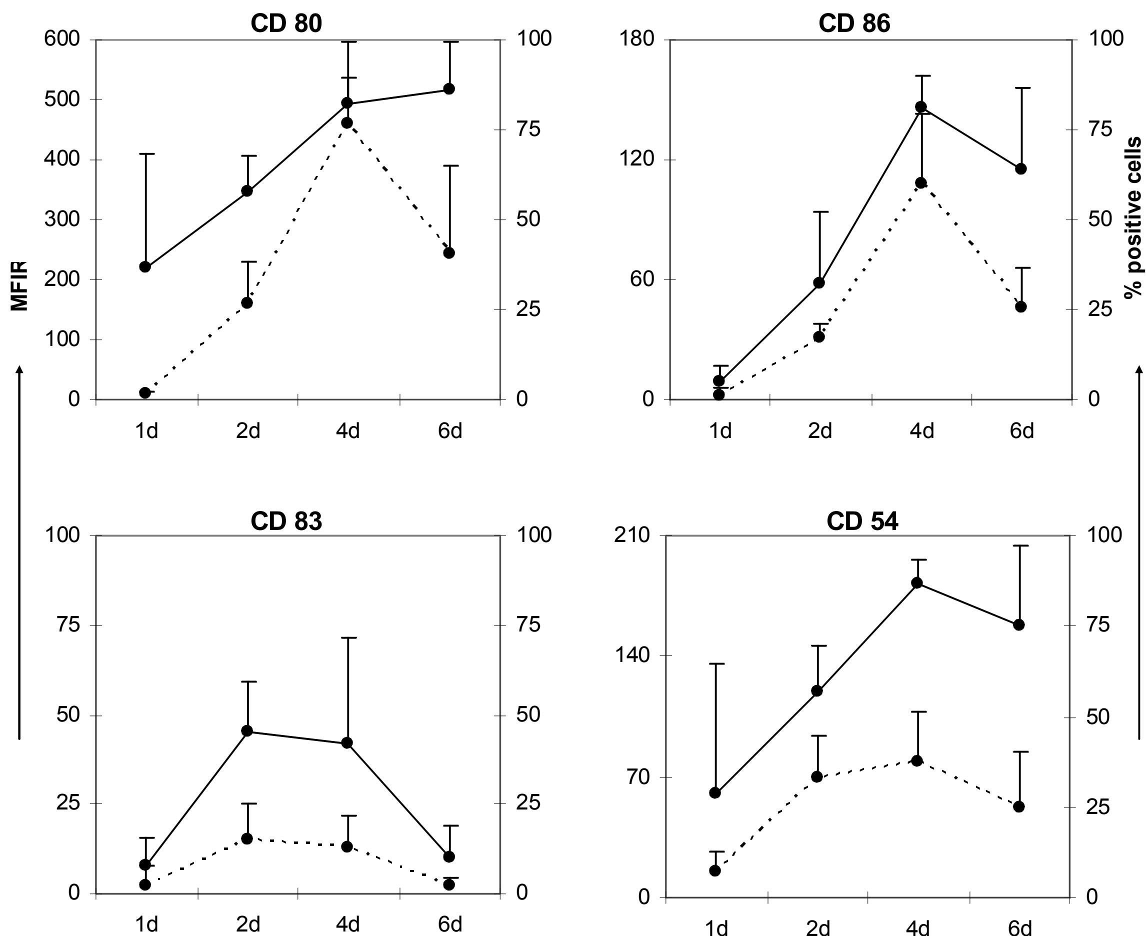

Figure 2. Kinetics of upregulation of costimulatory molecules on MCL cells activated by tCD40L. MCL cells were activated by tCD40L. MFIR and percentage of MCL cells positive for CD80, CD86, CD83 and CD54 were analyzed at different time points by flow cytometry. Data are shown as means ± SD for 6 patients. Straight lines indicate % positive cells, dotted lines represent MFIR.

Cytospin preparations of both unstimulated and tCD40L-activated MCL cells showed that after CD40 triggering, MCL cells increased 2-fold in size and obtained a dendritic cell-like morphology with a high degree of vacuolization. In conclusion, in the absence of CD40 activation minimal upregulation was observed in response to IL-10 and CpG. Stimulation of MCL cells by tCD40L for a period of 4 days caused the strongest upregulation of costimulatory and adhesion molecules, and modified MCL cells into morphological and phenotypical characteristic APC.

To examine the functional capacity of the different MCL APC to produce IL-10 and IL-12, supernatants from cultures of activated MCL cells after 96 hours were measured. Whereas unstimulated MCL and CpG activated MCL cells were not capable of producing significant amounts of IL-12 (median <15 pg/mL), high levels of IL-12 were produced by tCD40L-activated MCL cells with major variability between different patients (median 1,054 pg/mL; range 67-8,800 pg/mL, n=6). Neglectable amounts of IL-12p70 (mean 6 pg/mL) and IL-10 (mean 14 pg/mL) were produced by the CD40-activated MCL cells. Additional stimulation with CpG induced some increase in IL-12 production (median 1,370 pg/mL) and IL-12p70 (mean 31 pg/mL) but also induced the production of IL-10 (mean 299 pg/mL).

MLR using primary or MCL-APC as stimulator cells in the presence or absence of IL-10 and IL-12

To investigate the functional significance of IL-12 or IL-10 on the stimulatory capacity of MCL cells, a MLR was performed using primary MCL cells or MCL-APC as stimulator cells, and MNC from a donor as responder cells in medium with or without IL-10 or IL-12. As expected, primary MCL cells, derived from three different patients, had minimal allostimulatory capacity (see figure 3). IL-12 had a weak stimulatory effect on the proliferation of the allogeneic T cells, whereas IL-10 demonstrated some inhibition of T cell activation. In contrast, the stimulation index in response to MCL-APC as stimulators, was 25-50, illustrating the impressive immunostimulatory capacity of these malignant APC. As shown in figure 3, further increase of IL-12 concentrations did not enhance T cell proliferation nor did IL-10 suppress the induction of proliferation. In conclusion, tCD40L-activated MCL cells were considered the best MCL-APC, and were further used as stimulator cells in the subsequent experiments.

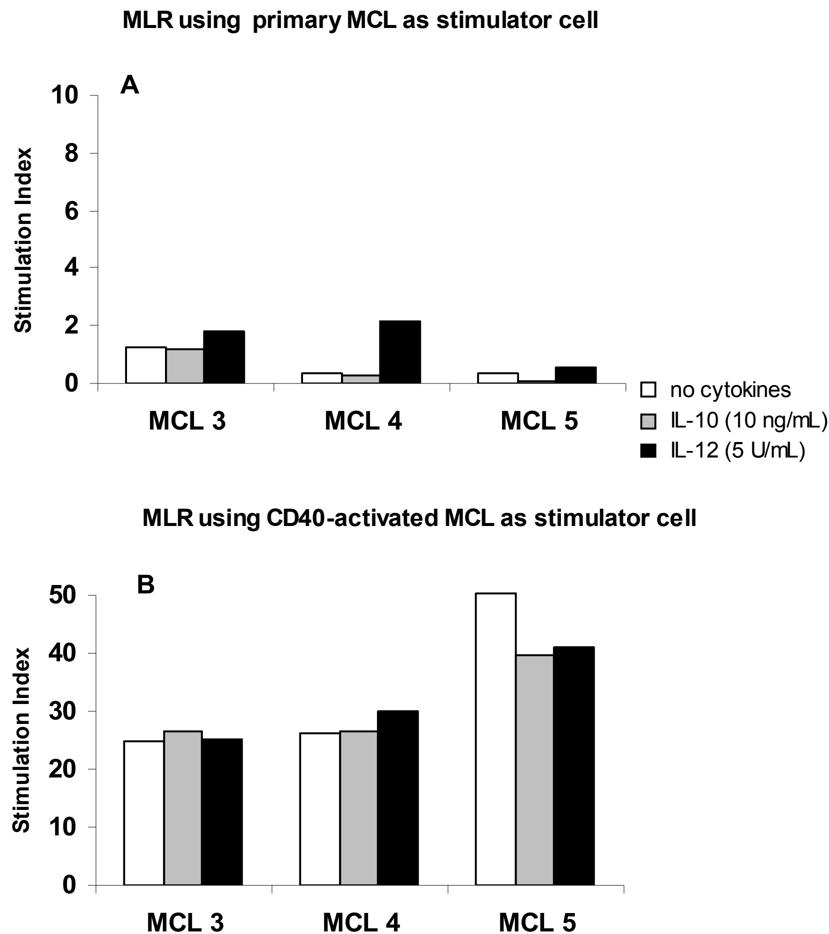

Figure 3. The proliferation and cytotoxicity of allogeneic CD8+ T cells in response to primary MCL and MCL-APC cells. (A) In contrast to primary MCL, MCL-APC cells as stimulators induced strong proliferation of CD8+ donor T cells in the URD anti-MCL-APC 3 and URD anti-MCL-APC 4 combinations.(B) cytotoxic activity of the CTL lines generated against MCL-APC 3 or MCL-APC 4 measured in a 12-hours CFSE-based cytotoxicity assay.

The generation of MCL-reactive CTL lines and clones from HLA-matched donors

To analyze the antigen-presenting capacity of primary MCL cells and tCD40L-activated MCL (MCL-APC) T cells from a fully HLA class I-matched donor and an unrelated HLA-A- and HLA-B-matched donor were stimulated with primary MCL or MCL-APC cells. CD4 depletion was performed at day 9 to eliminate allo-HLA class II responses. As demonstrated in figure 4, primary MCL cells were not capable of inducing T-cell proliferation and generating T cell lines. The cytotoxic activity of the CTL lines generated against MCL-APC was measured using the CFSE-based cytotoxicity assay [35] (see Material and methods) with CFSE-labeled primary MCL cells, MCL-APC cells, and PHA blasts from the patients and donors as target cells. As illustrated in figure 4, the $CD8^+$ CTL lines derived from the donors effectively killed the primary MCL cells, the MCL-APC, the PHA blasts from the patient, and not the PHA blasts from the donor in a 12 hours CFSE cytotoxicity assay.

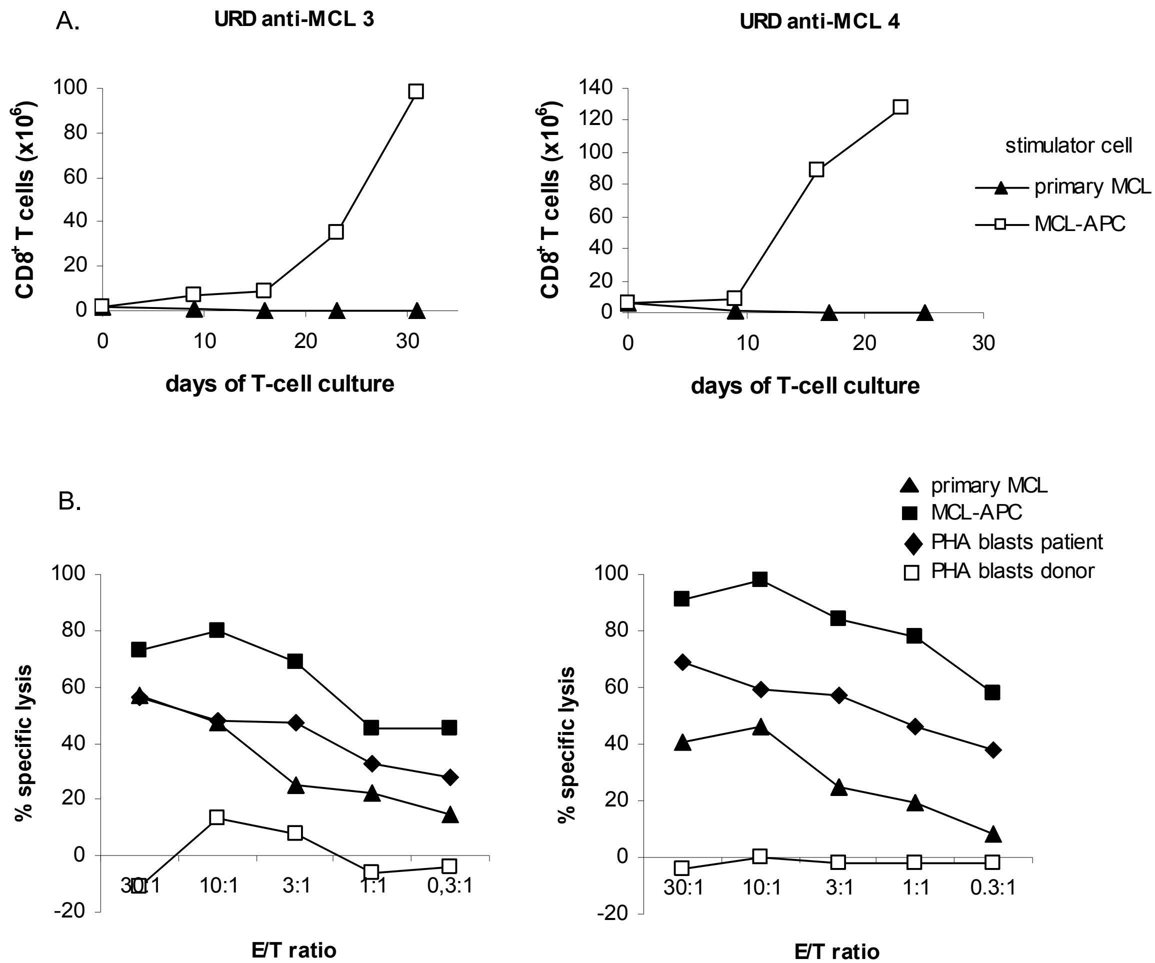

Figure 4. The proliferation and cytotoxicity of allogeneic CD8+ T cells in response to primary MCL and MCL-APC cells.(A) In contrast to primary MCL, MCL-APC cells as stimulators induced strong proliferation of CD8+ donor T cells in the URD anti-MCL-APC 3 and URD anti-MCL-APC 4 combinations.(B) cytotoxic activity of the CTL lines generated against MCL-APC 3 or MCL-APC 4 measured in a 12-hours CFSE-based cytotoxicity assay. Although there was an HLA-C locus mismatch in the MCL 4/donor combination and although the primary MCL 4 had some expression of CD80 and CD83 (see table 2) this was insufficient to overcome T cell anergy and to induce antigen-driven proliferation. In contrast, using MCL-APC cells as stimulators, vigorous expansion of $CD8^+$ T cells was observed in both patient/ donor combinations tested.

To estimate the precursor frequency of MCL-reactive T cells in the CTL lines, and to determine whether the cytotoxicity of the CTL lines was exerted by cytotoxic T cells with different specificities CTL clones were generated. In the URD α-MCL-APC 3 combination 13% of the 1 cell/well and 8% of the 0.3 cell/well showed proliferation. 29 out of 91 proliferating $CD8^+$T cell clones showed specific lysis of primary MCL at an E/T ratio of 10:1 (36 ± 14%, mean ± SD, n=29) in a 12 hours CFSE cytotoxicity assay. The plating efficiency in the second combination (URD α-MCL-APC 4) was 12%, resulting in 60 out of 89 $CD8^+$CTL clones, recognizing primary MCL cells (65 ± 20%, mean ± SD, n=60). These results indicated that in both CTL lines high frequencies of MCL-reactive T cells were present.

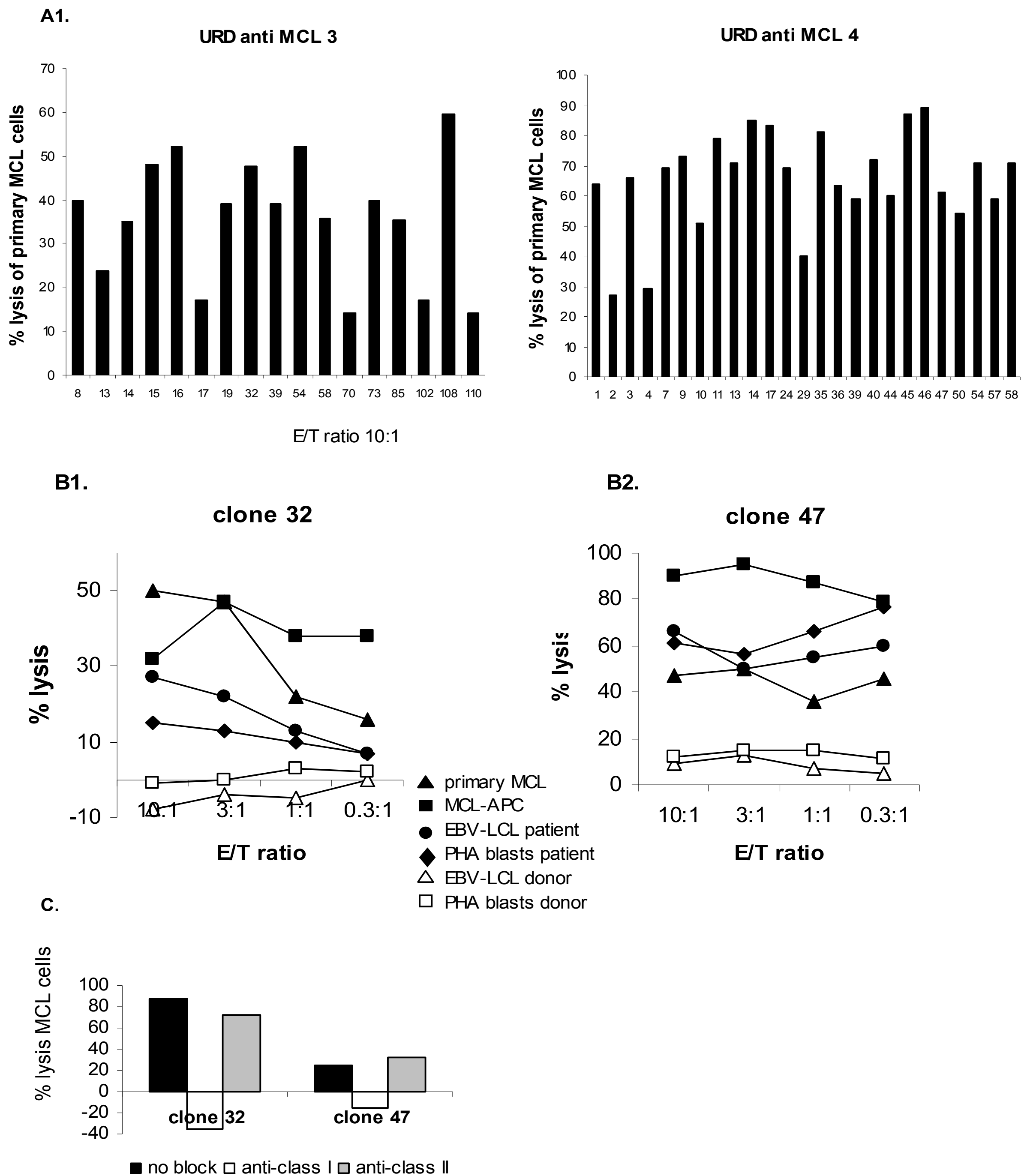

Figure 5. Cytotoxicity and specificity of representative MCL-reactive CD8+ CTL clones, derived from HLA-matched donors. (A1.and A2.) High numbers of CD8+ CTL clones recognizing primary MCL in a 12-hours CFSE cytotoxicity assay were generated from both URD (B1. and B2.) Four representative CTL clones from each donor/patient pair were analyzed for specific cytolytic activity against MCL, MCL-APC, and PHA blasts from donor and patient in a 12-hours CFSE cytotoxicity assay. All clones recognized MCL-specific as well as patient-derived targets and not PHA blasts or EBV-LCL from the donor. One representative example of these CTL clones from each combination is demonstrated. (C.). HLA class I-restriction of the cytotoxicity was demonstrated by blocking experiments using primary MCL as target at an E/T ratio of 10:1. Target cells were 30 minutes preincubated with anti-HLA class I (W6/32) or anti-HLA class II (PdV5.2) antibodies prior to the CTL assay using clone 32, derived from URD 3 and clone 47, derived from URD 4 as effector cells.

To analyze whether the MCL-reactive CTL clones were MCL-, B cell- or mHag-specific, four representative clones in both patient/donor pairs were tested for cytotoxicity against MCL-specific targets, patient- and donor-derived T lymphoblasts (PHA blasts) and B-cell-specific targets (EBV-LCL) from patient and donor. As shown in figure 5, CTL clones generated from the CTL lines from both donors were reactive against MCL, MCL-APC, and EBV-LCL of the patient, and not against donor-specific targets. The clones also killed T cell-derived targets indicating that these T cell clones were probably mHag-specific and that the recognized mHag is not B-lineage-restricted. To confirm HLA class I-restricted recognition of the targets by the $CD8^+$ CTL lines and clones, and to exclude HLA class II-restricted killing by contaminating CD4+ T cells, blocking studies were performed using representative examples of the generated MCL-reactive CTL lines and clones. Cytotoxicity of both CTL lines was completely blocked by anti-HLA class I or anti-CD8 antibodies and not by the addition of anti-HLA class II (data not shown). Cytolytic activity of the CTL clones could partially or completely be abrogated using the anti-HLA class I antibodies (figure 5).

Discussion

RIC allogeneic SCT is considered as a new promising treatment modality for patients with advanced MCL. After allogeneic SCT adoptive transfer of donor T cells, capable of killing MCL cells may eliminate residual malignant cells resulting in long-term remissions. In this context, this experimental study was performed to evaluate whether donor T cells can be triggered to preferential kill MCL-specific targets using MCL cells as stimulator cells. As illustrated in the results, we demonstrated that primary MCL cells as stimulator cells failed to induce proliferation and to generate a T-cell response, even in the presence of IL-12, probably due to the lack of expression of costimulatory molecules. Thus, to generate donor T cells with high avidity for MCL cells, modification of the MCL cells into professional APC with high expression of costimulatory and adhesion molecules was hypothesized to be essential. Treatment of MCL cells with proinflammatory cytokines, B-cell activating cytokines, and also the MCL-stimulating cytokine IL-10 did not significantly induce phenotypic changes of the MCL cells. In accordance with a recent report [36], we observed limited activation of MCL cells upon stimulation with CpG ODN 2006, probably due to the low expression of TLR9 on MCL cells.

Since CD40 engagement is the major signal that induces B cells to efficiently present antigen to T cells [16,18,37], and since malignant B cells strongly express CD40, activation through CD40 can be an effective tool to transform tumor B cells into an antigen-presenting phenotype [15,17,20,27,28]. In the present study, we show that CD40-ligation could modify MCL cells into phenotypically professional APCs with high expression of CD80, CD86, and the adhesion molecules, and induced some expression of CD83. Additional stimulation through TLR9 and cytokine receptors did not further enhance expression levels. In contrast with recent cytokine gene expression studies suggesting that both primary as well as CD40-activated MCL cells cannot produce IL-12p40 and assemble the IL-12 heterodimer [38], we illustrated in the present study the capacity of MCL cells to synthesize IL-12. MCL cells were only capable of producing IL-12 in response to CD40 stimulation. The MLR experiments

demonstrated that using MCL-APC as stimulator cells an increase in IL-12 concentrations did not further enhance T-cell proliferation.

Next, the stimulatory capacity of the generated MCL-APC to induce vigorous allogeneic MCL-specific T-cell responses was compared to primary MCL as stimulator cell in two allogeneic donors. In the HLA-C locus mismatched setting (URD αMCL 4) the unmodified MCL cells as stimulators did not induce any antigen-driven proliferation. Although MCL 4 showed some expression of the costimulatory molecules, this was apparently insufficient to induce an appropriate T-cell response over a major MHC mismatch barrier. In contrast, the MCL-APCs were capable of provoking vigorous expansion of the donor-derived T cells and of eliciting a sustained primary allogeneic MCL-reactive immune response. These results illustrate that primary MCL cells can be adequately killed by T cells generated against MCL-APCs. To evaluate whether MCL-specific $CD8^+$ T-cell responses could be generated in a fully HLA-class I-matched setting MNC from the URD were stimulated with primary MCL 3 or MCL 3-APC. Again no T-cell proliferation was observed against primary MCL and cytotoxic MCL-reactive $CD8^+$ T-cell lines and clones could be generated against MCL-APC, indicating that MCL-APCs are essential to induce an efficient anti-leukemic T-cell response. Allo-HLA-class II responses were eradicated by CD4 depletion and blocking experiments confirmed HLA class I-restricted recognition of the target cells. To further elucidate the different specificities of the leukemia-reactive T cells and to determine the precursor frequencies of these T cells in the CTL line, cloning experiments were performed. This resulted in high clonal expansion efficiency of 6-18% of the cells isolated, suggesting that MCL-APCs as stimulator cells enrich for MCL-reactive T cells. MCL-specific targets, but also patient-derived targets from T-cell (PHA blasts) and B-cell origin (EBV-LCL) were recognized by the CTL clones, indicating that the immunogenic antigen is not MCL- or B-cell-specific, but most likely mHag-specific. As we previously illustrated for patients with acute leukemia's [14,39,40], repeated stimulation of donor T cells with leukemic cells results in the generation of T-cell responses against mHag with relative specificity for the malignant cells. Therefore, although other patient-derived APC may be capable of eliciting mHag specific T-cell responses, MCL-derived APC likely will skew the immune response toward recognition of MCL-specific antigens or mHags highly expressed on the MCL cells thus leading to a more specific and efficient T-cell response.

In summary, our study shows that T cell anergy to primary MCL cells can be reversed by transforming MCL cells into professional malignant APCs using CD40 ligation and provides the first evidence that using these MCL-APCs as stimulators MCL-reactive mHag-specific $CD8^+$ CTL lines and clones can be readily generated from MNC of HLA class I-matched donors. Two recent reports demonstrated the feasibility of RIC allogeneic SCT as salvage therapy in patients with advanced MCL [41,42]. After allogeneic SCT sensible RT-PCR techniques for minimal residual disease and donor chimerism analysis enable to predict disease recurrence. The administration of DLI can lead to long-term remissions, but effectiveness may be limited due to low immunogenicity of MCL cells and the development of acute or chronic GvHD, caused by non-selected donor T cells. In this setting the application of mHag-specific CTLs, which preferently kill MCL cells and not non-hematopoietic targets

[14] could be of great value and could prevent or treat disease recurrence after allogeneic SCT. The magnitude and specificity of the immune response against MCL cells without causing concomitant GvHD may be maximized.

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Chapter 5

Characterization of graft-versus-leukemia responses in patients treated for advanced chronic lymphocytic leukemia with donor lymphocyte infusions after *in vitro* T-cell depleted allogeneic stem cell transplantation following reduced-intensity conditioning

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Submitted

Abstract

We here report the results of 12 patients suffering from chronic lymphocytic leukemia (CLL), treated with *in vitro* T-cell depleted reduced-intensity conditioning allogeneic SCT followed by donor lymphocyte infusions (DLI). Four patients achieved a complete remission (CR) after the administration of DLI demonstrating the curative potential of this procedure. In two representative patients graft-versus-leukemia activity was analyzed by isolating T cells producing IFNγ in response to CLL or CLL antigen-presenting cells (CLL-APC). From a patient suffering from progressive disease no leukemia-reactive T-cell responses could be isolated. Primary CLL-reactive T-cell responses were inducible in the unprimed donor only in response to CLL-APC but not to unmodified CLL. These results indicate that precursor CLL-reactive T cells were present in the DLI, but *in vivo* inadequately stimulated by the low immunogenic primary CLL. The other patient had a CR after DLI. In post-DLI experiments using peripheral blood, leukemia-reactive CTL clones with different specificities could be isolated, but only in response to CLL-APC indicating that CLL-reactive T cells were probably cross-primed by APC from recipient- or donor origin. These results suggest that persistent primary CLL after allogeneic SCT and DLI may escape from T-cell surveillance due to the lack of APC function.

Introduction

In spite of the spectrum of currently available chemotherapeutic agents and monoclonal antibodies (MoAb) [1-6], once CLL becomes therapy-resistent the prognosis is poor [2,3]. Adverse biological risk factors such as the mutation status of the immunoglobulin variable heavy (VH) genes, expression of ZAP-70 and unfavourable cytogenetics enable to early identify patients with a more rapid fatal course [7-11]. For these patients allogeneic stem cell transplantation (SCT) may be a potential curative treatment modality [12-14]. Recently, several reports have shown that allogeneic SCT following reduced-intensity conditioning (RIC) significantly diminished treatment-related mortality (TRM) while preserving the graft-versus-leukemia (GvL) effect [13,15-18]. However, considerable toxicity due to a high incidence of acute graft-versus-host disease (GvHD, 42%) and extensive chronic GvHD (>50%) was observed [16,17]. The use of alemtuzumab to deplete for donor and recipient T cells as part of the RIC strategy effectively reduces GvHD, while the administration of alemtuzumab with the stem cell graft may also result in a profound anti-CLL effect [13,19,20]. Once durable donor engraftment is achieved, adoptive immunotherapy with donor lymphocyte infusion (DLI) can be administered to promote and enhance the GvL effect. Since recipient-derived antigen-presenting cells (APC) appear to play an important role in the induction of GvHD the incidence and severity of GvHD after DLI may be less in T-cell depleted allogeneic SCT than the incidence of GvHD after unmodified allogeneic SCT following RIC [21]. We and others have demonstrated the feasibility of this approach [13,22].

Because CLL is a slowly progressive disease offering a time frame after allogeneic SCT to optimize the potential of adoptive immunotherapy, this transplantation protocol facilitates the analysis of graft-versus-CLL. Here, we report the result of a study including 12 patients with chemo-refractory CLL treated with this *in vitro* T-cell depleted RIC allogeneic SCT followed by the postponed administration of DLI. To characterize allogeneic immune reponses and the possible graft-versus-CLL activity, T cells from a patient successfully treated with DLI (patient 6), and T cells from a patient refractory to several applications of DLI after an initial partial response (patient 10) were studied. We have previously demonstrated that by CD40 triggering in the presence of IL-4, CLL cells can be modified into efficient malignant APC (CLL-APC) capable of inducing allogeneic T-cell responses [23]. In this study, T cells isolated from peripheral blood (PB) of the patients during clinical response to DLI were stimulated with primary CLL cells or CLL-APC derived from the patients. As described recently, T cells responding to the malignancy or the malignant APC by secreting interferon γ (IFNγ) were selected, directly cloned and expanded [24]. From the patient with progressive disease despite DLI (patient 10), no CLL-reactive T cells responding to stimulation by primary CLL or CLL-APC could be isolated. However, precursor CLL-reactive T cells were present in the unprimed donor but these T cells could only be induced to proliferate in response to CLL-APC, illustrating the lack of APC function of the unmanipulated leukemic cells. From the PB of the patient with an excellent clinical response after DLI (patient 6), leukemia-reactive CTL clones could be isolated. From this patient, stimulation with CLL-APC appeared to be more efficient to isolate CLL-reactive T cells. In accordance with our previous data, this study illustrates that the inappropriate APC function of primary CLL cells may result in T-cell

tolerance towards persistent CLL cells after allogeneic SCT [23]. The observed GvL reactivity resulting in good clinical responses may be mediated by T cells reactive against alloantigens expressed on recipient dendritic cells, or donor dendritic cells cross-primed with CLL cells.

Patients, materials and methods

Eligibility criteria

Patients with progressive CLL after conventional chemotherapy with ages between 18 and 70 years with an HLA-identical sibling donor or an HLA-compatible unrelated donor (MUD) were eligible for this study. This single center study was approved by the institutional Ethics Committee and patients gave written informed consent for participation in the protocol as well as for scientific research performed using their PB and bone marrow (BM) cells.

Patients characteristics

Between May 2000 and September 2005 twelve patients with advanced CLL were enrolled. Patients and transplantation characteristics are summarized in Table 1.

Table 1. Patient and transplant characteristics.

Characteristics		No.
Total patients		12
Men / women		8/4
Median age at transplantation, y (range)		56 (45-59)
Time diagnosis to alloSCT, y (range)		4.9 (2.3-9.7)
Previous chemotherapy regimen	≤ 3	5
	> 3	7
	Fludarabine refractory	11
Unfavourable cytogenetics (n=10)		5
Disease status after last therapy at transplantation	CR	1
	PR	9
	refractory / PD	2
Donor	HLA-identical related	10
	Matched unrelated	2
Stem cell dose	$CD34^+$ (10^6/kg): median (range)	12.3 (7.9-16.5)

The conditioning regimen consisted of fludarabine 30 mg/m^2/day intravenously on days –10 to –6, busulphan by intravenous infusion of 3.2 mg/kg/day on days –6 and –5, and antithymocyte globulin (horse, IMTIX Sangstat, Lyon, France) by intravenous infusion of 10 mg/kg/day on days –4 to –1. A dose of 15 x 10^6 $CD34^+$ cells/kg of the recipient body weight was targeted for SCT. T-cell depletion of the stem cell product was performed by incubation with 20 mg alemtuzumab for 30 minutes at room temperature under continuous agitation. No post-transplant GvHD prophylaxis was administered.

Donor lymphocyte infusions

In this study DLI was anticipated to be administered in all patients. Patients with persistent disease, minimal residual disease, or incomplete donor chimerism at six months after allogeneic SCT received 5 x 10^6 $CD3^+$ cells/kg. Escalating doses of $CD3^+$ T cells were administered at 3-6 months intervals in the absence of development of GvHD if mixed chimerism or CLL persisted.

Study endpoints and statistical analysis

Data were analyzed by April 1, 2006. The median follow-up was 21 months (range, 7–61 months). Neutrophil engraftment was defined as the first of 2 consecutive days with an absolute neutrophil count > 0.1 x 10^9/l. Platelet engraftment was defined as the first of 7 consecutive days with a platelet count of > 20 x 10^9/l without transfusion. Acute and chronic GvHD was graded according to consensus criteria [25]. Chimerism analysis was performed in BM samples at fixed time points (day +30, 60, 90, 180, 270, 365, 455, 545) as previously described [13]. Disease response was assessed using National Cancer Institute Working Group (NCI-WG) criteria [26] with the following additions. CR or PR required complete or at least 50% clearance of CD5/CD19-coexpressing CLL cells from both BM and PB as detected by flow cytometry, respectively. Patients receiving DLI were evaluable for response to therapy if they survived at least 90 days following the first infusion. Kaplan-Meier survival estimates were calculated from allogeneic SCT until death from any cause.

Isolation of *in vivo* primed CLL-reactive T cell clones

Two patients (patient 6 and patient 10) were selected for further analysis of the presence of GvL-mediating T cells. Both patients received DLI for persistent CLL and mixed chimerism. Patient 6 achieved a CR after DLI, patient 10 failed to respond to DLI. PB samples containing more than 90% of CLL cells, as assessed by flow cytometric analysis were obtained at diagnosis. In addition, BM and PB were obtained from the patients shortly before allogeneic SCT, and before and after the administration of DLI at regular time intervals. PB was also obtained from their HLA-identical sibling donors. Mononuclear cells (MNC) were isolated from the samples by Ficoll density separation, and cryopreserved. Patient- and donor-derived PHA blasts and donor-derived EBV-LCL were generated as described previously [27]. Due to very low (<1%) normal B-cell counts, no EBV-LCL could be generated from the patients. The CLL cells of the patients were modified into APC by IL-4 and CD40 stimulation as recently described [23]. After 4 days of stimulation the CLL cells were harvested, counted using eosin exclusion, analyzed by flow cytometry, and used as stimulator cells. T cells, isolated from the patient, were enriched from MNC by negative selection using the pan T isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Quantities of $0.5x10^6$-$2.5x10^6$ purified *in vivo* primed T cells were stimulated with irradiated (15 Gy) primary CLL or CLL-APC at a responder/stimulator (R/S) ratio of 10:1. After 16 hours of stimulation, IFNγ secreting T cells were stained using the IFNγ secretion assay (Miltenyi Biotec GmbH) according to the manufacturer's specifications, and isolated by single cell per well FACsorting as described previously [24]. Proliferating T cell clones were restimulated with feeder-mixture. From day 21 the clones were functionally analyzed. The nomenclature used for each T cell clone represents the patient number followed by clone number. For

flowcytometric analysis, cells were stained with FITC-conjugated anti-CD3 (BD), anti-CD4, PE-conjugated anti-CD8 (Caltag, Burlingame, USA) or anti-CD56 (BD), and Cy5-conjugated anti-CD8 (DAKO, Glostrup, Danmark), and analyzed on a FACScan (BD).

In vitro generation of CLL-specific CTL from the unprimed donors

Since non-synchronized production of IFNγ by T cells in primary anti-leukemia responses was found to hamper the effectiveness of isolation of these cells, the following procedure was adapted (Jedema et al., Blood 2003, 102, 722 abstract). After CD14 depletion MNC from the HLA matched sibling donors were plated at a concentration of 1 x 10^6 cell/well in 24-well plates (Costar), and stimulated with irradiated CLL or CLL-APC derived from the recipient at a responder/stimulator (R/S) ratio of 10:1. Low dose IL-2 (10 IU/mL, Chiron) was added at day 7 and 11 to ensure survival but to prevent non-specific proliferation of the T cells. At day 14 T cells were specifically restimulated with the irradiated CLL-APC or primary CLL. After 16 hours of stimulation the IFNγ secretion assay and the single cell per well cloning procedure was performed as described above. The proliferating T cell clones were functionally analyzed.

Cytotoxicity assay

To determine the cytotoxicity of the T cell clones standard 4 hours and overnight ^{51}Cr-release assays were performed as described previously [23]. Primary CLL cells, CLL-APC cells, PHA blasts from patient or donor, and/or EBV-LCL from unrelated individuals were used as target cells. T cell clones showing more than 10% specific lysis of target cells were considered cytotoxic. To determine HLA class I- and II-restriction of the recognition of the target cells, blocking studies were performed by incubating target cells with saturating concentrations of α-HLA class I antibodies (W6/32) or α-HLA class II antibodies (PdV5.2) for 30 minutes before effector cells added [23]. To further analyze the HLA class-restricting element of the CTL clones, the clones were tested for reactivity against a panel of EBV-LCL from unrelated individuals that shared an HLA molecule with the recipient. For blocking studies α-HLA A2 (SN66.E3), α-HLA A3 (MUL2.C6), α-HLA BC (B1.23.2), α-HLA DR (B8.11.2) and α-HLA DP (B7.21) antibodies (kindly provided by Dr. A. Mulder, Department of Immunohematology and Bloodtransfusion, Leiden) were used.

Analysis of cytokine production

For analysis of IFNγ and IL-4 production, 5,000 T cells were cocultured with 30,000 target cells in a final volume of 150 µl IMDM supplemented with 10% human serum and 50 IU/mL IL-2. After 24-hours, supernatant was harvested, and the concentration of IFNγ and IL-4 was measured in triplicate by ELISA (CLB, Amsterdam, The Netherlands). As a negative control T cells were incubated without target cells.

Results

Toxicity and graft-versus-host disease after allogeneic SCT and DLI

Patients received a median dose of 12.3 $x10^6$ $CD34^+$ cells/kg recipient body weight (range 7.9-16.5). All patients had sustained neutrophil (>0.1 x 10^9/L) and platelet recovery (>20 x 10^9/L) with a median time to engraftment of 12 days (range, 10-18 days and 11-18 days, respectively). No graft failures were observed. All patients were assessable for toxicity. As shown in table 2, prior to administration of DLI no significant GvHD was observed. Only one patient developed grade I acute GvHD of the skin. Of the 12 patients, one patient did not receive DLI because of infectious complications after allogeneic SCT. The indications for DLI were mixed chimerism (n=1), mixed chimerism and persistent disease (n=7) and progressive disease (n=3). According to the protocol, the DLI was given at a median of 6 months (range 5-13 months). Four patients received one dose of 5 x 10^6 $CD3^+$ T cells/kg, two patients received a lower dose (2.5 x 10^6 $CD3^+$ T cells/kg because of a MUD allogeneic SCT. Four patients received 2 doses and one patient (patient 10) received 5 doses of DLI with a maximum dose of 1.5x10^8 T cells/kg because of refractory disease. Four patients developed acute GvHD after DLI (1 grade I, 2 grade II and 1 grade IV). All four patients developed limited chronic GvHD. Two patients died of causes not related to relapse. One patient died at 8 months after allogeneic SCT due to sepsis of unknown origin and one patient died at 11 months due to CMV disease with concurrent GvHD after the administration of DLI (Table 2). The actuarial probability of TRM at 100 days was 0%, and at the end of follow-up (median 21 months) 19%.

Chimerism analysis and disease response to allogeneic SCT and to DLI

Sequential chimerism studies of BM cells, performed 6 months after allogeneic SCT and before the administration of DLI, showed sustained mixed chimerism with a median donor percentage of 68% (n=11 range, 1-99 %). Five patients showed conversion to full donor chimerism within 6 months after the administration of DLI, and maintained their full donor chimerism status at the time of last assessment (see Table 2). In three patients low percentages of donor chimerism after the allogeneic SCT and DLI was observed due to rapidly progressive disease. In most cases a reduction of CLL cells was observed in flow cytometric analysis of BM before and shortly after allogeneic SCT (data not shown). Six months after allogeneic SCT and before the administration of DLI one patient was in CR (8%), eight patients had persistent disease (perD, 67%) and three patients had progressive disease (PD, 25%). One patient (patient 3) was in CR at the time of allogeneic SCT and remained in CR during follow-up of 25 months. In four out of eight patients with perD, the elimination of CLL cells in BM was observed after the administration of DLI, resulting in an ongoing CR. In one of these four patients, the CR was unconfirmed because of persistent abdominal lymphadenopathy (patient 5). This patient died of infectious complications related to the DLI. Patient 1 had still perD 3 months after DLI. Patient 4 and 11 with perD were not evaluable for disease response because of too short follow-up after DLI and death, respectively. Patient 10 developed PD despite the administration of escalating doses of DLI. Three patients suffered from PD shortly after allogeneic SCT. None of them showed any disease response upon the cellular adoptive therapy. Two of these patients (patient 2 and 8) received a

Table 2. Clinical outcome after alloSCT and DLI

Patient no.	DLI			GvHD			Disease status		Chimerism (% donor)		Follow up after alloSCT (months)
	Indication	#	Initial dose (10^6 $CD3^+$/kg)	pre-DLI	post-DLI acute	post-DLI chronic	6 months after alloSCT	after DLI at last follow-up	pre-DLI	post-DLI	
1	MC / perD	2	5	no	no	no	perD	perD	67	61	11
2	PD	2	5	no	no	no	PD	PD †	8	3	19
3	MC	1	5	no	no	no	CR	CR	98	100	32
4	MC / perD	1	2.5*	no	NA	NA	perD	NA	89	NA	7
5	MC / perD	1	5	no	gr IV	limited	perD	CRu †	69	100	11
6	MC / perD	2	5	no	gr II	limited	perD	CR	91	100	42
7	MC / perD	1	2.5*	no	gr I	limited	perD	CR	67	100	23
8	PD	1	5	no	no	no	PD	PD	1	1	13
9	MC / perD	1	5	no	no	no	perD	CR	99	100	21
10	MC / perD	5	5	gr I	gr II	limited	perD	PD	68	80	61
11	not given	0	-	no	NA	NA	perD	NA †	NA	NA	8
12	PD	2	5	no	no	no	PD	PD	56	13	15

DLI, donor lymphocyte infusion; GvHD, graft-versus-host disease; MC, mixed chimerism; perD, persistent disease; PD, progressive disease; CR,complete response
CRu, complete response unconfirmed (flow cytometry BM negative for CLL cells, persistent abdominal lymphadenopathy); gr, grade; NA, not assessable; * MUD ; † , dead.

second transplant from the orginal donor with a myeloablative conditioning. Patient 2 ultimately died from PD. At a median follow-up time of 21 months (range, 5-61 months) nine patients are alive. Kaplan-Meier estimated probabilities of overall survival (OS) and event-free survival (EFS) are shown in Figure 1. At 2 years the OS was 67% and EFS was 33%.

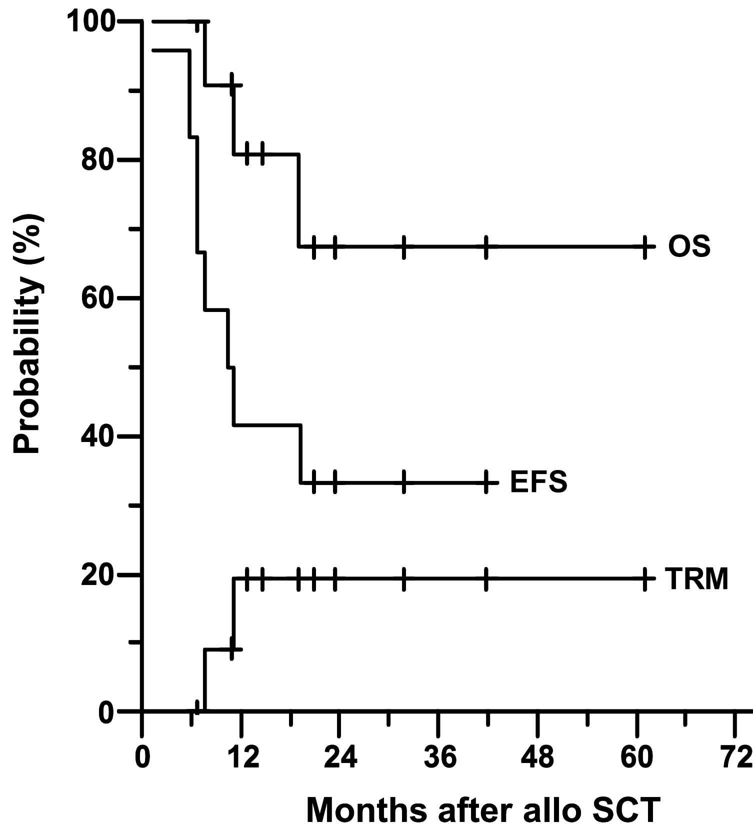

Figure 1. Overall survival, event-free survival and treatment-related mortality after *in vitro* T-cell depleted allogeneic SCT and the administration of DLI. OS, overall survival, ESF, event-free survival, TRM treatment-related mortality.

Isolation of CLL-reactive T cells in patients with clinical responses

To analyze graft-versus-CLL responses two patients were studied in detail. Patient 6 responded to DLI, whereas patient 10 failed. As shown in figure 2A, patient 6 converted to full donor chimerism after the application of two doses of DLI and flow cytometric analysis of the BM remained negative for CLL cells. In contrast, patient 10 initially partially responded to DLI but despite 5 doses of DLI (maximum dose 1.5×10^8 T cells/kg) he suffered from progressive disease (Figure 2B). He developed grade I GvHD of the skin for which local corticosteroid ointment was applied.

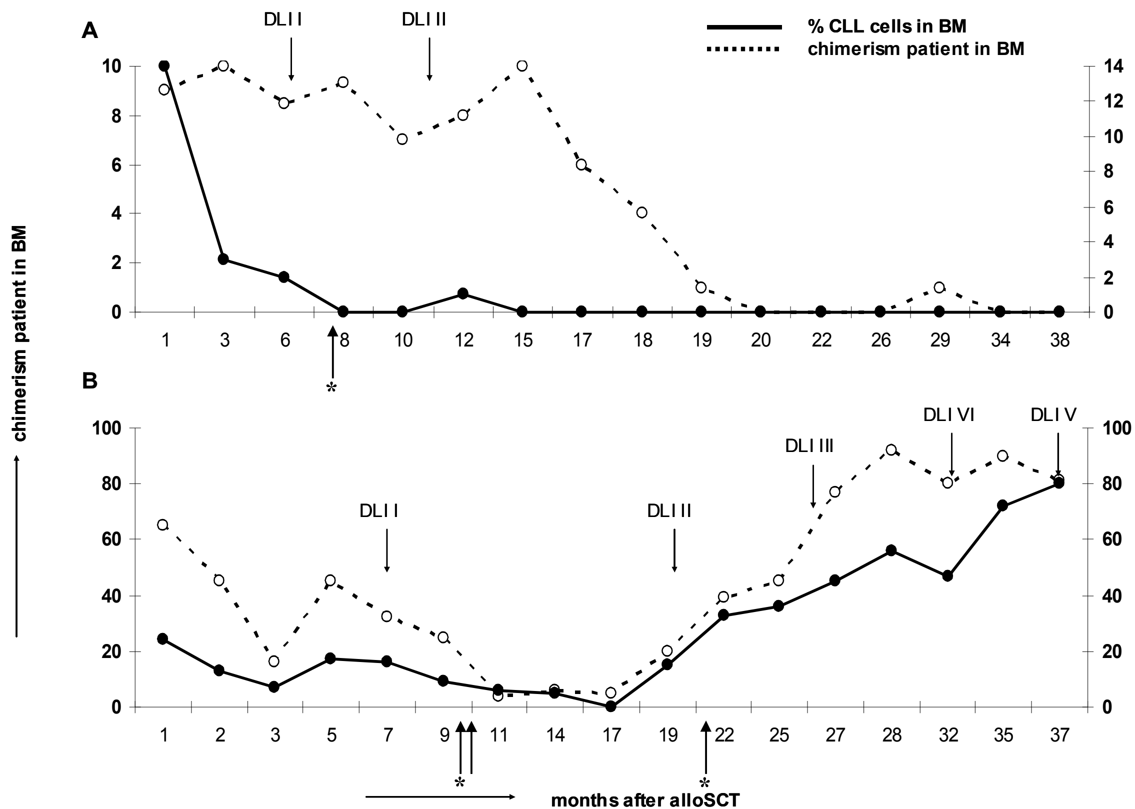

Figure 2. Patients chimerism, measured in BM and % of CLL cells in BM as determined by flow cytometry after allogeneic SCT and the administration of DLI. (A) In patient 6 disappearance of CLL cells in BM was observed after the administration of DLI and the patient converted to full donor chimera, resulting in a durable complete remission. (B) Patient 10 suffered from progressive disease after an initial response to DLI. (* timepoints of withdrawal of blood samples for *in vitro* postDLI experiments)

T cells responding to *in vitro* stimulation with the leukemia by the production of IFNγ were sorted one cell per well. As illustrated in figure 2, the T cells were isolated from the two patients 6-7 weeks after the application of DLI. The percentages of IFNγ secreting T cells in the purified T cell population from patient 6 after 16 hour of stimulation with primary CLL was 0.04% and with CLL-APC 0.22%. From patient 6, 23 proliferating clones out of 192 sorted T cells were obtained in response to primary CLL (plating efficiency 12%), and three of these clones exerted cytolytic activity against CLL-APC but not against primary CLL (data not shown). Using CLL-APC as stimulators, 148 T cell clones were isolated out of 1346 sorted T cells, resulting in a plating efficiency of 11%. As illustrated in Figure 3A, 13 out of these 148 proliferating clones showed specific lysis of CLL-APC (46% ± 14%, mean ± SD) and 8 of these CTL clones showed reactivity against primary CLL (24% ± 17%) in overnight ^{51}Cr assays indicating that they recognized CLL targets. IFNγ was produced by all of these cytotoxic clones in response to CLL and CLL-APC (255 ± 197pg/mL and 546 ± 375 pg/mL respectively, Figure 3B) whereas no production of IFNγ was found by randomly tested non-cytotoxic T cell clones. None of the CTL clones produced IL-4. All clones were CD8$^+$ except for two CD4$^+$ clones (clone 6.28 and 6.96). Three CTL clones (clones 6.15, 6.70 and 6.85) recognized PHA blasts of the patients (lysis 59%, 66% and 23% respectively). No reactivity with donor PHA blasts was observed.

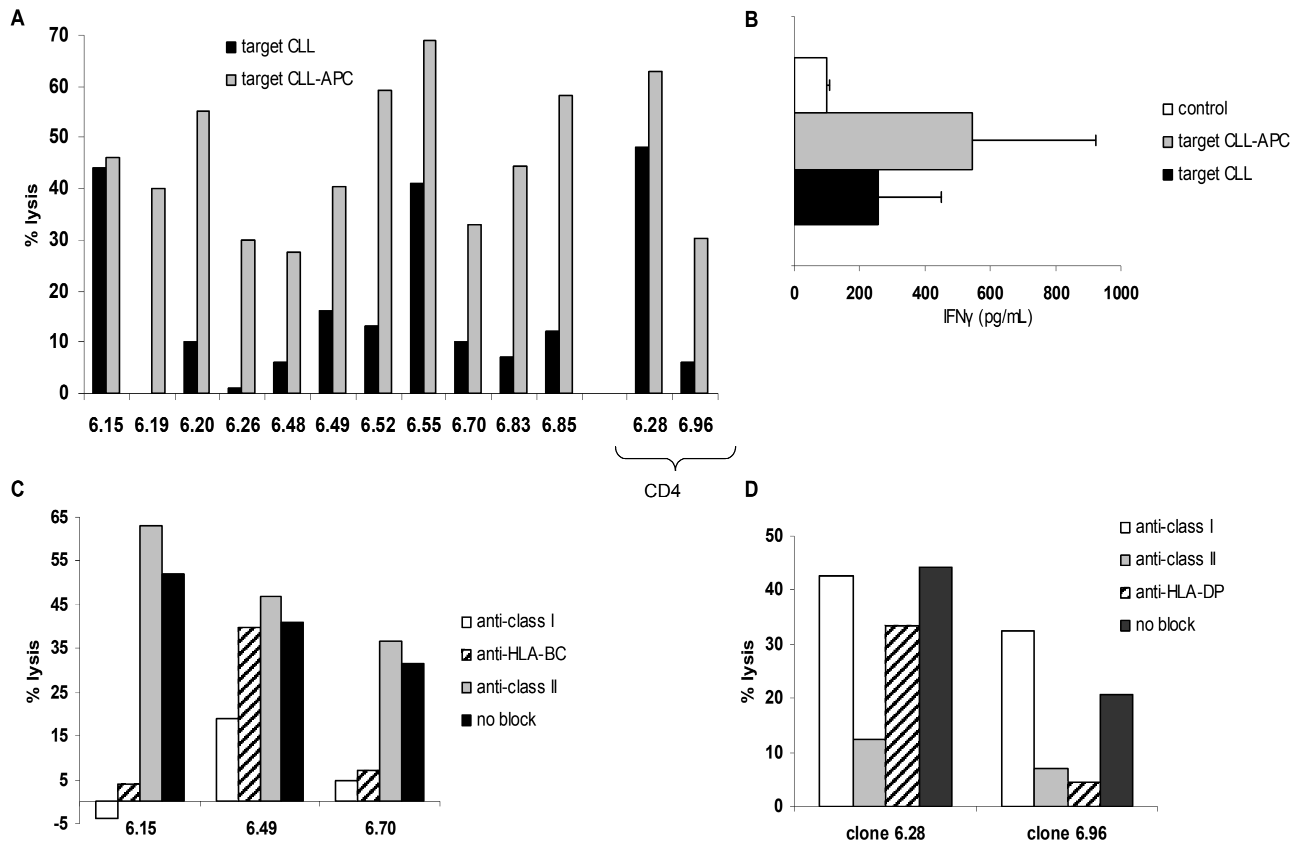

Figure 3. Characterization of CLL-reactive and mHag-specific CTL clones, derived from patient 6 six weeks after the application of DLI. (A) Cytolytic activity of CTL clones generated by stimulation of T cells, obtained from the patient six weeks after DLI with CLL-APC of the patient. All clones were $CD8^+$ except for the two $CD4^+$ clone 6.28 and clone 6.96. The percentage of lysis against different targets was measured in an overnight ^{51}Cr-assay at an E/T ratio of 10:1. (B) All cytotoxic clones (n=13) produced significant amounts of IFNγ in response to CLL and CLL-APC (255 ± 197 pg/mL and 546 ± 375 pg/mL, respectively). (C) HLA class I- and II-restricted recognition of the cytotoxicity was shown by blocking experiments using CLL-APC or PHA blasts of the patients (clone 6.15) as target at an E/T ratio of 10:1. Anti-HLA-BC MoAb blocked the recognition of clone 6.15 and 6.70 but not the cytotoxicity of clone 6.49. (D) Cytolytic activity of clone 6.28 and clone 6.96 was abrogated by anti-class II MoAb. The cytotoxicity of clone 6.96 could be blocked by anti-HLA-DP MoAb.

To further characterize the CTL clones obtained, blocking studies were performed. Cytolytic activity of three representative $CD8^+$ clones 6.15, 6.49 and 6.70 could be abrogated using the α-HLA class I MoAb (figure 3C) confirming HLA-restricted recognition. Additional experiments showed that α-HLA BC MoAb could block the recognition by clone 6.15 and 6.70 and not the cytotoxicity of clone 6.49 (figure 3C). A blocking study using α-HLA A2 MoAb (SN66.E3) showed HLA-A2-restricted recognition of clone 6.49 (data not shown). To further analyze and confirm the HLA-restricting molecule, clones 6.15, 6.49 and 6.70 were tested for reactivity against a panel of EBV-LCL from unrelated individuals that shared an HLA allele with the recipient. Clone 6.15 demonstrated HLA-B51-restricted recognition, clone 6.49 HLA-A2-restricted recognition and clone 6.70 exerted its reactivity in a HLA-B53-restricted way. The cytotoxicity of the $CD4^+$ clones 6.28 and 6.96 could be blocked by α-HLA class II MoAb (figure 3D). Cytolytic activity of clone 6.96 could be blocked by α-HLA DP MoAb (figure 3D). In patient

10 T cells obtained 6-7 weeks after the first, and 6-7 weeks after the second application of DLI were stimulated for 16 hours with primary CLL or CLL-APC in three independent experiments. The percentages of IFNγ secreting T cells in the purified T cell population were 0.07% to 0.11% in response to primary CLL and 0.09% to 0.16% in response to CLL-APC. After single cell per well sorting of the viable IFNγ-PE^+ cells, no proliferating clones were obtained in response to primary CLL and a total of 13 proliferating T cell clones were obtained using CLL-APC as stimulator cells. None of these T cell clones, recognized CLL-specific and/or patient-specific targets nor did they produce any IL-4 or IFNγ upon stimulation (data not shown). In summary, the experiments performed with the *in vivo* primed T cells from patient 6 showed that comparable percentages of proliferating T cell clones were obtained in response to primary CLL and CLL-APC. However, the stimulation with CLL-APC resulted in a more efficient isolation of CTL clones reactive to primary CLL, suggesting that primary CLL as weak immunogenic APC has limited capacity to activate CLL-reactive T cells. From patient 10 suffering from PD despite the administration of several doses of DLI, no CLL-reactive CTL clones could be obtained in response to primary CLL or CLL-APC.

***In vitro* generation of CLL-reactive CTL clones from the unprimed donors**

We have previously demonstrated that CLL-APC were immunogenic and capable of inducing mHag-specific and CLL-reactive T-cell responses in unprimed HLA-matched sibling donors (Hoogendoorn et al., Blood 2002, 100, 387abstract). To investigate whether a precursor CLL-reactive T-cell repertoire was present in the transplant donor of patient 10 despite the absence of a persistent clinical response to DLI in patient 10, a primary T-cell response against CLL-APC and primary CLL was generated. T cells derived from the specific donor were stimulated with CLL or CLL-APC. After 14 days of culture the T cells were specifically restimulated and responding T cells were selected on basis of their IFNγ production. Using CLL as stimulator cells, 67 proliferating clones could be tested for cytotoxicity resulting in one $CD4^+$ CTL clone with reactivity against CLL-APC (data not shown). After stimulation with CLL-APC 129 proliferating clones were obtained, reflecting a high plating efficiency of 44 %. Of these clones 35 (34 $CD8^+$ and one $CD4^+$ clone) showed cytotoxicity against CLL-specific and/or patient-specific targets. As illustrated In Figure 4A representative $CD8^+$ CTL clones and the $CD4^+$ CTL clone effectively lysed the primary CLL, the CLL-APC and PHA blasts from the patient and not donor PHA blasts in 4 hour and overnight ^{51}Cr assays indicating that the majority of these clones were mHag-specific. HLA-restricted recognition was demonstrated by blocking experiments (Figure 4B). Additional experiments demonstrated that several clones (e.g. clone 10.6, 10.9 and 10.37) had the same HLA-restriction element. The cytolytic activity of these clones could be abrogated with α-HLA-A3 antibodies (figure 4B). Other clones (clone 10.107 and clone 10.131) exerted their activity in a HLA-B35-restricted way (data not shown). In conclusion, we demonstrated the feasibility to generate a CLL-reactive mHag-specific CTL response from the stem cell donor of patient 10, illustrating the presence of precursor leukemia-reactive T cells in the unprimed donor. The T-cell responses could only be induced against CLL-APC indicating the limited APC function of the primary CLL which might in part underlie the absence of the clinical response.

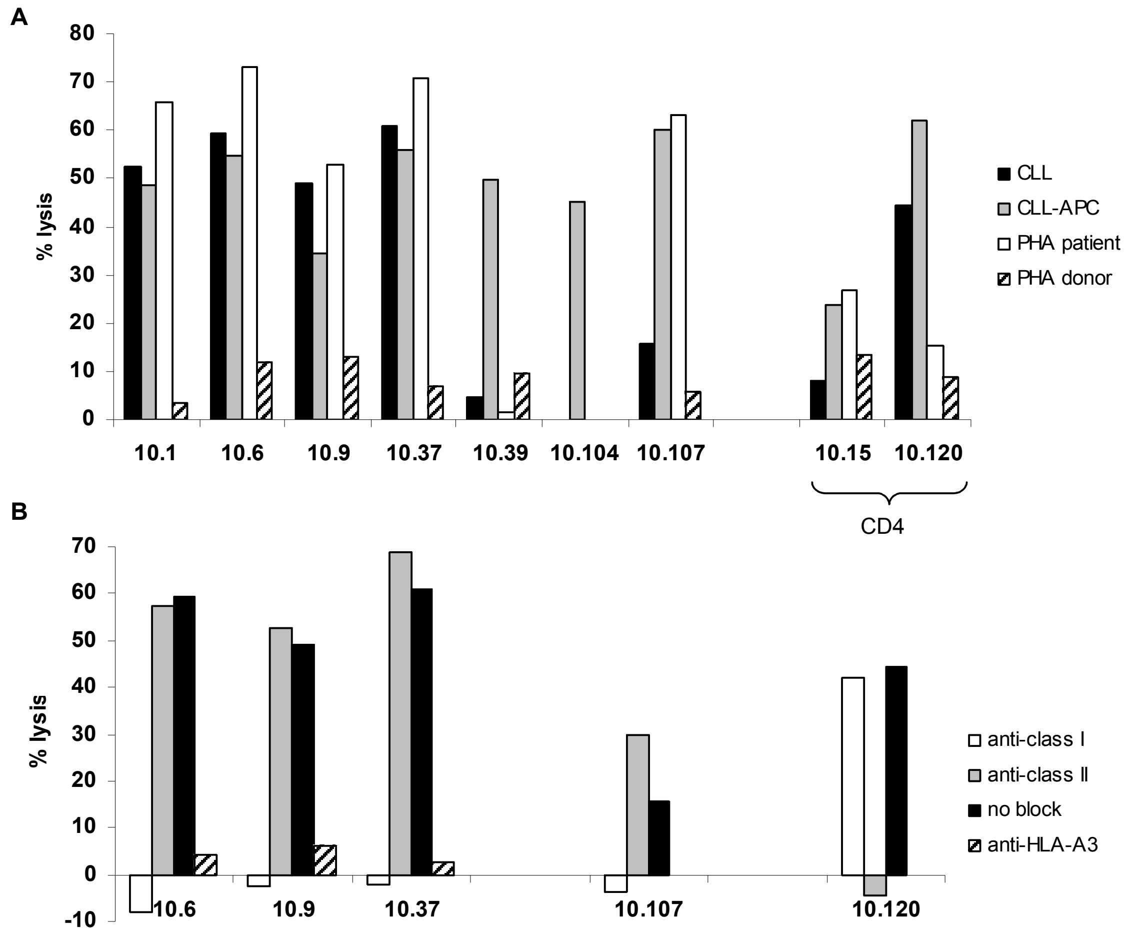

Figure 4. Cytotoxicity of CTL clones, derived from the unprimed donor of patient 10, using CLL-APC as stimulator cells. (A) Percentage lysis of CLL-specific targets and patient- and donor-derived PHA blasts in an overnight ^{51}Cr-assay at E/T ratio of 3:1. All clones were CD8^{+} except for the two CD4^{+} clones 10.15 and 10.120. (B) Blocking experiments confirmed HLA-restricted recognition by the CTL clones, and showed that the HLA-restricted element for clone 10.6, 10.9 and 10.37 was A3.

Discussion

Our study demonstrates that *in vitro* T-cell depletion using alemtuzumab "in the bag" and high doses of donor stem cells resulted in sustained donor engraftment and high percentages of donor chimerism after allogeneic SCT without causing significant GvHD. This T-cell depleted RIC regimen was well tolerated in this older patient population (median age, 56 years), and was associated with acceptable toxicity and no TRM after 3 months [19,20,22,28]. Nine patients had improvement of percentages of CLL cells in the BM after allogeneic SCT suggesting that alemtuzumab "in the bag" may exert its anti-CLL effect *in vivo* thus offering a window of 6 months after allogeneic SCT to allow postponed cellular adoptive therapy. This time frame may be necessary to replace the majority of the dendritic cells of the patient by donor dendritic cells thereby diminishing the occurrence of GvHD after DLI administration [21]. All patients were mixed chimeras and 11 out of 12 had persistent CLL cells in the BM 6 months after allogeneic SCT indicating that after the depletion of graft T cells by alemtuzumab, the application of

DLI is pivotal to achieve maximal disease responses and full donor chimerism. In our study DLI was projected to be administered in all patients. The sufficient high levels of donor chimerism and low incidence of GvHD after in-vitro T-cell depleted RIC allogeneic SCT was a prerequisite for this two-step approach.

Eleven out of 12 patients received DLI, and 6 out of these 11 patients could be evaluated after a long follow-up time post-allogeneic SCT (≥ 18 months) and post-DLI (≥ 12 months). Five patients converted to full donor chimerism after DLI. Four of these patients had an excellent and durable clinical response, clearly illustrating that CLL is susceptible to a GvL effect, and that GvL-mediated disease control is durable and has the potential to cure. Other patients experienced PD despite several administrations of DLI. To analyze the graft-versus-CLL effect and to elucidate discrepancies of clinical responses in patients subjected to DLI for persistent disease, we performed experiments using PB from patient 6 successfully treated with DLI and from patient 10, suffering from disease progression despite DLI. T cells reactive against primary CLL or the CLL-APC by producing IFNγ were isolated and cloned from the PB derived from the patients at the time of clinical response to DLI. We and others have demonstrated that this method enriches for tumor-reactive T cells, and allows characterization of the T-cell repertoire involved in the immune response against the original leukemia [24,29,30].

Our post-DLI experiments demonstrated that the stimulation of the *in vivo* primed T cells of patient 10 with primary CLL cells or CLL-APC did not result in the isolation of CLL-reactive CTL clones. The clinical course of patient 10 may be illustrative for patients with CLL, treated with a RIC allogeneic SCT in which persistent disease or relapses are frequently reported [19,20,30,31]. As demonstrated in figure 2B, after DLI donor chimerism initially gradually increased from 75% to 96% with concurrent very low percentages of CLL cells in the BM, but the initiated T-cell response was then apparently incapable of definitely eradicating the leukemia. These results suggest that the persistent primary CLL cells did not have the capacity to induce or further amplify a CLL-specific immune response. To further differentiate whether our observations in this patient can be explained by low immunogenicity of the CLL cells or whether precursor CLL-reactive and/or mHag-specific T cells were absent in the donor, a primary immune response was induced. Similar to results previously published [23], no CLL-reactive T-cell responses could be generated against primary CLL cells. As illustrated in figure 4, $CD8^+$ and $CD4^+$ CTL clones, reactive to CLL-specific targets could be isolated from the primary allogeneic T-cell response against CLL-APC. Several $CD8^+$ mHag-specific CTL clones, effectively killing CLL- and patient-derived targets were further analyzed, and were determined to be HLA-A3 (clone 10.6, 10.9 and 10.37) and HLA-B35 restricted (10.107 and 10.131). These results demonstrate that precursor CLL-reactive mHag-specific T cells present in the donor can not be adequately stimulated by the low immunogenic primary CLL cells. Thus, after allogeneic SCT and the application of DLI, the persistent or recurrent primary CLL cells may escape T-cell surveillance probably due to the lack of APC function, and this may result in the absence of a clinical response.

From PB derived from patient 6 six weeks after the application of DLI a diversity of CLL- and patient-reactive $CD4^+$ and $CD8^+$ CTL clones were isolated after *in vitro* stimulation with CLL-APC, and a few CTL clones reactive to CLL-APC were isolated after stimulation with primary CLL. Although stimulation with primary CLL cells did result in the isolation of a limited number of CLL-reactive T cells, a higher proportion of CLL-reactive T cells was induced to proliferate in response to CLL-APC resulting in a more effective isolation of these cells. As illustrated in figure 3, the CTL clones isolated in response to CLL-APC were probably directed against different mHags, and showed HLA-B51-(clone 6.15), HLA-B53-(clone 6.70) and HLA-A2-(clone 6.49) restricted recognition by the $CD8^+$ CTL clones, and HLA-DP-restricted recognition by a $CD4^+$ CTL clone (6.96). In accordance with our previous reports, this study confirms that T cells involved in efficient immune responses after the application of DLI are often directed against different distinct mHags [24,30]. Furthermore, these data suggest that the good clinical response after DLI in this patient is related to the presence of multiple CLL-reactive mHag-specific T cells. Our results provide direct evidence for the GvL reactivity, and illustrate the susceptibility of CLL cells to a GvL effect. However, our data also illustrate that the primary CLL cells may not efficiently stimulate the proliferation of the mHag-specific CLL-reactive T cells, even after *in vivo* priming. *In vivo* an adequate alloimmune response in this patient against patient-derived hematopoiesis including the primary CLL was observed, resulting in an excellent durable clinical response. The primary CLL cells from this patient were also not capable of inducing CLL-reactive T-cell responses *in vitro*. We hypothesize that the GvL effect in this patient is largely mediated by donor T cells, showing alloreactivity against mHag expressed on residual recipient APC, or engrafted donor APC efficiently cross-presenting alloantigens expressed on CLL cells.

In conclusion, in this study we illustrated the feasibility of *in vitro* T-cell depleted RIC allogeneic SCT followed by DLI in patients with advanced CLL. We characterized the GvL effect in a patient with a continuing CR and analyzed a patient with treatment failure both after the application of DLI. In the patient with progressive disease, although an initial partial response was suggested, the primary CLL cells ultimately escaped from T-cell mediated recognition. Experiments with *in vivo* primed T cells, isolated from that patient after DLI, illustrated the incapacity of these T cells of inducing an appropriate immune response against primary CLL and even against CLL-APC. These results showed that the inappropriate APC function of primary CLL might in part underlie the absence of a clinical response. We further demonstrated that precursor CLL-reactive T cells were present in the unprimed donor but these T cells could only be activated in response to CLL-APC. We hypothesized and provided evidence that the observed GvL effect in patient 6 was mediated by CLL-reactive mHag-specific T cells which were probably cross-primed by recipient- and/or donor APC. New cellular immunotherapeutic approaches to DLI necessary to improve specificity and efficacy of the GvL response, may be the *in vitro* generation of CLL-reactive T cell lines and clones from the donor using CLL-APC as stimulator cell [23]. The repetitive stimulation with CLL-APC may ultimately result in a relatively enrichment of leukemia-reactive T cells compare to GvHD-mediating T cells. Furthermore, by *in vitro* priming of CLL-reactive donor T cells against CLL-APC T-cell anergy towards primary CLL may be minimized.

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Chapter 6

Summary and General Discussion

Summary

Patients with chronic B-cell malignancies such as chronic lymphocytic leukemia (CLL) or mantle cell lymphoma (MCL), who have failed to respond to anthracycline- or fludarabine-based chemotherapy have a poor prognosis. For these patients new effective therapies with the potential to cure are needed. Prolonged remissions have been achieved with conventional allogeneic stem cell transplantation (SCT). Unfortunately, this procedure is hampered by a high treatment-related mortality (TRM) rate and thus only suitable for younger patients [1]. In patients treated with an allogeneic SCT long-term survival curves approach a plateau, and the achievement of a molecular remission is more likely than after autologous SCT, suggesting a graft-versus-leukemia (GvL) effect. Complete remissions after the administration of donor lymphocyte infusions (DLI) further provide evidence for susceptibility of CLL cells to a GvL effect [1-5] . Because the graft-versus-CLL effect is crucial for eradication of the disease, the intensity of the conditioning regimen may not be as important in CLL as in other diseases. In this regard, reduced intensity conditioning (RIC) regimens are being extensively investigated [3,6]. RIC regimen may reduce the TRM and extend the age limit of the procedure [6]. Donor-derived alloreactive T cells are not only responsible for GvL, but may also mediate graft-versus-host disease (GvHD) which accounts for considerable morbidity and mortality. The incorporation of alemtuzumab in the conditioning regimen to deplete recipient and donor T cells significantly reduces the risk for GvHD but may potentially impair the GvL effect. The early administration of sequential DLI after transplantation is then pivotal to achieve durable disease control [4,7-9]. Although DLI can be effective in reversing mixed chimerism in patients treated with a T-cell depleted RIC allogeneic SCT, it may not be sufficient to control relapsed or progressive disease [4,9]. One of the explanations for the low efficacy of DLI in CLL and MCL may be the lack of costimulatory signals on these malignant cells, resulting in an impaired T-cell activation and recognition. T cells recognize their targets through their antigen-specific T cell receptor (TCR) that recognizes peptides in the context of HLA molecules. For further T-cell activation and proliferation the expression of costimulatory and adhesion molecules on the stimulator cells are essential. In this thesis we investigated methods to modify primary CLL and MCL cells into an antigen-presenting cell (APC) phenotype capable of inducing an anti-leukemic T-cell response of donor T-cells *in vitro*. To further analyze graft-versus-CLL activity in patients treated with allogeneic SCT and DLI, T-cell responses after DLI were characterized.

In **chapter 2** we described the expression levels of several costimulatory and adhesion molecules on CLL cells of 14 patients and showed high expression of HLA class I and II, and CD40 but lack of expression of CD80, CD86 and CD83 and minimal expression of the adhesion molecules CD54 and CD58. To improve the immunogenicity of the CLL cells several cytokines were tested for their upregulating capacity. No significant upregulation of adhesion and costimulatory molecules with the cytokines or cytokine combinations was found. Since malignant B cells abundantly express toll-like receptor (TLR) 9 and since signalling through TLR can activates APC, CpG an agonist of TLR 9 was tested for its stimulatory capacity. CpG in combination with IL-4 induced an increased expression of costimulatory and adhesion molecules. However, CD40 activation by crosslinking using murine

fibroblasts transfected with human CD40L was superior in activating primary CLL cells and transformed these cells into characteristic APC phenotypes. The addition of IL-4 further enhanced expression levels. Time kinetic studies determined that 4 days of stimulation caused the strongest upregulation of costimulatory signals. After CD40 and IL-4 stimulation, the CLL cells were induced to produce significant amounts of IL-12. To test the allostimulatory capacity of the generated CLL-APC T cells from HLA class I-matched donors were repetitively stimulated with the primary CLL or CLL-APC. In contrast to primary CLL cells, CLL-APC as stimulator cells were capable of generating CLL-reactive cytotoxic T lymphocytes (CTL) lines and clones.

To investigate whether CD40 and IL-4 stimulated CLL cells are sufficiently immunogenic to initiate an adequate alloimmune response *in vitro* in a complete HLA-matched setting representing a clinical applicable transplantation model, primary induction of immune responses were performed using the generated CLL-APC as stimulator cells and using T cells from complete HLA-matched siblings. As described in **chapter 3**, only CLL-APC could stimulate precursor CLL-reactive donor-derived T cells to proliferate. In all three donor/patient pairs leukemia-reactive $CD8^+$ and/or $CD4^+$ CTL clones could be obtained, recognizing their targets in a MHC-restricted way. Further characterization of these clones revealed that primary CLL, CLL-APC as well as patient-derived targets such as phytohemagglutinin activated T cells (PHA blasts) and stable EBV transformed B cells (EBV-LCL) and no donor cells were recognized. Thus these clones were minor histocompatibility (mHag)-specific and the recognized mHag was not B lineage-restricted. Mhag are immunogenic alloantigens capable of eliciting an allogeneic T-cell response between HLA-matched individuals. For one $CD8^+$ CTL clone panel studies were performed, illustrating that the recognition of the targets by this clone was HLA-B8-restricted. These results showed the feasibility of CLL-APC to initiate CLL-reactive T-cell responses in HLA-matched settings thereby translating the results from **chapter 2** into a transplantation model mimicking clinical practice. Hence, based on these results, the application of *in vitro* generated CLL-reactive CTL clones as adoptive immunotherapy to treat disease recurrence after allogeneic SCT may be an effective potential curative approach.

In **chapter 4**, the modification of MCL cells into APC was analyzed. The MCL-specific cytokine IL-10 and B-cell specific cytokine IL-4, as well as CpG and CD40 activation were tested for their MCL-stimulating capacity. Ligation of CD40 on MCL cells was essential to upregulate costimulatory molecules and to induce the production of high amounts of IL-12. In response to these MCL-APC, from HLA class I-matched donors, high numbers of CTL clones could be generated capable of efficiently killing the MCL- and patient-specific targets and not donor-derived targets. Thus, the primary induction of MCL-reactive immune responses against MCL-APC was shown to be feasible.

In **chapter 5** we report the results of 12 patients with advanced CLL, treated with a T-cell depleted RIC allogeneic SCT, using alemtuzumab in the graft to deplete for donor and recipient T cells to reduce GvHD, followed by DLI. The administration of DLI was anticipated to promote GvL activity and achieve disease control. Using this transplantation protocol sustained donor engraftment without

GvHD prior to the administration of DLI was observed. It was shown that in some patients long durable remissions were obtained whereas in other patients persistent or progressive disease, despite the DLI was observed. To further characterize these differences in clinical response, *in vitro* experiments were performed using T cells, isolated from patients responding and non-responding to DLI and using T cells from the unprimed donor. The T cells were stimulated with the primary CLL or the CLL-APC. Enrichment of leukemia-reactive T cells was achieved using a new developed protocol, selecting IFN γ-producing T cells in response to the primary CLL or the CLL-APC. In accordance with data presented in chapter 2 and 3, we showed that primary CLL cells as stimulators could not activate *in vivo* primed CLL-reactive T cells. Only CLL-APC could induce CLL-reactive mHag-specific T-cell responses in the patient with a good clinical response, but not in the patient who failed to respond to DLI. In the unprimed donor from the nonresponding patient precursor CLL-reactive T cells could be activated in response to CLL-APC and CTL clones were obtained. These results demonstrated that precursor CLL-reactive T cells present in the donor could not be adequately stimulated by primary CLL illustrating the lack of APC function of the leukemic cells. The CLL-reactive T cells were therefore incapable of mounting an adequate immune response against residual CLL applying evidence for the clinical observations of continuous relapses after allogeneic SCT and the administration of DLI in patients suffering from CLL. From the patient with an excellent clinical response CLL-reactive mHag-specific T cell clones could be isolated in response to CLL-APC and not in response to primary CLL. We hypothesized that in this patient CLL-reactive T cells were probably stimulated by professional APC cells from donor or patient origin and that these alloreactive T cells had the capacity to definitely eradicate residual disease.

General Discussion

Several studies have shown that allogeneic SCT has the potential to cure patients with advanced B-cell malignancies. Survival curves of patients, treated with an allogeneic SCT show a plateau phase and complete molecular remissions have been reported after the application of DLI, suggesting the existence of GvL reactivity. Allogeneic SCT can be complicated by GvHD due to donor T cells in the graft, alloreactive to major or minor histocompatibility antigens (mHag). We and others have demonstrated that GvHD can be reduced by T-cell depletion of the stem cell graft but this also results in a higher incidence of relapse of the disease. The administration of donor T cells is then pivotal to mediate GvL activity and achieve disease control, but successful DLI is often associated with concurrent GvHD. Infusion of low numbers of DLI may reduce the incidence of GvHD while maintaining the GvL effect [7,10]. Ideally, selection, isolation and the application of donor T cells, that are specifically reactive with the malignant cells or with the recipient hematopoietic cells including leukemic cells may result in the separation of the beneficial GvL effect from the detrimental GvHD. In chapter 5 we reported the outcomes of 12 patients with CLL treated with an RIC allogeneic SCT followed by the infusions of escalating doses of DLI. In line with other reports we showed that some patients experienced an excellent clinical response, whereas other patients were unresponsive to DLI and suffered from continuous relapses [4,8,9]. *In vitro* experiments in an unresponsive patient, described in chapter 5, showed that CLL-reactive T-cell responses could be evoked by CD40 and IL-4 stimulated CLL (CLL-APC) cells and not by primary CLL in the unprimed donor. Experiments with T cells, isolated from that patient after DLI and thus donor T cells *in vivo* primed with primary CLL, illustrated the incapacity of these T cells of inducing an appropriate immune response against primary CLL and even against CLL-APC. These results showed that the inappropriate APC function of primary CLL might in part underlie the absence of a clinical response. Furthermore precursor CLL-reactive T cells might be silenced *in vivo* by primary CLL and could not be activated *ex vivo* by CLL-APC. In other patients excellent clinical responses were observed after allogeneic SCT and the application of DLI. In experiments using T cells isolated from a patient successfully treated with DLI, CLL-reactive T-cell responses could be evoked against CLL-APC but not against primary CLL suggesting that these leukemic-reactive T cells are probably *in vivo* stimulated by professional APC from the donor or the patient. These results illustrated that clinical responses to DLI are difficult to predict and that the efficacy of adoptive cellular immunotherapy in chronic B-cell malignancies needs to be improved.
An important reason for the limited GvL activity after DLI in CLL and/or MCL might be the inability of these malignant cells to properly function as APC and thus initiate an adequate T-cell response. In the chapters 2 and 4 several methods were analyzed to improve the immunogenicity of the neoplastic B cells. Primary CLL and MCL cells could be phenotypically and morphologically transformed into APC with high expression of costimulatory and adhesion molecules by CD40 stimulation using murine fibroblast cells transfected with human CD40L. The malignant APCs produced significant amounts of IL-12, a pivotal cytokine for T cell activation and neglectable amounts of IL-10. Since the transfected fibroblasts are not clinical grade, modification of the leukemic cells into APC using clinical grade available cytokines or microbial components, capable of triggering TLRs on the malignant cells, was

investigated. The CD40/CD40L pathway appeared to be critical for the stimulation of CLL and MCL cells. Therefore, effort should be put into the development of CD40 system under good manufacturing practice (GMP) conditions, permitting clinical application of the obtained malignant APCs. As alternative for the transfected murine fibroblasts, several candidates are under development such as anti-CD40 beads or humanized trimeric soluble CD40L [11,12]. Preliminary results showed that these products are inferior to the CD40L transduced fibroblasts. Autologous T cells activated to express CD40L can after engagement of CD40 on the leukemic cell induce upregulation of costimulatory molecules. Another approach to enhance the immunogenicity of primary CLL cells can be the direct gene transfer of CD40L into CLL cells via adenovirus vectors or the *in vitro* infection of CLL cells with vectors encoding for the costimulatory molecules [13,14].

The stimulatory capacity of the malignant APCs to induce tumor-reactive T-cell responses using HLA-matched donors was analyzed in chapter 2, 3 and 4. In contrast to the primary malignant B cells, CLL-APC and MCL-APC induced vigorous expansion of tumor-reactive $CD8^{+}$T cell lines in HLA class I-matched donors. Single cell per well sorting resulted in CLL-reactive and MCL-reactive T cell clones. These clones recognized also other patient-derived lymphohematopoietic targets and not donor cells indicating recognition of tumor-associated mHags. In chapter 4 we showed that the obtained CLL-APC were sufficient immunogenic to initiate an adequate alloimmune response *in vitro* in a complete HLA-matched setting representing a clinical applicable transplantation model. These results illustrate that by modifying the B-cell malignancies into appropriate malignant APCs, T-cell anergy can be overcome. Our method of repetitive *in vitro* stimulation of donor T cells with the generated malignant APC and the extensive culture period needed for the enrichment, selection, and expansion of leukemia-reactive T cells is time consuming, inefficient and may have lead to the selection of a population of T cells with a limited residual capacity to survive and expand *in vivo*. We have demonstrated the possibility to early detect and isolate leukemia-reactive T cells in peripheral blood of patient treated with DLI based on the production of interferon (IFN) γ in response to the leukemic cells [15]. This procedure using the IFNγ-secretion assay appeared to select for a population of T cells containing high frequency of leukemia-reactive T cells with direct cytolytic activity against hematopoietic cells from the patient. As illustrated in chapter 5, using the IFNγ-secretion assay and CLL-APC as stimulator cells, CLL-reactive T cells could be isolated from patients, treated with DLI. Recently, we adapted this procedure for the early detection and isolation of leukemia-reactive T cells from primary immune responses using phenotypically appropriate APC, generated from the primary leukemia. HLA-matched donor T cells were cocultured with the leukemic APC for two weeks under mild stimulatory conditions to enrich for the leukemia-reactive T cells. These T cells were then specifically restimulated with the leukemic APC resulting in a synchronized IFNγ by the responding T cells[16]. The feasibility of this strategy was illustrated by the experiments in chapter 5 demonstrating the generation of CLL-reactive T cells from an unprimed donor. Furthermore our data showed that the *in vitro* culture periods could be significantly shortened using this method.

As described in the chapters 2, 3 and 4, the CLL-reactive and MCL-reactive CTL clones were further characterized for their specific recognition of leukemic cells. The obtained CTL clones were cytotoxic against tumor-specific targets as well as patient-derived targets such as PHA stimulated lymphocytes and EBV-transformed B-cells patient- and not donor-specific targets, indicating that these clones were mHag-specific. Based on these experiments, we could exclude that the recognized mHags were B-cell lineage restricted. Blocking studies confirmed the HLA restricted recognition and in chapter 3 the restriction allele of a CTL clone could be determined using panel studies. In this thesis we illustrated the capacity of malignant APC of eliciting CLL- and MCL-reactive mHAg-specific T-cell responses *in vitro*, thereby showing the immunotherapeutic potential of such approach. However, the clinical outcome of T-cell responses against mHags may be largely determined by the tissue distribution of these mHags. T-cell responses against mHags that are selectively expressed in hematopoietic cells, including malignant cells such as HA-1, HA-2, BCL2A1, HB-1, endothelial-cell growth factor-1 (ECGF1) or PANE1 but not widely expressed in non-hematopoietic tissues may be preferentially associated with GvL activity [17-23]. Molecular characterization studies of human mHag showed that most non-sex-linked mHags are created by single nucleotide polymorphisms in the coding sequences of cellular genes. Since such polymorphisms are frequently encountered in the human genome, one might suggest that the total number of human mHags is likely to be quite large. Unfortunately, only a small number of mHags with the requisite tissue distribution to evoke a selective GvL response has been identified [19,21,22]. The allele frequency and the MHC restriction of the identified hematopoietic-restricted mHag further limit the clinical application. The identification of novel hematopoiesis-restricted mHags should have the highest priority. A strategy to partially circumvent these problems may be the retroviral transduction of T cells with T cell receptors that recognize hematopoiesis-resticted mHag. Unfortunately, this transduction technique is yet not clinical grade available [24]. Recently, a promising novel HLA-A3-restricted mHag, which is preferentially highly expressed in resting B-lymphoid cells including CLL cells, has been identified [20]. This mHag might be an attractive target for adoptive immunotherapy but the clinical implications may be limited due to infrequent donor/recipient disparity.

We and others have previously shown that low numbers of non-selected donor T cells, infused 6 months after an allogeneic SCT may cause minimal GvHD while GvL activity is achieved [7,10]. An explanation may be the replacement of recipients APC by APC of donor origin thereby diminishing the likelihood of development of GvHD caused by specific recognition of professional APC [25]. Once recipient hematopoiesis and the professional APC of the recipient are completely replaced by donor hematopoiesis, relapsing leukemic cells may be the only allogeneic trigger to activate alloreactive donor T cells. However, as illustrated in experiments performed in chapter 5, despite the present of a CLL-reactive mHag-specific T-cell repertoire in the donor, the naïve CLL cells were not recognized by these T cells probably due to low immunogenicity of the leukemic cells. Next, we demonstrated that the inability of the precursor CLL-reactive T cells to proliferate in response to the primary CLL cells can be overcome by the repetitive *in vitro* stimulation of these T cells against CLL-APC. We now hypothesize that these *in vitro* generated leukemia-reactive T cells may eradicate residual CLL cells *in*

vivo and may mediate only minimal or no GvHD. The repetitive stimulation with CLL-APC may ultimately result in a relatively enrichment of leukemia-reactive T cells compare to GvHD-mediating T cells. Furthermore by *in vitro* priming of CLL-reactive donor T cells against CLL-APC, T-cell anergy towards primary CLL may be minimized. In case of minimal residual disease this highly specific cellular immunotherapy may be appropriate to eliminate persistent tumor cells. However, for progressive disease after allogeneic SCT this intervention may not be sufficient and higher T cell doses may be necessary to increase the efficacy. In this setting several strategies to limit the occurrence of severe GvHD while preserving GvL activity can be considered. High doses of specified T cells or low dose DLI in combination with leukemia-reactive T cells can be administered. In addition, $CD8^+$ alloreactive T-cells are considered to be main effectors of GvHD and may not be necessary for the GvL reactivity [26]. Non-selected $CD4^+$T cells as DLI may exert some cytolytic activity against the neoplastic B cells and may in combination with *in vitro* generated tumor-reactive CTL provide activating cytokines and help for the cytotoxic T cells. Alternatively, the generation of purified $CD4^+$ leukemia-reactive cytotoxic T cells using the IFNγ capture assay may also be feasible.

Another immune-based strategy bypassing the risk for GvHD may be the induction of tumor-antigen-specific T-cell responses. Several tumor-associated antigens such as the tumor-specific idiotype, fibromodulin, murine double minute 2 (MDM2), Survivin or KW-13 can be expressed in CLL and MCL cells [27-32]. $CD34^+$-derived and/or $CD14^+$-derived APC from donor origin may be pulsed with the peptides, encoding for the tumor-antigens. Hence, antigen-specific donor T-cell responses can be induced and may be infused in the patient. However, probably due to low avidity of the T cell receptor for the specific tumor-antigen, these induced T-cell responses are often not robust and do not result in a powerful antileukemic effect. Furthermore tumor-antigen expression is variable among on the malignant cells, the tumor cells can escape recognition by specific T cells by downregulating the antigens or selection of leukemic cells lacking tumor-antigen expression may occur. Finally these procedures require extensive *in vitro* manipulation, are time consuming and are not clinical grade available yet. Vaccine strategies to induce leukemia-reactive T-cell responses *in vivo* are subject of many studies [13,33,34]. In autologus setting vaccinations with retroviral CD40L-transduced CLL cells showed an effective T-cell response against autologous leukemic cells but these T-cell responses were transient and no memory response could be evoked. Antileukemic vaccines composed of the leukemic blast fused with skin fibroblasts transduced to express human CD40L and IL-2, applied after allogeneic SCT was shown to be feasible without inducing GvHD [34]. Leukemia-reactive immune responses with a clinical response were observed. Several vaccine-based strategies in patients treated with a RIC allogeneic SCT for advanced chronic B-cell malignancies, using our *in vitro* generated CLL-APC or MCL-APC as vaccines can be developed. Because vaccination of the donor using vaccines composed of the malignant cells of the patients is unethical, the vaccines should be applied to the patient after the RIC allogeneic SCT. Most clinical trials with cancer vaccines conducted in patients with bulky or progressive disease are disappointing, suggesting that such a strategy reduces the likelihood of observing prolonged anti-tumor T-cell responses. According these observations, it may be therefore preferable to immunize patients with only minimal residual disease

after allogeneic SCT. Our T-cell depleted conditioning regimen followed by the postponed administration of DLI has the advantage that no immunosuppressive agents which may potentially impair the T-cell compartment are applied to the patient. However, due to prolonged T-cell suppression by the conditioning regimen vaccination of the patient should be performed concomitant with or shortly after the application of DLI. For the implementation of such a protocol, it is pivotal that disease control after allogeneic SCT is achieved for a longer period, thereby enabling the induction of efficient anti-leukemia T-cell responses through vaccination and DLI. Once complete remissions are obtained, vaccine boosts to induce re-expansion of the memory T-cell compartment may be useful. Whether such vaccine-based strategies using the *in vitro* generated malignant APC in combination with adoptive cellular therapy are ultimately capable of definitely eradicating minimal residual disease should be subject of future fase II and fase III studies.

In conclusion, in this thesis we illustrated both the curative potential of graft-versus-CLL activity by alloreactive T cells present in the allograft and/or in the DLI and the necessity to improve the efficacy of cellular adoptive immunotherapy because relapses of the disease after allogeneic SCT were frequently encountered. We showed the feasibility to transform primary CLL and MCL cells into malignant professional APC, capable of producing significant amounts of IL-12. The allostimulatory capacity of these APC was demonstrated by the induction of vigourous CLL- and MCL-reactive T-cell responses. The tumor-derived APC were sufficient immunogenic to evoke tumor-reactive CTL responses in HLA-matched sibling donors. Further characterization showed that these CTL were mHag-specific. The development of a clinical grade CD40 system is now pivotal to allow clinical application of these CTL lines. The further implementation of more sophisticated T cell selection techniques may result in the application of this highly specific, and probably highly effective adoptve cellular immunotherapy in the context of allogeneic SCT in patients with advanced chronic B-cell malignancies.

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Chapter 7

Nederlandse Samenvatting

Chronische B-cel leukemie

In het beenmerg bevinden zich hematopoietische stamcellen die de continue aanmaak van bloed- en afweercellen, ook wel hematopoiese, verzorgen. Bij maligne ontaarding van de hematopoietische stamcel kan de diagnose leukemie gesteld worden. Afhankelijk van de ontwikkelingsrichting, het ontwikkelingsstadium, waarin de maligne cel zich bevindt en de snelheid van deling van de kwaadaardige cel, kan leukemie verder geclassificeerd worden. Bij acute leukemie is er meestal sprake van een snelle proliferatie van de leukemiecellen in bloed en beenmerg met daarbij verdringing van de normale hematopoiese. Chronische vormen van leukemie, zoals chronische myeloïde leukemie (CML) en chronische lymfatische leukemie (CLL) kunnen zich in eerste instantie indolent gedragen. Dit proefschrift gaat over chronische leukemie waarbij de maligne kloon ontstaan is uit de rijpere B-cel lymfocyt.

Chronische lymfatische leukemie

Chronische lymfatische leukemie (CLL) is de meest voorkomende leukemie bij volwassenen en komt voornamelijk op oudere leeftijd voor. Deze chronische ziekte wordt gekarakteriseerd door klonale proliferatie en opeenhoping van maligne B-cellen in bloed, beenmerg en lymfeklieren. Dit kan leiden tot lymfadenopathie, splenomegalie, cytopenieën en een verhoogde gevoeligheid voor infecties. De ziekte gedraagt zich heterogeen. Een groot percentage van de patiënten met CLL is en blijft asymptomatisch, behoeft geen behandeling en heeft een goede prognose. In tegenstelling tot patiënten die bij presentatie symptomatisch zijn. Zij hebben ondanks behandeling met chemotherapie een snel progressieve ziekte. Met behulp van nieuwe moleculaire en cytogenetische technieken is het momenteel mogelijk om bij het stellen van de diagnose een inschatting te maken ten aanzien van de agressiviteit van de ziekte.

Ondanks dat het beschikbaar therapeutische arsenaal tegen CLL is uitgebreid met zeer effectieve chemotherapie en monoklonale antistof therapie, heeft dit in deze patiëntencategorie nog niet geleid tot genezing van de ziekte. Een nieuwe veelbelovende behandelmodaliteit die mogelijk kan leiden tot curatie van de CLL is een stamceltransplantatie (SCT) met stamcellen afkomstig van een gezonde donor (allogene SCT).

Mantel cel lymfoom

Mantel cel lymfoom (MCL) is een agressieve en ongewone vorm van non-Hodgkin lymfoom (NHL), die vooral optreedt bij mannen van middelbare leeftijd. Door een chromosomale translocatie (t(11;14)) van een B-cel die zich in de mantel van de lymfklier bevindt, ontstaat een sterk toegenomen celproliferatie met als eindresultaat een maligne ontaarding. Bij diagnose is er meestal sprake van uitgebreide ziekte met gegeneraliseerde lymfadenopathie. Veelvuldig is de ziekte leukemisch met uitgebreide accumulatie van maligne mantelcellen in bloed en beenmerg.De ziekte reageert initieel vrijwel altijd goed op agressieve chemotherapie. De ziekte keert echter bij vrijwel iedereen terug, met het

overlijden van de patiënt als resultaat. Om de overleving te verbeteren wordt nu bij de conventionele chemokuren specifieke monoklonale antistoftherapie in de vorm van rituximab gegeven. Als consolidatie behandeling wordt vervolgens een hoge dosis chemotherapie gegeven gevolgd door een autologe SCT. Deze intensieve therapie heeft geleid tot betere ziekte-vrije overleving, maar niet tot genezing. Allogene SCT wordt momenteel onderzocht als curatieve behandelingsoptie voor patiënten met MCL.

Cellulaire immuunreacties tegen CLL en MCL

Ondanks het feit dat CLL en MCL cellen rond circuleren in het bloed en voortdurend in contact zouden moeten komen met T cellen vindt er kennelijk geen adequate immuunreactie plaats en ontsnappen zij aan de specifieke afweer. Het ontbreken van een efficiënte T cel respons zou verklaard kunnen worden door het feit dat CLL en MCL cellen niet goed functioneren als antigeen presenterende cellen (APC). Zij ontberen expressie van costimulatoire moleculen op hun celoppervlak die noodzakelijk zijn voor T cel activatie. Verder worden ook geen relevante immuunresponsen tegen tumor-specifieke antigenen geobserveerd ondanks de aanwezigheid van die antigenen op de CLL cel. Aangezien in autologe setting immunotherapie tegen CLL en MCL niet succesvol is, kan het aantrekkelijk zijn om een compleet nieuw T cel repertoire te introduceren door middel van een allogene SCT.

Allogene stamceltransplantatie

Bij allogene SCT wordt de hematopoiese van de patiënt vervangen door donor hematopoiese. Om allogene donor stamcellen te laten prolifereren in de patiënt en om afstoting van het transplantaat te voorkomen dienen de T cellen van de patiënt op het moment van transplantatie geëradiceerd te worden middels een vorm van myeloablatieve behandeling met chemotherapie en/of totale lichaamsbestraling. Hoewel deze conditionering van de patiënt voor allogene SCT zeker een leukemie reducerend effect heeft, worden de immuunreacties die optreden na de allogene transplantaties, beschouwd als de belangrijkste oorzaak voor het curatieve effect van een allogene SCT. Indien in het stamceltransplantaat naast stamcellen ook donor T cellen aanwezig zijn, kunnen deze donor T cellen weefsels van de patiënt als lichaamsvreemd beschouwen en aanvallen. Deze immuunreactie kan ernstige schade geven aan huid en organen en staat bekend als graft-versus-host disease (GvHD). GvHD kan grotendeels voorkomen worden door de T cellen uit het transplantaat te halen (T cel depletie) voorafgaand aan de toediening. T cel gedepleteerde allogene SCT leidt echter tot een grotere kans op terugkeer van de leukemie. De T cellen in het transplantaat zijn dus blijkbaar in staat ook de leukemiecellen te herkennen die na de chemotherapie en bestraling nog aanwezig kunnen zijn. Deze gunstige reactiviteit van donor T cellen tegen leukemiecellen wordt graft-versus-leukemia (GvL) reactiviteit genoemd, en is waarschijnlijk het belangrijkste mechanisme voor het curatieve effect van een allogene SCT.

Cellulaire immunotherapie na allogene stamceltransplantatie

Veel onderzoek vindt plaats om enerzijds de toxiciteit en morbiditeit van GvHD na transplantatie te beperken en anderzijds de GvL-reactiviteit te behouden of verder te maximaliseren. Veel

transplantatie centra gebruiken protocollen waarbij donor T cellen bij het transplantaat worden toegediend teneinde optimale GvL-reactiviteit te verkrijgen met als prijs veel GvHD die vervolgens onderdrukt wordt met krachtige afweeronderdrukkende medicatie. Een alternatieve benadering bestaat uit het toedienen van een T cel gedepleteerd transplantaat na voorbehandeling van de patiënt waarbij de nadruk ligt op het vestigen van donor hematopoiese in de patiënt. Vervolgens kunnen rechtstreeks van de donor verkregen T cellen worden toegediend om de leukemiecellen te vernietigen. Deze vorm van cellulaire immunotherapie met allogene SCT als platform staat bekend als donor lymfocyten infusie (DLI). Na DLI kan echter ook GvHD optreden door alloreactiviteit van ongeselecteerde donor T cellen. Het selecteren en infunderen van donor T cellen die in staat zijn met name de leukemiecellen te herkennen en niet de normale weefsels van de patiënt, zou kunnen leiden tot optimale GvL-reactiviteit zonder het optreden van GvHD.

De immuunreactiviteit na allogene SCT kan worden veroorzaakt door de aanwezigheid van verschillen in HLA moleculen tussen donor en patiënt. Aangezien er een duidelijke correlatie tussen de immuunreacties en de mate van HLA verschillen tussen donor en patiënt is, wordt er bij allogene SCT naar gestreefd een HLA-identieke donor te vinden. Desondanks kunnen na HLA-identieke allogene SCT immuunreacties optreden tussen donor en patiënt. Dit wordt veroorzaakt door donor T cellen die minor histocompatibility antigenen (mHag), gebonden aan HLA moleculen, van de patiënt herkennen. Bij een HLA-identieke SCT kunnen de immunogene mHag dus verschillend tot expressie komen zowel als in patiënt en donor. De weefseldistributie en expressie van de mHag waartegen de T cel reactie is gericht, kan variëren. Sommige mHag komen op vrijwel alle cellen tot expressie. Een donor T-cel respons hiertegen zal dus zowel GvHD als GvL-reactiviteit kunnen induceren. Een ander type mHag komt alleen tot expressie op hematopoietische cellen inclusief leukemiecellen. Indien dit antigeen het doelwit is van de donor T cel kan dit leiden tot een GvL respons zonder het optreden van GvHD. Er is recent een mHag geïdentificeerd die selectief tot expressie komt op de normale en maligne B cel. Dit zou dus een aantrekkelijk doelwit kunnen zijn voor immunotherapie na allogene SCT.

Allogene stamceltransplantatie in patiënten met CLL of MCL

Patiënten met CLL of MCL die niet meer reageren op chemotherapie al dan niet in combinatie met monoklonale antistof therapie hebben een slechte prognose en zouden in aanmerking kunnen komen voor een allogene SC. De kans op terugkeer van ziekte na allogene SCT is kleiner dan na een autologe SCT en langdurige follow-up heeft laten zien dat er kans is op genezing. Dit betekent dus dat de CLL en MCL cellen target kunnen zijn van GvL-reactiviteit. Allogene SCT met standaard myeloablatieve conditionering middels totale lichaamsbestraling en hoge dosis chemotherapie leidt echter in deze oudere patiëntengroep tot hoge toxiciteit hetgeen resulteert in zeer hoge behandelingsgerelateerde mortaliteit. Met behulp van minder intensieve conditionering is het mogelijk gebleken de behandelingsgerelateerde mortaliteit sterk te reduceren. Hierdoor kunnen ook oudere patiënten in aanmerking komen voor deze in opzet curatieve therapie. Daarnaast heeft de incorporatie van alemtuzumab, een monoklonaal middel dat de donor T cellen in het transplantaat voor toediening

vernietigt, in het transplantatieprotocol geleid tot een aanzienlijke reductie van GvHD en dus morbiditeit. Zoals eerder weergegeven dient in dit transplantatieprotocol dan wel na allogene SCT cellulaire immunotherapie in de vorm van DLI gegeven te worden om GvL-reactiviteit te induceren en persisterende ziekte zo te bestrijden. Ondanks het feit dat er aanwijzingen zijn dat CLL en MCL gevoelig zijn voor GvL-reactiviteit na de toediening van DLI, laten de meeste studies zien dat de ziekte bij een groot percentage van de patiënten recidiveert. Om in deze patiëntengroep controle over de ziekte te bereiken, is het noodzakelijk om hogere doseringen DLI te infunderen met als gevolg aanzienlijke GvHD. Strategieën die de specificiteit van de immuunrespons tegen CLL en MCL verbeteren en resulteren in het versterken van de GvL-reactiviteit met daarbij reductie van GvHD zijn essentieel om te komen tot betere uitkomsten in deze patiëntengroep.

Dit proefschrift

De introductie van minder toxische conditioneringschemata (reduced intensity conditioning (RIC)) als voorbereiding op een allogene SCT heeft geleid tot sterk gedaalde behandelingsgerelateerde mortaliteit. Dit maakt het nu zinvol om RIC allogene SCT als potentiële curatieve behandeling aan te bieden aan patiënten met chronische B-cel leukemie die onvoldoende gereageerd hebben op de conventionele behandelingen. Echter, na allogene SCT en ondanks toediening van DLI wordt bij veel patiënten recidiverende ziekte geconstateerd. De maligne B cellen zijn schijnbaar in staat om te ontkomen aan de donor T-cel gemediëerde reactiviteit. Verder zijn de herhaaldelijk toediening van steeds hogere doseringen DLI noodzakelijk om persisterende ziekte na allogene SCT te behandelen hetgeen resulteert in aanzienlijke morbiditeit door GvHD. De verminderde T-cel herkenning na transplantatie zou kunnen worden verklaard door het ontbreken van expressie van belangrijke costimulatoire en adhesie moleculen. In dit proefschrift zijn de APC functie van de CLL en MCL cellen bestudeerd en is geanalyseerd wat de mogelijkheden zijn om deze maligne B cellen te transformeren in professionele APC cellen. Vervolgens is onderzocht in hoeverre deze maligne APC cellen in staat waren een T-cel respons tegen de CLL of MCL te induceren met donor T cellen afkomstig van zowel onverwante HLA-gematchte als verwante HLA-identieke donoren. In dit proefschrift zijn verder T-cel responsen bestudeerd bij patiënten met CLL die behandeld zijn met een T-cel gedepleteerde RIC allogene SCT gevolgd door cellulaire immunotherapie middels DLI.

Transformatie van CLL cellen in maligne antigeen presenterende cellen

In **hoofdstuk 2** worden de expressieniveaus van verschillende costimulatoire en adhesie moleculen op CLL cellen van 14 verschillende patiënten beschreven. CLL cellen hadden een hoge expressie van HLA klasse I, II en het costimulatoir molecuul CD40 op hun celmembraan. Expressie van de belangrijke costimulatoire moleculen CD80, CD86 en CD83 ontbrak echter volledig. Ook adhesie moleculen kwamen minimaal tot expressie. CLL cellen kunnen daardoor, ondanks dat het van oorsprong B cellen zijn, slecht functioneren als APC cellen. Om de APC functie te verbeteren, werd geanalyseerd in hoeverre bepaalde stimulerende cytokines in staat waren het expressieniveau van de costimulatoire moleculen te verhogen. Dit was niet succesvol. Aangezien normale maar ook maligne B cellen bepaalde receptoren (toll-like receptor) op hun celmembraan hebben die na activatie de APC functie van de B cel kan verbeteren, werd stimulatie van die toll-like receptor getest middels zijn agonist CpG. CpG in combinatie met het cytokine IL-4 resulteerde in enige opregulatie maar nog steeds geringe expressie van de belangrijkste costimulatoire en adhesie moleculen. Echter, stimulatie van het CD40 molecuul op de CLL cel met zijn stimulator CD40 ligand (CD40L) resulteerde in superieure activatie van de CLL cel. Dit gebeurde door gebruik te maken van muizefibroblasten die middels transfectie het humane CD40L hoog tot expressie hebben. Het toevoegen van IL-4 maximaliseerde de expressie van alle costimulatoire moleculen en adhesie moleculen. Na vier dagen van CD40 stimulatie in aanwezigheid van IL-4 was de expressie het hoogst en veranderde de morfologie van de CLL cellen in dendritisch-achtige cellen. Deze maligne APC cellen bleken functioneel in staat om het belangrijke immunostimulatoire cytokine IL-12 te produceren. Samengevat

kon geconcludeerd worden dat stimulatie van de CD40-receptor op de CLL cel in de aanwezigheid van IL-4 voor een periode van vier dagen resulteerde in transformatie van de CLL cel in een morfologische en fenotypische karakteristieke CLL-APC cel die de capaciteit had om significante hoeveelheden IL-12 te produceren.

Inductie van CLL-reactieve T cel responsen

Door donor T cellen *in vitro* te stimuleren met gemanipuleerde leukemiecellen van de patiënt kunnen populaties van zogenaamde cytotoxische T lymfocyten (CTL) gegenereerd worden die preferentieel leukemie-reactief zijn. Het toedienen van *in vitro* gegenereerde en geëxpandeerde leukemie-reactieve CTL lijnen aan de patiënt na allogene SCT zou kunnen leiden tot een versterkte GvL-reactiviteit en mogelijk verminderde GvHD. De proof of principle is aangetoond bij een patiënt met CML in geaccelereerde fase, die na toediening van CML-reactieve donor T cellen na allogene SCT in een voortdurend complete respons bleef. In hoeverre deze behandelstrategie ook bij patiënten met CLL mogelijk is, was onderwerp van studie in **hoofdstuk 2 en 3**. Allereerst werd onderzocht of de primaire ongemanipuleerde CLL cellen en/of de gegeneerde CLL APC cellen zoals beschreven in hoofdstuk 2 in staat waren om donor T cellen afkomstig van onverwante HLA klasse I-gematchte donoren te stimuleren. Aangezien er een HLA klasse II mismatch bestond, vond bij de inductie van de T-cel respons depletie plaats van de $CD4^+$ donor T cellen. In alle drie onderzochte donor/patiënten koppels konden primaire CLL cellen als stimulator cellen de donor T cellen niet activeren. Stimulatie van de donor T cellen met de CLL-APC cellen leidde echter tot sterke proliferatie van die T cellen in alle onderzochte koppels. De gegeneerde T cel lijnen vertoonde cytotoxie tegen de primaire CLL en de CLL APC cellen gemeten in een standaard ^{51}Cr release assay. De cytotoxie kon volledig geblokkeerd worden door toevoeging van antistoffen tegen HLA klasse I aangevend dat herkenning HLA gerestricteerd was. In aanvullende experimenten in een donor/patiënten paar werden uit een cytotoxische T cellijn door middel van limiting dilution CTL klonen verkregen. Deze CTL klonen herkenden naast de primaire CLL en de CLL-APC ook andere patiënt-afkomstige targets en herkenden niet targets afkomstig van de donor. Deze resultaten gaven aan dat in tegenstelling tot de primaire CLL CLL-APC als stimulator cellen in staat zijn om CLL-reactive CTL lijnen en klonen te genereren uit onverwante HLA klasse I-gematchte donoren. Om tot klinische implementatie van deze immunotherapische strategie te kunnen komen, was het vervolgens noodzakelijk om te onderzoeken in hoeverre het induceren van CLL-reactieve T-cel responsen in complete HLA-gematchte familiedonoren mogelijk was.

In **hoofdstuk 3** werd gedemonstreerd dat ook in HLA-indentieke setting het induceren van CLL-reactieve T-cel responsen door gebruik te maken van de stimulerende capaciteit van de gegenereerde CLL APC haalbaar was. Zowel $CD4^+$ als $CD8^+$ CTL klonen konden gegeneerd worden. Deze waren in staat CLL-specifieke targets maar ook andere B-cel targets en T cel targets van patiëntenorigine te doden. Deze klonen herkenden dus mHag die verschillend tot expressie werden gebracht in patiënt en donor. Het door de klonen herkende mHag op de patiënt was dus niet CLL-, B-cel, of T cel-specifiek. Een mHag-specifieke kloon werd verder geanalyseerd. Deze kloon bleek HLA-B8 gerestricteerd. Door

gebruik te maken van mesenchymale stamcellen, gekweekt uit het beenmerg van de patiënt, konden vervolgens experimenten verricht worden om te analyseren in hoeverre de door de kloon herkende mHag ook in niet-hematopoietisch weefsel tot expressie kwam. De CTL kloon herkende wel alle hematopoietische targets en niet de niet-hematopoietische target, de mesenchymale stamcel. Dit zou kunnen impliceren dat deze gegenereerde kloon in staat is primaire CLL te doden zonder significante GvHD te induceren doordat het herkende mHag vooral tot expressie komt op hematopoiese en niet op andere weefsels. Deze resultaten illustreerden de haalbaarheid om met behulp van de CLL-APC als stimulators te komen tot initiatie van CLL-reactieve en mHag-specifieke T-cel responsen, die relatief hematopoiese-specifiek zijn in een HLA-identieke setting. Deze studie liet verder zien dat de gebruikte limiting dilution methode zeer arbeidsintensief was, en leidde tot langdurige *in vitro* kweekperiodes. Om in de kliniek tot toediening van CLL-reactieve CTL klonen na transplantatie te komen, dienen meer efficiëntere methodes ontwikkeld te worden die kunnen resulteren in vroege selectie en isolatie van CLL-reactieve T cellen.

Genereren van MCL-reactieve T cel responsen

In **hoofdstuk 4** bleken uit experimenten de expressieniveaus van costimulatoire en adhesie moleculen op primaire MCL cellen conform primaire CLL cellen onvoldoende te zijn om MCL-reactieve donor T-cel responsen op te wekken. Er werd getracht de primaire MCL cellen te transformeren in professionele APC cellen door CD40 activatie en door middel van stimulatie met het MCL-specifieke cytokine IL-10, met IL-4, CpG. Ligatie van het CD40 molecuul op de MCL cel was essentieel voor de opregulatie van costimulatoire moleculen. Vier dagen van CD40 stimulatie resulteerde in de meest optimale expressie van costimulatoire en adhesie moleculen en in een hoge productie van het belangrijke immunostimulatoire cytokine IL-12. Deze MCL APC cellen als stimulator cellen waren vervolgens in staat om MCL-reactieve T-cel responsen op te wekken in HLA klasse I-gematchte donoren. Dit resulteerde in de generatie van grote aantallen mHag-specifieke CTL klonen, die in staat waren de primaire MCL cellen zeer effectief te elimineren.

Het karakteriseren van graft-versus-CLL responsen

In hoofdstuk 5 werden de resultaten en uitkomsten besproken bij twaalf patiënten met agressieve CLL, die behandeld werden met een T-cel gedepleteerde RIC allogene SCT, gebruikmakend van alemtuzumab in het transplantaat dat depleteerde voor donor en ontvanger T cellen. Onderdeel van het protocol was het toedienen van DLI na transplantatie om GvL activiteit te induceren en zo persisterende ziekte te eradiceren. Na transplantatie werd bij alle patiënten persisterend donor chimerisme waargenomen zonder toegenomen incidentie van transplantaat afstoting met verder minimale en acceptabele GvHD. Na toediening van DLI voor gemengd chimerisme en/of persisterende ziekte werden in sommige patiënten langdurige remissies verkregen, illustrerend dat CLL cellen gevoelig zijn voor het GvL effect. Echter, bij andere patiënten werd persisterende of progressieve ziekte geobserveerd ondanks het toedienen van oplopende doseringen van DLI. Om deze verschillen in klinische responsen verder te karakteriseren, werden *in vitro* experimenten verricht gebruikmakende van T cellen die verkregen zijn van op DLI responderende en niet op DLI responderende patiënten. Ook werden T cellen verkregen van de oorspronkelijke donor. De T cellen

werden gestimuleerd met de primaire CLL cellen en de CLL-APC cellen. Indien T cellen geactiveerd worden door de stimulatorcel kunnen zij immuunmodulerende cytokines zoals interferon gamma (IFNγ) produceren. In deze studie werden leukemie-reactieve T cellen geïsoleerd op basis van hun IFNγ productie in respons op primaire CLL en CLL-APC door middel van de IFNγ secretie assay. In overeenstemming met de experimenten in hoofdstuk 2 en 3, bleken primaire CLL cellen onvoldoende stimulatoire capaciteit te hebben om CLL-reactive T cellen te stimuleren. CLL-APC cellen konden CLL-reactive mHag-specifieke T-cel responsen induceren bij de patiënt met een goede klinische respons na allogene SCT en de toediening van DLI. Dit kon niet bij de patiënt die progressieve ziekte vertoonde ondanks oplopende doseringen van DLI. Vervolgens werden de experimenten herhaald met de T cellen van de oorspronkelijke donor van de niet-responderende patiënt om te onderzoeken in hoeverre voorloper CLL-reactive T cellen überhaupt wel aanwezig waren in de donor. Na activatie met CLL-APC konden met gebruik van de IFNγ secretie assay CLL-reactieve CTL klonen worden verkregen. Deze resultaten demonstreerden dat voorloper CLL-reactive T cellen die aanwezig zijn in de donor *in vivo* onvoldoende gestimuleerd werden door de primaire CLL. Dat berustte waarschijnlijk op het ontbreken van een APC fenotype. CLL-reactieve T cellen zijn dus niet in staat om een adequate immuun respons tegen persisterende CLL cellen op te wekken. Dit zou de reden kunnen zijn voor de klinische observaties van continue recidieven na allogene SCT en na de toediening van DLI.

Inzichten en vooruitzichten

Inzichten

De resultaten in dit proefschrift laten zien dat primaire CLL en MCL cellen onvoldoende in staat waren om te functioneren als APC cellen door het ontbreken van voldoende expressie van costimulatoire en adhesie moleculen. De primaire CLL en MCL cellen waren daarom niet in staat een relevante immuunrespons te induceren. CD40 stimulatie transformeerde deze leukemie cellen in professionele maligne IL-12 producerende APC cellen. Deze CLL-APC en MCL-APC cellen hadden de stimulatoire capaciteit om CLL- en MCL-reactieve T-cel responsen te initiëren in HLA-gematchte donoren. Repetitieve *in vitro* stimulatie van donor T cellen met de maligne APC cellen resulteerde in de generatie van leukemie-reactieve mHag-specifieke CTL klonen die preferentieel hematopoiese-specifieke targets herkenden. De relevantie van deze resultaten werd gedemonstreerd in een studie die de uitkomsten beschreef van patiënten met agressieve CLL die behandeld waren met een T-cel gedepleteerde RIC allogene SCT gevolgd door toediening van DLI voor het initiëren van GvL reactiviteit. Sommige patiënten hadden een zeer goede klinische respons wat illustreerde dat deze behandeling curatieve potentie heeft. Andere patiënten hadden echter continue recidieven na allogene SCT en DLI. De *in vitro* experimenten in een niet-responsieve patiënt demonstreerden dat voorloper CLL-reactive T cellen aanwezig waren in de donor maar na transplantatie *in vivo* niet geactiveerd konden worden door de persisterende primaire CLL cellen. Deze observaties suggereerden dat de inadequate APC functie van primaire CLL cellen waarschijnlijk deels verantwoordelijk was voor de

afwezigheid van een klinische respons. In deze groep patiënten is het daarom noodzakelijk de effectiviteit van adoptieve cellulaire immuuntherapie te verbeteren.

Vooruitzichten

In dit proefschrift werd geïllustreerd dat gemodificeerde leukemiecellen als stimulator cellen in staat zijn om donor T-cel reactiviteit tegen de primaire leukemiecellen te induceren. Met behulp van de IFNγ secretie assay was het vervolgens mogelijk om voorloper leukemie-reactieve T cellen vroegtijdig te detecteren en isoleren. Verdere efficiënte proliferatie kon bewerkstelligd worden door repetitieve stimulatie met de maligne APC cellen. Deze *in vitro* gegeneerde leukemie-reactieve T cellen zouden na toediening aan de patiënt in staat kunnen zijn om persisterende CLL of MCL cellen te eradiceren zonder inductie van uitgebreide GvHD. Immers de repetitieve stimulatie met leukemie APC cellen zou kunnen resulteren in een relatieve verrijking van leukemie-reactieve T cellen in vergelijking met GvHD-mediërende T cellen.

Met deze nieuw verworven inzichten zijn verschillende cellulaire adoptieve immuuntherapeutische benaderingen die het optreden van ernstig GvHD voorkomen en de GvL-reactiviteit versterken te overwegen. *In vitro* gegenereerde leukemie-reactieve T cellen zouden in lage doseringen direct na allogene SCT toegediend kunnen worden om toekomstige minimale residuale ziekte te controleren en elimineren. Als alternatief zouden hoge doseringen van deze specifieke T cellen later na allogene SCT na aantonen van persisterende ziekte toegediend kunnen worden. In hoeverre deze toedieningen gecombineerd dienen te worden met lage dosis DLI voor aanvullende immuuntherapeutische ondersteuning zou onderwerp van toekomstige studies kunnen zijn. Combinaties van lage dosis DLI, waaruit GvHD-inducerende T cellen zijn verwijderd, met GvL-inducerende *in vitro* gegenereerde T cellen zouden zeer synergistisch kunnen werken. Als alternatief zou het identificeren van nieuwe hematopoietische-gerestricteerde, bij voorkeur B-cel-gerestricteerde, mHag prioriteit moeten hebben. Immers dan zouden mHag-specifieke donor T cellen zonder de inductie van GvHD, gezien het ontbreken van expressie van de mHag op niet-hematopoietisch weefsel, na toediening de patiënt kunnen genezen van zijn ziekte. Een andere benadering zou kunnen zijn om patiënten na RIC allogene SCT te vaccineren met CLL-APC of MCL-APC als vaccin. Aangezien vaccinatiestudies bij patiënten met uitgebreide ziekte teleurstellend zijn, zou dit bij voorkeur al gegeven moeten worden bij minimaal residuale ziekte. Bij goede respons zou de vaccinatie herhaald kunnen worden om tot expansie van het memory T-cel compartiment te komen.

De uitkomsten van de *in vitro* experimenten beschreven in dit proefschrift zullen nu vertaald dienen te worden naar de klinische setting. In hoeverre de *in vitro* aangetoonde effectiviteit van de leukemie-reactieve T cellen ook *in vivo* in de patiënt werkzaam en relevant zijn, zou onderwerp van toekomstige studies en protocollen moeten zijn. Verder zal geëvalueerd moeten worden welke dosis van specifieke T cellen en welk tijdspad van toediening na allogene SCT het meest effectief is.

Nawoord

Dit proefschrift is mede tot stand gekomen door de enthousiaste medewerking en steun van velen. Het laboratorium voor Experimentele Hematologie was voor mij een inspirerende, boeiende en warme plek om te werken. Het creëren van zo'n open, collegiale en wetenschappelijke atmosfeer is een verdienste van alle medewerkers. Graag wil ik een aantal mensen in het bijzonder bedanken.

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Lieve Herminka, leven met jou is eindeloos. Zullen we weer …. *Let's get lost, lost in …….*

Curriculum Vitae

Mels Hoogendoorn werd geboren op 19 december 1965 te Zoetermeer. Na het behalen van het eindexamen Atheneum B aan het Oranje Nassau College te Zoetermeer in 1984, werd wegens uitloting voor de studie Geneeskunde gestart met de studie Biologie aan de Rijksuniversiteit Leiden. Na het behalen van het propedeutische examen kon alsnog aangevangen worden met de studie Geneeskunde aan dezelfde universiteit. In 1992 werd het artsexamen afgelegd na een half jaar onderzoek verricht te hebben in de Faulkner Hospital te Boston, USA (prof.dr. R.L. Murphy). Na een jaar als arts assistent chirurgie in het kader van de tropenopleiding gewerkt te hebben in het Spaarne Ziekenhuis te Heemstede (opleider dr. H.W.R. Siebbeles), werkte hij aanvankelijk als arts assistent niet-in-opleiding voor de interne geneeskunde in het Bronovo ziekenhuis te Den Haag (opleider dr. R. Bieger). In datzelfde ziekenhuis werd in 1996 de opleiding Inwendige Geneeskunde aangevangen en deze werd afgerond in het Leids Universitair Medisch Centrum (opleider prof.dr. A.E. Meinders). De registratie als internist vond plaats in 2001.

Vanaf 2001 tot 2004 was hij werkzaam als internist op de afdeling hematologie van het Leids Universitair Medisch Centrum (opleider prof. R. Willemze). In 2004 volgde registratie als internist met het aandachtsgebied hematologie. Vanaf 2001 werd het in dit proefschrift geschreven onderzoek verricht op het laboratorium Experimentele Hematologie onder begeleiding van prof.dr. J.H.F. Falkenburg. Vanaf december 2004 is hij werkzaam als internist-hematoloog in het Medisch Centrum Leeuwarden.