Allogeneic cellular immunotherapy for chronic B-cell leukemia

Hoogendoorn, M.

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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