Allogeneic cellular immunotherapy for chronic B-cell leukemia

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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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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

	DLI			GvHD			Disease status		Chimerism (% donor)		
Patient no.	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	Follow up after alloSCT (months)
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%.

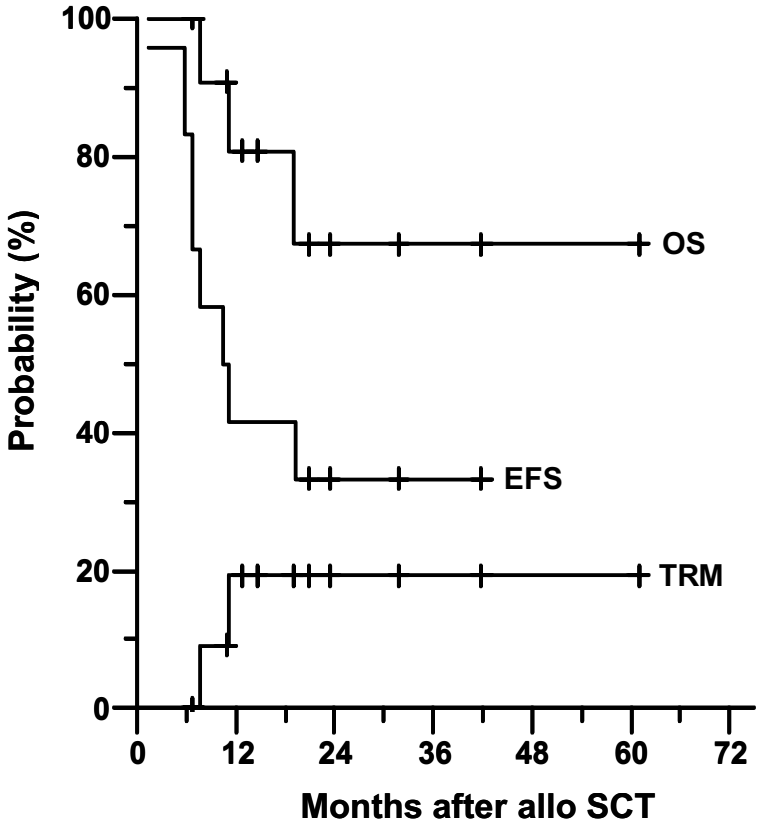

Figure 1. Overall survival, event-free survival and treatment-related mortality after *in vitro* T-cell depleted allogeneic SCT and the administration of DLI. OS, overall survival, ESF, event-free survival, TRM treatment-related mortality.

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 $1.5x10^8$ 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.

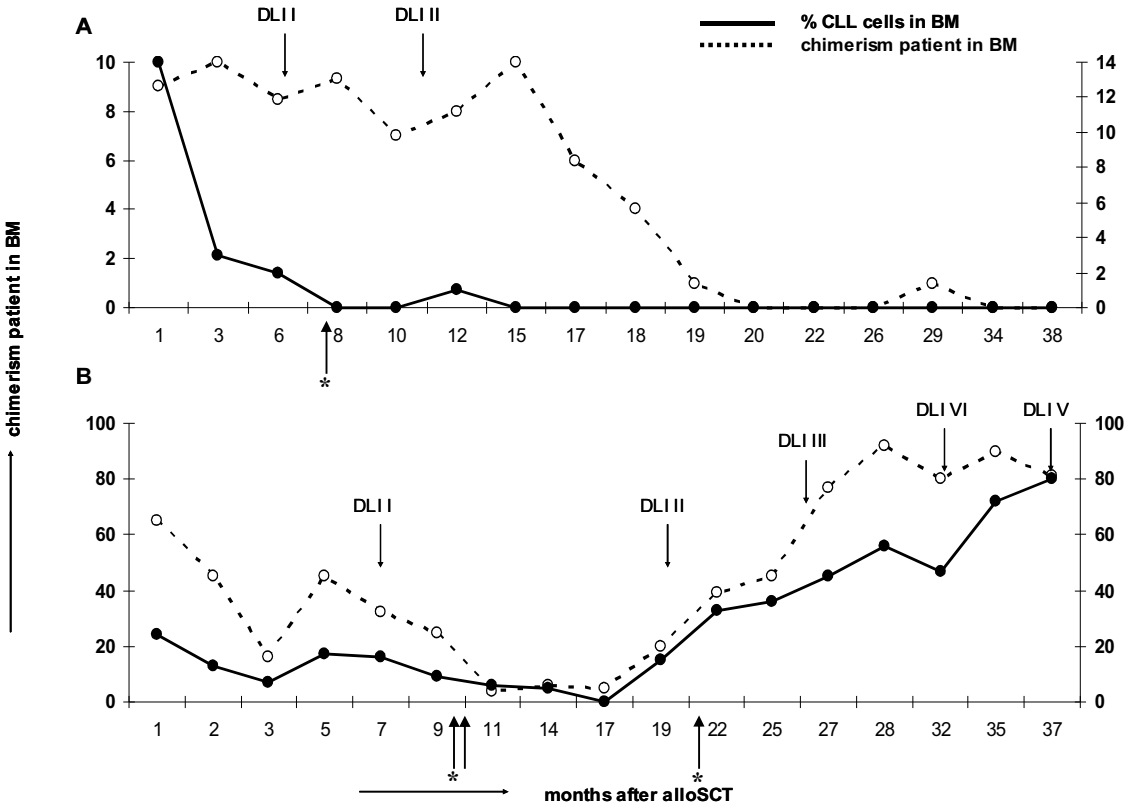

Figure 2. Patients chimerism, measured in BM and % of CLL cells in BM as determined by flow cytometry after allogeneic SCT and the administration of DLI. (A) In patient 6 disappearance of CLL cells in BM was observed after the administration of DLI and the patient converted to full donor chimera, resulting 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.

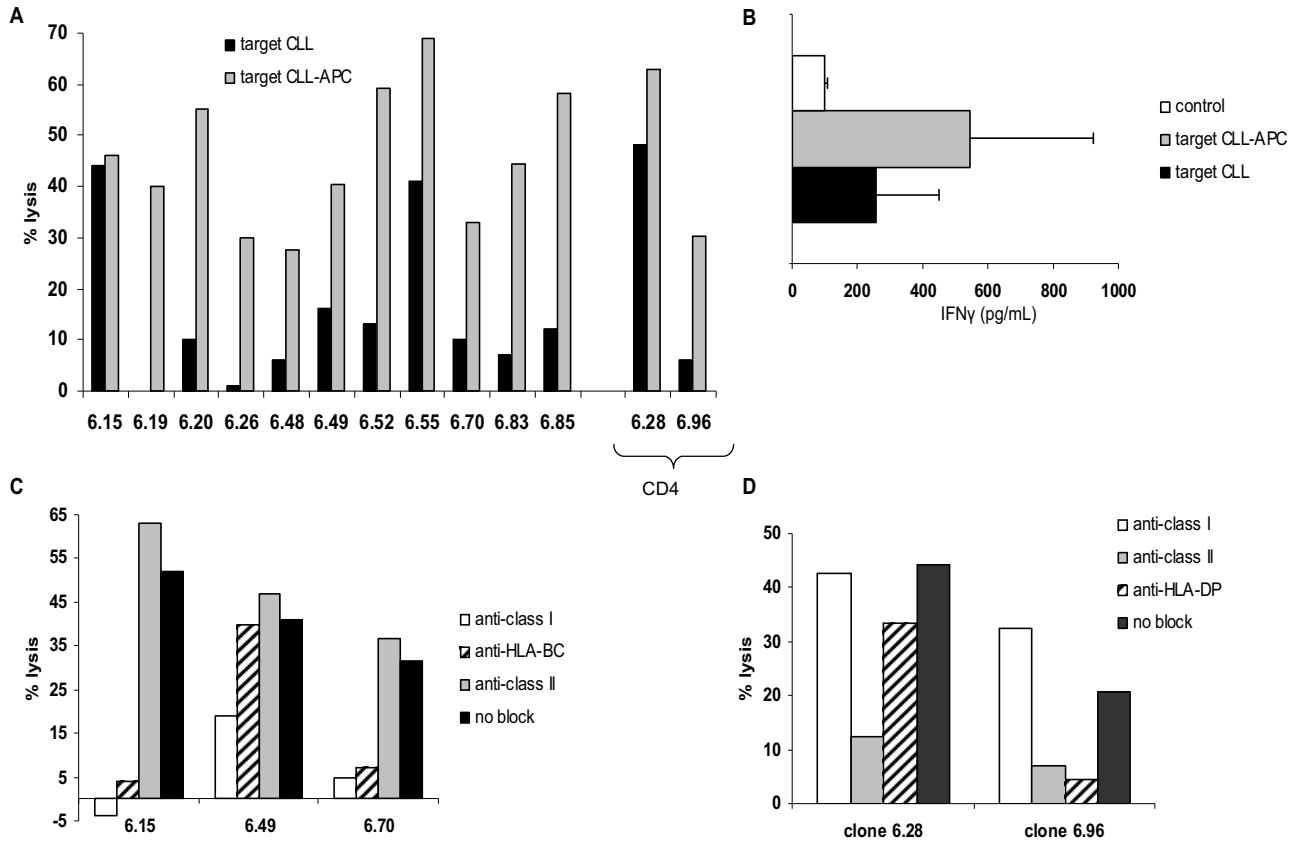

Figure 3. Characterization of CLL-reactive and mHag-specific CTL clones, derived from patient 6 six weeks after the application of DLI. (A) Cytolytic activity of CTL clones generated by stimulation of T cells, obtained from the patient six weeks after DLI with CLL-APC of the patient. All clones were $CD8^+$ 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.

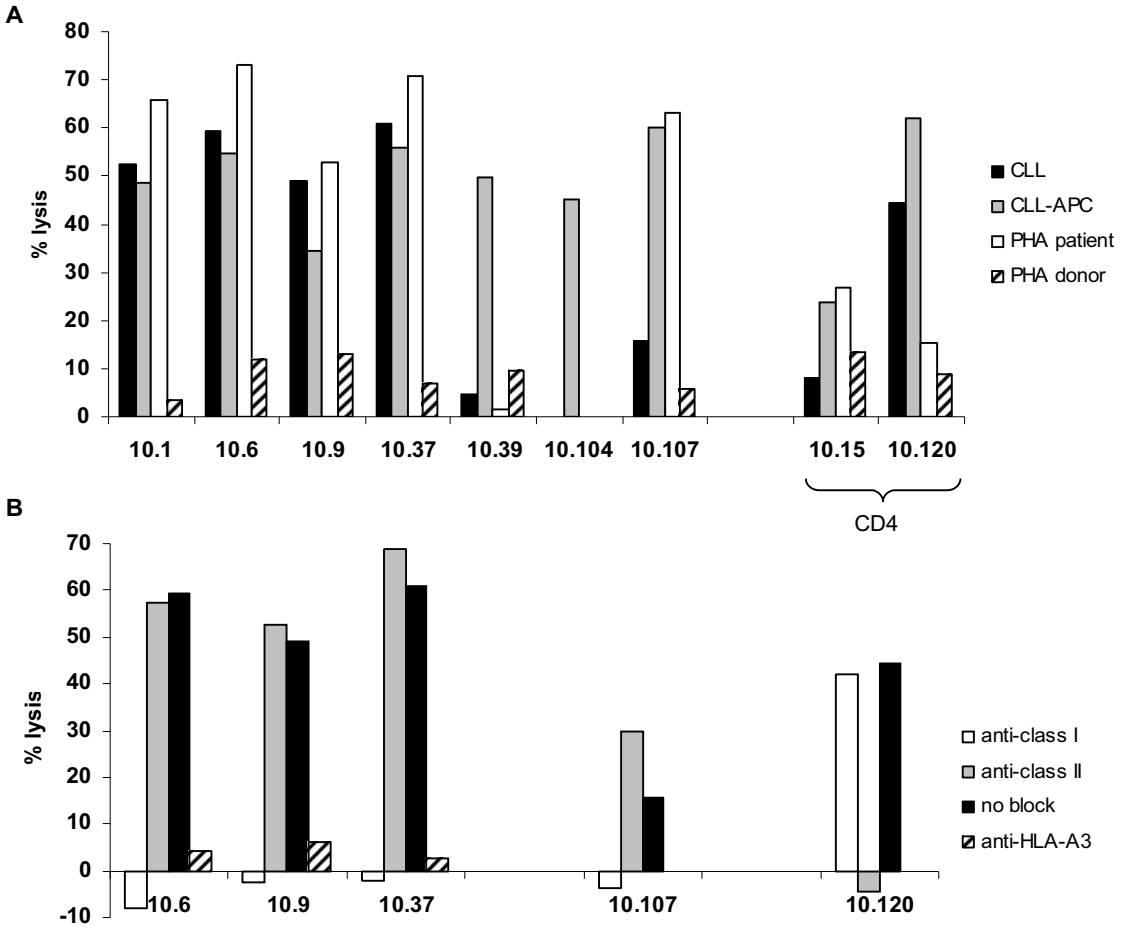

Figure 4. Cytotoxicity of CTL clones, derived from the unprimed donor of patient 10, using CLL-APC as stimulator cells. (A) Percentage lysis of CLL-specific targets and patient- and donor-derived PHA blasts in an overnight ^{51}Cr-assay at E/T ratio of 3:1. All clones were CD8$^+$ except for the two 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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