Phase I/II feasibility study evaluating the generation of leukemia-reactive cytotoxic T lymphocyte lines for treatment of patients with relapsed leukemia after allogeneic stem cell transplantation

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ABSTRACT

Background and Objectives

Graft-versus-host-disease may be avoided and the likelihood of a graft-versus-leukemia reaction increased by infusion of *in vitro*-generated, leukemia-reactive, cytotoxic T lymphocyte (CTL) lines as treatment for patients with relapsed leukemia after allogeneic stem cell transplantation, instead of donor lymphocyte infusion. The aim of this phase I/II study was to assess the feasibility of large-scale *in vitro* generation of leukemia-reactive CTL for clinical use.

Design and Methods

Using a modified limiting dilution culture system donor T cells were stimulated with HLA-identical leukemic antigen-presenting cells. Feasibility experiments demonstrated that in 16 of 27 donor-recipient pairs tested a CTL line could be generated. Twelve of these 16 patients developed a relapse and for 11 of these 12 patients a CTL line was generated under Good Manufacturing Practice conditions.

Results

The CTL lines showed moderate to high cytotoxic activity against original recipient leukemic cells *in vitro*. Eight patients with a relapse received from one to seven CTL lines. One patient entered a complete remission after CTL infusion only, one entered a complete remission after combined CTL infusion and donor lymphocyte infusion, two patients had temporarily stable disease, and in four patients no response was observed.

Interpretation and Conclusions

Although the current procedure to generate these CTL lines is feasible, the strategy is logistically complex and time-consuming, and needs further improvement.

Keywords: cellular immunotherapy, CTL, leukemia, allogeneic stem cell transplantation.

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Relapsed leukemia after allogeneic stem cell transplantation can be treated with donor lymphocyte infusion.[1] Complete remissions are obtained in 80-90% of patients with chronic myeloid leukemia (CML) in chronic phase[2] but only in 10-30% of patients with relapsed acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS), or multiple myeloma.[1,3] This may be due to inadequate antigen presentation by the malignant cells[4] and the production of inhibitory cytokines by leukemic cells suppressing an efficient anti-leukemic response.[5,6] *In vitro* stimulation of donor T cells with recipient leukemic antigen-presenting cells may bypass the inadequate *in vivo* induction of an anti-leukemic response, and thus increase the specificity and efficacy of cellular immunotherapy. We previously showed that CML-reactive cytotoxic T lymphocyte (CTL) lines could be generated *in vitro* between HLA-identical siblings.[7,8] Furthermore, we reported the administration of CML-reactive CTL lines resulting in a molecular complete remission in a patient who was refractory to donor lymphocyte infusion.[9] However, the generation of AML- or ALL-reactive CTL lines was less frequently successful. To effectively initiate an immune response, T cells require stimulation by professional antigen-presenting cells, expressing co-stimulatory and adhesion molecules as well as HLA class I and II molecules.[10] Recently, we and others showed that culturing AML cells in the presence of cytokines can result in increased expression of adhesion and co-stimulatory molecules and improved stimulatory capacity.[11-13] To enhance the reproducibility of the generation of leukemia-reactive CTL lines we modified the limiting dilution assay,[14] and adapted this method to generate leukemia-reactive CTL lines under Good Manufacturing Practice (GMP) conditions. Here, we report the results from a phase I/II feasibility study analyzing the possibility of large scale, *in vitro* generation of leukemia-reactive CTL lines to treat patients with relapsed leukemia after allogeneic stem cell transplantation. We assessed the adverse events and the potential anti-leukemic efficacy of these CTL lines.

Study design

Patients with a relapse of AML, MDS, ALL, or CML refractory to donor lymphocyte infusion after allogeneic stem cell transplantation and from whom sufficient leukemic cells could be harvested at diagnosis to generate at least three leukemia-reactive CTL lines were eligible. Patients with grade 3-4 graft-versus-host disease (GVHD) who were treated with high-dose corticosteroids, chemotherapy or immunosuppressive drugs or who had leukocyte counts over 100×10^9/L or clinical symptoms of leukostasis at the time of CTL treatment were not eligible. Informed consent was obtained from each patient according to the rules and regulations of the Leiden University Medical Center. CTL lines were generated under GMP conditions and freshly infused as described in the Materials and Methods section. The aim was to administer six CTL lines with a projected dose of $1\text{-}2\times10^9$ cells per CTL line. Infusion-associated side effects and adverse events were evaluated according to standard WHO criteria. GVHD was scored according to the Glucksberg criteria.[15] Anti-leukemic efficacy was evaluated by blood and bone marrow morphological examination, quantification of BCR/ABL load by real-time polymerase chain reaction (PCR) and/or by BCR/ABL fluorescence *in situ* hybridization (FISH), and by determination of percentage donor chimerism. Complete remission was defined as a reduction of blasts in bone marrow to <5% and disappearance of malignant blasts from peripheral blood and, if applicable, disappearance of the BCR/ABL signal or malignant metaphases from the bone marrow. Stable disease was defined as the absence of progression during CTL treatment. The characteristics of the patients included in study are presented in Table 1.

Table 1. Patients' characteristics.

Patient	Disease status at alloSCT*	Donor†	Sex patient/donor	Age at alloSCT (years)	GVHD post alloSCT‡	Interval alloSCT-relapse§
1	CML-CP	MUD	M/M	41	a II/III	16
2	CML-CP	MUD	F/M	8	chr limited	54
3	CML-CP	MUD	M/F	29	no	6
4	CML-CP	HLA-id niece	F/F	46	a II	12
5	CML-CP	MUD	M/M	32	a II/III	19
6	CML-CP	Sib	F/M	37	no	65
7	CML-CP	Sib	F/M	36	no	12
8	CML-CP	Sib	M/M	40	a I	11
9	CML-CP	Sib	M/F	32	no	6
10	CML-CP	Sib	M/F	31	no	4
11	B-ALL-CR1	MUD	M/F	22	a II	3
12	B-ALL-CR1	Sib	F/F	56	a I	NA°
13	T-ALL-CR1	HLA-id nephew	M/M	22	no	6
14	B-ALL-CR1	Sib	F/M	39	no	11
15	B-ALL-CR1	Sib	M/M	19	no	10
16	AML-CR1	Sib	M/M	54	no	7
17	AML-CR1	Sib	F/F	39	no	NA
18	AML-CR1	Sib	F/M	35	a I	8
19	AML-CR1	Sib	M/M	28	no	17
20	refractory AML	Sib	F/F	53	no	1
21	AML-CR1	Sib	M/F	31	no	4
22	AML-CR1	Sib	F/F	29	no	9
23	AML-CR1	Sib	F/F	55	a II	NA
24	AML-CR1	Sib	F/M	29	a II	NA
25	AML-CR1	Sib	F/M	20	no	NA
26	AML-CR1	Sib	F/M	36	no	4
27	AML-CR1	Sib	M/M	17	no	NA

*alloSCT: allogeneic stem cell transplantation; *CML-CP: chronic myeloid leukemia-chronic phase; ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; CR1: first complete remission; †MUD: HLA-phenotypically identical matched unrelated donor; sib: HLA-genotypically identical sibling donor; ‡a: acute GVHD, roman numbers designate grade; chr: chronic GVHD; §months °NA: not applicable; these patients were considered to be at high risk of relapse but have remained in continuous complete remission.*

Design and Methods

Collection of bone marrow and peripheral blood cells from patients and donors

After informed consent 100-200 mL of bone marrow were harvested at diagnosis from each patient, and collected in HBSS 1640 (BioWhittaker, Verviers, Belgium) using heparin as an anticoagulant. In addition, from each patient leukocytes were isolated from 500 mL peripheral blood by apheresis at diagnosis. Prior to allogeneic stem cell transplantation leukocytes were isolated from 500 mL peripheral blood by apheresis from each donor. Mononuclear cells from blood and bone marrow were isolated under GMP conditions by Ficoll density gradient separation, washed twice in IMDM (BioWhittaker) containing 1 g/L human albumin (CLB, Amsterdam, The Netherlands), and cryopreserved in IMDM containing 20 g/L human albumin and 10% dimethylsulphoxide (DMSO) in liquid nitrogen. Donor heparin plasma (250 mL) was collected, and frozen at –20°C under GMP conditions. Immediately before use mononuclear cells were thawed and resuspended in IMDM supplemented with 3 mmol/L L-glutamine (BioWhittaker), 100 U/mL penicillin, and 100 μg/mL streptomycin plus 10% heat-inactivated prescreened heparin plasma from healthy blood-bank donors.

Generation of leukemic antigen-presenting cells under GMP conditions

To induce differentiation towards leukemic antigen-presenting cells, malignant cells from patients with AML, ALL, or CML in lymphatic blast crisis were cultured for 4 days at concentrations of 10^5-10^6 cells/mL in medium containing 10% donor heparin plasma in the presence of clinical grade granulocyte-macrophage colony-stimulating factor 100 ng/mL (Novartis, Basel, Switzerland), stem cell factor 20 ng/mL (a kind gift from Amgen, Thousand Oaks, USA), interleukin-4 10 ng/mL (kindly provided by Schering-Plough, Innishammon, Cork, Ireland), and tumor necrosis factor-α 10 ng/mL (kindly provided by Bender Wien, Boeringer Ingelheim, Vienna, Austria) in 6-well tissue culture plates (Costar, Cambridge, MA, USA) as described previously.[12] The immunophenotype of the leukemic antigen-presenting cells was analyzed using a FACSCalibur (Becton Dickinson (BD), San Jose, CA, USA) after staining the cells with fluoroscein isothiocyanate-labeled IgG1 isotype control (CLB, Amsterdam, The Netherlands), CD86 (BD Pharmingen), CD40 (Serotec, Oxford, UK), CD54 (CLB), CD58 (SBA, Birmingham, AL, USA), HLA-DR, or CD45 (BD) monoclonal antibodies, and phycoerythin-labeled IgG1 isotype control (CLB), CD80, CD14, CD33, CD3, CD19, CD11c, CD14 (BD), GPA, CD83 (Sanbio, Uden, The Netherlands), or CD123 (BD) monoclonal antibodies.

Feasibility assessment to generate leukemia-reactive CTL lines

To avoid unnecessary waste of clinical grade reagents and cell populations, feasibility experiments were first performed for each donor-patient combination to determine the optimal culture conditions for the generation of leukemic antigen-presenting cells, and the optimal ratio between responder and stimulator cells prior to generation of clinical grade CTL lines. Furthermore, the most suitable read-out system to test the cytotoxicity of donor T cells was defined. Using a pipetting robot (Biomek 2000, Beckman, Mijdrecht, The Netherlands) to diminish the risk of errors or contamination with micro-organisms, serial, 2-fold dilutions from 160,000 cells/well down to 20,000 cells/well of donor responder mononuclear cells were cultured in 48 replicates per concentration in 96-well U-bottom plates (Costar, Cambridge, MA, USA). Each well was stimulated with 20,000 irradiated (25 Gy) leukemic antigen-presenting cells from the patient. As references, 24 wells were cultured containing only stimulator cells, or responder cells. The cells were cultured in IMDM supplemented with 10% donor heparin plasma. On day 6, 120 IU of interleukin-2/mL (Chiron, Amsterdam, The Netherlands) were added, and on day 9 all wells were re-stimulated with 20,000 irradiated leukemic antigen-presenting cells. Twice a week 50% of the medium was refreshed. After 16-25 days of culture 12.5-25% of effector donor T cells from each well were tested for cytotoxicity against leukemic target cells. Original stimulator CML, ALL, and AML cells were used as target cells in a 4-hour ^{51}Cr-release assay. Wells were considered to contain cytotoxic effector cells when ^{51}Cr-release exceeded the mean plus three times the standard deviation (SD) of control wells, containing target cells only. CML and AML cells were also tested as targets in the previously described liquid progenitor cell growth inhibition assay.[8,16] ALL cells could not be tested in this assay since no significant proliferation in response to hematopoietic growth factors was detected. Results from the two cytotoxicity read-out systems were compared to determine the best cytotoxicity assay to measure anti-leukemic reactivity, and to determine the optimal concentration of responder cells per well for the generation of CTL lines in the GMP facility. Positive wells were pooled and the immunophenotype of CTL line was analyzed by staining the cells with fluoroscein isothiocyanate-labeled IgG1 isotype control, CD3, TCRαβ, TCRγδ (BD) monoclonal antibodies, and phycoerythin-labeled IgG1 isotype control, CD4, CD8 (Caltag, Burlingame, CA, USA), CD14, CD19, CD56 (BD) monoclonal antibodies. To further determine the specificity, the pooled CTL lines were tested against recipient CML or AML cells using the liquid progenitor cell growth inhibition assay, against ^{51}Cr-labeled recipient CML, ALL, or AML cells and against recipient and donor PHA blasts.

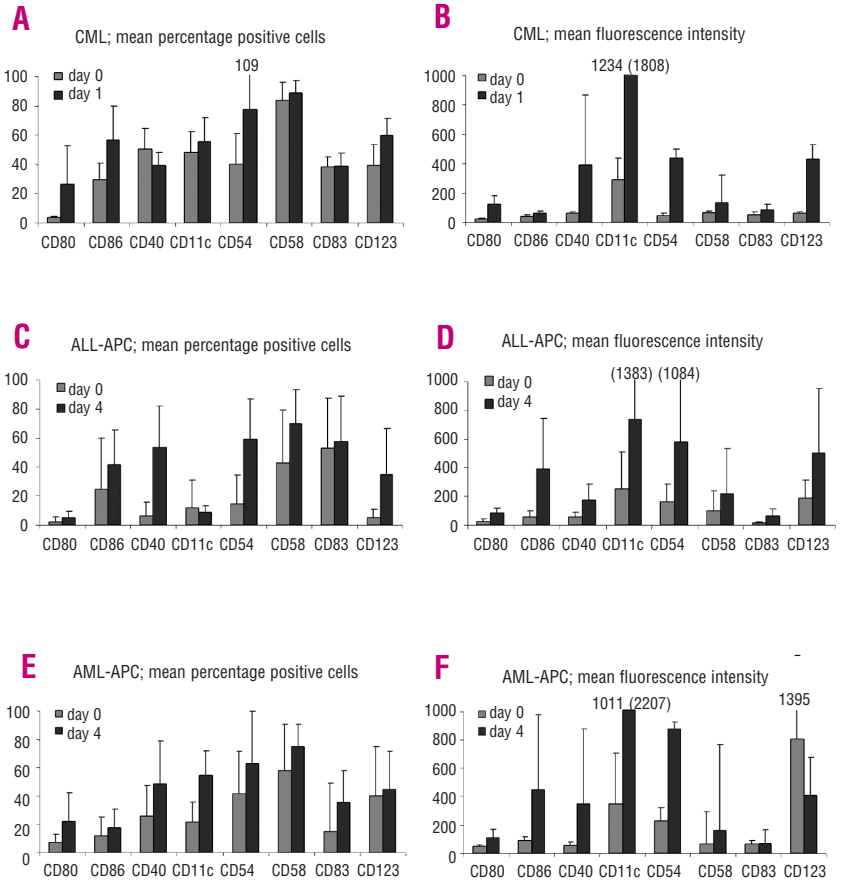

Figure 1. Expression of co-stimulatory and adhesion molecules on leukemic cells. Mean percentage (±standard deviation [SD]) of cells expressing co-stimulatory and adhesion molecules (A) and mean fluorescence intensity (MFI) (B) from seven CML chronic phase patients after overnight culture in culture medium plus serum, and from six ALL patients (C) and (D) and five AML patients (E) and (F) after 4 days of culture in culture medium plus interleukin-4, stem cell factor, granulocyte-macrophage colony stimulating factor, and tumor necrosis factor-α (day 4) compared to overnight culture in culture medium only (day 0).

Generation of leukemia-reactive cytotoxic T cell lines for in vivo administration

The CTL lines were generated in the GMP laboratory of the Leiden University Medical Center by stimulating the optimal concentration of responder cells per well, as determined in the pilot experiments with 20,000 irradiated leukemic antigen presenting cells, in 10-18 replicates of 96-well microtiter plates, and cultured as described in the previous paragraph. After performing the appropriate cytotoxicity read-out analysis the positive wells were pooled, and samples were taken for immunophenotypic analysis, GMP quality assurance testing, analysis in the ^{51}Cr-release assay, and in the progenitor cell growth inhibition assay in the case of CML or AML target cells. The CTL lines were kept in culture for one night prior to infusion and were subsequently prepared for infusion. CTL lines that were not immediately infused were frozen in liquid nitrogen for future use.

GMP quality assurance testing

CTL lines were tested for the presence of >90% CD3$^+$ T cells and the absence of anti-donor cytotoxicity, which was an absolute prerequisite for infusion, and for reactivity with recipient leukemic cells and recipient PHA blasts in the ^{51}Cr-release assay. The sterility of the CTL lines was checked by a direct Gram stain and overnight bacterial culture. The donor origin of the CTL lines was established by chimerism analysis as previously described.[17]

Infusion of leukemia-reactive CTL lines

The CTL lines were washed once, counted and resuspended in 300-500 mL NaCl 0.9% supplemented with 10% human albumin in a 1000 mL polystyrene transfusion bag (Cellgenix, Freiburg, Germany). All cultured CTL with a maximum of 10^8 CTL/kg body weight were administered per CTL line. In the case of fever and chills blood cultures were taken and the patient received pethidine, a synthetic short-acting morphine analog.

Results

Expression of co-stimulatory and adhesion molecules on leukemic antigen-presenting cells

Figure 1A shows the mean percentage (±standard deviation) of CML chronic phase cells expressing the co-stimulatory and adhesion molecules from seven patients after overnight culture in IMDM plus heparin plasma. Expression of CD80 increased from 4±1% to 27±26% of the cells, that of CD86 from 30±11% to 57±23%, that of CD54 from 40±22% to 77±32%, and that of CD123 from 40±14% to 59±12%. The percentage of cells expressing

CD40, CD11c, CD58, and CD83 remained constant. The mean fluorescence intensity (MFI) (± standard deviation) of CD86, CD58, and CD83 showed no relevant increase in contrast to the MFI of CD40, CD11c, CD54, and CD123 (Figure 1B). Figure 1C shows the mean percentage of leukemic cells from five ALL patients expressing co-stimulatory and adhesion molecules measured on day 0 and after 4 days of culture in the presence of cytokines. No relevant differences were observed between percentages of ALL cells expressing CD80, CD86, CD58, CD11c, and CD83 on day 0 and day 4. The percentage of cells expressing CD40, CD54, and CD123 increased from 7±9% to 53±29%, from 15±20% to 59±28%, and from 5±5% to 34±32%, respectively, after the 4-day culture period. The MFI of CD86, CD11c, CD54, and CD123 on ALL cells increased after the culture period in contrast to the MFI of CD80, CD40, CD58, and CD83 (Figure 1D). For the cells from six AML patients no relevant differences were observed between percentages of cells measured expressing CD86, and CD123, on day 0 and day 4, as shown in Figure 1E. The percentage of cells expressing CD80, CD40, CD11c, CD54, CD58, and CD83 increased from 7±6% to 22±20%, from 26±22% to 48±31%, from 22±14% to 55±18%, from 41±31% to 63±37%, from 58±33% to 75±16%, and from 15±34% to 35±23%, respectively. The MFI of CD86, CD40, CD11c, and CD123 on AML cells increased after 4 days of culture in contrast to the MFI of CD80, CD58, and CD83; the MFI of CD123 decreased (Figure 1F). Thus, although the percentage of cells expressing co-stimulatory and adhesion molecules increased in a number of cases the overall expression pattern of these molecules on the leukemic cells remained suboptimal.

Table 2. Results of feasibility experiments.

Patient	Disease status at relapse*	Donor†	Read-out system‡	Percentage positive wells§	Minimum percentage lysis or growth inhibition	CTL line
1	CML-AP	MUD	PIA	25	≥8	yes
2	CML-CP	MUD	PIA	27	≥30	yes
3	CML-CP	MUD	PIA	73	≥38	yes
4	CML-AP	HLA-id niece	PIA	56	≥33	yes
5	CML-CP	MUD	PIA	50	≥73	yes
6	CML-CP	sib	PIA	0	–	no
7	CML-CP	sib	PIA	0	–	no
8	CML-CP	sib	PIA	0	–	no
9	CML-BC	sib	PIA	0	–	no
10	CML-BC	sib	^{51}Cr-RA	8	≥9	yes
11	B-ALL	MUD	^{51}Cr-RA	65	≥10	yes
12	NA°	sib	^{51}Cr-RA	50	≥10	yes
13	T-ALL	sib	^{51}Cr-RA	0	–	no
14	B-ALL	sib	^{51}Cr-RA	0	–	no
15	B-ALL	sib	^{51}Cr-RA	0	–	no
16	refractory AML	sib	PIA	25	≥58	yes
17	NA	sib	PIA	27	≥30	yes
18	refractory AML	sib	PIA	31	≥25	yes
19	refractory AML	sib	^{51}Cr-RA	25	≥ 6	yes
20	refractory AML	sib	PIA	27	≥16	yes
21	refractory AML	sib	^{51}Cr-RA	0	–	no
22	refractory AML	sib	^{51}Cr-RA	0	–	no
23	NA	sib	PIA	33	≥26	yes
24	NA	sib	PIA	33	≥30	yes
25	NA	sib	^{51}Cr-RA	0	–	no
26	refractory AML	sib	^{51}Cr-RA	10	≥9	yes
27	NA	sib	PIA	0	–	no

**CML-CP: chronic myeloid leukemia-chronic phase; CML-AP: chronic myeloid leukemia-accelerated phase: CML-BC: chronic myeloid leukemia-blast crisis; ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; †MUD: HLA-phenotypically identical matched unrelated donor; sib: HLA-genotypically identical sibling; ‡PIA: liquid progenitor cell growth inhibition assay; ^{51}Cr-RA: ^{51}Cr-release assay; §48 wells tested; °NA: not applicable; these patients were considered to be at high risk of relapse but have remained in continuous CR.*

Assessment of the feasibility of generating leukemia-reactive CTL lines for individual patients and subsequent production for in vivo use

First, feasibility experiments were performed to generate CTL lines in combinations of donors-recipients from whom sufficient leukemic cells had been harvested at diagnosis or at relapse. For ten patients with CML, five patients with ALL, and 12 patients with AML sufficient numbers of cells were harvested. A CTL line could be generated in CML patients 1-5, in CML patient 10 with a B-lymphatic blast crisis (resembling a pre-B-ALL), in ALL patients 11 and 12, and in AML patients 16-20, 23, 24, and 26 (Table 2). The success rate for patients with CML, ALL and AML was 50%, 42% and 66%, respectively. The read-out system for the five CML patients was the liquid progenitor cell growth inhibition assay. In the feasibility experiments different responder cell concentrations were analyzed. The optimal responder cell concentration induced significant growth inhibition of CML precursor cells in 25-73% of the wells. For the three patients with ALL/CML-blast crisis and two with AML, the ^{51}Cr-release assay showed that significant lysis was present in 8, 50, 65, 25, and 10% of the wells. In six AML patients 25-33% of the wells showed significant growth inhibition of clonogenic AML in the liquid progenitor cell growth inhibition assay. Twelve of 16 patients for whom the feasibility experiments had been successful developed a relapse and for 11 of these patients two or more CTL lines were generated under GMP conditions (Figure 2). No CTL line was generated for the 12th patient because of rapid deterioration of her clinical condition. Four of the 16 patients remained in complete remission. Patients 12 and 17 were considered to be at a high risk of relapse at the time of allogeneic stem cell transplantation and therefore a CTL line was generated. However, these two patients are still in complete remission 5 and 6 years, respectively, after their transplant (Table 2). Thus, a CTL line was generated for 13 patients under GMP conditions, and eight patients were finally treated with these lines.

Table 3 shows the characteristics of the CTL lines, presenting the number of CTL lines generated for each patient, the initial and final numbers of viable cells, the fold increase of cells during the culture period and the cellular composi-

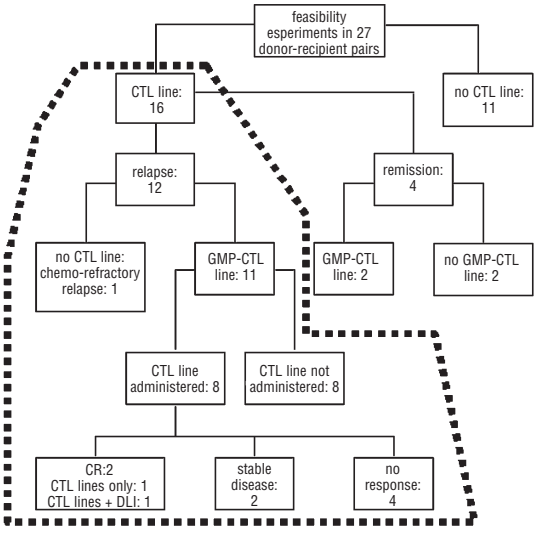

Figure 2. **Overview of patients included in the study, results of feasibility experiments, generation and administration of leukemia-reactive CTL lines. The dotted line surrounds the patients for whom the feasibility experiments were successful and who also developed a relapse, and were thus eligible for treatment with leukemia-reactive CTL lines when their clinical condition allowed treatment.**

tion. CTL lines were administered to patients 1-4, 10, 16, 18, and 20. No CTL line was administered to patient 5 who was first treated with donor lymphocyte infusion for his molecular relapse, or to patients 11 and 19 who died shortly after chemotherapy for their relapses.

Cytotoxicity testing and quality assurance of clinical grade CTL lines prior to infusion

Before infusion the CTL lines were tested according to GMP quality assurance qualifications. The CTL lines were functionally tested in the ^{51}Cr-release assay at an effector:target (E:T) ratio of 30:1, and/or when applicable in the liquid progenitor cell growth inhibition assay at an E:T ratio of 10:1. Cytotoxicity of the CML-reactive CTL lines, which were generated using the liquid progenitor cell growth inhibition assay to select positive wells, showed 90% growth inhibition in this assay and approximately 25% lysis of original CML cells in the ^{51}Cr-release assay. From 2- 40% of the patients' PHA blasts were lysed (Figure 3A). The ALL-reactive CTL lines showed from 25-54% lysis of the original ALL cells in the ^{51}Cr-release assay. The percentage lysis of patients' PHA blasts ranged from 2-49% (Figure 3B). Cytotoxicity of the ALL-reactive CTL lines was not determined in the progenitor cell growth inhibition assay since ALL target cells did not proliferate in this assay. Figure 3C shows that the CTL lines for patients 16-20 with AML displayed 60-94% growth inhibition of the original leukemic progenitor cells in the growth inhibition assay. Except for the CTL line for patient 17 and one CTL line for patient 18, lysis of unmodified leukemic cells or patients' PHA blasts by AML-reactive CTL lines was not detected in the ^{51}Cr-release assay. Chimerism analysis showed that all CTL lines were of donor origin and none of the CTL lines recognized donor PHA blasts. No bacterial contamination was detected. All CTL lines contained >90% CD3$^+$ T cells (Table 3).

Administration of leukemia-reactive CTL lines and clinical outcome

Each CTL line infusion was accompanied by fever and chills which responded well to pethidine. Blood cultures revealed no bacterial growth. Patient 10 received the second CTL line together with an unmodified donor lymphocyte infusion and developed grade II GVHD. None of the other 7 patients treated with leukemia-reactive CTL lines

Table 3. **Characteristics of leukemia-reactive CTL lines produced under GMP conditions.**

Patient	N. of CTL lines	Responder cell number at start of culture*	Viable number of CTL for infusion*	Fold increase	CD3	CD4	CD8	Phenotype of CTL lines† TCR αβ	TCR γδ	CD56	CD19
1	2	280 (270-289)	386 (246-526)	1.4	97	91	36	95	5	6	2
2	3	81 (65-114)	491 (170-670)	6	94	27	25	52	42	1.8	<1
3	7	47 (10-85)	226 (72-581)	4.8	98	53	36	79	19	1.1	<1
4	3	20	1117 (225-1700)	56	97	90	11	97	0	<1	<1
5	1	106	2507	24	97	61	18	79	18	1	<1
10	2	48	125 (114-136)	2.6	93	57	36	93	0	10	1
11	1	290	4074‡	14	95	90	5	94	1	<1	<1
12	1	136	181‡	1.3	95	73	14	87	8	<1	<1
16	2	101 (98-104)	86§	0.8	98	86	9	95	2	1.1	1.5
17	1	186	326‡	1.8	99	69	20	89	9	<1	<1
18	6	144 (120-160)	223 (82-421)	1.5	97	75	17	92	2	<1	2.6
19	2	22 (18-25)	144 (90-198)‡	6.5	97	81	9	90	7	1	<1
20	2	25	194 (111-276)	7.8	98	58	34	92	6	<1	<1

**mean cell number x 10^6 (range); †mean percentage of positive cells; ‡harvested and frozen in the vapor phase of nitrogen; §one CTL line infused and one CTL line harvested and frozen in the vapor phase of nitrogen.*

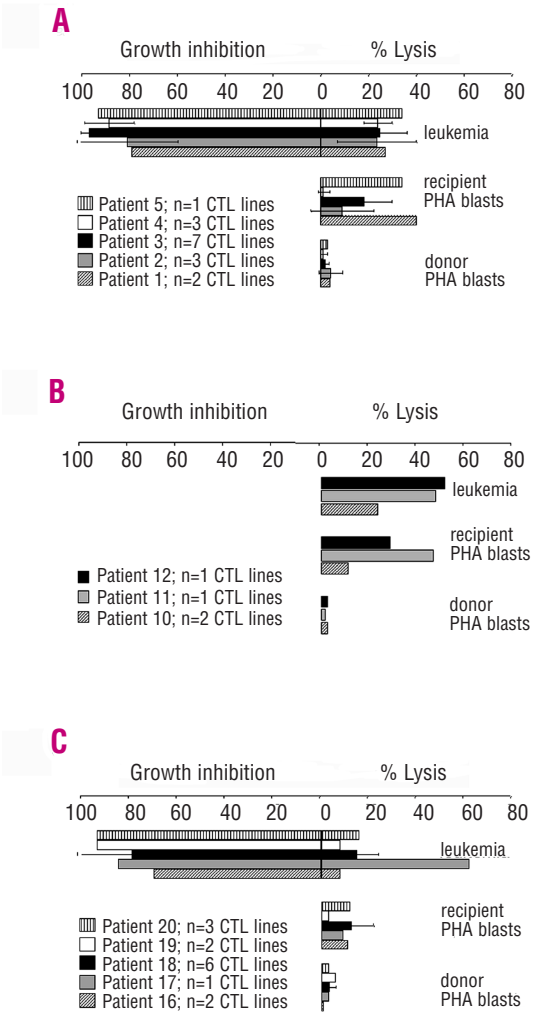

Figure 3. Target cell lysis and progenitor cell growth inhibition by leukemia-reactive CTL lines. Mean percentages of growth inhibition and lysis (±SD) of the CTL lines for patients 1-5 with CML (A), patient 10 with a B-lymphatic blast crisis of CML resembling ALL, patients 11 and 12 with B-ALL (B), and patients 16-20 with AML. The liquid progenitor cell growth inhibition assay was performed using unmodified leukemic progenitor cells of CML and AML patients as target cells. CTL lines were also tested against unmodified leukemia cells, and PHA blasts from donors and patients in the ^{51}Cr release assay. The bars represent different patients. CTL lines were tested at an effector:target ratio of 10:1 in the liquid progenitor cell growth inhibition assay and 30:1 in the ^{51}Cr release assay.

only developed GVHD. Anti-leukemic reactivity was observed in four patients. Two patients entered a complete remission (Table 4). Patient 4 with CML in accelerated phase obtained a complete remission, confirmed by a negative BCR/ABL PCR, after infusion of the third CTL line. This response occurred more than 1 year after a previously unsuccessful donor lymphocyte infusion.[9] Patient 10 with a CML B-lymphatic blast crisis received the second CTL line together with a donor lymphocyte infusion. Two weeks later GVHD grade II developed that was treated with 20 mg prednisolone. Four weeks after treatment a cytological complete remission, a negative FISH-BCR/ABL assay, and complete donor chimerism was detected. Since the interval between the combined CTL and donor lymphocyte infusion and the subsequent complete remission was very short, we hypothesize that the cultured antigen-specific donor T cells may have accelerated the immune response of naïve donor T cells present in the donor lymphocyte infusion. Stable disease was observed in two patients. In patients 3 and 18 this consisted of temporary control of leukemic cell growth without hydroxyurea treatment during a period of approximately 3 weeks after each infusion. Thereafter the patients had to be treated again with hydroxyurea to control the leukocyte count until the next infusion.

Discussion

Here, we present the results of a phase I/II feasibility study assessing the logistics of generating leukemia-reactive CTL lines under GMP conditions, and the potential toxicity and efficacy after administration to patients with relapsed leukemia after allogeneic stem cell transplantation. Leukemic cells were used to stimulate HLA-identical donor responder T cells. CML cells were used after overnight culture without cytokines, since we have shown previously that stimulation of donor T cells with unmodified CML cells reproducibly resulted in CML-reactive CTL lines.[8] Furthermore, due to the low percentage of CD34$^+$ cells in bone marrow samples from patients with CML-chronic phase not enough cells could be isolated for subsequent differentiation induction toward leukemic antigen-presenting cells. To optimize their stimulatory capacity, AML and ALL cells were first cultured in the presence of cytokines for 4 days. Although expression of the co-stimulatory molecules CD80, CD86, and the maturation marker CD83 could be increased only to a limited extent, CTL lines could be reproducibly generated within the same donor-recipient pairs. This has been described previously by us and others.[11-13] More effective maturation of leukemic antigen-presenting cells is currently not possible under GMP conditions, since clinical grade CD40 ligand trimers are not available and CD40-ligand transfected mouse fibroblasts cannot be used in a GMP-laboratory.

Prior to the large scale generation of clinical grade CTL lines small scale feasibility experiments were performed to analyze for which donor-recipient pairs CTL lines could be reproducibly generated, thus preventing the unnecessary use of clinical grade reagents and valuable cell populations. In these experiments we also determined the optimal responder to stimulator cell ratio and the most effective read-out system. We preferentially used the progenitor cell growth inhibition assay as a method to analyze growth inhibition of malignant precursor cells since we previously demonstrated the correlation of this assay with a clinical response in patients with CML treated with donor lymphocyte infusion.[8] Furthermore, this assay reflects

Table 4. Clinical responses after infusion of leukemia-reactive CTL lines.

Patient	*Diagnosis at relapse**	*Therapy prior to 1st CTL infusion†*	*Interval prior therapy-1st CTL treatment*	*CTL lines per patient‡*	*Clinical response§ (number of weeks after CTL infusion)*	*Additional treatment (weeks after CTL infusion)§*	*Outcome*
1	CML-AP	Ara-C/D+ DLI	9 months	2	NR (6)	imatinib (6)	alive
2	CML-CP	HU	5 days	3	NR (6)	imatinib (6)	alive
3	CML-CP	HU	5 days	7	SD (10)	DLI (10)	alive
4	CML-AP	DLI	10 months	3	CR (52)	–	myocardial infarction; deceased
10	CML-BC	VAAP	5 days	2	CR (5)	DLI (with 2nd CTL line)	aspergillosis; deceased
16	AML	Ara-C/D	2.5 months	1	NR (6)	–	died
18	AML	Ara-C/D	1 month	6	SD (8)	–	died
20	AML	HU	5 days	2	NR (6)	–	died

**CML-AP: chronic myeloid leukemia-accelerated phase; CML-CP: chronic myeloid leukemia-chronic phase; ALL: acute lymphatic leukemia; AML: acute myeloid leukemia; CR1: first complete remission †Ara-C/D: cytarabine/daunorubicin; DLI: donor lymphocyte infusion; VAAP: vincristine, asparaginase, cytarabine, prednisolone; HU: hydroxyurea; ‡when more than one CTL line was administered the interval between infusions was 4-5 weeks unless stated otherwise; §NR: no response; SD: stable disease; CR: complete response. §patients receiving more than one CTL line were treated with hydroxyurea when necessary to keep peripheral blood leukocyte counts between 5-10×10⁹/L.*

recognition of precursor cells, both malignant and non-malignant, in contrast to the ^{51}Cr release assay, in which lysis of more mature cells may obscure recognition of precursor cells.

In vitro generation of leukemia-reactive CTL lines for *in vivo* administration was feasible for 16 out of 27 donor-recipient pairs with high reproducibility, resulting in 33 CTL lines in total. In contrast, the total number of T cells generated per CTL line was not predictable and varied both within individual donor-recipient pairs as well as between different pairs. Since the culture conditions were kept identical it can be hypothesized that differences in numbers of T cells obtained at the end of the culture period may have been due to both quantitative and qualitative differences in responder T cells at the beginning of the cell culture including the T-cell precursor frequency, activation state, and naive or memory phenotype. In addition to the high numbers of cells obtained for patient 4, CTL lines generated for patients 5 and 11 also yielded high cell numbers. However, since these two patients have not relapsed, they have not been treated with CTL lines making it impossible to evaluate the anti-leukemic effect of these lines.

The phenotype of the majority of CTL lines consisted predominantly of CD4$^+$ T cells with up to a maximum of one third CD8$^+$ T cells. The high percentage of CD4$^+$ T cells may be due to the fact that the majority of the leukemic stimulator cells expressed HLA class II antigens (*data not shown*), preferentially stimulating CD4$^+$ T cells. Previously, we showed that cytotoxicity of CD4$^+$ T-cell clones against leukemic target cells, which was restricted by HLA-class II antigens, could be blocked by both anti-CD4- and anti HLA-class II antibodies, and is dependent on cell-cell contact.[7,14,9]

The high levels of cytotoxicity were reproducible for all leukemia-reactive CTL lines, as depicted in Figure 3. The potential recognition of normal recipient hematopoietic precursor cells by the CTL lines would not result in pancytopenia since the transplanted donor stem cells would ensure normal hematopoiesis.[18] We speculated that treatment with *in vitro*-generated, leukemia-reactive, but not leukemia-specific, CTL lines instead of unmanipulated donor lymphocyte infusion has the advantage of both circumventing inadequate antigen presentation *in vivo* and inducing relative specificity for leukemic or recipient hematopoietic cells. Recipient PHA blasts, which were recognized by the CTL lines from most patients, are hematopoietic cells expressing both hematopoiesis-restricted minor histocompatibility antigens, e.g. HA-1 and HA-2, and broadly expressed ones (e.g. HY). CTL lines recognizing predominantly hematopoiesis-restricted minor histocompatibility antigens would be expected to induce little or no GVHD.

Evaluation of the clinical effects of infused CTL lines showed that after repeated CTL infusions alone a complete remission occurred in one of eight treated patients. This patient received significantly higher numbers of CTL than did the other patients. As reported previously, we demonstrated an increased frequency of leukemia-reactive CTL in the peripheral blood of this patient suggesting that a memory T-cell response had been induced.[9] Another patient who obtained a complete response was treated with two CTL lines of which the second line was combined with donor lymphocyte infusion. Since the complete response developed already after 4 weeks, we assume that the CTL line may have accelerated the immune response of the unmodified donor lymphocyte infusion and therefore contributed to the anti-leukemic response.

We hypothesize that an *in vivo* anti-leukemic effect can only be obtained when either very large numbers of CTL can be administered at short intervals of approximately 1 week, or when leukemia-reactive T cells are able to proliferate *in vivo* after infusion. However, the logistics involved

in the generation of leukemia-reactive CTL in the GMP laboratory were complex and time-consuming resulting in an interval of up to 10 weeks before infusion of the first CTL line. In addition, the intervals between subsequent infusions were 4-5 weeks, which is too long for patients with rapidly expanding acute leukemia. Consequently, the majority of patients received other types of treatment in addition to the CTL lines to control the growth of leukemic cells relatively soon after the CTL infusions. Another drawback of our culture protocol may have been the long *in vitro* culture time of 4 or more weeks, which may have negatively influenced the *in vivo* functional activity and/or proliferative capacity of the CTL lines. Recently, we and others described mouse models for immunotherapy of acute leukemia using CTL lines cultured *ex vivo*, supporting this hypothesis.[19,20] These *in vitro* models showed an inverse correlation between the duration of the cultures and the proliferative and cytotoxic capacity of the CTL. Thus, the *in vivo* anti-leukemic efficacy of leukemia-reactive CTL may be improved by culturing the cells *in vitro* for only a limited period of time. Recently, we showed that it is possible to isolate donor T cells recognizing recipient antigens expressed on both mature and immature hematopoietic cells using an interferon-γ capture assay.[21] Infusion of such enriched leukemia-reactive, CTL lines that have undergone only short-term culture may result in a better anti-leukemic response and in the development of effector memory cells, which may be necessary for long-term control of the leukemia.

Author Contributions

EM, MvdH, CvB, RB, SvL-H, JvdM, JOW, NvdW, RW, FF: acquisition of data, analysis and interpretation of data, drafting the article and final approval of the version to be published; AW: substantial contributions to design, revising the article critically for important intellectual content; and final approval of the version to be published.

Conflict of Interest

The authors reported no potential conflicts of interest.

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