

Clonal Expansion and Cytotoxicity of TCRV β Subfamily T Cells Induced by CML and K562 Cells

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OBJECTIVE To investigate the anti-leukemia effect, the distribution and clonal expansion of TCR V β subfamily T cells in T cells from cord blood and adult peripheral blood induced by CML cells and K562 cells in vitro.

METHODS Peripheral blood T cells from one adult donor and 3 cases of cord blood were stimulated with CML cells and K562 cells and further amplified by a suspended T cell-bulk culture, in order to induce CML specific cytotoxic T lymphocytes. The induced T cells were further analyzed for the specific cytotoxicity in CML by LDH assay, the phenotype identification by indirect immunofluorescence technique and the distribution and clonal expansion of TCR V β subfamily by using reverse transcriptase-polymerase chain reaction (RT-PCR) and genescan analysis, respectively.

RESULTS Oligoclonal and oligoclonal tendency T cells with higher specific cytotoxicity from cord blood and adult peripheral blood could be induced by stimulation with CML cells and K562 cells.

CONCLUSIONS Specific cytotoxic T cells for an anti-CML effect could be induced by CML cells and K562 cells. The induced T cells which have the characteristic of specific cytotoxicity against CML cells may come from the clonal expansion of TCR V β subfamily T cells.

KEYWORDS: CML, T cell receptor, V β subfamily, clonality, genescan.

Allogenic bone marrow transplants (BMT) are the only proven curative therapy for the treatment of chronic myelogenous leukemia (CML). It is well recognized that the current conditioning regimen can not completely eliminate leukemic cells, and virtually all patients after BMT have minimal residual disease (MRD) that may lead to relapse. Donor T lymphocyte infusion (DLI) has been shown to be pivotal in the prevention and induction of leukemia remission following relapse after BMT for CML. This suggested that T lymphocytes were critically important in mediating an anti-leukemic effect in vivo. Unfortunately, this approach has met with limited overall success due to the graft-versus-host disease (GVHD) induced by the alloreactive T cells. Acute and chronic GVHD, infection, myelosuppression and pancytopenia are the most common side effects following DLI. Identification of T cell populations which mediate a graft-versus-leukemia (GVL) effect with reduced GVHD may likely improve transplantation outcome^[1-4]. In the present study, we have successfully induced specific anti-leukemia CTL from PBL and cord blood stimulated by CML cells or K562 cells in vitro. We further investigated the characters of distribution and clon-

al expansion of T cell receptor V beta (TCR V β) repertoire in T cell induction by CML cells and K562 cells.

MATERIALS AND METHODS

Samples

Peripheral blood mononuclear cells (PBMCs) from one patient with primary and untreated chronic myelogenous leukemia who expressed b3a2 BCR-ABL fusion gene, determined by RT-PCR, were used in this study. The diagnosis of CML was based on the morphology and genetics. Three cases of cord blood (Q1-Q3) were collected from the umbilical vein of neonates after normal full-term deliveries according to informed consent guidelines (maternal consent was obtained in all cases). No HBV, HCV or CMV infections were found in peripheral blood from the mothers before delivery. Peripheral blood was obtained from a healthy adult blood donor. K562 and Raji cell lines were provided from the Hematology Lab of Nan Fang Hospital or Wuhan University Cells Bank, respectively.

Mononuclear cell isolation

Cord blood and adult peripheral blood mononuclear cells (CBMC or PBMC) were isolated by density gradient centrifugation (Ficoll-Hypaque density 1.077 g/ml). CD3⁺ cells (CML cells) were negatively separated by using a Mini magnetic bead sorter according to the manufacturer's instructions (MACs, Miltenyi Biotec, Germany).

Mixed Lymphocyte-leukemia cell cultures

CBMC or PBMC at a concentration of 1×10^6 /ml were divided into three groups respectively. Each group was stimulated respectively with K562 cells or CML cells, or without any additional cells, at a responder-stimulator ratio of 10:1. CML cells and K562 cells used as stimulators were treated with 50 mg/ml mitomycin C (MMC) for 1 hour and washed 4 times by RPMI-1640 containing 15% human male AB serum. Cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂, in a total volume of 2 ml/well of RPMI-1640 supplemented with 100 IU penicillin, 100 μ g/ml streptomycin, 10% human serum albumin, 1 μ g/ml anti-CD3 McAb, 500 U/ml IL-2, 50 μ mol/L 2-ME, 2 mmol/L L-glutamine and 1 μ g/ml anti-CD28 McAb. On day 3-5 of incubation, half of the medium was exchanged for culture medium supplemented with 100 IU penicillin, 100 μ g/ml streptomycin, 2% human serum albumin, 500 U/ml IL-2, and 2 mmol/L L-glutamine. The second and third stimulations were performed at 5-

day intervals with MMC-treated K562 cells or CML cells respectively and half medium exchanged simultaneously.

Target cell culture

Raji and K562 cell line, CML cells selected by mini MACS were cultured at 1×10^6 /ml with RPMI-1640 media (Gibco, BRL) containing 10% human male AB serum, 100 IU penicillin and 100 μ g/ml streptomycin.

T cell phenotype analysis

T cell phenotype was analyzed by indirect immunophenotyping fluorescein dyeing using anti-CD3, CD4 and CD8 antibody and fluorescence microscopy.

T-cell cytotoxicity assay CTL

T-cell cytotoxicity was measured in vitro using the lactate dehydrogenase (LDH) release assay. Target cells were cocultured with effector T cells at effector-target cell ratio of 30:1 for 4 hours in 96-well round-bottom plates. Spontaneous release of effector and target cells was controlled by separate incubation of the respective populations. Maximal LDH enzyme release was measured after lysis of the target cells with 0.5% Triton X-100 (Sigma). Cell-free 100 μ l supernatants were incubated in a separate 96-well plate with LDH 100 μ l substrate for 30 min. at room temperature and measured by a microplate reader (Elx-800) at 490 nm with 650 nm reference. The percentage of cytotoxicity was calculated according to the following formula: $n = \frac{[E - St - Se]}{[M - St]} \times 100 (\%)$, (E: LDH release by effector-target co-culture, St: the spontaneous release by target cells, Se: the spontaneous release by effector cells, M: the maximal release by target cells).

RNA extraction and cDNA synthesis

RNA was extracted according to the direction of the Trizol Kit and reversely transcribed into the first single-strand cDNA with the use of random hexamer primer and reverse transcriptase Superscript II Kit (Gibco, BRL).

Polymerase chain reaction (PCR)

Nucleotide sequences of the 24 V β primers and a C β primer were used in unlabeled PCR. A fluorescent primer labeled at its 5' end with fam fluorophore (C β -fam) for runoff reaction was purchased from TIB MOLBIOL GmbH, Berlin, Germany^[5-6]. PCR was performed as described by Puisieux *et al*^[5-6]. Aliquots of the cDNA (1 μ L) were amplified in a 25 μ L reaction system with one 24 V β primer and one C β primer. The

final reaction mixture contained 0.5 $\mu\text{mol/L}$ sense primer(V β), 0.5 $\mu\text{mol/L}$ C β primer, 0.1 mmol/L dNTP, 1.25 U Taq polymerase (Perkin Elmer) and 1 \times PCR buffer containing 10 mmol/L Tris-HCl, pH 8.3, 50mmol/L KCl, 1.5 mmol/L MgCl₂ and 0.001% (w/v) gelatin. The amplification was performed on a DNA thermal cycler (Perkin Elmer). After 3 min of denaturation at 94°C, 40 PCR cycles were performed, each cycle consisting of reactions at 94°C for 1 min, 60°C for 1 min and 72°C, for 1 min, and a final polymerization step of 10 min at 72°C. The products were then stored at 4°C.

Analysis of T cell clonality

Runoff reactions (labeled PCR products) Aliquots of the unlabeled PCR product (2 μL) were separately added to a final 10 μL reaction system containing 0.1 $\mu\text{mol/L}$ C β -fam primer, 3 mmol/L MgCl₂, 0.2 mmol/L dNTP, 0.25 U Taq polymerase and PCR buffer (Perkin Elmer). After a 3 min denaturation at 94°C, 35 cycles of amplification were carried out (1 min at 94°C, 1 min at 66°C and 1 min at 72°C and a final 10 min elongation at 72°C)^[5-6].

Genescan analysis(CDR 3 length analysis) The fluorescent labeled PCR products (2 μL) were heat-denatured at 94°C for 4 min after addition of 2.5 μL formamide, 0.5 μL of Genescan-500 Tamra Size Standards (ABI, Perkin Elmer) and 0.5 μL of loading buffer (Dextran 50mg/ml, EDTA 25mmol/L, Genescan-500 Tamra Kit) and were then loaded on 6% polyacrylamide gel for size and fluorescence intensity determination by Genescan 672 analysis software on a 377A DNA sequencer. Since the positions of the V β and C β primers are fixed, the length distribution observed in the PCR V β -C β products depends only on the size of the rearrangement of V-D, D-J gene segment and the randomly inserted nucleotides (V_ND_NJ). After electrophoresis on an automated sequencer and subsequent computer analysis, the products of different size could be separated and expressed as different peaks^[5-6].

RESULTS

The change of cellular immunophenotype

The predominant phenotype of T cells in PBMC and CBMC were CD4⁺ T cells before culture, whereas CD8⁺ T cells were preferentially expanded after culture (Table 1).

Cytotoxicity analysis

T cells induced by CML cells or K562 cells could specifically recognize CML cells($P<0.05$) and could not recog-

nize Raji cells($P>0.05$). T cells from the three groups had non-specific cytotoxicity to K562 cells (Table 2).

Table 1. Change of T cell immunophenotype (percent)

Phenotype	T cells (PB)		T cells (CB)	
	BC	AC	BC	AC
CD4 ⁺	65.3	44.1	63.6	45.1
CD8 ⁺	36.7	55.9	37.4	54.9

BC: before culture, AC: after culture

Table 2. Cytotoxicity of different effector cells against CML, K562 and Raji cells

Effector cell	Target cell		
	CML cell	K562 cell	Raji cell
A	0.273 \pm 0.027	0.328 \pm 0.024	0.151 \pm 0.011
B	0.476 \pm 0.063	0.274 \pm 0.038	0.144 \pm 0.021
C	0.478 \pm 0.047	0.352 \pm 0.036	0.154 \pm 0.018

A:CD3AK B:T cells induced by K562 C:T cells induced by CML cells

TCR V β subfamily expression before and after MLTC

Only 6 and 9 TCR V β subfamilies were detected in T cells from two CBMC (Q1 and Q3) before cell culture (Q2 could not be detected due to inappropriate conservation of RNA). The number of detectable TCR V β subfamilies was increased in all cases after CBMC cultured with anti-CD3 antibody and IL-2 or stimulators. T cells in V β 2, V β 3, V β 5, V β 8, V β 13 and V β 21 subfamilies were frequently detected in most groups. V β 14 and V β 9 subfamilies T cells were newly expressed after culture (Table 3).

Clonal expansion of T cells after MLTC

Polyclonal expansion was the feature in all T cell V β subfamilies cultured with CD3 and IL-2. Clonal expansion or oligoclonal tendency of T cells could be identified in samples from 3 cases of CBMC and 1 case of PBMC induced by CML cells or K562 cells (Table 3). New appearance peaks and dominant peaks changes were also found in some V β subfamily T cells (Fig.1).

DISCUSSION

DLI is one of the best methods to eliminate MRD in leukemia patients. Although 70% to 80% complete remission was achieved in patients with relapsed CML after BMT, the incidence rate of acute GVHD was up to 50%-80%. Infusion of leukemia-specific T-cell clones is one of the best methods to reduce the incidence of

Table 3. TCR Vβ repertoire and clonality of T cells from CBMC and PBMC

Samples	Vβ1	Vβ2	Vβ3	Vβ4	Vβ5	Vβ7	Vβ8	Vβ9	Vβ10	Vβ12	Vβ13	Vβ14	Vβ15	Vβ16	Vβ21	Vβ22
Q1		P* ⁶	p		p		p				p				p	
cd3* ¹		p	p		p	p	p	p			p	p	p	p	p	p
CML* ²		P	P		Ot* ⁷		p	p			p	p	p	O* ⁸	B	p
K562* ³		p	p		p		p	p			p	p		O	O	
Q2																
cd3	p	p	p		p	p	p	p	p		p		p		p	
CML	p	p			p	p	p	O			p		p		B* ⁹	
K562	p	p	p		p	p	p		p		p		p		T* ¹⁰	
Q3																
cd3	p	p	p		p	p	p	p	p	p	p	p	p		p	
CML	p	p	p		ot		p		p							
K562		p	p		p						p	p	p			
CML* ⁴	p	p	p		p	p	p		p		p	p	p		O	
K562* ⁵	p	p	p			p	p		p	p		p	p	p	p	O
N	p	p	p		p	p	p		p		p					O
cd3		p	p			p	p	p	p		p	p		p	O	
CML	p	p	p		p	p	p	p			p	p		p		
K562	p	p	p		p	p	p	p			p	p				O
CML	p	p	p	p	Ot	p	p				p	p	Ot	p		
K562	p	p	p		Ot	p	p				p	p	Ot	Ot	O	

*¹: CD3AK, *²: stimulated by CML cell, *³:stimulated by K562, *⁴:stimulated by CML cell after 2 weeks, *⁵:stimulated by K562 after 2 weeks, *⁶:polyclonality, *⁷:Oligoclonality tendency, *⁸:Oligoclonality, *⁹:Biclonality, *¹⁰:triclinality.

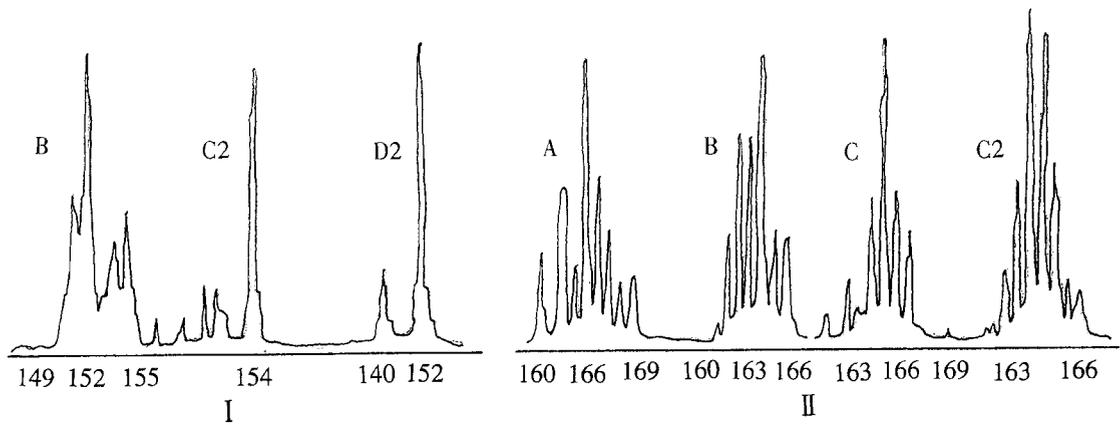


Fig. 1. CDR3 size patterns of Vβ21 and Vβ13subfamilies T cells of CBMC (Q3) before and after culture.

A: before culture. B: cultured with anti-CD3 antibody and IL-2. C: stimulated by CML cell 1 week. D: stimulated by K562 cell 1 week. C2: stimulated by CML cell 2 weeks. D2: stimulated by K562 cell 2 weeks. I : Oligoclonality proliferation of Vβ21 subfamily T cell in Q3. II : Vβ13 subfamily T cell in Q3 displayed different dominant peak induced by CML cell compare to cultured with anti-CD3 antibody and IL-2.

GVHD and still maintain the GVL effect [11-14]. And the basis of this promising specific immunotherapy strategy is to identify the specific anti-leukemia T cells, which can be performed by analysis of TCR Vβ sub-

family usage and clonality of T cells with RT-PCR and genescan techniques.

Recently, T cell receptor Vβ gene repertoire and clonality have been studied in patients with leukemia

and solid tumors. Our previous studies and others have reported skewed expression of the TCR V β repertoire and clonal expansion in T cells from patients with leukemia, melanoma, lung cancer and so on. The appearance of such clonal-expanded T cells may reflect the host's T cell response to tumor associated antigen^[5-8]. The presumption was confirmed by Farace *et al*, who showed that clonal expansion of V β 19 T cells from a case with B-CLL were isolated and amplified in vitro. These expanded V β 19 cells were shown to have specific cytotoxicity for autologous B-CLL cells. These specific expanded T cells may be used for immunotherapy^[9]. But it is difficult to establish an ideal method for expanding the autologous anti-leukemia T-cell clone in patients with leukemia due to the limited number of T cells of peripheral blood from untreated leukemia patients^[11].

Based on this restriction, development of specific allogeneic anti-leukemia T cells seems very important. In order to establish the induction of allogeneic anti-leukemia T cells, in the present study, T cells from cord blood and peripheral blood mononuclear cells were amplified with MMC treated CML or K562 cells, called mix lymphocyte tumor cell culture (MLTC). The results showed that T cells were successfully amplified with specific cytotoxicity for CML cells. TCR V β repertoire analysis showed that the induced T cells expressed only a part of V β subfamilies, and clonal-expanded T cells could be identified in some TCR V β subfamilies. The skewed expression of the TCR V β repertoire in induced T cells was thought to be related to some clonal-expanded T cells that responded for leukemia-associated antigen, leading to suppression of the proliferation of other T cell subfamilies. Since antigenic stimulation produces clonal expansion of T cells whose T cell receptors are specific for the antigen, clonally expanded T cells induced by CML cells and K562 cells would be driven by specific antigens, which may have the specific cytotoxicity for the primary CML cells, resulting in the GVL effect. It seems that selection and amplification of the clonal expanded TCR V β subfamily T cells may develop the specific GVL effect of T cells for anti-CML immunotherapy.

In the previous studies, it was difficult to determine a relationship between leukemia-associated antigen and its responding TCR V β subfamilies, whether in autologous or allogeneic clonal-expanded T cells. It might due to the individual immune response to leukemia-associated antigen^[11]. In the present study, oligoclonal-expanded T cells of TCR V β 16 and V β 21 subfamilies could be found in T cells from 2 cases of cord blood and 1 case of PBL induced by CML cells

and K562 cells, while T cell culture with CD3 McAb and IL-2, similar to normal individuals, displayed a broad distribution of the TCR V β repertoire with no predominant expression of V β segment. The results might suggest the tendency that the allogeneic anti-CML T cells expressed V β 16 or V β 21. The suggestion was supported by Kondo *et al*, who showed that clonal expanded V β 16 and V β 21 T cells could be detected in PBL of two CML patients treated with DLI for relapse after allogeneic BMT^[10].

It is well known that bcr-abl fusion genes are common in CML and that the bcr-abl peptide could elicit anti-CML specific CTL in vivo and in vitro^[11]. The oligoclonal expanded V β 16 and V β 21 T cells may be an immune response to bcr-abl fusion protein. It will be proved by further analysis of the distribution of TCR V β gene repertoire and clonality of T cells induced by the bcr-abl peptide.

REFERENCES

- 1 Li Y. Leukemia associated clonal expansion TCR V β subfamily T cells. *Hematol*, 2003;8:375-384.
- 2 Dazzi F, Szydlo RM, Goldman JM. Donor lymphocyte infusions for relapse of chronic myeloid leukemia after allogeneic stem cell transplant: where we now stand. *Exp Hematol*, 1999;27:1477-1486.
- 3 Jiang YZ, Mavroudis DA, Dermime S, et al. Preferential usage of T cell receptor (TCR) V beta by allogeneic T cells recognizing myeloid leukemia cells: implications for separating graft-versus-leukemia effect from graft-versus-host disease. *Bone Marrow Transplant*, 1997;19:899-903.
- 4 Patterson AE, Korngold R. Infusion of select leukemia-reactive TCR Vbeta+ T cells provides graft-versus-leukemia responses with minimization of graft-versus-host disease following murine hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*, 2001;7:187-196.
- 5 Puisieux I, Even J, Pannetier C, et al. Oligoclonality of tumor-infiltrating lymphocytes from human melanomas. *J Immunol*, 1994;153:2807-2818.
- 6 Li Y, Yang L, Chen S, et al. Clonal expansion T cells identified in acute monoblastic leukemia by CDR3 size analysis of TCR V β repertoire using RT-PCR and genescan analysis. *Chin Med J*, 2002;115:69-71.
- 7 Maccalli C, Farina C, Sensi M, et al. TCR γ -chain variable region driven selection and massive expansion of HLA class I restricted antitumor CTL lines from HLA-A0201 melanoma patients. *J Immunol*, 1997;158: 5902-5913.
- 8 Li Y, Li R, Yang L, et al. Study on clonal expansion and specific cytotoxicity of autologous and allogeneic T cells induced by M_{2b} cells. *Blood*, 2001; 99:122a.

- 9 Farace F, Orlanducci F, Dietrich PY, et al. T cell repertoire in patients with B chronic lymphocytic leukemia. Evidence for multiple in vivo T cell clonal expansions [J]. *J Immunol*, 1994;153:4281-4290.
- 10 Kondo Y, Shiobara S, Nakao S. Identification of T-cell clones showing expansion associated with graft -vs - leukemia effect on chronic myelogenous leukemia in vivo and in vitro. *Exp Hematol*, 2001;29:471-476.
- 11 Norbury LC, Clark RE, Christmas SE. b3a2 BCR-ABL fusion peptides as targets for cytotoxic T cells in chronic myeloid leukaemia. *Br J Haematol*, 2000; 109:616-621.