

Comparison of Wilms' tumor antigen 1-specific T lymphocyte generation soon after nonmyeloablative allogeneic stem-cell transplantation in acute and chronic leukemia patients

Wei Li · Zuo HongLi · Sun XueDong · Liu TieQiang · Guo Mei ·
Liu GuangXian · Sun QiYun · Qiao JianHui · Wang DanHong ·
Yu ChangLin · Hu KaiXun · Dong Zheng · Ai HuiSheng

Received: 9 November 2009 / Revised: 28 February 2010 / Accepted: 2 March 2010
© The Japanese Society of Hematology 2010

Abstract Clinical studies have shown that NST has better therapeutic results with chronic myelogenous leukemia (CML) than acute leukemia (AL), but whether the generation of graft-versus-leukemia (GVL) effects is different between AL and CML patients early after NST has not yet been studied, so we used a pentamer-staining technique in combination with ICCS to detect WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells in these two groups of patients. Results showed that the emergence time of WT1⁺CD8⁺CTL/WT1⁺Tc1 cells after NST in AL patients was similar to that in CML patients ($P = 0.58$), while the emergence time of WT1⁺Th1 cells after NST in AL patients was shorter than in CML patients ($P = 0.047$). Furthermore, the peak proportions of WT1⁺CD8⁺CTL/WT1⁺Tc1 ($P > 0.05$) and WT1⁺Th1 ($P > 0.05$) cells were similar between AL and CML patients, and the increased rates ($P > 0.05$) and elevated levels ($P > 0.05$) of WT1-specific T cells were not statistically different between the groups after G-CSF mobilized donor mononuclear cell infusion. In addition, the reconstruction of lymphocyte subsets ($P > 0.05$) and CD4/8 ratios ($P > 0.05$) in AL patients were not statistically different from those in CML patients within 180 days after NST. These results suggested that WT1 maybe induces similar GVL effects in both AL and CML patients early after NST.

Keywords Graft-versus-leukemia effect · Nonmyeloablative allogeneic stem-cell transplantation · Acute leukemia · Chronic myelocytic leukemia

1 Introduction

The graft-versus-leukemia (GVL) effect is one of the primary rationales for allogeneic stem-cell transplantation (Allo-SCT) to cure leukemia [1, 2]. Donor lymphocyte infusion (DLI) can induce GVL effects and successfully treat recurrent acute and chronic leukemias after bone marrow transplantation [3, 4]. The determinants of GVL effects have until recently been based on clinical therapeutic effects that lack sensitive and reliable quantitative indices. Rezvani et al. [5] used HLA-tetramers and intracellular cytokine staining to detect the emergence and proportion of WT1⁺CD8⁺CTL from peripheral blood samples of 10 acute lymphoblastic leukemia patients after Allo-SCT. They found that after transplantation, the number of WT1⁺CD8⁺CTL cells was significantly increased and negatively correlated with WT1 gene expression and leukemia relapse. They suggested that post-transplantation WT1⁺CD8⁺CTL proportion could be used as an indirect index for GVL effects. NST, a promptly established form in curing leukemia in last decade, has been citified to be an effective therapy platform on which elimination of MRD depends more on GVL effect, owing to the reduced cytotoxicity of conditioning regimes. A number of clinical studies have found that NST and DLI have better therapeutic effects in chronic myelogenous leukemia (CML) patients than in acute leukemia (AL) patients [1, 4, 6–9], but whether these differences are caused by distinct GVL effects generated in CML and AL patients requires further research. It has been reported that T lymphocytes (Tc1/Th1) that secrete IL-2 and IFN γ are important effector lymphocytes

W. Li · Z. HongLi · S. XueDong · L. TieQiang · G. Mei ·
L. GuangXian · S. QiYun · Q. JianHui · W. DanHong ·
Y. ChangLin · H. KaiXun · D. Zheng · A. HuiSheng (✉)
Department of Hematology, Affiliated Hospital of Academy
of Military Medicine Science, 100071 Beijing, China
e-mail: huishengai@163.com

W. Li
e-mail: weili75114cn2001@yahoo.com.cn

mediating GVL effects [10]. In a previous study, we used an HLA-tetramer to detect $WT1^{+}CD8^{+}CTL$ after NST [11]. In this study, a more sensitive pentamer-staining technique was used in combination with intracellular $IFN\gamma$ staining to study the generation and proportion of $WT1^{+}CD8^{+}CTL/WT1^{+}Tc1$ and $WT1^{+}Th1$ cells in AL and CML patients early after NST, and to investigate whether different GVL effects could be induced in different groups of patients early post-NST.

2 Patients, materials and methods

2.1 Materials

Anti-HLA-DR, anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CD16 and anti-CD56 antibodies and $IFN\gamma$ were purchased from Beckman Coulter Inc. PMA and Io were purchased from Sigma. WT1₁₂₆₋₁₃₄ (RMFPNAPYL) and HIV-Gag nine-peptide were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. HLA-A*0201/HIV-Gag-pentamer and HLA-A*0201/WT1-pentamer were purchased from Oxford Proimmune Ltd (UK). EPICSxL-MCL Flow Cytometer was from Beckman Coulter Inc.

2.2 Subjects studied

Fourteen HLA-A*0201-positive patients with AL or CML who received an HLA-identical NST between February 2007 and May 2008 were enrolled in this study. All the cases were diagnosed according to French–American–British classification, and confirmed by immunophenotyping and cytogenetics. Study participants included 7 AL patients and 7 CML patients. Follow-ups ranged from 2 to 17 months; none of the patients had recurrences during follow-up. The basic clinical characteristics of all the patients are shown in Table 1. NST conditioning, donor peripheral blood stem-cell mobilization and collection, and graft-versus-host disease (GVHD) prophylaxis were designed and performed according to the protocol recommended by the Chinese NST Collaborative Union [12].

Ten of the patients without acute GVHD (aGVHD) by 30 days post-NST were given a G-CSF-mobilized donor mononuclear cell infusion (GDMI) treatment with a dosage of $2\text{--}3.0 \times 10^8$ monocytes/kg [12].

2.3 Determination of donor-recipient chimerism after NST

Donor hematopoietic chimerism was evaluated using semi-quantitative polymerase chain reaction (PCR)-based analysis of short tandem repeats [12].

2.4 Detection of WT1 gene expression

The expressions of WT1 in both donor and recipient before and after NST were determined with RT-PCR [13]. GAPDH was used as an internal reference, and relative expression of WT1 was represented as $WT1/GAPDH$.

2.5 Detection of $WT1^{+}CD8^{+}CTL$ by WT1-specific HLA-pentamer staining

Peripheral blood samples were collected before transplantation and on post-transplantation days 14, 30, 60, 90, 120, 150 and 180, and subjected to HLA-A*0201/WT1-pentamer staining according to the manufacturer's instruction. For long-term follow-ups, the blood samples were collected at 30-day intervals until loss of follow-up. The HLA-A*0201/HIV-Gag-pentamer was used as a negative control. $CD8^{+}$ gated cells were further analyzed for pentamer staining, and cells double positive for HLA-A*0201/WT1-pentamer and $CD8$ staining were considered to be $WT1^{+}CD8^{+}CTL$. $WT1^{+}CD8^{+}CTL$ proportions were reported as WT1-specific cells among total $CD8^{+}T$ cells [5, 14]. The proportion of cell staining in the negative control was adjusted to 0%.

2.6 Flow cytometry analysis of intracellular $IFN\gamma$ expressions in $WT1^{+}Tc1$ cells

Peripheral blood samples were collected at the same time points as in pentamer staining. Intracellular cytokine detection was performed as described in previous research [14]. Briefly, after $WT1_{126-134}$ (RMFPNAPYL) stimulation, the intracellular $IFN\gamma$ protein levels were detected with flow cytometry using Beckman Coulter's EPICS XL-MCL. HIV-Gag nine-peptide (20 $\mu\text{g/mL}$) and PMA (10 $\mu\text{g/mL}$) plus Ionomycin (10 $\mu\text{g/mL}$) were used as negative and positive controls, respectively. $CD3^{+}$ cells were gated and the percentage of each T cell subset in the control group was adjusted to 0%. The Tc1 subset ($WT1^{+}Tc1$ cells) generated after WT1 stimulation was reported as the proportion of WT1-specific cells among $CD3^{+}CD8^{+}$ cells.

2.7 Absolute lymphocyte counting and determination of lymphocyte subsets

The proportions of $CD3^{+}T$, $CD3^{+}CD4^{+}T$, $CD3^{+}CD8^{+}T$, $CD4^{+}CD25^{+}Treg$, $CD3^{+}HLA-DR^{+}T$, $CD3^{-}CD16^{+}CD56^{+}$ and $CD3^{+}CD16^{+}CD56^{+}$ cell subsets in both AL and CML patients before NST and at post-transplantation days 30, 90 and 180 were determined by flow cytometry. The ratio of $CD4^{+}/CD8^{+}T$ cells was calculated at each time point.

Table 1 Basic clinical characteristics of 14 leukemia patients

Patients	Diagnosis	Age (years)	Gender	GDPB/LI	Presence of HLA-A*0201	Blood type	Status before NST	Chimerism type	GVHD (situation, grade, time) in observation period	Relapse(days) in observation period	Peak value of WT1 ⁺ CD8 ⁺ CTL proportion % (day after NST)		Peak value of WT1 ⁺ Tc1 proportion % (day after NST)		Peak value of WT1 ⁺ Th1 proportion % (day after NST)	
											Before NST	After NST	Before NST	After NST	Before NST	After NST
1	CML	28	F/F	44	Yes/yes	O/A	CP	FDC	Skin + liver (extensive, 130d)	None	0	0.13 (90)	0	1.34 (180)	0	0.49 (60)
2	CML	22	F/M	None	Yes/yes	A/A	CP	FDC	Skin (1,23d); extensive (6 m)	None	0	0.72 (180)	0.66	1.5 (90)	0	0.7 (180)
3	CML	42	M/M	35	Yes/yes	A/O	CP	FDC	Skin(II,45d)	None	0	0.32 (60)	0	4.49 (14)	0	1.65 (30)
4	CML	36	M/M	35	Yes/Yes	A/O	CP	FDC	None	None	0	0.08 (14)	0	0.3 (30)	0.07	0.19 (30)
5	CML	31	M/M	None	Yes/yes	O/O	CP	FDC	Skin(1,14d)	None	0	0.08 (90)	0	0.9 (120)	0	0.28 (60)
6	CML	43	M/M	35	Yes/Yes	A/A	AP	FDC	Skin + oral cavity (extensive, 5 m)	None	0	0.51 (30)	0	1.57 (150)	0	0.55 (60)
7	CML	50	M/M	35	Yes/Yes	O/O	CP	FDC	Skin (1,47d,105d)	None	0	0.14 (90)	0	0.75 (150)	0.12	2.76 (150)
8	AML	10	M/M	None	Yes/yes	B/B	CR2	FDC	None	None	0.26	0.25 (14)	0.2	2.75 (14)	0.29	2.3 (14)
9	AML	54	M/M	35	Yes/yes	O/O	CR1	FDC	Oral cavity (limited, 110d)	None	0.06	0.13 (180)	0.1	0.19 (90)	0	0.23 (60)
10	AML	47	M/M	35	Yes/yes	AB/A	CR1	FDC-MC-FDC	Skin (limited,120d)	None	0	0.11 (90)	0	0.27 (180)	0	0.87 (180)
11	AML	43	M/M	None	Yes/yes	A/A	CR1	MC-FDC	Skin (1,25d)	None	0	0.22 (30)	0	0.49 (30)	0	0
12	AML	48	M/F	38	Yes/yes	O/O	CR1	MC-FDC	Skin (limited,155d)	None	0	0.1 (150)	0	0.38 (150)	0	0.5 (150)
13	ALL	22	M/F	35	Yes/yes	O/A	CR2	FDC	Intestinal tract (II,100d)	None	0	0.31 (30)	0	2.7 (14)	0.2	2.94 (14)
14	ALL	34	M/F	36	Yes/yes	A/A	CR1	MC-FDC	Intestinal tract (I,80d;III,110d)	None	0	0.45 (14)	0	0.39 (120)	0	0.9 (60)

GDM/IL G-CSF-mobilized donor mononuclear cell infusion, *FDC* full donor chimerism, *MC* mixing chimerism, *FDC-MC-FDC* donor cell chimerism status changes from FDC to MC and then to FDC at last, *MC-FDC* donor cell chimerism level changes from MC to FDC

2.8 Statistical analysis

Each positive rate was analyzed using chi-square and Fisher's exact tests. Comparisons of AL and CML groups were performed using rank sum tests. In two-sided tests, differences with P values lower than 0.05 and 0.01 were considered statistically different and significantly different, respectively. The peak proportions of WT1-specific T cells between AL and CML patients were analyzed using rank sum tests.

3 Results

3.1 Expression of WT1 gene before NST in donors and recipients

Of 14 donors, 2 donors (Nos. 2 and 6) had low levels of WT1 gene expression (WT1/GAPDH ratios 3.1×10^{-7} and 1.4×10^{-7}), but WT1 gene expression was not found in any of the remaining 12 patients. The 14 patients had varying degrees of WT1 gene expression (WT1/GAPDH ratios were from 1.65×10^{-5} to 1.2×10^{-4}) before NST.

3.2 WT1⁺CD₈⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells were increased in recipients after NST, compared to levels prior to NST or levels in donors

WT1⁺CD₈⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells were not detected in donors using pentamer and intracellular IFN γ -staining techniques. Among the 14 patients, 2 AML patients (Nos. 8 and 9) had low levels of WT1⁺CD₈⁺CTL (0.26 and 0.06%) cells prior to NST; 2 CML and 2 AML patients (Nos. 2, 3, 8 and 9) had low levels of WT1⁺Tc1 cells (0.66, 0.1, 0.2 and 0.1%) and WT1⁺Th1 cells (0.07, 0.12, 0.29 and 0.2%) before NST. No other patients had WT1⁺CD₈⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells prior to NST.

WT1⁺CD₈⁺CTL cells (with proportions ranging from 0 to 0.72%) and WT1⁺Tc1 cells (with proportions ranging from 0 to 4.49%) were detected in all patients post-NST using pentamer and intracellular IFN γ staining, and WT1⁺Th1 cells were found in 13 patients (range 0.19–2.94%). Post-NST proportions of WT1⁺CD₈⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells in all patients were significantly higher than those prior to NST ($P < 0.01$) and those in donors ($P < 0.01$) (Fig. 1). The proportion of WT1⁺CD₈⁺CTL/WT1⁺Tc1 ($P > 0.05$) and WT1⁺Th1 ($P > 0.05$) cells after NST in AL patients was not significantly different from those in CML patients.

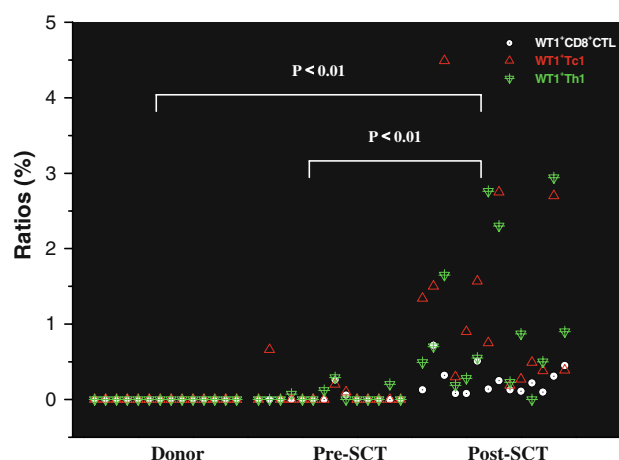


Fig. 1 Levels of WT1-specific T lymphocytes in donor and recipient before and after NST. WT1⁺CD₈⁺CTL, WT1⁺Tc1 and WT1⁺Th1 cells could not be detected in donors, while low levels were detected in leukemia patients prior to transplantation. After transplantation, all three types of cells were significantly increased in recipients ($P < 0.01$)

3.3 First detection time distributions of WT1⁺CD₈⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells in AL and CML patients after NST

After NST, generation of WT1⁺CD₈⁺CTL cells in AL patients was first detected at day 14 (2 patients), day 30 (3 patients), day 90 (1 patient) and day 150 (1 patient). These cells were detected in CML patients at day 14 (1 patient), day 30 (3 patients), day 60 (1 patient), day 90 (2 patients). Within 30 days after NST, WT1⁺CD₈⁺CTL cell levels in AL and CML patients were 71.43 and 57.14%, respectively. Although the proportion of WT1⁺CD₈⁺CTL cells in patients with AL was slightly higher than in CML patients, the difference was not statistically significant ($P = 0.58$).

WT1⁺Tc1 cells were generated in all AL and CML patients within 30 days after NST. Generation of WT1⁺Tc1 cells in the AL patients was first detected at day 14 (2 patients), and day 30 (5 patients). These cells were first detected in CML patients at day 14 (3 patients), and day 30 (4 patients), similar to the results in AL patients.

In addition, 6 out of 7 AL patients were generating WT1⁺Th1 cells within 30 days after NST, including 2 patients at day 14 and 4 patients at day 30. WT1⁺Th1 cells were induced in all CML patients, and first detected at day 14 (1 patient), day 30 (3 patients) and day 60 (3 patients). Statistical analysis indicated that the positive proportion of WT1⁺Th1 cells generated within 30 days after NST was significantly higher in AL patients than in CML patients ($P = 0.047$).

3.4 Induced levels of $WT1^{+}CD8^{+}CTL/WT1^{+}Tc1$ and $WT1^{+}Th1$ cells in AL and CML patients after NST

Pentamer and intracellular cytokine staining identified $WT1^{+}$ -specific T lymphocytes from blood samples at days 14, 30 and 60 for all patients, at day 90 for 12 patients, and at days 120, 150 and 180 for 11 patients. The proportion of $WT1^{+}CD8^{+}CTL/WT1^{+}Tc1$ and $WT1^{+}Th1$ cells at different time points after NST in each group was calculated (Fig. 2a–c). As shown in Fig. 2, proportions of $WT1^{+}CD8^{+}CTL/WT1^{+}Tc1$ and $WT1^{+}Th1$ cells at different time points within 180 days after NST were similar between AL and CML patients. Proportions of $WT1^{+}CD8^{+}CTL/WT1^{+}Tc1$ ($P > 0.05$) and $WT1^{+}Th1$ ($P > 0.05$) cells at different time points in AL patients were not significantly different from those in CML patients.

The peak proportions of $WT1^{+}$ -specific T lymphocytes in AL and CML patients following NST were then examined. As shown in Table 1, $WT1^{+}CD8^{+}CTL$ cells peaked at 0.1–0.45% and 0.08–0.72% among AL and CML patients, respectively. Peak levels of $WT1^{+}CD8^{+}CTL$ cells higher than 0.4% were found in 1 AL and 2 CML patients. Average peak values of $WT1^{+}CD8^{+}CTL$ were not significantly different between AL and CML patients ($P = 0.52$).

Intracellular $IFN\gamma$ staining indicated that $WT1^{+}Tc1$ cells peaked at 0.19–2.75% and 0.30–4.49% among AL and CML patients, respectively. Peak levels higher than 1.0% were found in 2 AL and 4 CML patients. $WT1^{+}Th1$ cells peaked at 0.23–2.94% (AL) and 0.19–2.76% (CML); peak values higher than 1.0% were found in 2 AL and 2 CML patients. Peak value differences between the groups were not significant ($P = 0.391$).

3.5 Impact of GDMI on generation of $WT1^{+}CD8^{+}CTL/WT1^{+}Tc1$ and $WT1^{+}Th1$ cells

Ten patients (5 AL and 5 CML) received GDMI at post-NST day 35. Pentamer staining at post-NST days 30, 60 and 90 indicated that 2 out of these 5 AL patients (Nos. 10 and 14) had increased $WT1^{+}CD8^{+}CTL$ proportions after GDMI, and these levels increased from 0 to 0.11% at post-NST day 90 and 0 to 0.10% at day 60 (Fig. 3a). Similarly, $WT1^{+}CD8^{+}CTL$ cells in 3 out of 5 CML patients (Nos. 1, 3, and 7) were significantly increased after GDMI, from 0 to 0.13% at post-NST day 90, from 0.07 to 0.32% at day 60 and from 0 to 0.14% at day 90 (Fig. 3a). Differences between the two groups were not statistically different ($P = 0.83$; $P = 0.54$).

Intracellular cytokine staining indicated that $WT1^{+}Tc1$ cells were elevated in 4 out of 5 AL patients (Nos. 9, 12, 13 and 14) after GDMI, from 0.05 to 0.19% at day 90, 0.08 to 0.19% at day 60, 0 to 0.17% at day 90, and from 0.11 to

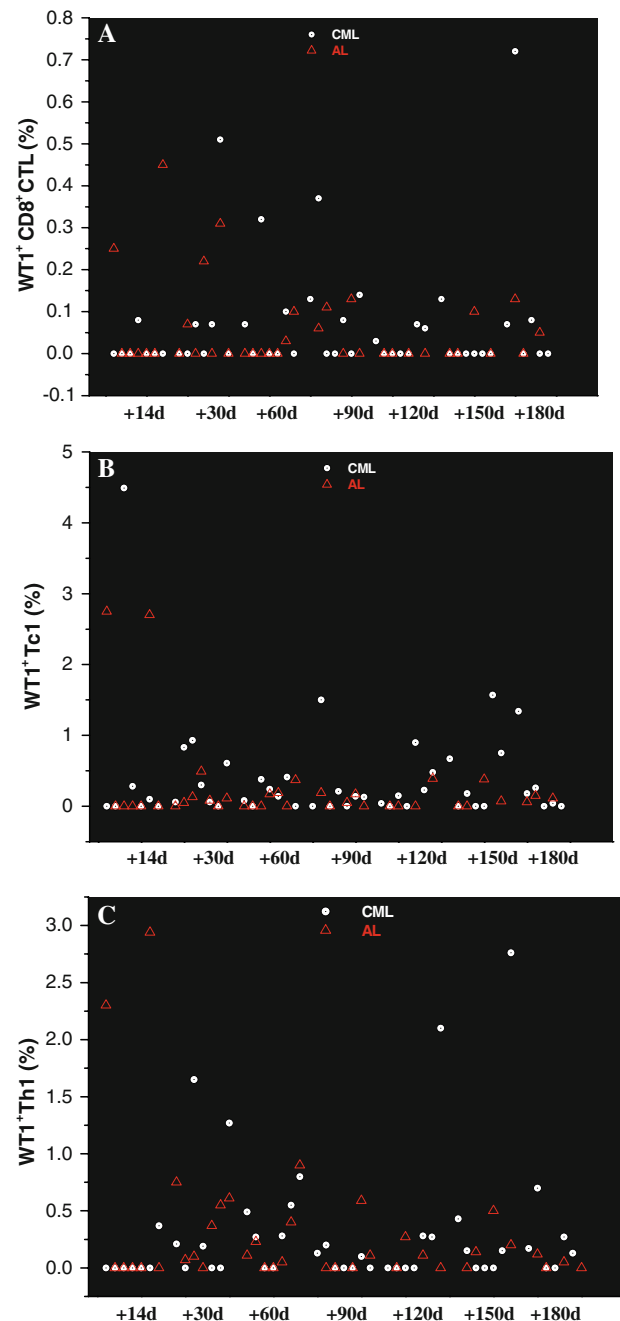


Fig. 2 Detection of $WT1$ -specific T lymphocytes in AL and CML patients soon after NST. **a** Proportion of $WT1^{+}CD8^{+}CTL$ cells at different time points within 180 days after NST. After NST, the induced generation of $WT1^{+}CD8^{+}CTL$ cells were similar between AL and CML patient groups. **b** $WT1^{+}Tc1$ cells at different time points within 180 days post-NST. After NST, the induced generations of $WT1^{+}Tc1$ cells were similar between groups. **c** $WT1^{+}Th1$ cells at different time points within 180 days after NST. Generation of $WT1^{+}Tc1$ cells in AL patients was similar to CML patients

0.37% at day 60. $WT1^{+}Tc1$ cells were elevated in 2 out of 5 CML patients (Nos. 1 and 6), from 0.06 to 0.08% at day 60 and 0 to 0.41% at day 60 (Fig. 3b). Group differences were not statistically different ($P = 0.20$; $P = 0.49$).

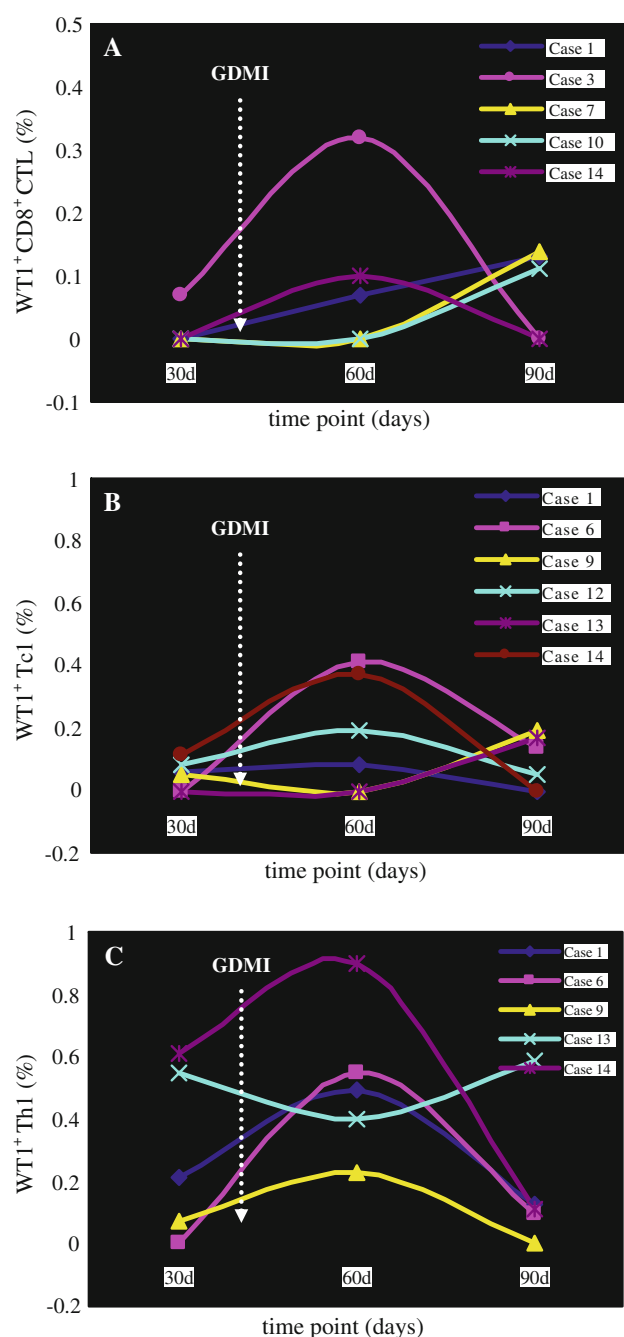


Fig. 3 The impact of GDMI on generation of WT1-specific T lymphocytes in AL and CML patients. **a** WT1⁺CD8⁺CTL cells after GDMI. Pentamer staining was performed at 30, 60 and 90 days post-NST, and increases in WT1⁺CD8⁺CTL were found in 2 AL patients (Nos. 10 and 14) and 3 CML patients (Nos. 1, 3 and 7). **b** WT1⁺Tc1 cells after GDMI. Intracellular cytokine detection indicated that increases in WT1⁺Tc1 cells were found in 4 AL patients (Nos. 9, 12, 13 and 14) and 2 CML patients (Nos. 1 and 6). **c** Generation of WT1⁺Th1 cells after GDMI. WT1⁺Th1 cells were significantly increased in 3 AL patients (Nos. 9, 13, 14) and 2 CML patients (Nos. 1 and 6)

WT1⁺Th1 cells were elevated in 3 of 5 AL patients (Nos. 9, 13 and 14), from 0.07 to 0.23% at day 60, 0.55 to 0.59% at day 90, and 0.61 to 0.90% at day 60. WT1⁺Th1

cells were elevated in 2 of 5 CML patients (Nos. 1 and 6), from 0.21 to 0.49% and from 0 to 0.55% at post-NST day 60 (Fig. 3c). Differences between groups were not significantly different ($P = 0.53$; $P = 0.77$).

3.6 WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells were negatively correlated with WT1 gene expression

WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells increased rapidly after NST and then fluctuated at high levels in all patients. Conversely, WT1 gene expressions were rapidly down-regulated after NST and then maintained at extremely low levels (WT1/GAPDH values ranged from 10^{-6} to 10^{-5}). As shown in Fig. 4, the phases of WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells were not exactly the same, and while both types of cells fluctuated after NST, the levels of WT1/GAPDH fluctuated at low levels in both AL and CML patients (Fig. 4).

3.7 Comparison of immune reconstitution between AL and CML patients soon after NST

Blood samples were taken prior to NST and at post-NST days 30, 90 and 180. Flow cytometry analysis indicated that the proportions of CD3⁺T, CD3⁺CD4⁺T, CD3⁺CD8⁺T, CD4⁺CD25⁺Treg, CD3⁺HLA-DR⁺T, CD3⁺CD16⁺CD56⁺ and CD3⁺CD16⁺CD56⁺ cell subsets and the CD4/8 ratios during the early stages after NST were not statistically different between AL and CML patients ($P > 0.05$). As shown in Fig. 5, the distribution of lymphocyte subsets within 180 days after NST in AL patients was similar to those of CML patients.

4 Discussion

NST can effectively induce GVL effects in leukemia patients. We found that after NST, WT1⁺CD8⁺CTL/WT1⁺Tc1 cells were significantly elevated in 14 patients, and WT1⁺Th1 cells were increased in 13 patients ($P < 0.01$). GDMI promoted an increase in WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 in some patients. In addition, generation of WT1-specific T lymphocytes was correlated with a down-regulation of WT1 gene expression. These results were consistent with the findings reported by Rezvani et al., which demonstrated that after NST, WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells play important roles in mediating GVL effects [5].

Previous clinical studies indicated that the 3- and 5-year disease-free survival (DFS) rates of CML patients after transplantation are 80 ~ 90% and 70 ~ 80%, respectively, significantly higher than those of AL patients [1, 7,

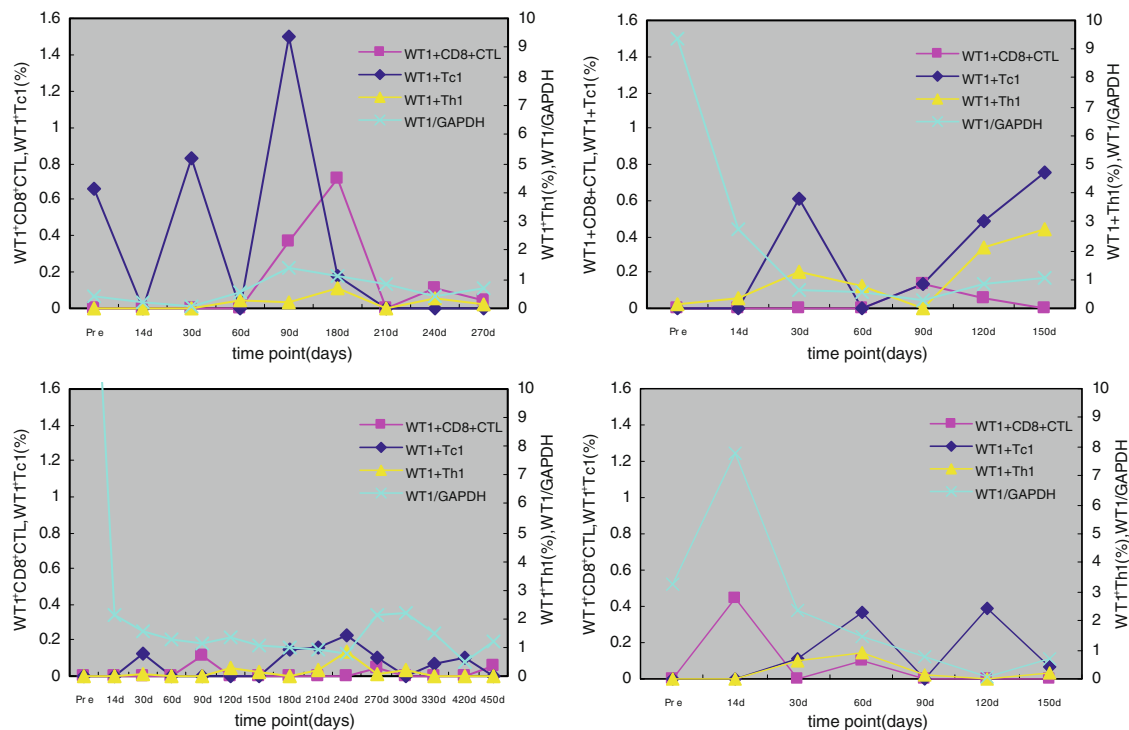


Fig. 4 WT1 gene expression was negatively correlated with generation of WT1-specific T lymphocytes after NST. WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells and expression levels of WT1 gene in No. 2 (upper left panel), No. 7 (upper right panel), No. 10 (lower left panel) and No. 14 (lower right panel) at time points after NST.

8, 15, 16]. These results suggested that after transplantation, GVL effects might be more potent in CML patients than in AL patients. In this study, through pentamer and intracellular IFN γ staining, we found that WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells were first detected within 30 days after NST in both AL and CML patients. Although the generation of WT1⁺Th1 cells began earlier in AL patients ($P = 0.047$), generation rate and fluctuation of WT1⁺CD8⁺CTL/WT1⁺Tc1 cells were not significantly different between the two groups ($P > 0.05$). During the first 180 days post-NST, average and highest levels of WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells were slightly but not significantly higher in CML patients than in AL patients ($P > 0.05$). These results suggest that after transplantation, generation of WT1-specific T cells may be similar between AL and CML patients, which indicated that NST does not have different GVL effects in the two groups.

It has been reported that 70 ~ 80% of the hematological and genetic recurrence of CML after transplantation can be avoided by sequential treatments of DLI or GDMI, but only 20 ~ 40% of recurrence in AL patients can be prevented by the same treatments, suggesting that DLI and GDMI have better therapeutic effects in CML patients than in AL patients [4, 6, 9, 17]. However, whether the different

WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells were significantly increased at different phases after NST, and this increase was correlated with down-regulations of WT1/GAPDH ratios in both AL and CML patients

therapeutic effects are associated with distinct GVL effects generated by DLI or GDMI is not clear. Marijt et al. used hematopoiesis-restricted minor histocompatibility antigen HA-1- or HA-2-specific T cells to treat relapsed hematopoietic malignancies (two CML and one multiple myeloma [MM]), and found that cells positive for tetramer staining could be detected 5–7 weeks after transfusion [18]. Because GDMI was performed post-NST day 35, we compared the proportions and levels of WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells before and after GDMI at post-NST days 30, 60 and 90 in AL and CML patients. We found that after GDMI, the proportion of WT1⁺CD8⁺CTL cells increased in 2 of 5 AL and 3 of 5 CML patients, and the proportion of WT1⁺Tc1 cells were elevated in 4 of 5 AL and 2 of 5 CML patients. Elevated levels of WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells were not statistically different between AL and CML patients ($P > 0.05$). These results demonstrate that GDMI can effectively promote generation of WT1-specific T lymphocytes in both AL and CML patients. These results are similar to the induced generation of WT1-specific T lymphocytes after NST. In addition, in this study, detection of WT1⁺CD8⁺CTL/WT1⁺Tc1 and WT1⁺Th1 cells after GDMI took place slightly earlier than reported by Marijt et al., which may be associated with different sensitivities

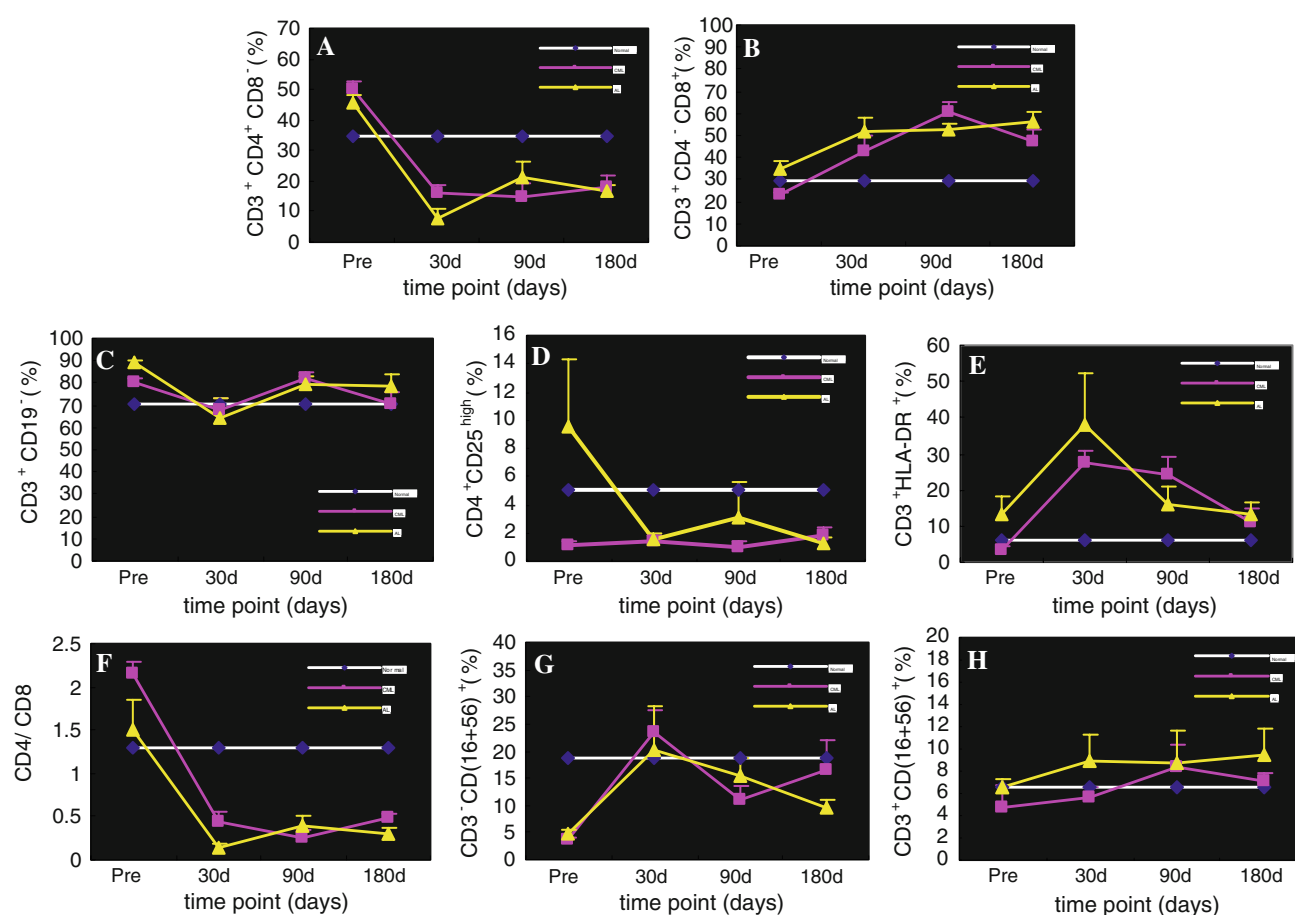


Fig. 5 Reconstitution of lymphocyte subsets within 180 days after NST in AL and CML patients. Lymphocyte subset distributions within 180 days after NST were similar between AL and CML patient groups, but not statistically different ($P > 0.05$). **a** $CD3^+CD4^+CD8^+$ T cells;

b $CD3^+CD4^+CD8^+$ Treg; **c** $CD3^+CD19^+$ T cells; **d** $CD4^+CD25^{high}$ Treg; **e** $CD3^+HLA-DR^+$ T cells; **f** $CD4/CD8$; **g** $CD3^+CD16^+CD56^+$ -NK cells; **h** $CD3^+CD16^+CD56^+$ -NKT cells

of detection methods and differences in immune environments in leukemia patients after NST.

Furthermore, since lymphocytes are a key factor in regulation of the therapeutic effects of DLI [19], they may play an important role in the generation of GVL effects after transplantation. We analyzed the reconstitution of lymphocyte subsets in 14 patients within 180 days after NST and found that the distribution of these subsets and CD4/8 ratios at time points after NST were not significantly different between AL and CML patients, in accordance with the alterations of $WT1^+CD8^+CTL/WT1^+Tc1$ and $WT1^+Th1$ cells between these groups after NST and GDMI.

In summary, although generation phases and levels of $WT1^+CD8^+CTL/WT1^+Tc1$ and $WT1^+Th1$ cells after NST and GDMI were different between groups and each group showed slightly different responses to GDMI, these differences may be not significant. After NST, $WT1$ -induced GVL effects are not statistically different between groups of AL and CML patients. Differential therapeutic effects of

NST and DLI in AL and CML patients may be caused by inherent sensitivities of AL and CML towards a similar GVL effect [1, 4, 18, 20, 21], rather than by induction of different GVL effects. However, as the sample size was small and follow-up duration relatively short, expanded study of induced GVL effects is necessary.

References

- Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*. 1990;75(1):555–62.
- Weiden PL, Flournoy N, Thomas ED, Prentice R, Fefer A, Buckner CD, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. *N Engl J Med*. 1979;300:1068–73.
- Van Rhee F, Kolb H-J. Donor leukocyte transfusions for leukemic relapse. *Curr Opin Hematol*. 1995;2:423–9.
- Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, et al. Graft-versus-leukemia effect of donor

- lymphocyte transfusions in marrow grafted patients. *Blood*. 1995;86:2041–50.
5. Rezvani K, Yong AS, Savani BN, Mielke S, Keyvanfar K, Gostick E, et al. Graft-versus-leukemia effects associated with detectable Wilms tumor-1 specific T lymphocytes after allogeneic stem-cell transplantation for acute lymphoblastic leukemia. *Blood*. 2007;110(6):1924–32.
6. Michallet AS, Nicolini F, Fürst S, et al. Outcome and long-term follow-up of alloreactive donor lymphocyte infusions given for relapse after myeloablative allogeneic hematopoietic stem cell transplantations (HSCT). *Bone Marrow Transplant*. 2005;35(6):601–8.
7. Liubimova LS, Savchenko VG, Mendeleva LP, et al. Effectiveness of allogeneic bone marrow transplantation in patients with acute leukemia in complete remission and in patients with chronic myeloid leukemia during the chronic phase. *Ter Arkh*. 1999;71(7):27–32.
8. Apperley JF, Mauro FR, Goldman JM, et al. Bone marrow transplantation for chronic myeloid leukemia in first chronic phase: importance of a graft-versus-leukemia effect. *Br J Haematol*. 1988;69:239–45.
9. Huang XJ, Liu DH, Liu KY, et al. Donor lymphocyte infusion for the treatment of leukemia relapse after HLA-mismatched/haploidentical T-cell-replete hematopoietic stem cell transplantation. *Haematologica*. 2007;92(3):414–7.
10. Fowler DH, Breglio J, Nagel G, Hirose C, Gress RE. Allospecific CD4+, Th1/Th2 and CD8+, Tc1/Tc2 populations in murine GVL: type I cells generate GVL and type II cells abrogate GVL. *Biol Blood Marrow Transplant*. 1996;2(3):118–25.
11. Sun WJ, Guo ZK, Ai HS. The antitumor activity of G-CSF-mobilized peripheral blood mononuclear cells activated by IL-2 alone or in combination with IL-12. *Zhongguo Shi Yan Xue Ye Xue Za Zhi*. 2005;13:687–91.
12. Hui-Sheng A, Chun-Hua Zhao R, Guo M, et al. Mixed hematopoietic chimerism—a new strategy for preventing GVHD in nonmyeloablative allogeneic stem cell transplantation. *Exp Hematol*. 2002;30:119.
13. Asemisen AM, Keilholz U, Tenzer S, Müller M, Walter S, Stevanovic S, et al. Identification of a highly immunogenic HLA-A*01-binding T cell epitope of WT1. *Clin Cancer Res*. 2006;12:7476–82.
14. Rezvani K, Brenchley JM, Price DA, Kilical Y, Gostick E, Sewell AK, et al. T-Cell responses directed against multiple HLA-A*0201-restricted epitopes derived from Wilms' Tumor 1 protein in patients with leukemia and healthy donors: identification, quantification, and characterization. *Clin Cancer Res*. 2005;11:8799–807.
15. Chen MH, Chiou TJ, Lin PC, et al. Comparison of myeloablative and nonmyeloablative hematopoietic stem cell transplantation for treatment of chronic myeloid leukemia. *Int J Hematol*. 2007;86(3):275–81.
16. Tauro S, Craddock C, Peggs K, et al. Allogeneic stem-cell transplantation using a reduced-intensity conditioning regimen has the capacity to produce durable remissions and long-term disease-free survival in patients with high-risk acute myeloid leukemia and myelodysplasia. *J Clin Oncol*. 2005;23(36):9387–93.
17. Choi SJ, Lee JH, Lee JH, et al. Treatment of relapsed acute myeloid leukemia after allogeneic bone marrow transplantation with chemotherapy followed by G-CSF-primed donor leukocyte infusion: a high incidence of isolated extramedullary relapse. *Leukemia*. 2004;18(11):1789–97.
18. Marijt WA, Heemskerk MH, Kloosterboer FM, et al. Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proc Natl Acad Sci USA*. 2003;100:2742–7.
19. Shaw BE, Byrne JL, Das-Gupta E, Carter GI, Russell NH. The impact of chimerism patterns and predonor leukocyte infusion lymphopenia on survival following T cell-depleted reduced intensity conditioned transplants. *Biol Blood Marrow Transplant*. 2007;13(5):550–9.
20. Smit WM, Rijnbeck M, van Bergen CAM, et al. Dendritic cells generated from FACS sorted chronic myeloid leukemia (CML) precursor cells express BCR/ABL, and are potent stimulators for allogeneic T cells. *Br J Haematol*. 1996;93:313a.
21. Mittermueller J, Kolb HJ, Gerhartz HH, Wilmanns W. In vivo differentiation of leukemic blasts and effect of low dose ara-c in a marrow grafted patient with leukemic relapse. *Br J Haematol*. 1986;62:757–62.