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RESEARCH ARTICLE

T-helper-1 (Th1) and Th2 cytokines after allogeneic hematopoietic stem cell transplantation (allo-HSCT)

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ABSTRACT

Dysregulation of T-helper (Th)1 (IFN-γ, IL-2, TNF-α) and Th2 (IL-4, IL-5, IL-10) type cytokines has been suggested in the pathogenesis of graft versus host disease (GvHD). We analyzed intracellular cytokine expression in Th ($CD4^+$) lymphocytes in 23 patients undergoing allo-hematopoietic stem cell transplantation (HSCT) both in the week of neutrophil engraftment (S1) and on the posttransplant 100^{th} day (S2). $CD4^+TNF\text{-}\alpha^+$ cells increased from 22% at S1 to 26% at S2 in the allo-peripheral blood (PB) group. $CD4^+IL\text{-}2^+$ cells at S1 in the bone marrow (BM) group had a tendency to be higher than in the allo-PB group and also higher than at S2. IL-2 and TNF-α expressions at S1 were significantly higher and IL-2 expression at S2 was significantly lower in patients with grade II-IV acute GvHD compared with grade 0-I GvHD. IFN-γ expression tended to be higher at S2 in the chronic GvHD group compared with the patient without GvHD. Consequently, the data in this study support the role of Th1 cytokines in GvHD.

Key words: Cytokines, T-helper-1, T-helper-2, allogeneic transplantation

ÖZET

Allojeneik hematopoetik hücre transplantasyonu (allo-HHT) sonrası T-helper-1 (TH1) ve TH2 sitokinler

Th1 (IFN-γ, IL-2, TNF-α) ve Th2 (IL-4, IL-5, IL-10) tip sitokinler graft versus host hastalığı(GvHH) patogenezinden sorumlu olduğu düşünülmektedir. Allo-HKHT yapılan 23 hastada Th ($CD4^+$) lenfositlerde hücre içi sitokinlerin ekspresyonu hem nötrofil engraftman haftasında (S1) hem de transplantasyon sonrası 100. günde (S2) değerlendirildi. $CD4^+TNF\text{-}\alpha^+$ hücreler allo-periferik kan (PK) grubunda S1'de % 22'den S2'de % 26'ya art-

tı. Kemik iliği (Kİ) grubunda $CD4^+IL\text{-}2^+$ hücreler allo-PK grubuna göre daha yüksek olma eğiliminde idi ve S2'dekinden daha yüksekti. Düzey II-IV GvHH olan hastalarda düzey 0-1 GvHH'lilere göre S1 de IL-2 ve TNF-α ekspresyonları anlamlı yüksek ve S2 de IL-2 ekspresyonlarında anlamlı oranda düşük olduğu görüldü. Kronik GvHH'li hastalarda S2'de IFN-γ ekspresyonununda artış olma eğilimindeydi. Sonuçta, bu çalışmadan elde edilen veriler GvHH'da Th1 sitokinlerin rolünü desteklemektedir.

Anahtar kelimeler: Sitokinler, T-helper-1, T-helper-2, allojeneik transplantasyon

INTRODUCTION

Allo-hematopoietic stem cell transplantation (HSCT) is a widely used therapeutic modality in various hematological and non-hematological diseases. The most common and serious cause of posttransplant non-relapse morbidity and mortality is the development of acute severe or chronic graft versus host disease (GvHD). Acute GvHD usually develops in the first 100 days of stem cell infusion in 30-60% of recipients of HLA-matched sibling allografts and the mortality rate may reach 50% [1-4]. Chronic GvHD develops in 35–50% of patients after transplant either as an extension or after resolution of acute GvHD, or without preceding acute GvHD. The incidence of chronic GvHD in the patients surviving after the 100^{th} day of transplantation has been reported to be 40–60% [5]. GvHD results from destruction of host tissues by donor T lymphocytes expanded against foreign host antigens. Dysregulation of the cytokine networks has been suggested as a primary cause for the pathogenesis of GvHD [2,3,6,7].

The two major T cell subgroups are the T-helper ($CD4^+$) and cytotoxic killer T cells ($CD8^+$); each is further divided into type-1 and type-2 cytokine producing subgroups. T-helper 1 (Th1) cells produce gamma-interferon (IFN-γ), interleukin 2 (IL-2) and tumor necrosis factor alfa (TNF-α). T-helper 2 (Th2) cells on the other hand produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 [8].

T-helper cells respond to exogenous antigens, which are presented by antigen presenting cells with the major histocompatibility complex (MHC) Class II. Th1 cells are responsible for delayed type hypersensitivity reactions and are important in defense against infectious microorganisms. Macrophages and dendritic cells, when exposed to foreign antigens, migrate to the lymphoid organs where they present the antigen to naive T cells and produce proinflammatory cytokines such as IL-12, which induces the Th1 phenotype. These Th1 cells produce IFN-γ and activate the neighboring macrophages and facilitate the progression of type 1 responses. Activated Th1 cells also help the B-lymphocytes to produce IgG responses. Moreover, the Th1 cells are an important source of IL-2, which is important for the cytotoxic $CD8^+$ T cells during the antiviral and antitumor responses [9,10].

It has been suggested that the Th1/Th2 polarization plays an important role in development of GvHD. A wide range of cytokines are differentially expressed by activated T cells in acute and chronic GvHD [11]. Both human and experimental studies suggest that Th1 cytokines increase the risk of GvHD [12-16]. On the other hand, Th2 cytokines are thought to possess immunosuppressive effects and inhibit GvHD [11,17-21]. There are some conflicting reports about the role of cytokines, especially the Th1/Th2 balance, on the pathogenesis of GvHD [22]. Some studies report that both Th1 and Th2 cytokines contribute to the pathogenesis of GvHD [23]. However, some other studies emphasize the protective effects of IL-2 and IL-12 against GvHD [24,25].

Chronic GvHD, on the other hand, has many similarities with autoimmune diseases and it is thought to be a T-cell-mediated autoreactive disorder [5,24]. Th2 cytokines were shown to be

predominant in chronic GvHD [2,11].

The aim of this study was to determine the specific cytokine expression representing the Th1 (IFN-γ, IL-2 and TNF-α) and Th2 (IL-4, IL-5 and IL-10) balance in 23 consecutive patients undergoing allo-HSCT from their HLA full-matched siblings and association of these cytokines with the occurrence of acute and chronic GvHD. Moreover, the effect of the stem cell source, peripheral blood (PB) versus bone marrow (BM), on these parameters was also investigated.

MATERIALS AND METHODS

Patients and Allo-HSCT Procedures

Twenty-three consecutive patients (Female:Male=8:15; median age=25 years, range 14 to 45 years) undergoing allo-HSCT from their HLA-matched siblings were enrolled in this study. The study protocol was approved by the Ethics Committee, and written informed consent was obtained from every patient.

The demographic characteristics of the patients and the donors are summarized in Table 1. Of the 23 patients, 11 received PB-derived stem cells and 12 received BM as the stem cell source. The majority of the patients (n=21) received busulphan 4 mg/kg/day for 4 days orally or 4 equal intravenous doses and intravenous cyclophosphamide 60 mg/kg/day for 2 days as conditioning regimen.

Table 1. Characteristics of the patients, the donors and the allogeneic HSCT

Patients	
Gender (Female/Male)	8/15
Age (Median year; range)	25 (14-45)
Diagnosis (n)	
AML/CML/ALL/MM	11 / 9 / 2 / 1
Donors	
Gender (Female/Male)	12/11
Age (mean years; range)	30 (11-49)
Transplantation	
Stem cell source (PB/BM)	11 /12
Gender mismatch (n)	12
F→ M → MÆF	4 / 8
Blood group mismatch (n)	11
Major/Minor/Rh	4 / 5 / 2
Mean $CD34^+x10^6$/kg (Range)	3.58 (0.32-8.07)
Mean $MNCx10^8$/kg (Range)	4.10 (0.35-9.98)
Mean $CD3+x10^8$/kg (Range)	1.22 (0.05-3.54)
Mean $CD3+CD4^+$x108/kg (Range)	0.65 (0.03-2.19)

PB: Peripheral blood. BM: Bone marrow

HSCs collected from the healthy donors were infused to the patients through central venous catheter at day 0. PB-derived stem cells were mobilized by subcutaneous administration of recombinant human granulocyte colony-stimulating factor (G-CSF) (rhG-CSF; Neupogen, Amgen-Roche, USA) 10 μg/kg/day for five days. On the morning of the fifth day, 2 h after rhG-CSF, PB-derived stem cells were collected using continuous flow cell separation device (Baxter CS3000 plus, Fenwal, IL). Apheresis procedure was performed once or twice until ≥ 4 x 10^6/kg recipient (BW) $CD34^+$ stem cells were collected.

In all cases, the protocol for GvHD prophylaxis was cyclosporin A (CsA 3 mg/kg/day starting iv on day (-1) and continuing 5 mg/kg/day, orally) and short-term methotrexate ($+1^{st}$ day 15 mg/m^2/day, $+3^{rd}$ and $+6^{th}$ days 10 mg/m^2/day, iv). In patients who received BM as the stem cell source, G-CSF (5 μg/kg/day, iv) was started on the $+1^{st}$ day until the eutrophil count was > $0.5x10^9$/L on three consecutive days. Neutrophil and platelet engraftments were defined as a first day of neutrophil count of ≥ 0.5 x 10^9/L for 3 consecutive days and nontransfused platelet count of ≥ 20 x 10^9/L for 3 consecutive days, respectively. Acute and chronic GvHD were defined according to the time of occurrence of GVHD before and after the first 100 days of transplantation, respectively.

METHODS

Blood samples were drawn through the brachial veins into the heparinized vacutainer tubes in the week of neutrophil engraftment (S1) and on the posttransplant 100th day (S2) and were processed immediately at the department's quality control flow cytometry laboratory.

Isolation of Mononuclear Cells

Venous blood samples in the heparinized vacutainer tubes were diluted with PBS (phosphate buffered saline) at a ratio of 1:1. Mononuclear cells were obtained by density gradient centrifugation. Briefly, 4-6 milliliters (ml) of the diluted blood was overlayed on the top of 4 ml Ficoll-Hypaque and was centrifuged at 300 x g for 20 min at room temperature (RT). The buffy coat was collected by a sterile pipette and then the collected cells were washed twice by using sterile PBS and centrifuging at 500 x g for 5 min. The samples were diluted in RPMI-1640 in sterile tubes.

Activation of Lymphocytes

For lymphocyte activation, phorbol-myristate acetate (10 ng/ml) (PMA) and ionomycin (0.4 μg/ml) were pipetted into each tube containing patient cell samples. At the last step, 1 ml/ml Brefeldin A (Golgiplug; Pharmingen Becton Dickinson, USA) was added to the samples to inhibit cytokine release from the cells. Tubes were incubated at 37°C for 6 h. The control tubes containing the cells in the RPMI-1640 without additional treatment were incubated at +4°C for 6 h.

Table 2. The outcome of transplantation according to stem cell source

	BM (n=12) Mean±SE	PB (n=11) Mean±SE	p
Engraftment kinetics (days)			
Neutrophil >0.5x10^9/L	17±1.0	15±0.7	0.09**
Platelet >20 x10^9/L	24±2.0	13±3.5	<0.001*
Acute GvHD n (%)	7 (63.6)	7 (60.9)	0.58
Grade I/II-IV	6/1	3/4	0.49
At the time of sampling	4 (33.3)	1 (9.1)	0.18
Grade I/ II-IV	2/2	1/0	0.47
Chronic GvHD (%)	5 (41.7)	8 (72.7)	0.32
Limited/Extensive	1/4	7/1	0.04*
At the time of sampling	4 (33.3)	1 (9.1)	0.26
Limited/Extensive	2/2	1/0	0.35
Relapse n (%)	3 (25)	1 (9)	0.59
Death n (%)	3 (25)	1 (9)	0.59

PB: Peripheral blood. BM: Bone marrow.
*p<0.05=significant; **p=0.05-0.10=tendency to significanc

Table 3. T-helper lymphocytes and the cytokine expressions at the time of engraftment (S1) and on the post-transplant 100th day (S2)

		S1 (%) Mean±SE	S2 (%) Mean±SE	p
T helper Cells	CD4	32.6±5.1	24.3±12.6	0.15
	CD4+-27+	12.0±2.8	4.6±1.2	0.03*
	CD4+-27-	20.3±4.6	19.9±2.7	0.93
Th1 Cytokines	CD4-IFN-γ	24.2±4.3	26.8±4.07	0.60
	CD4-TNF-α	23.8±4.7	20.1±3.3	0.56
	CD4-IL-2	15.1±4.6	11.5±2.3	0.54
Th2 Cytokines	CD4-IL-4	10.2±4.1	10.1±4.0	0.99
	CD4-IL-5	1.8±0.5	2.8±0.6	0.27
	CD4-IL-10	2.4±0.7	5.3±2.4	0.26

*p<0.05=significant; **p=0.05-0.10=tendency to significance

Staining for Th1 and Th2 Cytokines

Fluorescein isothiocyanate (FITC) labelled IFN-γ and IgG1 monoclonal antibodies (Immunotech Coulter, France), phycoerythrin (PE)-labelled CD69 (Becton Dickinson, San Jose, USA), CD27, TNF-α, IL-2, IL-4 and IgG2 monoclonal antibodies (Immunotech Coulter, France) and IL-5, IL-10 (PharMingen, San Diego, CA) and peridinin chlorophyll (PerCP)-labelled CD3, CD4 and IgG1 monoclonal antibodies (Immunotech Coulter, France) were used in the study.

Activated lymphocytes were incubated with the monoclonal antibodies for 15 min at RT in dark. Then, 2 ml lysis solution was added followed by 10 min incubation at RT in dark. After centrifuging at 500 x g, the first tube (CD3/CD27) was washed once with sterile wash buffer (PBS; at +4°C) and the cells were fixed with 500 ml cell-fix (1% paraformaldehyde). 500 mL FACS permeabilization solution (Becton Dickinson, San Jose, USA) was added to the other tubes and incubated for 15 min at RT in dark followed by a wash step with 2 ml wash-

Table 4. Comparison of Th1 and Th2 cytokine expressions according to the stem cell sources at S1 and S2

			BM(%) Mean±SE	PB(%) Mean±SE	p
T helper Cells	CD4+	S1	32.6±7.6	1.2±5.7	0.89
		S2	23.0±4.9	25.5±2.9	0.67
		p	0.26	0.39	
	CD4+-27+	S1	7.1±3.0	14.7±4.6	0.17
		S2	5.0±1.5	4.3±2.0	0.78
		p	0.38	0.05*	
	CD4+-27-	S1	25.2±7.2	16.6±4.5	0.32
		S2	19.5±3.2	20.3±4.5	0.87
		p	0.62	0.59	
Th1 Cytokines	CD4-IFN-g	S1	23.3±4.1	25.9±6.8	0.76
		S2	25.8±5.2	27.9±6.7	0.80
		p	0.65	0.86	
	CD4-TNF-a	S1	27.5±6.3	21.7±5.5	0.49
		S2	14.2±3.3	26.0±5.3	0.07**
		p	0.16	0.02*	
	CD4-IL-2	S1	20.4±6.8	7.0±1.8	0.08**
		S2	8.1±2.6	15.2±3.6	0.13
		p	0.07**	0.11	
Th2 Cytokines	CD4-IL4	S1	8.8±5.9	9.5±3.4	0.91
		S2	11.6±5.7	8.4±5.8	0.67
		p	0.58	0.68	
	CD4-IL5	S1	2.6±0.8	2.6±0.9	0.14
		S2	2.6±0.8	2.9±1.0	0.76
		p	0.72	0.17	
	CD4-IL10	S1	2.6±1.0	2.3±1.1	0.73
		S2	5.1±3.2	5.4±3.8	0.94
		p	0.47	0.42	

*p<0.05=significant; **p=0.05-0.10=tendency to significance

buffer. For the intracytoplasmic cytokine analysis, the labelled monoclonal antibodies (IgG2, IFN-γ and IL-4, TNF-α, IL-2, IL-5, IL-10) were added and incubated for 30 min at RT. The samples were washed again with the wash buffer and the cells were fixed by addition of 500 ml cell-fix solution (Becton Dickinson, San Jose, USA) to each tube.

Flow Cytometric Analysis

All samples were run at 450 nm argon laser FacSort Flow Cytometry (Becton Dickinson Immunoscience, San Jose, USA) in 24 hours' time and analyzed with Soft Ware Quest program. Lymphocytes were gated according to their side scatter (SSC) and forward scatter (FSC) properties. At the first step, lymphocyte activation was evaluated by dot blot analysis of control sample versus CD3/CD69 labelling. Other tubes were analyzed by gating of the T-helper lymphocytes according to CD4 expression. Cytokine expression was evaluated in each tube by using the samples not exposed to any stimulus as control group and after stimulation with PMA and ionomycin.

Statistical Analysis

Analysis was done by using the SPSS 10.0 software. The frequency of cytokine secreting cells was analyzed as mean percentage ± standard error (SE). To evaluate the intra- and intergroup differences, chi-square test, Fisher's exact test, Student's t-test, Mann-Whitney U test and one-way ANOVA test were used. A p value less than 0.05 was regarded as statistically significant, and a value between 0.05 and 0.10 was regarded as indicating tendency to significance.

RESULTS

Hematopoietic cell content and the outcome of allo-HSCT.

Absolute number of cells per recipient's body weight (mean ± SD $x10^6$ cells/kg) was significantly higher ($p<.001$) in PB stem cells compared to the BM stem cells: 5.0±5.0 versus 2.2±0.4 for CD34+ cells ($p<.0001$); 6.5±0.8 versus 1.9±0.9 for mononuclear cells ($p=0.001$) and 2.3±0.04 versus 0.3±0.4 for lymphocytes ($p<.0001$). Consistent with the high amount of lymphocytes, higher number of T-helper ($CD3^+CD4^+$) cells were also infused with PB stem cells compared to the BM stem cells (1.2±0.2 versus 0.1±0.02, $p<.0001$).

The transplantation outcomes are summarized in Table 2. Both neutrophil and platelet engraftment were achieved in all patients. The PB and BM groups did not significantly differ from each other in terms of neutrophil engraftment

Table 5. Association between the cytokine expressions and GvHD

				Th 1 Cytokines			Th 2 Cytokines		
	CD4+	$CD4^+$-CD27$^+$	$CD4^+$-CD27-	$CD4^+$-IFN$^+$	$CD4^+$-IL-2$^+$	$CD4^+$-TNF-α$^+$	$CD4^+$-IL-4$^+$	$CD4^+$-IL-5$^+$	$CD4^+$-IL-10$^+$
Acute GvHD (S1)									
Grade II-IV (n=5)	36.0±5.5	11.6±3.1	24.2±5.3	32.1±7.8	30.0±15	42.3±12	17.8±14	3.5±1.4	2.8±2.1
Grade 0-I (n=18)	17.3±5.1	7.8±6.5	9.7±1.7	22.5±4.3	9.5±1.8	19.8±3.6	6.7±2.32	1.5±0.4	2.8±0.8
p	0.03*	0.58	0.02*	0.32	0.08**	0.02*	0.18	0.22	0.97
Acute GvHD (S2)									
Grade II-IV (n=3)	12.5±2.2	4.5±1.4	10.3±2.9	22.9±13	0.7±0.6	10.9±5.8	18.9±18	1.9±0.8	1.9±0.9
Grade 0-I (n=18)	26.3±3.0	5.5±2.9	21.6±2.7	27.5±4.4	13.5±2.4	15.2±3.7	8.4±3.6	2.9±0.7	5.8±2.7
p	0.04*	0.76	0.07**	0.68	<0.001*	0.25	0.35	0.57	0.58
Chronic GvHD (S2)									
Present	22.5±4.0	4.1±1.7	18.0±3,5	32.3±5.8	11.7±2.9	21.3±4.5	14.0±6.1	2.8±0.8	4.5±2.9
Absent	27.5±3.3	4.9±1.7	23.4±4,0	17.3±4.2	11.2±3.9	17.9±4.8	3.7±1.4	2.6±0.9	5.9±4.9
p	0.74	0.76	0,34	0.07**	0.91	0.63	0.10	0.73	0.79

*p<0.05=significant; **p=0.05-0.10=tendency to significance

(15±0.7 vs 17±1.0 days, p=0.09), but the platelet engraftment was earlier (13±3.5 vs 24±2.0 days, p<0.001) in the PB group.

Patients were followed up for a mean of 343 (55-560) days. The incidences of both acute and chronic GvHD were similar in both groups. While the incidence of limited chronic GvHD was higher in the PB than in the BM group (63.8% vs 8.3%), extensive chronic GvHD was more common in the BM group (21.7% vs 9.1%, p=0.04). Of the 23 patients, only 3 patients relapsed and a total of 4 patients died. The causes of death were relapse of the disease (n=1), acute GvHD grade III and IV (n=2) and chronic extensive GvHD (n=1).

Posttransplant T-helper lymphocytes and the cytokine expression

The proportion of T-helper ($CD4^+$) cells and the expression of cytokines were evaluated both at S1 and at S2 (Table 3). The proportions of T-helper cells did not differ between the two sampling periods except from the $CD4^+CD27^+$ lymphocytes, which decreased from 12% at S1 to 4.6% at S2 (p=0.03). In addition, there was no significant difference between S1 and S2 samples in terms of Th1 and Th2 cytokines. The cytokine expression profile was compared between the sources of stem cells both at S1 and S2 (Table 4). The $CD4^+CD27^+$ T cells were decreased in the PB group at S2 compared to S1, which tended to be statistically significant (S1=14.7%, S2=4.3%, p=0.05). There was no significant difference in the cytokine expression profiles except $CD4^+TNF\text{-}\alpha^+$ cells, which increased from 21.7% at S1 to 26% at S2 in the PB group (p=0.02). Higher levels of $CD4^+TNF\text{-}\alpha^+$ cells were found at S2 samples in the PB group compared with the BM group, which tended to be significant (PB=26%; BM=14.2%, p=0.07). $CD4^+IL\text{-}2^+$ cells at S1 were higher in the BM group compared with the PB group (BM=20.4%; PB=7.0%, p=0.08) and also compared with S2 in the BM group (20.4% vs 8.0%, p=0.07). No significant differences in Th2 type cytokines were detected.

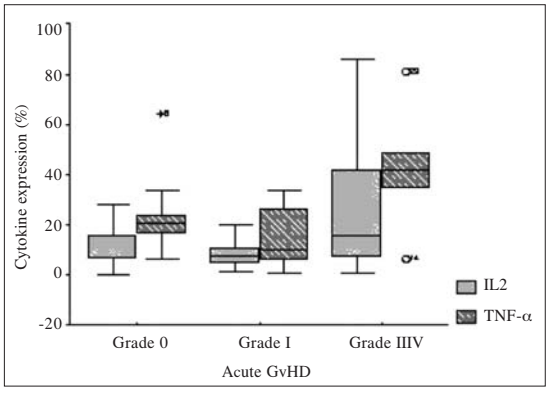

Figure 1. TNF-α and IL-2 expression at S1 according to acute GvHD grade (p=0.04 and p=0.08, respectively)

Cytokines and GvHD

Of all the patients transplanted, 14 had acute GvHD (grade I=9 and grade II-IV=5). When grade II-IV acute GvHD was compared with grade 0-I acute GvHD in terms of T- helper cells, the $CD4^+$ and $CD4^+CD27$- cells were higher at S1 but lower at S2 (Table 5). The expression of intracellular cytokines did not significantly differ except from TNF-α and IL-2. Both IL-2 and TNF-α expression at S1 was higher (IL-2: 30% vs 9.5%, p=0.08 and TNF-α: 42.3% vs 19.8%, p=0.02) and IL-2 expression at S2 was significantly lower (0.65% vs 13.5%, p<0.001) in grade II-IV compared with grade 0-I GvHD. When we compared the cytokine expressions by using one-way ANOVA test in grade 0, grade I and grade II-IV acute GvHD at S1 and S2, the change in the TNF-α expression at S1 was significant (p=0.04) and the change in IL-2 expression at S1 tended to be significant (p=0.08) (Figure 1).

A total of 13 patients had chronic GvHD. The S1 cytokine expressions did not significantly differ except from IFN-γ expression, which tended to be significantly higher in the chronic GvHD group compared with the group without chronic GvHD (32% vs 17%, p=0.07). When the chronic GvHD was evaluated together with limited and extensive GvHD, there was no significant difference in univariate analysis between S1 and S2 in terms of T- helper cells and cytokine expressions (Data not shown).

When the surviving patients (n=19) were compared with the deceased (n=4) patients, the $CD4^+CD27^+$ cells were significantly lower (12.5% vs 2%, p=0.004) and TNF-α expression was significantly higher (20.8% vs 43.4%, p=0.04) in the deceased group at S1. Similarly, in transplant-related mortality, the proportions of $CD4^+$ and $CD4^+CD27^+$ cells were significantly lower ($p<0.001$), and TNF-α as well as IL-2 and IL-5 expressions were significantly higher (p=0.007, 0.02 and 0.005, respectively). There were no significant differences between relapsed cases and the others at S1 and S2 in terms of cytokine expressions.

Th 1 / Th 2 ratios

Th1/Th2 ratio was evaluated based on the ratio of IFN-γ to IL-4 cytokine expression (26). The ratios were similar at S1 and S2 periods (26.8 ± 7.7 and 24.9 ± 10.5, p=0.87). When evaluated according to the grading of acute GvHD and also to the presence or absence of chronic GvHD, the Th1/Th2 ratio did not differ significantly ($p>0.05$). Th1/Th2 ratio at S1 period tended to be lower in transplant-related deaths compared with the total patients (3.38 ± 1.5 vs 28.4 ± 7.2, p=0.09 respectively).

DISCUSSION

Dysregulation of T cell cytokine networks has been proposed as the primary cause of GvHD [2,3,6]. Allogeneic PBSC grafts contain about 10 times more T and B cells than BM grafts. Especially the $CD4^+$ T cell counts are significantly higher in the PBSC recipients [27,28]. The majority of the memory $CD4^+$ T cells also express surface marker CD27. On extended stimulation, this molecule is lost from the surface. Thus, the $CD4^+CD27^-$ cells are considered to be functionally differentiated memory T cells [29,30]. When analyzed in the week of the engraftment (S1) in our study, approximately one-third of the T helper ($CD4^+$) cells were also $CD27^+$. The proportions of T-helper cells as well as the Th1 and Th2 phenotypes did not differ between S1 and the 100th day (S2). The only significant difference between S1 and S2 samples in terms of T-helper cell subgroups was a decrease in $CD4^+CD27^+$ cell ratio in S2. Thus, the decreased levels of $CD4^+CD27^+$ cells at the post- transplant 100th day may be a finding of immunological activation. This change between S1 and S2 samples was more prominent in allogeneic PB rather than BM transplantation. This result parallels with distinct immunological activation and the clinical frequency of chronic GvHD in the allogeneic PBSC transplantation [5].

In the present study, there was no significant difference in the Th2 phenotype between S1 and S2 samples in either stem cell source, between the various degrees of acute GvHD, or between the presence or absence of chronic GvHD. However, significant changes were observed in the Th1 phenotype. The proportion of $CD4^+TNF\text{-}\alpha^+$ cells in the PB group at S2 was significantly higher than at S1. When the $CD4^+TNF\text{-}\alpha^+$ cells at S2 were compared between the PB and BM groups, the PB group tended to have higher proportions of $CD4^+TNF\text{-}\alpha^+$ cells. On the other hand, in the BM group, the proportion of $CD4^+IL\text{-}2^+$ cells was lower at S2 compared with S1. At the posttransplant 100th day, the decreased proportion of $CD4^+IL\text{-}2^+$ cells in the BM group, where chronic GvHD is less frequent, and the increased proportion of $CD4^+TNF\text{-}\alpha^+$ cells in PB group, where the incidence of chronic GvHD is higher, seems to be important in the pathogenesis of chronic GvHD. This may be the result of differential response of Th1 cells to various stimuli [10,31].

Acute GvHD is an immunological event in which Th1 cytokines are thought to play a pivotal role. In spite of the fact that there are also conflicting reports, most clinical and non-clinical data support this assumption. In the present study, our findings support the leading role of Th1 phenotype in acute GvHD. Compared with grade 0-I acute GvHD, the cases who had grade II-IV acute GvHD had higher proportions of the $CD4^+$ and the $CD4^+CD27^-$ cells at S1. These

cases also had significantly higher proportions of $CD4^+IL\text{-}2^+$ and $CD4^+TNF\text{-}\alpha^+$ cells at S1. Since acute GvHD follows the engraftment, these parameters at the time of the engraftment may be included in the predictivity analysis in larger series. Determining these parameters at the time of engraftment might have prognostic significance. The cases with acute GvHD did not have any significant changes in the Th2 phenotype (IL-4, IL-5, IL-10). It has been demonstrated that G-CSF decreases T cell proliferation responses and also causes a switch from Th1 to Th2 cytokines [32]. Despite the fact that the BM recipients were administered rhG-CSF until neutrophil engraftment, we did not observe any Th2 dominance at S1, which is another evidence for the over-activation of Th1 responses. Furthermore, the significant decrease in the level of IL-2 expression at the 100th day in the grade II-IV GvHD group may be the consequence of intense treatment of GvHD with immunosuppressive agents.

The experimental data using the parent-into-F1 model of GvHD has shown that production of IL-2 by donor $CD4^+$ T cells is an early, initiating event. The changes observed in acute GvHD mice could be reversed or significantly inhibited by anti IL-2 treatment [30,33]. The level of soluble IL-2R is a sensitive determinant of both the initiation and the severity of acute GvHD [34,35]. In one study, it was demonstrated that Th1 cytokines (IL-2 and IFN-γ) were greatly increased in patients who received donor lymphocyte infusion, in particular, the changes in IL-2 gene expression correlated well with disease progression, suggesting its critical role [13]. On the other hand, Wang *et al.* [36] showed in their fully MHC and multiple minor Ag mismatched murine BM transplant model that a short course of high-dose IL-2 begun on the day of transplant protects against GvHD, and this inhibitory effect is directed against donor $CD4^+$ cells. Although the differential activation of Th1 and Th2 cells has been implicated in murine models of GvHD, it is unclear whether the Th1/Th2 paradigm is relevant to human clinical disease [37].

A strong correlation between the occurrence of severe acute GvHD after allogeneic PBSC transplantation and the preceding increases in TNF-α serum levels has been found, suggesting a major role of cytokine release in the pathogenesis [15,38]. During the course of pretransplant conditioning, the contribution of inflammatory cytokines released from the host tissues as a result of early tissue damage and induction of GvHD after allogeneic BM transplantation has been shown in experimental models. The prophylactic application of monoclonal anti-TNF-α has been reported to improve the severe cases of GvHD resistant to conventional treatment [39]. Tanaka *et al.* [40] demonstrated by using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) a significant association of increased IL-1b, IL-6 and TNF-α expression with the development of GvHD and the severity of disease. However, in their study, IL-2 expression was not detected at all and IFN-γ expression was only minimally changed during GvHD. It has been reported that Th1 cell numbers prior to allo-HSCT might determine patients with high risk of acute GvHD [41]. Thus, our findings support the leading role of Th1 phenotype in acute GvHD.

It has been reported that both Th1 and Th2 cytokines contribute to the development of end organ damage in acute GvHD. While Th2 cells are required for induction of both hepatic and skin GvHD, both Th1 and Th2 cells are required for intestinal pathology [23]. We have demonstrated here a predominance of $IFN\text{-}\gamma^+$ cells in S2 samples in chronic GvHD cases. Frequent transcription of IFN-γ has been found in skin biopsies of cutaneous chronic GvHD patients, suggesting its potential role in mediating the associated tissue injury [42]. When our findings are taken into account, it can be assumed that different subgroups in Th1 type cells may be important in the pathogenesis of acute and chronic GvHD.

Four patients died of either transplant-related causes or the recurrence of the hematological malignancy. When the cytokine expression of these cases was compared with the surviving patients, the level of TNF-a expression was found to be higher in S1 samples, with an additional increase in IL-2 levels in the transplant-related mortalities. It can be assumed that IL-2 and TNF-α might reflect the severity of GvHD.

The Th1/Th2 ratio in the whole group was measured based on the ratio of IFN-γ/IL-4 and was found to be approximately 25 in both S1 and S2 samples. A very low ratio shifts the balance to Th2 cytokine profile while a very high ratio shifts the balance to Th1 cytokine profile [26]. This ratio did not change when evaluated according to the presence or absence of acute or chronic GvHD. The only significant difference was very low ratio in S1 samples of GvHD- related deaths compared with the surviving patients (3.38 vs 28.4, p=0.09). The insufficient number of cases restricts the healthy evaluation of this data.

In conclusion, some important data were gained in this study. The level of CD27 expressing Th cells, which was high during the engraftment, pointing to the immunological inactivation, was decreased on the 100th day due to activation in chronic GvHD and some other causes. Th1 phenotype, especially the TNF-α levels, was found to be dominant during the engraftment before acute GvHD. The levels of $CD4^{+}IFN\text{-}\gamma^{+}$ cells tended to be increased on the 100th day in cases having chronic GvHD. The Th1/Th2 ratio was found to be far below the mean level especially in the transplant-related deaths. Besides the data supporting the role of Th1 cytokine in occurrence of GvHD, some important data were obtained in this study highlighting the differences between the stem cell sources with respect to cytokine expression profiles.

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