RESEARCH ARTICLE

Early pre-engraftment, functional, *in vitro* responsiveness of T lymphocytes in allotransplanted, acute leukemia patients: proliferation and release of a broad profile of cytokines, possibly predictive of graft-*versus*-host disease

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ABSTRACT. Previous studies of T cell reconstitution following allogeneic stem cell transplantation have described long-lasting T cell defects, including decreased levels of autocrine proliferating CD4+ T cells. However, T cell functions during the early phase of conditioning-induced, pre-engraftment pancytopenia have not been characterized previously. We used a whole blood assay to investigate T cell proliferation and cytokine release during the period of pre-engraftment cytopenia. The study included 13 acute leukemia patients receiving myeloablative conditioning followed by transplantation of G-CSF-mobilised peripheral blood stem cells derived from HLA-matched family donors. Maximal proliferation and cytokine release could not be reached by anti-CD3 stimulation alone, but was dependent on the presence of additional costimulation with anti-CD28. Circulating T cells showed a broad cytokine release profile after activation, and the highest levels were detected for IFNγ, GM-CSF and IL-6. Correlation analyses showed that TNFα/IL-4/IL-5/IL-13 in particular were released as a separate cluster, IFNγ and GM-CSF correlated strongly, whereas IL-17 showed a weak correlation to IL-6 only. The capacity of circulating T cells derived during pre-engraftment cytopenia to release high levels of IFNγ, IL-6 and IL-17 in response to *in vitro* activation with anti-CD3+anti-CD28 showed statistically significant correlations with later acute GVHD. We conclude that allotransplanted patients have a functional T cell system even during the pre-engraftment period of severe pancytopenia.

Keywords: allogeneic stem cell transplantation, pancytopenia, T cells, IFNγ, IL-17

Allotransplantation with peripheral blood stem cells (PBSC) is used in the treatment of younger patients with acute leukemia [1]. The use of PBSC instead of bone marrow stem cells is associated with rapid engraftment and thereby a shorter period of severe, treatment-induced neutropenia [2-4]. Thus, the use of PBSC alters myeloid regeneration, and the higher frequency of graft-versus-host disease (GVHD) in these patients [5, 6] suggests that the lymphoid reconstitution is also modulated. Myeloablative conditioning, combined with allogeneic stem cell transplantation, is followed by a period of severe T cell defects that may last for several months [7-10]. Additionally, the rate of reconstitution differs between T cell subsets [11, 12]. The level of circulating autocrine proliferating $CD4^+$ T cells is often decreased for at least six months [13, 14]. There is a predominance of memory-type $CD45RO^+$ cells and a relative absence of naive-type $CD45RA^+$ cells among these $CD4^+$ cells [15-17]. In contrast, the absolute number of $CD8^+$ T cells is often increased early after the transplantation [11], and the number of $CD16^+CD56^+$ NK lymphocytes usually returns to normal within six weeks [18]. Thus, the post-transplant, quantitative lymphocyte abnormalities have been extensively studied, but the qualitative or functional T cell characteristics during the early period of severe pancytopenia, have not been investigated.

Rapid lymphoid reconstitution after allotransplantation is associated with a decreased risk of post-transplant AML relapse [19], an observation suggesting that immunological events during this early post-transplant period are important for the anti-leukemic T cell reactivity. Targeting of the remaining T cells during lymphopenia should therefore be considered to modulate graft-versus-host reactivity, increase defence against infections, or enhance post-transplant anti-leukemic reactivity. In this context, we investigated the function of circulating T cells derived from acute leukemia patients with severe pancytopenia following myeloablative conditioning and allotransplantation

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with PBSC derived from family donors. Our intention was to examine the total cytokine release capacity of circulating T cells, and for this reason we used experimental models based on mitogenic and not antigen-specific, T cell activation.

METHODS AND MATERIALS

Patients and samples

The regional Ethics Committee (REK III, University of Bergen, Norway) approved the studies. Blood samples were collected after informed, written consent from consecutive patients with acute leukemia over a 21-month period. The clinical details of the patients are summarized in *table 1*. All patients received myeloablative conditioning chemotherapy with busulfan and cyclophosphamide, without total body irradiation. Busulfan was administered intravenously as 0.8 mg/kg over two hours every sixth hour from day - 7 to day - 4 pretransplant, and cyclophosphamide was administered intravenously as 60 mg/kg on days - 3 and - 2. Patients with acute lymphoblastic leukemia or AML M4/M5, in addition received 12 mg of methotrexate as an intrathecal injection on day - 4. The patients received intravenous GVHD prophylaxis with cyclosporine and methotrexate. Cyclosporine was started on day -1 and the targeted serum level was 250-350 ng/mL. Methotrexate was administered intravenously as 15 mg/m^2 on day + 1 and 10 mg/m^2 on days + 3, + 6 and + 11 post-transplant. All patients were transplanted with PBSC derived from HLA-identical family donors. The stem cell donors received G-CSF 10 µg/kg/day subcutaneously once daily for five days, before harvesting by leukapheresis and transplantation of the stem cell graft.

Blood samples were collected during the post-transplant period of pre-engraftment pancytopenia. All patients then developed severe cytopenia with total leukocyte counts below 0.5 x 10^9/L, and a dependency on regular platelet transfusions to maintain peripheral blood platelet counts above 10-20 x 10^9/L. The median duration from transplantation until the first day with cytopenia was three days (range one-six days), and the median duration of neutropenia (total leukocyte counts below 0.5 x 10^9/L) was 8.5 days (range four-17 days).

All samples were collected between 08:00 and 09:00 a.m., through a central venous catheter. Thirteen consecutive patients were sampled regularly during the cytopenic period with three-five day intervals. At the time of sampling, all patients had neutrophil counts < 0.5 x 10^9/L or lymphocyte counts ≤ 0.2 x 10^9/L. Lymphocyte counts below 0.2 x 10^9/L were observed for 23 out of 31 samples. Patients were regularly screened for cytomegalovirus (CMV) infections, but none of them showed clinical or laboratory (CMV-PCR screening) signs of viral reactivation or infection.

In vitro *T cell examination*

Heparinized blood samples were diluted with growth medium within one hour, and in vitro cultures were prepared within two hours of sampling (see below). Previous methodological studies have demonstrated that a delay of up to eight hours before preparation of cultures does not have any major influence [20]. Cultures were prepared with serum-free X-vivo 10® medium (BioWhittaker, Walkersville, MA, USA) with 100 µg/mL of gentamicin. Recombinant human IL-2 (Peprotech, Rocky Hill, NJ, USA) was used at a final concentration of 20 ng/mL [20, 21]. Anti-CD3 (1.5 mg/mL; mouse IgE Moab; CLB-T3/4.E) [21] and anti-CD28 (2 mg/mL; mouse IgG1 Moab, CLB-CD28/1) were purchased from The Central Laboratory of the Netherlands Red Cross Blood Transfusion Services (Amsterdam, The Netherlands) [20, 21]. These antibodies were diluted in the culture medium (0.1 mL antibody diluted up to 12.5 mL, final dilution

Table 1
Clinical and biological characteristics of the acute leukemia patients involved in the study

Patient	Age (years)	Gender (male/female)	Donor	Diagnosis[a]	Duration of neutropenia[b]	Engraftment day[c]	Transplanted CD34+ cell dose[d]	Number of infused lymphocytes[e]
1	50	M	Brother	AML	8	11	14.0	4.94
2	41	M	Brother	AML	12	15	13.8	5.90
3	25	F	Sister	AML	8	13	7.0	8.29
4	43	F	Sister	AML	10	14	7.2	4.49
5	56	M	Brother	AML	17	19	5.2	4.11
6	61	M	Brother	AML	11	15	5.7	2.52
7	48	M	Brother	AML	7	13	11.0	6.69
8	17	M	Brother	T-ALL	9	13	3.2	4.34
9	52	M	Brother	AML	16	23	7.2	3.88
10	35	M	Brother	AML	8	12	3.7	2.98
11	57	M	Brother	AML	4	11	7.2	8.32
12	57	M	Son	AML	7	11	6.6	11.7
13	61	M	Sister	AML	13	19	11.5	8.21

[a] Patients were classified according to the WHO criteria. According to the FAB classification patients 5 and 9 would have been classified as refractory anemia with an excess of blasts in transformation.
[b] Neutropenia was defined as a neutrophil count below 0.5 x 10^9/L.
[c] Number of days from transplantation to engraftment. Engraftment was defined as a sustained neutrophil count of at least 0.5 x 10^9/L.
[d] x 10^6/kg body weight.
[e] x 10^8/kg body weight.

1:125), and these stock solutions were stored at - 70°C [20]. The anti-CD3 Moab was further diluted to 1:500 and anti-CD28 to 1:250, before being used in the experiments [20, 21].

The whole-blood cytokine release assay is a modification of the *in vitro* technique previously characterized in detail by Wendelbo *et al.* [20]. Two ml of heparinized blood was diluted with 4.6 mL of medium before cultures were prepared in tissue culture tubes (Falcon tubes 3033, 15 mL, BD, NJ). Into each tube we then added: *i)* 500 µL of medium-diluted blood; *ii)* 1,000 µL of culture medium eventually supplemented with exogenous IL-2; *iii)* 200 µL of anti-CD3 and eventually 200 µL of anti-CD28. Supernatants were harvested after four days of incubation at 37°C in a humidified atmosphere of 5% CO_2. Alternatively, 50 µL of diluted blood was added to U-bottomed microtiter plates (Costar 3796, Cambridge, MA, USA) together with 100 µL of medium eventually supplemented with IL-2 20 ng/mL, 20 µL of anti-CD3 and 20 µL of anti-CD28. ^{3}H-thymidine (Amersham, UK; TRA 310, 37 kBq/well) was added to the microtiter cultures after three days and nuclear radioactivity determined 18 hours later [20]. Because we used a T cell-specific activation signal (anti-CD3), we refer to these responses as T cell responses. Control experiments showed no detectable response in anti-CD28/IL-2 control cultures.

Cytokine levels were determined either by ELISA (IFN-γ, GM-CSF, IL-17; Quantikine ELISA kits, R&D Systems, Abingdon, UK) or by multiplex analyses (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13 and TNFα; Cytokine 10-Plex for Luminex, Biosource International, CA, USA). All analyses were performed strictly according to the manufacturers' instructions. Standard curves were constructed using the mean of duplicate determinations, and differences between duplicates were generally < 10% of the mean. The minimal detectable levels were: IFNγ 8 pg/mL, GM-CSF 3 pg/mL, TNF-α 10 pg/mL, IL-1β 15 pg/mL, IL-2 6 pg/mL, IL-4 5 pg/mL, IL-5 and IL-6 3 pg/mL, IL-10 5 pg/mL, IL-12 4 pg/mL, IL-13 32 pg/mL and IL-17 15 pg/mL.

Analysis of the data

The statistical analyses were performed using a standard software package (SPSS 15.0, SPSS, Chicago, IL, USA). Most parameters displayed a skewed distribution, and only non-parametric tests were used. Unless otherwise stated, all samples were regarded as independent biological events because the clinical and biological status differed even when samples were collected from the same patient at different times. The Kendall test was used for the correlation analyses. Wilcoxon's 2-tailed test was used for analyses of paired observations, and the Mann-Whitney U test for comparison of independent samples.

Proliferation was assayed in triplicate, and the mean counts per minute (cpm) were used in all statistical comparisons. Significant proliferation was defined as ^{3}H-thymidine incorporation corresponding to > 1,000 cpm [20]. For the analyses of cytokine levels in GVHD, only the first sample collected from each patient was included.

RESULTS

Proliferative T cell responses are detected early after allogeneic stem cell transplantation

We investigated the proliferative T cell responsiveness in the whole blood assay for 31 samples derived from 13 consecutive, allotransplanted patients. The overall results are presented in *figure 1A*. The T cells showed no spontaneous proliferation when incubated in medium alone or with anti-CD3 alone (nuclear ^{3}H-thymidine incorporation corresponding to < 1,000 cpm). Detectable ^{3}H-thymidine incorporation was observed only for six of 31 samples (derived from five different patients), when T cells were activated with anti-CD3 + IL-2. In contrast, proliferation was significantly increased when the cells were stimulated with anti-CD3 + co-stimulatory anti-CD28 (16 out of 31 samples with detectable responses) compared with anti-CD3 alone ($p < 0.001$). The responses could be even further increased by adding exogenous IL-2 together with anti-CD3 + anti CD28 ($p = 0.002$). Thus, detectable T cell proliferation can be induced before hematopoietic reconstitution even for these severely immunocompromized patients.

Approximately half of the samples were collected during the first five days of the cytopenic period and the other half after 6-15 days of cytopenia. We found no statistically significant differences in T cell proliferation between early and late samples (data not shown). Furthermore, the proliferative responsiveness was not different for samples collected within the first 24 hours after methotrexate GVHD prophylaxis (data not shown).

A broad T cell cytokine response is detected during pre-engraftment cytopenia

Both clinical and experimental studies suggest that cytokines are important in the pathogenesis of GVHD. This is true both for IFNγ that is released at high levels after activation both by normal and post-engraftment T cells [22-24], and for IL-17, which seems to cooperate with IFN-γ [25, 26]. We therefore investigated an extended T cell cytokine release profile for 28 unselected samples derived from 12 consecutive patients. A total of 10 T cell-secreted cytokines were examined. The overall results are summarized in *table 2*, and the results for IFNγ, GM-CSF and IL-17 are presented in detail in *figure 1*. It can be seen that *i)* the highest cytokine levels were generally detected after activation with anti-CD3 + anti-CD28 + IL-2, the only exception being IL-17; *ii)* the highest concentrations were observed for IL-6, GM-CSF and particularly IFNγ; *iii)* lower levels were detected for IL-2, TNFα and the immunosuppressive cytokines IL-4, IL-10 and IL-13; *iv)* detectable levels of the T cell growth factor IL-2 were observed for most samples, whereas detectable IL-17 was seen for less than half of the samples. Furthermore, low or undetectable IL-1β and IL-12 levels were observed in the culture supernatants (data not shown). Similar results were observed when the statistical analyses only included the first sample collected from each patient (data not shown). No spontaneous release was observed for any cytokine except for IL-6; undetectable or very low levels were generally

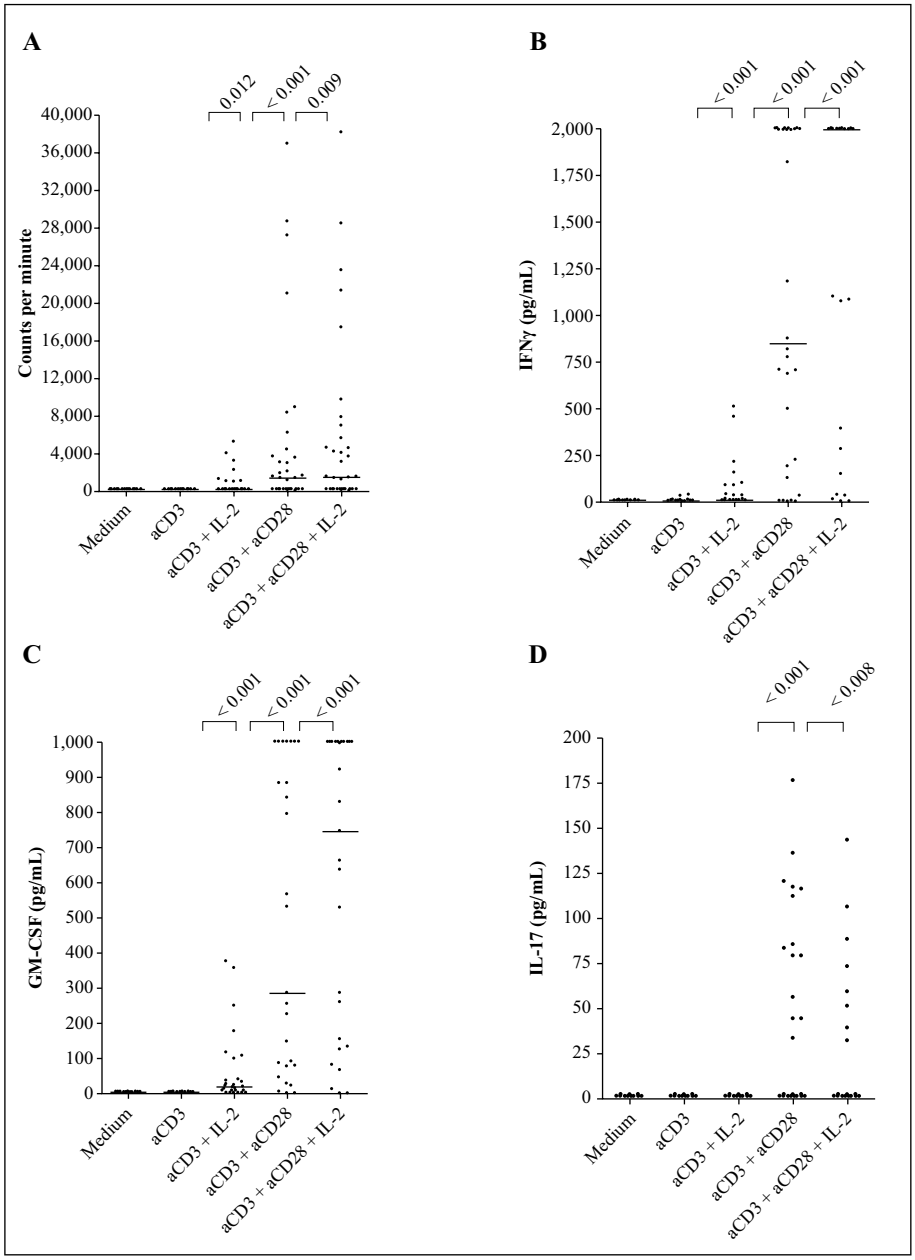

Figure 1

The proliferative T cell responsiveness (**A**), the release of IFNγ (**B**), GM-CSF (**C**) and IL-17 (**D**) for allotransplanted, acute leukemia patients examined during the period of severe cytopenia before hematopoietic reconstitution. P-values are indicated at the top of the figures; the median levels are also indicated in the figure.

detected with anti-CD3 alone and relatively low levels of all cytokines were also detected with anti-CD3 + exogenous IL-2. Thus, circulating T cells derived during the early period of severe, pre-engraftment pancytopenia are able to release a wide range of cytokines in response to an optimal activation signal.

We compared the cytokine release and leukocyte counts in peripheral blood at the time of sampling, and IL-17 was the only cytokine that showed a significant correlation with the number of circulating leukocytes ($r = 0.388$, $p = 0.031$). Finally, we compared the cytokine release for the severely cytopenic patients with samples derived from three patients with higher leukocyte counts as a sign of the start of hematopoietic reconstitution; IL-17 was then the only cytokine that differed significantly and showed higher levels for samples with higher counts (data not shown).

We did not find any difference in cytokine release when comparing samples derived early and late during cytopenia (data not shown). However, significantly higher GM-CSF levels were observed for samples collected the day after a methotrexate infusion ($p = 0.045$), whereas no such difference was observed for the other cytokines (data not shown).

The pre-engraftment release of different cytokines shows strong correlations

We investigated the correlations between the capacity to release various T cell cytokines. For these studies, we used the maximal responses induced by anti-CD3 + anti-CD28 + IL-2. The overall results are summarized in *figure 2*. Strong correlations, corresponding to $p < 0.01$, were observed particularly for a cytokine

Table 2
Cytokine release after mitogenic T cell activation; a summary of the overall results for allotransplanted patients with pre-engraftment cytopenia after myeloablative chemotherapy conditioning[a]

Cytokine	Negative Control[b]	Anti-CD3 stimulated activation		Anti-CD3 + IL-2	Anti-CD3 + anti-CD28	Anti-CD3 + anti-CD28 + IL-2
		Frequency of detectable level[c]	Cytokine level median (range)			
IFNγ	< 8	1/28	< 8 (< 8-38)	< 8 (< 8-509)**	774 (< 8-> 2,000)**	> 2,000 (< 8-> 2,000)**
GM-CSF	< 3	1/24	< 3 (< 3-5)	19 (< 3-376)**	285 (< 3-> 1,000)**	746 (< 3-> 1,000)**
IL-17	< 15	0/31	< 15	< 15 (< 15)	< 15 (< 15-176)**	< 15 (< 15-106)*
IL-2	< 6 (< 6-26)	1/28	< 6 (< 6-26)		204 (< 6-1,925)	
IL-4	< 5 (< 5)	0/28	< 5 (< 5)	< 5 (< 5-78)	18 (< 5-69)*	40 (< 5-139)**
IL-5	< 3 (< 3-33)	5/28	< 3 (< 3-37)	4 (< 3-274)*	122 (< 3-4,354)**	219 (9-2552)*
IL-6	66 (< 3-1005)	26/28	99 (< 3-> 18,619)	182 (< 3-> 18,619)*	732 (77-> 18,619)*	1,332 (93-> 18,619)**
IL-10	7 (< 5-16)	22/28	7 (< 5-16)	27 (< 5-250)**	32 (< 5-272)	65 (12-422)**
IL-13	< 32 (< 32)	0/28	< 32 (< 32)	< 32 (< 32-173)	162 (< 32-2,406)**	244 (< 32-5,535)**
TNFα	< 10 (< 10)	0/28	< 10 (< 10)	< 10 (< 10-25)	14 (< 10-179)*	23 (< 10-225)*

[a] We investigated 24-31 unselected samples derived from 12 consecutive patients. The results are presented as the median cytokine concentrations (pg/mL) and with corresponding variation range in brackets. In the statistical analyses we compared*: i)* Anti-CD3 *versus* negative control; *ii)* anti-CD3 + IL-2 *versus* anti-CD3 alone; *iii)* anti-CD3 + anti-CD28 *versus* anti-CD3 + IL-2; *iv)* anti-CD3 + anti-CD28 + IL-2 *versus* anti-CD3 + anti-CD28. (* $p < 0.01$; ** $p < 0.001$).
[b] Negative controls were cells incubated in medium alone.
[c] Number of samples with detectable levels relative to the number of samples tested.

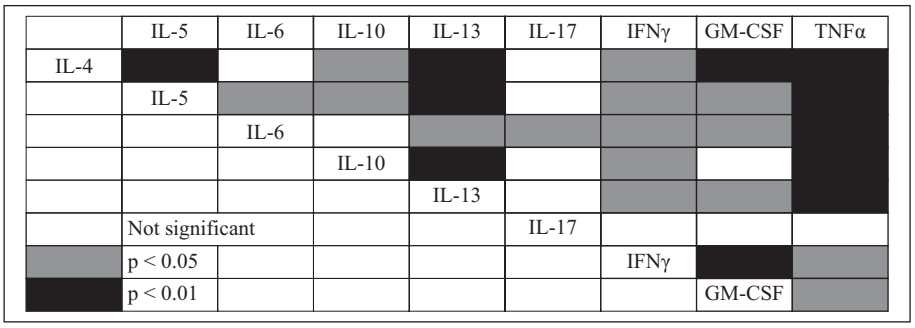

Figure 2
Correlation analyses of the capacity of post-transplant T cells to release cytokines in response to activation with anti-CD3 + anti-CD28 + IL-2. T cells were derived from 12 patients (1 sample per patient) during the period of pre-engraftment cytopenia.

cluster including TNFα/IL-4/IL-5/IL-13. IFNγ and GM-CSF showed a strong correlation, whereas IL-17 levels showed only a weak correlation with IL-6; similar results were also observed when comparing the results for anti-CD3 + anti-CD28 (data not shown). T cell proliferation showed statistically significant correlations with IL-4 ($p = 0.029$), IL-5 ($p = 0.002$), IL-6 ($p = 0.036$), IL-13 ($p = 0.011$) and TNFα ($p = 0.043$).

The early IFN-γ response is influenced by the number of lymphocytes in the allograft

A major portion of circulating lymphocytes in healthy individuals are T cells [27], and most lymphocytes in peripheral blood stem cell grafts will therefore be T cells [28]. The number of T lymphocytes in our stem cell grafts was not available, and for this reason we had to compare the cytokine responses with the total number of infused lymphocytes (lymphocytes per kg of body weight). We then compared the lymphocyte numbers *(table 1)* with cytokine levels after activation with anti-CD3 + anti-CD28. This activation signal was chosen because cytokines are then released at relatively high, but not maximal levels (higher levels when IL-2 is also added) for most patients. In this analysis, we included only the first sample collected for the 12 consecutive patients. A significant correlation was observed between the number of infused lymphocytes and the IFNγ response ($r = 0.567$, $p = 0.049$), but not for the other cytokine responses. Thus, the post-transplant cytokine release profile for circulating T cells is influenced by the number of infused T cells.

Costimulatory T cell signalling differs for acute leukemia patients, with treatment-induced cytopenia due to conventional chemotherapy and myeloablative conditioning

We compared our present results for T cell release of IFNγ and GM-CSF with a group of acute leukemia patients receiving conventional intensive chemotherapy [22]. Neither IFNγ nor GM-CSF levels differed between these two groups after stimulation with anti-CD3 alone, anti-CD3 + anti-CD28 and anti-CD3 + anti-CD28 + IL-2 (data not shown). In contrast, a statistically significant decrease in IFNγ levels was seen after stimulation with anti-CD3 + IL-2 for allotransplanted patients (median level < 8 pg/mL, range < 8 - 509 pg/mL, $p < 0.001$), compared with conventionally-treated patients (median level 65 pg/mL, range < 8 - > 2,000 pg/mL). Similarly, GM-

CSF levels were also decreased for the allotransplanted patients (median 23 pg/mL, range < 3 - 376 pg/mL, p = 0.022) compared with conventionally treated patients (median 154 pg/mL, range < 3 - > 1,000 pg/mL), after stimulation with anti-CD3 + IL-2. To summarise, T cells derived from allotransplanted patients showed relatively low cytokine responses in the absence of additional CD28-mediated costimulation even when the T cell growth factor IL-2 was added in excess, whereas maximal cytokine responsiveness for conventionally-treated patients was not dependent on additional anti-CD28-induced costimulation, and could be reached after anti-CD3 stimulation alone if exogenous IL-2 was present. These observations demonstrate that even though these two patient groups show comparable quantitative T cell defects, there are qualitative T cell differences between the groups (*i.e.* different dependency on CD28-mediated costimulation).

Patients with later, acute GVHD show increased T cell cytokine release during pre-engraftment cytopenia

Previous studies have demonstrated that IFNγ is important for the development of acute GVHD [29, 30], and recent studies suggest that IL-17-releasing Th17 cells also contribute to the pathogenesis of this complication [25, 31, 32]. We therefore compared the cytokine release for patients with later, acute GVHD (four patients) and patients without GVHD (seven patients). A total of 12 patients were included in the cytokine studies, but one of these patients could not be included in the analyses because of early death from septicaemia. The onset and degree of GVHD are detailed for each patient in *table 3*. Only the first sample collected for each patient was included in these statistical analyses. We compared cytokine levels after stimulation with anti-CD3 + anti-CD28 ± IL-2 because these two activation signals caused the highest cytokine release. Those cytokine/activation signal combinations that showed a significant difference between patients with and without acute GVHD are presented in *table 4* and *figure 3*. It can be seen that high release of IFN-γ, IL-6 and IL-17 were associated with later, acute GVHD. The amount of infused lymphocytes did not differ significantly between patients with or without acute GVHD.

DISCUSSION

Several previous studies have investigated T cell reconstitution after allogeneic stem cell transplantation [33-35], but T cell functions during the early period of pre-engraftment cytopenia has not been studied either in patients receiving blood or marrow stem cells. Immunological events during this early period may be important for graft-versus-leukemia effects since rapid lymphoid reconstitution is associated with reduced risk of leukemia relapse [19]. In this context, we examined T cell functions in allotransplanted, acute leukemia patients with pre-engraftment pancytopenia. We then used experimental strategies with mitogenic T cell activation to evaluate the total capacity of cytokine release, but the models

Table 3
Development of GVHD in the 11 unselected acute leukemia patients included in the statistical analyses

Patients[a]	Development of acute GVHD (aGVHD)			Survival	Cause of death
	Onset	Organs involved	GVHD stage[b]		
3	No aGVHD			> 22 months	
4	No aGVHD			> 21 months	
5	Day 33	Skin, gut, liver, mucosa	IV	5 months	Sepsis
6	Day 50	Gut, liver	II	> 18 months	
7	Day 40	Skin, gut	II	> 12 months	
8	No aGVHD			> 12 months	
9	No aGVHD			3.5 months	Sepsis, heart failure
10	No aGVHD			> 6 months	
11	Day 20	Skin, mucosa	II	> 6 months	
12	No aGVHD			4 months	Leukemia relapse
13	No aGVHD			5 months	Sepsis

[a] A group of 11 consecutive patients were prospectively examined for T cell cytokine responsiveness. Patients are numbered according to *table 1*; cytokine responses were not examined for patient 1 and patient 2 was excluded from the analysis because he died from leukemia relapse 18 days post-transplant.
[b] GVHD was staged according to the Glucksberg grading system.

Table 4
Cytokine release after T cell activation in patients that did or did not develop acute GVHD[a]

Cytokine	Activation signal	Acute GVHD	No acute GVHD	P-value
IFN-γ	aCD3 + aCD28	2,000 (2,000)[b]	685 (13-2,000)	0.0343
IL-6	aCD3 + aCD28 + IL-2	4,985 (1,839-18,619)	886 (117-4,068)	0.0233
IL-17	aCD3 + aCD28 + IL-2	81 (0-106)	0 (0)	0.0116

[a] Samples were collected during pre-engraftment cytopenia, and only the first sample from each patient was included.
[b] Values are given as median (variation range).

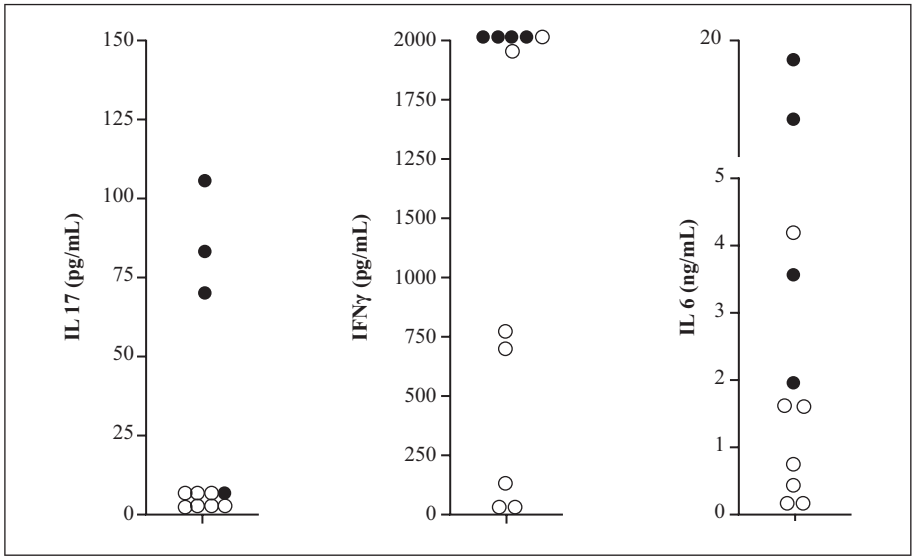

Figure 3
Cytokine levels in allotransplanted patients with and without acute GVHD. T cell cytokine release was tested during the early phase of post-transplant cytopenia before hematopoietic engraftment. IFNγ was tested after stimulation with anti-CD3 + anti-CD28, whereas IL-6 and IL-17 were tested after stimulation with anti-CD3 + anti-CD28 + IL-2. The data are presented as the cytokine concentrations in the supernatants for patients with later, acute GVHD (●) and patients without (○) this complication.

were based on activation signalling initiated through physiological pathways (CD3, CD25, CD28). Antigen-specific activation was not used, and the influence of previous antigen-exposure and antigen-specific immunoregulatory effects were thereby avoided.

Our use of a standardised, whole blood assay for analysis of T cell responses has several advantages [20]. Firstly, a well-characterized IgE anti-CD3 antibody was used for T cell activation. Secondly, the assay is suitable for repeated examination of T cell functions for severely ill and leukopenic patients, when limited blood sample volumes are available. Thirdly, proliferative responses and cytokine release can be quantified, and by using this assay our results are comparable with previous studies of other patient groups [21, 36-38]. The major disadvantage is that the responses are determined both by qualitative and quantitative characteristics [20].

We observed detectable T cell proliferation in most samples during pre-engraftment cytopenia. However, induction of a detectable response was usually dependent on exogenous anti-CD28-mediated costimulation, suggesting that other immunocompetent cells cannot initiate optimal co-stimulatory signalling in these severely immunocompromized patients.

We investigated T cell cytokine responsiveness in our whole blood assay without T cell enrichment because only small sample volumes were available, and enrichment procedures may, incidentally, influence the functional characteristics of the cells. However, T cell-specific activation signals were used, and for this reason we refer to the detected responses as T cell responses. The absence of spontaneous cytokine release in control cultures also supports this, the only exception being IL-6 that was also detected in control cultures. This release may have been caused by monocytes, but it should be emphasized that these levels were relatively low compared with the levels reached after optimal T cell activation.

Circulating T cells derived from these severely immunocompromized patients were able to release a wide range of cytokines, and the highest levels were generally reached when T cell activating anti-CD3 was present, together with exogenous costimulatory anti-CD28 and the T cell growth factor IL-2. High levels were detected especially for IFNγ, GM-CSF and IL-6. The ability to release these cytokines is probably influenced both by quantitative and qualitative characteristics of the patients; but only IFNγ levels showed a significant correlation with the number of infused donor T cells, whereas high GM-CSF/IL-6 levels were detected independently of this number. The capacity to release high levels of GM-CSF and IFNγ has also been observed for circulating T cells derived from normal individuals, as well as allotransplanted leukemia patients when examined after the hematopoietic reconstitution [23, 24]. Furthermore, the immunoregulatory cytokines TNFα and IL-5, together with the anti-inflammatory IL-4/IL-10/IL-13, were released at lower levels and seemed to form a separate cluster, together with IL-6, with relatively strong correlations between the maximal concentrations reached *(figure 3)*. Finally, the proinflammatory IL-17 levels showed no strong correlations to any other cytokines.

We found significantly higher GM-CSF release for samples collected the day after methotrexate infusion, whereas the other cytokine responses and the proliferative T cell responses were not altered. This increase is possibly due to an effect of methotrexate on the accessory cells; it is unlikely that this reflects a general T cell stimulatory effect because methotrexate is used for T cell-suppressive GVHD prophylaxis. This observation rather illustrates that the influence of various clinical parameters differs between the cytokine responses; GM-CSF is affected by methotrexate, IFN-γ is more dependent on the number of infused T cells, and IL-17 levels depend on the number of circulating leukocytes.

Previous studies have shown that the risk of GVHD depends on the number of transplanted donor T cells [5, 39], and a recent study demonstrated that post-transplant T cell development is also important for the GVHD risk [40]. Our present study is the first to suggest that T cell cytokine responsiveness during the early period of severe pre-engraftment cytopenia is important for the risk of later GVHD in addition to the transplanted T cell number and post-transplant T cell expansion.

Our present results suggest that the early capacity to release IL-6, IL-17 and IFNγ influences the risk of later GVHD, and probably these cytokines have independent effects because their levels showed no significant correlations. These results have to be interpreted with great care because of the low number of patients involved. However, other observations also suggest that these particular cytokines are important in the development of GVHD. Firstly, an IL-6 genotype associated with high post-engraftment serum IL-6 levels, is a risk factor for later development of GVHD [41], and our present study suggest that the early, post-transplant T cell capacity to release IL-6 is important. Secondly, a recent study described increased numbers of circulating proinflammatory Th17 cells prior to development of GVHD after hematopoietic reconstitution [42]; our present results suggest that this Th17/IL-17 effect is established before engraftment. Finally, IFNγ seems to be involved in the pathogenesis of acute GVHD [29], and high plasma levels of IFNγ are observed during this complication [43]. Thus, both our own results as well as other observations suggest that these three cytokines are important; our present results indicate that the T cell capacity to release these cytokines during the early pre-engraftment period, reflects a risk of later, acute GVHD.

The immunosuppressive, regulatory T cells and the proinflammatory Th17 (IL-17 releasing) T cell subsets have been recently characterized in detail. IL-17 is a family of cytokines that is produced almost exclusively by a distinct lineage of proinflammatory Th17 cells [44-46]. The two subsets are related and Th17 cells can be differentiated from regulatory T cells [47]. Several studies suggest that regulatory T cells can down-regulate or even prevent acute GVHD [25, 48-50], but despite the developmental connection between the two subsets, the previous studies have not investigated whether Th17 cells contribute to the development of GVHD. In the present study, we did not investigate regulatory T cells, but our results suggest that there is an association between later, acute GVHD and the pre-engraftment capacity to release IL-17. Recent observations in animal models also suggest a role for IL-17/Th17 cells in the development of acute GVHD [32, 51].

The GVHD-associated cytokine release profiles (high IL-6, IL-17 and IFNγ), were detected during early pre-engraftment cytopenia before the development of clinical disease. However, it is not surprising that such early parameters are associated with later development of acute GVHD, because even serum biomarkers reflecting initial GVHD-associated tissue damage can be elevated at least one week before clinical symptoms appear [52].

To conclude, even for acute leukemia patients receiving myeloablative conditioning therapy, functional T cells remain in the circulation during the period of severe cytopenia before hematopoietic reconstitution, and the cytokine release capacity of these cells may be involved in the development of GVHD.

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REFERENCES

1. Suciu S, Mandelli F, de Witte T, *et al.* Allogeneic compared with autologous stem cell transplantation in the treatment of patients younger than 46 years with acute myeloid leukemia (AML) in first complete remission (CR1): an intention-to-treat analysis of the EORTC/GIMEMAAML-10 trial. *Blood* 2003; 102: 1232.
2. Couban S, Simpson DR, Barnett MJ, *et al.* A randomized multicenter comparison of bone marrow and peripheral blood in recipients of matched sibling allogeneic transplants for myeloid malignancies. *Blood* 2002; 100: 1525.
3. Vellenga E, van Agthoven M, Croockewit AJ, *et al.* Autologous peripheral blood stem cell transplantation in patients with relapsed lymphoma results in accelerated haematopoietic reconstitution, improved quality of life and cost reduction compared with bone marrow transplantation: the Hovon 22 study. *Br J Haematol* 2001; 114: 319.
4. Vose JM, Sharp G, Chan WC, *et al.* Autologous transplantation for aggressive non-Hodgkin's lymphoma: results of a randomized trial evaluating graft source and minimal residual disease. *J Clin Oncol* 2002; 20: 2344.
5. Ho VT, Soiffer RJ. The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation. *Blood* 2001; 98: 3192.
6. Aversa F, Tabilio A, Velardi A, *et al.* Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. *N Engl J Med* 1998; 339: 1186.
7. Savage WJ, Bleesing JJ, Douek D, *et al.* Lymphocyte reconstitution following non-myeloablative hematopoietic stem cell transplantation follows two patterns depending on age and donor/recipient chimerism. *Bone Marrow Transplant* 2001; 28: 463.
8. Derdouch S, Gay W, Negre D, *et al.* Reconstitution of the myeloid and lymphoid compartments after the transplantation of autologous and genetically modified CD34+ bone marrow cells, following gamma irradiation in cynomolgus macaques. *Retrovirology* 2008; 5: 50.
9. Bomberger C, Singh-Jairam M, Rodey G, *et al.* Lymphoid reconstitution after autologous PBSC transplantation with FACS-sorted CD34+ hematopoietic progenitors. *Blood* 1998; 91: 2588.
10. Soiffer RJ, Bosserman L, Murray C, Cochran K, Daley J, Ritz J. Reconstitution of T-cell function after CD6-depleted allogeneic bone marrow transplantation. *Blood* 1990; 75: 2076.
11. Autran B, Malphettes M, Dhedin N, Gorochov G, Leblond V, Debre P. Studies of T cell reconstitution after hematopoietic stem cell transplant. *Hematol Cell Ther* 1997; 39: 252.
12. Crooks GM, Weinberg K, Mackall C. Immune reconstitution: from stem cells to lymphocytes. *Biol Blood Marrow Transplant* 2006; 12: 42.
13. Talmadge JE. Lymphocyte subset recovery following allogeneic bone marrow transplantation: CD4(+)-cell count and transplant-related mortality. *Bone Marrow Transplant* 2008; 41: 19.

14. Rozans MK, Smith BR, Burakoff SJ, Miller RA. Long-lasting deficit of functional T cell precursors in human bone marrow transplant recipients revealed by limiting dilution methods. *J Immunol* 1986; 136: 4040.

15. Mackall CL, Hakim FT, Gress RE. Restoration of T-cell homeostasis after T-cell depletion. *Semin Immunol* 1997; 9: 339.

16. Mackall CL, Bare CV, Granger LA, Sharrow SO, Titus JA, Gress RE. Thymic-independent T cell regeneration occurs via antigen-driven expansion of peripheral T cells resulting in a repertoire that is limited in diversity and prone to skewing. *J Immunol* 1996; 156: 4609.

17. Mackall CL, Granger L, Sheard MA, Cepeda R, Gress RE. T-cell regeneration after bone marrow transplantation: differential CD45 isoform expression on thymic-derived versus thymic-independent progeny. *Blood* 1993; 82: 2585.

18. Ashihara E, Shimazaki C, Yamagata N, *et al.* Reconstitution of lymphocyte subsets after peripheral blood stem cell transplantation: two-color flow cytometric analysis. *Bone Marrow Transplant* 1994; 13: 377.

19. Kumar S, Chen MG, Gastineau DA, *et al.* Effect of slow lymphocyte recovery and type of graft-versus-host disease prophylaxis on relapse after allogeneic bone marrow transplantation for acute myelogenous leukemia. *Bone Marrow Transplant* 2001; 28: 951.

20. Wendelbo O, Bruserud O. Functional evaluation of proliferative T cell responses in patients with severe T lymphopenia: characterization of optimal culture conditions and standardized activation signals for a simple whole blood assay. *J Hematother Stem Cell Res* 2003; 12: 525.

21. Bruserud O, Ulvestad E. Acute myelogenous leukemia blasts as accessory cells during in vitro T lymphocyte activation. *Cell Immunol* 2000; 206: 36.

22. Ersvaer E, Hampson P, Hatfield K, *et al.* T cells remaining after intensive chemotherapy for acute myelogenous leukemia show a broad cytokine release profile including high levels of interferon-gamma that can be further increased by a novel protein kinase C agonist PEP005. *Cancer Immunol Immunother* 2007; 56: 913.

23. Bruserud O, Hamann W, Patel S, Ehninger G, Schmidt H, Pawelec G. IFN-gamma and TNF-alpha secretion by CD4+ and CD8+ TCR alpha beta + T-cell clones derived early after allogeneic bone marrow transplantation. *Eur J Haematol* 1993; 51: 73.

24. Bruserud O, Ulvestad E, Berentsen S, Bergheim J, Nesthus I. T-lymphocyte functions in acute leukaemia patients with severe chemotherapy-induced cytopenia: characterization of clonogenic T-cell proliferation. *Scand J Immunol* 1998; 47: 54.

25. Socie G, Ratajczak P, Leboeuf C, *et al.* Th17/Treg in Human Graft-Versus-Host-Disease (GvHD) of the Gastro-Intestinal (GI) Tract. *Blood (ASH Annual Meeting Abstracts)* 2008; 112.

26. Broxmeyer HE, Starnes T, Ramsey H, *et al.* The IL-17 cytokine family members are inhibitors of human hematopoietic progenitor proliferation. *Blood* 2006; 108: 770.

27. Lenkei R, Bratt G, Holmberg V, Muirhead K, Sandstrom E. Indicators of T-cell activation: correlation between quantitative CD38 expression and soluble CD8 levels in asymptomatic HIV+ individuals and healthy controls. *Cytometry* 1998; 33: 115.

28. Barge RM, Brouwer RE, Beersma MF, *et al.* Comparison of allogeneic T cell-depleted peripheral blood stem cell and bone marrow transplantation: effect of stem cell source on short- and long-term outcome. *Bone Marrow Transplant* 2001; 27: 1053.

29. Krenger W, Ferrara JL. Dysregulation of cytokines during graft-versus-host disease. *J Hematother* 1996; 5: 3.

30. Dickinson AM, Cavet J, Cullup H, Wang XN, Sviland L, Middleton PG. GvHD risk assessment in hematopoietic stem cell transplantation: role of cytokine gene polymorphisms and an in vitro human skin explant model. *Hum Immunol* 2001; 62: 1266.

31. Kappel LW, Goldberg GL, King CG, *et al.* IL-17 contributes to CD4-mediated graft-versus-host disease. *Blood* 2009; 113: 945.

32. Yi T, Zhao D, Lin CL, *et al.* Absence of donor Th17 leads to augmented Th1 differentiation and exacerbated acute graft-versus-host disease. *Blood* 2008; 112: 2101.

33. Talmadge JE, Reed E, Ino K, *et al.* Rapid immunologic reconstitution following transplantation with mobilized peripheral blood stem cells as compared to bone marrow. *Bone Marrow Transplant* 1997; 19: 161.

34. Biggar WD, Park BH, Good RA. Immunologic reconstitution. *Annu Rev Med* 1973; 24: 135.

35. Sharp JG, Kessinger A, Lynch JC, Pavletic ZS, Joshi SS. Blood stem cell transplantation: factors influencing cellular immunological reconstitution. *J Hematother Stem Cell Res* 2000; 9: 971.

36. Ersvaer E, Hampson P, Wendelbo O, Lord JM, Gjertsen BT, Bruserud O. Circulating T cells in patients with untreated acute myelogenous leukemia are heterogeneous and can be activated through the CD3/TCR complex. *Hematology* 2007; 12: 199.

37. Bruserud O, Ulvestad E. Cytokine responsiveness of mitogen-activated T cells derived from acute leukemia patients with chemotherapy-induced leukopenia. *J Interferon Cytokine Res* 2000; 20: 947.

38. Wendelbo O, Nesthus I, Sjo M, Ernst P, Bruserud O. Cellular immune responses in multiple myeloma patients with treatment-induced cytopenia early after high-dose chemotherapy and autologous peripheral blood stem cell transplantation. *Leuk Res* 2004; 28: 461.

39. Prentice HG, Blacklock HA, Janossy G, *et al.* Depletion of T lymphocytes in donor marrow prevents significant graft-versus-host disease in matched allogeneic leukaemic marrow transplant recipients. *Lancet* 1984; 1: 472.

40. Thiant S, Yakoub-Agha I, Terriou L, Jouet J, Dessaint J, Labalette M. Correlation between plasma interleukin 7 and interleukin 15 levels and acute graft versus host disease and relapse in patients undergoing myeloablative allogeneic stem cell transplantation. *Bone Marrow Transplantation* 2009; 43 (Suppl. 1): S15 (abstract 153).

41. Cavet J, Dickinson AM, Norden J, Taylor PR, Jackson GH, Middleton PG. Interferon-gamma and interleukin-6 gene polymorphisms associate with graft-versus-host disease in HLA-matched sibling bone marrow transplantation. *Blood* 2001; 98: 1594.

42. Dlubek D, Jaskula E, Sedzimirska M, Lange J, Lange A. Increased proportion of TH17+ cells in patients post hsct heralds overt aGVHD. *Bone Marrow Transplantation* 2009; 43 (Suppl. 1): S122 (abstract 527).

43. Yeh SP, Liao YM, Chiu CF, Lo WJ, Lin CL. The frequencies of T helper 1, T helper 2 in CD4+ T cells and plasma interleukin-10 are good biomarkers of graft *versus* host disease. *Bone Marrow Transplantation* 2009; 43 (Suppl. 1): S124 (abstract 533).

44. Gaffen SL, Kramer JM, Yu JJ, Shen F. The IL-17 cytokine family. *Vitam Horm* 2006; 74: 255.

45. Kramer JM, Gaffen SL. Interleukin-17: a new paradigm in inflammation, autoimmunity, and therapy. *J Periodontol* 2007; 78: 1083.

46. Chen Z, O'Shea JJ. Th17 cells: a new fate for differentiating helper T cells. *Immunol Res* 2008; 41: 8.

47. Radhakrishnan S, Cabrera R, Schenk EL, *et al.* Reprogrammed FoxP3+ T regulatory cells become IL-17+ antigen-specific autoimmune effectors *in vitro* and *in vivo*. *J Immunol* 2008; 181: 3137.

48. Wolf D, Wolf AM, Fong D, *et al.* Regulatory T-cells in the graft and the risk of acute graft-versus-host disease after allogeneic stem cell transplantation. *Transplantation* 2007; 83: 1107.

49. Edinger M, Powrie F, Chakraverty R. Regulatory mechanisms in graft-versus-host responses. *Biol Blood Marrow Transplant* 2008; 15: 2.

50. Engelhardt B, Jagasia M, Rock MT, *et al.* Circulating Gut- or Skin-Homing Regulatory T Cells (Tregs) Predict Whether Acute Graft-Versus-Host Disease (aGVHD) Occurs in Gut or Skin Following Allogeneic Stem Cell Transplantation (ASCT). *Blood* 2008; 112 (Suppl. 1): S11 (abstract 717).

51. Carlson MJ, West ML, Coghill JM, Panoskaltsis-Mortari A, Blazar JS. *In vitro*-differentiated TH17 cells mediate lethal acute graft-*versus*-host disease with severe cutaneous and pulmonary pathologic manifestations. *Blood* 2009; 113: 1365.

52. Paczesny S, Krijanovski OI, Braun TM, *et al.* A biomarker panel for acute graft-*versus*-host disease. *Blood* 2009; 113: 273.