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Cytokine production during myeloablative and reduced intensity therapy before allogeneic stem cell transplantation

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A B S T R A C T

Background and Objectives. The aim of this study was to elucidate the effect of the type of chemo-radio therapy given before allogeneic hematopoietic stem-cell transplantation (HSCT) on cytokine release.

Design and Methods. We analyzed serum cytokine levels during pre-transplant therapy in 178 HSCT recipients. Samples were drawn daily during the treatment and serum levels of tumor necrosis factor-α (TNF-α) were analyzed by automated chemo-luminescence immunoassay. Conventional high-dose myeloablative therapy was given to 119 patients, while 59 patients received reduced intensity therapy (RIC). Most patients had a hematologic malignancy: their median age was 37 years (range 1-67). Anti-thymocyte globulin (ATG) was given to 126 patients as part of the pre-transplant treatment.

Results. The use of ATG significantly increased the TNF-α levels in the last four days before transplantation. We found significantly higher TNF-α levels, days -4 to -2 before transplant, in patients given RIC compared to myeloablative therapy independently of ATG treatment. No effect of age or disease stage was found. In patients not given ATG, we found a correlation between high TNF-α levels on day –2 and moderate-to-severe acute graft-versus-host disease (GVHD).

Interpretation and Conclusions. To conclude, during the pre-transplant treatment for HSCT, patients receiving RIC and those treated with ATG had increased levels of TNF-a.

Key words: cytokines, HSCT, reduced intensity conditioning.

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Allogeneic hematopoietic stem-cell transplantation (HSCT) is now well established to be a curative treatment for many hematologic malignancies and some non-malignant disorders.[1-3]

The pre-transplant chemo-radiotherapy in HSCT plays an important role in determining the outcome. The aim of the pre-transplant therapy is to eradicate malignant cells and to suppress the immune system. Following HSCT, graft-versus-host disease (GVHD) has been found to contribute to an anti-leukemic effect.[4,5] An alloresponse by donor T lymphocytes is probably the cause of GVHD and the graft-versus-leukemia (GVL) effect,[4,5] but it is now known that cytokines are also intimately involved in the development and maintenance of GVHD.[6-8] A recent study showed that the tissue damage of GVHD may be mediated by an associated inflammatory response rather than a direct cellular immunologic attack.[9] Piguet and co-workers were the first to report that tumor necrosis factor α (TNF-α) was an important mediator of GVHD.[10] Increased levels of TNF-α during pre-transplant treatment were found with correlate to moderate-to-severe GVHD and transplant-related mortality (TRM).[11,12] Infusion of donor cells into an inflammatory environment may cause activation of donor T lymphocytes, further cytokine release and the development of GVHD.[7]

The routine for several decades has been to administer profoundly myeloablative pre-transplant therapy to eradicate leukemic cells and induce marked immunosuppression to pave the way for the donor immuno-hematopoietic system.[13,14] However, this approach has been challenged by using lower doses and less toxic treatments

to induce immunosuppression and take advantage of the GVL effect later rather than relying solely on the anti-leukemia effect of the pre-transplant chemo-radiotherapy.[15-18]

The aim of this study was to elucidate the effect of the type of pre-transplant therapy on cytokine release. We wanted to study whether non-myeloablative, or reduced intensity therapy (RIC), also had effect on cytokine release before transplantation.

Design and Methods

Patients

One hundred and seventy-eight consecutive patients undergoing allogeneic haematopoietic stem cell transplantation (HSCT) at Huddinge University Hospital were included in this study. Most patients had a hematologic malignancy (n=147), but 15 patients with a non-malignant disorder and 16 with solid tumors were also included. Among the patients with a hematologic malignancy, 80 were in first complete remission (CR 1) and 67 in later stages at the time of transplantation. The median age of the series was 37 years (range 1-67 years). There were 110 males and 68 females. The patients' characteristics are shown in Table 1. The Research Ethics Committee at Huddinge University Hospital approved the study.

Table 1. Diagnoses and other characteristics of the 178 patients receiving either myeloablative or reduced intensity therapy (RIC) before hematopoietic stem cell transplantation. Median (range) or absolute numbers are given. *$p<0.001$.

	RIC	*Myeloablative*
N of patients	59	119
Age	53 (7-67)	26 (1-60)*
Gender (male/female)	36/23	74/45
Diagnosis		
Non-malignant disorder	5	10
Acute myeloid leukemia CR1/>CR1	5/3	27/14
Acute lymphoid leukemia CR1/>CR1	0	21/15
Chronic myeloid leukemia CP/not in CP	14/0	16/2
Myelodysplastic syndrome	7	9
Lymphoma	4	4
Myeloma	5	0
Solid tumor	16	0
ATG during conditioning	47	79
Stem cell source (BM/PBSC)	12/47	49/70
Nucleated cell dose ($\times 10^8$/kg)	9.1 (1-25)	6.2 (0.4-55)
$CD34^+$ cell dose ($\times 10^6$/kg)	6.1 (0.8-26)	7.1 (0.2-45)
Donor		
HLA-identical sibling	27	41
HLA-matched unrelated donor	32	71
Mismatched donor	0	7
Acute GVHD		
No	33 (58%)	31 (27%)
Grade I (mild)	10 (18%)	38 (33%)
Grade II (moderate)	10 (18%)	30 (26%)
Grades III-IV (severe)	4 (7%)	16 (13%)

CR: complete remission; CP: chronic phase; ATG: anti-thymocyte globulin; BM: bone marrow; PBSC: peripheral blood stem cells; HLA: human leukocyte antigen; GVHD: graft-versus-host disease.

Pre-transplant treatment

One hundred and nineteen patients received myeloablative chemo-radio therapy. Fifty-five patients received fractionated total body irradiation (TBI)(4×3 Gy) combined with cyclophosphamide 60 mg/kg/day on two consecutive days. Sixty-four patients were given busulfan, 4 mg/kg/day, on four consecutive days followed by Cy as mentioned above.[19]

Reduced intensity conditioning was given to fifty-nine patients. Fludarabine 30 mg/m^2/day for three days (sibling donor) or five days (unrelated donor) was combined with 2 Gy TBI (n=18)[16] or fludarabine was given for six days in combination with busulfan, 4 mg/kg/day, for two consecutive days (n=27) or cyclophosphamide, 30 mg/kg/day for 2 consecutive days (n=14).[18] Anti-thymocyte globulin (ATG, Thymoglobulin®, IMTIX-Sangstat, Lyon, France), 2 mg/kg/day, was given for 2-4 days to 126 patients with unrelated donors, those with a diagnosis of severe aplastic anemia (SAA) and those receiving the fludarabine + busulfan protocol.[20] The day of graft infusion was designated as day 0. The different treatment protocols are shown in Table 2.

Cytokine analysis

Blood samples from the patients were drawn every morning during the pre-transplant treatment. Samples were allowed to clot and then centrifuged and the serum was frozen and stored at -20°C until analyzed. As all blood samples were drawn early in the morning, the effect of all drugs was seen on the sample the day after (e.g. the effect of ATG given day -4 was seen on day -3). Cytokine assays were performed approximately monthly. TNF-α was analyzed using an automated chemo-luminescence immunoassay (IMMULITE®, DPC, Los Angeles, CA, USA) with a sensitivity of 1.7 pg/mL, an intra-assay coefficient of variation (CV) of 3.2% and an inter-assay CV of 5.1%.

Statistics

The analysis was performed on August 15, 2003. Mean differences in TNF-α levels between groups at various time-points before HSCT were tested using analysis of variance (ANOVA) with ATG and type of

Table 2. Different pre-transplant therapy protocols given to 178 hematopoietic stem-cell transplant recipients at Huddinge University Hospital.

Protocoll	*RIC*	*N*	*Treatment*								
			d-8	*d-7*	*d-6*	*d-5*	*d-4*	*d-3*	*d-2*	*d-1*	*D0*
FTBI + Cy±ATG	No	38/17				Cy	Cy ±ATG	TBI±ATG	TBI±ATG	TBI±ATG	TBI
Bu + Cy±ATG	No	41/23	Bu	Bu	Bu	Bu	Bu±ATG	Cy±ATG	Cy±ATG	±ATG	
Flu + Bu±ATG	Yes	27	Flu	Flu	Flu	Flu	Bu±ATG	Bu±ATG	ATG	ATG	
Flu + TBI±ATG	Yes	9/9			Flu	Flu	Flu±ATG	Flu±ATG	Flu±ATG	±ATG	TBI
Flu + Cy±ATG	Yes	11/3	Flu	Flu	Flu	Flu	Cy±ATG	Cy±ATG	±ATG	±ATG	

Bu: busulfan 1 mg/kg × 4/day; Flu: fludarabine 30 mg/m²/day; Cy: cyclophosphamide 60 mg/kg/day (30 mg/kg/day in RIC); ATG: antithymocyte globulin 2 mg/kg/day; FTBI: fractionated total-body irradiation, 3 Gy/day.

treatment (RIC vs. myeloablative) as *fixed* factors. All cytokine levels were log-transformed to meet the basic conditions on which the theory of ANOVA is based. The logistic regression method was used when analyzing factors associated with acute GVHD grades II-IV, and multiple linear regression when analyzing factors associated with TNF-α levels. Factors included in the multivariable linear regression analysis were: age, type of pre-transplant treatment, ATG, diagnosis and disease stage. The geometrical mean was calculated on antilog. All measurements of variation are expressed as a geometrical mean with a 95% confidence interval (95% CI). All calculations were computed with Statistica/W© software (StatSoft Inc., Tulsa, OK, USA).

Results

ATG and treatment regimen

Anti-thymocyte globulin, given during the pre-transplant therapy, significantly increased the serum levels of TNF-α on days -3, -2, -1 and 0 before HSCT ($p<0.001$) (Figure 1). TNF-α levels slowly decreased over time in patients not given ATG, while in ATG-treated patients the levels of TNF-α showed a sharp increase after ATG was given. The effect of ATG on TNF-α release was distinct in patients treated with either RIC or myeloablative therapy (Figure 1). We found significantly higher levels of TNF-α on days –4 to –2 in patients treated with RIC than in those given myeloablative therapy regardless of treatment with ATG (Figure 2).

In multivariable analysis, corrected for disease stage, patient's age and diagnosis, we found that RIC (days –4 to –2) and ATG (days –3 to 0) were the only factors associated with increased TNF-α levels (Table 3). The cytokine patterns in two myeloablative protocols with and without ATG are illustrated in Figure 3. The effect of ATG seems to be more distinct in recipients of the busulfan + cyclophosphamide protocol than in those receiving fractionated TBI + cyclophosphamide.

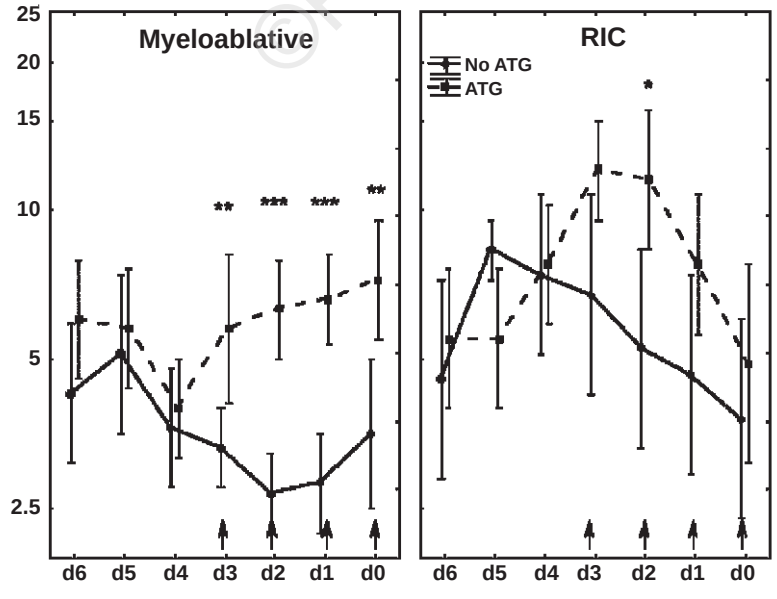

Figure 1. Serum TNF-α levels during the pre-transplant therapy in patients treated or not with ATG who received a) myeloablative or b) reduced intensity therapy (RIC). Geometrical means and 95% CI. The *p*-value indicates differences between the two groups at different time points. ***$p<0.001$, **$p<0.01$, *$p<0.05$. Arrows indicate the effect of ATG on TNF-α levels.

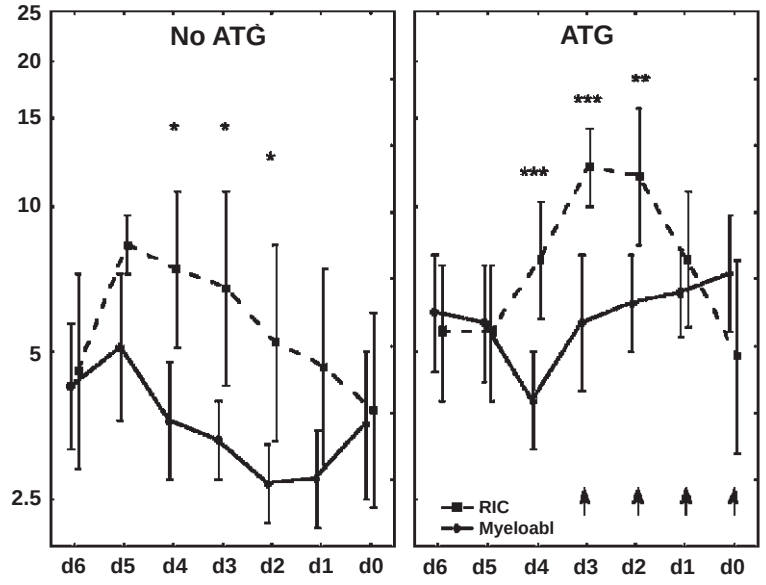

Figure 2. Serum TNF-α levels during the pre-transplant therapy in patients given RIC and myeloablative therapy, a) not given ATG and b) given ATG. Geometrical means and 95% CI. The value indicates differences between the two groups at different time points. ***$p<0.001$, **$p<0.01$, *$p<0.05$. Arrows indicate the effect of ATG on TNF-α levels.

Table 3. Results from multivariable linear regression analysis of factors associated with increased TNF-α levels. Corrected for patient's age, diagnosis and disease stage.

Factor	*Day -6*	*Day –5*	*Day -4*	
RIC vs. Myeloablative	OR=0.96, (p=0.7)	OR=0.96, (p=0.7)	OR=1.32, (p=0.003)	
ATG vs. no ATG	OR=1.17, (p=0.2)	OR=1.06, (p=0.5	OR=1.05, (p=0.5)	
Factor	*Day -3*	*Day –2*	*Day -1*	*Day 0*
RIC vs. Myeloablative	OR=1.33 (p=0.001)	OR=1.20, (p=0.03)	OR=1.05, (p=0.54)	OR=1.09, (p=0.37)
ATG vs. no ATG	OR=1.16, (p=0.04)	OR=1.54, (p<0.001)	OR=1.53, (p<0.001)	OR=1.29, (p=0.003)

RIC: reduced intensity therapy; ATG: anti-thymocyte globulin.

Diagnosis

Patients with non-malignant diseases had slightly higher levels of TNF-α during therapy than did patients with chronic leukemia who, in turn, had slightly higher levels than patients with acute leukemia. However, these differences could only be detected at the beginning of the conditioning therapy, namely day –8 to day –5. In the multivariate analysis, diagnosis influenced TNF-α levels only on day –7 (p=0.01) and day -6 (p=0.04).

Other factors

Disease stage and patient's age were not found to have an effect on TNF-α levels.

Graft-versus-host disease

The incidence of acute GVHD in patients given myeloablative therapy and RIC are shown in Table 1.

Among patients who did not receive ATG during the pre-transplant therapy we found a correlation between high levels of TNF-α on day –2 and moderate-to-severe acute GVHD (grades II-IV) (p=0.02). This correlation (OR 1.29, CI 1.01-1.64, p=0.03) was still apparent when correcting for confounding factors such as donor type and HLA-matching. Mean TNFα levels were 3.3 pg/mL (CI: 2.5-4.1 pg/mL) and 5.5 pg/mL (CI: 3.8-7.3 pg/mL) in patients with no or only mild acute GVHD and in those with moderate-to-severe acute GVHD, respectively. No such correlation was seen among patients given ATG during conditioning. Corresponding figures for patients receiving ATG were 7.9 pg/mL (6.3-10.0 pg/mL) and 6.2 pg/mL (4.6-8.3 pg/mL), respectively.

Discussion

ATG significantly increased TNF-α serum levels in the last four days before transplantation (Figure 1). This effect was seen both during myeloablative therapy and RIC. Furthermore, patients given RIC showed higher TNF-α levels than did patients receiving myeloablative therapy whether they were or were not given ATG (Figure 2).

ATG binds to T cells, causing their activation, cytokine release and subsequent elimination.[21] ATG was given on the last 2 to 4 days of the pre-transplant therapy, after several days of chemotherapy. Given that RIC is a milder treatment, patients conditioned with such a protocol may harbor more T cells at the time of ATG treatment than patients given myeloablative therapy. We also

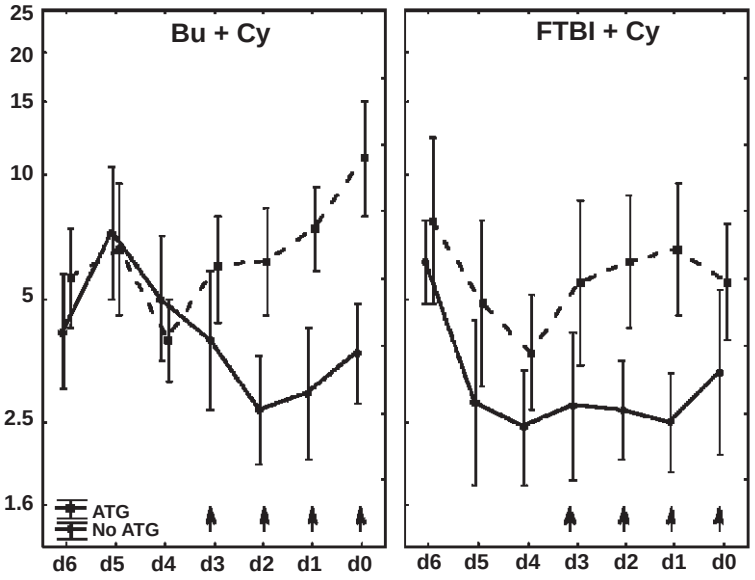

Figure 3. Serum TNF-α levels during the pre-transplant therapy in patients given two different myeloablative therapy regimens. Geometrical means and 95% CI. Arrows indicate the effect of ATG on TNF-α levels.

found higher TNF-α levels during RIC than during conventional myeloablative therapy in patients not receiving ATG (Figure 3). The therapy used in RIC is largely the same (except fludarabine) as that in myeloablative therapy, but administered at lower doses. The high doses of busulfan and cyclophosphamide used in myeloablative therapy effectively reduce the number of leukocytes, thus reducing the source of cytokine release, while the milder treatment in RIC spares leukocytes and therefore possibly maintains the number of cells that may release cytokines. Fludarabine and the lower doses of busulfan and cyclophosphamide may also stimulate lymphocytes to release inflammatory mediators while higher doses kill them. Fludarabine might have toxic effects on endothelial cells, which could add to the level of TNF-α secretion. However, RIC is often reserved for patients who are sicker and sicker patients may be more prone to higher TNF-α release than healthier patients. We cannot role out that this difference in the groups of patients may account for at least some of the difference in the TNF-α levels observed. We found that patients with non-malignant diseases had higher levels of TNF-α than those with chronic leukemia, who in turn had higher levels than patients with acute leukemia. This effect was mainly seen during the first days of therapy, while no effect was seen in the last four days. This may indicate that patients who had had multiple cycles of intensive chemotherapy before the pre-transplant treatment are much more depleted of immune effector cells and macrophages which then leads to lower TNF-α levels. In the multivariate analysis, diagnosis was correlated with higher TNF-α levels only days –7 and –6 before transplantation. Disease stage was not found to have any effect.

That cytokines have a role in the development and maintenance of GVHD after HSCT has been proven in many studies, both experimental and clinical.[11,12,22-25] The pre-transplant treatment creates an environment that has been altered to promote activation and proliferation of inflammatory cells.[7] Donor cells rapidly encounter not only a foreign environment, but also one that has been altered to promote the activation and proliferation of inflammatory cells by the increased expression of adhesion molecules, cytokines and cell-surface recognition molecules.[7] High serum levels of TNF-α during the pre-transplant therapy have been shown to be associated with moderate-to-severe acute GVHD.[11,12] In line with previous studies[11,12] we found a correlation between high TNF-α levels and moderate-to-severe GVHD, but only in patients not given ATG. No such correlation was found in patients treated with ATG. ATG causes *in vivo* T-cell depletion, thus very few T-cells remain to be activated. It has previously been shown that inclusion of ATG in the pre-transplant therapy reduces the incidence and severity of GVHD.[20,26-27] ATG induces an increased release of a broad range of cytokines which may include not only inflammatory cytokines but also anti-inflammatory cytokines such as IL-10, which could down-modulate GVHD.

When milder, less toxic therapy was introduced, it was thought that the incidence and severity of GVHD would be lower. Less toxic treatment was thought to cause less tissue damage, less intestinal permeability with a low incidence of acute GVHD. However, as we have now learnt, this is not the case. GVHD is still a problem when using reduced intensity therapy and similar to that after conventional myeloablative therapy.[28-29] Our finding that TNF-α levels are higher in patients receiving RIC than in those treated with myeloablative therapy may be one, among various other reasons, for

the similar incidence of acute GVHD after these two types of pre-transplant therapies. Recently it has been proposed that recipient antigen presenting cells (APC) play a major role in the activation of donor cells.[30] Experimental data suggest that full intensity therapy might be more efficient than reduced intensity therapy in rapidly depleting APC. This may partly explain the similar incidence of GVHD in patients treated with either RIC or myeloablative therapy. Other factors, including polymorphism of cytokine genes and HLA minor histocompatibility antigens, may also play a role in the development of GVHD.[31-34] These factors were not investigated in the present study. Certain cytokine genes (e.g. TNF2, TNFd3d3) have been correlated with severe GVHD after HSCT,[34] the mechanism probably being greater cytokine production. To conclude, during the pre-transplant treatment for HSCT, patients given RIC and those treated with ATG had increased levels of TNF-α. Less intensive pre-transplant therapy may be less toxic to the patients, but it does not reduce cytokine release during the treatment.

MR designed the study, performed some of the cytokine analysis, analyzed the material statistically, wrote the manuscript and designed figures 1-3 and tables 1-3. BS contributed to the design, performed most of the cytokine analyses, critically revised the manuscript and approved the final version. MR takes primary responsibility for the paper. MR created all Tables and Figures.

The authors reported no potential conflicts of interest.

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