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Prognostic significance of increased IL-10 production in patients prior to allogeneic bone marrow transplantation

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Summary:

IL-10 is a potent immunosuppressant which inhibits allo-antigen-specific T cell responses. In addition, IL-10 is a strong endogenous anti-inflammatory cytokine. To investigate the role of IL-10 in the induction of acute GVHD following allogeneic bone marrow transplantation (BMT) we performed a prospective study on spontaneous IL-10 production by peripheral blood mononuclear cells (PBMNC) in 84 patients admitted for allogeneic BMT. High spontaneous IL-10 production by PBMNC at the time of admission and prior to any preparative treatment correlated with a subsequent low incidence of GVHD and transplant-related mortality (8%), as compared to patients with low or intermediate IL-10 production (50%, $P < 0.01$). Our data demonstrate the prognostic significance of increased IL-10 production in BMT patients and suggest a major role of IL-10 in maintaining immunobalance in the setting of allogeneic BMT. *Bone Marrow Transplantation* (2000) **25,** 237–241.

Keywords: bone marrow transplantation; IL-10; GVHD; TNF-α

Release of proinflammatory cytokines such as TNF-α and IL-1 in the course of pretransplant conditioning has been shown to be involved in the induction of acute graft-versus-host disease (GVHD) following experimental[1,2] as well as clinical[3] bone marrow transplantation (BMT). An additional role of these soluble mediators has been documented in the effector phase of acute GVHD[4] which led to preclinical and clinical studies using cytokine antagonists such as IL-1 receptor antagonist (IL-1ra) or monoclonal anti-TNFα antibodies[5,6] for treatment of aGVHD. Within the cytokine network, however, activation of proinflammatory mediators is followed by increased production of endogenous inhibitory molecules including antagonistic cytokines. Among these endogenous antagonists, IL-10 has raised substantial interest: IL-10 is produced by both monocytes/macrophages and T cells belonging to the Th0, Th1 and Th2 subsets. IL-10 suppresses the production of proinflammatory monokines[7] and modulates the expression of important costimulatory molecules such as CD80[8] on antigen presenting cells (APC). In addition, it inhibits alloantigen-induced T cell activation.[9] These biological characteristics suggested that IL-10 might be a suppressive mediator involved in preventing aGVHD and in inducing T cell tolerance in the setting of BMT. This hypothesis was supported by the observation that increased IL-10 production by mononuclear cells is associated with tolerance in SCID patients following haploidentical BMT.[10] However, several experimental studies using recombinant IL-10 as an exogenous antagonist *in vivo* failed to show beneficial effects.[11,12]

To date, few reports have analyzed IL-10 production in the course of clinical BMT[13,14] demonstrating an association of increased IL-10 production with transplant-related complications. We have now performed a prospective study on the cellular release of IL-10 by PBMNC at the time of admission for allogeneic as well as autologous BMT. Results show that the prognostic significance of increased IL-10 production strongly depends on the time of analysis. At the time of admission, ie during steady-state conditions prior to any treatment, increased IL-10 production predicted uneventful courses of the transplants and absence of aGVHD.

Materials and methods

Patients

Ninety-eight consecutive patients receiving either autologous (n = 14) or allogeneic (n = 84) BMT between December 1993 and June 1995 were included in the present study. The median age of patients receiving autografts was 35 years (range 19–55), the median age of patients receiving allografts was 40.5 years (range 16–56). Among the allografted patients, 51 were transplanted from HLA-identical sibling donors, 28 from HLA-identical unrelated donors, and five from related, one HLA-antigen different donors. Bone marrow was used as a stem cell source in all patients. Further patient characteristics are given in Table 1. Peripheral blood mononuclear cells (PBMNC) were obtained at the time of admission, ie before application of

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Table 1 Univariate analysis of patients' characteristics and history in different groups of spontaneous IL-10 production by PBMNC at the time of admission

	Low IL-10	*Intermediate IL-10*	*High IL-10*	*P*
Diagnosis:				
CML	27	14	8	
Acute leuk.	13	11	4	NS
Others	3	0	0	
Age (years)	38.8 (1.4)	38.2 (1.8)	39.2 (2.9)	NS
Sex: M/F	23/22	12/16	4/8	NS
Stage at BMT:				
stage I	20	12	10	0.04
> stage I	25	16	2	
pos. for EBV	100%	85%	83%	NS
pos. for CMV	42%	39%	50%	NS
monocytes (% positive cells)	2.2 (0.8)	3.4 (1.2)	2.5 (1.4)	NS
HLA-DR 3				
pos.	10	4	3	NS
neg.	35	24	8	
Spontaneous TNF-α production (pg/ml)	252 (189)	374 (87)	1678 (754)	0.009

Groups were formed according to low ($\leq$100 pg/ml), intermediate (101–1000 pg/ml) and high (>1000 pg/ml) IL-10 levels observed in supernatants of 24 h cultures of PBMNC in medium. Incidences were compared by chi-square analysis, parametric variables were analyzed by *t*-tests. For parametric variables, mean and s.e.m. (in ( ) brackets) are shown.
CML = chronic myelogenous leukemia; Acute leuk. = acute leukemias; Pos for EBV/CMV = positive recipient serology for Epstein–Barr and cytomegalovirus at admission; Stage I = chronic phase of CML or first remission of acute leukemia; > stage I = all other stages; HLA-DR3 = at least one DR locus was DR3; NS = not significant.

any preparative regimen or other interventions. Collection of blood was explained to all patients and relatives, and informed consent was given prior to admission. Pretransplant conditioning (TBI/CY or BUS/CY), prophylaxis (cyclosporin and a short course of methotrexate) as well as treatment (prednisolone as first-line treatment) for aGVHD and supportive care were performed as previously described.[3,14]

Peripheral blood mononuclear cell cultures

Heparinized blood was obtained prior to the first application of cytotoxic drugs or the first day of total body irradiation for pretransplant cultures. After Ficoll–Hypaque (Pharmacia, Freiburg, Germany) centrifugation, MNC were washed twice in PBS buffer and adjusted to 1×10^6/ml in RPMI 1640 medium, supplemented with 10% FCS, 1% penicillin-streptomycin and 1% L-glutamine (all reagents from Gibco, Karlsruhe, Germany). Mononuclear cell separations were in the range of 80–96% pure, and monocyte contents were in the range of 2–10%, as determined by flow cytometric analysis (not shown). To analyze spontaneous cytokine production, 2×10^6 cells/ml were cultured for 24 h in a 5% CO_2 incubator at 37°C in medium (see above). Triplicate cultures were performed in microwell plates (Nunc, Wiesbaden, Germany). Subsequently, supernatants from individual patients' cultures were collected and frozen at −20°C until use. In 19 patients, additional serum samples were collected at the time of PBMNC isolation, and frozen at −20°C until use.

Analysis of IL-10 and TNF-α

IL-10 was analyzed in supernatants of PBMNC cultures as well as in sera using a sensitive ELISA. Briefly, two different monoclonal antibodies (MoAbs) highly specific for human IL-10 (coating MoAb: J9D7; detecting MoAb: 12G8) were used for capture and detection of IL-10 contained in the samples. Detection was performed by a peroxidase-coupled goat-anti-rat IgG antibody (J4, all antibodies provided by DNAX Research Institute, Palo Alto, CA, USA). The detection limit of the IL-10 assay was 20 pg/ml for cell culture supernatants. In addition to IL-10, TNF-α was analyzed in all supernatants using our previously described ELISA.[15] In the first cohort of patients, additional serum samples had been cryopreserved throughout the period of pretransplant conditioning and were evaluated for TNF-α levels using the same protocol. For both IL-10 and TNF-α levels in supernatants, at least duplicate assays were performed.

Clinical and statistical analysis

Patients were grouped according to the type of BMT and complications occurring during the aplastic phase or following engraftment, actuarial transplant-related mortality (TRM) and severity of acute and chronic GVHD as determined by clinical criteria. For pretransplant culture, the following parameters were analyzed: age, sex, underlying disease, stage at the time of BMT, cytomegalovirus- and EBV-titers and HLA-DR haplotypes. IL-10 and TNF-α levels in supernatants of cultures performed prior to BMT, also obtained in these different groups, were summarized and compared by Mann–Whitney tests. Incidences were analyzed by chi-square analysis. Multivariate analyses and Kaplan–Meier analysis to calculate actuarial TRM were performed by using the NCSS statistical program.

Results

IL-10 production in PBMNC cultures obtained prior to conditioning

The analysis of spontaneous IL-10 production by PBMNC of asymptomatic patients obtained at the time of admission showed a clear correlation between increased IL-10 production and subsequent uneventful courses. In 26 patients receiving allogeneic BMT and developing neither GVHD requiring immunosuppressive treatment nor other severe or lethal complications, mean spontaneous IL-10 production was as high as 2368 (±787 s.e.m.) pg/ml, whereas 23 patients with GVHD or nonlethal complications had a mean IL-10 production of 211 (±74) pg/ml, and 35 patients dying from TRC showed a mean production of 462 (±294)

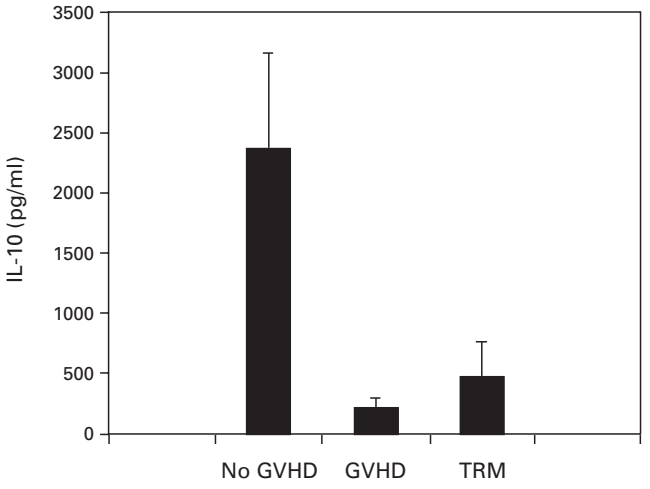

Figure 1 Mean and s.e.m. of spontaneous IL-10 production by PBMNC of patients admitted to allogeneic BMT. Patients were grouped according to outcome following BMT: No GVHD = patients never developed GVHD requiring immunosuppressive treatment (n = 26); GVHD = surviving patients with GVHD grade II or more (n = 23); TRM = patients dying from any form of transplant-related complications (n = 35). Differences in mean IL-10 production were highly significant between patients without GVHD and all other patients ($P < 0.005$).

pg/ml ($P < 0.005$, Figure 1). None of the patients with IL-10 production >1000 pg/ml had aGVHD grade II or more following BMT while the percentage of aGVHD rose to 58% in patients with intermediate and to 63% in patients with low IL-10 production ($P = 0.007$, data not shown). This resulted in significantly lower actuarial TRM in high IL-10 producers ($P = 0.01$), as compared to intermediate or low IL-10 producers (Figure 2).

Analysis of serum levels

In 19 patients, IL-10 serum levels were analyzed at the time of PBMNC collection. In 14 patients with low cellular (<100 pg/ml) IL-10 production, mean IL-10 serum levels were 102 (±26) pg/ml, whereas the remaining five patients

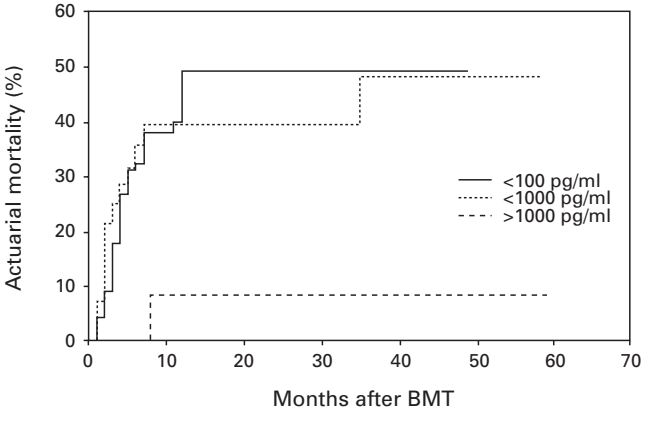

Figure 2 Actuarial transplant-related mortality and spontaneous IL-10 production by PBMNC at the time of admission in patients receiving allogeneic BMT. TRM is significantly lower for patients with IL-10 production >1000 pg/ml as compared to patients with low (<100 pg/ml) and intermediate (100–1000 pg/ml) IL-10 production ($P = 0.01$, log rank test).

with high cellular IL-10 production (>1000 pg/ml) had increased IL-10 serum levels (mean 792 ± 502 pg/ml, $P < 0.02$). These data at least suggest simultaneous occurrence of cellular as well as systemic IL-10 production. In 48 patients, TNF-α serum levels were analyzed throughout the period of pretransplant conditioning: in 11 patients with high IL-10 production (>1000 pg/ml) prior to conditioning, *de novo* release of TNF-α during conditioning was virtually absent (19 ± 7 pg/ml at admission, 22 ± 4 pg/ml maximum during conditioning), while in 37 patients with intermediate or low IL-10 production (<1000 pg/ml) at admission, TNF-α serum levels rose from 19 ± 5 pg/ml to 49 ± 9 pg/ml ($P < 0.01$).

Analysis of risk factors of increased IL-10 production

A variety of parameters were analyzed in order to identify factors associated with increased cellular IL-10 production at the time of admission (Table 1). Age, sex, diagnosis, HLA-DR haplotypes, infections, treatment prior to admission and monocyte counts in PBMNC fractions proved to be without any correlation with the levels of spontaneous IL-10 production. There was a trend to a higher proportion of patients in an early stage of disease (ie CML chronic phase or first remission of acute leukemia) in the group of patients with high IL-10 production, but the most significant finding associated with increased IL-10 production was high spontaneous TNF-α production in the same cultures: in all cultures performed, the correlation between TNF-α and IL-10 production was 0.670. In multivariate analysis of factors associated with increased IL-10 production, simultaneous TNF-α production was the only significant factor ($P < 0.03$).

The 14 patients receiving autologous BMT were analyzed separately. In this subgroup of patients, IL-10 production at admission showed no correlation with outcome as only one patient died from complications. Among these patients, high cellular IL-10 production was more frequently observed in patients with advanced lymphoma as compared to patients with AML ($P < 0.05$).

Discussion

Our study addresses several aspects of the biology of IL-10 in the setting of clinical BMT. The observation of a strong association of increased IL-10 production at the time of admission with subsequent uneventful courses, which was highly significant in a large series of patients, suggests a protective role of IL-10. Indeed, systemic TNF-α release during pretransplant conditioning, which was previously reported to be an indicator of poor outcome,[15,16] was prevented in patients with high IL-10 production at the time of admission. Thus, the absence of complications after BMT might be explained by IL-10-mediated down-modulation of proinflammatory cytokine production.

However, the absence of GVHD in patients with increased IL-10 production suggests effects of IL-10 beyond suppression of inflammatory cytokines which might even result in donor–host tolerance. High levels of IL-10 produced prior to BMT may down-regulate tissue

expression of ICAM-1 and ICAM-1-mediated transendothelial migration of inflammatory cells which occurs after irradiation or LPS activation.[17,18] In addition, downmodulation of other costimulatory molecules including CD80 on antigen presenting cells such as dendritic cells by IL-10 may result in tolerance.[19] Finally, presence of high IL-10 production at the time of donor cell infusion might induce a host-specific anergic state in donor T lymphocytes as reported *in vitro* in mixed lymphocyte reactions carried out in the presence of IL-10.[8]

Analysis of factors associated with spontaneous IL-10 production at the time of admission revealed a correlation between increased IL-10 and early stage of disease and spontaneous TNF-α production. Disease-associated IL-10 production has been shown in several lymphatic malignancies[19,20] and more recently also for patients with CML.[21] In our laboratory, we have observed a decreased capacity for cytokine production in macrophages derived from patients with advanced stages of these diseases which might reflect exhaustion of immune regulation or an effect of more aggressive previous cytotoxic treatment. This is in line with our present observation that increased IL-10 production occurs mainly in the early phases of disease.

The association of high spontaneous IL-10 and TNF-α production observed in our patients suggests that IL-10 release occurs as a consequence of activation of TNF-α, which is consistent with experimental data demonstrating that TNF-α is up to 100-fold more potent with regard to IL-10 induction than any other cytokine.[22] These results are in line with our previous observation that a small subgroup of patients with high spontaneous steady-state TNF-α production at the time of admission is protected from transplant-related complications[23] in contrast to patients with *de novo* TNF-α release during conditioning. In that study, we speculated that mechanisms resembling TNF-α desensitization mediate this protection. The present data suggest that IL-10 is the crucial protective cytokine responsible for the phenomenon. This hypothesis is supported by experimental and clinical studies indicating that IL-10 is involved in LPS desensitization[24] and by recent experiments using neutralizing IL-10 and TNF-α antibodies in PBMNC cultures of patients admitted to BMT performed in our laboratory.

The background of this protective steady-state production at the time of admission is still poorly understood. Immunogenetic differences might be an important explanation for the differences in spontaneous cytokine production of cells obtained during steady-state conditions. Cytokine promoter polymorphisms have been described for both TNF-α and IL-10 and data reported by Middleton *et al*[25] as well as preliminary data from our group[26] suggest that these variables are of relevance in the setting of clinical GVHD.

In addition to the pretransplant IL-10 production it will be most interesting to investigate systemic and cellular IL-10 release in the course of BMT. As one might expect from our experience with release of TNF-α and as indicated in the few published studies on IL-10 levels[13,14] post transplant, reactive IL-10 release in the course of clinical complications has a completely different prognostic significance and is associated with increased transplant-related mortality. Thus, for both TNF-α and IL-10, steady-state and reactive production indicate a completely different biological status.

In summary, our data suggest a major role of IL-10 in maintaining immunobalance in the setting of BMT and are in accordance with previous studies reporting an association of tolerance with increased IL-10 production in SCID patients receiving HLA mismatched grafts.[10] Besides the possible systemic effects of increased IL-10 production by recipient cells, the generation of tolerizing regulatory donor T cells, as recently described,[27] can be responsible for the phenomenon. Whether a similar mechanism occurs also in our patients in whom high host cell-related IL-10 production may still be present at the time of donor T cell infusions is an attractive but still speculative explanation which needs further analysis.

Failure to detect significant protection from GVHD by prophylactic application of exogenous IL-10 in animal models also points to more complex underlying immunological mechanisms. Indeed, studies on administration of IL-10 indicate that timing and dosage of IL-10 are crucial as IL-10 may protect from LPS-mediated shock but enhance mortality in other infectious models.[28,29] This narrow range between sufficient and excess immunosuppression induced by IL-10 may also be relevant for IL-10 in the setting of BMT, and it is reminiscent of the difficulties of identifying therapeutic windows for other cytokine antagonists such as monoclonal anti-TNF-α antibodies. The possibilities of identifying subgroups of patients on the basis of altered cytokine regulation may help to target groups of patients at high risk who are more likely to benefit from exogenous administration of cytokine antagonists. Besides identification of low and high risk patients, further analysis of patients protected by high spontaneous TNF-α and IL-10 production will provide new insights into cytokine regulation in the setting of BMT.

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