

ORIGINAL ARTICLE

Production of IL-10 by alloreactive sibling donor cells and its influence on the development of acute GVHD

LE Weston^{1,3}, AF Geczy^{1,3} and H Briscoe²

¹Department of Cellular Tissue Typing, Australian Red Cross Blood Service-NSW/ACT, Sydney, Australia; ²Infectious Disease & Immunology, Central Clinical School, University of Sydney, Sydney, Australia and ³Research Unit of Transfusion Medicine and Immunogenetics, Faculty of Medicine, University of Sydney, Sydney, Australia

Graft-versus-host disease (GVHD) is a major complication of allogeneic hematopoietic stem cell transplantation. Pretransplant conditioning regimes cause release of proinflammatory cytokines that stimulate alloreactive donor T cells to attack recipient tissues. IL-10 has been shown to directly downregulate CD4⁺ T cells by suppressing IL-2 secretion and a critical role played by regulatory T cells has been demonstrated in animal models. One defining cytokine profile for regulatory T cells is the production of IL-10. Release of specific cytokines (IL-10, IL-4 and IFN- γ) was detected using ELISPOT technology, following stimulation of donor peripheral blood mononuclear cells by recipient (human leukocyte antigen-matched sibling) alloantigen or by mitogen. Correlation between the frequency of cytokine-releasing cells and the development of acute GVHD was investigated. A high frequency of donor cells producing IL-10 in response to recipient alloantigen stimulation correlated with absence of acute GVHD after bone marrow transplant (BMT), while low frequency was strongly associated with severe GVHD. This study presents strong evidence that estimating the frequency of donor alloreactive cells producing IL-10 in response to recipient antigens will provide valuable information prior to BMT regarding potential transplant outcome.

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Introduction

The role of activated donor-derived T cells in the induction of graft-versus-host disease (GVHD) has been established

together with evidence to implicate T-cell-derived and other proinflammatory cytokines.^{1,2} In response to the toxicity of the conditioning regimen of a bone marrow transplant (BMT), a cascade of primarily proinflammatory cytokines has been shown to amplify target organ injury and increase the expression of human leukocyte antigen (HLA) molecules, which, in turn, activates antigen-specific T cells. Analysis of cytokine networks has demonstrated their release in response to conditioning. Initially, TNF- α , IL-1 and IL-6 are released by recipient tissue, with subsequent release of IFN- γ and IL-2 by activated donor T cells, accompanied by the endogenous release of IL-10.³ IL-10 suppresses the production of proinflammatory cytokines⁴ and regulates the expression of costimulatory molecules on antigen-presenting cells.⁵ IL-10 has been shown to directly affect CD4⁺ T cells by suppressing IL-2 secretion.⁶ Groux *et al.*⁷ demonstrated that IL-10 induced a long-lasting antigen-specific unresponsiveness against allogeneic antigens that could not be reversed by IL-2 or CD28 stimulation.

Using a murine model, Hoffmann *et al.*⁸ demonstrated rescue of recipients from lethal GVHD using freshly isolated CD4⁺CD25⁺ from unprimed mice. The protection was dependent on IL-10 production and only donor-type CD4⁺CD25⁺ T cells were capable of preventing death of the host animal. Jonuleit *et al.*⁹ observed that human regulatory T cells, produced high levels of IL-10 when induced by immature dendritic cells. Further, T regulatory type 1 (Tr1) cells were shown to mediate their regulatory function by producing a distinct profile of immunosuppressive cytokines, including IL-10 and TGF- β , with little or no IL-2 or IL-4 production.¹⁰

The present study sought to determine the relationships between frequency of cytokine-secreting donor cells and incidence and severity of acute GVHD following sibling HLA-matched BMT. The frequency of IL-10-, IL-4- and IFN- γ -producing cells was detected using an ELISPOT assay adapted specifically for the purpose of detecting responses to membrane-bound alloantigens. We demonstrated a strong negative correlation between the frequency of IL-10-producing cells detected in the blood of HLA-matched sibling donors when challenged by peripheral blood mononuclear cells (PBMC) from their potential recipients and the probability of developing severe acute

Correspondence: Dr LE Weston, Department of Cellular Tissue Typing, Australian Red Cross Blood Service-NSW/ACT, 153 Clarence Street, Sydney, NSW 2000, Australia.

E-mail: lweston@arcbs.redcross.org.au

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GVHD post-transplant. These data have significant implications for informed donor selection, improved outcome and for guiding immunotherapy in HLA-matched sibling BMT recipients.

Patients and methods

Transplant donor–recipient population

The study group of 26 recipients from six transplant centers received BMTs from HLA-identical sibling donors as treatment for their hematological disorders over the period January 1990–June 2000. Recipients received a myeloablative pretransplant treatment regimen, and GVHD prophylaxis was with methotrexate and cyclosporin A (CSA). Transplanted marrow was not manipulated prior to grafting. The age range for recipients was 19–55, with a mean age of 38 years. For donors, the age range was 15–51, with a mean of 36 years. Gender, disease and outcome data are detailed in Table 1. For participation in this study, each donor–recipient pair had to have available PBMC taken from the recipient before immune ablation therapy and from the donor before bone marrow extraction. These cells were cryopreserved and stored in liquid nitrogen.

Table 1 Details of recipients and donors and transplantation outcome

ID	Gender		Disease	Cells producing IL-10/10 ⁶ PBMC	Grade acute GVHD
	Recipient	Donor			
BM-1	F	F	NHL	5649	0
BM-2	M	M	CML	None detected	3
BM-3	F	M	CML	675	0
BM-4	M	F	CML	4504	0
BM-5	M	M	MM	None detected	2
BM-6	F	M	ALL	824	4
BM-7	M	F	CML	53	2
BM-8	M	M	Myelodysplasia	88 000	0
BM-9	M	M	CML	None detected	2
BM-10	F	M	CML	1353	0
BM-11	M	F	MM	274	3
BM-12	M	M	NHL	263	3
BM-13	M	F	Myelodysplasia	3246	0
BM-14	M	M	CML	2136	3
BM-15	M	F	CML	76 923	3
BM-16	M	F	AML	250 000	0
BM-17	M	M	Myelodysplasia	841	3
BM-18	F	F	AML	2808	0
BM-19	M	M	CML	100 000	0
BM-20	F	M	AML	1280	0
BM-21	M	F	CML	558	3
BM-22	M	F	Solid tumour	908	2
BM-23	M	M	CML	942	1
BM-24	F	M	AML	None detected	1
BM-25	M	F	CML	None detected	4
BM-26	M	F	CML	1748	1

F = female; M = male; CML = chronic myelogenous leukaemia; AML = acute myeloid leukaemia; NHL = non-Hodgkin's lymphoma; ALL = acute lymphoblastic leukaemia; MM = multiple myeloma. Grade of acute graft-versus-host disease was assessed according to Seattle Criteria.¹⁸

Assessment of acute GVHD

The relevant transplant center provided assessment of the transplant outcome. Determination of acute GVHD grade, classified into 5 grades (0–4) of increasing severity, was according to the Seattle criteria.¹¹

Preparation of human PBMC for ELISPOT analysis

The frequency of IL-4-, IL-10- and IFN- γ -expressing cells was estimated retrospectively in the 26 BMT donor–recipient sibling pairs described above. Cells from all donor/recipient pairs were viable at the time of testing, as demonstrated by their ability to produce cytokine in response to phytohemagglutinin (PHA).

Cryopreserved PBMC were thawed using 20% pooled normal human serum in HEPES-buffered RPMI 1640 (Roswell Park Memorial Institute), pH 6.8–7.4, washed, and prepared for ELISPOT assay at 1×10^6 cells/ml in HEPES-buffered RPMI 1640 supplemented with 7.5% heat-inactivated (56°C) pooled human serum, 10^{-5} M 2-mercaptoethanol (2ME), 4 IU Heparin/ml.

Activation of alloreactive effector cells

Preincubation of PBMC with antigen or mitogen is necessary to initiate cytokine production by effector cells.¹² Standard ELISPOT methodology was adapted to allow prestimulation of donor PBMC with the alloantigens of the corresponding recipient PBMC as follows: four serial two-fold dilutions of donor PBMC, prepared as above, were made in 96-well U-bottom plates. The highest concentration of responding cells was 50 000 cells per well. PBMC from the corresponding recipient were gamma-irradiated (50 G) and 50 000 cells were added to all wells. Plates were incubated overnight at 37°C with 5% CO₂. Positive control for viability of the preserved cells and for cytokine production was achieved by mitogenic stimulation of cell populations with PHA 5 μ l/ml (Becton Dickinson, Sparkes, MD, USA, stock concentration undisclosed). PHA stimulation was also applied to the irradiated recipient cells to assure the effectiveness of irradiation and ensure that this cell population was not contributing to cytokine production. All cell populations were cultured in medium alone to assess the background levels of cytokine production.

Enumeration of cytokine-secreting cells

Each of the 96 wells in Multiscreen HA 0.45 μ m plates (Millipore, France) was coated with 100 μ l of monoclonal anti-human cytokine antibody, 15 μ g/ml in coating buffer (0.1 M Na₂HPO₄, pH 9.0) 4°C overnight. All antibodies were obtained from Pharmingen (San Diego, CA, USA). For IL-4, the mouse anti-human antibody clone was 8D4-8 and the biotinylated rat-anti human antibody clone was MP-25D2. For IL-10, respective clones were JES3-19F1 and JES3-12G8 and for IFN- γ they were NIB42 and 4S.B3. The antibody-treated nitrocellulose plates were washed, then blocked using 1% BSA in PBS (filtered through a 0.2- μ m filter). The stimulated test, positive and negative control cells from step 1, were thoroughly resuspended and transferred to the antibody-coated test plates (two replicates per well per cytokine), and incubated in a 'vibration-free' incubator for 24 h to detect IFN- γ , or 48 h to detect

IL-4 and IL-10. Cells were removed and plate wells were washed with 0.05% v/v Tween 20 in phosphate-buffered saline (PBST) prior to addition of 100 μ l 1 μ g/ml biotinylated monoclonal α -human-cytokine antibody (diluted in PBST) for 3 h at RT with gentle agitation. After washing, the plate was incubated with 100 μ l 1 μ g/ml streptavidin-alkaline phosphatase (diluted in PBST) for 2 h at RT with gentle agitation. The presence of cytokine-secreting cells was revealed by addition of 100 μ l BCIP/NBT mixture (BioRad Hercules, CA, USA) for 30 min and the reaction stopped by washing with excess tap water. The plate was dried and spots counted under a dissecting microscope ($\times 40$ magnification). Only spots with a clear diffusion pattern were counted.

Statistical analysis

χ^2 analysis was performed using Fischer’s exact test (Table 2). The diagnostic value of the tests was expressed in terms of sensitivity, specificity and predictive value (Table 3). The formulae for calculating these are based on the following premises: sensitivity – the percent positive in the disease (true positive divided by true positive plus false negative); specificity – the percent negative in health (true negative divided by true negative plus false positive); predictive value of a positive result – percent true positives (true positive divided by true positive plus false positive); predictive value of a negative result – percent true negatives (true negative divided by false negative plus true negative); the test efficiency – the percentage of times the test returns a correct diagnostic answer compared with the total number of tests (sum of true positive and negatives divided by total number of tests performed).

Results

Frequency of IL-10-producing donor cells in response to recipient alloantigen

The number of IL-10-producing cells per million PBMC detected when the donor population was stimulated by irradiated recipient PBMC is represented by the bars in Figure 1. Of 26 sibling donor–recipient pairs tested in this way, 21 (80%) produced detectable numbers of cells

Table 2 Comparison of frequency of IL-10-producing cells and development of acute GVHD after BMT

IL-10 response to sibling alloantigen ^a	Grade 0–I ^b	Grade II–IV ^b	Total
IL-10 < 1000/10 ⁶	3	11	14
IL-10 > 1000/10 ⁶	10	2	12
Total	13	13	26

^aLow (< 1000/10⁶ PBMC) and high (> 1000/10⁶) production of IL-10 by donor cells when stimulated by their respective irradiated sibling PBMC.

^bLow (0–I) and severe (II–IV) grades of acute GVHD were compared using Fisher’s exact test: two-sided *P*-value 0.0048.

Table 3 IL-10 as predictor of development of acute GVHD after BMT

Predictive parameter	IL-10 assay for acute GVHD (%)
Sensitivity	85
Specificity	77
Predictive value of positive result	79
Predictive value of negative result	83
Test efficiency	84

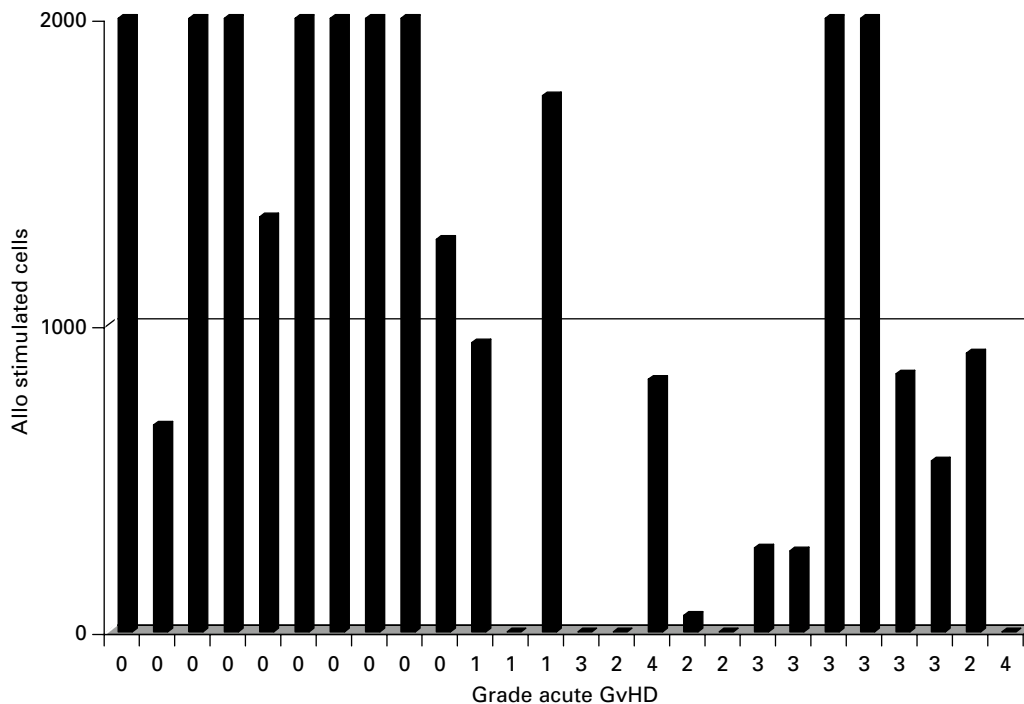


Figure 1 Responder (donor) cytokine production determined by ELISPOT technology. Pretransplant pairs were assessed to determine the frequency of donor cells producing specific cytokines in response to stimulation by alloantigen in the form of irradiated recipient PBMC. The pairs are classified for this figure into the grade of acute GvHD determined after transplant, based on the Seattle Criteria.

secreting IL-10. Cells from each of the 26 donors generated IL-10-secreting cells after PHA stimulation (positive control, not shown). No IL-10 was produced by unstimulated, donor cells or irradiated recipient cells alone (negative control) or by irradiated recipient cells stimulated with PHA (negative control) (data not shown). Further, we observed that the frequency of cells secreting IL-10 in response to recipient alloantigens did not correlate with the frequency observed for the same donor cells stimulated with mitogen (PHA). In 12/26 of the pairs tested IL-10 frequency was higher after mitogen stimulation; in 7/26 pairs IL-10 was lower after mitogen; in 7/26 pairs frequency of IL-10-secreting cells was similar after mitogen or alloantigen stimulation. Thus, the efficacy of the ELISPOT assay for detecting numbers of IL-10-secreting donor cells was established.

Frequency of IL-4- and IFN- γ -producing donor cells in response to recipient alloantigen

In all, 20 donor/recipient pairs were assessed to determine the number of donor cells that produced IFN- γ when stimulated with irradiated recipient PBMC.

IFN- γ -secreting cells were detected in only four (20%) donors stimulated with alloantigen, but in 19 (96%) donors stimulated with PHA (positive control). There was no correlation between cells that produced maximum PHA responses and maximum responses to sibling stimulation. No IFN- γ -secreting cells were detected when cells were cultured in medium alone (negative control).

A similar result was seen when 26 donor/recipient pairs were assessed for IL-4 secretion. Only four donors (15%) produced detectable numbers of IL-4-secreting cells when simulated by sibling alloantigen, compared with 100% of donors secreting IL-4 in response to PHA. Donors BM19–23 produced detectable but low numbers of IL-4-secreting cells in response to PHA. There was no correlation between maximal PHA response and maximal sibling response.

Predictive value of IL-10 assay

A major aim of the present study was to correlate the frequency of donor cells producing cytokine in response to recipient alloantigen with the grade of GVHD that followed BMT between the sibling pairs. Failure to observe IFN- γ - or IL-4-producing donor cells in response to sibling alloantigen precluded statistical analysis of the impact of cells producing these cytokines on post-transplant development of acute GVHD. However, in 21/26 donors IL-10 secretion was induced and statistical analysis was possible. To establish a cutoff value ($1000/10^6$), the mean and three standard deviations of the background level of IL-10 production were determined for all cohort donor PBMC. Data were grouped by frequency of IL-10-secreting cells as low ($<1000/10^6$ PBMC) and high ($>1000/10^6$ PBMC), and by grade of acute GVHD as, acceptable (0–I) and actionable (II–IV). Table 2 demonstrates a correlation between the number of IL-10-producing cells, detected in the donor in response to allostimulation by the sibling recipient, and the grade of acute GVHD which developed after transplant. χ^2 analysis (Table 2) using Fisher's exact test gave the two-sided *P*-value of 0.0048.

In contrast, using the same definition of high and low frequency as above, the frequency of IL-10-producing cells after mitogen stimulation did not correlate with acute GVHD development (data not shown). These data indicate a strong, statistically significant association between high frequency of alloantigen-specific IL-10-secreting cells and low grades of acute GVHD and low IL-10 frequency with the development of severe grades of acute GVHD.

Sensitivity and specificity and predictive values of IL-10 assay

The value of the IL-10 assay was further investigated by determining the sensitivity and specificity of the assay for the disease along with the predictive value of the positive and negative results. The high level of sensitivity (85%), specificity (77%), the positive and negative predictive values (79 and 83%, respectively) and the overall test efficiency (84%) are shown in Table 3.

Discussion

The work presented in this study demonstrates a strong correlation between the frequency of IL-10-producing cells detected in the blood of HLA-matched sibling donors when challenged by PBMC from their potential recipients and the probability of developing acute GVHD post-transplant. Cells from those recipients who developed acute GVHD (grade II or higher) after transplant stimulated a low frequency ($<1:1000$ PBMC) of donor cells producing IL-10, whereas those recipients who did not develop a severe grade of GVHD (0–I) generally received bone marrow from donors whose PBMC had a high frequency ($>1:1000$) of IL-10-producing cells. BMT pairs were randomly selected for IL-10 assessment, the sole criterion was cell availability. Outcome data were not available until the study had been completed.

The positive and negative predictive values associated with this assessment are influenced by the prevalence of the disease in the study population. These predictive values reflect the probability of accurate prediction of the development or lack of acute GVHD on the basis of the IL-10 production. The test efficiency is a calculation of the percentage of times the IL-10 assessment accurately predicts acute GVHD compared to the total number of assessments in this study (Table 3).

Statistical analysis of the number of IL-10-producing cells indicates a significant negative correlation with the severity of acute GVHD post-transplant, as demonstrated in Table 2. In addition, we have demonstrated previously a strong positive correlation between the high frequency of IL-2-producing cells detected in the donor and development of severe acute GVHD post-BMT.¹³ Despite the small number of transplant pairs and their diverse hematological disease profiles, statistical analysis of the relevance of this assay to GVHD is strong.

Current literature suggests that IL-10 may affect CD4 + T cells, downregulating IL-2 secretion and inducing allo-antigen-specific unresponsiveness.^{7,8} Further, in an *in vitro* proliferation assay in response to allostimulation

using human PBMC, Taga and Tosato¹⁴ demonstrated reduced production of IFN- γ and IL-2 in the presence of IL-10. These authors suggested that the biological activities of IL-10 implicate it as a potent negative regulator of immunoproliferative and inflammatory responses, including alloreactivity. Taken together, these observations provide an explanation for the protective role of IL-10-producing cells in subsequent GVHD after transplantation.

Data from studies using mitogen stimulation strengthen this conclusion; the frequency of cells producing IL-10 in response to PHA stimulation did not correlate with the frequency of cells responding to allo-stimulation or GVHD. Our experimental work demonstrated no correlation between individuals with a maximal response to mitogenic stimulation (PHA) and those who responded with maximal production of cytokine, either IL-10 or in the small number of positive individuals, IL-4 or IFN- γ , in response to allostimulation by the matched sibling recipient.

Donor response to mitogen stimulation demonstrated the ability of the cellular populations sampled to produce IFN- γ and IL-4. However, when cocultured with sibling alloantigens, only a small number of individuals produced detectable numbers of IFN- γ - or IL-4-producing cells. Perhaps the alloantigen stimulus was suboptimal or the amount of cytokine secreted was below the level of detection in the conditions of this ELISPOT assay.

Previous reports have noted a correlation between IL-10 production and acute GVHD.^{3,15,16} Holler *et al.*³ measured the spontaneous release of IL-10 by BMT recipients at the time of admission, that is, before any preparative regimen or other interventions. Using ELISA to detect the spontaneously released IL-10, they noted that high IL-10 production correlated with a low incidence of GVHD. Chen *et al.*¹⁷ reported the induction of alloantigen-specific tolerance and generation of regulatory cells by pretreatment with IL-10/TGF- β . This study also identifies IL-10-producing cells that respond to allostimulation. The test efficiency of the assay in this report was 84%, the IL-10 being of donor origin. The cytokine-releasing profile of the donor pretransplant is of great relevance, as it is the donor's hematological competency that the recipient assumes.

Considering the mechanism of IL-10 effector function, Rafiq *et al.*¹⁸ investigated IL-10 production in cultures of freshly isolated human T cells from healthy normal volunteers and found that a rise in intracellular calcium and CD28 triggering were important signals for IL-10 induction. Further, the total inhibition of IL-10 production in the presence of CsA indirectly implicated the involvement of the calcium-calcineurin pathway. Moreover, Groux *et al.*⁷ previously demonstrated that IL-10 interfered with proximal events in the TCR signalling pathway, probably at the level of Ras-microtubule-associated protein (MAP) kinase activation. These findings, together with the present data, have significance in transplant situations where the patient receives post-transplant CsA. Given the importance of IL-10 in reducing GVHD severity, CsA treatment would prevent the activity of a 'naturally' produced suppressive or tolerizing activity of recipient cells. Our data suggest that monitoring the frequency

of IL-10 and IL-2 cytokine-producing host-specific cells may identify recipients for whom a reduction in immunosuppressive therapy is possible and highlight a dilemma in treatment of patients.

Strong evidence, presented by this study, indicates that estimating the frequency of alloreactive IL-10-producing cells in the donor will provide valuable information prior to a BMT regarding potential transplant success. Assay turnaround time for a clinical purpose was 5 days, which includes drying the plates, counting the spots and calculation time. The number of the PBMCs required from both donor and recipient and consumables was one-quarter that for current limiting dilution analysis.

The mechanism of IL-10 activity has not been addressed in this study. Whether IL-10 induces anergy in all T cells or is effective only on a subset that differentiates into Tr₁ cells that apparently induce anergy in the remaining T cells is yet to be determined. It is essential for IL-10 to be present in the environment when the initial and repetitive presentation of alloantigen occurs.¹⁵ For those transplant pairs in whom a high frequency of cells are capable of producing IL-10 (detected prior to transplant), this cytokine-rich environment would exist at the time of repetitive presentation of antigen (the transplant) and would be predicted to result in no or lesser grades of acute GVHD.

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