The impact of acute and chronic graft-versus-host disease on normal and malignant B-lymphoid precursors after allogeneic stem cell transplantation for B -lineage acute lymphoblastic leukemia

Joaquín Sánchez-García
Josefina Serrano
Pedro Gómez
Francisco Martínez
Carmen Martín
José Román-Gómez
Antonia Rodríguez
Concepción Herrera
José M. García
Miguel A. Alvarez
Antonio Torres

Background and Objectives. The development of graft-versus-host disease (GVHD) after allogeneic stem cell transplantation (SCT) for B-lineage acute lymphoblastic leukemia (B-ALL) is associated with a lower probability of leukemia relapse. However, mechanisms by which this GVHD-associated graft-versus-leukemia effect is exerted are poorly understood. In this study, we simultaneously traced the kinetics of normal donor-derived and leukemic recipient-derived B-lymphopoiesis comparing patients with or without GVHD.

Design and Methods. We used multiparameter flow-cytometry to quantify pro-B (CD19+CD10+CD34+), pre-B (CD19+CD10+CD34−) precursors and malignant lymphoblasts identified by leukemia-associated markers in 161 prospective marrow samples from 39 consecutive B-ALL patients after allogeneic SCT. Chimerism analysis was performed by quantitative real-time polymerase chain reaction of null alleles and insertion/deletion (indel) polymorphisms.

Results. Acute GVHD of grades II-IV is associated with a strong inhibition of normal donor-derived pro-B and pre-B precursors at days +30 and +60 post-SCT. Patients who develop chronic GVHD have lower percentages of marrow B-cell precursors during the first year after SCT. Likewise, recipient-derived leukemia B cells were absent at days +30 and +60 in patients with acute GVHD grades II-IV and were less likely to be detected in patients with chronic GVHD. Induction of GVHD as treatment of increasing amounts of leukemia cells causes inhibition of both normal and malignant B compartments even in the absence of steroid therapy.

Interpretation and Conclusions. We conclude that the development of GVHD after allogeneic SCT is associated with a non-specific inhibition of B-lymphopoiesis.

Key words: B-lymphopoiesis, stem cell transplantation, GVHD.

All authors from the Hematology and Bone Marrow transplantation Department, University Hospital "Reina Sofía", Córdoba, Spain

Correspondence:
Joaquin Sanchez Garcia, MD, PhD, Hematology Department, University Hospital Reina Sofía, Avda. Menendez Pidal s/n, 14004 Córdoba, Spain. E-mail: joaquin.sanchez@cheerful.com

From mid-gestation throughout life, the bone marrow is the exclusive site of human B lymphopoiesis. A common lymphoid progenitor differentiates into the early B cells and subsequently into pro-B-cells, which comprise a well-defined marrow population co-expressing CD34, CD19, CD10, TdT and V-to-DJ rearrangements. Pro-B cell differentiation into the pre-B compartment is characterized by the loss of CD34 and TdT with acquisition of cytoplasmic ζ heavy chains.[1] This complex process is strictly governed by regulatory signals provided after adherence of B-cell precursors to bone marrow stromal cells.[1-3] B-lineage acute lymphoblastic leukemia (B-ALL) arises as a consequence of the disruption of this hierarchical process leading to a transformed clone resistant to apoptosis and incapable of undergoing differentiation.[4] Following allogeneic hematopoietic stem cell transplantation (SCT), grafted donor-derived precursor cells must recapitulate the ontogeneic process of B-lymphopoiesis. Indeed, an early post-SCT period of severely depressed peripheral

B-cell counts is followed by a marked increased in the percentage of immature B-cells.[5,6] Progressive normalization of B-cell values occurs within the first year after transplant. However, the development of acute or chronic graft-versus-host disease (GVHD) after allogeneic SCT is associated with a prolonged B-cell-mediated humoral immunodeficiency because of the decrease or even absence of marrow B-cell precursors.[7-10] On the other hand, allogeneic SCT can potentially cure a subgroup of high-risk patients diagnosed with B-ALL,[4] but leukemia relapse still remains the main cause of treatment failure. Leukemia relapse occurs in up to 60% of patients depending on the phase of disease, age at the time of transplant, and most importantly the incidence of graft-versus-host disease (GVHD).[11,12] Based on clinical observations in large series,[13-16] it is widely recognized that the combination of acute and chronic GVHD confers the best protective effect against leukemia relapse. Furthermore, in some studies, the induction of GVHD has

been successfully used to treat leukemia reappearance after SCT,[17,18] reinforcing the idea that GVHD can play a pivotal role in the development of a graft-versus-leukemia reaction. Although the cytotoxic mechanisms of GVHD and the graft-versus leukemia effect are now being differentiated in murine models,[19,20] the separation of the two effects after SCT for B-lineage ALL in humans is still a challenging issue.[21,22] In this sense, quantification of donor-derived normal B-lymphopoiesis and recipient-derived leukemic B lymphoblasts post-SCT might serve to identify a differential specific anti-leukemia effect or a non-specific effect affecting both compartments. In this study, we examined the kinetics of normal B-cell lymphopoiesis recovery and the presence of B leukemia cells after SCT in order to determine the impact of GVHD as well as other potential clinical factors. For this purpose, we used multi-parameter flow cytometry, which can unequivocally distinguish normal from malignant B lymphoid precursors based on the presence of leukemia-associated markers.[23-26]

Design and Methods

Patients

Thirty-nine consecutive patients diagnosed with B-ALL (12 BI and 27 BII cases according to the EGIL classification) who underwent allogeneic SCT were enrolled in this study. Their clinical characteristics and details of the procedures are listed in Table 1. The allogeneic transplants were performed from March 1999 to June 2005 in the University Hospital Reina Sofía, Cordoba, Spain. At the time of transplantation, all cases had less than 5% lymphoblasts, as evaluated by light microscopy. None of the Philadelphia-chromosome-positive ALL patients had received imatinib prior to SCT. GVHD prophylaxis, consisted mostly of cyclosporine A (3 mg/kg) starting on day –1 with or without a short course of methotrexate (15 mg/m²). The cyclosporine was withdrawn at a rate of 20% weekly starting on day +100. Acute GVHD was defined and staged using standard criteria,[27] and chronic GVHD was defined, based on the clinicopathological classification,[28] as limited (limited skin involvement and/or hepatic dysfunction) or extensive (either the former plus involvement of the eyes, salivary glands or any other target organ, or generalized skin involvement). Acute GVHD was initially treated with prednisone (2 mg/Kg) for 2 weeks, then the dose was gradually tapered off. Two patients needed antithymocyte globulin (15 mg/day for 5 days) as treatment for grade IV acute GVHD. Chronic GVHD was treated with oral prednisone (1mg/Kg) and cyclosporine (6mg/Kg every other day). Overt bone marrow relapse (morphological relapse) was defined as the presence of ≥5% of lymphoid blast cells, assessed by optical microscopy. No patient developed EBV-lymphoproliferative disorders and thus none received anti-CD20 antibodies. Appropriate institutional ethical committees approved all procedures.

Methods

Bone marrow aspirates (n=161) were obtained prior to the conditioning regimen, at day +30 or at the time of neutrophil recovery, whichever occurred first (n=29), and

Table 1. Clinical characteristics of the 39 ALL patients.

Parameters	N
Total n. of patients	39
Median age years at SCT (range)	15 (2-49)
Sex Male/Female	20/19
Median WBC at diagnosis×10⁹/L (range)	21.4 (1-574)
t(9;22) or BCR-AB	13
Status at SCT	
1st CR	17
2nd CR	15
>2nd CR	7
Donor type	
HLA identical sibling	16
Matched unrelated	21
Haploidentical related*	2
Graft Source	
Bone marrow	29
Peripheral blood	6
Umbilical cord blood	4
Preparative Regimen	
TBI+CY	24
BU+CY+VP	13
TBI+FLU+TT	2
GVHD Prophylaxis	
CsA	6
CsA+MTX	31
CD34⁺	2
Acute GVHD (II-IV grades)	18
Chronic GVHD	11
Bone marrow relapse post-SCT†	12

CR, complete remission; TBI, total body irradiation (13Gy); CY, cyclophosphamide (120 mg/kg); BU, busulfan (16mg/kg); VP, etoposide 60 mg/kg; FLU: fludarabine (200 mg/m²); TT, thiotepa (10 mg/kg); CsA: cyclosporine (3mg/kg); MTX, methotrexate (15 mg/m²); GVHD, graft-versus-host disease. * Patients grafted from haploidentical sibling donors were conditioned with TBI+FLU+TT and received purified peripheral blood CD34 cells without further GVHD prophylaxis.† Additionally, three patients developed extramedullary relapse (central nervous system).

at days +60 (n=31), and +90 (n=21). Subsequent samples were taken every two months (n=24, n=18, n=15, n=13 and n=10 at months +5,+7,+9,+11 and +15, respectively). Bone marrow aspirates were collected in preservative-free heparin or in EDTA at the intervals described above. Mononuclear marrow cells were isolated by Ficoll-Hypaque (Lymphoprep, Nycomed, Oslo, Norway) density gradient centrifugation and washed twice in phosphate-buffered saline containing 0.2% bovine serum albumin and 0.2% sodium azide (PBSA). Human immunoglobulins (Flebogamma, Grifolls, Barcelona, Spain) were added to saturate Fc receptors.

Quantification of normal B-cell precursors

Normal B-cell precursors were quantified at two different stages:[1,3] first, as pro-B cells, defined as the population expressing CD10, CD34 and CD19 (CD19⁺CD10⁺CD34⁺) and second, as pre-B cells, characterized by the loss of CD34 (CD19⁺CD10⁺CD34⁻). Cells were stained with CD19 (IgG1 Clone HD37) conjugated with phycoerythrin, CD34 (IgG1 Clone HPCA2), conjugated with peridin-chlorophyll or fluorescein isothiocyanate), and CD10 (clone HI10a) conjugated with allophycocyanin, (all from Becton-Dickinson, San Jose, CA, USA) or CD10 (IgG1, Clone ALB1) conjugated with Pcy5 (Immunotech, Marseille, France). Isotype-matched controls were used to establish regions.

Table 2. Immunophenotypic combinations used to assign leukemia-associated immunophenotypes.

Combination	Percentage*
CD45/CD19/CD34-CD10	48%
CD38/CD19/CD34-CD10	45%
CD58/CD19/CD34-CD10	37%
CD13/CD19/CD34-CD10	29%
CD66/CD19/CD34-CD10	30%
CD33/CD19/CD34-CD10	22%
TdT/μcyt/CD34	22%
CD21/CD19/CD34-CD10	7%
CD15/CD19/CD34-CD10	3%

Percentage of patients who displayed these specific leukemia-associated immunophenotype-combinations based on differences of antigen intensity expression or aberrant expression compared to normal lymphoblasts. All patients were traced with at least two combinations.

Figure 1. Quantification of normal and leukemic B-lymphoid precursors by multiparametric flow cytometry. Mononuclear marrow cells were incubated with monoclonal antibodies against human CD19, CD10 and CD34. The first gate (R1) was drawn around lymphoid events based on forward and side-scattered signals and the second gate (R2) over B-lymphoid events on a dot plot CD19-PE vs. SSC. All dot plots represented are gated on R1 and R2. The expression of CD10 and CD34 allows pro-B cells (CD19⁺CD10⁺CD34⁺) contained within the R4 region and pre-B cells (CD19⁺CD10⁺CD34⁻) contained within the R3 region to be distinguished. Gray dots represent phenotypically normal B-lymphoid precursors and black dots represent B-lymphoid precursors with aberrant leukemic phenotypic profiles (R5 region). A patient with normal precursors without leukemia detection is represented in (A) (complete donor chimerism). A patient with minimal residual disease after allo-SCT is represented in (B), with a mixture of normal and malignant precursors expressing CD58 (mixed chimerism). This patient eventually relapsed with complete replacement of normal donor-derived precursors by recipient's leukemia cells (complete recipient chimerism) (C).

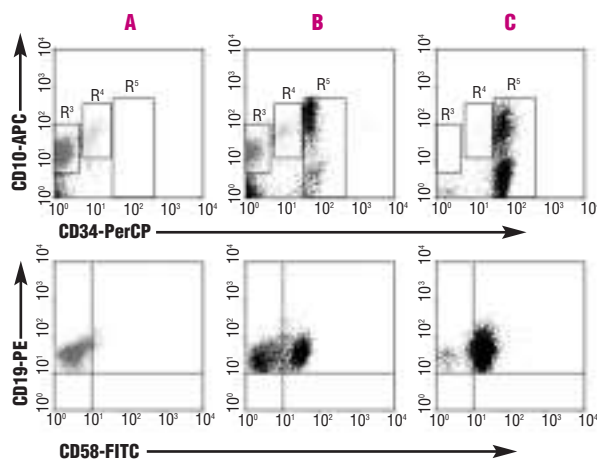## Quantification of leukemic B-cell precursors

The definition of leukemia-associated immunophenotypes has been previously published.[23,24] Briefly, these immunophenotypes are based on over-expressed normal antigens (CD10 or CD34), aberrant expression of myeloid antigens or an altered maturation pattern (Table 2). Commercially available reagents used were mostly mouse anti-human antibodies, in all cases labeled with fluorescein isothiocyanate: CD38 (IgG1, clone TI16), CD21 (IgG1, Clone BL13), CD58 (clone AICD589) from Immunotech, Marseille, France), CD45 (clone HI30), CD15 (IgM, Clone MMA) (from Pharmingen-Becton Dickinson San Jose, CA, USA), anti-TdT (Clone HT-6), CD66 (IgG1, Clone Kat4c), anti μ chain (polyclonal rabbit anti-human F(ab)₂), CD13 (IgG1 Clone WM-47) and CD33 (IgG1, Clone WM-54) (from Dako, Denmark).

Flow cytometry, acquisition and analysis

A dual laser FACScalibur™ flow cytometer with Cell Quest Software (Becton Dickinson) was used with triple or four-color staining protocols. A first record of light scattering and fluorescence signals of 10,000 events, was made, enabling us to draw one gate around lymphoid cells and a second gate to define immature feature, such as CD19-CD34/CD10. Ten thousand events were stored. To record results, forward versus side-scatter gating first eliminated cellular debris and non-viable cells. Total B-lymphoid events were selected by CD19-phycoerythrin versus side-scatter gating and re-analyzed to assess the percentages of normal B-cell precursors, as described above, as well as the percentage of B-cell precursors expressing patient-specific leukemia-associated immunophenotypes (Figure 1) with gating strategies specific for each patient. Multiparametric flow cytometry for minimal residual disease (MRD) in ALL is capable of unambiguously detecting one leukemic cell among 10,000 normal marrow cells.

Chimerism analysis

Molecular chimerism analysis was based on amplification by quantitative real-time polymerase chain reaction (qrt-PCR) of null alleles and insertion/deletion (indel)

polymorphisms as reported previously by our group.[29] Four null alleles (GSTM1, GSTT1, RhD and SRY) and ten indels (Xq28, rs4399, DCP1, R271, FVII, THYR, MID-1039, MID-1335, MID-1385 and MID-2062) were quantified by qrt-PCR using specific primers and probes for LightCycler technology. Sensitivity studies (using a mixture of donor and recipient cells) showed a high level of sensitivity (up to 0.01%) for all markers.

Statistical analysis

The results were analyzed in September 2005. The median follow-up was 38 months (range: 3-71). Disease-free survival was calculated according to the Kaplan-Meier method and results were compared using the log rank test. Percentages of normal and malignant B-cells were analyzed if marrow samples had been taken while the patient was in complete remission and no therapy had been given to treat MRD. Results are expressed as mean values ± the standard error of mean. The non-parametric test for independent samples (U Mann-Whitney) or Student's t-test was used to compare mean values of continuous variables. Differences in the incidence of GVHD and categorical variables were compared with Fisher's exact test. Multiple regression analyses were used to evaluate the relationships between normal marrow B-lymphoblast counts at days +30 and +60 (for chronic GVHD at months +7 and +9) with the clinical transplantation
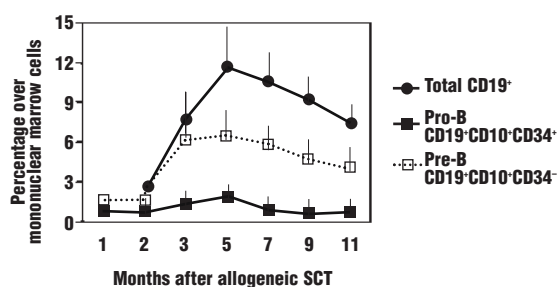
Figure 2. Kinetics of B-lymphopoiesis after allogeneic SCT. Mean values (±SE) of total CD19 events, Pro-B cells (CD19⁺CD10⁺CD34⁺) and pre-B cells (CD19⁺CD10⁺CD34⁻) contained in 161 consecutive marrow aspirates obtained from 39 B-ALL patients during the first year after SCT are displayed.

variables. A stepwise selection procedure was used with 5% entry and 10% exit criteria. Logistic regression was used to evaluate the relationships between MRD detection at any point and the clinical transplantation variables. Patients transplanted with CD34-purified grafts (n=2) were not included in these analyses.

Results

The recovery of B-lymphopoiesis is a time-dependent process and thus, for the global series, marrow CD19⁺ cells progressively reappeared after SCT, reaching a peak value at month +5 (11.6 ± 2.5% over mononuclear marrow cells, respectively). Thereafter, marrow CD19 cells decreased to normal levels by about one year post-SCT (Figure 2). During the first three months post SCT, most of the CD19⁺ cells consisted mainly of immature pro-B precursors (CD19⁺CD10⁺CD34⁺) and pre-B (CD19⁺CD10⁺ CD34⁻) resembling a physiological ontogeny. From month +5, the percentages of both immature precursors smoothly declined to baseline levels, while more mature B cells, lacking CD10 and CD34 markers, emerged.

Impact of acute GVHD on the recovery of normal B-lymphopoiesis

By the end of the study period, 18 patients had developed acute GVHD grades II-IV Patients receiving hematopoietic progenitors from donors other than HLA identical siblings had a higher incidence of acute GVHD grades II-IV (52.7% vs. 37.5% p=0.3) and in our series, patients undergoing allogeneic SCT in first complete remission were more likely to develop acute GVHD grades II-IV (66.6% vs. 28.6 p=0.04), mainly due to an increased use of alternative donors and sex-disparity between donor and recipient in this subset of patients. The recovery of normal B lymphopoiesis in the first three months after allo-SCT is severely affected by the presence of allogeneic GVHD grades II-IV. Thus, the percentages of pro-B and pre-B cells, which included most of the CD19⁺ cells, were profoundly decreased in patients with acute GVHD grades II-IV compared to the percentages in those with grades 0-I. These differences reached statistical sig-
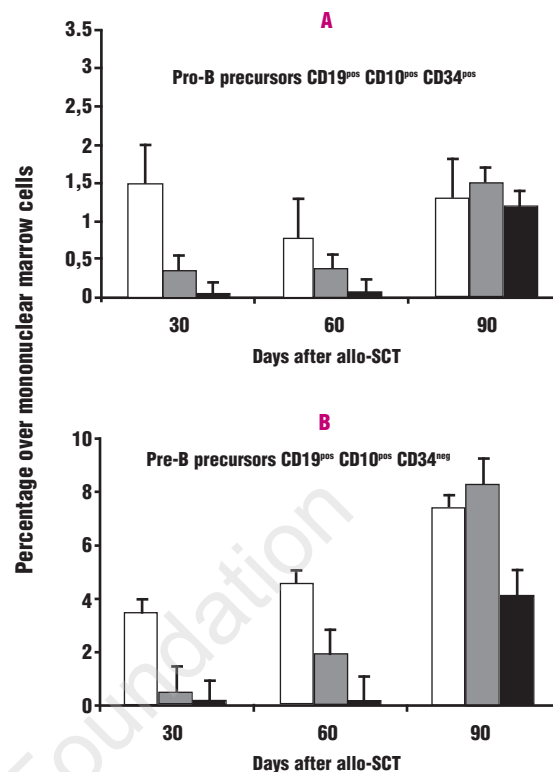
Figure 3. Percentages of normal B-cell precursors (pro-B and pre-B) in patients with or without acute GVHD. (**A**) Percentages of pro-B cells (CD19⁺CD10⁺CD34⁺) and (**B**) pre-B cells (CD19⁺CD10⁺ CD34⁻) (mean±SE) over mononuclear marrow after allogeneic SCT comparing patients without acute GVHD (white bars), those with with grade I acute GVHD (gray bars) and those with grades II-IV (black bars).

nificance for pro-B precursors at days + 30 (0.015±0.006 vs. 0.88±0.37%, p=0.04) and +60 (0.047±0.03 vs. 0.6± 0.15%, p<0.01) as well as for pre-B precursors (0.02±0.006 vs. 1.9±1.4%, p<0.01) at day +30 and at day +60 (0.1±0.03 vs. 3.36±1.1%, p<0.01). When we analyzed patients with acute GVHD grade I and those with grades II-IV separately, but all of them receiving steroid treatment, we found a correlation between the intensity of GVHD and the degree of inhibition of normal B-lymphopoiesis (Figure 3). At day +90, we did not observe statistical differences, probably due to the tapering of the steroid treatment. In the univariate analysis, we found that the percentages of B-cell precursors were higher in marrow samples obtained from patients receiving a marrow graft from an HLA-identical sibling donor than those obtained from patients grafted from alternative donors. Likewise, patients who underwent SCT for ALL second or subsequent complete remission had significantly higher levels of normal pro-B cells than those grafted in first complete remission. However, in the multiple regression analysis, only acute GVHD remained statistically significant (Table 3). We did not observe statistical differences in the normal B-cell precursors when comparing patients who did or did not relapse, since we only analyzed marrow samples in complete morphological remission.

Table 3. Multivariate analysis of clinical factors influencing recovery of normal B-lymphopoiesis and MRD.

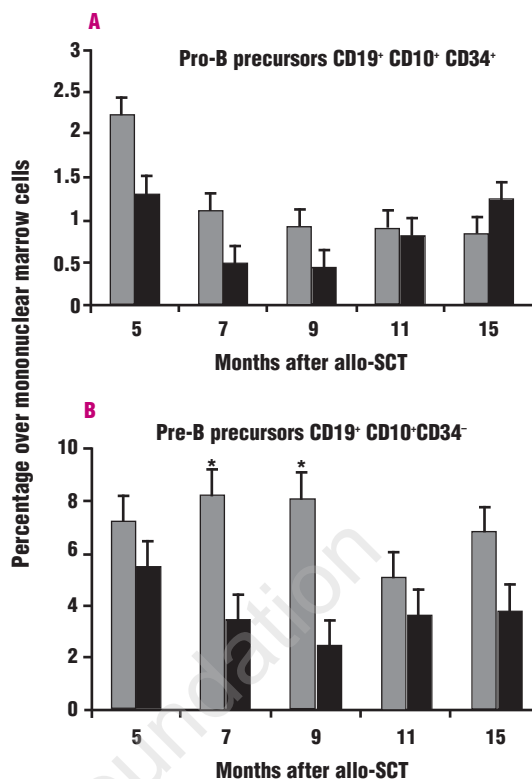|	Percentage of normal pro-B cells		Percentage of normal pre-B cells		
	Regression coefficient	p*	Regression coefficient	p*	MRD detection p†
Acute GVHD	1.27	0.02	3.39	0.02	0.04
Donor type	0.15	0.68	-0.58	0.58	0.10
Recipient's age	<0.01	0.98	-0.04	0.34	0.54
Disease status	-0.02	0.96	2.23	0.07	0.50
Chronic GVHD	–	–	4.91	0.03	0.09

Acute GVHD: acute graft-versus-host-disease (grades 0-I vs. II-IV). MRD: minimal residual disease. Donor type: familiar sibling vs. unrelated donor (haploidentical transplants were excluded). Recipient's age: continuous variable. Disease status: 1ˢᵗ complete remission (CR) vs. 2ⁿᵈ CR. Chronic GVHD: chronic graft-versus-host disease. Univariate analysis of other clinical factors included: donor age; conditioning regimen with or without radiotherapy, number of infused cells, or presence of Philadelphia chromosome; all of which were without statistical significance. *Multiple regression analyses at days +30 and +60 (for chronic GVHD at months +7 and +9); †Logistic regression analysis for MRD detection at any point.

### Impact of chronic GVHD on the recovery of normal B-lymphopoiesis

At the end of the study period, 11 out of 32 patients who had survived for more than 3 months post-SCT, developed limited (n=6) or extensive (n=5) chronic GVHD. Nine patients had previously had acute GVHD grades II-IV and two patients acute GVHD grade I. The use of donors other than HLA identical siblings (p=0.03) and the presence of acute GVHD grades II-IV (p<0.01) were major clinical risk factors for the development of chronic GVHD, whereas recipient's age or disease status did not have an influence. As shown in Figure 4, the percentages of pro-B and pre-B precursors were lower in patients with chronic GVHD. These differences reached statistical significance for pre-B precursors at month +7 (8.2±1.6% vs. 3.4±1.4%, p=0.04) and month +9 (8.08±1.7% vs. 2.4±0.7%, p<0.01). Pro-B precursors were present in bone marrow at equal amounts in both groups approximately one year post-SCT. However, when considering only patients with active extensive chronic GVHD one year post-SCT (n=3), the percentages of pro-B (0.08±0.06% vs. 0.8±0.6%) and pre-B precursors (0.63±0.5% vs. 6.2±4.5%) were much lower than those in patients with limited or no GVHD.

### Impact of acute and chronic GVHD on the detection of B-leukemia cells

At the end of the study period, 12 patients (30.7%) had progressed to overt marrow leukemia relapse at a median of 7 months (range: 2.5-9) post-SCT. MRD analysis in these patients had shown detectable levels of leukemia cells in at least one previous scheduled marrow aspirate. As expected, the development of acute GVHD and chronic GVHD had a positive effect on disease-free survival (Figure 5). Advanced disease status (second or subsequent complete remission) and MRD detection before transplant were clinical factors adversely affecting disease-free survival (p=0.02 and p<0.01, respectively), while recipi-

Figure 4. Percentages of normal B-cell precursors (pro-B and pre-B) in patients with or without chronic GVHD. A. Percentages of pro-B cells (CD19⁺CD10⁺CD34⁺) and (B) pre-B cells (CD19⁺CD10⁺CD34⁻) (mean ± SE) over mononuclear marrow after allogeneic SCT comparing patients with (black bars) or without (grey bars) chronic GVHD. Statistically significant differences by the Student's t test or the U Mann Whitney test are marked with an asterisk (p value was ≤0.05).
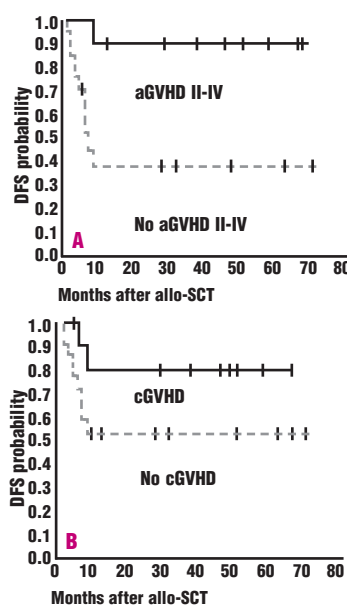
Figure 5. Probability of leukemia-free survival after allogeneic SCT according to GVHD status. (A) disease-free survival in patients developing acute GVHD grades II-IV (n=18) was significantly higher than that in patients with grade 0-I (n=21) (90.9±8.6% vs. 38.5±11.9% respectively, p<.01 log rank test). (B) Accordingly, disease-free survival in patients developing limited or extensive chronic GVHD (n=11) was higher than that in patients lacking symptoms (n=21) (80±12.6% vs. 53.1±11.8%, respectivel; p=0.12 log rank test).
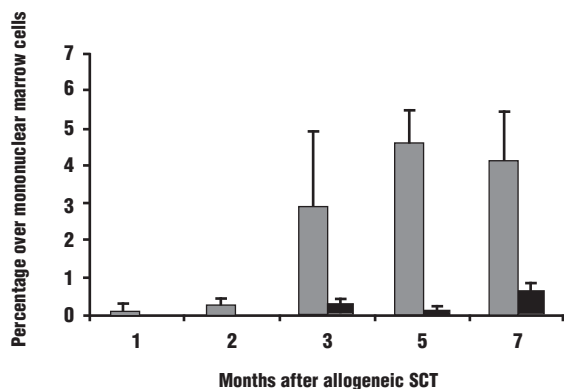
Figure 6. Percentages of leukemia B-cell precursors in patients with or without GVHD post-SCT. A: Percentages of leukemia cells (mean + SE) over mononuclear marrow after allogeneic SCT comparing patients with (black bars) or without (gray bars) chronic GVHD.

ent's age and donor type had no impact. Patients who developed acute GVHD had a high probability of surviving without disease (91±8.6%) and thus, no marrow leukemia cells were detected at days +30 and +60. Interestingly, patients who did not develop acute grades II-IV (disease-free survival of 38.5±11.9%) were more likely to have detectable leukemic cells at days +30 (0.058±0.05%), +60 (0.24±0.14%) and +90 (2.8±2.3%) post-SCT (Figure 6). Patients developing chronic GVHD had fewer marrow leukemia cells than patients lacking clinical symptoms, at month +5 (0.38±0.37 vs. 4.5v3.4) and month +7 (0.66±0.66% vs. 4.07±4.01%). In the logistic regression analysis, only acute GVHD remained a significant clinical factor influencing MRD (Table 2). Detection of marrow leukemia cells was consistently associated with a molecular mixed chimerism, while those patients with only phenotypically normal B-cell precursors in their marrow were always in complete donor chimerism. Therefore, B-cell precursors with aberrant leukemia markers were always of recipient origin and phenotypically normal B-cell precursors of donor origin. Neither autologous reconstitution nor leukemia of donor origin was observed in this series.

Induction of GVHD as a treatment of MRD

Due to the very high risk of overt marrow relapse,[24,26] eight patients with detectable levels of leukemic cells in bone marrow and mixed chimerism following SCT were induced to produce a graft-versus-leukemia reaction by abrupt discontinuation of immunosuppression avoiding steroid treatment. Five patients did not respond and rapidly progressed to overt relapse. In three patients diagnosed with Bcr-Abl-positive B-ALL, the leukemia responded to this strategy, as shown by a bone marrow aspirate examined one month later, concomitantly with development of skin GVHD. Interestingly, the percentage of phenotypically normal B-cell precursors was also observed in all three patients. Thus, in patient UPN 376 leukemia cells decreased from 2.3% to 0.3%, pro-B cells decreased from 0.9% to 0.03% and pre-B cells decreased
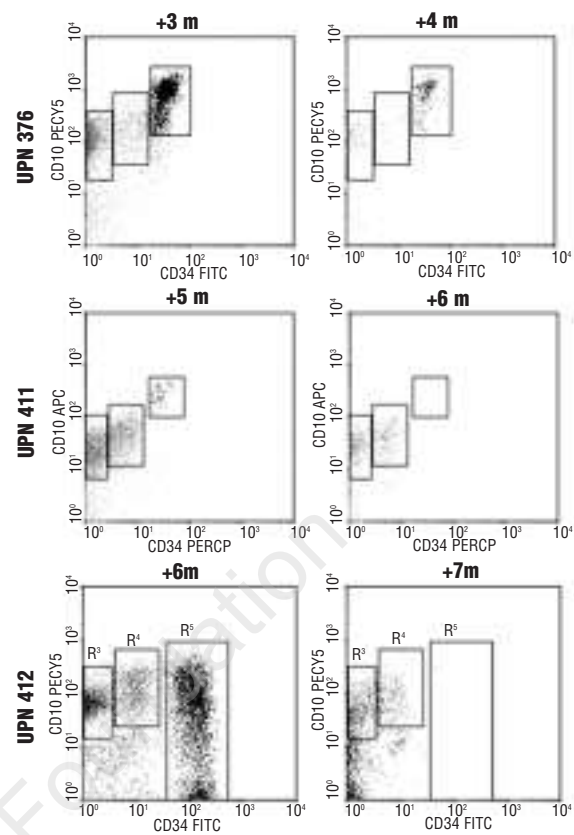
Figure 7. Quantification of normal and leukemic B-lymphoid precursors by multiparametric flow cytometry before and after GVHD induction. Patients UPN 376, 411 and 412 were induced to develop GVHD as treatment of leukemia cell detection and mixed chimerism avoiding the use of steroids. Left row shows dot plots with regions including pre-B, pro-B and leukemic lymphoblasts prior to GVHD induction. Right row displays dot plots from a marrow aspirate obtained one month later showing partial or complete leukemia remission and simultaneously a lower percentage of normal B-precursors.

from 3.3% to 1.46%. The response was transient, lasting for 2 months, and required a single donor lymphocyte infusion to achieve a complete cytometric and molecular response, which persisted until the end of the follow-up (+48 months). Patient UPN 411 showed a complete response (leukemic cells from 0.09% to undetectable levels) with pro-B cells decreasing from 0.1% to 0.05% and pre-B cells from 0.7% to 0.48%. The response was stable and the patient remained in complete remission at the last follow-up (+30 months). Patient UPN 412 also showed a complete response (leukemic cells from 9% to undetectable levels) with pro-B cells decreasing from 2.4% to 0.11% and pre-B cells from 6.4% to 0.4%. The marrow response was stable but relapse was detected in the central nervous system at month + 11 while still showing complete remission in the bone marrow. Representative dot plots from these patients are presented in Figure 7.

Discussion

In this study, taking advantage of the unique ability of flow cytometry to measure MRD and normal hematopoi-

etic function simultaneously, we demonstrated, in a large number of prospective collected samples, that GVHD is a major clinical factor causing a non-specific inhibition of both normal and malignant B-cell precursors. This suppression is more profound in advanced grades of acute GVHD and in extensive chronic GVHD.

While it is widely known that peripheral B-lymphocytes are decreased post-SCT in patients with GVHD,[30] prospective analyses of the kinetic behavior of marrow B-cell precursors after allogeneic SCT are very scarce.[6,8-10] The largest series reported to date included marrow samples obtained at days 30, 80 and 365 from 93 allograft recipients with heterogeneous diagnoses.[8] Our results are in agreement with those of Storek *et al*.[8] who reported lower numbers of marrow B-cell precursors in patients with acute GVHD grades II-IV and extensive chronic GVHD, although B-cell precursors were defined only as early (CD45$^+$CD19^{low}) and late (CD45$^+$CD19^{high}). Molecular techniques, have it has also indicated that GVHD is associated with a lower probability of MRD post-SCT in patients with B-ALL.[13] However, to the best of our knowledge, ours is the first prospective study to analyze normal and malignant B-cell precursors simultaneously after allogeneic SCT. Both normal and malignant B-cell precursors are inhibited during acute and chronic GVHD, even in the absence of steroid treatment. The putative mechanisms that mediate this non-specific effect might include the destruction of supportive marrow stromal cells or the production of inhibitory cytokines by activated donor T cells.

The successful development of mammalian B-lineage cells relies on marrow stromal cell-derived molecules that promote survival, growth and differentiation signals to common lymphoid and B-cell progenitors.[1] Likewise, ALL cells may also show adhesion-dependent survival on stromal cell layers.[31] It has been postulated that as a consequence of conditioning regimens with chemo/radiotherapy, there is stromal damage[32] with reduced expression of vascular cell adhesion molecule-1 (VCAM-1) resulting in an impaired capacity to support B-lymphopoiesis.[33] However, our findings highlight that this alteration in the hematopoietic environment is particularly severe only in patients developing GVHD. In fact, most patients without acute GVHD grades II-IV showed prompt reconstitution of pro-B and pre-B cell precursors, as well as a better survival of likely aggressive ALL cells.[34] This finding suggests that, despite the use of high doses of chemo/radiotherapy as a conditioning regimen, the stromal microenvironment is highly functional early post-SCT, enabling B-lymphopoiesis to be supported in patients with acute GVHD grades 0-I.

On the other hand, GVHD arises after HLA-matched allogeneic SCT as a consequence of the expansion of alloreactive donor T cells probably targeting minor histocompatibility antigens (mHag). Thus, tetrameric HLA-mHag ubiquitous peptide complexes can be detected in peripheral blood samples from patients with GVHD.[35] Likewise, a mHag (HB1) restricted to leukemia B cells capable of inducing allogeneic T-cell leukemia-specific responses has also been described.[36] Thus, it is tempting to speculate that, in addition to an altered stromal environment, normal donor-derived B-lymphopoiesis could be suppressed *in vivo* by the expansion of donor T cells targeting mHag.[37] These mHag-specific alloreactive T cells could produce large amounts of interferon-gamma, interleukin-1 or tumor necrosis factor, all cytokines with the ability to suppress normal donor-derived B-lymphopoiesis.[38,39]. In conclusion, our findings suggest a GVHD-associated non-specific inhibition of B-lymphopoiesis. It is tempting to speculate that this might play a role in controlling leukemia relapse.

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