Early and Late Extensive Chronic Graft-versus-Host Disease in Children Is Characterized by Different Th1/Th2 Cytokine Profiles: Findings of the Children’s Oncology Group Study ASCT0031

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Numerous mechanisms underlie chronic graft-versus-host disease (cGVHD), including skewing of Th1/Th2 cytokine expression. There are biological differences between early-onset and late-onset cGVHD. To test whether different Th1/Th2 cytokines are associated with early- or late-onset cGVHD, peripheral blood was collected from 63 children enrolled on the Children’s Oncology Group Phase III trial ASCT0031 evaluating hydroxychloroquine therapy for newly diagnosed extensive cGVHD. mRNA expression of interferon (IFN)-γ and interleukin (IL)-2, -4, and -10 from stimulated peripheral blood mononuclear cells was evaluated by quantitative polymerase chain reaction. Early-onset cGVHD (n = 33) was characterized by decreased expression of IFN-γ and IL-2 mRNA after nonspecific phorbol 12-myristate 13-acetate–ionomycin stimulation. In contrast, late-onset cGVHD (n = 11) was characterized by decreased expression of IL-4 and IL-2 mRNA after anti-CD3 stimulation of T cells. Receiver-operating characteristic curve analysis revealed that IFN-γ expression was correlated with the absence of early cGVHD (area under the curve [AUC] = 0.77) and that IL-4 (AUC = 0.89) and IL-2 (AUC = 0.84) expression was correlated with the absence of late cGVHD. There was no correlation between cytokine expression and a specific immune cell subset. Increased expression of Foxp3 mRNA was seen in early-onset cGVHD and late controls. The different time-dependent cytokine profiles in patients with newly diagnosed cGVHD suggests that the mechanisms underlying cGVHD are temporally regulated. Although larger validation studies are needed, our data suggest that cytokine profiles have a potential use as biomarkers for the diagnosis of cGVHD.

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INTRODUCTION

Myeloablative allogeneic blood and marrow transplantation (BMT) is the only successful cellular-based immunotherapy for high-risk hematopoietic malignancies. It also is the only curative treatment for numerous marrow failure syndromes [1], nonmalignant blood disorders [2], primary immunodeficiencies [3], autoimmune diseases [4], and inherited metabolic diseases [5]. However due to the increased usage of unrelated donors, more than one-half of patients who receive an allogeneic BMT will develop chronic graft-versus-host disease (cGVHD) [6], which has become the leading cause of transplantation-related morbidity and mortality [7]. In adults with cGVHD, mortality is 60% after 8 years [8], and in children...
with cGVHD, mortality is 20% after 15 years [9]. Numerous possible mechanisms of cGVHD have been investigated, but previous human clinical studies have been hindered by a number of factors, including (1) the insidious onset and multiple organ involvement of cGVHD, (2) samples obtained at different times in the course of the disease and from patients taking a wide variety of immunosuppressants, (3) failure to consider time of onset, and (4) lack of proper controls to account for patterns of normal immune recovery post-BMT.

Our group previously reported evidence suggesting that the biology of cGVHD is temporally different and influenced by immune reconstitution after BMT, and that there are different patterns of biomarkers in early-onset (3-8 months post-BMT) and late-onset (>9 months post-BMT) cGVHD [10]. Soluble B cell activation factor (sBAFF), anti-dsDNA antibody, soluble interleukin -2 receptor α (sIL-2Rα), and soluble CD13 (sCD13) were elevated in patients with early-onset cGVHD compared with controls. sBAFF and anti-dsDNA were elevated in patients with late-onset cGVHD. These previous findings suggest that the pathophysiology of cGVHD is heterogeneous, with different mechanisms operative at different times after BMT. The present study aimed to further characterize the differences between early-onset and late-onset cGVHD.

A number of different effector cell types are thought to be important in the pathophysiology of cGVHD, including B cells, regulatory T (Treg) cells, and effector memory T cells. B cells have been increasingly recognized as having an important role in the pathophysiology of cGVHD, as was initially identified in a murine model by our group [11]. Later human data confirmed the importance of B cells in cGVHD by establishing a role for autoantibodies, such as HY antibodies in male recipients with female donors correlating with cGVHD development [12-14], high levels of sBAFF [10,15], increased plasma cell populations [16], and CD21-CD27+ B cells [17]. Their importance also is clinically supported by the successful treatment of steroid-refractory cGVHD with rituximab, an anti-CD20 (B cell antigen) monoclonal antibody [18-20].

The role of Tregs in cGVHD is less clear. Mouse models show that Tregs play an important role in prevention of GVHD [21], and that adoptive transfer of freshly isolated or ex vivo expanded CD4+CD25+ T cells can prevent GVHD [22,23]. In humans, there are conflicting data as to the importance of Tregs in cGVHD, with different studies showing decreased, unchanged, or increased numbers of these regulatory cells [24-27].

The roles of other T cell populations appear to be equally varied and unclear. cGVHD has been associated with a preponderance of interferon (IFN)-γ, IL-4, IL-5, and IL-2-producing CD4+ effector memory cells [28,29] and with infiltration of CD8+ T cells in skin [30] and intestine [31]. OX40, an activation marker on both CD4+ and CD8+ T cell populations, may be associated with cGVHD onset [32]. A predominance of cytokine-producing Th1/Th2 immune responses has also been postulated. A review of the current literature yields contradictory results regarding the synthesis of cytokines, such as IFN-γ. In some studies, increased IFN-γ mRNA expression has been associated with extensive cGVHD [33,34], whereas others have shown that patients with microsatellite polymorphisms within the first intron of the IFN-γ gene associated with decreased production have higher rates of cGVHD [35]. In mouse models, high IFN-γ production by natural killer (NK) T cells results in lower rates of cGVHD [36,37]. There are no data on this mechanism in children.

Based on our findings showing different biomarker profiles in early-onset and late-onset cGVHD, we hypothesized that distinctive Th1/Th2 cytokine profiles are associated with early and late cGVHD. To verify our hypothesis, we made use of a well-controlled Children’s Oncology Group study, ASCT0031, evaluating hydroxychloroquine therapy in children with newly diagnosed extensive cGVHD between 2002 and 2005. As part of the biological studies associated with this clinical trial, we prospectively measured mRNA levels of IFN-γ, IL-2, IL-4, and IL-10 in peripheral blood mononuclear cells (PBMCs) after either nonspecific mitogen stimulation with phorbol 12-myristate 13-acetate (PMA)-ionomycin (PI) or T cell stimulation with anti-CD3 in newly diagnosed cGVHD patients and comparing these levels with those in time-matched BMT control subjects who did not develop cGVHD. We also collected data on the percentage and absolute counts of immune cell subsets, and retrospectively analyzed mRNA levels of Foxp3 in resting or anti-CD3-stimulated PBMCs to investigate the temporal role of regulatory T cells in cGVHD.

**PATIENTS AND METHODS**

**Patients**

Samples were obtained from patients enrolled in the Children’s Oncology Group trial ASCT0031. All enrolled patients provided signed informed consent approved by each individual center’s Institutional Review Board in accordance with the Declaration of Helsinki. The study was a Phase III randomized, placebo-controlled, double-blinded trial evaluating 2 treatment regimens for patients age 1-30 years with newly diagnosed extensive cGVHD. The patients received a standard regimen of cyclosporine and alternate-day prednisone with either hydroxychloroquine or placebo. Eligibility requirements allowed concurrent steroid therapy for treatment
and/or prophylaxis of acute GVHD (aGVHD) if the prednisone dose was ≤2 mg/kg/day (or equivalent) at study entry. Enrollment was entirely voluntary at each center, and there was no attempt to establish matched controls for the patients with cGVHD. The decision to allow a center to participate in control enrollment was based not on the characteristics of the patients enrolled, but rather on the resources available to identify and follow such individuals. Newly diagnosed extensive cGVHD was documented with biopsy confirmation of at least one organ system (eg, lip, skin, liver) and either generalized skin involvement or localized skin involvement and/or liver dysfunction plus at least one of the following: liver histology showing chronic aggressive hepatitis, bridging necrosis, or cirrhosis; eye involvement (Schirmer’s test with <5 mm wetting); involvement of minor salivary glands or oral mucosa on lip biopsy; or involvement of any other target organs or of at least 2 target organs. Peripheral blood samples in patients with cGVHD were collected at study entry and at 2 months, 6 months, and 12 months after study entry. For controls, peripheral blood was collected from patients without documented cGVHD at 6 and 12 months after transplantation. A total of 82 patients were enrolled in the original Phase III trial. Forty-four patients with cGVHD and 19 control patients without cGVHD were included in this part of the study for a total of 63 patients, representing 77% of the original sample size. The samples collected were used for a number of different studies, and the present study was limited by the amount of sample received for some patients. Control samples were collected at 6 months and 12 months post-BMT from patients without cGVHD to address the potential confounding factor of immune reconstitution after BMT. A time-matched comparison was performed, with days post-BMT at the time of cGVHD diagnosis used to divide the patients into an early-onset group (3-8 months) and a late-onset group (≥9 months) to allow comparison with samples from control patients drawn at 6 months and 12 months post-BMT, respectively. All blood samples were collected before initiation of protocol therapy.

**Patient Characteristics**

Patients with cGVHD and controls at each time point were compared for differences in age, sex, donor source, donor type, aGVHD, GVHD prophylaxis, and concurrent steroid use (≤2 mg/kg/day) (Table 1). There appeared to be a clinical difference in unrelated donors, peripheral blood source, and aGVHD at the early time point, as expected given that all are associated with higher rates of cGVHD, but there was no apparent statistically significant difference between the groups at each time point.

| Table 1. Comparison of Patients with cGVHD and Controls for Differences in Age, Sex, Donor Type, Donor Source, aGVHD, GVHD Prophylaxis, and Concurrent Steroid Therapy |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Age, years                      | Early Controls  | Early cGVHD     | Difference (95% CI) | Late Controls  | Late cGVHD     |
| Median (range)                  | 9.0 (1.3-20.7)  | 11.3 (2.0-21.0) | 7.3 (1.2-15.4)  | 13.5 (3.2-21.8) |
| Mean ± standard deviation       | 9.7 ± 6.0       | 12.0 ± 5.7      | 7.0 ± 4.8       | 13.2 ± 5.5      |
| Sex, n (%)                      | Male 12 (67)    | 23 (70)         | 7 (64)          | 8 (73)          |
|                                | Female 6 (33)   | 10 (30)         | 4 (36)          | 3 (27)          |
| Donor source, n (%)             | Bone marrow 11 (61) | 13 (39) | 21.7% (-6 to 49) | 7 (64) | 7 (64) |
|                                | Peripheral blood 4 (22) | 14 (42) | 20.2% (-45 to 5) | 2 (18) | 2 (18) |
|                                | Umbilical cord blood 3 (17) | 6 (18) | 2 (18)          | 2 (18) |
| Donor type, n (%)               | Sibling 13 (72) | 15 (45)         | 27% (0-54)      | 7 (64) | 5 (45) |
|                                | Unrelated 5 (28) | 18 (55) | 27% (-53 to 0) | 4 (36) | 6 (55) |
| aGVHD, n (%)                    | Yes 6 (33)      | 20 (61)         | 27% (54 to 0)   | 4 (36) | 6 (55) |
|                                | No 12 (66)      | 13 (39)         | 27% (0-54)      | 7 (64) | 5 (45) |
| GVHD prophylaxis, n (%)         | Cyclosporine 14 (78) | 26 (79) | 10 (91) | 9 (82) |
|                                | Methotrexate 13 (72) | 22 (67) | 8 (73) | 6 (55) |
|                                | Steroids 6 (33) | 8 (24)          | 4 (36)          | 3 (27) |
|                                | Tacrolimus 6 (33) | 7 (21) | 3 (27) | 2 (18) |
|                                | Mycophenolate mofetil — | 2 (6) | — | — |
|                                | Antithymocyte globulin — | 2 (6) | — | — |
| On prednisone or equivalent (≤2 mg/kg/day) at study entry | Yes 8 (44) | 16 (48) | 5 (45) | 3 (27) |
|                                | No 10 (56)      | 17 (48)         | 6 (55)          | 8 (73) |

CI indicates confidence interval.
Quantitative Polymerase Chain Reaction

Th1 (IL-2 and IFN-γ) cytokine, Th2 (IL-4 and IL-10) cytokine, and Foxp3 mRNA levels were determined by quantitative polymerase chain reaction (qPCR). Blood from cGVHD and control subjects was collected in heparinized tubes and then shipped overnight at room temperature to the reference laboratory at the University of Iowa. PBMC (0.5-1.0 × 10^6), isolated by Ficoll-Hypaque separation, were maintained in complete RPMI medium (10% fetal calf serum, 1000 U/mL penicillin, 1000 U/mL streptomycin, and 20 mM glutamine), then placed in Costar plates with medium alone or stimulated with PMA (75 ng/mL; Sigma-Aldrich, St. Louis MO) plus ionomycin (1 μM, Sigma-Aldrich) or with plate-bound anti-CD3 (biotinylated anti-CD3; Pharmingen, San Diego, CA). After an 18-hour incubation, cells were lysed and RNA was isolated with the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was generated using the Gibco BRL Superscript First-Strand Synthesis system (Invitrogen, Carlsbad, CA). qPCR was performed according to the manufacturer’s instructions as described previously [38]. TaqMan primers and probes for human cytokines were purchased from Applied Biosystems (Foster City, CA), and samples were analyzed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). For each sample, mRNA expression level was normalized to the level of glyceraldehyde-3-phosphate dehydrogenase housekeeping genes. The value of each sample was an average of 3 independent qPCR measurements. Data from the qPCR experiments were analyzed using the comparative CT method for multiplex reactions, as described previously [39].

Flow Cytometric Analysis of Immune Cell Populations

PBMCs were isolated by Ficoll-Hypaque gradient centrifugation. After isolation, cells were washed twice in FACS staining buffer (phosphate-buffered saline, 0.1% sodium azide, 1% fetal calf serum), and then stained with a panel of fluorescein-conjugated antibodies. Expression of cell surface markers was quantified with a FACScan analyzer (BD Biosciences Immunocytometry Systems, San Jose, CA) and Cellquest V3.2 software (BD Biosciences, Mountain View, CA), as described previously [40]. Mouse anti-human monoclonal antibodies (mAbs), conjugated with fluorescein isothiocyanate, phycoerythrin, or peridinin chlorophyll protein complex and reacting with T cell markers (CD3, CD4, and CD8), B cell markers (CD19), NK cell markers (CD16/56), and various other markers (ie, CD25, CD57, CD69, HLA-DR), were purchased from BD Biosciences.

Statistical Analysis

Descriptive statistics for all data were generated using Prism version 4 (GraphPad Software, San Diego, CA). The percentage change in cell surface molecule expression was defined as the difference in the percentage of positive cells after culture with stimulation and the percentage of positive cells after culture in otherwise identical conditions with no stimulation. The degree of dispersion from the mean was calculated as standard deviation (σ). The significance of observed changes was determined using the Mann-Whitney U test. The α (P) value was set at .05, making all P values <.05 statistically significant. Even when a P value was <.05, the results were not considered biologically significant unless the difference was either 50% higher or lower in the GVHD value compared with the control value. The receiver-operating characteristic (ROC) curve methodology was used to assess the ability of cytokine expression to predict the likelihood of cGVHD. The ability to discriminate between the presence and absence of cGVHD was evaluated using the area under the ROC curve (AUC). An AUC value of 0.5 indicates that the measurement is no better than chance in discriminating between the 2 conditions, whereas a value of 1 indicates perfect discriminatory power. ROC analysis allowed us to determine cutoffs for cytokine levels to maximize accuracy.

RESULTS

IFN-γ, IL-4, IL-2, and IL-10 Concentrations in Early Chronic GVHD

We evaluated cytokine mRNA expression from PBMCs after activation with a nonspecific mitogen, PI, which stimulates all T cells, NK cells, monocytes, and B cells, and compared these findings with cytokine expression after T cell–specific stimulation with anti-CD3 mAb. We found decreased IFN-γ production in patients with early cGVHD compared with early controls after PI stimulation (Figure 1A; P = .002), but no difference in IFN-γ production after anti-CD3 mAb stimulation (Figure 1B). Similarly, we found decreased IL-2 production in patients with early cGVHD versus early controls after PI stimulation (Figure 1C; P = .03), but no significant difference after anti-CD3 mAb stimulation. No differences were seen for either PI or anti-CD3 mAb-stimulated IL-4 production in early cGVHD versus early controls after PI stimulation (Figure 1D; P = .002), but no difference in IFN-γ expression after anti-CD3 mAb stimulation (Figure 1B). IL-10 production was assessed only after anti-CD3 mAb stimulation. There appeared to be a higher expression in patients with early cGVHD, but the difference was not statistically significant (Figure 1G; P = .12). A previous history of aGVHD was not significantly associated with the expression of any cytokine.

IFN-γ, IL-4, IL-2, and IL-10 Concentrations in Late cGVHD

Neither PI nor anti-CD3 mAb stimulation resulted in a significant difference in IFN-γ expression
Figure 1. (A) IFN-γ mRNA production at early and late time points in controls and patients with cGVHD after PI stimulation for 18 hours. (B) IFN-γ mRNA production at early and late time points in controls and patients with cGVHD after anti-CD3 mAb stimulation for 18 hours. (C) IL-2 mRNA production at early and late time points in controls and patients with cGVHD after PI stimulation for 18 hours. (D) IL-2 mRNA production at early and late time points in controls and patients with cGVHD after anti-CD3 mAb stimulation for 18 hours. (E) IL-4 mRNA production at early and late time points in controls and patients with cGVHD after PI stimulation for 18 hours. (F) IL-4 mRNA production at early and late time points in controls and patients with cGVHD after anti-CD3 mAb stimulation for 18 hours. (G) IL-10 mRNA production at early and late time points in controls and patients with cGVHD after anti-CD3 mAb stimulation for 18 hours.
in patients with late-onset cGVHD versus controls (Figure 1A and B). IL-2 production, which was lower in patients with late-onset cGVHD versus late controls after PI stimulation, became significant only after stimulation with anti-CD3 mAb (Figure 1C; \( P = .007 \)). Similarly, there was decreased IL-4 production after PI stimulation in patients with late cGVHD versus late controls (Figure 1E; \( P = .07 \)), although the difference was not statistically significant. This difference became significant after T cell–specific anti-CD3 mAb stimulation (Figure 1F; \( P = .004 \)). IL-10 production was assessed only after anti-CD3 mAb stimulation. Expression appeared to be higher in patients with late cGVHD, but again the difference was not statistically significant (Figure 1G; \( P = .10 \)). A previous history of aGVHD was not associated with a significant effect on expression of any cytokine.

**ROC Analysis of Cytokine Levels to Predict the Absence of cGVHD**

We used ROC curve analyses to explore whether and if so, at what level, cytokine mRNAs might be useful in predicting the presence or absence of cGVHD. PI-induced IFN-\( \gamma \) production was found to be potentially useful in predicting the absence of GVHD at the early time point, with an AUC value of 0.77 (Figure 2A; \( P = .002 \)), with 80% specificity at a value of 20,544 (sensitivity, 73%). Analysis of PI-induced IL-2 production at the early time point did not yield a sufficiently high AUC value to be predictive (AUC = 0.69; \( P = .03 \)). We found no significant association for either IFN-\( \gamma \) (AUC = 0.62; \( P = .18 \)) or IL-2 (AUC = 0.57; \( P = .40 \)) after T cell–specific stimulation with anti-CD3 mAb. IL-4 had no predictive value at the early time point.

At the late time point, ROC analysis revealed a strong predictive value for the absence of cGVHD of anti-CD3–induced IL-4 production (Figure 2B; AUC = 0.89; \( P = .004 \)), with 80% specificity at a value of 170.5 (sensitivity, 91%), as well as IL-2 production (Figure 2C; AUC = 0.84; \( P = .006 \)), with 80% specificity at a value of 189.5 (sensitivity, 82%) in the late cGVHD population. PI-induced IL-4 production (AUC = 0.74; \( P = .06 \)) was not significantly predictive of the absence of late cGVHD. Contrary to the early cGVHD group, the significant IL-2 results seen in the late cGVHD group were obtained only with T cell–specific anti-CD3 mAb stimulation.

**Evaluation of Other Factors That May Affect Cytokine Production**

The cytokine patterns in early-onset and late-onset cGVHD were not correlated with the donor type (related or unrelated), graft source (cord blood, peripheral blood, or bone marrow), or organ involvement by cGVHD (data not shown).

**Foxp3 mRNA Concentrations in Early-Onset and Late-Onset Chronic GVHD**

At the time of our retrospective analysis, no consistent Foxp3 antibody was available for flow cytometry; thus, we measured mRNA expression in unstimulated and anti-CD3 stimulated PBMC samples. We found a nearly 10-fold increase in Foxp3 mRNA expression in patients with early-onset cGVHD, although the difference did not reach statistical significance in either the unstimulated (Figure 3A; \( P = .08 \)) or anti-CD3–stimulated (Figure 3B; \( P = .27 \)) sets. Conversely, at the later time point, there was an increase in Foxp3 mRNA expression in control patients that was
significant in the anti-CD3–stimulated set (Figure 3B; \(P = .04\)).

### Absolute Lymphocyte Count and Correlation of Immune Cell Subsets with Cytokine Production

We measured the absolute lymphocyte count (ALC) at the early and late time points for all 4 combinations of aGVHD (A) and cGVHD (C): A–C–, A1C–, A–C1, and A1C1. At the early time point, the highest ALC was in the A1C1 group, with 2339 ± 3245/μL, followed by A–C1 with 2141 ± 1280/μL, A1C– with 1586 ± 1122/μL, and A–C with 1144 ± 477/μL. There was a statistically significant difference between the patients with cGVHD and without cGVHD and no history of aGVHD (\(P = .009\)). There were no significant differences between groups at the late time point (A–C–, 2013 ± 1669/μL; A1C–, 2312 ± 1402/μL; A–C1, 2274 ± 3055/μL; and A1C1, 2729 ± 2174/μL). There also were no statistically significant differences in the percentages of cell phenotypes—T cells (CD31, CD41, CD41CD571, CD81, CD81CD571), NK cells (CD31CD161CD561), and B cells (CD191)—in patients with cGVHD and controls at both time points in unstimulated samples. We also compared the percentages of activated T cell lymphocyte subsets (CD41CD251, CD81CD251, CD41CD691, CD81CD691, CD41HLA-DR1, and CD81HLA-DR1) in unstimulated versus anti-CD3–stimulated samples and found no significant differences. Furthermore, we found no correlation between mRNA expression of all cytokines and the percentage and absolute cell counts of anti-CD3–stimulated T cell lymphocytes evaluated at both time points (data not shown).

### DISCUSSION

One of the most significant findings from the previously published Children’s Oncology Group ASCT0031 study is that there are immunologic differences between cGVHD occurring early after BMT (3-8 months) and that occurring later (≥9 months) [10], and that immune reconstitution post-BMT can significantly affect the interpretation of cGVHD biomarkers. In the current analysis, the presence of early cGVHD was characterized by a decreased Th1 cytokine mRNA response, whereas the presence of late cGVHD was characterized by a decreased Th2 cytokine mRNA profile. Despite our small number of patients, we found significant patterns that require subsequent validation in larger prospective clinical trials.

To better understand the immunopathogenesis of cGVHD, PBMCs isolated from patients with new-onset cGVHD and time-matched controls were stimulated with a nonspecific lymphocyte mitogen, PI, or T cell–specific anti-CD3 mAb. In patients with early-onset cGVHD, IFN-γ and IL-2 mRNA expression was significantly decreased only after PI stimulation, and not after anti-CD3 mAb stimulation, suggesting that the initial effector cell populations might not be T cells. One possibility is that these effectors are NK cells, which are capable of producing inflammatory cytokines and mediating cytolytic activity [41]. The concept of an NK-mediated regulatory function is supported by the observation that a higher number of bone marrow NK cells is associated with a decreased incidence of cGVHD after HLA-identical sibling bone marrow transplantation in humans [42]. Recent findings indicate that this regulatory function can be mediated through dendritic cells (DCs). Through the production of IFN-γ, NK cells can enhance the function of DCs, induce expression of costimulatory molecules and production of IL-12 and TNF-α, and skew the T cell stimulatory capacity.
of DCs toward Th1-type responses. It has been suggested that NK cells mediate reduction of cGVHD by direct lysis of T cells, inhibition of donor T cell proliferation, and induction of T cell apoptosis [43].

There is much less evidence suggesting a protective role for B cells in cGVHD. Effector B cells (Be1 and Be2 cells) can secrete cytokines, such as IFN-γ, IL-12, IL-4, and IL-2, that reinforce and stabilize the cytokine profiles of both effector Th1 and Th2 cells [44].

Our findings suggest that the ability to generate a Th1/Tc1 response (IFN-γ and IL-2) early post-BMT protects against cGVHD. Unfortunately, we did not find any correlation between IFN-γ and IL-2 mRNA expression and the number of NK or B cells. A limitation of the present study was the lack of functional studies on immune cell subsets. It will be important in future trials to determine which immune cells are responsible for the production of our cytokines of interest.

The decreased IL-4 mRNA expression in patients with late-onset cGVHD supports the widely accepted hypothesis that a Th2 response is protective. In contrast to the earlier time point, there was significant elevation with both PI- and anti-CD3- induced IL-4 production, suggesting that the effector cell population may be a CD3+ T cell population. One promising candidate is the Tregs, as described previously [21-23]. We also observed a significant decrease in IL-2 mRNA synthesis in patients with early-onset and late-onset cGVHD. IL-2 is known to play a central role in Th2 differentiation [45], and is reportedly necessary for the production and expansion of CD4+CD25+ Tregs [46]. Mice deficient in either IL-2 or elements of its receptor develop a massive lymphoproliferative syndrome accompanied by severe autoimmunity [47,48]. IL-2 is necessary for B cell tolerance, and its absence could contribute to the unchecked expansion of autoreactive B cells. A role for B cells in the pathophysiology of cGVHD has been suggested, and as part of this study we identified increased numbers of B cells, primed for a Toll-like receptor 9 (TLR9) response [49]. However, we did not find a difference in the absolute CD19+ B cell count between control and GVHD patients, although our study was limited by the lack of specific phenotype labeling (such as IgM memory B cells [50] or CD21− B cells [17]) and functional data.

To better address the potential role of regulatory T cells in cGVHD, we retrospectively measured Foxp3 mRNA expression from archived frozen cell lysates. During the initial design of the study, a proper Foxp3 antibody was not available, and data on this cell type were limited. At the early time point, we were able to show a trend toward increased Foxp3 mRNA expression in anti-CD3-stimulated cell lysates from patients with cGVHD. In contrast, at the late time point, we found an increase in Foxp3 mRNA expression in anti-CD3-stimulated cell lysates from patients without cGVHD. Although these results were not statistically significant, they could suggest the presence of different activated and/or regulatory T cell populations that are pathogenic at the early time point and protective at the late time point. We now know that Foxp3 expression is not uniquely associated with regulatory T cells [51,52]. We can hypothesize that at the early time point, there is a pathological population of activated cytotoxic CD8+ T lymphocytes. In support of this hypothesis, D’Asaro et al. [53] reported a correlation between increases in the effector memory subset (TEMRA, CCR7−, CD45RA−) in CD8+ T cells and the occurrence of cGVHD in patients who underwent allogeneic PBSC transplantation. In addition, multiparous females have measurable levels of circulating HY, the female-specific minor H antigen, and specific CD8+ T cytotoxic lymphocytes can be readily expanded in vitro. It is noteworthy that the use of multiparous females as donors significantly increases the risk of GVHD [54].

The discovery of temporally regulated cytokine profiles in cGVHD has 2 potential applications that merit further investigation. First, it is possible that certain cytokine expression levels could serve as biomarkers in cGVHD. It is important that expression levels of all of the cytokines discovered in this study be applied prospectively to a new cohort of patients with cGVHD to validate these cytokines as biomarkers. This effort is currently under way. Cytokine expression can serve as a biomarker by (1) providing another test to help confirm a diagnosis of cGVHD when there is uncertainty; (2) directing the choice of therapy, that is, choosing a therapy that targets pathogenic immune subsets involved in the production of the cytokines; and (3) determining whether a patient with cGVHD is responding to therapy if cytokine levels change. Second, determining the cells of origin of these cytokines in future prospective studies will provide insight into the pathophysiology of cGVHD.

Combining our previous findings with the present results led us to generate a tentative model in which T cell activation with sIL2Rz and sBAFF up-regulation are part of early-onset cGVHD, and IFN-γ and IL-2 production is required to inhibit CD8+ T cell and B cell expansion and antibody production. Late-onset cGVHD is characterized by a lack of Th2 shift and the development of activated high TLR9−expressing B cells responding to up-regulated BAFF expression from monocyte lineages. TLR9 activation in combination with sBAFF results in increased autoantibody production and makes these B cells potent antigen-presenting cells for T cells in the periphery. IL-4 down-regulates TLR9 transcripts and proteins in naive B cells [55].

Data from the present study differ from that reported previously. The present study involved children and adolescents, and whether there are any immunologic differences in cGVHD between these
populations is unclear. Children have a lower incidence of cGVHD compared with adults. Zecca et al. [56] reported a cumulative probability of cGVHD of 27%, less than one-half of the estimated probability in adults. This was true even for children undergoing transplantation with bone marrow stem cells, contesting the theory that the overall incidence of cGVHD in children is lower due to the increased use of cord blood stem cells. It has been assumed that the mechanisms underlying cGVHD are the same in children and adults as their clinical manifestations, and that responses to therapy are similar as well. Moreover, laboratory findings, including sBAFF levels, are comparable in studies involving both populations [10,15].

One possible limitation of the present study is that both cyclosporin A and tacrolimus can have a potent dose-dependent inhibitory effect on cytokine expression from lymphocytes after stimulation with PI [57]. Puzik et al. [57] reported that the effect of calcium calmodulin inhibitors was dependent on donor age. In cord blood lymphocytes, the inhibitory effect was dose-dependent, whereas the function of adult lymphocytes was impaired only at high doses of both compounds. In the present study, the lack of statistical difference among the experimental groups in terms of GVHD prophylaxis and cord blood donors limits this confounding factor.

The cytokine expression results presented here provide novel evidence that there are potentially different immune mechanisms underlying the timing of onset of cGVHD. Furthermore, the different cytokine profiles may explain the numerous contradictions in the literature regarding cytokine expression and immune dysregulation during cGVHD, particularly when the timing of disease onset is not taken into account.

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