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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

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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 and 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 cGHVD.

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