Chronic graft-versus-host disease is associated with increased numbers of peripheral blood CD4\(^+\)CD25\(^{high}\) regulatory T cells

Fiona J. Clark, Richard Gregg, Karen Piper, Debbie Dunnion, Lisa Freeman, Mike Griffiths, Gulnaz Begum, Premini Mahendra, Charles Craddock, Paul Moss, and Ronjon Chakraverty

Chronic graft-versus-host disease (cGVHD) is characterized by a state of profound immunodeficiency in association with alloreactive and autoimmune phenomena. These observations indicate an impairment of immunologic tolerance that could involve both central and peripheral mechanisms. Defective thymic function may contribute to dysregulation of central tolerance, but few studies have addressed peripheral tolerance. Recently a population of CD4\(^+\)CD25\(^+\) T cells (T\(_{reg}\) cells) has been characterized, which controls immunologic reactivity in vivo and which on transfer can prevent experimental acute GVHD. We investigated the number and function of peripheral blood CD4\(^+\)CD25\(^{high}\) T cells in patients more than 100 days after allogeneic hematopoietic stem cell transplantation. Patients with cGVHD had markedly elevated numbers of CD4\(^+\)CD25\(^{high}\) T cells as compared to patients without GVHD. CD4\(^+\)CD25\(^{high}\) T cells derived from patients in both groups were of donor origin, lacked markers of recent activation, and expressed intracellular CD152. In contrast to controls, CD4\(^+\)CD25\(^{high}\) T cells derived from patients with cGVHD were characterized by lower surface CD62L expression. In vitro, CD4\(^+\)CD25\(^{high}\) T cells were hyporesponsive to polyclonal stimulation and suppressed the proliferation and cytokine synthesis of CD4\(^+\)CD25\(^-\) cells, an effect that was independent of interleukin 10. These results indicate that chronic graft-versus-host injury does not occur as a result of T\(_{reg}\) cell deficiency.

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Introduction

Chronic graft-versus-host disease (cGVHD) remains the most frequent complication following allogeneic hematopoietic stem cell transplantation (HSCT), occurring in 30% to 70% of long-term survivors. Profound immune dysregulation leads to both immunodeficiency and autoimmunity, suggesting defects in central or peripheral immunologic tolerance. Impairment of thymic function observed in patients with cGVHD could lead to the release into the periphery of T cells with autoreactive potential. Although such perturbations in central tolerance may be important, data relating to experimental autologous GVHD induction or to models in which thymic, negative selection is prevented, suggest that autoreactive T cells only induce tissue injury when there are additional defects in peripheral tolerance.

Recently a population of naturally occurring regulatory CD4\(^+\) T cells (T\(_{reg}\) cells) that constitutively express the α chain of the receptor for interleukin 2 (IL-2; CD25) has been characterized that can suppress immune responses both in vitro and in vivo (for reviews, see Maloy and Powrie, Shevach and Wood and Sakaguchi). In humans, T\(_{reg}\) cells appear to be enriched within the 1% to 2% of peripheral blood CD4\(^+\) T cells that are CD25\(^{high}\) and in vitro, inhibit the function of effector T cells via a mechanism that requires prior T-cell activation, involves cell-to-cell contact, and is independent of cytokine secretion. In contrast, in vivo studies indicate that cytokines such as IL-10 or transforming growth factor β (TGF-β) may be required for the T\(_{reg}\) cell function, a discrepancy that may be explained by the capacity of T\(_{reg}\) cells to induce CD4\(^+\)CD25\(^+\) T cells to develop cytokine-dependent regulatory function. CD4\(^+\)CD25\(^+\) T cells inhibit proliferation of CD4\(^+\)CD25\(^-\) cells in response to alloantigen and their selective depletion in vivo leads to increased severity of acute GVHD in mice following bone marrow transplantation. Consistent with this, cotransfer of large numbers of naturally occurring or ex vivo expanded CD4\(^+\)CD25\(^+\) T cells prevents or ameliorates acute GVHD and requires IL-10 production. These experiments have raised the possibility that transfer of T\(_{reg}\) cells could be exploited therapeutically in the prevention of GVHD. However, no studies have examined to what extent, if any, T\(_{reg}\) cells modulate chronic graft-versus-host injury.

We have performed a cross-sectional study of patients at least 100 days after allogeneic HSCT to determine the number of CD4\(^+\)CD25\(^{high}\) T cells circulating in peripheral blood. Here we demonstrate that patients with cGVHD had markedly elevated numbers of peripheral blood CD4\(^+\)CD25\(^{high}\) T cells as compared to those without cGVHD. As in controls, CD4\(^+\)CD25\(^{high}\) T cells derived from patients with cGVHD lacked markers of recent activation, expressed intracellular cytotoxic T lymphocyte–associated antigen 4 (CTLA-4/CD152), and displayed regulatory activity in vitro. These results indicate that chronic graft-versus-host injury does not occur as a result of T\(_{reg}\) cell deficiency.
Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>cGVHD, n = 17</th>
<th>Controls, n = 23</th>
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<tbody>
<tr>
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<td>43 (range, 29-55)</td>
<td>41 (range, 18-54)</td>
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<td>Acute leukemia</td>
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<td>5</td>
</tr>
<tr>
<td>Myelodysplastic syndrome</td>
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<td>1</td>
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<tr>
<td>Donor*</td>
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<td>HLA-matched VUD</td>
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<td>Preparative regimen†‡</td>
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<td></td>
</tr>
<tr>
<td>Standard</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Reduced-intensity</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Source of HSC</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>12</td>
</tr>
<tr>
<td>PBSC</td>
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<td>11</td>
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<tr>
<td>In vivo T-cell depletion§</td>
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<td>8</td>
</tr>
<tr>
<td>Acute GVHD grade II–IV</td>
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<td>1</td>
</tr>
<tr>
<td>Immunosuppression at time of analysis</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>Time after HSCT for analysis, mo</td>
<td>17 (range, 4-71)</td>
<td>11 (range, 4-110)</td>
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</tbody>
</table>

VUD indicates volunteer unrelated donor; HSC, hematopoietic stem cells; BM, bone marrow.

*HLA typing was performed by molecular techniques for HLA class I A, B, and C and for class II HLA DRB1 and DQB1, in all donors and recipients.
†Standard conditioning was performed using total body irradiation (TBI) and cyclophosphamide, TBI and melphalan, or busulfan and cyclophosphamide. Reduced intensity conditioning was performed using fludarabine and melphalan or BCNU, cytarabine arabinoside, etoposide, and melphalan.
‡GVHD prophylaxis was with cyclosporine/methotrexate in the majority of patients (n = 36), with the exception of 4 patients who received cyclosporine alone.
§In vivo T-cell depletion with alemtuzumab was administered before transplantation to a subset of patients who were recipients of volunteer unrelated HSCs or underwent reduced intensity conditioning.
| | | |

Results

Patients

A total of 40 adult patients treated by allogeneic HSCT for hematologic malignancy were recruited into the cross-sectional study (Table 1). Of these, 17 patients had cGVHD and 23 patients had no current or previous cGVHD. Of the patients with cGVHD, 13 had extensive and 4 had limited disease. At diagnosis of cGVHD, composite prognostic scores based on the presence of thrombocytopenia, progressive-type onset, or extensive skin involvement stratified patients into low risk (score 0, n = 7), intermediate risk (score < 2, n = 7), or high risk (score ≥ 2, n = 3) groups. A greater proportion of patients with cGVHD had a history of antecedent acute GVHD and required immunosuppressive treatment at the time of sampling (Table 1).

CD4⁺CD25high T cells are increased in patients with cGVHD

Using the gating strategy shown in Figure 1A, flow cytometric enumeration of peripheral blood revealed significantly increased numbers of CD4⁺CD25high T cells in patients with cGVHD compared to patients without this complication, expressed both as a percentage of CD4⁺ T cells (cGVHD, 3.9% ± 0.7%; no GVHD, 2.0% ± 0.3%; P = .02) or as absolute counts (cGVHD, 19.0 ± 3.4 × 10⁶/L; no GVHD, 5.9 ± 1.6 × 10⁶/L; P < .001; Figure 1B). As shown in Table 2, multiple regression analyses demonstrated cGVHD to be the most significant independent factor influencing CD4⁺CD25high T-cell numbers expressed as absolute counts (P = .0002) or as a percentage of CD4⁺ T cells (P = .02).
Trends toward a positive correlation between younger recipient age and higher absolute CD4<sup>+</sup>CD25<sup>high</sup> T-cell numbers (P = 0.09) and between earlier time from transplantation and higher CD4<sup>+</sup>CD25<sup>high</sup> T-cell numbers expressed as a percentage of CD4<sup>+</sup> cells (P = 0.07), were noted. The increase in CD4<sup>+</sup>CD25<sup>high</sup> T-cell numbers in patients with cGVHD was matched by an increase in the total number of CD4<sup>+</sup>CD25<sup>+</sup> T cells (incorporating both CD25<sup>low</sup> and CD25<sup>high</sup> populations), expressed both as a percentage of CD4<sup>+</sup> T cells (cGVHD, 29% ± 2.7%; no GVHD, 16% ± 1.5%; P < 0.001) and as an absolute CD4<sup>+</sup>CD25<sup>+</sup> count (cGVHD, 165.0 ± 26.0 × 10<sup>6</sup>/L; no GVHD, 43.0 ± 11.2 × 10<sup>6</sup>/L; P < 0.001). There was, however, no significant difference in the absolute numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells between the groups (cGVHD, 415 ± 66 × 10<sup>9</sup>/L; no GVHD, 284 ± 69 × 10<sup>9</sup>/L; P = 0.20). Patients with cGVHD had higher numbers of CD4<sup>+</sup>CD25<sup>high</sup> T cells expressed as a percentage of CD4<sup>+</sup> T cells than an age-matched healthy volunteer group (cGVHD, 3.9% ± 0.7%; healthy controls, 1.8% ± 0.3%; P = 0.04), but there was no significant difference in terms of absolute numbers (cGVHD, 19.0 ± 3.4 × 10<sup>6</sup>/L; healthy controls, 13.9 ± 2.9 × 10<sup>6</sup>/L; P = 0.31).

The results of the above cross-sectional study prompted us to evaluate prospectively the kinetics of CD4<sup>+</sup>CD25<sup>high</sup> T-cell expansion after transplantation in 9 patients with or without cGVHD. With a median follow-up of 72 weeks after transplantation (range, 37-88 weeks), 4 patients developed cGVHD (all patients evaluated are shown in Figure 2). In each case, onset of cGVHD was associated with a significant increase in CD4<sup>+</sup>CD25<sup>high</sup> T-cell numbers followed by a subsequent decline in association with significant responses to treatment with cyclosporine or prednisolone or both. In sharp contrast, 4 patients without significant acute or cGVHD showed no major increases in the number of circulating CD4<sup>+</sup>CD25<sup>high</sup> T cells during the study period. In the remaining patient (UPN038), onset of immune thrombocytopenia was preceded by moderate rises in CD4<sup>+</sup>CD25<sup>high</sup> T-cell numbers, although at the time of study this did not meet the criteria for cGVHD.

**CD4<sup>+</sup>CD25<sup>high</sup> T cells from individuals with cGVHD express less surface CD62L than controls**

CD4<sup>+</sup>CD25<sup>high</sup> T cells derived from patients with cGVHD had a broadly similar phenotype to equivalent populations in controls and healthy volunteers (Figure 3A-C and data not shown). In particular, we confirmed that cGVHD CD4<sup>+</sup>CD25<sup>high</sup> T cells had low forward scatter, low expression of CD45RA, 10,11,29 but expressed surface CD95 (CD71, CD95). 10,29 Furthermore, the CD4<sup>+</sup>CD25<sup>high</sup> fraction expressed intracellular CTLA-4 (CD152)10,12,27,29 (Figure 3A), but in contrast, CD25<sup>low</sup> T cells (incorporating both CD25<sup>lo</sup>/int and CD25<sup>low</sup>) were not demonstrable in cGVHD patients (Figure 3A and data not shown).

**Table 2. Multivariate analysis of clinical factors**

<table>
<thead>
<tr>
<th>Absolute count CD4&lt;sup&gt;+&lt;/sup&gt;CD25&lt;sup&gt;high&lt;/sup&gt;</th>
<th>SE</th>
<th>P</th>
<th>% CD4&lt;sup&gt;+&lt;/sup&gt;CD25&lt;sup&gt;high&lt;/sup&gt; of CD4&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Regression coefficient</th>
<th>SE</th>
<th>P</th>
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<tr>
<td>cGVHD</td>
<td>14.42</td>
<td>3.49</td>
<td>0.002</td>
<td>1.74</td>
<td>0.70</td>
<td>0.02</td>
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<tr>
<td>Recipient age</td>
<td>-0.32</td>
<td>0.19</td>
<td>0.09</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Time from HSCT, mo</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>-0.03</td>
<td>0.02</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Multiple regression analyses used a stepwise selection procedure with 10% entry and exit criteria. Univariate analyses of other potential clinical factors that may have influenced CD4<sup>+</sup>CD25<sup>high</sup> T-cell numbers included recipient age, time from transplantation; donor source, HLA-identical related donor/VUD; HSC source, bone marrow/PBSCs; conditioning protocol, standard myeloablative/reduced intensity, in vivo T-cell depletion; and prior acute GVHD. Treatment with immunosuppressive medication was not included in the analysis because few patients without cGVHD were on treatment at the time of the study. — indicates that no statistical correlation was found.
lacked markers of recent activation (CD40L, CD69; Figure 3B and data not shown). As shown in Figure 3C, we observed a moderate reduction in surface expression of CD62L by CD4+CD25<sup>high</sup> T cells of patients with cGVHD as compared to patients without GVHD (percent cells positive, cGVHD, 75% ± 4%; no GVHD, 91% ± 1%; P < .01; mean cellular fluorescence [MCF], cGVHD, 109 ± 27; no GVHD, 254 ± 36; P < .01) or healthy volunteers (percent cells positive, 88% ± 2%; P < .05; MCF, 289 ± 61; P = .01). An evaluation of other adhesion molecules and chemokine receptors found no other differences between CD4<sup>+</sup>CD25<sup>high</sup> T cells from cGVHD patients and controls, in that they expressed CCR4, CCR7, and cutaneous lymphocyte antigen, but stained negatively for CD103 (data not shown).<sup>10,29-31</sup> CD4<sup>+</sup>CD25<sup>high</sup> T cells from individuals with cGVHD display a regulatory phenotype

Flow-sorted, highly pure (> 97%) CD4<sup>+</sup>CD25<sup>high</sup> T-cell fractions from healthy volunteers and patients with or without cGVHD were evaluated to determine their functional regulatory activity in vitro. As shown in Figure 3D, the proliferative and IFN-γ cytokine response of CD4<sup>+</sup>CD25<sup>high</sup> T cells to polyclonal stimulation was considerably lower than that of CD4<sup>+</sup>CD25<sup>+</sup> T cells in both patients with cGVHD and healthy volunteers. When CD4<sup>+</sup>CD25<sup>high</sup> T cells were cocultured with CD4<sup>+</sup>CD25<sup>+</sup> cells at a 1:1 ratio, the proliferative responses to polyclonal stimulation were significantly suppressed and the production of IFN-γ completely inhibited (Figure 3D). Cell yields following coculturing of CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells were reduced by about 10% in comparison to the input cell number. The suppressive activity of CD4<sup>+</sup>CD25<sup>high</sup> T cells on CD4<sup>+</sup>CD25<sup>+</sup> cells was observed in all cGVHD patients tested and similar results were observed using CD4<sup>+</sup>CD25<sup>high</sup> T-cell populations derived from patients without GVHD (data not shown). Titrations indicated that the frequency of suppressor cells within the CD4<sup>+</sup>CD25<sup>high</sup> T-cell population was broadly equivalent in cGVHD patients and controls (Figure 3E). In healthy individuals, clonal analysis of the CD4<sup>+</sup>CD25<sup>high</sup> T-cell population has shown it to be heterogeneous, with only a subset of clones (~45%) displaying suppressive activity in vitro.<sup>26</sup> We derived multiple CD4<sup>+</sup>CD25<sup>high</sup> T-cell clones from a patient with cGVHD using published protocols.<sup>26</sup> All clones retained surface CD25 and intracellular CTLA-4 expression (data not shown), 7 of 8 were hyporesponsive to polyclonal stimulation and 6 of 8 induced suppression (~20% inhibition) of CD4<sup>+</sup> T-cell proliferation to immobilized anti-CD3 (Figure 3F). Freshly sorted CD4<sup>+</sup>CD25<sup>high</sup> T-cell cells from one of 5 cGVHD patients produced IL-10 in supernatants following ex vivo stimulation with immobilized anti-CD3 plus anti-CD28 (11 pg/mL), but no TGF-β was detectable. In contrast, no cytokines were detected in supernatants of PHA-stimulated cocultured CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD25<sup>+</sup>-cells. To rule out the possibility that inhibitory IL-10 was present in very low amounts or was consumed in the coculture experiments, we performed additional studies using neutralizing antibodies to IL-10. Inhibition of IL-10 activity had no effect on the suppression by CD4<sup>+</sup>CD25<sup>high</sup> T cells on the proliferation or IFN-γ production of CD4<sup>+</sup>CD25<sup>+</sup> T cells (Figure 3D). We confirmed that exogenous IL-10 was both inhibitory in this assay and that neutralizing antibody could reverse this inhibition (Figure 3G).

CD4<sup>+</sup>CD25<sup>high</sup> T cells derived from individuals with cGVHD are of donor origin

Experimental models that use T<sub>reg</sub> cells to suppress acute GVHD have indicated that donor but not host cells mediate the effect.<sup>15,21</sup> To determine the origin of these cells we sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells in 7 patients and performed chimerism studies by fluorescent in situ hybridization or short tandem repeat polymerase chain

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**Figure 2.** Prospective enumeration of CD4<sup>+</sup>CD25<sup>high</sup> T cells in patients after transplantation. (A) Nine patients were evaluated prospectively from time of transplantation (shown in weeks). Onset of cGVHD is denoted by the arrowhead and acute GVHD onset by the asterisk. CD4<sup>+</sup>CD25<sup>high</sup> T cells were enumerated and expressed as percent of CD4<sup>+</sup> lymphocytes (□) or as absolute peripheral blood counts × 10<sup>9</sup> (▲). Four patients (UPN011, UPN013, UPN017, UPN027) developed cGVHD during the course of the study. Three patients (UPN021, UPN036, UPN040) did not develop cGVHD, and one patient (UPN021) did not fulfill criteria for cGVHD during the course of the study. Three patients (UPN011, UPN013, UPN017, UPN027) developed cGVHD but developed immune thrombocytopenia at week 44. (B) Dot plots showing CD4<sup>+</sup>CD25<sup>high</sup> T-cell frequencies in patient UPN011 prior to the development of cGVHD (left, week 25), at its onset (middle, week 47) and following a subsequent response to treatment with cyclosporine and prednisolone (right, week 58). Percentages indicate CD4<sup>+</sup> cells gated as CD25<sup>high</sup>. Add-back experiments at week 47 confirmed that sorted CD4<sup>+</sup>CD25<sup>high</sup> T cells retained their suppressive activity in vitro (not shown).
We cannot determine from this study whether or not Treg cells have acquired regulatory function during thymic development or if Treg cell function has been acquired by CD4+ CD25+ T cells after encounter with antigen in the periphery. Patients with cGVHD have impaired thymic function and although it remains possible that aberrant thymic selection may be associated with an increased output of thymic Treg cells, this is unlikely in the setting of the reduced T-cell receptor excision circles levels typical of this condition. An alternative explanation is that despite their anergic phenotype in vitro, preexisting Treg cells undergo vigorous proliferation in vivo in response to antigen, as has been suggested in recent animal models. It is also possible that Treg cells may develop de novo within the periphery from mature CD4+ CD25+ T cells and although the specific requirements for this pathway are not known, antigen presentation in absence of costimulation and cytokines such as IL-10 or TGF-β have been
proposed as important determinants. Expansion of preexisting CD4+CD25+ T reg cell populations or their development from CD4+CD25- T cells would be supported by our prospective analyses of individual patients, which revealed a rapid increase in T reg cell number occurring in conjunction with the onset of cGVHD.

Lower CD62L expression was observed on CD4+CD25high T cells from patients with GVHD and, because CD62L is an important determinant of T-cell entry into lymph nodes, this might imply a more peripheral distribution of regulatory cells in the GVHD patient group and may also provide an additional explanation for their elevated numbers in peripheral blood. Regulatory T cells have been shown to persist at the site of the tolerated skin grafts and to be concentrated in synovial fluid in patients with active rheumatoid arthritis, suggesting that they locate at the site of the effector responses to mediate their host responses. The host tissues that are targeted by alloreactive or autoreactive donor lymphocytes in GVHD are clearly widespread and thus a peripheral distribution of T reg cells may be necessary in this setting. Reductions in CD62L expression have been observed in a subset of T reg cells undergoing rapid proliferation in response to target self-antigens and it is possible that this also applies to the T reg cell population in cGVHD. Although it is reported that only a subset of T reg cells expressing CD62L can prevent autoimmune disease in vivo, the implication that induction of tolerance occurs within secondary lymphoid tissue, induction and maintenance of immunologic tolerance may occur in different environments.

Active immune responses directed against host tissues are not completely suppressed in vivo in patients with cGVHD. Chronic tissue injury occurs despite the presence of regulatory T cells, although it should be noted that the ability of T reg cells to suppress responses to host antigens was not tested in this study. Although it is possible that such immune reactivity might be even greater in the absence of T reg cell populations, there may be a relative failure of T reg cells to modulate an established chronic inflammatory state that involves a complex interplay between T- and non-T-cell effectors, humoral responses, and changes in target tissue chemo-kine or cytokine expression. It is also conceivable that as a result of thymic injury, the repertoire of T reg cells is incomplete and lacks particular specificities that would be required for the prevention of tissue injury. Conversely, T reg cells may in fact have a causative role in the development of cGVHD, a possibility that might be suggested by the finding that allogeneic peripheral blood stem cell (PBSC) grafts containing higher numbers of CD4+CD25high T cells are associated with an increased risk of cGVHD or that patients with cGVHD show responses to therapy with daclizumab, a humanized monoclonal antibody directed against human IL-2 receptor alpha chain. T reg cell-mediated suppression of individual components of the effector immune response could prevent full resolution of the inflammatory state or alternatively T reg cell induction of cytokines such as TGF-β could exacerbate chronic tissue injury.

Our findings suggest that adoptive transfer of T reg cells may have limited clinical impact in the setting of cGVHD in which functional T reg cells are already present despite ongoing disease activity. It will be important for future experimental GVHD models to examine the effect of T reg cell in the setting of cGVHD and to include an assessment of the long-term effects of transfer. Nevertheless, T reg cells remain a potentially important target for therapeutic intervention. It may prove possible to increase the functional activity of T reg cells that already exist in vivo and the potential value of increasing the number of T reg cells in the early phase of transplantation remains unexplored in the human population.

Acknowledgments

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References


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