Specific recruitment of CD4⁺CD25⁺⁺ regulatory T cells into the allograft in heart transplant recipients

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CHRONIC SUBCLINICAL INFLAMMATION represents a major limitation to the long-term success of cardiac transplantation. Although the pathogenesis is multifactorial, the persistent presence of inflammatory cells suggests that continuous perivascular inflammation is the prime course of cardiac allograft vasculopathy (CAV) development (18). Inflammation contributes to endothelial injury, intimal hyperplasia, and proliferation of vascular smooth muscle cells (30, 39, 40, 42). After allograft transplantation, professional antigen-presenting cells, such as mature host dendritic cells, present alloantigens to T cells initiating the immune response (3). T-cell activation is associated with a prompt and sustained expression of the surface activation marker CD69 (26), which is increased during acute rejection in peripheral blood (32).

On the other hand, a specific subpopulation of so-called tolerogenic dendritic cells (14), enriched among the subgroup of plasmacytoid dendritic cells, has the potential to counteract immune responses (41). Tolerogenic dendritic cells promote heart allograft survival, either by deletion of alloreactive T cells or expanding immunosuppressive regulatory T cells (Treg) (20, 29, 37, 43). In humans, Treg are found in a subpopulation of T cells expressing CD4 and high levels of CD25 (31), the α-subunit of the IL-2 receptor (35). Whereas the expression of inhibitory markers, such as cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) (CD152), is a characteristic but not a specific feature for Treg (33), the recently identified forkhead/winged helix transcription factor FOXP3 (10, 17) is specifically expressed in Treg and so far is the most unambiguous marker to identify naturally occurring Treg (7, 31).

Treg exert their immunosuppressive properties by secretion of IL-10 (15), expression of transforming growth factor-β (TGF-β) (11), contact-dependent inhibition of alloreactive T cells (38), through induction of apoptosis (19) or destruction of T cells (13), or via conversion of neighboring T cells into Treg (47).

Moreover, after induction of Treg producing IL-10 in atherosclerotic-prone animals, the development of lesions can be reduced (27), pointing to an involvement of Treg in vascular pathogenesis. Since a predominance of activated T cells could be involved in the chronic perivascular inflammation, we investigated the potentially ongoing activation of T cells related to levels of tolerogenic Treg in the presence of effective long-term immunosuppression. Moreover, since there are no data regarding a potential recruitment of Treg into the human cardiac allograft, we measured the levels of distinct T-cell subpopulations and of dendritic cells after transcoronary passage through the allograft in patients after heart transplantation.

METHODS

Patient population. A total of 51 subjects were prospectively studied. Twenty-two patients were included 6 to 192 mo (median, 55.2 mo) after orthotopic heart transplantation, without acute rejection [≥grade 1B International Society for Heart and Lung Transplantation classification (4)] episodes at least 3 mo before inclusion, who...
underwent cardiac catheterization for routine biopsy or for their routine coronary angiography.

Eighteen healthy subjects, matched for age and sex, without any evidence of coronary artery disease (CAD) by history and physical examination, served as control group for comparison of circulating cells from peripheral blood samples. Control subjects were recruited from among personnel, friends, relatives, and the Frankfurt police choir.

For comparison of transcoronary gradients, eleven subjects who underwent routine angiography were studied. Of these, four had no abnormalities and seven had stable CAD, defined as angiographically documented CAD and the absence of acute coronary syndromes or acute myocardial infarction for 3 mo before blood samples were drawn. Since healthy controls and patients with CAD were matched for age and sex and showed comparable levels of the different subsets of T cells, patients with CAD, undergoing routine diagnostic catheterization, were considered as controls for the transcoronary gradients.

The baseline characteristics of the patients are shown in Table 1.

Exclusion criteria were clinical or biochemical evidence for the presence of concomitant inflammatory disease, chronic renal insufficiency (serum creatinine $\geq$ 2.4 mg/dl), impaired left ventricular ejection fraction ($<45\%$), autoimmune or malignant disease, or unwillingness to participate.

No anti-inflammatory medication, including nonsteroidal anti-inflammatory drugs, were taken by controls or patients with CAD. All study participants gave written informed consent, and the study was approved by the Ethical Committee of the J. W. Goethe University, Frankfurt. The investigation conformed with the principles outlined in the Declaration of Helsinki.

Information was obtained through a structured interview, physical examination, laboratory tests, and, in patients, review of medical records.

Catheter protocol. All heart recipients underwent sampling from the femoral vein and great cardiac vein for measurement of CD4+CD25++ Treg and dendritic cells, cytokine levels, and high sensitivity C-reactive protein (hs-CRP, ultrasensitive N LatexCRP kit, Behring) before an endomyocardial biopsy specimen was taken. In patients scheduled for coronary angiography, additional blood samples were obtained from the femoral artery and the aortic root before injection of the contrast agent. The transcoronary gradients were expressed in percent changes from values obtained from either the aortic root or, if not obtainable, the femoral vein.

Catheterization analysis. All patients had standard biplane coronary angiography 0 to 36 mo (median, 0 mo) before inclusion of the study. Coronary angiograms were evaluated for the presence of segmental stenosis. Stenoses were visually classified as $<25\%$, 25 to 50\%, 51% to 75\%, or $>75\%$. The most severe lesion was used to classify each patient. Semiquantified scoring of catheterization recordings was performed by two independent blinded observers (28).

Flow cytometry analysis. Human CD4+CD25++ Treg were analyzed by four-channel flow cytometry (FACSCalibur, Becton Dickinson, BD). Erythrocyte lysis was performed using commercially available lysis solution (BD). First, a regional gate was defined to exclude debris and platelets defined by forward/side scatter. Among the remaining cells, the number of CD3+ events in the morphological lymphocyte gate identified with directly allophycocyanin (APC)-conjugated monoclonal antibody (CD3-APC, BD) was quantified to normalize each measured cell population to the total number of T cells. Among the T-cell population, CD4+CD25++ T cells were quantified using anti-CD4 (FITC conjugated, conjugated) and anti-CD25 [phycoerythrin (PE) conjugated, eBioscience] (2) (Fig. 1A). PE-conjugated anti-CD69 was from BD. Isotype identical antibodies served as controls. Antibodies against surface molecules were incubated 20 min at 4° C in the dark.

The CD4+CD25++ T cells were further assessed for their intracellular CD152 expression (CTLA-4; PE conjugated, Cymbus Biotechnology) after permeabilization with Cytofix/Cytoperm (BD Biosciences) and washing with PermWash. CD125 staining of CD4+CD25++ T cells was done with a four-color staining protocol setting the first gate on CD3+ lymphocytes (APC conjugated, BD). Among the T lymphocytes, CD4+CD25++ T cells were stained with anti-CD4 (peridinin chlorophyll-α conjugated, BD) and anti-CD25 (FITC conjugated, BD).

Subpopulations of dendritic cells from peripheral blood samples were identified with the Blood Dendritic Cell Enumeration Kit (Miltenyi Biotec) using a modified protocol. Accordingly, plasmacytoid dendritic cells were CD4+CD19+ BDCA-2+.

Real-time quantitative RT-PCR. For measurement of FOXP3 mRNA from different T-cell populations, cells were first isolated with the human CD4+CD25++ regulatory T Cell Isolation Kit (Miltenyi Biotec). Genes of interest were quantified using TaqMan assays.

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Stable CAD</th>
<th>HTR</th>
<th>P</th>
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<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>11</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>53±12</td>
<td>54±17</td>
<td>57±10</td>
<td>NS</td>
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<tr>
<td>Men</td>
<td>16 (89%)</td>
<td>10 (91%)</td>
<td>17 (77%)</td>
<td>NS</td>
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<tr>
<td>Reason for heart transplantation</td>
<td></td>
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<tr>
<td>Idiopathic cardiomyopathy</td>
<td>15 (68%)</td>
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<tr>
<td>Coronary artery disease</td>
<td>5 (23%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Other</td>
<td>2 (9%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Recipient negative for CMV</td>
<td>5 (23%)</td>
<td></td>
<td></td>
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<tr>
<td>Immunosuppressive therapy</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CSA + MMF + Pred</td>
<td>18 (81%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CSA + Aza + Pred</td>
<td>2 (9%)</td>
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<td></td>
<td></td>
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<tr>
<td>TK + MMF + Pred</td>
<td>2 (9%)</td>
<td></td>
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<td></td>
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<tr>
<td>Transplant vasculopathy</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>None</td>
<td>12 (55%)</td>
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<tr>
<td>Mild</td>
<td>8 (36%)</td>
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<tr>
<td>Severe</td>
<td>2 (9%)</td>
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<tr>
<td>Diabetes</td>
<td></td>
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<tr>
<td>Statin therapy</td>
<td>4 (22%)</td>
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<tr>
<td>Creatinine, mg/dl</td>
<td>0.98±0.16</td>
<td>0.99±0.18</td>
<td>1.59±0.5</td>
<td>NS</td>
</tr>
<tr>
<td>hs-CRP, mg/dl</td>
<td>0.16±0.2</td>
<td>0.16±0.14</td>
<td>0.39±0.50</td>
<td>HTR vs. Co: &lt;0.001</td>
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Values are means ± SE [age, creatinine, and high-sensitivity C-reactive protein (hs-CRP)]. CAD, coronary artery disease; HTR, heart transplant recipient; CMV, cytomegalovirus; CSA, cyclosporine A; MMF, mycophenolate mofetil; Pred, prednisone; Aza, 5-azathioprine; TK, tacrolimus; NS, nonsignificant difference; Co, control.

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regulatory T cells in cardiac allografts

Results

The baseline characteristics of the 51 subjects are summarized in Table 1. Demographic characteristics were similar in all three groups. Indication for heart transplantation was idiopathic dilated cardiomyopathy in 68% of patients, ischemic cardiomyopathy in 23%, surgical complication in one patient, and restrictive cardiomyopathy in one patient. At inclusion into the study, 45% of heart recipients were diagnosed as having transplant vasculopathy.

Levels of Treg in heart transplant patients. Heart transplant patients had significantly lower numbers of systemically circulating CD4+CD25+ T cells both in terms of absolute numbers (8.9 ± 1.3/μl vs. 15.8 ± 1.6/μl, P = 0.002) as well as given as relative proportion of lymphocytes (P < 0.001) when compared with controls (Fig. 1, A and B).

FOXP3 is crucial for the differentiation and function of mouse Treg (10, 17, 24), and around 90% of CD25+ T cells in human tissue were shown to express FOXP3 (7). To confirm between groups were analyzed by t-test (two-sided); not normally distributed continuous variables were compared by the Mann-Whitney U-test. Correlation coefficients were calculated with the Spearman rho test. Comparison of categorical variables in the baseline characteristics was generated by the Pearson χ2-test. Statistical significance was assumed if a null hypothesis could be rejected at P ≤ 0.05. Data are presented as means ± SE. All statistical analysis was performed with SPSS software version 11.5 (SPSS).

Continous variables were tested for normal distribution with the Kolmogorov-Smirnov test. Comparisons of

Biotec) according to the manufacturers’ instructions. Treg were assessed to be >93% pure by fluorescence-activated cell sorting analysis. Total RNA was extracted with RNeasy micro kit (Qiagen, Hilden, Germany). TagMan RT-PCR was performed as described elsewhere (34). mRNA levels were quantitatively analyzed by comparing experimental levels to standard curves generated using serial dilutions of the same positive sample.

FOXP3 quantitative analysis was performed using Assay on Demand (Applied Biosystems, Warrington, United Kingdom), as described previously (6). GAPDH quantitative analysis was performed using predeveloped TaqMan assay reagents target kits (Applied Biosystems). GAPDH was used for normalization. No primer nor any probe did react with DNA.

Cytokine levels. Circulating levels of IL-2, IL-10, IL-12, and TGF-β were quantified from citrated plasma or serum (IL-12) with the Man assay reagents target kits (Applied Biosystems). Indication for heart transplantation was idiopathic dilated cardiomyopathy in 68% of patients, ischemic cardiomyopathy in 23%, surgical complication in one patient, and restrictive cardiomyopathy in one patient. At inclusion into the study, 45% of heart recipients were diagnosed as having transplant vasculopathy.

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that circulating CD4+CD25+ T cells in heart transplant recipients and controls were predominantly T_{reg}. FOXP3 was quantified from freshly isolated CD4+CD25−, CD4+CD25+, and CD4+CD25++ T cells (Fig. 1C). Real-time quantitative PCR showed strong expression of FOXP3 only in CD4+CD25++ T_{reg} isolated from blood. Furthermore, CD4+CD25++ T cells were positive for intracellular CD152 (CTLA-4), which has negative effects on T-cell activation (representative blot, Fig. 1D).

Subsets of dendritic cells and activated T cells. Tolerogenic dendritic cells display a plasmacytoid phenotype and are involved in induction of T_{reg} (14, 43). We therefore investigated whether the reduced numbers of T_{reg} in heart transplant recipients might relate to lower numbers of circulating plasmacytoid dendritic cells. However, similar levels were found in heart transplant patients when compared with controls (Fig. 2, A and B), indicating that the reduction of T_{reg} is caused by a different mechanism.

Despite effective immunosuppression, and in the absence of overt acute rejection in patients after heart transplantation, activation status of circulating CD4+ T cells, measured by the surface marker CD69, was slightly, but nonsignificantly, higher compared with that of healthy controls (0.65 ± 0.18% of CD4+ T cells (n = 6) vs. 0.47 ± 0.12% of CD4+ T cells (n = 8), P = 0.3), whereas the total numbers of CD4+ T cells was slightly reduced in patients after heart transplantation (Fig. 2C).

No correlation was found between number of T_{reg} and the severity of transplant vasculopathy, left ventricular function, or duration after the transplantation.

Correlation of T_{reg} with hs-CRP and cytokines. As shown in Table 1, hs-CRP, as an unspecific marker of inflammation, was significantly elevated in heart transplant recipients (P < 0.05). In contrast, there were no significant differences between control subjects (n = 18) and heart transplant patients (n = 22) with respect to plasma levels of IL-2 (6.6 ± 2.8 vs. 7.3 ± 2.4...
pg/ml), IL-10 (0.87 ± 0.1 vs. 0.86 ± 0.13 pg/ml), TGF-β (117 ± 17 vs. 167 ± 17 pg/ml), or serum levels of IL-12 (0.29 ± 0.12 vs. 0.94 ± 0.38 pg/ml, P = 0.2). In transplanted patients, there was a significant positive correlation between total numbers of circulating Treg and IL-10 or TGF-β plasma levels (r = 0.47, P = 0.013 and r = 0.49, P = 0.01, respectively). The correlation between total numbers of Treg and IL-10 was also present in the total study group (r = 0.44, P = 0.006), whereas for otherwise no correlation was observed between cytokine levels and number of circulating Treg or dendritic cells.

Transcoronary gradients of T-cell populations. To determine the fate of CD4⁺CD25⁺⁺ T cells, we measured their transcoronary gradients in heart transplant recipients and patients with CAD serving as controls (see METHODS). Only in heart transplant patients did the number of CD4⁺CD25⁺⁺ T cells decrease after transcoronary passage (P < 0.05), indicating that they were recruited into the allograft (Fig. 3A). In support of this finding, we observed an infiltration of FOXP3⁺ T cells into the myocardium (Fig. 3B) accounting for <1% of T cells in the absence of myocardial rejection. We also showed that all FOXP3⁺ cells also expressed CD3⁺ (n = 7).

In contrast, we found higher numbers of activated CD4⁺ T cells after passage through the allograft, as measured by the early activation marker CD69 from blood samples taken from the coronary sinus as compared with the systemic sample in heart transplant patients (see Fig. 3C). Transcardiac passage did not influence total numbers of CD4⁺ T cells (47 ± 14% vs. 50 ± 13%, P = 0.8) in transplant patients.

There was no difference between patients without CAV or with mild to severe CAV in the relative proportion (−19.6% ± 5.1% vs. −23.1% ± 8.9%, P = 0.7) of Treg remaining in the allograft after transcardiac passage. However, in the subgroups of patients with mild to severe CAV, there was a trend toward higher reduction of Treg after transcardiac passage (r = 0.63, P = 0.097) in patients with mild CAV compared with patients with severe CAV.

DISCUSSION

A large body of evidence suggests that Treg, particularly CD4⁺CD25⁺⁺ Treg, are key mediators in maintaining transplantation tolerance in animal models (23, 44, 45). Consistent with this concept, transfer of Treg leads to persistent tolerance of mismatched grafts. In humans, chronic inflammatory processes, despite effective immunosuppression, limit long-term success after cardiac allograft transplantation. Reinforcing tolerance in human allograft recipients could thus lead to enhanced allograft acceptance, diminishing ongoing chronic subclinical rejection.

Primarily, Treg are found in the lymphoid tissue and may exert their suppressive function by inhibiting T-cell priming (1). Nonetheless, there is increasing evidence that Treg integrate into grafts, which induces potent immunosuppression (12, 36). Although animal data demonstrate the involvement of Treg in the suppression of allograft rejection, a definite demonstration of naturally occurring Treg in humans after allograft transplantation is lacking. To our knowledge, the data of the present study for the first time provide in vivo evidence for the presence of Treg in human cardiac allografts. The negative transcardiac gradients of Treg suggest a constant recruitment and migration of circulating Treg into the allograft. Indeed, we
could confirm the presence of T<sub>reg</sub> in histological sections of human cardiac allograft biopsies by staining for the specific transcription factor FOXP3 (10, 17, 24) in the absence of acute rejection episodes. Clearly, the nature of our clinical study does not permit us to dissect the function or fate of these cells. Owing to the small number of patients with chronic allograft vasculopathy, no information regarding the influence of T<sub>reg</sub> can be drawn from this study, which represents a limitation to our study.

Although some reports have shown that the suppressive activity by T<sub>reg</sub> cells can be due to the release of IL-10 and/or TGF-β (22), in general, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells act via cell-to-cell contact (19, 21). Thus the observed correlation between IL-10 and TGF-β serum levels and the levels of circulating T<sub>reg</sub> cells may reflect either their production by CD4<sup>+</sup>CD25<sup>+</sup> cells or the contemporaneous activation of other T<sub>reg</sub> cell types mainly acting via the release of soluble cytokines (11, 15, 38). IL-10 has a strong vasculoprotective role in atherosclerosis (16) and has been shown to slow CAV (8, 9). Although the role for TGF-β in CAV remains controversial, higher levels of TGF-β in tolerated grafts also implicate a role for graft tolerance (22).

The reduced numbers of circulating CD4<sup>+</sup>CD25<sup>+</sup> in allograft recipients imply lower numbers of immunosuppressive T<sub>reg</sub>. This could be due to either the immunosuppressive regimes with a subsequent reduction of CD25 as activation marker on the surface on CD4<sup>+</sup> T cells or ongoing recruitment into the graft leading to an exhaustion of a presumably finite supply. Indeed, cyclosporine A was experimentally shown to reduce the number of T<sub>reg</sub> (46). However, the negative gradient of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> across the coronary circulation coinciding with a significant transcoronary increase in CD4<sup>+</sup> T cells bearing the activation marker CD69 suggests that T<sub>reg</sub> are specifically recruited into the graft as a potential attempt to foster immune privilege, even in the presence of systemic immunosuppressive therapy. Constant presentation of alloantigen by endothelial cell may account for the continuous activation of CD4<sup>+</sup> T cells (5). Indeed, preliminary data in patients with renal transplants did not reveal any transcoronary cardiac gradients despite similar immunosuppressive regimens (data not shown).

The type of stimulating dendritic cells is a critical factor determining the outcome of an allosemune response (14, 29). Therefore, we investigated the possibility of lower numbers of circulating T<sub>reg</sub> being a consequence of reduced numbers of plasmacytoid dendritic cells (14, 20, 25, 43). However, the lack of a direct correlation of plasmacytoid dendritic cells with T<sub>reg</sub>, as well as similar numbers of plasmacytoid dendritic cells in controls and heart transplant recipients, does not support a direct functional interaction between these two cell types. Nevertheless, it should be kept in mind that the vast majority of interactions between these cells will take place in lymphoid or non-lymphoid tissue, which was not accessible in our study subjects.

In addition to the reduced number of T<sub>reg</sub> after transcardiac passage, our study also showed an increase of activated T cells. Recurrent rejections are regarded as a stimulus for the development of allograft vasculopathy (18). During acute rejections, the activation marker CD69 has been shown to be increased on T lymphocytes (26, 32), whereas otherwise, it remains within normal levels in allograft recipients. Thus, although systemic levels were comparable with controls, we identified the allograft as a source for the activation of T lymphocytes even in the absence of acute rejections.

In summary, for the first time, we have demonstrated the presence of regulatory T cells in human heart allografts. The constant recruitment and migration into the allograft and the ongoing activation within the allotransplant may eventually lead to the dysbalance between regulatory and activated T cells, as observed in the study. Prospective data analyzing the development of CAV will have to confirm a potential influence of circulating or resident regulatory T cells on the outcome of this disease. Tools to increase regulatory T cells might become an interesting therapeutic option for induction or maintaining allograft tolerance in the management for long-term allograft recipients.

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GRANTS

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