Neutralizing IL-6 Reduces Human Arterial Allograft Rejection by Allowing Emergence of CD161+ CD4+ Regulatory T Cells

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Neutralizing IL-6 Reduces Human Arterial Allograft Rejection by Allowing Emergence of CD161⁺ CD4⁺ Regulatory T Cells

Birgit Fogal,* Tai Yi,* Chen Wang,* Deepak A. Rao,* Amir Lebastchi, † Sanjay Kulkarni, † George Tellides, † and Jordan S. Pober* *

Perioperative injuries to an allograft exacerbate graft rejection, which in humans is primarily mediated by effector memory T cells. IL-6 transcripts in human coronary artery segments rapidly increase posttransplantation into immunodeficient mouse hosts compared with those of pretransplant specimens and fall dramatically by 30 d. Adoptive transfer of human PBMCs allogeneic to the artery 2 d postoperatively results in T cell infiltration and intimal expansion 4 wk later. Ab neutralization of human IL-6 reduces the magnitude of intimal expansion and total T cell infiltration but increases the relative expression of CD161⁺ T cells in the arterial wall while decreasing other Th17 markers. Coculture of MHC class II-expressing human endothelial cells (ECs) with allogeneic CD4⁺ memory T cells results in T cell activation and EC secretion of IL-6. Neutralizing IL-6 in primary allogeneic T cell–EC cocultures results in enhanced T cell proliferation of CD161⁺ CD4⁺ T cells, reduces total T cell proliferation upon restimulation in secondary cultures (an effect dependent on CD161⁺ T cells), increases expression of FOXP3 in CD161⁺ T cells, and generates T cells that suppress proliferation of freshly isolated T cells. These data suggest that IL-6 released from injured allograft vessels enhances allogeneic T cell infiltration and intimal expansion in a model of human allograft rejection by inhibiting an increase in CD161⁺ regulatory T cells.

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Abbreviations used in this article: EC, endothelial cell; i-Treg, inducible regulatory T cell; MIF, migration inhibitory factor; n-Treg, natural regulatory T cell; PHD3, prolyl hydroxylase 3; qRT-PCR, quantitative real time PCR; RORc, retinoic acid receptor-related orphan receptor c; SDF1, stromal cell-derived factor 1; SMC, smooth muscle cell; Treg, regulatory T cell.

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niioc acid receptor-related orphan receptor c (RORc) in humans, that is associated with Th17 development (20). However, even though all IL-17–producing cells express CD161, not all CD161+ T cells will differentiate into Th17 cells (18, 20). Furthermore, most IL-17A–producing T cells isolated from human arteries also produce IFN-γ (21), implying that not all IL-17–producing T cells are actually Th17 cells. A fourth group of CD4+ T cells has been described that can suppress cytokine production by effector Th cells of various subsets. Such regulatory T cells (Tregs) are themselves heterogeneous. “Natural” regulatory T cells (n-Tregs) emerge directly from the thymus expressing high levels of FOXP3 and Helios transcription factors and are characterized by high surface expression of CD25 and low expression of CD127 (20, 22–27). n-Tregs respond to self-antigens and control autoimmune (24, 28, 29). Other populations of Tregs develop or convert from CD4+ effector T cells and are specific for non-self Ags, including alloantigens (30–34). These inducible regulatory T cells (i-Tregs) are Helios negative (27) and may or may not express FOXP3 (35); i-Tregs are likely responsible for the control of allograft rejection in the process described as “infectious tolerance” (36, 37). An i-Treg population has recently been described that can control acute arterial rejection in a mouse aortic allograft model (38).

The signals that determine whether a CD4+ T cell will become an effector Th cell or an i-Treg, and if an effector cell, of what Th subtype, are largely determined by the milieu in which that T cell is activated by Ag, and cytokines are a key component of this effect (39). Th1 cells are favored by IL-12 and IFN-γ, whereas Th2 cells are favored by IL-4. The development of Th17 cells requires TGF-β plus IL-6 or IL-1, although it has been proposed that TGF-β acts indirectly, suppressing Th1 and Th2 cells and thereby allowing Th17 cells to emerge (40). Some types of i-Tregs require TGF-β and an absence of IL-6 (41) to develop, whereas others may require IL-10 (42). Many of these conclusions are based on studies of naive T cell commitment, but human memory T cells, especially those expressing CD161, are also plastic, and their phenotype may be modulated by cytokines such as IL-23 (18, 43). A complete understanding of which cytokines act on which types of human memory T cells is lacking but has clear importance for transplant rejection.

Clinical observations have suggested that preoperative or perioperative injuries to an allograft result in more severe early allograft rejection and increase the risk of late graft failure (44, 45). We propose that nonimmune injuries to graft endothelium or smooth muscle cells (SMCs) do so by releasing cytokines that modify the host immune response, affecting Th or i-Treg differentiation. In support of this hypothesis, our group has previously shown that injured ECs in vitro express IL-10, which can increase the release of both IFN-γ and IL-17 from alloreactive CD4+ memory T cells in EC–T cell cocultures (46). Blockade of IL-1 function by IL-1 receptor antagonist, known as anakinra, reduces the intensity of intimal arteritis-like lesions in our humanized mouse model, although neutralization of IL-17, unlike our findings with neutralization of IFN-γ, influenced SMC chemokine production without reducing lesion intensity (47). IL-6, like IL-1, has also been associated with inflammatory disorders (48). Furthermore, increased IL-6 levels correlate with the severity of acute vascular rejection in primates (49) and with adverse outcome after transplantation in humans (50–52), although protective effects have also been reported (53, 54). Vascular ECs and SMCs are known to produce IL-6 in culture (55, 56). These considerations have led us to evaluate the possible role of IL-6 as a regulator of the T cell response to the artery wall. In this study, we report the presence of increased levels of IL-6 transcripts in human arterial segments after transplantation into immunodeficient mice that decrease to pretransplant levels by 30 d as the graft “heals in.” Neutralizing human IL-6 reduces the intensity of allograft rejection in this model. Activated CD4+ T cells induce allogeneic ECs to secrete IL-6 in vitro, largely through a TNF-dependent mechanism. Blockade of EC-derived IL-6 in CD4+ T cell–EC cocultures results in increased proliferation of allogeneic CD161+CD4+ memory T cells upon primary stimulation but reduced magnitude of the allosresponse upon restimulation in secondary culture; both responses depend upon the presence of CD161+ T cells. More importantly, CD4+ memory T cells expanded in primary culture in presence of an IL-6 neutralizing Ab can suppress the allosresponse of freshly isolated allogeneic CD4+ memory T cells, suggesting that there is an induction of a Treg phenotype from CD161+CD4+ T cells when activation occurs in absence of IL-6. These data identify vessel-derived IL-6 as a factor that can direct human T cell responses toward a more destructive response against allograft vasculature.

Materials and Methods

Human cell isolation and culture

All human cells were obtained using protocols approved by the Institutional Review Board of Yale University. PBMCs were isolated by density gradient centrifugation of leukapheresis products from healthy adult volunteer donors by using Lymphocyte Separation Medium (MP Biomedicals, Solon, OH) according to the manufacturer’s instructions and stored in 10% DMSO–90% FBS in liquid nitrogen until further purification.

To isolate CD4+ T cells, PBMCs were incubated in RPMI 1640 (Life Technologies Invitrogen, Carlsbad, CA) supplemented with 10% FBS, l-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) on tissue culture plates for 30 min at 37°C to deplete adherent cells. CD4+ T cells were then isolated from nonadherent PBMCs by positive selection using Dynabeads (Invitrogen, Carlsbad, CA) magnetic bead separation and were released from the beads with DETACHaBEAD (Invitrogen) according to the manufacturer’s recommendation. Activated T cells and monocytes were removed by negative selection with the following magnetic bead-coated beads: CD4+ (49) and with adverse outcome after transplantation in humans (49, 50, 51). Although protective effects have also been reported (53, 54). Vascular ECs and SMCs are known to produce IL-6 in culture (55, 56). These considerations have led us to evaluate the possible role of IL-6 as a regulator of the T cell response to the artery wall. In this study, we report the presence of increased levels of IL-6 transcripts in human arterial segments after transplantation into immunodeficient mice that decrease to pretransplant levels by
and 0.8 mM EDTA. Remaining cells were stained with FITC-conjugated saline. Successful lymphocyte engraftment was assessed 14 d after PBMC were washed, and viable cells were counted via trypan blue exclusion and

Malvern, PA)—a human anti–IL-6 IgG1/mouse anti–CD4 IgG2a antibody (clone UCHT1; Beckman Coulter, Miami, FL) and PE-conjugated anti-mouse CD45 (clone 30-F11; BD Biosciences, San Jose, CA), and the percentage of circulating human CD3+ murine CD45+ cells was determined. Engraftment was considered successful when a distinct population of human CD3+ cells was demonstrated, which typically ranged from 1 to 10% of total mononuclear cell population in the circulation of mice 14 d after inoculation.

To neutralize IL-6, CNT0136 (Centocor Research & Development, Malvern, PA)—a human anti–IL-6 IgG1/kb—was administered s.c. as a bolus (250 μg) 1 d after transplantation (1 d prior to inoculation with PBMCs). Thereafter, animals received 125 μg CNT0136 s.c. three times per week. Control animals were treated in the same way with human IgG1

against human

IL-6 neutralizing mAbs (30 μg/ml; clone 6708; R&D Systems, Minneapolis, MN) or a control mouse IgG1 (30 μg/ml; clone 11711; R&D Systems) during primary stimulation only.

To test for suppression, CD4+ CD45RA+ CD25- DR+ T cells were added at a density of 1.5 × 10^5 cells/ml to confluent cultures of allogeneic CIITA-transduced HUVECs plated into gelatin-coated wells of 24-well tissue culture plates. Cultures were supplemented with CNT0136 (30 μg/ml) or human IgG (30 μg/ml; Sigma) for 3 d before cells were collected, washed three times with RPMI 1640, and rested for 3 d in presence of IL-2 (10 IU/ml). Rested T cells (“suppressor T cells”) were collected, washed three times with T cell medium, and added to cocultures of CFSE-labeled, freshly isolated CD4+ CD45RA+ CD25- HLA-DR- T cells (1.5 × 10^5 cells/ml; responder T cells) and allogeneic CIITA-transduced HUVECs from the same donor as used in primary stimulation plated onto gelatin-coated 96-well tissue culture plates. T cells were added at the following ratios (responder T cells/suppressor T cells): 1:0, 1:0.5, 1:1, 1:3. Proliferation of CFSE-labeled responder T cells was assessed after 7 d in culture.

FACS analysis

Cells were collected by vigorous pipetting, washed once in PBS, and stained for cell surface markers in PBS supplemented with 1% BSA using PE- or allopurinol-conjugated anti-human CD4 mAbs (clone RPA-T4; BD Biosciences), PE-conjugated anti-human CD31 (clone W559; BD Biosciences), FITC-conjugated anti-human CD25 (clone B1.49.9; Beckman Coulter), Pacific blue-conjugated anti-human CD127 (clone A019D5; BD Biosciences), and PE-conjugated anti-human CD31 (clone WM59; BD Biosciences) during reverse transcription. RNA was purified from the tissue section lysates using RNeasy Mini kits (Qiagen) according to the manufacturer’s protocol. Quantitative real-time PCR was performed using the Multiscribe RT system protocol (Applied Biosystems, Carlsbad, CA). RT-PCR reactions were prepared with TaqMan 2× PCR Master Mix and pre-developed assay reagents from Applied Biosystems. Samples were analyzed on an iCycler 96 Real Time system using a 96-well chip according to the manufacturer’s instructions. Cells were analyzed on an LSR II (BD Biosciences) using FlowJo software (Tree Star). FOXP3 staining was performed using eBioscience FoxP3 Staining Kit following the manufacturer’s instructions. Cells were stained with an anti-human IL-6 mAb (clone MQ2-39C3; eBioscience). BrdU labeling was performed using the BrdU Flow kit (BD Biosciences) according to the manufacturer’s instructions.

ELISA

Cytokine secretion into culture supernatants was analyzed by ELISA specific for human IFN-γ, IL-2, IL-17A, IL-10, IL-5, IL-6, and TGF-β (all from eBioscience), according to the manufacturer’s instructions.

Statistical analysis

All experiments were repeated at least three times, and statistical analysis was performed using Prism software (GraphPad Software, San Diego, CA).

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as described in each figure legend. When appropriate, percentage data were first transformed (arc sin square root) before analysis because the data were non-normally distributed (59). In all experiments, significance was assessed at p < 0.05.

Results

Increased IL-6 levels after arterial injury in vivo

To test the hypothesis that injured or stressed vascular cells produce IL-6 and other mediators that can modulate alloreactive T cell responses to graft blood vessels, we used quantitative RT-PCR to identify transcripts that are altered in perioperative vascular tissue versus healed vascular tissues. Adjacent short segments of human coronary artery were implanted into paired immunodeficient SCID/beige mice as infrarenal aortic interposition grafts. One segment was harvested after 2 d (representing a freshly injured graft), the other after 30 d (representing a healed-in graft), and mRNA expression of a number of candidate genes associated with ischemia, inflammation, or healing was assessed (Table I). IL-6 levels were dramatically higher at 2 d than at 30 d after transplantation when normalized to GAPDH (Table I). A more complete time course of IL-6 expression revealed that large increases in IL-6 transcript levels compared with those of untransplanted vessels were detectable as early as 6 h posttransplantation and remained elevated for at least 3 d before returning to baseline levels after 30 d (Fig. 1). These data support the conclusion that IL-6 levels are increased in response to perioperative injuries and return to baseline as the artery “heals in.” In contrast, expression levels of TGF-β, migration inhibitory factor (MIF), stromal cell-derived factor 1 (SDF1; also designated as CXCL12), prolyl hydroxylase 3 (PHD3), and IFN-induced protein of 10 kDa (IP-10; also designated as CXCL10) were significantly lower at 2 d than at 30 d after transplantation when normalized to GAPDH (Table I). There was no significant change in expression of IL-1α, vascular endothelial growth factor A (VEGF-A), IL-8 (also designated as CXCL8), and inducible chemokine of T cell activation (I-TAC; also designated as CXCL11) between 2 and 30 d after transplantation (Table I).

IL-6 neutralization reduces T cell-mediated injury in human artery allografts

To determine the role of IL-6 in modulating human T cell responses to allogeneic human artery grafts in vivo, we used our established human–mouse chimeric model of intimal arteritis-like lesions in artery allografts to allogeneic human artery grafts in vivo, we used our established human–mouse chimeric model of intimal arteritis-like lesions in human artery allografts. To determine whether IL-6 modulates human T cell responses to allogeneic artery grafts in vivo, allogeneic PBMCs were adoptively transferred into mice 2 d after artery transplantation, at which time point IL-6 mRNA levels were elevated. Mice received a bolus dose of control IgG or a human anti–IL-6 IgG1 mAb (CNT0136) 1 d prior to inoculation with PBMCs and were treated thereafter with the same anti-human IL-6 mAb or human IgG three times/week for 28 d. Treatment with anti-human IL-6 did not alter the number of circulating human T cells after PBMC transfer at 2 wk after inoculation (IgG 3.92 ± 1.13% versus anti–IL-6 5.56 ± 2.12%; n = 5 pairs of animals, p = 0.3636); however, IL-6 blockade significantly reduced both the total vessel area and the intimal area and increased the lumen area (Fig. 2A). Additionally, neutralization of human IL-6 reduced the total number of intimal T cells in rejecting artery grafts (Fig. 2B).

We next examined whether neutralizing human IL-6 altered the characteristics of the immune response in addition to effects on its magnitude. To do so, we measured expression of T cell effector cytokine transcripts within rejecting artery grafts normalized for expression of a T cell marker CD3ε to correct for extent of infiltration. Blockade of human IL-6 did not significantly alter the expression of human IFN-γ or IL-17A although a trend toward reduced IL-17A expression was apparent in the anti-human IL-6–treated group (Table II). Surprisingly, neutralization of human IL-6 resulted in reduced expression of human IL-10 when normalized to CD3ε (Table II). Furthermore, levels of the transcription factors Tbet, RORc, and FOXP3—typically associated with Th1, Th17, or certain Treg populations, respectively—were not significantly altered in the arterial grafts by blockade of human IL-6 (Table II). In humans, IL-17–producing cells are also characterized by expression of CD161, CCR6, and CCR4 (18, 61). CD161 expression relative to CD3ε was increased as a consequence of IL-6 neutralization (Table II), CCR6 was significantly decreased, and CCR4 was not altered in anti–IL-6 recipients. These data imply that graft-derived IL-6 promotes allogeneic human T cell infiltration and alters the nature of the T cell response by modulating the phenotype of the infiltrating T cells. Specifically, CD161 expression is increased in the graft, suggesting either that infiltrating T cells have a higher expression of CD161 or T cells expressing CD161 are relatively increased among the infiltrating T cells. However, the characteristics of these CD161+ T cells may not cor-

Table I. Changes in gene expression after arterial injury in vivo

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Day 2</th>
<th>Day 30</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>0.061</td>
<td>0.007</td>
<td>0.0101</td>
</tr>
<tr>
<td>TGF-β</td>
<td>0.173</td>
<td>0.533</td>
<td>0.0013</td>
</tr>
<tr>
<td>MIF</td>
<td>1.836</td>
<td>2.643</td>
<td>0.0161</td>
</tr>
<tr>
<td>IL-1α</td>
<td>4.4 × 10^{-5}</td>
<td>4.6 × 10^{-5}</td>
<td>0.5815</td>
</tr>
<tr>
<td>Chemokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL8</td>
<td>0.089</td>
<td>0.050</td>
<td>0.0974</td>
</tr>
<tr>
<td>CXCL10</td>
<td>7.3 × 10^{-5}</td>
<td>6.5 × 10^{-4}</td>
<td>0.0118</td>
</tr>
<tr>
<td>CXCL11</td>
<td>0.029</td>
<td>0.042</td>
<td>0.0927</td>
</tr>
<tr>
<td>SDF1</td>
<td>0.176</td>
<td>0.384</td>
<td>0.0017</td>
</tr>
<tr>
<td>Hypoxia-induced proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHD3</td>
<td>0.002</td>
<td>0.0104</td>
<td>0.0001</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.031</td>
<td>0.0473</td>
<td>0.1822</td>
</tr>
</tbody>
</table>

Real-time RT-PCR analysis of inflammation, hypoxia, and repair genes in human artery grafts 2 or 30 d posttransplantation. Gene expression was normalized to GAPDH, and ratio paired t test analysis was performed on four to six pairs of animals to assess statistical significance (p < 0.05).
respond to conventional Th17 cells. To address the possibility that the protective effect of IL-6 blockade is mediated by an altered and/or expanded CD161+ T cell population, we attempted to determine the effect of depleting CD161+ T cells from the adoptively transferred population on the degree of protection conferred by anti–IL-6 administration. Although we effectively reduced the number of CD3+ CD161+ T cells from 25 to 1.5% in the PBMC inoculum by means of negative selection with magnetic beads, the percentage of circulating human T cells that express CD161 rapidly rebounded after inoculation. At 2 wk, the percentage of circulating CD3+ CD161+ T cells in mice that received CD3+ CD161+ T cell-depleted PBMCs had increased to 7.4% compared with 12.5% in animals that received nondepleted PBMCs, and at 4 wk, mice that received CD3+ CD161+ T cell-depleted inoculum by means of negative selection with magnetic beads, the percentage of circulating human T cells that express CD161 rapidly rebounded after inoculation. At 2 wk, the percentage of circulating CD3+ CD161+ T cells in mice that received CD3+ CD161+ T cell-depleted PBMCs had increased to 7.4% compared with 12.5% in animals that received nondepleted PBMCs, and at 4 wk, mice that received CD3+ CD161+ T cell-depleted inoculum were indistinguishable from mice that received nondepleted PBMCs in the percentage of circulating CD3+ CD161+ T cells (18.4% depleted versus 16.9% nondepleted). This rapid expansion of CD161+ human T cells after adoptive transfer into recipient mice precluded our efforts to test the effects of anti–IL-6 in the absence of these cells.

IL-6 release from HUVECs is increased by coculture with allogeneic CD4+ T cells in a TNF-dependent manner

To investigate further how IL-6 neutralization could alter CD4+ T memory cell responses to vascular cells, we examined the role of IL-6 in cocultures of purified CD4+ T cell with allogeneic MHC class II+ HUVECs. Consistent with previous reports, cultured human ECs constitutively expressed low levels of IL-6 (Ref. 55 and Fig. 3A). Expression of IL-6 was significantly increased by addition of allogeneic CD4+ T cells after 24 h in culture (Fig. 3A), but the increase in IL-6 induced by the CD4+ T cells was dependent upon reinduction of MHC class II molecule expression on the ECs (Fig. 3B). Because CD4+ T cell activation in response to ECs depends upon MHC class II expression, the requirement for MHC class II expression implies that the T cells must be activated to generate IL-6, and T cell activation in response to ECs depends upon MHC class II expression (62). To determine which cell type was responsible for the increase in IL-6 production, brefeldin A was added to cocultures after 20 h to prevent cytokine release. Four hours later, CD4+ T cells were collected by vigorous pipetting, HUVECs were collected via trypsinization, and each cell
suspension was further depleted of the other cell type via magnetic bead depletion using anti-human CD31 or anti-human CD4 mAbs, respectively. IL-6 levels were measured in the cell lysates via ELISA. Both unstimulated and cocultured CD4+ T cells expressed similar low levels of IL-6. The majority of IL-6 protein was associated with ECs, and EC production was increased by coculture with allogeneic CD4+ T cells (Fig. 3C). We suspected that activated T cells induced ECs to produce IL-6 by secretion of a cytokine known to have this effect, namely TNF (63). We confirmed this hypothesis by showing that induced expression of IL-6 in ECs was decreased by addition of a TNF neutralizing Ab, although constitutive expression of IL-6 was not affected (Fig. 3D). Furthermore, CD4+ T cells expressed TNF after coculture with MHC class II+ HUVECs, but not in coculture with class II– HUVECs [Fig. 3E and as previously reported (63)], and addition of TNF to HUVECs resulted in a concentration-dependent increase of IL-6 release (Fig. 3F). Collectively, these observations suggest that CD4+ T cells, activated by recognition of allogeneic MHC class II molecules on the ECs, release TNF, which in turn causes ECs to synthesize and secrete IL-6.

IL-6 neutralization regulates the proliferation of activated memory CD4+ CD161+ T cells

To examine the effects of EC-secreted IL-6 on the human allogeneic T cell response, cocultures of CD4+ T cells and allogeneic

| Table II. Neutralization of IL-6 alters infiltrating T cells |
|----------------|----------------|----------------|
|               | IgG            | Anti–IL-6      | p Value       |
| Cytokines     |                |                |               |
| IFN-γ         | 1.0100         | 1.1140         | 0.1981        |
| IL-17         | 0.0019         | 0.0004         | 0.1452        |
| IL-10         | 0.0488         | 0.0138         | 0.0110        |
| Transcription factors |          |                |               |
| Tbet          | 0.5050         | 0.3995         | 0.2432        |
| RORγ           | 0.0316         | 0.0205         | 0.1805        |
| FOXP3          | 0.0114         | 0.0204         | 0.0938        |
| Phenotype markers |            |                |               |
| CD161          | 0.7403         | 1.0200         | 0.0435        |
| CCR6           | 0.1275         | 0.0210         | 0.0040        |
| CCR4           | 0.3457         | 0.2432         | 0.1473        |

Real-time qRT-PCR analysis of candidate genes in artery grafts undergoing T cell-mediated rejection treated with control IgG or anti-IL-6 mAb. Gene expression was normalized to CD3ε, and ratio paired t test analysis was performed on six to eight pairs of animals (from three to four independent experiments) to assess statistical significance (p < 0.05).
MHC class II-expressing HUVECs were treated with a mouse anti-human IL-6 mAb (30 μg/ml) or control IgG (30 μg/ml). Blockade of IL-6 in vitro resulted in decreased IFN-γ production from allogeneic CD4+ T cells after 24 h in culture compared with controls, whereas IL-2 levels were unaffected (Fig. 4A). IL-17, IL-10, and IL-5 were not detected in either group (data not shown). In contrast, proliferation of allogeneic CD4+ T cells increased significantly after 7 d in culture. We had previously reported that memory but not naive CD4+ T cells can be activated by allogeneic MHC class II-expressing ECs (6). Therefore, the same experiment was repeated with cocultures of HUVECs with purified memory CD4+ T cells, defined by absence of CD45RA, again resulting in increased proliferation in presence of an IL-6 neutralizing Ab (Fig. 4B).

Given the selective increase in CD161 expression in rejecting artery grafts after IL-6 neutralization, we hypothesized that IL-6 may differentially affect the proliferation of CD161+ and CD161− CD4+ T cells and tested whether the CD161+ T cell population was responsible for the increase in proliferation when IL-6 is neutralized in our coculture system. Indeed, an increased percentage of the proliferating cells express CD161. We next separated memory CD4+ T cells into CD161+ and CD161− T cell populations and cocultured each separately with MHC class II-expressing HUVECs in presence of anti-human IL-6 or control IgG as described above. Strikingly, an increase in proliferation, assessed by CFSE dilution was observed in the CD161+ T cell population but not in the CD161− T cell population (Fig. 4C), suggesting that IL-6 exerts its effects specifically on memory CD4+ CD161+ T cells. Similarly sized effects of anti–IL-6 induced increases in proliferation detected by CFSE dilution are observed when assessing proliferation by incorporation of BrdU (data not shown). Thus, our in vitro assays suggest that the relative increase in CD161 mRNA we had observed within the arterial wall was due to more T cells expressing this protein. Unfortunately, we were not able to detect human CD161 in tissue sections by immunofluorescence microscopy to confirm directly this conclusion.

Despite the similar enrichment of CD161 expression in our in vivo and in vitro systems, there is still a seeming inconsistency in that we observed a reduction of T cell numbers within the artery wall after IL-6 neutralization in vivo and a net increase of total T cells as a result of IL-6 blockade in vitro. One possible explanation is that our in vivo experiment is of 28-d duration following introduction of T cells, whereas our in vitro cultures are of only 7-d duration, and it might require time for an i-Treg population to be generated before it could act to suppress T cell responses. To address this possibility, memory CD4+ T cells were cocultured with allogeneic MHC class II-expressing HUVECs in presence of an anti–IL-6 neutralizing Ab or control IgG for 3 d, rested for 3 d, and then restimulated by fresh ECs from the same donor as in the primary stimulation in absence of Abs. CD4+ T cells that were cocultured with ECs in presence of anti–IL-6 during the primary stimulation showed a decrease in the overall allogeneic response in secondary cultures. Specifically, production of IFN-γ, IL-17, IL-5, and IL-10 by allogeneic CD4+ T cells were all decreased after 24 h of restimulation (Fig. 5A), and proliferation of allogeneic memory CD4+ T cells was decreased after 3 d of restimulation (Fig. 5B). To determine whether this effect was dependent on CD161+ T cells as observed in the primary stimulation, the effect of IL-6 blockade on proliferation of memory CD4+ T cells upon restimulation was compared between cocultures with complete CD4+ memory T cells or with memory populations depleted of CD161+ T cells. We found that IL-6 neutralization during the primary stimulation resulted in reduction of proliferation upon restimulation only when CD161+ T cells were present (Fig. 5C).

**IL-6 neutralization induces Tregs**

The secondary culture experiments suggested that allogeneic MHC class II-expressing ECs can induce a population of CD161+ Tregs

**FIGURE 4.** IL-6 neutralization increases proliferation of CD4+ CD161+ memory T cells during the anti-EC allogeneic response in vitro. A. Effects of supplementing MHC class II+ HUVEC–CD4+ T cell cocultures with an anti-human IL-6 neutralizing Ab (30 μg/ml) versus control IgG on proliferation after 7 d in culture as assessed via BrdU incorporation (n = 3 independent experiments) and on release of IFN-γ or IL-2 after 24 h as assessed by ELISA (n = 3 independent experiments). Paired t test analysis was performed to assess statistical significance: p < 0.05. B. Effect of supplementing MHC class II+ HUVEC–CD4+ T cell memory cocultures with an anti-human IL-6 neutralizing Ab (30 μg/ml) versus control IgG on proliferation after 7 d in culture as assessed via BrdU incorporation. Paired t test analysis was performed to assess statistical significance (n = 3 independent experiments): p < 0.05. C. Effects of supplementing MHC class II+ HUVEC–CD4+ CD161+ or CD161− memory T cells with an anti-human IL-6 neutralizing Ab (30 μg/ml) versus control IgG on proliferation after 7 d in culture as assessed via CFSE dilution. Statistical significance was assessed via two-way ANOVA followed by Bonferroni post hoc test: p < 0.05.
when IL-6 is neutralized. To test this possibility, we performed suppressor assays, where memory CD4+ T cells were cocultured with allogeneic MHC class II-expressing ECs in presence of anti-human IL-6 mAb (30 μg/ml) or control IgG (30 μg/ml) for 3 d, rested, and then added to primary cocultures of CD4+ memory T cells and ECs from the same donor pairs used to generate suppressor T cells, but in absence of Abs. The addition of memory CD4+ T cells previously stimulated in presence of IL-6 blockade suppressed the proliferation of freshly isolated memory CD4+ T cells, whereas addition of memory CD4+ T cells previously stimulated in presence of an irrelevant isotype control had no effect (Fig. 6A, 6B). The suppressive effect increased with the addition of increasing numbers of restimulated cells (Fig. 6A, 6B), consistent with the generation of a Treg population after primary stimulation in absence of IL-6. To characterize further the nature of the CD161+ T cells that expanded in vitro, we examined gene expression in CD161– and CD161+ CD4+ T cells after 7 d of coculture with MHC class II+ HUVECs in presence of anti–IL-6 or an irrelevant control Ab. Consistent with the induction of a Treg population, FOXP3 mRNA expression was increased in anti–IL-6 treated cocultures containing CD161+ CD4+ T cells, but not in cocultures containing CD161+ CD4+ T cells compared with cul-

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**FIGURE 5.** IL-6 neutralization decreases T cell proliferation and cytokine production upon restimulation by allogeneic HUVEC. CIITA transduced HUVECs were cocultured with CD4+ memory T cells for 3 d in presence of either control IgG or an anti–IL-6 neutralizing mAb (30 μg/ml). CD4+ memory T cells were then rested for 3 d and restimulated with allogeneic CIITA-transduced HUVECs from the same donor as in the primary culture. A, ELISA analyses of media collected from cocultures 24 h after restimulation (n = 12 from three independent experiments, *p < 0.05). B, CD4+ memory T cells were labeled with CFSE after rest, and proliferation was assessed 4 d after restimulation with allogeneic ECs via CFSE dilution. Shown are representative FACs blots from one experiment (left) and quantitative data pooled from three independent experiments (right). Paired t test analysis was performed to assess statistical significance: *p < 0.05. C, Cells were treated as in B, but proliferation was compared between complete population of CD4+ memory T cells and CD161+ T cell-depleted CD4+ memory T cells. Statistical significance was assessed by two-way ANOVA followed by Bonferroni post hoc test: *p < 0.05.
tures treated with an irrelevant isotype control (Fig. 6C). In contrast, the expression of mRNA encoding transcription factors Tbet or RORc did not change after anti–IL-6 treatment (Fig. 6C). No changes in expression of IFN-γ, IL-17A, IL-10, or TGF-β mRNAs were observed after anti–IL-6 treatment in either CD161+ or CD161– CD4+ T cell–HUVEC cocultures (data not shown). To characterize further the CD161+ FOXP3+ T cell population expanding in coculture with MHC class II+ HUVECs when IL-6 is neutralized, we determined expression of CD25, CD127, FOXP3, and Helios on the CD161+ subset before and after coculture in presence of anti–IL-6 or an irrelevant control Ab. In agreement with the quantitative real time PCR (qRT-PCR) results, neutralizing IL-6 resulted in an increased percentage of FOXP3-expressing CD161+ T cells compared with that in control Ab-treated cultures. (Fig. 7A). Consistent with an induction of a Treg population, the CD161+ FOXP3+ expressing T cells also express CD25 but not CD127 (Fig. 7A, 7B), but we did not observe an increase in the percentage of Helios-expressing CD161+ T cells (Fig. 7B).

**Discussion**

Using in vivo and in vitro approaches, we show a role for vascular graft cell-derived IL-6 in modulating the human anti-graft adaptive immune response, resulting in increased T cell-mediated injury to allogeneic vasculature. Specifically, we demonstrate that recently transplanted human artery segments interposed into the infrarenal aortae of immunodeficient C.B-17 SCID/beige mice express increased levels of IL-6 compared with those of healed-in artery segments harvested 4 wk later. Adoptive transfer of allogeneic human T cells to animals bearing freshly transplanted human arterial grafts produces lesions that resemble acute cell-mediated vascular rejection (“intimal arteritis”). Neutralization of human IL-6 reduces the number of intimal T cells, neointima formation, and vascular remodeling, suggesting a protective effect of IL-6 blockade on acute rejection. Despite the reduction in the total numbers of infiltrating T cells, the proportion of infiltrating T cells expressing CD161 appears to increase as inferred from transcript levels, although we cannot rule out increased mRNA by an equiv-
We have not established the cellular source or sources of human IL-6 in our in vivo model. Specifically, we cannot exclude the possibility that T cell-derived IL-6 contributes to the nature of the rejection response, although our in vitro experiments suggest that vessel-derived IL-6 may be less important for healthy than injured organs. To test this idea, we attempted to examine if neutralizing IL-6 still had a protective effect when PBMC adoptive transfer was delayed until 30 d after transplantation. Notably, the lesions that developed in such arteries were much smaller than expected, consistent with our hypothesis but precluding an evaluation of the effects of anti–IL-6. Studies are in progress to test this idea further in a rigorous manner.

We observed that IL-6 blockade significantly diminished T cell-mediated graft injury, as assessed by T cell intimal infiltration and intimal expansion. It is possible that IL-6 affects T cell recruitment, survival, or retention in addition to effects on activation and proliferation studied in this work. Previously, we had established that IFN-γ release by T cells is the major cause of arterial injury and pathological remodeling in this model (15). Therefore, it may seem initially surprising that IL-6 blockade reduces neointimal expansion without reducing IFN-γ expression by those T cells that do infiltrate the vessel wall. However, the significant reduction in the total number of infiltrating T cells into the neointima would undoubtedly result in a net decrease of the total amount of IFN-γ released within the intima despite absence of changes in IFN-γ production by individual T cells.

Our study points to CD161-expressing memory T cells as the primary target of IL-6. CD161, a lectin receptor originally described as an NK cell receptor, is also expressed on 10–25% of primary target of IL-6. CD161, a lectin receptor originally described as an NK cell receptor, is also expressed on 10–25% of infiltrating adult memory T cells (19). The exact role of this CD161+ subset of memory T cells is not clear. Recently, CD161 has been described as a marker of all IL-7–producing T cells (20); that is, all IL-7–producing T cells are exclusively contained in a population of effector memory T cells that express CD161 (18, 20). However, it is important to note that not all CD161+ T cells will differentiate into Th17 cells (18). Significantly, a potential immune-suppressive activity of CD4+ CD161+ T cells has been reported in cancer patients (79). We do not know if IL-17A–producing and –suppressive CD4+ CD161+ T cells are distinct or the same population of cells whose functions are shifted by environmental cues. Notably, Treg differentiation and development of inflammatory Th17 cells are tightly linked (80–83), and IL-6 appears to play a role in the plasticity of human memory Tregs, inducing secretion of IL-17A (84). It is possible that a similar plasticity exists for CD161+ CD4+ memory cells. The cytokine
milieu has been suggested to be important in regulating the Th1/Th17 plasticity (85), and similar effects may pertain to Th17/i-Treg conversions. Our in vivo data showed that CCR6 levels in the infiltrating T cells were decreased upon IL-6 neutralization, and a downward trend could be seen in several other Th17-associated genes investigated (IL-17A, RORc, CCR4) along an upward trend of FOXP3 expression. IL-17A levels were just barely above the detection level, resulting in high variability of the measurements, the likely explanation for failure to reach statistical significance. This problem was compounded by the fact that the total number of infiltrating T cells was reduced in anti–IL-6–treated animals. Because only 10–25% of memory T cells express CD161 (18, 19) and because it is possible that only a subset of these cells is affected by IL-6, changes in genes expressed by the IL-6–responsive population may be too small to detect within the overall T cell infiltrate, despite a measurable increase in CD161. Unfortunately, attempts to enrich for these cells by adoptively transferring purified CD4+ CD161+ T cells into our animals have not been successful. Similarly, CD3+ CD161+ cells depleted from the PBMCs prior to inoculation rapidly increase in the circulation within 2 wk after adoptive transfer into SCID/bg mice, so that mice receiving CD3+ CD161+ T cell-depleted PBMCs are eventually indistinguishable from mice receiving nondepleted inocula, precluding us from testing the idea that the protective effect of IL-6 blockade in vivo would be lost in the absence of CD161+ T cells. These data might also suggest that CD161 expression is induced in some T cells upon activation and may not truly be a marker of cell lineage. Nevertheless, our in vitro data suggest a specific effect of IL-6 to inhibit expansion of CD161+ CD4+ memory T cells, although we cannot exclude IL-6–mediated modulation of CD161 expression itself or of CD161+ CD8+ T cells present in vivo, especially in light of a recent report that CD161 is expressed by CD8+ T cells that can differentiate into IL-17–producing cells (20).

We demonstrate that CD4+ T cells can be induced by cultured allogeneic ECs to become suppressive when IL-6 signaling is blocked. In pilot experiments, depletion of CD161+ T cells during suppressor cell generation prevented the development of suppressor cells (B. Fogal, C. Wang, and J.S. Pober, unpublished observations), suggesting that the Treg population generated is CD161+. This finding is consistent with our observations that FOXP3 mRNA is selectively increased in the CD161+ subpopulation under conditions of IL-6 blockade. Although the suppressive effects we have observed are reproducible and statistically significant, their magnitude is small, perhaps because the alloresponse to ECs is itself small, involving fewer than 1% of the total memory cells. Furthermore, while we refer to the mixed populations in our suppressor assays as containing a 1:1 to 1:3 ratio of responder/suppressor populations, we do not actually know the frequency of Tregs in the “suppressor” population. Thus, when the suppressor and responder populations are mixed, Tregs may not be in close proximity to responding cells, and we do not know whether the suppressive effects of these Tregs requires cell contact, relies on soluble mediators, or is the result of depletion of activating cytokines. IL-6 neutralization results in decreased IL-10 levels in vitro and in vivo, so it is unlikely that IL-10 is responsible. Additionally, TGF-β transcript levels were unchanged in vivo after IL-6 blockade (B. Fogal and J.S. Pober, unpublished observations), although in this case T cell–associated changes may be masked by vessel cell–derived TGF-β. It is also important to note that the suppressive effect of i-Tregs we demonstrate involves memory T cells as responders, a population thought to resist regulation in mice (86). However, it is possible that species-specific differences exist, and ex vivo-expanded n-Tregs have recently been shown to reduce arterial injury in a humanized mouse model similar to our own (87). If the protective effect we have observed in vivo does depend upon i-Tregs, then anti–IL-6 therapies may be a way to promote such responses in the perioperative transplant period.

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Disclosures
The authors have no financial conflicts of interest.

References
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IL-6 INHIBITS CD161+CD4+ Treg DEVELOPMENT


