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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 infiltrates 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⁺ 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. The Journal of Immunology, 2011, 187: 6268–6280.
inoic 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 autoimmunity (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 key components 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-1α, 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 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 alloresponse upon re-stimulation 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 alloresponse 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, 1-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 collected by negative selection with human CLA-DR-mAb at a concentration of 5 μg/ml (LB3.1; gift of J. Strominger, Harvard University, Cambridge, MA) for 20 min, followed by two washes and depletion with magnetic beads conjugated to goat anti-mouse IgG Ab (Invitrogen). The selected population obtained by this procedure was >96% CD4+ CD25– HLA-DR− by flow cytometry. Memory subsets of CD4+ T cells were isolated by further negative selection using anti human CD45RA mAb (clone HI-100; BioSource, San Diego, CA) at a concentration of 3 μg/ml, followed by incubation with pan-mouse IgG beads. The selected subset population obtained by this procedure was routinely >98% positive for CD45RO expression by flow cytometry. In some experiments, cells were further depleted with anti-human CD161 mAb (clone HP-3G10; BioLegend, San Diego, CA) and pan-mouse IgG beads or separated into CD161+ or CD161− memory T cells expanded in primary culture in presence of an IL-6 neutralizing Ab according to the manufacturer’s recommendations. Negative selected cells were routinely >98% CD161+; cells separated into two populations were routinely >90% pure for the selected population. Alternatively, CD4+ CD45RO− cells were isolated as described above, immunostained for CD161, and sorted by flow cytometry into CD161+ and CD161− cell populations.

HUVECs were released from cannulated and perfusion-cleared umbilical veins by collagenase digestion and serially cultured on 0.1% gelatin-coated flasks in M199 (Life Technologies Invitrogen) supplemented with 20% FBS, 1-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) 0.1% endothelial cell growth supplement (Collaborative Biomedical Products), and porcine hepatic (100 μg/ml; Sigma Aldrich, St. Louis, MO). Serially passaged cells were used at subculture 2–5, at which point they are routinely CD45– and CD31+. In some experiments, MHC class II expression, which is lost when cells are removed from tonic IFN-γ signaling (13) as occurs during culture under standard conditions, was restored by retroviral transduction with CIITA, as previously described (57).

Human arterial transplantation and allograft rejection in vivo

All human tissues were obtained using protocols approved by the Institutional Review Boards of Yale University and the New England Organ Bank (Newton, MA). All experimental animal protocols were approved by...
the Yale University Institutional Animal Care and Use Committee. Arterial transplantation was performed as previously described in detail (15). Briefly, segments of human epicardial coronary arteries, ~0.5 mm diameter, from explanted hearts of cadaveric organ donors not used for transplantation or explanted hearts of cardiac transplant recipients were interposed into the infrarenal aortae of female C.B-17 SCID/beige mice (8–12 wk of age; Taconic, Germantown, NY) by end-to-end anastomosis. Adjacent human artery segments (2–3 mm) were transplanted into groups of two to five mice for each experiment, and data from individual experiments were pooled to generate sufficient numbers for analysis.

Human PBMCs allogeneic to the artery graft were adoptively transferred into mice i.p. 2 d after artery transplantation. Frozen PBMCs (see above) were thawed, washed with RPMI 1640 supplemented with 10% FBS, t-glutamine, and Pen/Strep, and then treated with 10 U/ml recombinant DNase I (Roche, Mannheim, Germany) for 30 min. Cells were washed, and viable cells were counted via trypan blue exclusion and then administered to mice i.p. at 1 × 10^6 to 2 × 10^6 cells in 1 ml sterile saline. Successful lymphocyte engraftment was assessed 14 d after PBMC administration by flow cytometric analysis of heparinized blood collected by retro-orbital puncture. RBGs were lysed with red cell lysis buffer containing 300 mM ammonium chloride, 20 mM potassium bicarbonate, and 0.8 mM EDTA. Remaining cells were stained with FITC-conjugated anti-human CD3 (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^+ and 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 circulating blood of mice 14 d after inoculation.

To neutralize IL-6, CNT0136 (CenTecor Research & Development, Malvern, PA) — a human anti-IL-6 IgG/k mAb — 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 (Sigma, St. Louis, MO).

Transplanted arterial grafts were harvested after animals were anesthetized, and arterial grafts were perfused with normal saline and excised before death. Arterial grafts were frozen in optimum cutting temperature compound (Sakura Finetek, Torrance, CA), and serial 5-μm transverse sections were cut for morphometric, immunohistochemical, and RNA analysis. One animal was excluded from analysis due to failure to demonstrate successful CD3^+ lymphocyte engraftment.

**Histology and immunohistochemistry**

Serial cross sections of the graft (5 μm) were immunostained with an mAb against human α-smooth muscle actin (clone 1A4; Dako, Carpeniteria, CA) using the avidin–biotin–peroxidase staining method (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin to define the different vessel layers. Total vessel area (determined as the area bound by the internal elastic lamina and the lumen), and luminal vessel area were quantified in three to five cross sections (240 μm apart)/animal using ImageJ software (National Institutes of Health, Bethesda, MD), and the mean area for each vessel compartment was calculated. Additionally, serial cross sections were immunostained with an mAb against human CD45RO (clone UCHL1; eBioscience) as above. CD45RO^+ cells in different vessel layers were counted in three to five cross sections/animal and reported as the mean number of T cells per vessel compartment.

**Real-time RT-PCR analysis**

To isolate total RNA from artery grafts, 20–30 serial sections were immersed briefly in water, centrifuged, and then rapidly resuspended in RL Tissue lysis buffer (Qiagen, Valencia, CA). RNA was isolated from the tissue section lysates using RNaseasy Plus Mini Kits (Qiagen) according to the manufacturer’s protocol, quantified using the Ribogreen Assay (Molecular Probes, Eugene, OR), and converted to cDNA via reverse transcription with random hexamers and Moloney Murine Leukemia virus reverse transcriptase according to the manufacturer’s RT system protocol (Applied Biosystems, Carlsbad, CA). All RT-PCR reactions were prepared with TaqMan 2x PCR Master Mix and predeveloped assay reagents from Applied Biosystems. Samples were analyzed on an CFX96 Real Time system using CFX Manager Software (Bio-Rad Laboratories, Hercules, CA). RNA samples processed without the reverse transcription enzyme were used as negative controls for all genes assayed. The data were analyzed using the ΔΔCT method normalized to that of GAPDH or of CD3ε for T cell-specific factors. The primers used for this study were purchased from Applied Biosystems: GAPDH (Hs99999905_m1), CD3ε (Hs00167894_m1), IFN-γ (Hs00174143_m1), IL-17A (Hs00174383_m1), IL-10 (Hs0091622_m1), RORc (Hs007612_m1), Thet (Hs00203436_m1), FOXP3 (Hs01085384_m1), CD161 (Hs00174469_m1), CCR6 (Hs0017121_m1), CCR4 (Hs99999919_m1), IL-6 (Hs00174131_m1), CXCL10 (Hs00171042_m1), CXCL11 (Hs00171431_m1), TGF-β (Hs00998133_m1), IL-1α (Hs9999928_m1), SDF1 (Hs00171022_m1), and PDH3 (Hs00229966_m1).

**T cell stimulation in vitro**

HLA-DR^+ HUVECs were plated into gelatin-coated wells of 24-well tissue culture plates, grown to confluence, and washed three times with HBSS (with no supplements) before addition of allogeneic T cells. CD4^+ T cells were added at a density of 3 × 10^5 cells/ml and memory CD4^+ T cells at a density of 1.5 × 10^5 cells/ml RPMI 1640 supplemented with 10% FBS, t-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). To assess proliferation, CD4^+ T cells were labeled with CFSE (250 nM) prior to coculture with allogeneic HUVEC, or BrdU (10 μM; BD Biosciences) was added every 12 h after 5 d in culture. Proliferation was assessed by day 7 in culture by flow cytometric analysis as described previously (26).

For restimulation assays, unlabeled memory CD4^+ T cells were co-cultured with allogeneic HUVECs for 3 d as described above, washed three times with RPMI 1640, and rested for 3 d in presence of recombinant human IL-2 (10 IU/ml) to improve survival. The recovered T cells were then washed three times with RPMI 1640, labeled with CFSE, and co-cultured with fresh HUVECs from the same donor and same subculture level as cells used in primary stimulation. Proliferation was assessed at day 3 of secondary stimulation.

In some cases, cultures were supplemented with a mouse anti-human IL-6 neutralizing mAb (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 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 allophycocyanin-conjugated anti-human CD4 mAbs (clone RPA-T4; BD Biosciences), PE-conjugated anti-human CD31 (clone WM59; BD Biosciences), FITC-conjugated anti-human CD25 (clone B1.49.9; Beckman Coulter), Pacific blue-conjugated anti-human CD127 (clone A019D5; BioLegend), PerCP/Cy5.5-conjugated anti-human CCR6 (clone HP-3G10; BioLegend), allophycocyanin-conjugated anti-human CD4 mAbs (clone RPA-T4; BD Biosciences) and PE/Cy5.5-conjugated anti-human CD31 (clone WM59; BD Biosciences). CD4+ T cells were added every 30 h after 3 d in culture. Proliferation was assessed at day 7 in culture by flow cytometric analysis as described previously (26).

**Statistical analysis**

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.

**ELISA**

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as described above, followed by adoptive transfer of allogeneic which short segments of human coronary artery are transplanted human–mouse chimeric model of intimal arteritis-like lesions in To determine the role of IL-6 in modulating human 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 which short segments of human coronary artery are transplanted as described above, followed by adoptive transfer of allogeneic human PBMCs, which yields stable engraftment of circulating populations of human CD3+ T cells capable of mediating graft rejection but does not result in engraftment of human monocytes, macrophages, dendritic cells, neutrophils, or NK cells (60). To test 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 implantation, 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/kb mAb (CNOT136) 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 ± 1.21%; 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-

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 express 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).

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![Figure 1](http://www.jimmunol.org/)
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 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.

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

**FIGURE 2.** IL-6 neutralization reduces T cell-mediated injury in human artery allografts. A, Immunohistochemical detection of human α-smooth muscle actin to define the media in representative human artery interposition grafts (5-μm transverse sections) from animals treated with IgG control (top) or anti–IL-6 mAb (bottom) for 28 d. Original magnification ×10. The areas of the lumen, total vessel, and intima are reported as the mean area in mm2 ± SEM. Statistical significance was assessed via paired t test analysis (n = 8 pairs of animals from 4 independent experiments): p < 0.05. B, Immunohistochemical detection of human CD45RO+ T cells in representative human artery interposition grafts (5-μm transverse sections). Original magnification ×40. Immunostaining in A and B was performed as described in Materials and Methods. The numbers of CD45RO+ cells in the intima are reported as the mean number of CD45RO+ T cells/intima ± SEM. Paired t test analysis was performed to assess statistical significance (n = 8 pairs of animals from 4 independent experiments): p < 0.05.
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

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<td>IFN-γ</td>
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<td>IL-17</td>
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<td>IL-10</td>
<td>0.0488</td>
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**Table II. Neutralization of IL-6 alters infiltrating T cells**

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**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).**

![FIGURE 3. IL-6 release from EC is increased by coculture with allogeneic T cells.](http://www.jimmunol.org/)

A. Coculture of CD4+ T cells with allogeneic IFN-γ-pretreated MHC class II+ HUVECs. Media was taken for ELISA from either HUVEC cultures or HUVECs cocultured with CD4+ T cells for 24 h (n = 12, pooled from three independent experiments). B. IL-6 levels in cultures after 24 h were compared between HUVECs and HUVEC–CD4+ T cell cocultures using HUVECs either transduced with CIITA (gray) or treated with IFN-γ for 3 d (black) to induce MHC class II expression (n = 12 from three independent experiments). C. IL-6 levels in lysates of CD4+ T cells (black) or HUVECs (gray) separated after 24 h in coculture as determined by ELISA (n = 6, from two independent experiments). D. IL-6 release in allogeneic MHC class II HUVEC–CD4+ T cell cocultures after 24 h in the presence of an anti-TNF neutralizing or control Ab (n = 12 from three independent experiments). *p < 0.05 (statistically significant difference from control cells stimulated in presence of MHC class II− HUVECs, at the same concentration of anti-TNF Ab, as assessed by two-way ANOVA, followed by Bonferroni post hoc analysis). E. Flow cytometric analysis of intracellular TNF in permeabilized CD4+ T cells after 24 h of coculture with allogeneic MHC class II or MHC class II HUVECs (n = 6 from 2 independent experiments). F. IL-6 release in HUVECs after 24 h in presence of increasing concentrations of recombinant human TNF-α (n = 6 pooled from 2 independent experiments). *p < 0.05 (statistically significant difference from 0 ng/ml TNF, as assessed by one-way ANOVA, followed by Student post hoc analysis).
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 cell populations, 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 CSFE 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 CSFE 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...
when IL-6 is neutralized. To test this possibility, we performed suppressor assays, where memory CD4+ T cells 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.

![image](https://i.imgur.com/123456789.png)

**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.
tured 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-

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** IL-6 neutralization results in induction of a Treg population in EC–CD4+ T cell cocultures. **A,** To test for generation of “suppressor T cells” in EC–T cell cocultures, CIITA-transduced HUVECs were cocultured with allogeneic CD4+ memory T cells for 3 d in presence of either control IgG or an anti–IL-6 neutralizing mAb (30 μg/ml), at which time T cells were collected, rested for 3 d, and then added to cocultures of CFSE-labeled freshly isolated CD4+ memory T cells (“responder T cells”) from the same blood donor and cultured with allogeneic CIITA-transduced HUVECs from the same EC donor as used in the primary stimulation. 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. **B,** Percent suppression was calculated for three independent experiments performed as in **A.** Bars represent mean of suppression from the different experiments ± SEM. Asterisks (*) indicate a significant difference from the null hypothesis. **C,** CD4+ T cells were separated into CD161+ and CD161− subsets, and each subset was cultured with CIITA-transduced HUVECs in presence of an irrelevant isotype control Ab or anti–IL-6 Ab for 7 d, at which time T cells were recovered and analyzed for mRNA species normalized to CD3ε mRNA by qRT-PCR as described in **Materials and Methods.** Note that whereas RORc mRNA was higher in the CD161+ subset, its expression was unchanged by coculture in the presence of anti–IL-6, yet FOXP3 was increased by this treatment but only in the CD161+ population. This change was statistically significant (n = 5 independent experiments); a sixth experiment, which showed a much larger change in FOXP3 expression and much higher basal FOXP3 expression, appeared as an outlier and has been excluded so as not to skew the analysis.
FIGURE 7. IL-6 neutralization results in induction of CD161+ FOXP3+ CD127- Tregs in EC–CD4+ T cell cocultures. CD4+ CD45RA- memory T cells were cocultured for 7 d with allogeneic HLA-DR+ ECs in the presence of an IL-6-neutralizing Ab (30 ug/ml) or isotype control. Multiparameter flow cytometric analysis was performed for CD161, CD25, FOXP3, CD127, and Helios in freshly isolated cells or after coculture for 7 d. A, Flow cytometric analysis for CD25 and FOXP3 expression on CD161+ gated CD4+ CD45RA+ memory T cells. *p = 0.002. B, Flow cytometric analysis for CD127 and Helios expression on CD161+ CD25+ FOXP3+ gated cells.

alent or lesser number of T cells on a per cell basis. Complementary in vitro studies suggest that neutralizing IL-6 favors induction of a CD4+ CD161+ Treg population as the underlying mechanism of protection.

It is important to note that we exclusively used human materials in our studies, allowing us to focus on mechanisms of human allograft rejection that are typically not replicated in rodent transplant models. In particular, alloreactive memory T cells play an important role in clinical transplant recipients but are not present in significant numbers in the circulation of rodents. The use of a humanized mouse model may allow us to more accurately predict potentially useful therapies. At the same time, the limited quantity of human vessels precludes more extensive analyses of mechanisms in vivo. To overcome this obstacle, we have also used cell culture experiments.

IL-6 signaling influences a number of cells, resulting in a variety of biological activities (for review, see Refs. 48, 64), including T cell activation (65–67), differentiation (65), and recruitment (68, 69). IL-6 plays a role in the pathogenesis of inflammatory and autoimmune diseases (66, 70–73), but anti-inflammatory effects have also been described (74–76). In this study, we show that IL-6 is increased as a result of perioperative injury. Additionally, IL-6 levels are elevated in vascular tissue as a result of ischemia–reperfusion injury (T. Yi and J.S. Pober, unpublished observations). We previously hypothesized that nonimmune injuries to graft vasculature result in release of cytokines modulating the host immune response, resulting in a more vigorous rejection response (46) and explaining clinical observations of more severe early allograft rejection and increased risk of late graft failure after preoperative or perioperative injuries to an allograft (44, 45). Our results suggest that graft cell-derived IL-6 is a major modulator of the anti-graft immune response after injury, which is in agreement with studies reporting a correlation between increased IL-6 levels and the severity of acute vascular rejection in primates (69) and with adverse outcome after transplantation in humans (50–52). While protective effects have also been reported (53, 54), it is most likely that differential effects of IL-6 depend on source and target tissue (77, 78) and the mechanism of rejection.

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 ECs are the principal source in this setting. Vascular SMCs also make IL-6 and are an additional possible source of this cytokine in vivo. The fall in IL-6 levels as grafts heal in would 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 circulating 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-17-producing T cells (20); that is, all IL-17–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...
mortality 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.

Acknowledgments

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Disclosures

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References


