Interleukin (IL)-1 promotes allogeneic T cell intimal infiltration and IL-17 production in a model of human artery rejection

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Interleukin (IL) 1α produced by human endothelial cells (ECs), in response to tumor necrosis factor (TNF) or to co-culture with allogeneic T cells in a TNF-dependent manner, can augment the release of cytokines from alloreactive memory T cells in vitro. In a human–mouse chimeric model of artery allograft rejection, ECs lining the transplanted human arteries express IL-1α, and blocking IL-1 reduces the extent of human T cell infiltration into the artery intima and selectively inhibits IL-17 production by infiltrating T cells. In human skin grafts implanted on immunodeficient mice, administration of IL-17 is sufficient to induce mild inflammation. In cultured cells, IL-17 acts preferentially on vascular smooth muscle cells rather than ECs to enhance production of proinflammatory mediators, including IL-6, CXCL8, and CCL20. Neutralization of IL-17 does not reduce T cell infiltration into allogeneic human artery grafts, but markedly reduces IL-6, CXCL8, and CCL20 expression and selectively inhibits CCR6+ T cell accumulation in rejecting arteries. We conclude that graft-derived IL-1 can promote T cell intimal recruitment and IL-17 production during human artery allograft rejection, and suggest that targeting IL-1 in the perioperative transplant period may modulate host alloreactivity.

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Abbreviations used: EC, endothelial cell; EVG, Elastica–van Gieson; ICAM, intercellular adhesion molecule; ICS, intracellular cytokine staining; IL-1Ra, IL-1R antagonist; ROR, retinoic acid–related orphan receptor; SMC, smooth muscle cell.

Allograft blood vessels are major targets of clinical rejection responses (1, 2). Acute vascular rejection may be mediated either by alloreactive T cells, producing a lesion called intimal arteritis, or by alloreactive antibodies (3, 4). Chronic vascular rejection, characterized by concentric intimal expansion and inadequate compensatory outward remodeling, resulting in luminal stenosis, is a major cause of late graft failure (1). Both of these forms of vascular rejection are particularly resistant to current therapies, and increased understanding of their pathogenesis may suggest new therapies.

Graft endothelial cells (ECs) appear to be a likely target of the host immune response against graft vasculature (5). Human ECs can directly activate allogeneic memory T cells, and effector memory T cells can directly injure the microvessels of allogeneic human skin (5, 6). Alloreactive memory T cells, which comprise roughly half of the total circulating alloreactive T cells in humans (7, 8), differ in their activation requirements and susceptibility to immunosuppression from naive T cells, and have been suggested to pose a significant barrier to the induction of transplant tolerance in humans (9). This component of the host antigraft immune response is frequently overlooked in transplant models using rodents, which typically lack significant numbers of circulating memory T cells.

Allograft endothelial injury is often initiated by perioperative ischemia-reperfusion injury, which predisposes allografts to both acute and chronic rejection (10, 11). We have hypothesized that such injury causes ECs to expose "alarmins" that promote a more destructive antigraft immune response. In support of this hypothesis, we have previously shown that freeze–thaw–injured ECs release IL-1α, which enhances alloreactive T cell IFN-γ and IL-17

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production in EC–T cell co-cultures (12). IL-1α and the related cytokine IL-1β are prototypical proinflammatory cytokines that elicit essentially indistinguishable responses through IL-1R1; however, unlike IL-1β, IL-1α stored intracellularly is both bioavailable and bioactive as a cytokine in its unprocessed form (13).

A pathological role for effector T cell–derived IFN-γ in vascular rejection is well supported experimentally (14); however, the contribution of IL-17 to vascular rejection remains unclear. T cell production of IL-17 plays a critical role in several mouse models of autoimmune disease, including experimental autoimmune encephalomyelitis, arthritis, and colitis, and has also been implicated in acute rejection of mouse heterotopic heart transplants (15–18). Elevated plasma or tissue levels of IL-17 have been reported in patients with autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and psoriasis (19–22). Additionally, increased numbers of T cells capable of inducing IL-17–dependent inflammation correlates with an increased risk of obliterative bronchiolitis, a form of chronic rejection in lung transplants (23). Although not completely specific, IL-17–producing T cells, sometimes referred to as "inflammatory" T helper cells (24), can be generally identified by expression of CCR6 (25, 26). IL-17 induces expression of inflammatory molecules such as IL-6, CXCL8 (also known as IL-8), and CCL20, the only known CCR6 ligand, in several human cell types in vitro (27); however, specific pathologic processes induced by IL-17 in intact human tissues have not yet been demonstrated.

The factors that promote T cell IL-17 production appear to differ between humans and mice. The combination of TGF-β and IL-6 is sufficient to induce Th17 differentiation in mouse but not human naive T cells (28–32). Although the effect of TGF-β on human T cells remains controversial, several groups have demonstrated a critical role for IL-1 in the differentiation of naive human T cells toward an IL-17–producing phenotype (31, 33–35). IL-17 production is more easily induced in memory T cells than naive T cells, and IL-1 enhances IL-17 production from alloreactive memory CD4+ T cells in human mixed lymphocyte–endothelial reactions (12, 32). However, the relevance of these factors to human T cell cytokine production in vivo is unknown.

In this paper, we demonstrate that ECs expressing high levels of cell-associated IL-1α, like soluble IL-1, can selectively skew alloreactive CD4+ memory T cells toward production of IL-17 in vitro. More significantly, in a human–mouse chimeric model of memory T cell–mediated allograft artery rejection, graft-derived IL-1 enhances allogeneic T cell infiltration into the vessel intima, causing intimal expansion, and specifically promotes IL-17 production from infiltrating human T cells. IL-17, in turn, drives an inflammatory cascade that selectively promotes recruitment of CCR6+ T cells into rejecting artery allografts, principally through its actions on smooth muscle cells (SMCs). These observations identify a central role for IL-1 in modulating human T cell responses to allograft vasculature.

RESULTS

IL–1α expression in human vascular cells

Because IL-1 can modulate human alloreactive T cell responses in vitro (12), we examined expression of IL-1α by the two principle cell types of the vessel wall, ECs and SMCs. Both ECs and SMCs typically express low levels of IL-1α in culture; however, IL-1α expression in both cell types could be markedly up-regulated by treatment with TNF (Fig. 1 A). TNF treatment increased the amount of IL-1α contained in EC or SMC lysates without causing accumulation of IL-1α in the culture media, consistent with previous observations that TNF-induced IL-1α remains cell associated in ECs (36). EC but not SMC expression of IL-1α was also increased after co-culture with allogeneic CD4+ T cells. In these experiments, EC MHC class II expression lost during culture was restored by retroviral transduction with class II transactivator (Fig. 1 B) or pretreatment with IFN-γ (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20081661/DC1) before co-culture. The increase in EC IL-1α expression induced by co-culture with alloreactive T cells could be inhibited by addition of a neutralizing antibody to TNF (Fig. 1 C), suggesting that allogeneic T cells enhance IL-1α expression in ECs by secreting TNF. In contrast, IL-1α expression in MHC class II–positive SMCs was not changed after co-culture with allogeneic CD4+ T cells (Fig. S1), consistent with the ability of ECs but not SMCs to induce cytokine production from resting allogeneic T cells (37).

EC–expressed IL–1α skews alloreactive CD4+ T cells toward IL–17 production

To examine effects of EC-expressed IL-1α on the allogeneic response, we co-cultured purified CD4+ memory T cells with allogeneic MHC class II–positive ECs either pretreated with TNF or left untreated. Similar to effects of exogenous IL-1 (12), ECs pretreated with TNF stimulated increased production of IFN-γ and IL-17 from allogeneic memory CD4+ T cells compared with untreated ECs (Fig. 1 D). Most of this enhanced immunogenicity was blocked by addition of a neutralizing antibody to IL-1α.

To determine whether EC-expressed IL-1α can polarize alloreactive memory CD4+ T cell responses, we performed restimulation assays in which CD4+ memory T cells were co-cultured with either TNF-pretreated or untreated allogeneic ECs for 3 d, rested, and then restimulated by co-culture with fresh, untreated ECs from the same donor as the primary culture. CD4+ T cells that were co-cultured with TNF-pretreated ECs during the primary stimulation selectively produced more IL-17 upon restimulation than did T cells initially co-cultured with untreated ECs, with no significant change in IFN-γ or IL-5 production (Fig. 1 E). Neutralization of IL-1α during the primary co-culture completely blocked the ability of TNF-treated ECs to skew the alloreactive population toward IL-17 production.

To confirm that EC-expressed IL-1α alters the number of T cells that produce IL-17 upon restimulation, T cell cytokine production was also analyzed by intracellular cytokine
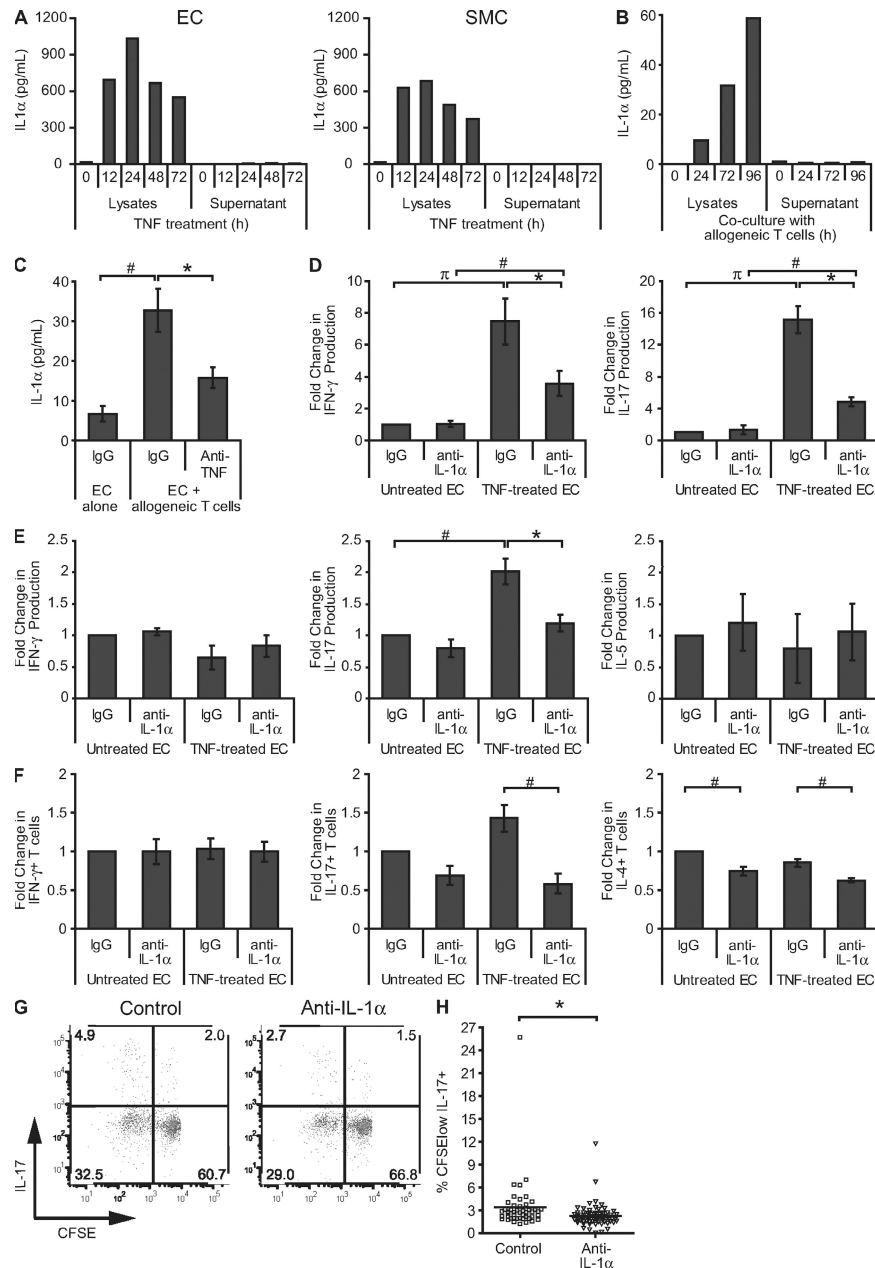
Figure 1. Human ECs use cell–associated IL–1α to promote alloreactive memory CD4+ T cell IL-17 production. (A) ECs or SMCs were treated with 10 ng/ml TNF for the indicated times, and cell lysates and supernatants were assayed for IL-1α by ELISA. (B) MHC class II–positive ECs were co-cultured with allogeneic CD4+ T cells for the indicated times, and then ECs were purified and assayed for IL-1α in EC lysates and supernatants by ELISA. For A and B, data from one of three experiments with similar results are shown. (C) MHC class II–positive ECs were co-cultured with CD4+ T cells for 48 h with a neutralizing anti-TNF antibody or isotype control, and IL-1α in purified EC lysates was measured by ELISA. Means ± SEM (n = 3 experiments) are shown. (D) Untreated or TNF-pretreated ECs were co-cultured with allogeneic memory CD4+ T cells with 5 µg/ml of a neutralizing anti–IL-1α antibody or isotype control. Culture medium was assayed for IFN-γ and IL-17 by ELISA at 24 h. (E) CD4+ T cells from primary co-cultures were restimulated with fresh ECs, and the culture medium was assayed for IFN-γ, IL-17, and IL-5 by ELISA at 24 h. Means ± SEM of fold changes relative to the untreated, isotype control group from seven (D) or three (E) experiments are shown. The ranges of values in the control group were 1–33 pg/ml (IFN-γ) and 1–10 pg/ml (IL-17) in D, and 301–645 pg/ml (IFN-γ), 5–128 pg/ml (IL-17), and 8–428 pg/ml (IL-5) in E. (F) Purified memory CD4+ T cells were co-cultured with CD32-transduced, anti-CD3 mAb–coated ECs that were either pretreated with TNF or left untreated, and then T cells were restimulated with PMA plus ionomycin and analyzed by ICS. A neutralizing antibody to IL-1α or isotype control was added to the primary co-culture as indicated. Means ± SEM of fold changes relative to the untreated, isotype control group from six different donors are shown. (G) CFSE-labeled memory CD4+ T cells co-cultured with allogeneic ECs for 14 d were restimulated with PMA and ionomycin and analyzed by ICS. Representative dot plots of CFSE dilution and IL-17 expression in CD45+-gated lymphocytes are shown (numbers indicate percentages). (H) Scatter plot of the fraction of CFSE-low, IL-17+ cells in individual microtiter co-cultures. Pooled data from two experiments with similar results are shown. Horizontal bars indicate means. *, P < 0.05; #, P < 0.01; π, P < 0.001.

staining (ICS). Because the frequency of human CD4$^+$ T cells that can be activated by allogeneic ECs is low, detection of cytokine-positive T cells by ICS after co-culture with allogeneic ECs is difficult. Therefore, we used a model, developed in our laboratory, in which ECs are transduced to express the FcγR CD32 and overlayed with anti-CD3 mAb (38). T cells co-cultured with these ECs are activated polyclonally by a defined TCR signal in the context of the costimulatory molecules expressed by human ECs. Purified memory CD4$^+$ T cells were co-cultured with CD32-transduced, anti-CD3 mAb–coated ECs that had been either pretreated with TNF to induce IL-1α or left untreated, and an anti–IL-1α antibody or an isotype control was added. After 3 d of primary co-culture, T cells were rested for 3 d and then restimulated with PMA plus ionomycin and evaluated by ICS. Consistent with the results generated by ELISA, neutralization of IL-1α during the primary co-culture of TNF-treated ECs and CD4$^+$ T cells significantly reduced the number of T cells that produced IL-17 upon restimulation (Fig. 1 F) but had no effect on the number of IFN-γ–producing T cells. In this system, neutralization of IL-1α appeared to slightly reduce the number of IL-17–producing cells in co-cultures in which the ECs were not pretreated with TNF before co-culture, most likely because the large number of activated T cells produce enough TNF during the primary culture to rapidly enhance IL-1α expression by the ECs. IL-1α neutralization also modestly reduced the number of IL-4–producing T cells, although we did not observe a similar pattern when measuring IL-5 production in allogeneic restimulation assays by ELISA.

In addition, we asked whether IL-1α produced by ECs in response to alloreactive T cells might in turn alter IL-17 production by the alloreactive T cell population. To examine this, CFSE-labeled memory CD4$^+$ T cells were co-cultured with allogeneic ECs in replicate microtiter wells with or without addition of an anti–IL-1α neutralizing antibody for 14 d and were then restimulated with PMA and ionomycin. Alloreactive T cells were identified as cells that had proliferated and diluted the CFSE dye, and the number of alloreactive cells expressing IL-17 in each well was quantified by ICS. Neutralization of IL-1α in these co-cultures did not affect total alloreactive T cell proliferation (unpublished data) but significantly reduced the number of alloreactive T cells that produced IL-17 upon restimulation (Fig. 1, G and H). Collectively, these results indicate that IL-1α expressed by human ECs, as previously shown for recombinant IL-1 (12), enhances early IFN-γ and IL-17 production from alloreactive CD4$^+$ T cells and further directs alloreactive CD4$^+$ memory T cells selectively toward production of IL-17.

Vascular cell–expressed IL-1 promotes T cell intimal infiltration and IL-17 production in vivo

We next examined the effect of IL-1 in directing human T cell responses to allogeneic human artery grafts in vivo using a human–mouse chimeric model of intimal arteritis. In this model, short segments of human coronary artery are im-

planted into immunodeficient SCID/beige mice as infrarenal aortic interposition grafts, and allogeneic human PBMCs are then adoptively transferred. Adoptive transfer of human PBMCs into SCID/beige mice 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, DCs, neutrophils, or natural killer cells (39).

Expression of IL-1α was consistently detected in the endothelium of human artery interposition grafts, as well as in occasional cells in the media and adventitia (Fig. 2 A). To test whether IL-1 modulates human T cell responses to allogeneic artery grafts in vivo, allogeneic PBMCs were adoptively transferred into mice recently grafted with human artery, and the mice were treated with IL-1R antagonist (IL-1Ra) or PBS daily for 21 d. In sequential experiments, two doses of IL-1Ra were used (50 and 150 mg/kg/day) that yielded similar effects; therefore, data from the two doses were pooled
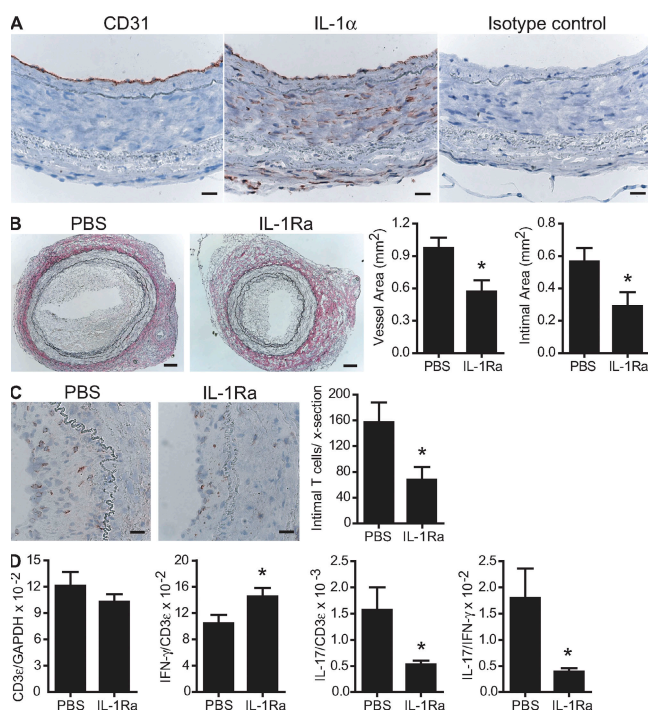
Figure 2. IL-1 blockade reduces T cell–mediated injury and IL-17 production in human artery allografts. (A) Immunohistochemical detection of human endothelium (CD31) and IL-1α in a human artery aortic interposition graft. Staining with an isotype-matched primary antibody is shown as a control. Bars, 25 μm. (B) 5-μm transverse sections were stained with EVG, and the total vessel area (area bounded by the external elastic lamina and the lumen) and the intimal area (area bounded by the internal elastic lamina and the lumen) were quantified using ImageJ software. Bars, 100 μm. (C) Immunohistochemical identification and quantification of human CD45RO$^+$ T cells in graft intimas. Bars, 25 μm. (D) RT-PCR analysis of CD3ε (normalized to GAPDH), IFN-γ, and IL-17 (normalized to CD3ε), and the ratio of IL-17/IFN-γ in artery grafts undergoing T cell–mediated rejection. For B–D, means ± SEM (n = 10 per group) are shown. *, P < 0.05.

to generate larger numbers for analysis. Treatment with IL-1Ra did not alter the number of circulating human T cells after PBMC transfer (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20081661/DC1); however, IL-1 blockade significantly reduced the total vessel area, the intimal area, and the number of intimal T cells in rejecting artery grafts (Fig. 2, B and C).

We also examined expression of T cell effector cytokines within the rejecting artery grafts. Blockade of IL-1 significantly decreased the expression of IL-17 in treated grafts when normalized either to the T cell marker CD3ε or to GAPDH (Fig. 2 D; and not depicted). In the same specimens, IL-1 blockade caused a corresponding increase in the amount of IFN-γ per T cell in the grafts, yielding a significantly altered ratio of IL-17/IFN-γ production from graft-infiltrating T cells.

In mouse systems, IL-17 is principally produced by a CD4$^+$ effector T cell subset that expresses retinoic acid–related orphan receptor (ROR) γT, designated as Th17 cells (40, 41). In humans, IL-17–producing CD4$^+$ T cells express CD161, IL-23R, CCR6, and RORC (the human equivalent of RORγT), but human cells expressing these markers may produce IL-17, IFN-γ, or both cytokines (25, 26, 42). Expression of these genes and of genes typically associated with classical Th1 cells, including CXCR3, IL-12Rβ2, and T-bet, could be detected in artery grafts infiltrated with allogeneic T cells but not in control grafts without T cells; however, no significant changes were observed in the expression of these genes per T cell as a result of IL-1 blockade (Fig. 3 A). Furthermore, IL-1 neutralization did not significantly alter expression of any of these genes in memory CD4$^+$ T cells activated in vitro by TNF-pretreated ECs despite alterations in IL-17 production (Fig. 3 B and Fig. 1 F). Expression of RORC appeared to be increased in T cells co-cultured with TNF-pretreated ECs in an IL-1–dependent manner; however, this change did not reach statistical significance when corrected for multiple comparisons. By immunofluorescence staining, we observed that ~10% of the CD3$^+$ T cells infiltrating the intimas of human artery grafts also express CD161, and IL-1 blockade did not alter the proportion of CD3$^+$ T cells that expressed CD161 (Fig. 3 C). These data indicate that graft-derived IL-1 promotes allogeneic T cell intimal infiltration and directs alloreactive T cells toward IL-17 and away from IFN-γ production in a model of human artery graft rejection in vivo. However, although IL-1 alters the cytokines produced by artery-infiltrating memory T cells, it does not cause obvious alterations in T cell markers associated with Th1- or Th17-like subsets.

IL-17 induces inflammatory responses in human vascular cells
Based on rodent studies, IL-17 is proposed to be a mediator of acute inflammation. To confirm this in human tissues, recombinant IL-17 was injected intradermally into human skin grafts transplanted onto immunodeficient mice, and the grafts were harvested 6 h later. In this model, healed-in human skin grafts transplanted onto CB.17 SCID/beige mice are per-

fused primarily through human microvessels, which anastomose with mouse vessels at the edges of the graft (39). Grafts injected with IL-17 showed a modest degree of infiltration with mouse neutrophils, as well as increased expression of IL-6 and CCL20 (Fig. 4, A and B). We also observed variable increases in CXCL1 and CXCL8 that did not reach statistical significance (unpublished data). In addition, occasional human E-selectin–positive vessels could be found in IL-17–injected grafts (Fig. 4 C). Thus, administration of IL-17 alone induces mild inflammation in an intact human tissue.

To investigate which cells within an artery wall respond to IL-17 secreted by infiltrating T cells, we tested the ability of IL-17 to induce inflammatory responses in cultured human vascular cells. Treatment of cultured ECs with IL-17 slightly increased transcript expression of IL-6, CXCL8, and CCL20 but did not alter protein secretion of these mediators or induce adhesion molecule expression (Fig. 5, A–C). The positive effect of TNF on E-selectin and intercellular adhesion molecule (ICAM) 1 protein in replicate cultures is clear cut (Fig. 5 C). The lack of adhesion molecule induction on cultured ECs by IL-17 suggests that the E-selectin expression observed on some vessels in IL-17–treated skin may be induced indirectly. In contrast to the limited EC response, SMCs treated with IL-17 increased transcript levels of IL-6, CXCL8, and CCL20 in a time- and dose-dependent manner and markedly increased secretion of these proteins (Fig. 5, D–F). The ability of IL-17 to induce CCL20 secretion in SMCs was relatively minor compared with that of TNF; however, IL-17 synergized with TNF to yield markedly elevated CCL20 secretion (Fig. 5 G). Microarray analyses of IL-17–treated SMCs identified additional cytokines and chemokines induced by IL-17, several of which were confirmed by RT-PCR (Table I and Table S1, available at http://www.jem.org/cgi/content/full/jem.20081661/DC1). Induction of most of these genes could not be prevented by cycloheximide, indicating that these genes are directly induced by IL-17 without the requirement for new protein synthesis.

T cell–derived IL-17 promotes inflammation and CCR6$^+$ T cell accumulation in vivo
We next investigated the role of IL-17 in this model of human artery allograft rejection. Human allogeneic PBMCs were again adoptively transferred into mice transplanted with human artery interposition grafts, and the mice were treated with a neutralizing antibody to IL-17 or an isotype-matched control antibody for 4 wk. Neutralization of IL-17 did not reduce intimal expansion and had no effect on total T cell infiltration into human artery grafts, as assessed by RNA and immunohistochemistry (Fig. 6, A and B). IL-17 neutralization also did not significantly alter IFN-γ or IL-17 expression within the grafts (Fig. 6 C). However, inhibition of IL-17 markedly diminished graft expression of IL-6, CXCL8, and CCL20, three genes that may participate in a potential inflammatory Th17 cytokine axis, but not expression of CXCL10, an IFN-γ–inducible chemokine (Fig. 6 C). The decrease in CCL20 expression appears to have functional

consequences, because IL-17 neutralization also reduced the transcript levels of CCR6, but not CXCR3, in rejecting grafts (Fig. 6 D). These data suggest that T cell production of IL-17, enhanced by IL-1, alters the inflammatory environment to further attract additional inflammatory CCR6+ T cells, but that IL-17 is not necessary for recruitment of CXCR3+ T cells, a likely source of IFN-γ.

DISCUSSION

Using models of human T cell responses to allogeneic vascular cells in vitro and in vivo, we demonstrate important roles for graft-derived IL-1 in shaping the host antigraft adaptive immune response and in promoting T cell–mediated injury to allogeneic vasculature. Specifically, we demonstrate that the EC lining of human artery segments interposed into the
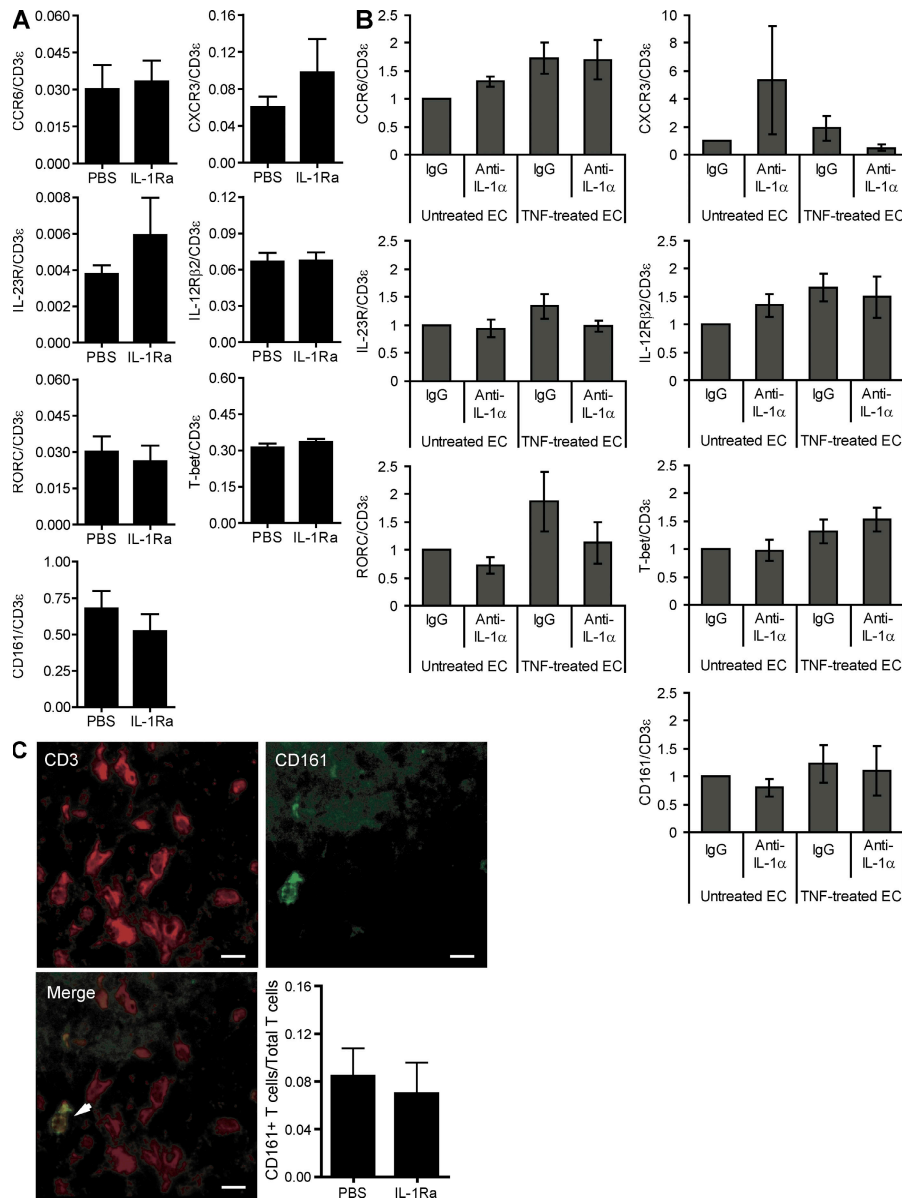
Figure 3. IL-1 blockade does not alter memory CD4+ T cell expression of Th17–associated markers. (A) RT-PCR analysis of T cell genes normalized to CD3ε in artery grafts undergoing T cell–mediated rejection treated with IL-1Ra or PBS. Means ± SEM (n = 10 per group) are shown. (B) RT-PCR analysis of T cells activated by co-culture with CD32-transduced ECs overlayed with anti-CD3 mAb. ECs pretreated with TNF or left untreated were overlayed with anti-CD3 mAb and co-cultured with purified, memory CD4+ T cells with an anti–IL-1α antibody for 3 d. T cells were then rested with 3 d and RNA was isolated. Gene expression was normalized to CD3ε, and means ± SEM of fold changes relative to the untreated, isotype control group from six different donors are shown. (C) Immunofluorescence microscopy of intimal T cells stained with anti-CD3 (red) and anti-CD161 (green). The arrowhead points to a cell positive for both CD161 and CD3. Quantification of CD161+/CD3+ double-positive cells is expressed as a fraction of total CD3+ T cells in the intimas of artery grafts treated with IL-1Ra or PBS. Means ± SEM (n = 7 per group) are shown. Bars, 10 μm.

infrarenal aortae of immunodeficient CB.17 SCID/beige mice expresses IL-1α and that blockade of IL-1 with IL-1Ra reduces T cell recruitment to the graft intima. Thus, blockade of IL-1 appears to protect allograft arteries from a process that resembles acute cell–mediated vascular rejection, sometimes described as "intimal arteritis."

The use of human materials in our work allows for two important advances beyond the insights gained from rodent transplant models. First, we focus on alloreactive memory cells, which are present in significant numbers in clinical transplant recipients but are typically lacking in rodents. Second, we can identify signals that alter human T cell responses (e.g., IL-17 production), which can differ significantly from those that act in mice. Translational studies using humanized mouse models, despite being limited by scarce materials and few tools for genetic manipulation, are likely to more accurately predict potentially useful therapies (43). Our study contributes several novel observations regarding the interactions of IL-1 and IL-17: (a) IL-1α localized on ECs can selectively enhance IL-17 production from human alloreactive memory CD4+ T cells, especially upon restimulation; (b) allograft–expressed IL-1 promotes IL-17 production from human T cells in vivo; (c) IL-17 drives inflammatory responses and inflammatory T cell recruitment during T cell–mediated human allograft rejection in vivo; and (d) the principle vascular cell target of IL-17 appears to be the SMCs rather than ECs, a striking difference from the inflammatory responses mediated by TNF or IL-1.

Although best known as an inducer of acute inflammation, IL-1 can also modulate adaptive immune responses. Recent studies have demonstrated a critical role for IL-1 in inducing Th17 differentiation in human naive T cells (31, 33–35). IL-1 can also participate in the differentiation of mouse Th17 cells (28, 44); however, some effects of IL-1 on mouse T cell differentiation appear to differ between mouse

strains (45). Although most studies have focused on differentiation of naive T cells, cytokine production from memory CD4+ T cells can also be modulated by the environment. For example, polarized Th1 cells from both humans and mice can be induced to produce Th2 cytokines by activation under strongly Th2-polarizing conditions and vice versa, although this plasticity may be more pronounced in human compared with mouse T cells (46–48). In addition, we have previously shown that addition of exogenous IL-1 to human EC–T cell co-cultures can selectively skew alloreactive memory CD4+ T cells toward IL-17 production (12). We now show that human ECs express sufficient bioavailable IL-1α, up-regulated either by treatment with exogenous TNF or by TNF produced by alloreactive memory CD4+ T cells, to selectively promote IL-17 production from alloreactive memory T cells. The presence of this cell-associated, bioactive IL-1α suggests that serum levels of IL-1, even when collected from a local site, may not accurately predict the activity of this mediator in modulating T cell–driven pathological processes.

Although several cytokines, including IL-1, IL-6, IL-23, TGF-β, and IL-21, have been suggested to contribute to the development of IL-17–producing T cells in vitro (12, 31–35, 49), little information exists on which factors may be relevant in human immune responses in vivo (50). Using a human–mouse model of human artery allograft rejection, we provide the first evidence that IL-1 promotes IL-17 production from human T cells in vivo. Blockade of IL-1 reduces IL-17 expression but increases IFN-γ expression in rejecting human artery allografts, suggesting that IL-1 promotes T cell production of IL-17 at the expense of IFN-γ in vivo. This change in cytokine expression could reflect a role for IL-1 in directing selective T cell recruitment or T cell differentiation in the periphery; however, we did not observe significant alterations in the expression of several genes that are preferentially expressed by human IL-17–producing T cells (CCR6, IL-23R,
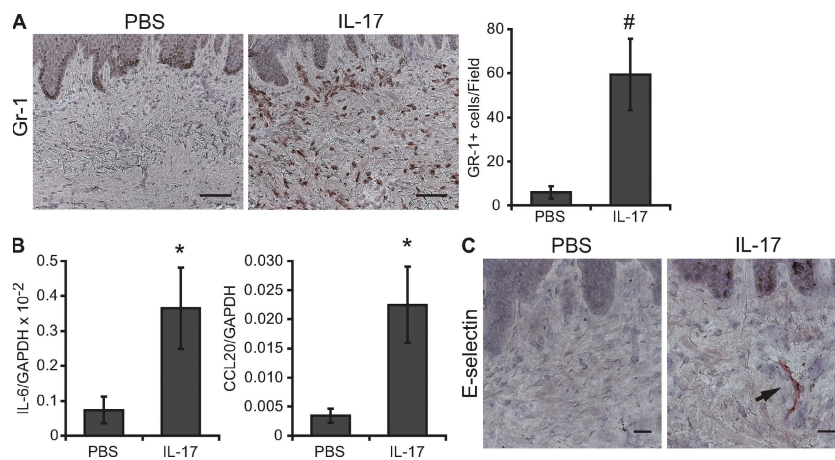
Figure 4.   IL–17 induces acute inflammatory responses in human skin grafts. Well-healed skin grafts on CB.17 SCID/beige mice were harvested 6 h after injection of 1 µg IL-17 or PBS. (A) Immunohistochemical quantification of Gr-1+ mouse neutrophils in human skin grafts. Bars, 100 µm. (B) Quantitative RT-PCR analysis of IL-6 and CCL20 expression normalized to GAPDH in injected grafts. (C) Immunohistochemical identification of human E-selectin+ vessels (arrow) in skin grafts. Bars, 25 µm. Data are pooled from two independent experiments with similar results, and means ± SEM (n = 6 per group) are shown in A and B. *, P < 0.05; #, P < 0.01.

and RORC) with IL-1 blockade in artery grafts in vivo or in purified memory CD4^{+} T cells activated with ECs expressing high levels of IL-1α in vitro. These observations suggest that, rather than having an effect on lineage specification, IL-1 may alter effector cytokine production in undifferentiated or partially differentiated human memory T cells, which show considerable plasticity in cytokine production phenotypes (47).

This possibility may be particularly relevant for IL-17, which can be produced by T cells that also make IFN-γ (25, 26). Indeed, human IL-17–producing and IFN-γ–producing T cell clones show significant overlap in expression of differentiation markers and have been suggested to share a common developmental program (26). Nevertheless, the data presented in this paper suggest that the effector responses of alloreactive
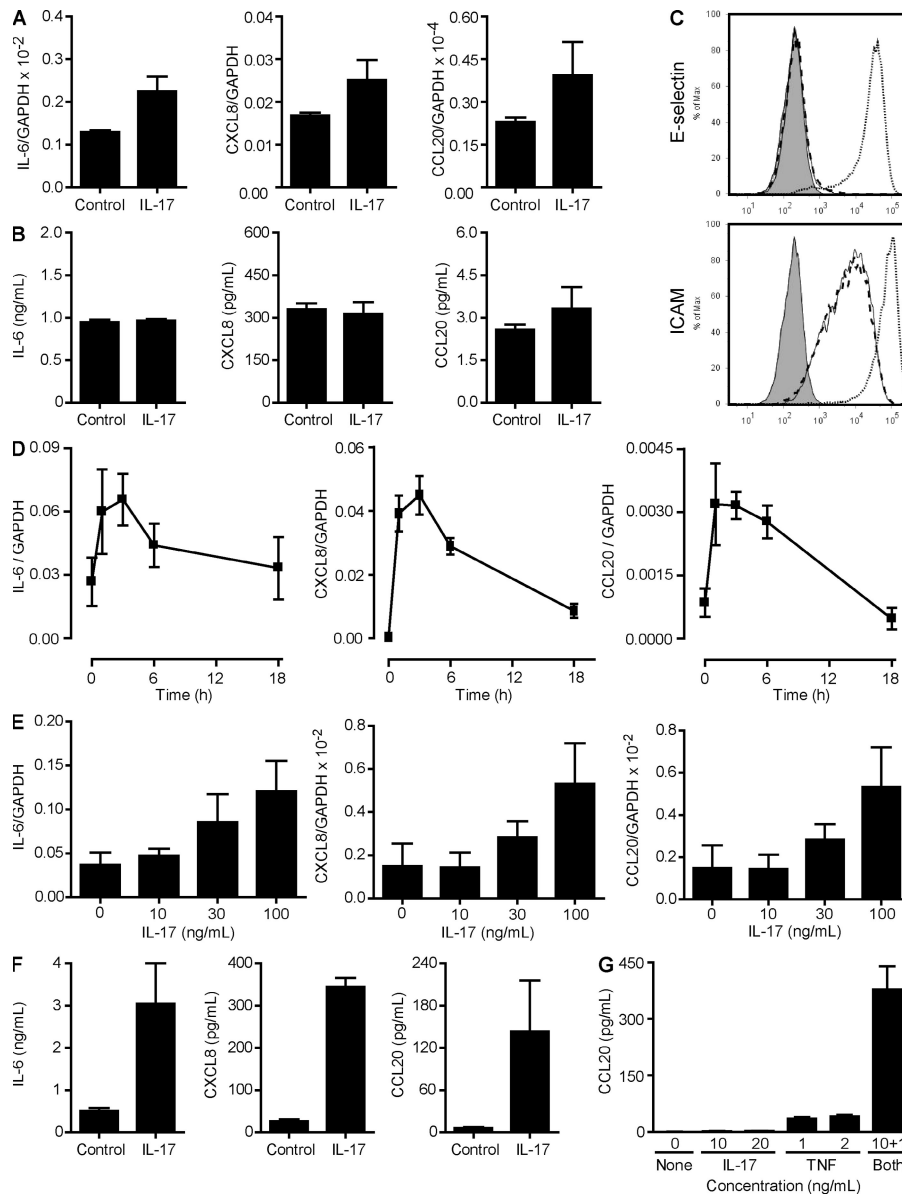
Figure 5. IL–17 induces inflammatory gene expression in cultured human SMCs. (A) ECs were treated with 100 ng/ml IL-17 for 6 h, and target gene expression was measured by quantitative RT-PCR. Data shown are means ± SD of triplicate samples from one of two independent experiments with similar results. (B) ECs were treated with 100 ng/ml IL-17, and the culture medium was assayed for IL-6, CXCL8, and CCL20 by ELISA. Means ± SEM (*n* = 3 experiments) are shown. (C) Measurement of surface E-selectin and ICAM-1 expression on ECs treated for 6 h with 100 ng/ml IL-17 (dashed line), 10 ng/ml TNF (dotted line), or vehicle control (continuous line) by flow cytometry. Shaded histograms represent staining with an isotype-matched control antibody. Representative data are from one of three independent experiments with similar results. (D and E) SMCs were treated with 100 ng/ml IL-17 for the indicated times (D) or with IL-17 at the indicated concentrations for 6 h (E), and target gene expression was measured by quantitative RT-PCR. (F) SMCs were treated with 100 ng/ml IL-17 for 24 h, and the culture medium was assayed for IL-6, CXCL8, and CCL20 by ELISA. (G) SMCs were treated with the IL-17 and TNF alone or in combination, and the culture medium was assayed for CCL20 by ELISA. Means ± SEM from four (E and G) or six (D and F) experiments are shown.

memory T cells, in this case production of IL-17, can be modulated by signals in the microenvironment of the target tissue (e.g., by IL-1α expressed on graft endothelium).

We also observed that in addition to decreasing IL-17 expression by artery-infiltrating T cells, IL-1 blockade significantly diminished T cell–mediated graft injury, as assessed by T cell intimal infiltration, neointimal expansion, and outward arterial remodeling. Our group has previously demonstrated a key role for T cell production of IFN-γ in promoting arterial injury and pathological remodeling in this model (51, 52); therefore, it may seem initially surprising that IL-1 blockade reduces neointimal expansion without reducing IFN-γ expression by those T cells that do infiltrate the vessel wall. The likely explanation is that the effect of IL-1 blockade on the extent of T cell infiltration into the neointima significantly reduces the total amount of IFN-γ in the intima. The effect on intimal T cell numbers by IL-1 blockade may relate to T cell recruitment, T cell retention, or T cell proliferation and survival, all of which could be influenced by IL-1. The failure to reduce IFN-γ by individual T cells in vivo differs from our observed effects in vitro, when T cells were co-cultured with allogeneic ECs. These differences may relate to an incomplete blockade of IL-1 by IL-1Ra in vivo, perhaps because of rapid clearance, or may suggest that alloreactive T cells within the wall more closely resemble our secondary stimulation of T cells in which the effects of IL-1 on IFN-γ during the initial activation are no longer observed.

The correlation between reduced intimal infiltration and reduced IL-17 expression caused by IL-1 blockade suggested a potential pathological role for IL-17; however, unlike IL-1 blockade, IL-17 neutralization failed to reduce T cell intimal infiltration and neointimal expansion. As IL-1 appears to be an upstream inducer of IL-17, it is possible that blockade of IL-1 may inhibit production of other Th17-derived cytokines, including IL-17F and IL-22, and may thus be more effective than blockade of individual Th17 effector cytokines in limiting Th17-mediated pathology. We were unable to evaluate this possibility because the expression levels of IL-17F and IL-22 were too low to accurately measure in these experiments. Alternatively, our inability to detect IL-17F and IL-22 may suggest that the IL-17A detected in the artery grafts is produced by undifferentiated memory T cells or by IFN-γ/IL-17 double producers rather than bona fide Th17 cells. It is also clear that IL-1 blockade has other protective effects beyond modulating T cell cytokine profiles, such as reducing the extent of T cell infiltration.

Elevated levels of IL-17 have been reported in several human autoimmune diseases; however, specific effects of human T cell production of IL-17 in vivo have yet to be reported. In the model of human artery graft rejection described in this paper, neutralization of IL-17 markedly reduces expression of certain proinflammatory molecules such as IL-6, IL-8, and CCL20 within the graft while leaving expression of CXCL10, an IFN-γ–inducible chemokine, unaffected. IL-6, CXCL8, and CCL20 are known IL-17–inducible genes (27); however, the efficacy of IL-17 neutralization is somewhat surprising given that IL-17 is far less potent at inducing expression of these molecules than IL-1 or TNF (unpublished data). These data indicate that in the absence of other human leukocytes, allogeneic T cell–mediated induction of these acutely inflammatory transcripts requires the action of IL-17. Our finding that IL-17 synergizes with TNF to induce CCL20 production by cultured SMCs suggests that these factors may act in concert within the vessel wall to drive chemokine expression and that interference with one single factor can strongly reduce CCL20 production.

The reduction in CCL20 expression caused by IL-17 neutralization appears functional because it is associated with a corresponding decrease in CCR6 expression within the graft, suggesting that alloreactive T cell–derived IL-17 propagates an inflammatory cascade that promotes further accumulation of inflammatory, CCR6+ T cells during human allograft rejection. Because our conclusion is based on mRNA levels, we cannot distinguish whether this reduction in CCR6+ cells represents a change in the number of CCR4+/CCR6+ T cells that produce only IL-17 or the number of cells in the CCR6+/CXCR3+ population that produce both IL-17 and IFN-γ (25). Interestingly, IL-1 blockade did not

**Table I.** IL-17–induced up-regulation of cytokines and chemokines in SMCs

Transcript	Microarray (fold increase)	qPCR (fold increase)	Inhibited by cycloheximide
BMP2	4.4/3.9	2.5	Yes
CCL20	5.5	7.7	No
CXCL1	2.2	13.7	No
CXCL3	4.9	7.9	No
IL-1β	2.6/2.6	11.1	No
IL-6	2.8	4.4	No
CXCL8	2.5/3.3	17.2	No
IL-11	20.8/3.6	3.7	Yes
LIF	4.1	2.7	No

SMCs were treated with 100 ng/ml IL-17 for 6 h in three independent experiments using different donors. The mean fold change of cytokine and chemokine transcript expression in response to IL-17 treatment was assessed by microarray (certain cytokines were represented by more than one probe) and quantitative RT-PCR (qPCR). Transcripts for which IL-17–mediated up-regulation was not diminished by 10 μg/ml cycloheximide, as assessed by RT-PCR, were considered primary gene targets.

cause a similar specific reduction in CCR6 expression in rejecting artery grafts, which may suggest that a more complete inhibition of IL-17, as achieved with antibody neutralization of IL-17 compared with treatment with IL-1Ra, is required to affect subsequent CCR6+ T cell recruitment. IL-17–induced production of chemokines that recruit neutrophils (CXCL8) and DCs (CCL20) observed in the experiments presented in this paper would be expected to contribute to the rejection of human allografts; however, more complete human–mouse chimeric models that allow for the engraft-

ment or development of human neutrophils and DCs will be required to further evaluate these effects.

The effects of neutralizing IL-17 on overall graft pathology are less than those observed by blocking IL-1 and much less than those observed by blocking IFN-γ (52). Indeed, IFN-γ still appears to be the central and nonredundant effector cytokine in our model. Nevertheless, our results suggest that IL-1 blockade may be a useful adjunct therapy to prevent vascular rejection, an idea supported by results in mouse and rat cardiac transplant models showing delayed acute rejection
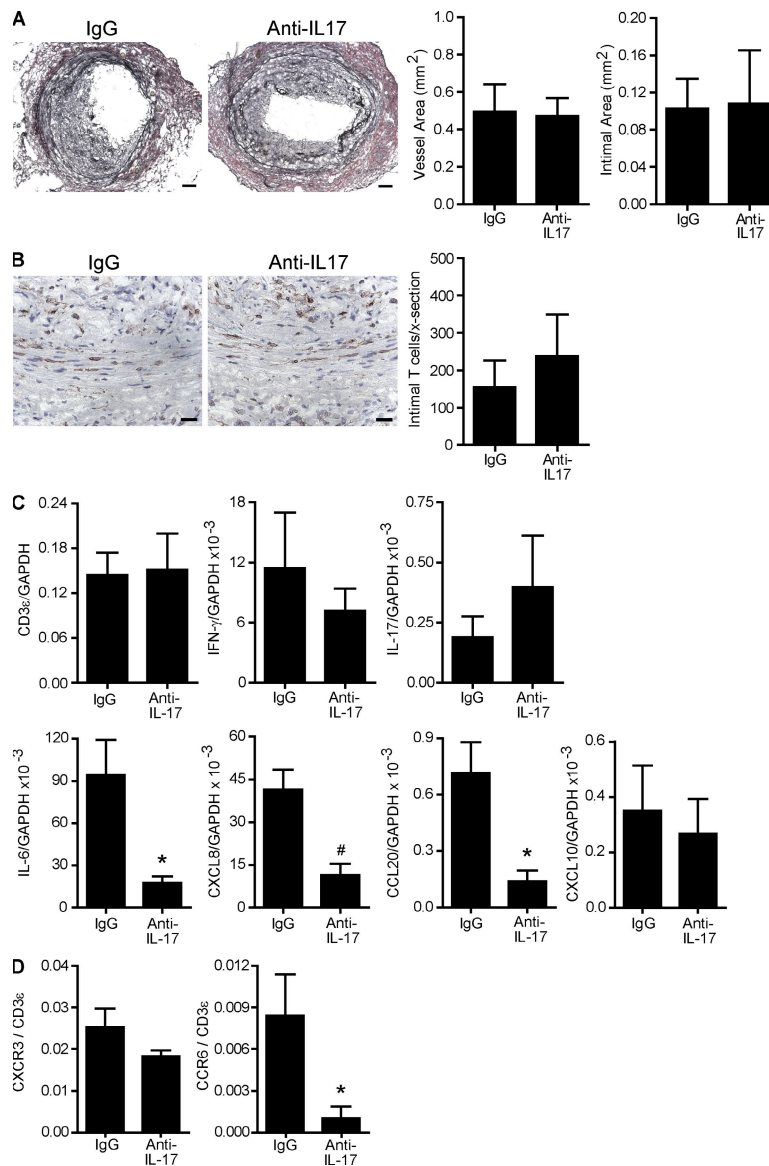
Figure 6.   IL-17 blockade reduces inflammatory responses during allogeneic T cell–mediated rejection of human artery grafts. Human PBMCs were adoptively transferred into mice bearing human artery interposition grafts, and mice were treated three times weekly with a neutralizing anti–IL-17 antibody or an isotype control. (A) 5-μm transverse sections were EVG stained, and the vessel area and the intimal area were quantified using ImageJ software. Bars, 100 μm. (B) Immunohistochemical identification and quantification of human CD45RO+ T cells in graft intimas. Bars, 25 μm. (C) RT-PCR analysis of cytokine and chemokine expression (normalized to GAPDH) in grafts from mice treated with an anti–IL-17 antibody or an isotype control. (D) RT-PCR analysis of chemokine receptor expression (normalized to CD3ε) in grafts from mice treated with an anti–IL-17 antibody or an isotype control. Means ± SEM are shown (IgG, n = 6; anti–IL-17, n = 5). *, P < 0.05; #, P < 0.01.

with IL–1 blockade (53, 54). As IL-1 is well known to be produced as a consequence of ischemia-reperfusion injury (55–57), we propose that IL-1 produced in the periphery as a consequence of perioperative injury promotes T cell–mediated inflammation locally within an allograft and further modulates the nature of the T cell response. Perioperative blockade of IL-1 thus represents an attractive new therapeutic strategy in clinical transplantation that will need to be tested experimentally. This role of IL-1 on T cell responses may also carry significant implications for autoimmunity, tumor immunotherapy, and stroke.

MATERIALS AND METHODS

Isolation and culture of human cells. All human cells and tissues, including skin and blood vessels, were obtained and used under protocols approved by the Institutional Review Groups of Yale University and the New England Organ Bank. CD4+ T cells were isolated from human PBMCs by positive selection using CD4 Dynabead (Invitrogen) magnetic bead separation and were released from the beads with DETACHaBEAD (Invitrogen), as previously described (12). Activated T cells and monocytes were routinely depleted from CD4-selected populations by further negative selection using a mouse anti–human HLA-DR antibody (clone LB3.1; a gift from J. Strominger, Harvard University, Cambridge, MA). Memory CD4+ T cells were isolated from CD4-selected populations by negative selection using anti-CD45RA antibody (eBioscience), followed by incubation with goat anti–mouse antibody–coated beads (Invitrogen).

Human umbilical vein ECs were isolated from umbilical cords and cultured as previously described (58). Such cultures are uniformly positive for von Willebrand factor and CD31, and lack detectable contamination by CD45-expressing leukocytes. In some experiments, MHC class II expression lost during culture was restored by retroviral transduction with class II transactivator, as previously described (38). In brief, ECs were washed with HBSS, overlaid with virus-containing medium for 4–6 h, and returned to complete media overnight. MHC class II expression was confirmed by flow cytometry, and four to five rounds of transduction routinely achieved >85% transduction efficiency.

Human aortic or coronary artery SMCs were isolated by explant outgrowth and serially cultured. No phenotypic differences were detected between the two types of SMCs, and cells were used at passages three to four. For quantitative RT-PCR and ELISA analyses, cells were serum deprived in 0.5% PBS for 48 h before treatment with cytokines. For microarray analyses, SMCs were maintained in 20% FBS-supplemented medium and treated with or without 100 ng/ml IL-17 (R&D Systems) for 6 h.

To measure changes in IL-1α in ECs after co-culture with T cells, class II transactivator–transduced ECs were co-cultured with allogeneic CD4+ T cells for 3 d, and adherent cells were collected with trypsin and depleted of contaminating T cells by negative depletion using anti-CD4+ beads (Invitrogen). In experiments comparing changes in IL-1α expression in ECs and SMCs after co-culture with T cells, MHC class II expression was induced in both ECs and SMCs by pretreatment with 50 ng/ml IFN-γ for 3 d before co-culture. ECs or SMCs after co-culture or cytokine treatment were lysed in 50 mM Tris (pH 7.4), 300 mM NaCl, 10% glycerol, 3 mM EDTA, 1 mM MgCl$_2$, 20 mM β-glycerophosphate, 25 mM NaF, 1% Triton X-100, 25 μg/ml leupeptin, 25 μg/ml pepstatin, and 3 μg/ml aprotinin, and lysates normalized to a concentration of 1 mg/ml were analyzed by ELISA.

T cell activation in vitro. Class II transactivator–transduced ECs were pretreated with 10 ng/ml TNF (R&D Systems), as indicated in the figures, and were washed three times with HBSS to remove TNF. Purified memory CD4+ T cells were added at a density of 1–2 × 10⁶ cells/ml in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). For allogeneic restimulation assays, memory CD4+ T cells were co-cultured with allogeneic, MHC class II–positive ECs for 3 d, rested for 3 d with 10 U/ml IL-2, and restimulated with

fresh ECs from the same donor as used in the primary co-culture. For polyclonal restimulation assays, ECs transduced with a retrovirus encoding CD32, as previously described, were pretreated with TNF and overlayed with 1 μg/ml anti-CD3 for 30 min before co-culture with purified memory CD4+ T cells (38). After 3 d of co-culture, T cells were rested for 3 d with 10 U/ml IL-2 and restimulated with 10 ng/ml PMA and 1 μM ionomycin (Sigma-Aldrich) for 5 h in the presence of 10 μg/ml brefeldin A (Invitrogen), and were analyzed by ICS. T cells were restimulated with PMA and ionomycin. For extended primary co-cultures, purified memory CD4+ T cells were labeled with 250 nM CFSE (Invitrogen) and were added to microtiter plates containing allogeneic ECs at a T cell/EC ratio of 3:1. 48–72 replicate wells were plated in each experiment, and a neutralizing antibody to IL-1α (5 μg/ml) was added to half of the wells. Co-cultures were incubated for 14 d with 10 U/ml IL-2 added on days 3, 7, and 10. On day 14, co-cultures were restimulated with PMA and ionomycin, as described, and were analyzed by ICS.

Arterial transplantation. All experimental animal protocols were approved by the Yale University Institutional Animal Care and Use Committee. Arterial transplantation was performed as previously described (52). In brief, 1–3-mm segments of human epicardial coronary arteries from explanted hearts of cadaveric organ donors or cardiac transplant recipients were interposed into the infrarenal aortae of female SCID/beige mice (Taconic) using an end-to-end microsurgical anastomotic technique. Adjacent human artery segments were transplanted in groups of two to five mice for each experiment, and data from individual experiments were pooled to generate sufficient numbers for analysis.

Where indicated in the figures, 3 × 10⁸ human PBMCs allogeneic to the artery graft were adoptively transferred into mice intraperitoneally 2–7 d after artery transplantation. Based on a pilot experiment, we have found that varying the day of transfer within the first week after transplant does not significantly affect the rejection response. Successful engraftment, achieved in all mice in this study, was defined as detection by flow cytometry of a distinct population of human CD3+ cells, typically ranging from 0.5–10% of the total mononuclear cell population, in the circulation of mice 7–14 d after inoculation. For IL-1 blockade, mice with artery grafts were injected daily subcutaneously with 50 or 150 mg/kg IL-1Ra (Amgen) or with PBS as a control starting on the day before PBMC transfer. For IL-17 neutralization, animals were injected intraperitoneally with a neutralizing mouse anti–human IL-17 antibody (clone 41809; R&D Systems) or an irrelevant IgG2b antibody (clone 20116; R&D Systems), starting with a 250-μg loading dose the day before PBMC transfer and then a subsequent dose of 125 μg three times per week.

To harvest transplanted arterial grafts, animals were anesthetized and arterial grafts were perfused with normal saline and excised before death. Arterial grafts were frozen in optimum cutting temperature (OCT) compound, and serial 5-μm transverse sections were cut for morphometric, immunohistochemical, and RNA analyses. The graft from one mouse treated daily with PBS for 21 d was excluded from analyses because an intravascular thrombus was discovered upon graft harvest, and the graft displayed abnormal morphology with no demonstrable T cell infiltration or signs of rejection.

Skin grafting. Two human split thickness skin grafts ∼1 cm² in area were orthotopically transplanted onto the dorsum of CB.17 SCID/beige mice, as previously described (59). Grafts were allowed to heal for 6 wk, and only similarly sized, well-healed grafts were used for experiments. Skin grafts were injected intradermally with 20 μl PBS alone or PBS containing 1 μg of recombinant human IL-17, and were harvested 6 h later. Skin grafts were embedded in OCT, and serial 8-μm sections were cut for immunohistochemical and RNA analyses.

Histology and immunohistochemistry. Microscopic morphometric evaluation was performed on Elastica–van Gieson (EVG)–stained 5-μm cross sections of artery grafts using ImageJ software (available at http://rsbweb.nih.gov/ij/). Serial sections were stained with mouse mAbs against CD31 (Dako) or IL-1α (R&D Systems) using the avidin–biotin–peroxidase staining method (Vector Laboratories). Graft-infiltrating human lymphocytes were quantified by staining for human CD45RO (BD) or human CD3 (Dako).

In pilot experiments, these two markers yielded indistinguishable results and were used interchangeably. For skin grafts, mouse neutrophils were quantified by staining 8-μm sections with rat anti–mouse Gr-1 (BD), and E-selectin expression was evaluated using mouse anti–human E-selectin (58). CD161^{+} cells in artery grafts were detected by immunofluorescence microscopy using a mouse anti–human CD161 (Miltenyi Biotec) and an Alexa Fluor 488–conjugated anti–mouse secondary antibody (Invitrogen). Double staining was performed with the addition of a rabbit anti–human CD3 antibody (Dako) plus an Alexa Fluor 594–conjugated secondary antibody (Invitrogen). An FITC filter was used to detect Alexa Fluor 488–stained cells, a tetramethylrhodamine isothiocyanate filter was used to detect Alexa Fluor 594–stained cells, and a DAPI filter was used to detect nuclei. The numbers of intimal CD3^{+} and CD161^{+}/CD3^{+} cells were counted in six 40× (original magnification) fields per graft.

Quantitative RT-PCR. To isolate total RNA from artery and skin grafts, serial sections of artery or skin grafts were immersed briefly in water, centrifuged briefly, and then rapidly resuspended in RLT lysis buffer (QIAGEN). RNA was isolated from co-cultured T cells or tissue sections using RNeasy mini kits (QIAGEN) according to the manufacturer's protocol. RT with random hexamer and oligo-dT primers was performed according to the Multiscribe RT system protocol (Applied Biosystems). All RT-PCR reactions were prepared with TaqMan 2× PCR Master Mix and predeveloped assay reagents from Applied Biosystems. Samples were analyzed on an iCycler or iQ5 (Bio-Rad Laboratories). RNA samples processed without the RT enzyme were used as negative controls for all genes assayed. The expression level of each target was normalized to that of GAPDH unless otherwise indicated in the figures.

Microarray analysis. Preparation of cRNA from RNA isolated from SMCs and hybridization to the human genechip set (U133 Plus 2.0 Array; Affymetrix) were performed according to the manufacturer's protocol. The stained chips were read and analyzed with a GeneChip Scanner 3000 and expression console (Affymetrix). RNA levels were quantified, and gene-specific probe sets categorized as present or marginal were further analyzed, whereas those categorized as absent were excluded. Data analysis software (GeneSpring GX 7.3; Silicon Genetics) using the robust multiarray average was used for normalization and fold-change calculations. The microarray analyses were performed using three independent SMC lines. For the purposes of this study, genes encoding cytokines or chemokines that were regulated by IL-17 more than twofold relative to untreated controls were considered for further analysis. Microarray data have been deposited in the Gene Expression Omnibus under accession no. GSE11367.

Flow cytometric analysis. For cell-surface expression of HLA-DR, ICAM-1, and E-selectin (Beckman Coulter), ECs or SMCs were collected by trypsinization, incubated with the antibody in PBS/BSA for 30 min, fixed in 4% paraformaldehyde, and analyzed. For ICS, activated T cells were stained with allophycocyanin-conjugated anti-CD45 (eBioscience) and fixed in Cytofix/Cytoperm (BD). Intracellular cytokines were detected using PE-conjugated anti–IL-17, PE-Cy7–conjugated anti–IFN-γ, or PE-conjugated anti–IL-4 antibodies (all from eBioscience). Cells were analyzed on an LSR II with a High Throughput Sampler attachment (BD) using FlowJo software (Tree Star, Inc.).

Measurement of cytokine production. Supernatants were collected from cell-culture experiments, as indicated in the figures, and were assayed by ELISA for IL-1α (PeproTech), IFN-γ (Invitrogen), IL-5, and IL-17 (both from eBioscience), and IL-6, CXCL8, and CCL20 (all from R&D Systems), according to the manufacturer's instructions.

Statistical analysis. Data were analyzed by unpaired Student's *t* tests or by analysis of variance using a Tukey posttest correction when more than two groups were present. For in vitro alloreactivity analyses, the significance of fold changes calculated relative to the control group was evaluated using log-transformed values to allow for a normal distribution of the data. $P < 0.05$ was considered significant.

Online supplemental material. The online supplemental figures provide information on the differential ability of human allogeneic CD4^{+} T cells to induce IL-1α expression in human ECs and SMCs (Fig. S1), and the lack of effect of IL-1Ra and anti–IL-17A mAb on the adoptive transfer of human T cells into immunodeficient SCID/beige mice (Fig. S2). Table S1 lists all of the genes up- or down-regulated more than twofold by IL-17 in human smooth muscles, as detected by the Affymetrix microarray analysis. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081661/DC1.

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