Interleukin-17 Accelerates Allograft Rejection by Suppressing Regulatory T Cell Expansion

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Background—Interleukin-17 (IL-17), which is predominantly produced by T helper 17 cells distinct from T helper 1 or T helper 2 cells, participates in the pathogenesis of infectious, autoimmune, and allergic disorders. However, the precise role in allograft rejection remains uncertain. In the present study, we investigated the role of IL-17 in acute allograft rejection using IL-17-deficient mice.

Methods and Results—Donor hearts from FVB mice were heterotopically transplanted into either C57BL/6J-IL-17-deficient (IL-17−/−) or -wild-type mice. Allograft survival was significantly prolonged in IL-17−/− recipient mice due to reduced local inflammation accompanied by decreased inflammatory cell recruitment and cytokine/chemokine expression. IL-17−/− recipient mice exhibited decreased IL-6 production and reciprocally enhanced regulatory T cell expansion, suggesting a contribution of regulatory T cells to prolonged allograft survival. Indeed, allografts transplanted into anti-CD25 mAb-treated IL-17−/− recipient mice (regulatory T cell-depleted) developed acute rejection similar to wild-type recipient mice. Surprisingly, we found that gamma delta T cells rather than CD4+ and CD8+ cells were key IL-17 producers in the allografts. In support, equivalent allograft rejection was observed in Rag-2−/− recipient mice engrafted with either wild-type or IL-17−/− CD4+ and CD8+ T cells. Finally, hearts transplanted into gamma delta T cell-deficient mice resulted in decreased allograft rejection compared with wild-type controls.

Conclusions—During heart transplantation, (1) IL-17 is crucial for acceleration of acute rejection; (2) IL-17-deficiency enhances regulatory T cell expansion; and (3) gamma delta T cells rather than CD4+ and CD8+ T cells are a potential source of IL-17. IL-17 neutralization may provide a potential target for novel therapeutic treatment for cardiac allograft rejection. (Circulation. 2011;124[suppl 1]:S187–S196.)

Key Words: acute allograft rejection ■ γδ ■ IL-17 ■ regulatory T cell ■ T cell

Despite advances in immunosuppressive agents, acute and chronic allograft rejection remains the major causes of graft failure after cardiac transplantation.1 During the first months after cardiac transplantation, almost 40% of adults will have at least 1 acute rejection episode with most patients experiencing rejection in the first 6 months.2 Acute allograft rejection is a T cell-dependent phenomenon and may be triggered by different types of helper T cells. T helper 1 (Th1) responses initiate allograft rejection by promoting cytotoxic T cell activities and interferon-γ (IFN-γ)-mediated delayed-type hypersensitivity reactions,3–5 whereas Th2 responses cause allograft damage through the recruitment of eosinophils induced by interleukin-4 (IL-4) and IL-5.6–7 Although Th1 responses were traditionally thought to play a central role in the development of acute allograft rejection,6,7 recent studies have reported that IFN-γ may not be essential for these responses, suggesting that other cytokines may contribute to the inflammation cascade and facilitate rejection.8,9

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Th17 cells is a newly identified T cell lineage that secretes the proinflammatory cytokine IL-17. Naive CD4+ T cells differentiate into Th17 cells in the presence of IL-6 and transforming growth factor-β. Th17 cells express IL-23 receptor, and IL-23, an IL-12 family member, is critical for their survival and proliferation. Orphan nuclear receptor ROR-γt is the key transcription factor that orchestrates differentiation of the Th17 lineage. IL-17 activates inflammatory, endothelial, and epithelial cells and induces a variety of proinflammatory cytokines, chemokines, and adhesion molecules. Excessive/inappropriate IL-17 production and/or Th17 cell activation have been reported to be involved in the development of autoimmune and allergic diseases, including arthritis, multiple sclerosis, contact dermatitis, and neutrophilic nonatopic asthma.

Recent clinical and experimental transplantation studies have suggested the involvement of IL-17 in allograft rejection. Increased IL-17 mRNA and protein levels were observed in patients with acute rejection after lung and kidney transplantation. In addition, IL-17 inhibition through administration of a recombinant soluble IL-17 (IL-17R-Fc) receptor blocking antibody attenuated acute rejection in heart and aortic transplant rodent models. However, the precise role of IL-17 in acute allograft rejection remains unclear. For example, multiple cell types can produce IL-17; therefore, it is conceivable that cell types other than Th17 are involved in these inflammatory responses. Furthermore, despite a reciprocal developmental pathway existing for the generation of pathogenic effector Th17 versus regulatory CD4+ T cells (Treg), the effect of IL-17 on Treg development in the cardiac transplantation milieu still remains poorly understood. Accordingly, in the present study, we used genetically IL-17 (IL-17A)-deficient C57BL/6 recipient mice to investigate the biological importance of IL-17 in acute allograft rejection after cardiac transplantation in mice.

**Methods**

**Animals**

C57BL/6J (H-2b), FVB (H-2q), and C57BL/6 TCRγ−/− mice were purchased from Jackson Laboratory (Bar Harbor, ME) or SLC Japan (Hamamatsu, Japan). C57BL/6-Rag−2−/− mice were obtained from Taconic (Hudson, NY). C57BL/6-IL-17−/−
mice were generated as described elsewhere.24 Male mice (6 to 10 weeks) were used in experiments. Animals were maintained in the animal care facility at Stanford University or Tokyo Medical University, and all experiments were approved by the Stanford University Institutional Animal Care and Use Committee or by Tokyo Medical University and performed in accordance with the Guide for the Care and Use of Laboratory Animals.25

Cell Culture
For mixed lymphocyte reaction, inguinal, axillary, and brachial lymph nodes cells from C57BL/6J-wild-type (WT) and IL-17−/− mice were prepared as responder cells. As stimulator cells, T cell-depleted spleen cells were prepared. Splenocytes from C57BL/6 or FVB mice were incubated with biotinylated antimouse CD4 (GK1/5; eBioscience, San Diego, CA) and antimouse CD8 (53 to 6.7; eBioscience) at 4°C for 15 minutes and then incubated with Streptavidin Particles Plus-DM (BD Biosciences, San Jose, CA) at 4°C for 15 minutes. T cell-depleted spleen cells were separated by the iMag system (BD Biosciences) and treated with mitomycin C (Sigma, St Louis, MO) at 37°C for 15 minutes. T cell-depleted spleen cells were separated by the iMag system (BD Biosciences) and treated with mitomycin C (Sigma, St Louis, MO) at 37°C for 10 minutes. Lymph node cells (5×10^5 cells/well) were cocultured with mitomycin C-treated T cell-depleted splenocytes (3×10^5 cells/well) in a 96-well flat-bottomed plate (200 µL/well) at 37°C for 72 hours. The proliferative responses were determined by using Cell Titer 96 AQ (Promega, Madison, WI).

For IL-17 stimulation, Rag-2−/− splenocytes (5×10^5 cells/well) were cultured in the presence or absence of rmIL-17 (R&D System, Minneapolis, MN), rmIL-1β (PeproTech, Rocky Hill, NJ), and/or rmTNF (PeproTech) in a 96-well plate at 37°C for 72 hours. IL-6 levels in the culture supernatants were determined by a Mouse IL-6 Enzyme-Linked Immunosorbent Assay Kit (eBioscience).

Heterotopic Heart Transplantation
Donor hearts were implanted into the recipient abdomen as described previously.26 Graft viability assessed by daily abdominal palpation. Allografts were harvested on postoperative Days (POD) 1, 3, 7, 50, and/or 100. For Treg depletion, IL-17−/− mice were intraperitoneally injected with 500 µg of antimouse CD25 mAb (PC61) or control rat IgG (Jackson ImmunoResearch, West Grove, PA) 1 day before transplantation. For adoptive T cell transfer, Rag-2−/− mice were intravenously injected with CD3 T cells (1×10^7 cells) purified from WT and IL-17−/− splenocytes as described subsequently. Splenocytes were incubated with biotinylated antimouse B220 (RA3-6B2), CD11b (M1/70), CD11c (HL3), CD25 (PC61.5), CD49b (DX5), CD119 (2B8), FceR1a (MAR-1), Gr-1 (RB6-8C5), Ter119 (Ter119), and γδ TCR (GL3) mAbs at 4°C for 15 minutes. These antibodies were obtained from eBioscience and BD Biosciences.
ences. After washing, the cells were incubated with Streptavidin Particles Plus-DM (BD Biosciences). Then CD3+ T cells (>95%) were isolated by negative selection using a BD IMag system (BD Biosciences).

Cytokine and Chemokine Analysis

For quantitative real-time polymerase chain reaction, total RNA in allografts was isolated using TRIzol (Invitrogen, Carlsbad, CA). Using the isolated RNA, cDNA was obtained by reverse transcription–polymerase chain reaction with a SuperScript First-Strand cDNA synthesis kit (Invitrogen). Then, a TaqMan real-time polymerase chain reaction assay was performed with the ABI Prism 7900 HT real-time Polymerase Chain Reaction System (Applied Biosystems, Carlsbad, CA). The primers for mouse glyceraldehyde-3-phosphate dehydrogenase, IFN-γ, tumor necrosis factor, IL-4, IL-6, IL-17, and IL-23 were purchased from Applied Biosystems. All samples were run in duplicate, and expression levels of these cytokines were normalized to corresponding glyceraldehyde-3-phosphate dehydrogenase expression. For protein measurement, protein concentration of allograft supernatants was determined by a bicinchoninic acid method. The levels of cytokines and chemokines in allograft supernatants were evaluated by a 26-plex mouse cytokine detection kit (Luminex; Millipore, Billerica, MA).

Flow Cytometry Analysis

Fluorescein isothiocyanate, phycoerythrin, or allophycocyanin-conjugated mAbs specific for mouse CD4 (GK1.5), CD8a (53-6-7), CD11b (M1/70), B220 (RA3-6B2), Gr1 (RB6-8C5), γδ TCR (GL3), CD45 (30-F11), Foxp3 (FJK-16s), IFN-γ (XMGI.2), IL-17 (TC11-18H10), and their isotype controls were purchased from BD Biosciences, eBioscience, or BioLegend (San Diego, CA). Cells in alloantigenic response and related proinflammatory cytokines in FVB donor hearts transplanted into C57BL/6 WT recipient mice during acute rejection, the levels of cytokines and chemokines in the homogenates of allografts (FVB hearts) transplanted into B6-WT and B6-IL-17−/− recipient mice on POD 7 were determined by Luminex system and presented as box plot graphs (each group: n=8). The line in the middle of the box represents the median. The lower and the upper edges of the box are the first and third quartiles, respectively. The whiskers show the remainder of the distribution (1.5×interquartile range). Outliers are shown as filled circles. *P<0.05 versus corresponding values for B6-WT recipients. IL-17 indicates interleukin-17; WT, wild-type; POD, post-operative day.

Results

Increased IL-17 Expression in Cardiac Allografts During Acute Rejection

To investigate the role of IL-17 in acute cardiac allograft rejection, we first examined the time course of IL-17 expression and related proinflammatory cytokines in FVB donor hearts transplanted into the C57BL/6 WT recipient mice (MHC full-mismatch; naïve heart: n=7; other time points: n=5 to 7). Increased mRNA expression of IL-6, IL-17, and IL-23 were observed in allografts on POD 1 (Figure 1A). By POD3, IFN-γ, IL-4, and tumor necrosis factor mRNA expression was also significantly upregulated (Figure 1A). Although IL-17 mRNA levels did not change significantly from POD1 to POD7, IL-23 mRNA was reduced after POD1 (Figure 1A). Overall, the expression of IFN-γ, tumor necrosis factor, and IL-6 mRNA was highest on POD 7 (Figure 1A).

After isolating graft infiltrating cells, total cell counts and profiles were characterized by flow cytometry (naïve heart:...
n=3; POD 3: n=3; POD 7: n=4). As shown in Figure 1B, the total number of CD4\(^+\), CD8\(^+\), CD11b\(^+\), and CD45\(^+\) cells was gradually increased on POD3 and then substantially increased by POD7. Gr1\(^+\) cells were upregulated on POD 3 and reached statistical significance by POD 7. Additionally, an increased number of IFN-\(\gamma\) and IL-17\(^+\) graft-infiltrating cells was observed by POD 7 (Figure 1B).

**IL-17 Accelerates Acute Rejection During Cardiac Transplantation**

To elucidate the contribution of IL-17 to the pathogenesis of acute rejection, donor hearts from FVB mice were transplanted into C57BL/6-WT and IL-17\(^{-/-}\) mice. Cardiac allograft survival was significantly prolonged in the donor hearts transplanted into IL-17\(^{-/-}\) recipients (median survival: 18 days; n=20) compared with WT recipients (median survival: 10 days; n=20; P<0.001, log-rank test; Figure 2A). Graft survival in almost all WT recipients (19 of 20) was ≤11 days, whereas a single allograft (1 of 20) survived 12 days. Of note, 30% of the allografts transplanted into IL-17\(^{-/-}\) recipients (6 of 20) showed remarkably prolonged graft survival (>50 days after transplantation and still beating at last follow-up), 45% (9 of 20) showed moderately prolonged graft survival (12 to 30 days), and the remaining 25% (5 of 20) showed shorter graft survival (8 to 11 days).

Although the importance of IL-17 in acute rejection could be attributed to its role in enhancing dendritic cell maturation and/or antigen-specific T cell activation,\(^{32}\) we found that the alloantigen-specific proliferative response of C57BL/6J-IL-17\(^{-/-}\) lymph node cells cocultured with FVB spleen cells were similar to C57BL/6J-WT lymph node cells (Figure 2B). These findings suggest that IL-17 may be important for the induction of inflammation at the local site rather than activation of alloantigen-specific T cells in lymph nodes. Supporting this hypothesis, local inflammation in allografts transplanted into IL-17\(^{-/-}\) recipients was decreased compared with that into WT recipients on POD 7 (Figure 2C). Although the percentage of CD4\(^+\), CD8\(^+\), and CD11b\(^+\) cells was similar between the 2 groups, the total number of CD4\(^+\), CD8\(^+\), and CD11b\(^+\), and Gr1\(^+\) in allografts transplanted into IL-17\(^{-/-}\) recipient mice was significantly reduced compared with WT on POD 7 (each group: n=6; Figure 2D–E). The frequency and total number of B220\(^+\) cells were similar between the 2 groups (data not shown).

Correspondingly, the levels of proinflammatory cytokines (IL-1\(\beta\), IL-6, IL-12p40, and tumor necrosis factor) and chemokines (CXCL1/KC and CCL2/monocyte chemotactic protein-1) were also significantly decreased in the homogenates from allografts transplanted into IL-17\(^{-/-}\) recipient mice, whereas the levels of IFN-\(\gamma\), IL-10, IL-23, and...
transforming growth factor-β1 were similar between groups (each group: n=8; Figure 3). In contrast to the mRNA and fluorescence-activated cell sorter analyses (Figure 1A), the levels of IL-17 protein were below the limit of detection of Luminex systems in both groups (data not shown). These results indicate that IL-17 accelerates acute rejection by enhancing local inflammation.

γδ T Cells Are Key IL-17-Producing Cells During Acute Rejection

To determine the source of IL-17, graft infiltrating cells were isolated on POD 2 and POD 7 and characterized through intracellular cytokine staining. On POD 2, a small number of IL-17-producing CD4^+^, CD8^+^, and γδ T cells were identified (Supplemental Figure I; http://circ.ahajournals.org). On POD 7, the proportions of IFN-γ-producing CD45^+^ cells were similar in the donor graft and spleen between WT mice and IL-17^-/-^ mice (Figure 4A). Interestingly, few IL-17-producing CD4^+^ and CD8^+^ T cells were detectable even in cardiac allografts transplanted into WT recipient mice (Figure 4B). On the other hand, we discovered that γδ T cells preferentially produced IL-17 in the allografts from WT recipients (Figure 4C-D), although the number of γδ T cells in cardiac allografts was comparable between WT and IL-17^-/-^ recipient mice (each group: n=6; Figure 4E).

Corroborating this finding, allografts transplanted into Rag-2^-/-^ mice, which had been previously injected with γδ T cell-depleted, CD4^-^ and CD8^-^ T cells from IL-17^-/-^ mice (median survival: 9 days; n=8), had similar severity of acute rejection compared with those hearts transplanted into Rag-2^-/-^ mice injected with γδ T cell-depleted CD4^-^ and CD8^-^ T cells from WT mice (median survival: 8.5 days; n=8; P=0.83, log-rank test; Figure 5). Of note, allograft survival was significantly prolonged in intact nonreconstituted Rag-2^-/-^ mice (n=8, P<0.001, log-rank test; Figure 5), as reported previously.28 Finally, to confirm that γδ T cell-derived IL-17 production was important for the development of acute rejection, donor hearts were transplanted into γδ TCR-deficient recipients. Importantly, 17% of the allografts transplanted into γδ TCR-deficient recipients (3 of 18) showed remarkably prolonged graft survival (>30 days after transplantation and still beating at last follow-up), 28% (5 of 18) showed moderately prolonged graft survival (12 to 30 days), and the remaining 55% (10 of 18) showed shorter graft survival (8 to 11 days). As a result, graft survival was significantly prolonged in γδ TCR-deficient recipients compared with WT controls (median survival: 10 days; n=20 versus γδ TCR^-/-^, median survival: 11 days; n=18, P=0.02, log-rank test). Although not as effective as the IL-17^-/-^ recipients (median survival: 18 days; n=20), the difference was not significant (P=0.08, log-rank test; Figure 6). These experiments suggest that γδ T cell IL-17 production (not CD4/CD8^-^ T cell-derived IL-17 alone) participates in the development of acute cardiac allograft rejection.

Prolonged Allograft Survival Is Associated With Enhanced Treg Expansion

Tregs have been described to prolong allograft survival in both acute and chronic rejection.29 We also found that the number of CD4^-^CD25^-^Foxp3^-^ Tregs was increased in the allografts transplanted into IL-17^-/-^ recipient mice with prolonged survival (Figure 7A–B). Of note, Tregs were not significantly upregulated in allografts from WT recipients; however, the allografts survive only approximately 9 days.

Several investigators have established that IL-6 inhibits Treg differentiation, yet induces Th17 cells, in the presence of transforming growth factor-β1.30 Reciprocally, IL-17 can induce IL-6 production by various types of cells such as fibroblasts in the presence and/or absence of tumor necrosis factors.
factor and/or IL-1.\textsuperscript{17,31} In our model system, IL-1β, tumor necrosis factor, IL-6, but not transforming growth factor-β1, was reduced in allografts transplanted into IL-17\textsuperscript{-/-} recipient mice (Figure 3). We also noted that the combination of IL-17, IL-1β, and tumor necrosis factor promotes the largest significance increase in IL-6 production by splenocytes from Rag-2\textsuperscript{-/-} mice in vitro (Figure 7C). Therefore, IL-17 deficiency may result in enhanced Treg expansion through IL-6 reduction by a non-T/B cell population, contributing to the prolongation of allograft survival. Supporting this hypothesis, allografts transplanted into anti-CD25 mAb-injected, Treg-depleted IL-17\textsuperscript{-/-} recipient mice resulted in more severe acute rejection compared with control IgG-injected IL-17\textsuperscript{-/-} recipient mice (control IgG, median survival: 11 days; n=8 versus anti-CD25 mAb, median survival: 8 days; n=8, $P=0.005$, log-rank test; Figure 8). These observations suggest that increased Treg cell expansion contributed to the prolongation of allograft survival in IL-17\textsuperscript{-/-} recipient mice.

We and other investigators have previously shown that IL-17 contributes to the development of chronic rejection or cardiac allograft vasculopathy.\textsuperscript{32,33} However, the precise role of IL-17 in acute cardiac transplant rejection still remains unclear, although a recent study reported that IL-17 blockade with an anti-IL-17 antibody resulted in prolongation of allograft survival in a skin transplantation model.\textsuperscript{34} In the present study, using C57BL/6J-IL-17\textsuperscript{-/-} recipient mice, we demonstrated that (1) IL-17 expression is upregulated in cardiac allografts during acute rejection; (2) IL-17 deficiency significantly attenuates acute cardiac allograft rejection by decreasing graft inflammatory cell infiltration and cytokine and chemokine production; (3) γδ T cell-derived IL-17 production participates in the development of acute rejection; and (4) IL-17 deficiency promotes Treg expansion, resulting in the attenuation of acute rejection.

In contrast to the findings reported in this study, Gorbacheva et al\textsuperscript{35} recently reported that IL-17 deficiency did not

**Discussion**
modify cardiac allograft survival despite reduced CXCL1 and CXCL2 expression and delayed allograft neutrophil and T cell recruitment; importantly, these findings were in a different strain combination (A/J donor and BALB/c IL-17−/− recipients). Similarly, C57BL/6 corneal allograft survival was comparable between BALB/c-WT and IL-17−/− recipient mice, although the onset of rejection was delayed in BALB/c-IL-17−/− mice.36 Finally, using murine graft versus host disease model systems, varied immune responses have been observed between BALB/c and C57BL/6 mice; injection of C57BL/6-splenocytes (H-2b) to BDF1 mice or CBF1 mice (H-2bkd) results in the development of acute graft versus host disease, whereas injection of DBA/2- or BALB/c-splenocytes (H-2d) to BDF1 or CBF1 mice results in chronic graft versus host disease.37,38 Therefore, phenotypic differences in distinct strain combinations likely explain the disparate role of IL-17 in acute rejection in heart transplants. An alternative explanation includes absence of immunosuppression in our model system. Immunosuppressive agents may directly alter IL-17 production as well as inhibit regulatory signals by Tregs, thereby explaining the dissimilar observations.

In the present study, mRNA IL-17A expression in the allografts was upregulated as early as POD 1 despite the small number of graft infiltrating cells. Using a different strain combination, Gorbacheva et al also detected early IL-17 mRNA expression in allografts on POD 2, reaching peak levels by Day 4 and declining by the time of graft rejection.35 Because IL-17 mRNA was only slightly increased in syngenic grafts, alloantigen recognition seems to be required for local IL-17 production.35 Similar to our findings, early IL-17 mRNA was detected in the setting of very few graft inflammatory cells.35

Interestingly, IL-17 produced by CD4+ and/or CD8+ T cells has been reported to accelerate acute and chronic allograft rejection in T-bet−/− mice.33,39,40 Conversely, we hypothesize that IL-17 produced by γδ T cells is important for the development of acute rejection, whereas CD4+ and CD8+ T cell-derived IL-17 alone is not sufficient when adoptively transferred to Rag-2−/− murine recipients. This theory is further supported by (1) IL-17 being preferentially produced by γδ T cells rather than CD4+ and CD8+ T cells; and (2) γδ T cell-deficient recipient mice showing prolonged cardiac allograft survival compared with controls.41 In addition to CD4+ and CD8+ T cells, several lines of evidence show that γδ T cell-derived IL-17 plays pathogenic roles in various diseases such as infectious and autoimmune diseases.42–45 As well, γδ T cell-derived IL-17 has been shown to participate in the pathogenesis of ischemia–reperfusion injury in the brain.46 Intriguingly, ischemia–reperfusion injury is also a well-known alloantigen-independent risk factor for acute allograft rejection and subsequent cardiac allograft vasculopathy.47,48 Although not directly tested in this study, other IL-17-producing cells likely participate during the pathogenesis, because the acute allograft rejection noted in γδ T cell-deficient mice was not as severe as WT controls. These findings support potential development of immunosuppressive agents directed at γδ T cells.

Tregs are potent immunosuppressive cells reported to prolong allograft survival.29,49 In the present study, we found that prolonged allograft survival in IL-17−/− recipient mice was associated with increased Tregs and decreased IL-6 expression in allografts. IL-6 is known to induce IL-17-producing Th17 cell differentiation, yet reciprocally inhibit Treg differentiation in the presence of transforming growth factor-β.11,12 Corroborating these findings, Lei et al recently reported that anti-IL-6 mAb treatments resulted in the down-regulation of Th17, expansion of Tregs, and consequently, prolongation of cardiac allograft survival.50 In addition to synovial fibroblasts, chondrocytes, and osteoblasts,15 we found that IL-17 could enhance IL-6 secretion by non-T or B immune cells when in the presence of IL-1β and/or tumor necrosis factor. Similar to reported arthritic models,51 IL-6 may act downstream of IL-17, contributing to the acceleration of acute allograft rejection not only (1) enhancing inflammation as a proinflammatory cytokine; but also (2) inhibiting Treg differentiation.

In summary, we clearly demonstrate that IL-17 plays an important role in acute rejection during cardiac transplantation. In this model system, γδ T cells appear to be the key IL-17 producers. Finally, prolonged graft survival in the absence of IL-17 may be related to increased allograft Treg expansion. Overall, these findings suggest that neutralization of IL-17 may be a potential therapeutic target for acute allograft rejection.

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Disclosures
None.

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On line Supplement Figure I.

IL-17 expression profiles of T cells from FVB donor heart transplanted into C57BL/6-WT recipient mice on POD 2.