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+ T 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,3,5 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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Presented at the 2010 American Heart Association meeting in Chicago, IL, November 12–16, 2010.

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Circulation is available at http://circ.ahajournals.org

DOI: 10.1161/CIRCULATIONAHA.110.014852

S187
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-H-2d T cell (TCR γ−) 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-J-IL-17−/−
mice were generated as described elsewhere. 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.

**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 10 minutes. T cell-depleted spleen cells were incubated with biotinylated antimouse CD4 (GK1/5; eBioscience) 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 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).

**Heterotopic Heart Transplantation**

Donor hearts were implanted into the recipient abdomen as described previously. 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 CD220, CD11b, CD11c, CD49b, CD119, and/or CD40 (BD Biosciences) 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 the 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).

Histology

Grafts (POD 7 or 100) were fixed in 20% buffered formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin. The frozen sections of grafts (POD 100) were prepared and incubated with rat antimouse Foxp3 (FJK-16a; eBioscience) after blocking. Then, the sections were incubated with Alexa Flour 488-conjugated goat antirat IgG (Invitrogen). Images were captured using a confocal laser scanning microscope (DM400B; LEICA, St Gallen, Switzerland).

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), γδTCR (GL3), CD45 (30-F11), Foxp3 (FJK-16a), IFN-γ (XMG1.2), IL-17 (TC11-18H10), and their isotype controls were purchased from BD Biosciences, eBioscience, or BioLegend (San Diego, CA). Cells in allografts were prepared by digestion with 2 mg/mL Collagenase D (Worthington Bio, Lakewood, NJ) and 10% fecal calf serum in RPMI 1640 media for 2 hours at room temperature. After filtration, cells were first incubated with inactivated normal hamster and mouse sera (Jackson ImmunoResearch) and 5 μg/mL anti-CD16/32 mAb (2.4G2; BD Bioscience), then stained with antimonium CD4, CD8, CD11, B220, γδTCR, CD45, and/or CD25 mAb for 30 minutes at 4°C. Subsequently, 7-amino-actinomycin D (BD Bioscience) was added and incubated for 10 minutes to exclude the dead cells. Then, cells were fixed and permeabilized with PharMingen Perm/Fix solution (BD Bioscience) for 30 minutes at 4°C and incubated with antimouse IFN-γ, IL-17, or Foxp3 mAb. Expression of markers was determined by FACS Calibur (BD Bioscience) and FlowJo software (Tree Star, Ashland, OR).

Statistical Analysis

Statistical analyses were performed with SPSS 17.0 for Windows software (SPSS, Inc, Chicago, IL). Graft survival data were plotted by the Kaplan-Meier method using the log-rank test to analyze differences in graft survival between groups. Comparisons between 2 groups were performed with the use of the Mann-Whitney rank sum test. Multiple comparisons were addressed using Bonferroni correction. P<0.05 was considered statistically significant.

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 was observed in allografts on POD 7 (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-γ 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,\(^2^\) 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^+ cells was similar between the 2 groups, the total number of CD4^+, CD8^+, and CD11b^+ cells was significantly reduced compared with WT on POD 7 (each group: n=6 to 8; 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-1β, IL-6, IL-12p40, and tumor necrosis factor) and chemokines (CXCL1/KC and CCL2/monocyte chemoattractant protein-1) were also significantly decreased in the homogenates from allografts transplanted into IL-17^--/-- recipient mice, whereas the levels of IFN-γ, 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

**Figure 6.** Graft survival was significantly prolonged in γδ TCR-deficient recipients compared with wild-type controls, although not as effective as the IL-17−/− recipients. Allograft survival after transplantation of FVB hearts into C57BL/6J-wild-type (B6-WT), IL-17-deficient (B6-IL-17−/−; each group: n=20) or γδ TCR-deficient (B6-TCRγγ−/−; n=18). Graft survival was significantly prolonged in γδ TCR-deficient recipients compared with wild-type controls (P=0.02, log-rank test). Although not as effective as the IL-17−/− recipients, the difference did not reach statistical significance (P=0.08, log-rank test). IL-17 indicates interleukin-17.

**Figure 5.** CD4+ and CD8+ T cell-derived IL-17 is not crucial for acute rejection. Allograft survival after transplantation of FVB hearts into Rag-2−/− recipient mice and Rag-2−/− recipient mice, which had been previously transferred with γδ T cell-depleted CD4+ and CD8+ T cells from B6-WT or B6-IL-17−/− mice (each group: n=8). *P<0.05. IL-17 indicates interleukin-17; WT, wild-type.
factor and/or IL-1. 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−/− 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−/− 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−/− recipient mice resulted in more severe acute rejection compared with control IgG-injected IL-17−/− 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−/− recipient mice.

**Discussion**

We and other investigators have previously shown that IL-17 contributes to the development of chronic rejection or cardiac allograft vasculopathy. 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. In the present study, using C57BL/6J-IL-17−/− 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 recently reported that IL-17 deficiency did not
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/cA 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. 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-2b/d) 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. 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 and/or CD8 T cells when in the presence of IL-1 cytokines. Similar to reported arthritic models, 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.

**Sources of Funding**

This work was supported by the Falk Research Fund for the Department of Cardiothoracic Surgery at Stanford University Medical School, Stanford, CA; American Association for Thoracic Surgery (Norman E. Shumway award to M.P.F.); by grants from the National Institute of Biomedical Innovation (ID 05-24; H.S.); by Jichi Medical University Young Investigator Fellowship Award (S.I.); by Grants-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (S.I.); and by Program for Improvement of Research Environment for Young Researchers, The Special Coordination Funds for Promoting Science and Technology of the Ministry of Education, Culture, Sports, Science, and Technology (S.N.).

**Figure 8.** Tregs contribute to the prolonged allograft survival in IL-17−/− recipient mice. Allograft survival after transplantation of FVB hearts into B6-IL-17−/− recipient mice treated with anti-CD25 mAb or control IgG (each group; n=8). *P<0.05. Tregs indicate regulatory T cells; IL-17, interleukin-17.
Disclosures

None.

References


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Circulation. 2011;124:S187-S196
doi: 10.1161/CIRCULATIONAHA.110.014852

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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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.