CCL17 Aggravates Myocardial Injury by Suppressing Recruitment of Regulatory T Cells

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BACKGROUND: Recent studies have established that CCR2 (C-C chemokine receptor type 2) marks proinflammatory subsets of monocytes, macrophages, and dendritic cells that contribute to adverse left ventricle (LV) remodeling and heart failure progression. Elucidation of the effector mechanisms that mediate adverse effects of CCR2+ monocytes, macrophages, and dendritic cells will yield important insights into therapeutic strategies to suppress myocardial inflammation.

METHODS: We used mouse models of reperfused myocardial infarction, angiotensin II and phenylephrine infusion, and diphtheria toxin cardiomyocyte ablation to investigate CCL17 (C-C chemokine ligand 17). We used Ccl17 knockout mice, flow cytometry, RNA sequencing, biochemical assays, cell trafficking studies, and in vivo cell depletion to identify the cell types that generate CCL17, define signaling pathways that controlled its expression, delineate the functional importance of CCL17 in adverse LV remodeling and heart failure progression, and determine the mechanistic basis by which CCL17 exerts its effects.

RESULTS: We demonstrated that CCL17 is expressed in CCR2+ macrophages and cluster of differentiation 11b+ conventional dendritic cells after myocardial infarction, angiotensin II and phenylephrine infusion, and diphtheria toxin cardiomyocyte ablation. We clarified the transcriptional signature of CCL17+ macrophages and dendritic cells and identified granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling as a key regulator of CCL17 expression through cooperative activation of STAT5 (signal transducer and activator of transcription 5) and canonical NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) signaling. Ccl17 deletion resulted in reduced LV remodeling, decreased myocardial fibrosis and cardiomyocyte hypertrophy, and improved LV systolic function after myocardial infarction and angiotensin II and phenylephrine infusion. We observed increased abundance of regulatory T cells (Tregs) in the myocardium of injured Ccl17 knockout mice. CCL17 inhibited Treg recruitment through biased activation of CCR4. CCL17 activated Gq signaling and CCL22 (C-C chemokine ligand 22) activated both Gq and ARRB (β-arrestin) signaling downstream of CCR4. CCL17 competitively inhibited CCL22 stimulated ARRB signaling and Treg migration. We provide evidence that Tregs mediated the protective effects of Ccl17 deletion on myocardial inflammation and adverse LV remodeling.

CONCLUSIONS: These findings identify CCL17 as a proinflammatory mediator of CCR2+ macrophages and dendritic cells and suggest that inhibition of CCL17 may serve as an effective strategy to promote Treg recruitment and suppress myocardial inflammation.

Key Words: chemokine CCL17 ◼ dendritic cells ◼ inflammation ◼ macrophages ◼ monocytes ◼ T-lymphocytes, regulatory

Myocardial inflammation is associated with heart failure incidence and progression in multiple patient populations.1,2 After myocardial infarction (MI), peripheral monocyte abundance and expression of proinflammatory cytokines and chemokines predict adverse left ventricle (LV) remodeling and cardiovascular mortality. In the context of chronic heart failure, cytokine levels are associated with heart failure severity and...
Clinical Perspective

What Is New?
- CCL17 (C-C chemokine ligand 17) is an inflammatory mediator expressed in CCR2+ (C-C chemokine receptor type 2) macrophages and CD11b+ conventional dendritic cells recruited to the heart after myocardial injury.
- Granulocyte-macrophage colony-stimulating factor signaling is a key regulator of CCL17 expression through cooperative activation of STAT5 (signal transducer and activator of transcription 5) and canonical NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling.
- Ccl17 deletion attenuates inflammatory cytokine/chemokine expression, left ventricular remodeling, and myocardial fibrosis in mouse models of reperfused myocardial infarction and hypertrophy through increased recruitment of regulatory T cells to the injured heart.

What Are the Clinical Implications?
- Identification of effector molecules that mediate adverse effects of CCR2+ monocytes, macrophages, and dendritic cells provides insight into therapeutic strategies to suppress myocardial inflammation.
- Inhibition of CCL17 expression or neutralization of CCL17 signaling may serve as an effective strategy to promote regulatory T-cell recruitment and suppress myocardial inflammation.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>AKT</td>
<td>protein kinase B</td>
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<tr>
<td>AngII/PE</td>
<td>angiotensin II and phenylephrine</td>
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<tr>
<td>ARRB</td>
<td>β-arrestin</td>
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<tr>
<td>CCL17</td>
<td>C-C chemokine ligand 17</td>
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<tr>
<td>CCL22</td>
<td>C-C chemokine ligand 22</td>
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<tr>
<td>CCR2</td>
<td>C-C chemokine receptor type 2</td>
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<tr>
<td>CCR4</td>
<td>C-C chemokine receptor type 4</td>
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<tr>
<td>DT</td>
<td>diphtheria toxin</td>
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<tr>
<td>DTR</td>
<td>diphtheria toxin receptor</td>
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<tr>
<td>FOXP3</td>
<td>forkhead box P3</td>
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<td>G1</td>
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<td>gate 3</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>IR</td>
<td>ischemia–reperfusion</td>
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<tr>
<td>JAK2</td>
<td>Janus kinase 2</td>
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<tr>
<td>LV</td>
<td>left ventricle</td>
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<tr>
<td>LY6C</td>
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<td>LY6G</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MHC II</td>
<td>major histocompatibility complex II</td>
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<tr>
<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
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<tr>
<td>STAT5</td>
<td>signal transducer and activator of transcription 5</td>
</tr>
<tr>
<td>Tnnt2</td>
<td>troponin T2</td>
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<tr>
<td>Treg</td>
<td>regulatory T cell</td>
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<tr>
<td>ZBTB46</td>
<td>zinc finger and BTB domain-containing protein 46</td>
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Chemokines play an important role in heart inflammation by directing trafficking of infiltrating leukocytes and thus represent promising therapeutic targets.3,17 CCL17 has previously been reported to influence helper T cell and regulatory T cell (Treg) recruitment by activating the G protein–coupled CCR4 (C-C chemokine receptor type 4).1,18 CCL17 is associated with inflammatory diseases including allergic asthma,20 dermatitis,21 colitis,22 arthritis,23 and atherosclerosis.24 CCL17 is yet to be investigated within the heart.

We used mouse models of reperfused MI, angiotensin II and phenylephrine (AngII/PE) infusion, and diphtheria toxin (DT) cardiomyocyte ablation to investigate the role of CCL17 in heart failure pathogenesis. We reveal that CCL17 is expressed in CCR2+ macrophages and CD11b+ conventional dendritic cells that infiltrate the heart and are marked by the expression of CCR2 (C-C chemokine receptor type 2).11,12 Inhibition of monocyte recruitment is sufficient to prevent accumulation of proinflammatory CCR2+ monocytes, macrophages, and dendritic cells and suppress adverse LV remodeling across heart failure models.13-15 The effector mechanisms that mediate adverse effects of CCR2+ monocytes, macrophages, and dendritic cells remain poorly defined.

We previously identified CCL17 (C-C chemokine ligand 17 [originally named TARC (thymus- and activation-regulated chemokine)]) as a chemokine selectively expressed in mouse and human CCR2+ macrophages.11,16

mortality.3,4 Despite extensive evidence that inflammation contributes to heart failure pathogenesis in preclinical models,6,8 the therapeutic potential of targeting myocardial inflammation is yet to be realized.

Recent advances in cardiac immunology have reinvigorated efforts to target inflammation in the injured and failing heart.5,7 High-dimensional flow cytometry, genetic lineage tracing, and single-cell RNA sequencing studies have identified populations of monocytes, macrophages, and dendritic cells that generate proinflammatory cytokines, chemokines, and damaging oxidative products.9-10 These populations originate from monocytes recruited to the heart and are marked by the expression of CCR2 (C-C chemokine receptor type 2).11,12 Inhibition of monocyte recruitment is sufficient to prevent accumulation of proinflammatory CCR2+ monocytes, macrophages, and dendritic cells and suppress adverse LV remodeling across heart failure models.13-15 The effector mechanisms that mediate adverse effects of CCR2+ monocytes, macrophages, and dendritic cells remain poorly defined.

We previously identified CCL17 (C-C chemokine ligand 17 [originally named TARC (thymus- and activation-regulated chemokine)]) as a chemokine selectively expressed in mouse and human CCR2+ macrophages.11,16
heart after myocardial injury. We define signaling pathways that regulate CCL17 expression and demonstrate that Ccl17 deficiency is sufficient to suppress adverse LV remodeling and heart failure progression. We provide evidence that CCL17 prevents Treg migration by biasing signaling events downstream of the CCR4 receptor and show that the protective effects of Ccl17 deficiency on myocardial inflammation and adverse LV remodeling are dependent on the presence of Tregs. These findings implicate CCL17 as a proinflammatory mediator of CCR2+ macrophages and dendritic cells and highlight the therapeutic potential of inhibiting CCL17 in the injured or failing heart.

METHODS

Complete detailed Methods are provided in the Supplemental Material. Raw data that support the findings of this study are available from the corresponding author on reasonable request.

Animal Studies

Mice were bred and maintained at the Washington University School of Medicine and all experimental procedures were done in accordance with the animal use oversight committee guidelines. Mouse strains included Ccl17GFP/25, Foxp3-GFP/DTR/26, Zbb449pp/27, and Tnnt2-DTR/16. In the Tnnt2-DTR model, cardiomyocyte injury was induced by administering 20 ng DT through intraperitoneal injection. For closed-chest ischemia–reperfusion, myocardial injury was induced by administering 20 ng DT through intraperitoneal injection. For closed-chest ischemia–reperfusion injury, ischemia was induced for 90 minutes. For vehicle controls (Figure 1A), Injection of DT into Tnnt2-DTR Ccl17GFP/+ mice revealed GFP (green fluorescent protein) expression in CD68+ cells (macrophages and dendritic cells) within the myocardium (Figure 1B).

Ccl17 mRNA expression was also elevated within the LV myocardium 2, 4, and 7 days after IR injury compared with sham controls. Ccl17 expression returned to baseline levels on days 14 and 28 after IR injury (Figure 1C), which was consistent with other inflammatory chemokines and cytokines (Figure S1A). GFP and CD68 immunostaining of Ccl17GFP/+ hearts revealed that GFP was expressed in CD68+ cells within the infarct (Figure 1D). AngII/PE infusion also induced myocardial Ccl17 expression compared with saline infused controls. Ccl17 mRNA expression was found to be elevated beginning 2 days after pump implantation with peak expression observed on days 4 and 7. Ccl17 mRNA expression returned to baseline values on days 14 and 28 (Figure 1E). GFP and CD68 immunostaining of Ccl17GFP/+ hearts demonstrated the presence of GFP-expressing CD68+ cells within the myocardium of mice infused with AngII/PE (Figure 1F). Flow cytometric analysis indicated that 0.5% to 1.5% of CD45+ cells expressed GFP across injury models (Figure 1G and Figure S1B–S1D). Immunostaining of human myocardial samples obtained from donor controls or patients with acute MI or ischemic cardiomyopathy confirmed the presence of CCL17+ CD68+ cells after acute MI (Figure 1H and Table S1). These data indicate that CCL17 is expressed in a subset of CD68+ cells within the myocardium after cardiac injury.

CCL17 is Expressed in Subsets of Monocyte-Derived Macrophages and Conventional Dendritic Cells Recruited to the Injured Heart

To define the precise immune cell types that express CCL17 within the injured heart, we performed flow cytometry and RNA sequencing using Tnnt2-DTR Ccl17GFP/+ mice. Flow cytometry 4 days after DT injection revealed that GFP was expressed in cell populations marked by CD11b, CD64, CD11c, and MHC II (major histocompatibility complex II). We did not detect GFP expression in T cells (CD3), B cells (CD19), natural killer cells (NK1.1), or neutrophils (LY6G [lymphocyte antigen 6 complex, locus G6D]). These data suggest that monocytes, macrophages, and/or dendritic cells express Ccl17 (Figure S2A). Flow cytometry revealed 2 distinct populations of CCL17+ cells: CD64hiLY6Chi (gate 1 [G1]) and CD64loLY-
**Figure 1. CCL17 is expressed after myocardial injury.**

A. Quantitative reverse transcription polymerase chain reaction measuring Ccl17 (C-C chemokine ligand 17) mRNA expression in hearts of Tnnt2-DTR mice 2 and 4 days after diphtheria toxin (DT; n=4) or normal saline (vehicle, n=4) administration. **B.** Immunostaining for CCL17-GFP (white), CD68 (red), and DAPI (4',6-diamidino-2-phenylindole; blue) shows the spatial distribution of GFP+ (green fluorescent protein) CCL17-expressing cells in hearts of Tnnt2-DTR Ccl17 GFP/+ mice 4 days after DT or saline administration. **C.** Quantitative reverse transcription polymerase chain reaction measuring Ccl17 mRNA expression in hearts of wild-type (WT) mice 2, 4, 7, 14, and 28 days after closed chest ischemia–reperfusion (IR) surgery (n=4) or sham surgery (n=4). **D.** Immunostaining for CCL17-GFP (white), CD68 (red), and DAPI (blue) (Continued)
6C (gate 2 [G2]) cells (Figure 2A). We identified similar populations of Ccl17-expressing CD64£LY6C (G1) and CD64£LY6C (G2) cells in the hearts of mice subjected to IR injury and AngII/PE infusion (Figure 2B). CD64£LY6C (G1) and CD64£LY6C (G2) cells expressed different levels of Ccl17 (Figure 2C) and displayed different sizes and morphologies (Figure 2D), suggesting that they likely represent distinct cell types.

Previous studies have suggested that CCL17 is expressed in dendritic cells. To determine whether CCL17+ G1 and G2 cells are macrophages or dendritic cells, we generated Tnt2-DTR Zbtb46GFP/ mice. ZBTB46 (zinc finger and BTB domain-containing protein 46) is specifically expressed in dendritic cells and ZBTB46+ dendritic cells (gate 3 [G3]) within the heart by flow cytometry (Figure 2E and Figure S1E). ZBTB46+ dendritic cells (G3) displayed low levels of CD64 and LY6C (lymphocyte antigen 6 complex, locus C) expression. Comparison of CD64 and LY6C expression between CCL17+CD64£LY6C (G1), CCL17+CD64£LY6C (G2), and ZBTB46+ (G3) dendritic cells indicated similarities between CCL17+CD64£LY6C (G2) and ZBTB46+ (G3) dendritic cells. CCL17+CD64£LY6C (G2) and ZBTB46+ (G3) dendritic cells also displayed similar MHC-II and CCR2 expression and physical properties (forward and side scatter; Figure 2F).

We performed RNA sequencing to substantiate whether CCL17+CD64£LY6C (G1) and CCL17+CD64£LY6C (G2) cells represent macrophages and dendritic cells, respectively. We compared CCL17+CD64£LY6C (G1) and CCL17+CD64£LY6C (G2) cells with bona fide populations of ZBTB46+GFP+ dendritic cells (G3) and CD64+ macrophages (G4; Figure 2G) in the Tnt2-DTR injury model. Principal component analysis revealed that CCL17+CD64£LY6C (G1) cells clustered with CD64+ macrophages (G4) and were transcriptionally distinct from ZBTB46+GFP+ dendritic cells (G3). CCL17+CD64£LY6C (G2) cells clustered independent of the other populations (Figure 2H). Quantification of genes associated with macrophages and dendritic cells demonstrated that CCL17+CD64£LY6C (G1) cells expressed high levels of macrophage markers (Cd64, Cd68, F4/80, MerTK) and high levels of dendritic cell markers (Zbtb46, Cd26, Cd11c, Cd103). In contrast, CCL17+CD64£LY6C (G2) cells expressed low levels of macrophage markers (Cd64, Cd68, F4/80, MerTK) and high levels of dendritic cell markers (Zbtb46, Cd26, Cd11c, Cd103).

Hierarchical clustering and differential gene expression analysis comparing CCL17+CD64£LY6C (G1) and CCL17+CD64£LY6C (G2) confirmed that these populations were transcriptionally distinct and identified 914 differentially expressed genes (>2-fold change; false discovery rate P<0.05; Figure 2J and 2K). CCL17+CD64£LY6C (G1) expressed high levels of several chemokines (Ccl2, Ccl3, Ccl6, Ccl7, Ccl24, Cxcl1, Cxcl2, Cxcl3) and cytokines (TNF, II10). CCL17+CD64£LY6C (G2) expressed higher levels of Klb1b, Fcgr2b, Irf4, Cdh1, F11r, Icam1, Adnf, Stat1, Bcl11a, and Mink1 (Figure 2L and Figure S2B).

To determine whether CCL17+ macrophages and dendritic cells originate from recruited populations of monocytes, we treated Tnt2-DTR Ccl17 GFP/+ mice with RS504393 (CCR2 inhibitor; Figure 2M). After DT injection, we observed reduced CCL17 expression in the hearts of mice treated with RS504393 compared with vehicle-treated controls (Figure 2N). Flow cytometry revealed that the numbers of total CCL17+ cells, CCL17+CD64£LY6C (G1) macrophages (G1), and CCL17+CD64£LY6C (G2) dendritic cells within the myocardium were also reduced in RS504393-treated mice compared with vehicle-treated controls (Figure 2O). These results suggest that CCL17+CD64£LY6C (G1) and CCL17+CD64£LY6C (G2) likely originate from monocytes or monocyte-like progenitors that infiltrate the injured heart.

GM-CSF Signaling Regulates CCL17 Expression in Cardiac Macrophages and Dendritic Cells

To identify upstream activators of CCL17 expression, we exposed mouse bone marrow–derived macrophages to hypoxia, interleukin 1β, interleukin 4, polycytidylic acid, lipopolysaccharides, CpG oligodeoxynucleotides, and granulocyte-macrophage colony-stimulating factor (GM-CSF).
Figure 2. CCL17 is expressed in CCR2+ macrophages and conventional dendritic cells recruited to the injured heart.

A. Flow cytometry identified 2 populations of CCL17+ (C-C chemokine ligand 17) cells in hearts of Tmrt2-DTR Ccl17 GFP/+ mice 4 days after diphtheria toxin (DT) administration: CCL17+CD64hiLy6Chi CCL17+ cells (gate 1 [G1]) and CCL17+CD64loLy6Clo (gate 2 [G2]). B. Flow cytometry demonstrating CCL17+CD64hiLy6Chi (G1) and CCL17+CD64loLy6Clo (G2) cells in hearts of CCL17 GFP/+ mice 4 days after ischemia-reperfusion (IR) and angiotensin II and phenylephrine (AngII/PE) injury. C. Quantitative reverse transcription polymerase chain reaction comparing Ccl17 mRNA expression in CCL17+ G1 and G2 cells from DT-injured hearts (n=4 per experimental group). D. Representative Hema-3 stained images of CCL17+ G1 (n=20) and G2 (n=20) cells from DT-injured hearts (left panel) and quantification of cell area (right panel). (Continued)
GM-CSF demonstrated the strongest effect on Ccl17 mRNA expression and secreted protein abundance (Figure S3A and S3B). GM-CSF signaling activates several intracellular signaling pathways, including PI3K/AKT (phosphatidylinositol 3-kinase/protein kinase B), MAPK (mitogen-activated protein kinase), canonical NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), and JAK2 (Janus kinase 2)/STAT5 (signal transducer and activator of transcription 5). To define the requirement for each pathway, we stimulated bone marrow–derived macrophages with GM-CSF in the presence of the JAK1/2 inhibitor ruxolitinib, inhibitor of nuclear factor κB kinase subunit β (TPCA), a MAPK inhibitor (U0126), and the AKT inhibitor miltefosine. Ccl17 mRNA expression and secreted protein abundance was markedly reduced by TPCA and ruxolitinib. U0126 and miltefosine modestly reduced Ccl17 expression (Figure S3C). Western blot and immunostaining demonstrated that GM-CSF promoted accumulation of NF-κB subunits p65 and p50 in the nucleus and phosphorylation of STAT5, which were prevented by TPCA and ruxolitinib, respectively (Figure S3D–S3H). These results are consistent with predicted binding sites for NF-κB and STAT5 in the Ccl17 promoter and suggest that canonical NF-κB and STAT5 pathways are required for GM-CSF to induce Ccl17 expression (Figure S3I).

To decipher whether GM-CSF regulates Ccl17 in vivo, we treated Ccl17GFP/+ mice who underwent DT cardio-myocyte ablation, AngII/PE infusion, and myocardial IR injury with either an isotype control antibody or GM-CSF neutralizing antibody. Reverse transcription polymerase chain reaction was used to measure myocardial Ccl17 mRNA expression. Flow cytometry was used to quantify the percentage and absolute number of CCL17+ macrophages and dendritic cells as well as STAT5 phosphorylation in CCL17+ leukocytes and were not reduced further by GM-CSF neutralizing antibody treatment.

Ccl17 Deletion Attenuates Heart Remodeling After Reperfused MI and AngII/PE Infusion

To delineate the functional relevance of Ccl17 in the context of myocardial injury, we investigated control (Ccl17+/+) and Ccl17-deficient mice (Ccl17−/−) in clinically relevant models of reperfused MI and AngII/PE infusion. Compared with littermate controls, Ccl17−/− mice demonstrated attenuated LV remodeling after 90 minutes of MI injury. Ccl17−/− mice showed reduced heart/body weight ratio compared with controls 28 days after reperfused MI injury. Echocardiography revealed that deletion of Ccl17 resulted in improved LV ejection fraction and smaller left ventricular volumes 28 days after myocardial IR injury. No differences were observed between control and Ccl17−/− mice that underwent the sham procedure (Figure 4A–4F). Wheat germ agglutinin and trichrome staining revealed reduced cardiomyocyte cross-sectional area and interstitial fibrosis within the border zone of Ccl17−/− mice compared with controls 28 days after myocardial IR injury (Figure S4A–S4C). Ccl17−/− mice showed smaller infarcts 28 days after myocardial IR injury compared with control mice (Figure 4G). The initial infarct area was equivalent between control and Ccl17−/− mice as measured by echocardiography 1 day and triphenyl tetrazolium chloride staining 4 days after myocardial IR injury (Figure S4D and S4E), suggesting that Ccl17 deletion influences infarct expansion.

To investigate the role of Ccl17 in a nonischemic injury model, we infused control and Ccl17−/− mice with either saline (sham) or AngII/PE (Figure 4H). Quantification of heart/body weight ratio demonstrated reduced size of Ccl17−/− hearts compared with controls 28 days after AngII/PE infusion. No differences were observed between control and Ccl17−/− hearts infused with saline.
Figure 3. GM-CSF-STAT5 signaling is essential to specify CCL17+ cells.

A. Tnnt2-DTR Ccl17GFP/+ mice were treated with normal saline (vehicle) or diphtheria toxin (DT) on day 0, followed by isotype control (ISO-Nab) or GM-CSF (granulocyte-macrophage colony-stimulating factor) neutralizing antibody (G-Nab) on days 1 and 3, and hearts harvested on day 4 for analysis.

B. Quantitative reverse transcription polymerase chain reaction measuring Ccl17 (C-C chemokine ligand 17) mRNA expression in hearts.

C. Flow cytometry quantifying the percentage of CCL17+ immune cells (left panel) and the number of CCL17+ immune cells per milligram heart tissue (right panel).

D through G. Flow cytometry shows levels of pSTAT5 (phosphorylated signal transducer and activator of transcription 5) in all CCL17+ cells (D), CCL17+ G1 cells (E), CCL17+ G2 cells (F), and CD45+CCL17- cells (G) isolated from hearts. (Continued)
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Figure 3 Continued. H, Ccl17<sup>−/−</sup> mice were treated with saline (sham) or angiotensin II and phenylephrine (AngII/PE) infusion through osmotic minipump on day 0 and administered ISO-Nab or G-Nab on days 1 and 3. Hearts were harvested on day 4 for analysis. I, Quantitative reverse transcription polymerase chain reaction measuring Ccl17 mRNA expression in hearts. J, Flow cytometry quantifying the percentage of CCL17<sup>+</sup> immune cells (left panel) and the number of CCL17<sup>+</sup> immune cells per milligram heart tissue (right panel). K, through N. Flow cytometry shows levels of pSTAT5 in all CCL17<sup>+</sup> cells (K), CCL17<sup>+</sup> G1 cells (L), CCL17<sup>+</sup> G2 cells (M), and CD45<sup>+</sup>CCL17<sup>+</sup> cells (N) isolated from the hearts of mice treated with AngII/PE and ISO-Nab or G-Nab. O, Ccl17<sup>−/−</sup> mice underwent closed-chest ischemia–reperfusion (IR) injury (90 minutes of ischemia) on day 0 and were administered ISO-Nab or G-Nab on days 1 and 3. Hearts were harvested on day 4 for analysis. P. Flow cytometry quantifying the percentage of CCL17<sup>+</sup> immune cells (left panel) and the number of CCL17<sup>+</sup> immune cells per milligram heart tissue (right panel). Q through T, Flow cytometry shows levels of pSTAT5 in all CCL17<sup>+</sup> cells (Q), CCL17<sup>+</sup> G1 cells (R), CCL17<sup>+</sup> G2 cells (S), and CD45<sup>+</sup>CCL17<sup>+</sup> cells (T) isolated from hearts. D through G, K through N, Q through T. Black: IgG control; red: pSTAT5 antibody and G-Nab treatment. For comparisons between 2 groups, unpaired t tests were performed. For multiple comparisons, 1-way analysis of variance followed by Tukey test was performed; n=4 per experimental group and all data are mean±SD. *P<0.05, **P<0.01, ***P<0.001.

Recruitment of Cardiac Tregs in the Absence of Ccl17

To determine the chemotactic function of Ccl17 in the heart, we first examined infiltration of innate immune cells. Flow cytometry and immunostaining did not reveal any differences in monocyte, macrophage, or neutrophil abundance within the heart between control and Ccl17<sup>−/−</sup> mice 4 days after reperfused MI or AngII/PE infusion (Figure S5A–S5C and S5F–S5H). Ccl17 selectively binds to CCR4, which is expressed on T cells. We observed increased accumulation of CD4<sup>+</sup> Tregs (CD3<sup>+</sup>CD4<sup>+</sup>FOXP3<sup>+</sup> [forkhead box P3]) in the hearts of Ccl17<sup>−/−</sup> mice that underwent reperfused MI or AngII/PE infusion compared with controls (Figure 4J and 4K and Figure S4F–S4I). Taken together, these data indicate that Ccl17 deletion attenuates LV remodeling after myocardial injury.

CCL17 and CCL22 Are Competitive Biased CCR4 Ligands That Differentially Modulate Treg Chemotaxis

To assess mechanisms by which Ccl17 deficiency leads to Treg recruitment, we examined whether CCL17 influences Treg differentiation, proliferation, or chemotaxis. CCL17 had no effect on the differentiation of na"ive CD4<sup>+</sup> T cells into Tregs in vitro (induced Tregs) after stimulation with interleukin-2, transforming growth factor-β, and anti-CD3/CD28 antibodies (Figure S6A). Flow cytometry revealed that Tregs displayed minimal proliferation rates in both control and Ccl17<sup>−/−</sup> hearts 7 days after AngII/PE infusion (Figure S6B). To assess potential effects on chemotaxis, we first sought to identify whether other CCR4 ligands are present within the injured heart. Quantitative reverse transcription polymerase chain reaction revealed that Ccl22 (C-C chemokine ligand 22) was expressed in the myocardium after IR injury, AngII/PE infusion, and DT cardiomyocyte ablation (Figure 6A and Figure S6C). RNA in situ hybridization showed co-staining of Ccl17 and Ccl22 mRNA in CD68<sup>+</sup> macrophages (Figure 6B).

On the basis of these findings, we examined the relative effect of CCL17 and CCL22 on Treg chemotaxis. Transwell cell migration assays revealed that CCL17 inhibited CCL22-induced chemotaxis of induced Tregs and CCR4-expressing BW5147.3 T cells in a concentration-dependent manner (Figure 6C and 6D). Increasing doses of CCL22 were sufficient to augment Treg chemotaxis induced by CCL17 in induced Tregs (Figure 6E) and BW5147.3 T cells (Figure S6E). These findings suggest that CCL17 and CCL22 are competitive agonists and that CCL22 has a greater capacity to elicit chemotaxis, consistent with previous findings regarding a competitive relationship between CCL17 and CCL22. Induced Tregs and BW5147.3 T cells expressed CCR4 on their cell surface and did not display evidence of internalization after CCL17 treatment (Figure S6D and S6K). CCR4 antagonists (C021, AZD2098) abrogated Treg migration toward CCL17 and CCL22 (Figure 6F and 6G). These data suggest that the relative balance of CCL17 and CCL22 is an important determinant of Treg chemotactic capacity.

To investigate the mechanistic basis by which CCL17 and CCL22 competitively regulate Treg chemotaxis, we examined major second messenger signals downstream of CCR4 including G<sub>q</sub>α (cAMP), G<sub>q</sub>α (intracellular Ca<sup>2+</sup>), and ARRB (β-arrestin). Compared with vehicle control, CCL17 and CCL22 increased intracellular calcium levels in induced Tregs and BW5147.3 cells. The combination of CCL17 and CCL22 had a synergistic effect (Figure S6F...
Deficiency of Ccl17 attenuates left ventricular remodeling after myocardial infarction and angiotensin II and phenylephrine infusion.

A, Control mice (Ccl17+/+) and Ccl17−/− mice underwent closed-chest ischemia–reperfusion (IR) injury or sham surgery on day 0. Hearts were harvested on day 28 after myocardial infarction (MI). B, Measurement of heart weight to body weight ratio (HW/BW) in control and Ccl17−/− mice after sham surgery (n=6) or MI (n=12). C, Representative end systolic echocardiographic images of control and Ccl17−/− hearts 28 days after MI. Regional left ventricle (LV) displacement throughout the cardiac cycle is displayed. D through F, Quantification of LV ejection fraction (LVEF), LV end-diastolic volume (LVEDV), and LV end-systolic volume (LVESV), respectively, 28 days after IR surgery. G, Representative images of infarct area identified by trichrome staining (left panel) and quantification of infarct area (right panel). H, Control and Ccl17−/− mice were implanted with osmotic minipumps containing angiotensin II and phenylephrine (AngII/PE; n=6) or normal saline (sham; n=6) on day 0. Hearts were harvested on day 28 for analysis. I, Measurement of HW/BW in control and Ccl17−/− mice implanted with osmotic minipumps containing AngII/PE or saline (sham). J, Representative wheat germ agglutinin–stained images (mid-LV) shows cardiomyocytes in cross-section (red, left panels) and quantification of cardiomyocyte cross-sectional area (right panels). K, Representative low magnification trichrome staining images to visualize interstitial fibrosis (left panels) and quantification of fibrotic area based on trichrome staining (right panels). All data are mean±SD. For comparisons between 2 groups, unpaired t test was performed. For multiple comparisons, 2-way analysis of variance with Geisser-Greenhouse correction followed by Sidak test was performed. *P<0.05, **P<0.01, ***P<0.001.
and S6H). Neither CCL17 nor CCL22 increased cAMP levels compared with vehicle control in induced Tregs or BW5147.3 cells (Figure S6G and S6l). To examine ARRB signaling, we focused on BW5147.3 cells, given their ability to be transfected. To measure physical interactions between CCR4 and ARRB, we used the NanoBiT system, which assesses intracellular protein–protein interactions by structural complementation of luciferase (Figure 6H). BW5147.3 T cells were transiently transfected with CCR4-SmBiT and ARRB2-LgBiT. Compared with vehicle control, CCL17 did not lead to binding of ARRB2 to CCR4. In contrast, robust luminescence was detected after application of CCL22, indicating binding of ARRB2 to CCR4. CCL17 abrogated CCR4-ARRB2 binding in a dose-dependent fashion (Figure 6I and 6J).

To decipher the relative requirement for ARRB signaling in Treg chemotaxis, we transfected BW5147.3 T cells with ARRB1 (Si-ARRB1) and ARRB2 (Si-ARRB2) siRNA. Quantitative reverse transcription polymerase chain reaction and Western blot analysis confirmed the knockdown efficiency of each siRNA (Figure S6L). ARRBI and ARRB2 siRNA inhibited chemotaxis of BW5147.3 T cells induced by CCL22 but not CCL17 (Figure 6K). Application of U73122 (phospholipase C inhibitor), which blocks agonist-induced Ca<sup>2+</sup> efflux from endoplasmic reticulum, suppressed accumulation of intracellular calcium and BW5147.3 T-cell chemotaxis induced by either CCL17 or CCL22 (Figure 6L and Figure S6M). These findings suggest that CCL22 promotes Treg chemotaxis through concomitant ARRB and Ca<sup>2+</sup> signaling. The presence of CCL17 dampens Treg chemotaxis by inhibiting ARRB signaling (Figure 6M). This concept is consistent with previous reports that ARRB2 deletion reduces the number of CD4<sup>+</sup>FOXP3<sup>+</sup> Tregs in experimental autoimmune encephalitis<sup>27</sup> and that CCL22 selectively induces concentration-dependent coupling of CCR4 to ARRB2.<sup>38</sup>

### CCL17 Deficiency Suppresses Heart Remodeling Through a Treg-Dependent Mechanism

To determine whether Treg recruitment is responsible for the protective effect of CCL17 deficiency on heart remodeling, we depleted Tregs from control and CCL17<sup>−/−</sup> mice. To selectively deplete Tregs, we crossed control and CCL17<sup>−/−</sup> mice to Foxp3-DTR/GFP mice. Flow cytometry confirmed efficient depletion of Tregs in the resultant progeny mice after DT treatment (100 ng/mouse, IP every 2 days; Figure S7A and S7B). For these experiments, we chose to focus on the AngII/PE model. We implanted osmotic minipumps containing AngII/PE into control and CCL17<sup>−/−</sup> Foxp3-DTR/GFP mice and either saline or DT was injected to deplete Tregs (Figure 7A). Echocardiography performed 28 days after AngII/PE infusion revealed that compared with control Foxp3-DTR/GFP (control + saline) mice, CCL17<sup>−/−</sup> Foxp3-DTR/GFP (CCL17<sup>−/−</sup> + saline) mice displayed smaller LV chamber dimensions, increased LV fractional shortening, and reduced heart weight to body weight ratio. Depletion of Tregs from CCL17<sup>−/−</sup> Foxp3-DTR/GFP (CCL17<sup>−/−</sup> + DT) mice led to increased LV chamber dimensions, decreased fractional shortening, and increased heart weight to body weight ratios compared with CCL17<sup>−/−</sup> Foxp3-DTR/GFP (CCL17<sup>−/−</sup> + saline) mice. We did not observe any significant difference between Foxp3-DTR/GFP mice treated with saline or DT (Figure 7B–7D and Table S2). We also examined cardiac chamber dimensions, increased LV fractional shortening, and reduced heart weight to body weight ratio. Depletion of Tregs from CCL17<sup>−/−</sup> Foxp3-DTR/GFP mice led to greater cardiomyocyte cross-sectional area and increased interstitial fibrosis compared with CCL17<sup>−/−</sup> Foxp3-DTR/GFP mice that did not undergo Treg depletion. No significant differences were observed between Foxp3-DTR/GFP hearts treated with saline or DT (Figure 7E and 7F and Figure S7C and S7D). These data indicate that CCL17 deficiency attenuates LV remodeling in a Treg-dependent manner.

### Treg Recruitment Suppresses Macrophage-Associated Inflammation

To determine the mechanistic basis by which Treg recruitment confers protection in CCL17-deficient mice, we first performed RNA sequencing on CD4<sup>+</sup>FOXP3<sup>+</sup> cells isolated by fluorescence activated cell sorting from control and CCL17<sup>−/−</sup> hearts 7 days after AngII/PE infusion. Principal component analysis and differential gene expression analysis did not demonstrate any significant differences between Tregs isolated from the hearts of control and CCL17<sup>−/−</sup> hearts (Figure SBA–SBC). These data suggest that CCL17 deficiency influences the relative number of Tregs in the heart as opposed to stimulating their transcriptional activation.

Tregs are potent negative regulators of inflammation in various biological contexts and inhibit proinflammatory properties of macrophages.<sup>39</sup> To examine this possibility, we quantified macrophage abundance and proinflammatory gene expression 7 days after AngII/PE infusion in control and CCL17<sup>−/−</sup> hearts treated with either saline or DT (Figure 8A). We did not detect any significant differences in macrophage abundance between experimental groups (Figure 8B). Next we isolated cardiac macrophages (CD64<sup>+</sup>Ly6C<sup>−</sup>) by fluorescence activated cell sorting and measured the expression of proinflammatory cytokines and chemokines in macrophages. Quantitative reverse transcription polymerase chain reaction demonstrated that compared with Foxp3-DTR/GFP mice treated with saline, macrophages isolated from CCL17<sup>−/−</sup> Foxp3-DTR/GFP hearts treated with saline displayed reduced Ccl3, Ccl4, Il1β, and TNFα mRNA expression. Deple-
Figure 5. Ccl17 deficiency increases cardiac regulatory T-cell abundance.
A, Control (Ccl17−/−) mice and Ccl17−/− mice were subjected to closed-chest ischemia–reperfusion (IR) injury on day 0 and hearts were harvested on day 4 for analysis. B, Flow cytometry gating scheme used to identify CD4+ FOXP3+ (forkhead box P3) regulatory T cells (Tregs). C, Ccl17 deficiency led to expansion of Tregs in hearts of IR injured mice. Flow cytometry shows the proportion of cardiac FOXP3+ Tregs among total CD4+ cells (left panel) and the number of FOXP3+ Tregs to identify CD4+ FOXP3+ (forkhead box P3) regulatory T cells (Tregs).

We then assessed the expression of Ccl3, Ccl4, II1β, and TNFα mRNA expression in macrophages isolated from Foxp3-DTR/GFP and Ccl17−/− Foxp3-DTR/GFP hearts treated with DT. No significant differences were observed in Ccl17−/− Foxp3-DTR/GFP mice hearts treated with saline or DT (Figure 8C).

We report that CCL17, a chemokine specifically expressed in CCR2+ macrophages and dendritic cells, orients Tregs in the myocardium of Foxop3-DTR/GFP mice and Ccl17−/− Foxp3-DTR/GFP mice treated with saline or DT. Each of these proinflammatory mediators was increased by AngII/PE infusion. Compared with salinetreated Foxp3-DTR/GFP mice infused with AngII/PE, salinetreated Ccl17−/− Foxp3-DTR/GFP mice infused with AngII/PE exhibited blunted expression of Ccl3, Ccl4, II1β, and TNFα mRNA in the myocardium. Depletion of Tregs reversed the protective effects of Ccl17 deficiency, resulting in expression levels of Ccl3, Ccl4, II1β, and TNFα that were equivalent to control mice infused with AngII/PE (Figure 8D). Gm-csf, Il6, and Il1y were modestly affected by Ccl17 deletion and no effects were observed with respect to Ccl9 expression (Figure S8D and S8E). Together these findings demonstrate that Tregs suppress the expression of macrophage-derived proinflammatory mediators in Ccl17-deficient mice.

DISCUSSION
Within the mouse and human heart, CCR2 expression identifies monocytes, macrophages, and dendritic cells with robust inflammatory potential.11,12,40–45 The relevant effector molecules by which CCR2+ monocytes, macrophages, and dendritic cells orchestrate cardiac inflammation remain to be elucidated. We investigated the functional significance of CCL17, a chemokine specifically expressed in CCR2+ macrophages and dendritic cells. We report that Ccl17 is expressed in CCR2+ macrophages and type II conventional dendritic cells recruited to the injured heart. Within these populations, GM-CSF signaling induced CCL17 expression. Deletion of Ccl17 was sufficient to improve LV systolic function and attenuate key components of LV remodeling including LV dilation, cardiomyocyte hypertrophy, and fibrosis in response to MI and AngII/PE infusion. Ccl17 deficiency conferred protection through enhanced Treg cell recruitment and subsequent attenuation of inflammatory macrophage gene expression. We identified CCL17 and CCL22 as biased CCR4 ligands with opposing effects on Treg migration. These data establish CCL17 as a proinflammatory mediator of CCR2+ macrophages and dendritic cells and suggest that inhibition of CCL17 may serve as an
Figure 6. CCL17 and CCL22 are competitive biased CCR4 ligands that differentially modulate regulatory T-cell chemotaxis.

A, Quantitative reverse transcription polymerase chain reaction measuring mRNA levels of Ccl17 (C-C chemokine ligand 17), Ccl22 (C-C chemokine ligand 22), and CCR4 (C-C chemokine receptor type 4) in hearts of wild-type mice 4 days after implantation of osmotic minipump containing angiotensin II and phenylephrine (AngII/PE; n=4) or normal saline (sham; n=4).

B, Representative images of DAPI (blue), Cd68 (orange), Ccl17 (white), and Ccl22 (magenta) in hearts of AngII/PE infused mice by RNA in situ hybridization.

C and D, Chemotaxis of primary induced regulatory T cells (Tregs; C) and BW5147.3 T cells (D) in response to CCL22 (10 ng/mL) was suppressed by increasing concentrations of CCL17 (1, 10, 100, 1000 ng/mL).

E, Chemotaxis of primary induced Tregs in response to single dose of CCL17 (10 ng/mL) (Continued)
Ccl17 deletion reduced proinflammatory chemokine Ccl17 effects of deficiency. Within the injured heart, Treg depletion was sufficient to reverse the protective NF-κB. Through mechanistic studies, we showed that CCL17 signals through CCR4 expressed on CD4+ helper T cells. We and others observed that Ccl17 deletion selectively increases Treg abundance in the diseased heart, aorta, and colon. Tregs attenuate inflammation through effects on macrophages, effector T cells, and fibroblasts. In each of these scenarios, Treg depletion was sufficient to reverse the protective effects of Ccl17 deficiency. Within the injured heart, Ccl17 deletion reduced proinflammatory chemokine and cytokine expression in macrophages in a Treg-dependent manner. RNA sequencing of Tregs isolated from injured control and Ccl17–/– hearts did not reveal evidence of altered gene expression or function, indicating that Ccl17 deletion affected Treg recruitment and not activation. Together these findings suggest a generalized role for CCL17 in regulating Treg trafficking and identify Ccl17 as a therapeutic target to expand Tregs, limit tissue inflammation, and improve organ function.

Little is understood regarding the mechanisms by which Tregs are recruited to the heart and no effective strategies exist to expand Tregs within the injured myocardium. We provide insights into the mechanism by which CCR4 ligands (CCL17 and CCL22) regulate Treg recruitment. CCL17 and CCL22 are coexpressed after myocardial injury. However, their relative concentration within the infarct are unknown. Using primary Tregs and a Treg cell line, we show that CCL17 and CCL22 are competitive biased ligands for CCR4. CCL17 selectively activated Gq signaling; CCL22 simultaneously activated Gq and ARRB signaling downstream of CCR4. We demonstrated that CCL17 competitively inhibited CCL22-stimulated ARRB signaling and Treg chemotaxis. We cannot exclude the possibility that CCL17 inhibits CCL22 through additional mechanisms that are independent of CCR4 in vivo. CCL22 promotes interactions between dendritic and regulatory T cells within lymph nodes and regulates immunity. These findings provide an explanation for how CCL17 might inhibit Treg recruitment in the injured heart and highlight the possible use of biased CCR4 agonists as therapeutic agents to increase Treg recruitment and suppress inflammation.

This study is not without limitations. Although we exclusively detected CCL17 expression in macrophages and dendritic cells within the heart, we cannot exclude the possibility that CCL17-expressing cells outside of the heart contributed to the observed protection after cardiac injury. Conditional Ccl17 knockout mice, cardiac macrophages, and dendritic-specific Cre recombinases are not available. Further-
Figure 7. Ccl17 deficiency suppresses left ventricle remodeling through a regulatory T-cell–dependent mechanism.

A, Control (Ccl17+/−; C-C chemokine ligand 17) and Ccl17−/− mice were crossed with Foxp3-DTR/GFP to generate control Foxp3-DTR/GFP and Ccl17−/− Foxp3-DTR/GFP mice in which regulatory T cells (Tregs) are deleted after diphtheria toxin (DT) administration. Osmotic minipumps containing angiotensin II and phenylephrine (AngII/PE) were implanted into control Foxp3-DTR/GFP and Ccl17−/− Foxp3-DTR/GFP mice. Mice were treated with DT or normal saline every 2 days. Echocardiography was performed on day 28 and hearts were harvested thereafter.

B, Representative echocardiographic images of control + saline, control + DT, Ccl17−/− + saline, and Ccl17−/− + DT hearts 28 days after AngII/PE infusion.

C, Quantification of left ventricular internal diameter (LVID) at end diastole (left panel) and at end systole (center panel) and left ventricular fractional shortening (right panel) 28 days after AngII/PE infusion.

D, Quantification of heart weight to body weight ratio (HW/BW).

E, Wheat germ agglutinin staining (red) examining the effects of Treg depletion on cardiomyocyte hypertrophy of control mice and Ccl17−/− mice 28 days after AngII/PE infusion (left panels) and quantification of cardiomyocyte cross-sectional area on the basis of wheat germ agglutinin staining (right panel).

F, Low magnification trichrome-stained images (left panels) examining the effects of Treg depletion on myocardial interstitial fibrosis in control and Ccl17−/− mice 28 days after AngII/PE injury. Quantification of fibrotic area based on trichrome staining is shown in the right panel.

N=6 per group and all data are mean±SD; 2-way analysis of variance with Geisser-Greenhouse correction followed by Sidak test was performed. *P<0.05, **P<0.01, ***P<0.001.
more, whereas we included multiple models of cardiac injury, it is possible that CCL17 deletion may not be protective in all cardiac pathologies. Future studies are needed to delineate the potential benefit of altering the balance of CCL17 and CCL22 signaling in vivo and assess the therapeutic window of CCL17 inhibition.

Our findings identify CCL17 as a proinflammatory mediator of CCR2+ macrophages and dendritic cells and suggest that inhibition of CCL17 may serve as an
effective strategy to promote Treg recruitment, suppress myocardial inflammation, and attenuate LV remodeling.

**ARTICLE INFORMATION**

Received May 26, 2021; accepted January 7, 2022.

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**Acknowledgments**

The authors thank the Genome Technology Access Center at Washington University School of Medicine. The Center is partially supported by National Cancer Institute Cancer Center Support Grant P30 CA91842 to the Siteman Cancer Center and by Institute for Clinical and Translational Science/ Clinical and Translational Science Award Grant UL1TR002345 from the National Center for Research Resources, a component of the National Institutes of Health, and National Institutes of Health Roadmap for Medical Research. The authors thank the mouse cardiovascular phenotyping core for performing echocardiography. Dr Feng performed the immunostaining, cell culture, tetrazolium chloride, histology, RNA sequencing, flow cytometry, and cell culture experiments. Drs Bajpai and Bredemeyer assisted with the flow cytometry experiments. Drs Ma, Koenig, and L. Lai assisted with the RNA sequencing analyses. I. Lokshina assisted with animal treatments, breeding, and harvests. Dr Förster provided the Ccl17+/− mice. Dr Kreisel provided Foxp3-GFP/DTR mice and assisted with experimental design and critical review of the manuscript. Dr Lavine is responsible for all aspects of this article, including experimental design, data analysis, and manuscript production.

**Sources of Funding**

Dr Lavine is supported by the National Institutes of Health (grants R01 HL138466, R01 HL139714, R01 HL151078, and R35 HL161185), Leducq Foundation Network (grant 20CDV02), Burroughs Welcome Fund (grant 1014782), Children’s Discovery Institute of Washington University and St Louis Children’s Hospital (grant CHL-1995-462, CHL-2017-628, and PM-LI-2019-829), Amgen, and Foundation of Barnes-Jewish Hospital (grant 8038-88).

**Disclosures**

None.

**Supplemental Material**

Methods Figures S1–S8 Tables S1 and S2

**REFERENCES**


