Activated T Lymphocytes are Essential Drivers of Pathological Remodeling in Ischemic Heart Failure

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Background—Inappropriately sustained inflammation is a hallmark of chronic ischemic heart failure (HF); however, the pathophysiological role of T lymphocytes is unclear.

Methods and Results—Permanent coronary ligation was performed in adult C57BL/6 mice. When compared with sham-operated mice, mice with HF (8 weeks after ligation) exhibited the following features: (1) significant (P<0.05) expansion of circulating CD3+CD8+ cytotoxic and CD3+CD4+ helper (Th) T lymphocytes, together with increased Th1, Th2, Th17, and regulatory T-cell (Treg) CD4+ subsets; (2) significant expansion of CD8+ and CD4+ T cells in failing myocardium, with increased Th1, Th2, Th17, and Treg CD4+ subsets, marked reduction of the Th1/Th2 ratio, augmentation of the Th17/Treg ratio, and upregulation of Th2 cytokines; and (3) significantly increased Th1, Th2, Th17 cells, and Tregs, in the spleen and mediastinal lymph nodes, with expansion of splenic antigen-experienced effector and memory CD4+ T cells. Antibody-mediated CD4+ T-cell depletion in HF mice (starting 4 weeks after ligation) reduced cardiac infiltration of CD4+ T cells and prevented progressive left ventricular dilatation and hypertrophy, whereas adoptive transfer of splenic CD4+ T cells (and, to a lesser extent, cardiac CD3+ T cells) from donor mice with HF induced long-term left ventricular dysfunction, fibrosis, and hypertrophy in naive recipient mice.

Conclusions—CD4+ T lymphocytes are globally expanded and activated in chronic ischemic HF, with Th2 (versus Th1) and Th17 (versus Treg) predominance in failing hearts, and with expansion of memory T cells in the spleen. Cardiac and splenic T cells in HF are primed to induce cardiac injury and remodeling, and retain this memory on adoptive transfer. (Circ Heart Fail. 2017;10:e003688. DOI: 10.1161/CIRCHEARTFAILURE.116.003688.)

Key Words: adaptive immunity | adoptive transfer | heart failure | inflammation | T lymphocytes

Heart failure (HF) is a state of chronic inflammation, characterized by heightened levels of circulating and myocardial proinflammatory cytokines that promote pathological left ventricular (LV) remodeling.1,2 Although preclinical and early human studies suggested a therapeutic role for cytokine antagonism in HF,1 large-scale clinical trials of antibody-based cytokine neutralization in HF failed to show benefit.3–5 The failure of immunomodulation trials suggests an incomplete understanding of the nature of inflammation in HF. Specifically, as plasma cytokine levels are in large part determined by the combined influences of innate and adaptive immune system activation, they may be less-sensitive indexes of underlying disease pathogenesis than the direct effects (both locally and systemically) of specific immune cell populations on the heart. In this regard, we have recently demonstrated robust expansion of monocytes/macrophages and dendritic cells (DCs) in chronic ischemic HF in the context of a proinflammatory cardiосplenic axis, with activated splenocytes homing to failing heart to induce tissue injury.6 These observations further suggested that directly targeting specific leukocyte populations might represent a more fruitful approach to immunomodulation in HF.

See Clinical Perspective

The primary function of DCs is to process and present antigens to T cells, thereby inducing T-cell activation.7 Therefore, the presence of global DC expansion in HF8 implies concomitant global T-cell activation. Several T-cell populations, including CD4+ T cells,4 Foxp3+ regulatory T cells (Tregs),8 and invariant natural killer T cells10 have been shown to favorably regulate inflammation, immune cell trafficking, angiogenesis, and cardiac remodeling early after myocardial infarction (MI). CD4+ T cells also promote macrophage recruitment and arteriogenesis in acute hindlimb ischemia models.11 However, whether and how T cells affect the pathophysiology of chronic ischemic cardiomyopathy and HF, which exhibit substantially different profiles of inflammatory activation and tissue injury than acute MI, are unknown. Notably, in murine models of nonischemic, pressure-overload HF, activated CD4+ T cells have been shown to infiltrate the...
heart and play a crucial role in promoting cardiac fibrosis, hypertrophy, and remodeling. In human HF secondary to either ischemic or nonischemic causes, a low circulating total lymphocyte count is predictive of poor outcomes and prognosis; however, the impact of specific lymphocyte subpopulations, and their relationship to HF disease stage, are poorly understood.

CD4+ T cells are composed of a heterogeneous family of adaptive immune cells with specialized functions. These include proinflammatory T-helper (Th)1 cells that produce interferon-γ (IFN-γ) and interleukin (IL)-2, anti-inflammatory Th2 cells that produce IL-4, IL-5, and IL-13, proinflammatory Th17 cells that secrete IL-17, and immunomodulatory regulatory T cells (Tregs) that globally suppress the activation of immune responses. Given that ischemic HF is a chronic inflammatory state, it follows that alterations in the activation and spatiotemporal distribution of T-cell subsets during the development of HF may have considerable impact on the progression of pathological LV remodeling. Accordingly, we characterized temporal changes in circulating T-lymphocyte profiles during the development of ischemic HF, the status of various CD4+ T-cell subsets in the heart, mediastinal lymph nodes (LNs), and spleen in chronic HF 8 weeks after MI, and, using a combination of antibody-mediated depletion and cardiac and splenic T-cell adoptive transfer, delineated the pathophysiological role of T cells in HF progression. Our results indicate that T cells are globally expanded and activated in chronic ischemic HF and are indispensable for the progression of pathological LV remodeling.

**Methods**

All animal studies were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee and were compliant with the NIH Guide for the care and use of Laboratory Animals (DHHS publication No. 85-23, revised 1996). A total of 138 mice were used for all the studies.

**Mouse Model and Surgical Protocol**

Male, 8- to 10-week-old C57BL/6 mice (Jackson Laboratories, stock no. 000664) underwent left thoracotomy and either sham surgery (n=28) or left coronary artery ligation (n=80) to induce MI and ischemic HF, as previously described. Peripheral blood was collected serially (1, 2, 4, 6, and 8 weeks post MI) from the facial vein, and all the mice were euthanized at 8 weeks post MI. Heart, spleen, and heart-draining mediastinal LNs were collected at the time of euthanasia, and mononuclear cells were isolated either for T-cell analysis by flow cytometry or for adoptive transfer studies (see below).

**Echocardiography**

Murine echocardiography was performed under 1% to 2% isoflurane anesthesia using a VisualSonics Vevo 770 High-Resolution System, a heated, bench-mounted adjustable rail system (Vevo Imaging Station) and RMV707B scanhead as previously described. Blood pellets were resuspended in staining buffer and incubated with a cocktail of antimouse CD3-FITC/PE (BD Biosciences), CD4-Quintos650NC/PECY7/eVolve 605/PerCP-Cy5.5 (eBioscience), CD8-Quintos605NC/eVolve 655 (eBioscience), and NK1.1-PE/AF700 (eBioscience) (and in separate experiments CD44-PE from BD Biosciences and CD62L eVolve 605 and CD69-AF700 from eBioscience) fluorochrome-conjugated antibodies for 1 hour. After washing with phosphate-buffered saline, the cells were fixed using 1% paraformaldehyde, permeabilized for 30 minutes with 0.5% v/v tween-20 and stained with fluorochrome-conjugated antibodies (eBioscience) targeting the intracellular cytokines IFN-γ, IL-4, and IL-17, and the transcription factor Foxp3. In some experiments, cells were fixed before staining with antibodies for cell surface markers. After 1 hour, the cells were washed with phosphate-buffered saline and data were acquired by BD FACSDiva LSR-II Flow cytometer (BD Biosciences). FlowJo software version 10.0.6 was used for data analysis. From a separate group of mice, 100 μL of blood, 15 to 20 mg of spleen, and 1 mediastinal LN were analyzed for cell counts, whereas whole tissue was digested for absolute counts of cardiac T cells. AccuCount beads were used to adjust for differences in events recorded by the flow cytometer. The total live cell side scatter/forward scatter (SSC/FSC) gate was used to normalize cell populations in the spleen, mediastinal LNs, and heart, whereas the total lymphocyte–monocyte gate was used in peripheral blood. The pan T-cell marker CD3 was used to identify all lymphocytes. Helper and cytotoxic T cells were defined as CD3+CD4+ and CD3+CD4-CD8+, respectively. Among CD4+ T cells, the presence of intracellular IFN-γ, IL-4, IL-17, and Foxp3 was used to identify Th1, Th2, Th17, and Treg subsets, respectively. Also, CD3+CD4+CD44+ T cells were considered antigen-experienced, CD44hiCD69+ were further defined as activated effector T cells, CD44hiCD69+ as total memory T cells, and CD44hiCD62Lhi as central memory T cells.

**Measurement of Circulating Cytokines**

Blood was collected from the facial vein, clotted and centrifuged at 4000g (10 minutes at 4°C), and serum was separated and stored at −80°C for analysis. Simultaneous analysis of circulating T-lymphocyte–related cytokines (IFN-γ, tumor necrosis factor-α, IL-17, IL-6, IL-10, and IL-4) was performed using the mouse Th1/Th2/Th17 Cytometric Bead Array (CBA) kit (BD Biosciences) following the manufacturer’s protocol. Briefly, 50 μL of serum or standard was incubated with 50 μL of bead mixture and PE-conjugated detection antibody at room temperature. After 2 hours, excess PE-conjugated reagent was removed by washing with 1 mL of wash buffer, and samples were subsequently analyzed on a BD LSRII flow cytometer. FCAP Array software was used to measure mean fluorescence intensity of each cytokine, and serum concentrations were calculated from standard curves prepared simultaneously.

**Cardiac and Splenic Gene Expression by Quantitative Real-Time PCR**

RNA extraction from LV tissue (encompassing remote and border zone myocardium), cDNA synthesis, and quantitative real-time PCR were performed as previously described. Methodology for the determination of splenocyte gene expression has been previously described. Briefly, splenic mononuclear cells were isolated by layering on a Ficoll-Paque gradient and incubated in serum-free Dulbecco’s Modified Eagle Medium overnight. Adherent cells were collected and stored at −80°C in TRIzol reagent (Invitrogen) for subsequent RNA extraction and measurement of mRNA transcript levels. Gene expression was determined for IL-2, IL-4, IL-5, IL-10, IL-13, IL-17,
IL-18, IL-33, IL-12Rβ2, IFN-γ, transforming growth factor-β, and C–C chemokine receptor type 5. The forward and reverse primer pairs used to determine gene transcript levels are provided in Table I in the Data Supplement. Gene expression was normalized to β-actin for LV tissue and 18s rRNA expression for splenocytes using the ΔΔCt comparative method and expressed as fold-changes. Th1/pro-inflammatory mediators considered were IFN-γ (Th1 T-cell–specific cytokine), IL-12Rβ2, and C–C chemokine receptor type 5 (receptors induced during Th1 polarization). IL-18 (promotes Th1 polarization), IL-17 (produced by Th17 cells), and IL-2 (released by T cells). For anti-inflammatory markers, we measured IL-4, IL-5, and IL-13 (cytokines released by Th-2 cells), transforming growth factor-β and IL-10 (anti-inflammatory cytokines released by Tregs and Th-2 T cells). Histological staining was performed as previously described.

Masson’s trichrome was used to evaluate tissue fibrosis and Alexa Fluor 488–conjugated wheat germ agglutinin (Invitrogen) to assess myocyte area, as quantified from 5 to 6 high-power fields per section in noninfarcted myocardium using Metamorph software version 6.3r5 (Molecular Devices).

To evaluate for tissue abundance of CD4+ and CD8+ T cells, heart sections were embedded in OCT compound (Tissue-Tek OCT) and then kept at −80°C until sectioning. Sections (7 μm thickness) were fixed with 4% paraformaldehyde in phosphate-buffered saline, and labeled with either rat antimouse CD4 (Clone GK1.5; eBiosciences) or CD8 antibody (Clone 4SM15; eBiosciences). Goat antirat antibody conjugated with Alexa Fluor 555 and 488 (Life Technologies) were used as secondary antibodies for CD4 and CD8, respectively, and fluoroshield-containing DAPI (Sigma-Aldrich) was used as the mounting medium. CD4+ or CD8+ cells were quantified using confocal microscopy from 5 to 6 sections and 3 to 4 mice from each group. All measurements were conducted in a blinded manner. Confocal microscopy was performed on an LSM710 microscope (Zeiss).

Antibody-Mediated In Vivo CD4+ T-Cell Ablation
Four weeks after coronary ligation, C57BL/6 mice with comparable weights were euthanized and tissues harvested for gravimetric and histological analysis by flow cytometry.

Adoptive Transfer Studies
CD3+ T cells from the heart and CD4+ T cells from the spleen were harvested from naive control (n=6) and HF mice (n=6; 8 weeks post MI) and used for adoptive transfer into naive recipient mice as described below.

Spleenic CD4+ T Cells
Mononuclear splenocytes from naive control and HF mice were isolated as described previously. Splenic CD4+ T cells were separated and collected using the mouse CD4+ T-cell isolation kit (Miltenyi) following the manufacturer’s protocol. Cell recovery was 12×10^6 cells per spleen, with 85% to 90% purity of the CD4+ T-cell population by flow cytometry and 90% to 95% cell viability by trypan blue exclusion.

Cardiac CD3+ T Cells
Naive control and HF hearts were minced and digested with collagenase, and cardiac mononuclear cells were isolated as described previously. Cells were labeled with CD3-FITC (BD Biosciences) for 30 minutes at 4°C. Viable CD3+ T cells were identified by exclusion of the fluorochrome 7-aminocyaninomycin D (7-AAD) and then sorted by flow cytometry.

Because of the labor-intensive experimental setup, tissues from 3 animals were processed per day. Cells isolated from either 3 HF or control mice each day were pooled, divided into equal aliquots, and adoptively transferred intravenously to syngeneic naive C57BL/6 recipient mice. The interday variability in the number of transferred splenic CD4+ T cells was negligible, ranging from 11 to 13×10^6 cells. However, the number of cardiac CD3+ T cells adoptively transferred to recipients varied on different days because of inconsistent yield from control and failing hearts. The first group (n=5) of naive recipients administered failing cardiac T cells were given ≈7300 CD3+ cells intravenously, whereas the second group (n=3) received ≈48 100. Naive recipients administered control cardiac T cells were given between 15 400 and 18 200 CD3+ cells (n=6 total). Recipient mice were followed by serial echocardiography performed at baseline (day 0) and 4 and 8 weeks after cell transfer. After 8 weeks, mice were euthanized and tissues harvested for gravimetric and histological analysis.

Statistical Analysis
All quantitative analyses are means±SD. For statistical analyses, the variance of data sets was first assessed using the F-statistic, and the D’Agostino–Pearson test was used to assess normality. Statistical comparisons between HF and sham/control groups were then performed using the unpaired Student’s t test with equal or unequal variance, for normally distributed data. For non-normally distributed data (observed with quantitative data of absolute T-cell number in the myocardium), logarithmic transformation was first performed to satisfy the assumption of normality, followed by analysis using the unpaired Student’s t test with equal or unequal variance. Repeated-measures ANOVA and Bonferroni post-test were used to analyze the adoptive transfer studies. GraphPad Prism version 7.0a was used for all analyses. A P value of <0.05 was considered significant.

Results
Profound Structural LV Remodeling in Chronic Ischemic HF
Cardiac structural and functional parameters for control and HF mice 8 weeks after sham surgery or coronary ligation are shown in Figure I in the Data Supplement. Representative gross heart specimens and M-mode echocardiograms of sham and failing hearts demonstrated cardiac enlargement, LV dilatation, and LV systolic dysfunction in HF mice. As seen in the group data (Figure IA in the Data Supplement), HF mice exhibited markedly increased LV end-diastolic and end-systolic volume, severely reduced LV ejection fraction, and significantly increased heart and wet lung weights consistent with cardiac hypertrophy and pulmonary congestion (Figure IB in the Data Supplement). Increased spleen size and weight, along with increased total counts of splenic CD45+ leukocytes and CD3+ T cells, were also evident in HF mice (Figure IC in the Data Supplement), consistent with splenic remodeling occurring in HF, as demonstrated in our earlier study. Hence, there was substantial pathological LV remodeling 8 weeks post MI and evidence of chronic HF.

CD4+ T Lymphocytes Are Globally and Progressively Expanded in HF
CD3+CD4+ (helper) and CD3+CD4−CD8+ (cytotoxic) T lymphocytes were serially measured in peripheral blood after...
As evident from Figure 1A, 1B and Figure II A in the Data Supplement, both sham and HF mice exhibited time-dependent reduction in the frequency of circulating CD4+ and CD8+ T cells after operation, approximating levels in nonsurgical control mice by 2 weeks, a response likely associated with the surgical procedure.
However, in ligated mice, blood CD4⁺ T-cell frequency was significantly higher than sham at nearly all time points after MI (only the 2 weeks post MI interval did not reach significance, \(P=0.064\)), and measured absolute circulating CD4⁺ cell count was elevated at both 1 and 8 weeks after MI (Figure 1B). Similarly, the frequency of CD8⁺ T cells was significantly increased over sham mice both early (1 week) post-MI and in established HF (≥24 weeks after MI), as was the absolute circulating CD8⁺ T-cell count 8 weeks after MI (Figure IIA in the Data Supplement).

Circulating CD4⁺ T-cell subsets were characterized as Th1 (CD3⁺CD4⁺NK1.1⁻IFN-\(\gamma\)⁺), Th2 (CD3⁺CD4⁺NK 1.1⁻IL-4⁺), Th17 (CD3⁺CD4⁺NK1.1⁻IL-17⁺), and Tregs (CD3⁺CD4⁺Foxp3⁺). As invariant natural killer T cells also produce both IFN-\(\gamma\) and IL-4, only NK1.1⁺ cells were considered for Th1, Th2, and Th17 cell characterization. Similar to total CD4⁺ T cells, the frequency of circulating Th1, Th2, and Th17 cells was increased in HF versus sham mice at all time points after MI (Figure 1C). Furthermore, absolute Th1, Th2, and Th17 cell counts were all significantly increased in HF mice when compared with sham at 8 weeks post MI (Figure I1D). Th1 and Th2 cell counts were also elevated early (1 week) post-MI. In contrast, the profile of circulating Tregs, an immunomodulatory CD4⁺ T-cell subset, was biphasic in HF mice, with no change in either frequency or absolute counts early (1 week) post-MI, and then augmented levels onward from 2 weeks post MI, including increased total counts at 8 weeks when compared with sham mice. Consistent with the cell data, mice with HF (8 weeks post MI) exhibited the elevated serum levels of cytokines that induce either Th1 cells (IFN-\(\gamma\) and tumor necrosis factor-\(\alpha\)), Th2 cells (IL-6 and IL-10) or Tregs (IL-10)³⁴,³⁵ when compared with sham, but without observed differences in serum IL-17 and IL-4 (Figure 1E).

CD4⁺ T Lymphocytes Are Augmented in the Failing Heart

CD4 and CD8 immunostaining was performed in hearts harvested 8 weeks after coronary ligation or sham operation (Figure 2; Figure IIB in the Data Supplement). Both CD4⁺ and CD8⁺ T cells were readily observed in failing hearts, at nearly 3- to 5-fold higher levels than in sham hearts (\(P=0.018\) and 0.022, respectively). Cardiac T-cell subpopulations were quantified by flow cytometry. As depicted in Figure 3A, >95% of cardiac mononuclear cells were viable and excluded the membrane impermeant fluorochrome 7-AAD. Scatter plots for CD4⁺, CD8⁺, and Foxp3-stained CD3⁺ T cells indicated significantly augmented populations of CD4⁺ helper, CD8⁺ cytotoxic, and Foxp3⁺ regulatory T cells in failing hearts (Figure 3B; Figure IIC in the Data Supplement). Of the Th subsets, cardiac Th1, Th2, and Th17 populations were all robustly increased in HF when compared with sham (Figure 3C). The cardiac Th1/Th2 ratio was decreased in HF, indicating a Th2 predominant milieu in failing myocardium and was accompanied by an augmented Th17/Treg ratio (Figure 3D). This cell profile was accompanied by diffuse mRNA upregulation of multiple Th2 (and Treg) polarizing inflammatory mediators in border and remote zone failing myocardium (IL-4, IL-13, IL-5, IL-16, IL-22, IL-10, IL12R\(\beta\)2, C–C chemokine receptor type 5, and IL-2; Figure 3E).

IL-33, transforming growth factor-\(\beta\), and IL-10) but only select overexpression of Th1 and Th17 polarizing mediators when compared with sham (IL-18 and IL-17; no difference in IFN-\(\gamma\), IL12R\(\beta\)2, C–C chemokine receptor type 5, and IL-2; Figure 3E).

CD4⁺ T Lymphocytes Are Broadly Activated in the HF Spleen and Mediastinal LNs

In HF mice, the hypertrophied spleen exhibited expansion of both CD4⁺ and CD8⁺ T cells when compared with sham (Figures 4A; Figure IIDD in the Data Supplement) and significant elevation of all CD4⁺ T-cell subsets examined—Th1, Th2, Th17, and Tregs (Figure 4A and 4B). Splenocyte gene expression in established HF (8 weeks post MI) exhibited the upregulation of both Th1 polarizing mediators (IFN-\(\gamma\), IL12R\(\beta\)2, C–C chemokine receptor type 5, IL-18, IL-17, IL-2) and Th2 (and Treg) polarizing mediators (IL-4, IL-5, IL-10; no change in IL-13, IL-33, transforming growth factor-\(\beta\); Figure 4E). Splenic antigen-experienced CD4⁺ T cells, defined by high CD44 expression,²⁵ were also expanded significantly in HF mice (Figure 4D). This expansion was composed of increased levels of both CD44⁺CD69⁺ activated effector T cells and CD44⁺CD69⁻ total memory CD4⁺ T cells²⁷ (Figure 4E). Splenic central memory T cells²⁷ as identified by CD44⁺CD62L⁺ expression were also significantly elevated in HF mice when compared with sham (Figure 4F). Like the spleen, mediastinal LNs in HF mice were enlarged when compared with sham mice, with similar increases in cell frequencies and counts of CD4⁺ T cells and the Th1, Th2, Th17, and Treg subsets, consistent with immune activation (Figure III in the Data Supplement). The total cell number of CD8⁺ T cells was also increased significantly in HF mediastinal LNs (Figure...
Bansal et al  T cells in Heart Failure

At 4 weeks post MI, HF mice with equivalent degrees of LV dilatation and dysfunction (Figure IV in the Data Supplement) were randomly assigned to receive either anti-CD4 antibody or isotype IgG control antibody, and LV remodeling and T-cell subsets were assessed at 8 weeks (Figure 5A). Anti-CD4 treatment selectively depleted CD4+ T cells in the blood, spleen, mediastinal LNs, and heart, without affecting circulating CD8+ T cells (Figure 5B). Moreover, CD4+ T-cell depletion reduced LV and splenic remodeling, as evidenced by lower normalized heart, LV, and spleen weight when compared with control antibody (Figure 5C). In addition, echocardiography revealed progressive LV chamber dilatation in HF mice treated with control antibody that was prevented by the administration of anti-CD4 antibody (Figure 5D), without significant effects on LV ejection fraction. Hence, CD4+ T cells are necessary for the progression of pathological structural remodeling.

CD4+ and CD3+ T Cells Are Sufficient to Induce Cardiac Remodeling

Splenic CD4+ T cells and cardiac CD3+ T cells isolated from HF mice (8 weeks post MI) or naive control mice were adoptively transferred (separately) into naïve recipient mice (Figure 6A). Splenic CD4+ T cells were selected based on our previous work.6
Figure 4. A, Example SSC/FSC live cell gate and scatter plots for CD3−CD4+ and CD3−CD4+Foxp3+ Tregs in mononuclear splenocytes isolated from sham and heart failure (HF) mice, and quantitative group data for the same, cell frequency previously published in Ismahil et al. B, Flow cytometry scatter plots and frequency and cell count group data for CD4+ T-cell subsets in the spleen. C, Th1- and (Continued)
that demonstrated significant cardiac dysfunction in recipient mice after adoptive transfer of HF-activated splenocytes. Cardiac CD3+ T cells were also tested as a T-cell population presumably enriched for activation against cardiac antigens. Recipient mice were followed for 8 weeks, with serial echocardiographic assessment of LV function every 4 weeks. As illustrated in Figure 6B, transfer of HF-activated splenic CD4+ T cells induced significant LV systolic dysfunction (increased end-systolic volume and reduced ejection fraction) and LV hypertrophy (increased LV wall thickness) during this time, but without chamber dilatation (no change in end-diastolic volume), when compared with mice that received control splenic CD4+ T cells. Gravimetric analysis 8 weeks after cell transfer revealed a significant increase in normalized heart weight (Figure 6C), and histological analysis indicated a mild but significant augmentation of interstitial fibrosis and robust myocyte hypertrophy (Figure 6D) in mice receiving HF-activated (versus naïve) splenic CD4+ T cells. Similar effects on LV function and interstitial fibrosis, but with less pronounced effects on cardiac hypertrophy, were observed on adoptive transfer of HF-activated cardiac CD3+ T cells into naïve recipients (Figure V in the Data Supplement). The milder responses engendered by cardiac CD3+ T-cell transfer may be either a consequence of fewer and greater variability of transferred T-cells in this group (see Methods section of this article) or heterogeneity in the transferred T-cell subset pool between cardiac and splenic cells (ie, cardiac cells included CD8+ cells and were more enriched for Th2 and Th17 T cells when compared with splenic T cells). Collectively, however, these results indicate that T cells in ischemic HF are primed to induce myocardial injury and contribute importantly to progressive LV remodeling.

Discussion

There are several key and novel findings in this study. First, in chronic ischemic cardiomyopathy, there is systemic expansion of CD4+ and CD8+ T cells and CD4+ Th1, Th2, Th17, and Treg subsets, which encompasses the failing heart, circulation, and lymphoid organs. Second, there is temporal divergence in the behavior of Th subsets during the progression of ischemic HF and organ-localized differences in Th-cell distribution in

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**Figure 4 Continued.** Th2-related gene expression in splenocytes harvested from sham and HF mice (n=5 per group). Fold changes are shown using 18S rRNA as the endogenous control. D, Representative flow histograms for splenic CD3+CD4+CD44+ T cells (left), scatter plots for identification of antigen-experienced CD44hi cells in sham-operated and HF mice (middle), and corresponding quantitative group data (right). E, Flow cyometry scatter plots and group data for CD4+CD44+CD69− activated (effector) T cells and CD4+CD44+CD69+ total memory T cells, and (F) CD4+CD44+CD62Lhi central memory T cells in splenocytes from sham-operated and HF mice (n=5–6/group). IFN-γ indicates interferon-γ; IL, interleukin; and TGF, transforming growth factor.
established disease, with the failing myocardium exhibiting Th2 (when compared with Th1) and Th17 (when compared with Tregs) predominance (see below). Third, we have established that activated CD4+ T cells are necessary for disease progression in ischemic HF, as CD4+ T-cell ablation alleviated pathological LV remodeling. Fourth, we have demonstrated that antigen-experienced splenic effector and memory T cells are expanded in HF. Finally, via adoptive transfer, we have provided direct evidence that both splenic CD4+ and cardiac CD3+ T cells in HF are biologically active and primed for myocardial injury, and sufficient for the production of pathological remodeling. Taken together, we conclude that systemic CD4+ T-cell activation, presumably directed toward cardiac antigens, plays an indispensable role in the progression of adverse cardiac remodeling in chronic ischemic HF.

Chronic Ischemic HF Is a Systemic CD4+ T-Cell–Activated State

Multiple immune cell types infiltrate the acutely infarcted heart in a phasic manner during early post-MI remodeling.36–40 Following an early wave of neutrophils, infiltrating proinflammatory (Ly-6C+) monocytes promote tissue digestion early (1–4 days) after MI and subsequently differentiate into reparative (Ly-6C−) macrophages that resolve inflammation and promote fibrosis over several days. In concert with DCs,41 CD4+ T cells,8 including Foxp3+ Tregs,9 infiltrate the heart and promote wound healing after MI by favorably influencing monocyte/macrophage trafficking and differentiation and by facilitating collagen matrix formation and angiogenesis. Indeed, effective myocardial repair and remodeling after ischemic injury are critically linked to tissue-level responses mediated by both innate and adaptive immune cells.39 Importantly, early post-MI remodeling is ultimately marked by inflammation resolution and quiescence; consequently, the aforementioned studies provide little insight as to how T cells might affect ischemic cardiomyopathy and HF, a disease state with a substantially different inflammatory profile.

We recently demonstrated that ischemic cardiomyopathy (well after completion of early post-MI remodeling) is characterized by sustained expansion of proinflammatory...
Activated CD3+CD4+ T cells impart detrimental effects on LV overload-induced HF have indicated that heart infiltrating, this regard, recent studies of murine nonischemic (pressure overload-induced) HF have indicated that heart infiltrating, activated CD3+CD4+ T cells impart detrimental effects on LV remodeling.12,13 However, the role of CD4+ T cells, and the behavior of various CD4+ T-cell subsets, in chronic ischemic HF, in which the heart exhibits much more robust initial cell death, has heretofore been poorly understood.

Our data establish (to our knowledge for the first time) that in chronic ischemic HF, there is robust T-cell expansion of both CD4+ and CD8+ T cells, and CD4+ Th1, Th2, Th17, and Tregs, both within the failing myocardium and globally in the lymphoid organs and circulation. Interestingly, although all CD4+ T-cell subsets were expanded in the failing heart, the Th1/Th2 ratio was significantly diminished (classically anti-inflammatory), whereas the Th17/Treg ratio was increased (classically proinflammatory). Moreover, HF was characterized by robust proinflammatory gene expression in spleen, and augmented levels of splenic CD4+CD44++CD69+ antigen-exposed effector T cells,25,27 CD4+CD44++CD69+ total memory T cells27 and CD4+CD44++CD62L+ central memory T cells, consistent with T-cell activation presumably in response to self-antigens during MI or post-MI remodeling, with potential for reactivation of heart-directed adaptive immunity on repeated exposures. These findings indicate that HF is not easily categorized as a “Th1” or “Th2” inflammatory state, but rather that T-cell alterations are spatiotemporally complex in the context of disease. This notion is in line with the understanding that CD4+ T cells are composed of a heterogeneous family of pro- and anti-inflammatory T cells with diversely varying properties.15 In the failing heart, the reduced Th1/Th2 cell ratio suggests that expanded cardiac Th2 cells may induce local damage by secreting Th2 cytokines, which are known fibrogenic factors.12 In this regard, studies in human HF have demonstrated that the urinary levels of IL-4 correlate with structural LV remodeling.43 Also, analogous to the failing heart in our study, the aging rodent heart exhibits a Th2-type phenotype, with augmented IL-4 and IL-13 levels that enhance collagen synthesis and interstitial fibrosis.44 Hence, Th2 T cells may represent a therapeutic target in chronic ischemic HF to reduce tissue fibrosis and inflammation.

Our assessment of the T-cell network during the development of ischemic HF highlights two additional caveats. Tregs, immunomodulatory T cells that suppress effector T-cell responses,45 exhibited a biphasic profile in the circulation, initially decreased in frequency early (1 week) after MI (without change in absolute cell counts), with subsequently elevated levels thereafter when compared with sham mice. Although we did not specifically examine the role of Tregs in HF, in light of their potent immunoregulatory features, Treg modulation may represent a viable approach to indirectly modify CD4+ T-cell behavior and chronic inflammation. Moreover, we observed time-dependent alterations in circulating CD4+ T cells and helper T-cell subsets in sham-operated mice, when compared with nonsurgical control mice, which generally approximated normal levels after 2 to 4 weeks. Presumably these changes represent a generalized response to thoracotomy and subsequent postoperative healing (and not MI per se) and should be considered in the design of future experimental studies.

**HF-Activated CD4+ T Cells Are Both Necessary and Sufficient for Pathological LV Remodeling**

Our findings of global CD4+ T-cell activation in murine ischemic HF are consistent with the previous demonstration of increased circulating CD4+ T cells and CD4/CD8 T-cell ratios in human HF.46,47 To evaluate the pathophysiological role of T-cell expansion, we first evaluated whether CD4+ T cells are required for LV remodeling. Anti-CD4 antibody administration in established HF halted progressive LV dilatation (without affecting systolic function) and improved chamber hypertrophy, indicating that activated CD4+ T cells are obligatory for the progression of structural disease. Notably, early after acute MI, Hoffman et al48 have shown that CD4+ T cells are beneficial for infarct wound healing and tissue repair. In contrast, our results show that in chronic ischemic HF, CD4+ T cells impart proinflammatory and detrimental effects on the heart that promote adverse remodeling. Interestingly, antibody-mediated CD3+ T-cell depletion49 and genetic CD4+ (but not CD8+) T-cell disruption50 in the setting of pressure overload prevented the long-term development of systolic dysfunction and fibrosis, but did not affect hypertrophy, underscoring important differences about T-cell-mediated remodeling responses in ischemic vis-a-vis nonischemic HF.

Finally, to evaluate whether HF-activated T cells are sufficient to drive tissue remodeling, we defined the effects of adoptive transfer of splenic CD4+ T cells, and cardiac CD3+ T cells, isolated from HF mice. Interestingly, the transfer of HF-activated T-cells recapitulated cardiac injury in naive recipients over an extended follow-up period, with LV systolic dysfunction, chamber or myocyte hypertrophy, and interstitial fibrosis. Moreover, splenocytes exhibited marked upregulation of a broad array of proinflammatory mediators. These results are consistent with our previous work demonstrating a robust tissue–injurious cardiосplenic axis in ischemic HF,6 and also suggest that heart-localized T cells are responsible for detrimental tissue responses. The cardiac phenotype induced in recipient mice varied depending on splenic CD4+ versus cardiac CD3+ T-cell transfer. As alluded to above, this heterogeneity in response may be related to differences in the overall number of T-cells transferred, as well as disparities in the composition of T-cell subsets within transferred cardiac CD3+ T cells and splenic CD4+ T cells. Nonetheless, despite these limitations, these studies provide important proof-of-concept for T cells as disease mediators in HF.

The spleen and LN play several important roles in antigen-specific T-cell immunity, including naive T-lymphocyte recruitment, the screening of antigens, and antigen-loaded DCs in blood and lymph delivered from inflamed sites, the provision of a suitable microenvironment for inducing effector responses.
cells. Most importantly, activated CD4+ T cells are obligatory
Th17 cell predominance when compared with Th1 cells and
Th1, Th2, Th17, and Treg cells. Failing hearts exhibit Th2 and
fer. Hence, immunomodulation of CD4+ T cells may represent
activated in HF to induce cardiac tissue injury on adoptive trans-
mon of cardiac-injurious effects of splenic CD4+ T cells from HF
none. We gratefully acknowledge the excellent technical assistance provid-
mg of Health grants 1K99HL132123 (to Dr Bansal) and 1R01HL125735
uT cells in Heart F ailure

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**Disclosures**

None.

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Therefore, we propose that ischemic cardiomyopathy should be thought of, in part, as an immune-mediated disease, as CD4+ subsets, including Th1, Th2, Th17, and regulatory T cells, were increased in the heart, together with expansion of splenic levels. In this study, we demonstrate a central role for T cells in the progression of cardiac remodeling after myocardial goal. In particular, clinical trials of proinflammatory cytokine neutralization failed to show benefit. Classically, cytokine Although heightened inflammation is a hallmark of heart failure, therapeutic immunomodulation remains an unrealized approach. On the basis of these results, we further propose that circumscribed inhibition of CD4+ T cells may represent a more fruitful therapeutic strategy in chronic ischemic heart failure. Our findings should stimulate an analogous evaluation immunomodulation as a viable approach to alleviate pathological cardiac remodeling.
Activated T Lymphocytes are Essential Drivers of Pathological Remodeling in Ischemic Heart Failure

Shyam S. Bansal, Mohamed Ameen Ismahil, Mehak Goel, Bindiya Patel, Tariq Hamid, Gregg Rokosh and Sumanth D. Prabhu

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Supplemental Table 1. Forward and reverse primer sequences used for RT-PCR.

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**Supplemental Figure Legends**

**Figure S1.** *(A)* Representative M-mode echocardiograms and quantitative group data for LV ejection fraction (EF), end-diastolic volume (EDV), and end-systolic volume (ESV) from heart failure (HF) and sham mice 8 w after permanent coronary ligation or sham surgery (n = 5-6 per group). *(B)* Representative gross images of a sham-operated and failing heart, and group gravimetric data for heart, and wet lung weights. *(C)* Representative gross image of spleens isolated from a HF and sham mouse and group quantitation of organ weight, and total cell counts for CD45+ leukocytes and CD3+ T-cells per spleen. All weights normalized to tibia length (n = 4-6 per group).

**Figure S2.** *(A)* Representative flow cytometry scatter plots for CD3+CD4+ helper and CD3+CD8+ cytotoxic T-cells and group quantitation of CD8+ T-cell frequency in the circulation at different time intervals post-MI, and absolute cell counts at 8 w post-MI (HF) or sham surgery. *(B)* Representative confocal microscopy images of CD8+ T-cells *(green)* in sham-operated and failing hearts (8 w post-MI) and group quantitation. *(C)* Representative flow cytometry density plots and group quantitation of CD3+CD8+ T-cells in hearts, *(D)* spleens, and *(E)* lymph nodes (LNs) isolated from sham-operated and HF mice (n = 5-10).

**Figure S3.** *(A)* Example gross images of heart-draining mediastinal lymph nodes (LNs) from a sham-operated and HF mouse *(Inset)*, along with flow cytometry scatter plots for live cell SSC/FSC gate and CD3+CD4+ helper and CD3+CD4+Foxp3+ regulatory T-cells in mediastinal LNs and *(B)* corresponding group quantitation. *(C)* Group quantitation of the frequency and absolute number of CD4+ T-cell LN subsets: Th1 (IFN-γ+), Th2 (IL-4+), and Th17 (IL-17+) (n = 4-6 per group).
**Figure S4.** Group quantitation of baseline end-diastolic and end-systolic volume (EDV and ESV), and ejection fraction (EF) in HF mice 4 w post-MI prior to randomization to treatment with either isotype IgG or anti-CD4 antibody (n = 6-10 per group).

**Figure S5.** Naïve mice were adoptively transferred with cardiac CD3⁺ T-cells sorted from either naïve control or HF mice and followed for 8 w, as outlined in Figure 6A. **(A)** Quantitative group data for changes in LVEF, EDV, and ESV, in recipient mice 4 and 8 w after cardiac CD3⁺ T-cell transfer, along with measured septal and posterior wall (PW) thickness at 8 w. **(B)** Gravimetric data for the same experimental groups at 8 w after transfer. Representative Masson’s trichrome stains and quantitation of fibrosis **(C)**, and wheat germ-agglutinin stains (cell membranes green, nuclei blue with DAPI counterstain; scale bar 200 μm) and quantitation of myocyte size **(D)** in mouse hearts 8 w after transfer of naïve control or HF-activated cardiac CD3⁺ T-cells. Arrows indicate representative myocytes used for measurement of cross-sectional area.
Baseline LV parameters prior to antibody treatment