Inflammation

Increasing Regulatory T Cells With Interleukin-2 and Interleukin-2 Antibody Complexes Attenuates Lung Inflammation and Heart Failure Progression

Huan Wang,* Lei Hou,* Dongmin Kwak, John Fassett, Xin Xu, Angela Chen, Wei Chen, Bruce R. Blazar, Yawei Xu, Jennifer L. Hall, Jun-bo Ge, Robert J. Bache, Yingjie Chen

See Editorial Commentary, pp 27–29

Abstract—Congestive heart failure (CHF) is associated with an increase of leukocyte infiltration, proinflammatory cytokines, and fibrosis in the heart and lung. Regulatory T cells (Tregs, CD4+CD25+Foxp3+) suppress inflammatory responses in various clinical conditions. We postulated that expansion of Tregs attenuates CHF progression by reducing cardiac and lung inflammation. We investigated the effects of interleukin–2 (IL–2) plus IL–2 monoclonal antibody clone JES6-1 complexes (IL2/JES6-1) on induction of Tregs, transverse aortic constriction–induced cardiac and lung inflammation, and CHF progression in mice. We demonstrated that end-stage CHF caused a massive increase of lung macrophages and T cells, as well as relatively mild left ventricular (LV) leukocyte infiltration. Administration of IL2/JES6-1 caused an ∼6-fold increase of Tregs within CD4+ T cells in the spleen, lung, and heart of mice. IL2/JES6-1 treatment of mice with existing transverse aortic constriction–induced LV failure markedly reduced lung and right ventricular weight and improved LV ejection fraction and LV end-diastolic pressure. Mechanistically, IL2/JES6-1 treatment significantly increased Tregs; suppressed CD4+ T-cell accumulation; dramatically attenuated leukocyte infiltration, including decreasing CD45+ cells, macrophages, CD8+ T cells, and effector memory CD8+; and reduced proinflammatory cytokine expressions and fibrosis in the lung of mice. Furthermore, IL2/JES6-1 administered before transverse aortic constriction attenuated the development of LV hypertrophy and dysfunction in mice. Our data indicate that increasing Tregs through administration of IL2/JES6-1 effectively attenuates pulmonary inflammation, right ventricular hypertrophy, and further LV dysfunction in mice with existing LV failure, suggesting that strategies to properly expand Tregs may be useful in reducing CHF progression. (Hypertension. 2016;68:114-122. DOI: 10.1161/HYPERTENSIONAHA.116.07084.)

* Online Data Supplement

Key Words: fibrosis heart heart failure inflammation lung regulatory T cell

In clinic, patients with congestive heart failure (CHF) usually do not seek treatment until the symptoms of left ventricular (LV) dysfunction occur. Additionally, in many cases, CHF inevitably transits from LV failure to right ventricular (RV) failure and turns into the end-stage even with a proper therapy to improve the heart function. Therefore, in addition to improving the LV function, more effective therapies to attenuate the progression of existing LV failure is desired.

End-stage CHF exhibits increased pulmonary venous pressure, increased lung weight and lung leukocyte infiltration, pulmonary arterial hypertension, and subsequent RV hypertrophy and failure.1-3 Inflammation plays an important role in CHF development.4 The expression of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β are upregulated in cardiac tissues and blood of patients and experimental animals with CHF.4 T cells and CD4+ T cells accumulate in the LV tissues of mice with CHF and contribute to the pathogenesis of CHF.5,6 We recently demonstrated that end-stage CHF is associated with a profound lung leukocyte infiltration in mice,7 suggesting that inflammation plays an important role in LV failure–induced lung remodeling and the transition from LV failure to RV failure.

CD4+CD25+Foxp3+ regulatory T cells (Tregs) are a subpopulation of T cells that modulate immune tolerance and suppress inflammatory responses in various clinical diseases.4 IL-2
is a critical cytokine for the survival and functional competence of Tregs. Administration of IL-2 alone results in a robust expansion of Tregs in vitro, but only a mild induction of Tregs in vivo because of its rapid enzymatic degradation. A particular IL-2 monoclonal antibody clone JES6-1 binds to a site on IL-2 that could reduce IL-2 degradation in vivo and selectively lead to vigorous stimulation of CD25⁺ cells. Administration of IL-2 plus IL-2 monoclonal antibody clone JES6-1 complexes (IL2/JES6-1) could result in a robust increase of endogenous Tregs in experimental animals. 

We demonstrated that end-stage CHF in mice is associated with a dramatic accumulation of lung immune cells, including macrophages and T cells. Administration of IL2/JES6-1 can selectively expand Tregs ≈6-fold and attenuate the progression of transverse aortic constriction (TAC)–induced CHF by suppressing cardiac and lung and systemic inflammation in mice. Our findings suggest that cytokine therapy to selectively expand Tregs or cell therapy by transplantation of Tregs may be an attractive new therapeutic approach in treating CHF. An intense interest has been developed in using Tregs for immunotherapy, and efficacy has already been shown in suppressing immune inflammation-related diseases. Therefore, the translational potential of treating CHF with Tregs is high.

In this study, we treated the mice when they already had LV failure induced by TAC. Our experimental strategy is to examine the effect of IL2/JES6-1 complexes on the LV and lung in mice with existing LV failure, which is more clinically relevant.

Methods
Detailed methods are available in the online-only Data Supplement.

Animals and Experiment Design
IL2/JES6-1 complexes (1 μg mouse IL-2 plus 5 μg anti-IL-2 antibodies clone JES6-1; Biolegend) were administrated (IP) to mice on 3 consecutive daily doses every 6 days during the study. PBS was used for vehicle control.

To determine the effect of IL2/JES6-1 on mice with existing CHF, male Balb/c mice (4–5 weeks of age) from the Jackson Laboratory were used because Balb/c mice develop LV dysfunction rapidly in response to TAC. Briefly, male Balb/c mice were subjected to a TAC procedure created with a 27G needle, a model that mimics clinical heart failure, which is more clinically relevant.

Statistics
A normality test (Shapiro–Wilk) provided by SigmaPlot was used to determine whether data were normally distributed. If data were normally distributed, the data were presented as mean±SEM. A Student’s t test was used to test for differences between 2 groups. A 2-way ANOVA followed by a Bonferroni correction post hoc test was used to test for differences among >2 groups. If mouse physiological data were not normally distributed or the sample size in one of the experimental groups was <10, a nonparametric test (Mann–Whitney or Kruskal–Wallis) followed by a Bonferroni post hoc correction was performed. All pairwise P values are 2-sided. The null hypothesis was rejected at P<0.05.

Results

End-Stage CHF Exhibits Dramatic Leukocyte Infiltration in Lungs and Mild Leukocyte Infiltration in Hearts
We examined the relative inflammatory status in the lungs and hearts of mice with end-stage CHF produced by TAC. As shown in Figure 1A and 1B, ratios of LV and lung weight to tibial length were significantly increased 2.2-fold and 2.3-fold in CHF mice, respectively. Echocardiography and hemodynamic measurement showed that mice with CHF exhibited a significant decrease of LV EF (34% in CHF versus 77.6% in control group; Figure 1C) and a dramatic increase (12.3-fold) of LV end-diastolic pressure (Figure 1D), indicating that TAC caused end-stage CHF in these mice.

LV and lung leukocyte infiltration was significantly increased in CHF mice, as revealed by immunostaining and flow cytometry analysis of CD45⁺ cells. Notably, CD45⁺ cell infiltration was 0.15×10⁶ cells per LV and 10.1×10⁶ cells per lung in CHF mice (Figure 1E and 1F). In addition, flow cytometry analysis showed that CD3⁺ T cells were significantly increased in both the LV and the lung of CHF mice, whereas CD3⁺ T cell infiltration was 13.8×10⁶ cells per LV and 1780×10³ cells per lung in CHF mice (Figure 1G). Both CD4⁺ and CD8⁺ T cells were 2.2-fold and 2.7-fold increased, respectively, in the lung of CHF mice (Figure 1H and 1I). Flow cytometry gating strategies are presented in Figures S1 and S2 in the online-only Data Supplement. These data indicate that end-stage CHF is associated with a dramatic lung inflammation and relatively mild LV inflammation. Similar results were observed in both C57/B6 and Balb/c background mice.

IL2/JES6-1 Treatment Was Effective in Increasing Endogenous Tregs in Mice
Flow cytometry gating strategies are presented in Figures S2 and S3. IL2/JES6-1 treatment resulted in an ≈6-fold increase of Tregs in the spleen and lung after 5 days, whereas total numbers of CD4⁺ and CD8⁺ T cells were not altered (Figure S4A–S4F). Foxp3 immunostaining and quantitative data also demonstrated a significant increase of Tregs in the LV and lung in mice treated with IL2/JES6-1, whereas Tregs were rarely found in the LV compared with those in the lung (Figure S4G and S4H). At the end point of the treatment for 12 weeks, Tregs still maintained an ≈6-fold increase in the spleen with IL2/JES6-1 treatment without altering the total number of CD4⁺ or CD8⁺ T cells (Figure S4I–S4K). These data demonstrate that IL2/JES6-1 treatment can efficiently expand Tregs in mouse lungs and hearts.

IL2/JES6-1 Treatment Effectively Attenuated CHF Progression in Mice With Existing LV Failure
To examine whether IL2/JES6-1 treatment affected the progression of CHF in Balb/c mice exhibiting LV dysfunction, we used Balb/c mice because they usually develop LV dysfunction rapidly in response to TAC. Briefly, IL2/JES6-1 treatment was commenced to mice when LV EF reduced to 58% 10 days after TAC. Final studies were performed 4 weeks after TAC (Figure 2A).
Echocardiographic measurements showed that mice exhibited a significant decrease of LV EF (32% in CHF versus 78% before TAC) and a 2.1-fold increase in LV end-systolic diameter 4 weeks after TAC. IL2/JES6-1 treatment significantly attenuated the further loss of LV function, as indicated by significantly less reduction of LV EF and less increase of LV end-systolic diameter (Figure 2A and 2B). LV end-diastolic diameter was also increased 29% 4 weeks after TAC, whereas it was comparable between PBS- and IL-2/JES6-1-treated mice (Figure 2C). Moreover, hemodynamic measurements showed that LV end-diastolic pressure was significantly (6.3-fold) increased by TAC, and IL2/JES6-1 treatment significantly reduced this increase (Figure 2D). IL2/JES6-1 treatment also significantly attenuated TAC-induced reductions of LV maximum rate of rise of pressure \((\frac{dP}{dt_{\text{max}}}})\) and LV maximum rate of decline of pressure \((\frac{dP}{dt_{\text{min}}}})\) with similar aortic systolic pressures (Figure 2E–2G). Furthermore, ratios of LV, LA, lung, and RV weight to tibial length were, respectively, increased 1.4-fold, 14.5-fold, 2.2-fold and 1.3-fold 4 weeks after TAC, suggesting severe LV hypertrophy and dysfunction and intense pulmonary congestion because of TAC (Figure 2H–2K and Table S1). Interestingly, although ratios of LV weight to tibial length were not significantly different (Figure 2H and Table S1), ratios of lung and RV weight to tibial length were remarkably reduced in IL2/JES6-1-treated mice as compared with PBS-treated mice (Figure 2J and 2K and Table S1), suggesting that IL2/JES6-1 treatment might have exerted a prominent role in reducing lung remodeling and RV hypertrophy in these mice.

**IL2/JES6-1 Treatment Significantly Reduced Lung Inflammatory Cytokines and Mildly Affected LV Inflammatory Cytokines in Mice With Existing LV Failure**

To determine whether IL2/JES6-1 treatment plays a role in pulmonary or myocardial inflammation with existing LV failure, intraperitoneal IL2/JES6-1 treatment was administered to mice with LV failure and the effect on pulmonary and myocardial inflammatory cytokine levels was assessed. The results showed a significant reduction in lung inflammatory cytokines, indicating a potential role for IL2/JES6-1 in reducing pulmonary inflammation in LV failure.

**Table. Outline of Experiment Design**

<table>
<thead>
<tr>
<th>Study Purpose*</th>
<th>Mice Background</th>
<th>Needle Used for TAC</th>
<th>Time to Give IL2/JES6-1</th>
<th>Time to Collect Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol 1</td>
<td>Balb/c</td>
<td>27G</td>
<td>10 days after TAC</td>
<td>4 wk after TAC</td>
</tr>
<tr>
<td>Protocol 2</td>
<td>C57/B6</td>
<td>26G</td>
<td>3 days before TAC</td>
<td>12 wk after TAC</td>
</tr>
</tbody>
</table>

IL2 indicates interleukin 2; IL2/JES6-1, IL-2 plus IL-2 monoclonal antibody clone JES6-1 complexes; LV, left ventricular; TAC, transverse aortic constriction; and Treg, regulatory T cells.

*The purpose of protocol 1 was to determine the effect of induction of Tregs in treating mice with existing LV failure. The purpose of protocol 2 is to determine the effect of induction of Tregs in attenuating or preventing the development of LV failure. The degree of TAC is calibrated by a 27G needle (severe TAC) or a 26G (moderate TAC).
failure, lung and LV mRNA levels of inflammatory cytokines were measured by quantitative reverse transcriptase polymerase chain reaction. The percentage of Tregs and the total number of Tregs in the lung were 1.9-fold and 5.1-fold increased in CHF mice as compared with those in control mice. As expected, IL2/JES6-1 treatment resulted in a robust increase of Tregs in CHF mice (Figure 3A and 3B). As shown in Figure 3C, mRNA levels of IL-1β, TNF-α, and monocyte chemoattractant protein-1 were, respectively, upregulated 8.4-fold, 1.7-fold, and 4.8-fold in the lung of CHF mice, whereas IL-10 was reduced 81% as compared with that in control mice. IL2/JES6-1 treatment extensively suppressed IL-1β, TNF-α, and monocyte chemoattractant protein-1 by 85%, 33%, and 68%, respectively, and significantly (5.1-fold) increased IL-10 in CHF mice (Figure 3C). These data demonstrate that IL2/JES6-1 treatment increases Tregs and blunts proinflammatory cytokine expression in the lung of mice with existing LV failure.

Furthermore, as shown in Figure 3D, Foxp3 mRNA level was similar in the LV of CHF and control mice. IL2/JES6-1 treatment markedly increased Foxp3 mRNA level in CHF mice. The mRNA level of IL-1β in the LV was upregulated 1.8-fold, whereas TNF-α did not change in CHF mice. IL2/JES6-1 treatment did not alter IL-1β or TNF-α mRNA level. However, LV IL-10 mRNA level was significantly increased by IL2/JES6-1 treatment. These data suggest that IL2/JES6-1 treatment did not significantly affect the contents of proinflammatory cytokine IL-1β or TNF-α mRNA, but increased anti-inflammatory IL-10 mRNA in mice with existing LV dysfunction.

IL2/JES6-1 Treatment Suppressed Macrophage, CD45+, CD4+, and CD8+ Cell Infiltration in the Lung of Mice With Existing LV Failure

Inflammatory stimuli accumulate a variety of immune cells, including macrophages and cytotoxic CD8+ T cells, which generate inflammatory cytokines that are important for the outcome of tissue inflammation. To understand the potential mechanism for beneficial effects of IL2/JES6-1 on the lung inflammation because of CHF, we examined macrophages, CD4+, and CD8+ T cells in the lung using flow cytometry analysis. CHF mice exhibited dramatic macrophage accumulation in the lung. IL2/JES6-1 treatment significantly reduced macrophage accumulation in the lung with existing LV failure (Figure 4A and 4B), which was also consistent with the observation of Mac2 staining in the lung (Figure S5A). Besides, leukocyte infiltration, including CD45+ cells, CD4+, CD8+ T cells, and effector memory CD8+ T cells, was markedly increased in the lung of CHF mice. IL2/JES6-1 treatment significantly attenuated these increases in the lung of mice with existing LV failure (Figure 4C–4F). These data reveal that IL2/JES6-1
treatment reduces immune cell accumulation in the lung of mice with existing LV failure.

**IL2/JES6-1 Treatment Attenuated Pulmonary Fibrosis in Mice With Existing LV Failure**

Proinflammatory cytokines are known to accelerate the accumulation of collagen after the inflammatory response, which causes pulmonary fibrosis. As shown in Figure 5A and Figure S5B, lung fibrosis as identified by Sirius red and Fast green staining was remarkably increased (7.2-fold) in the lung of CHF mice as compared with that in control mice. IL2/JES6-1 treatment significantly attenuated TAC-induced profound lung fibrosis. Consistently, the increased transforming growth factor-β, a profibrogenic cytokine mRNA in the lung of CHF mice, was also significantly reduced by IL2/JES6-1 treatment (Figure 5B), which was in line with collagen-III deposition results (Figure 5C). These data demonstrate that IL2/JES6-1 treatment attenuates lung fibrosis in mice with existing LV failure.

**IL2/JES6-1 Treatment Attenuated TAC-Induced LV Inflammatory Responses and Hypertrophy**

Because IL2/JES6-1 treatment tended to decrease the LV hypertrophy in mice with existing LV failure (Figure 2H), we subsequently determined whether IL2/JES6-1 treatment attenuated TAC-induced LV inflammatory responses and hypertrophy. Briefly, IL2/JES6-1 treatment was commenced 3 days before a moderate TAC created with a 26G needle, and final studies were performed 12 weeks after TAC. As expected, IL2/JES6-1 treatment significantly attenuated TAC-induced increases in ratios of LV, LA, and lung weight to tibial length (Figure 6A–6C). IL2/JES6-1 treatment also attenuated TAC-induced decrease of LV EF (Figure 6D) and the increase of LV end-systolic diameter (Figure 6E), whereas LV end-diastolic diameter was not significantly affected (Figure 6F). Furthermore, IL2/JES6-1 treatment prevented TAC-induced increases of LV atrial natriuretic peptide and β-myosin heavy chain protein content (Figure 6G). In addition, IL2/JES6-1 treatment significantly attenuated TAC-induced increases of LV CD45+ cell infiltration (Figure 6H) and mRNA levels of IL-1β, TNF-α, IL-10, and Foxp3 mRNA levels in LV lysates. n=4 to 6 per group. IL2/JES6-1 indicates IL-2 plus IL-2 monoclonal antibody clone JES6-1 complexes.

**Discussion**

We demonstrate that induction of Tregs with IL2/JES6-1 treatment is effective in attenuating cardiac and lung inflammation, RV hypertrophy, and further LV functional deterioration in mice with existing LV failure. Mechanistically, we demonstrated that end-stage CHF is characterized by a profound immune cell accumulation in the lung and relatively mild inflammation in the heart. Cytokine therapy with IL2/JES6-1 results in significant increases of Tregs, consequently reduces infiltration of macrophages and CD8+ T cells, and decreases proinflammatory cytokine expression in the lung of mice with existing LV failure. IL2/JES6-1 treatment prior TAC also attenuates the development of LV hypertrophy and dysfunction. The potential underlying mechanism is summarized in Figure 6I and online Figure S6. These findings indicate that therapeutic approaches to...
increase Tregs are useful in treating CHF by attenuating cardiac, lung, and systemic inflammation.

In clinical conditions, patients usually do not seek treatment until LV-dysfunction symptoms occur. Therefore, therapies to attenuate the transition from LV failure to RV hypertrophy/failure are clinically significant. We found that IL2/JES6-1 treatment continuously caused systemic Treg induction in mice under control conditions and after TAC. IL2/JES6-1 treatment significantly reduced pulmonary inflammation and fibrosis and attenuated RV hypertrophy in mice with existing LV failure. IL2/JES6-1 also significantly attenuated further decrease of LV dysfunction in mice with existing LV failure. These data indicate that increasing Tregs can be a promising therapeutic approach to attenuate the transition from LV failure to RV hypertrophy/failure. The findings that IL2/JES6-1 treatment dramatically reduced lung inflammation/remodeling and RV hypertrophy without significantly attenuating LV hypertrophy in mice with existing LV failure suggest that IL2/JES6-1 treatment might exert more impact on the lung and RV remodeling in these mice. The decreased lung remodeling after IL2/JES6-1 treatment in mice with existing LV failure is likely a collective effect of the increased expression of anti-inflammatory cytokine IL-10, the reduced accumulation of inflammatory leukocytes (such as CD4+ T cells, CD8+ T cells, and macrophages), and the reduced expression of proinflammatory cytokines (such as IL-1β, TNF-α, and monocyte chemoattractant protein-1) in lung tissues. The reduced RV hypertrophy is likely a result of reduced lung inflammation and remodeling, as well as the reduced ventricular inflammatory response because of the induction of Tregs.

![Figure 4. IL2/JES6-1 treatment suppresses immune cell infiltration in the lung of mice with existing left ventricle (LV) failure. Data were collected from mice under basal conditions (Ctr) or treated with IL2/JES6-1 or PBS under transverse aortic constriction (TAC) conditions (CHF-IL2/JES6-1 or CHF-PBS). A. Flow cytometry plots and quantitative data represent the percentage of macrophages (F4/80+) within the CD45+ cell population of lungs. B. Quantitative data of flow cytometry represent the total number of macrophages in the lung. C–F. Quantitative data of flow cytometry represent the total number of CD45+ cells, CD4+ T, CD8+ T cells, and effector memory (CD44highCD62Llow) CD8+ T cells in the lung. n=4 to 6 per group. IL2 indicates interleukin 2; and IL2/JES6-1, IL-2 plus IL-2 monoclonal antibody clone JES6-1 complexes.](http://hyper.ahajournals.org/)

![Figure 5. IL2/JES6-1 treatment attenuates lung fibrosis in mice with existing left ventricle (LV) failure. Data were collected from mice under basal conditions (Ctr) or treated with IL2/JES6-1 or PBS under transverse aortic constriction (TAC) conditions (CHF-IL2/JES6-1 or CHF-PBS). A. Quantitative data of Sirius red/Fast green staining for detection of fibrosis in lungs. B. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) results of transforming growth factor (TGF)-β mRNA levels in lung lysates. C. Western blot of collagen III and β-actin (loading control) in lung lysates. n=5 per group. IL2 indicates interleukin 2; and IL2/JES6-1, IL-2 plus IL-2 monoclonal antibody clone JES6-1 complexes.](http://hyper.ahajournals.org/)
In addition, IL2/JES6-1 treatment before TAC also effectively attenuated TAC-induced LV hypertrophy and dysfunction, suggesting that IL2/JES6-1 treatment attenuates CHF progression not only by attenuating lung inflammation observed in mice with end-stage CHF, but also by attenuating the low-grade LV inflammation at least partially through the collective effect of the increased expression of IL-10, the reduced accumulations of CD3+ T and macrophages, and the reduced expression of proinflammatory cytokines in LV tissues. Our findings that induction of Tregs attenuates tissue inflammation and CHF progression are conceptually consistent with the important role of Tregs in suppressing inflammation in other diseases, such as type-1 diabetes mellitus, organ transplantation-induced inflammation and rejection, myocardial infarction-induced cardiac remodeling, and high fat diet-induced atherosclerosis. Acute depletion of Tregs resulted in a rapid development of autoimmune diseases and mice died within 3 weeks. Depletion of Tregs with Foxp3-DTR transgene in adult mice resulted in an increased infarct size, exacerbated myocardial inflammation, and worse clinical outcome. Depletion of Tregs using anti-CD25 antibodies also resulted in increases of infarction-induced LV dilation and remodeling. Although the detailed molecular mechanism by which Tregs exert their suppressor/regulatory activity has not been definitively characterized, studies indicate that Tregs exert their biological actions by suppressing proliferation and cytokine production of T helper cells, reducing the maturation and function of antigen-presenting cells, and raising the secretion of anti-inflammatory cytokines, such as IL-10 and transforming growth factor-β.

IL-2 is required for the proliferation, functional competence, and stability of Tregs and plays a critical role in regulating immune tolerance. Inhibition of IL-2 signaling by IL-2 neutralization, IL-2 gene deletion, or IL-2 receptor deletion causes autoimmune diseases, such as diabetes mellitus and colitis, by reducing proliferation of Tregs. IL-2 administration drives Treg expansion both in vitro and in vivo. However, because of a rapid enzymatic degradation of IL-2, administration of IL-2 alone in vivo could only lead to a mild increase of Tregs. Nevertheless, the biological activity of IL-2 in vivo can be greatly enhanced by administration of IL-2 with IL-2 monoclonal antibody JES6-1, which binds to a site on IL-2 that reduces IL-2 degradation and selectively leads to vigorous stimulation of CD25+ cells. The 6-fold increase of Tregs in response to IL2/JES6-1 treatment is largely consistent with the previous reports in other disease models.
It should be mentioned that severe LV failure results in an increase of pulmonary venous pressure, a critical factor in CHF-induced lung remodeling and RV hypertrophy. Previous studies have reported lung inflammation in swine after pulmonary venous banding and in patients with increased pulmonary venous pressure secondary to mitral valve diseases or LV failure. In the present study, induction of Tregs in mice with existing LV failure only partially inhibits the further decrease of LV EF and lung inflammation in these mice, indicating that ameliorating inflammation alone cannot totally block CHF progression.

We demonstrate that end-stage CHF is associated with a profound lung leukocyte infiltration and a relatively mild LV leukocyte infiltration, suggesting an important role of lung inflammation in CHF progression and the transition from LV failure to RV hypertrophy/failure. Unfortunately, there are no available experimental models to unambiguously define the precise impact of lung inflammation/remodeling on CHF progression at the present time.

In summary, cytokine therapy using IL2/JES6-1 treatment is effective in selective induction of Tregs and in attenuating the progression of CHF by reducing cardiac, pulmonary, and systemic inflammation.

**Perspectives**

End-stage CHF is often accompanied by severe inflammatory responses in lungs and moderate inflammation in hearts. CD4+CD25+Foxp3+ Tregs suppress activation of the immune system and contribute to the maintenance of immunologic self-tolerance. Cytokine therapy with IL-2/anti-IL-2 antibody (JES6-1 complexes (IL2/JES6-1) selectively expands and maintains CD4+CD25+Foxp3+ regulatory T cells and attenuates development and progression of atherosclerosis.

**Sources of Funding**

This study was supported by US Public Health Service Grants HL021872, HL098669, HL098719, HL102597, HL089249, R01HL105406, HL11879, and T32HL069764 from the National Institutes of Health and Research Grant 09GRNT2260175 from the American Heart Association.

**Disclosures**

None.

**References**


What Is New?

- Induction of regulatory T cells is effective in attenuating lung inflammation and in the transition from left ventricular to right ventricular hypertrophy in mice with existing left ventricular failure.

What Is Relevant?

- End-stage congestive heart failure is often accompanied by severe inflammatory responses in lungs and moderate inflammation in hearts. Thus, induction of regulatory T cells may be an attractive new therapeutic approach for treating end-stage congestive heart failure.

Novelty and Significance

<table>
<thead>
<tr>
<th>What Is New?</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induction of regulatory T cells is</td>
<td>Selective induction of regulatory T cells is effective in attenuating</td>
</tr>
<tr>
<td>effective in attenuating lung</td>
<td>lung inflammation and fibrosis, right ventricular hypertrophy, and the</td>
</tr>
<tr>
<td>inflammation and in the transition</td>
<td>further decrease of left ventricular function in mice with existing</td>
</tr>
<tr>
<td>from left ventricular to right</td>
<td>left ventricular failure, suggesting that strategies to properly</td>
</tr>
<tr>
<td>ventricular hypertrophy in mice</td>
<td>expand regulatory T cells may be useful in treating congestive heart</td>
</tr>
<tr>
<td>with existing left ventricular</td>
<td>failure.</td>
</tr>
<tr>
<td>failure, suggesting that strategies</td>
<td></td>
</tr>
<tr>
<td>to properly expand regulatory T</td>
<td></td>
</tr>
<tr>
<td>cells may be useful in treating</td>
<td></td>
</tr>
<tr>
<td>congestive heart failure.</td>
<td></td>
</tr>
</tbody>
</table>

Downloaded from http://hyper.ahajournals.org/ by guest on November 12, 2017
Increasing Regulatory T Cells With Interleukin-2 and Interleukin-2 Antibody Complexes Attenuates Lung Inflammation and Heart Failure Progression

Huan Wang, Lei Hou, Dongmin Kwak, John Fassett, Xin Xu, Angela Chen, Wei Chen, Bruce R. Blazar, Yawei Xu, Jennifer L. Hall, Jun-bo Ge, Robert J. Bache and Yingjie Chen

Hypertension. 2016;68:114-122; originally published online May 9, 2016;
doi: 10.1161/HYPERTENSIONAHA.116.07084

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/68/1/114

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2016/05/09/HYPERTENSIONAHA.116.07084.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Increasing regulatory T cells with interleukin-2 and interleukin-2 antibody complexes attenuates lung inflammation and heart failure progression

Short title: Tregs and progression of congestive heart failure

1 Huan Wang, PhD; 2 Lei Hou, MD; 1 Dongmin Kwak, Ph.D; 1 John Fassett, PhD; 1 Xin Xu, PhD; 1 Angela Chen, 3 Wei Chen PhD; 3 Bruce R. Blazar, MD; 2 Yawei Xu, M.D; 1 Jennifer L. Hall, PhD; 2 Jun-bo Ge, M.D; 1 Robert J. Bache, MD; 1 Yingjie Chen, PhD.

1 Cardiovascular Division and Lillehei Heart Institute, University of Minnesota Medical School, Minneapolis, MN 55455, USA

2 Shanghai Tenth People’s Hospital of Tongji University, Shanghai, China

3 Division of Blood and Marrow Transplantation, Department of Pediatrics, Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455, USA

*These authors contributed equally to this work.

Address for correspondence: Yingjie Chen, PhD

Lillehei Heart Institute, University of Minnesota
Cancer & Cardiovascular Research Building (CCRB)
2231 6th Street SE, 4-135; Minneapolis, MN 55455
Tel: (612) 626-0613; Fax: (612) 626-4411
Email: chenx106@umn.edu
Supplementary Material and Methods

Animals and experimental design: To determine the effect of IL2/JES6-1 complexes (1 µg recombinant mouse IL-2 plus 5 µg anti-IL-2 mAb (JES6-1), Biolegend) on the progression of transverse aortic constriction (TAC)-induced congestive heart failure (CHF), Balb/c background male mice 4-5 weeks of age were subjected to TAC created with a 27G needle.1 When left ventricular (LV) ejection fraction (EF) reached to 58% in mice 10 days after TAC, IL2/JES6-1 complexes were administrated (i.p.) on 3 consecutive daily doses every 6 days in mice. Treatment was initiated from this time point because our previous data showed that a dramatic TAC-induced lung remodeling occurs when LV EF reached to ~55%.2 A control group was treated with PBS. Samples were collected 4 weeks after TAC when LV EF of mice treated with PBS dropped to 32%. LV hypertrophy, cardiac function and pulmonary congestion were assessed. The main purpose of this experimental strategy was to examine the effect of IL2/JES6-1 complexes on the LV and lung in mice with existing LV failure.

To evaluate the effect of IL2/JES6-1 complexes on the development of TAC-induced LV hypertrophy and cardiac dysfunction, C57/B6 background male mice 4-5 weeks of age were subjected to a moderate TAC created with a 26G needle. IL2/JES6-1 treatment was commenced 3 days before TAC. Samples were collected 12 weeks after TAC when LV EF of mice treated with PBS dropped to 63%. Pulmonary congestion was mild. The main purpose of this experimental strategy was to define the effect of IL2/JES6-1 complexes on the LV response to TAC-induced systolic overload. All mouse studies were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Echocardiography and evaluation of LV hemodynamics: Echocardiographic images were obtained with a Visualsonics Vevo system as previously described.1 For aortic pressure and LV hemodynamics, a 1.2-F pressure catheter was used as described before.1

Sample collection: Mouse heart and lung samples for protein analysis were collected as previously described.1

Western blotting: Western blot analysis was processed as previously described.1 Antibody against atrial natriuretic peptide (ANP) was purchased from Peninsula Laboratories Inc.; Myosin heavy chain (slow skeletal muscle, β-MHC) was from Sigma and collagen III, β-actin and vinculin were from Santa Cruz Biotechnology, Inc..

Histological staining: Sections of 5 µm thickness were sliced. Immunostaining was conducted to identify CD4+CD25+Foxp3+ regulatory T cells (Tregs) in mouse LV and lung. Antibody against Foxp3 was purchased from eBioscience; Foxp3 staining was visualized by using an avidin/biotin peroxidase-linked detection system (Vector Laboratories). Antibody against CD45 (R&D systems) was used to detect mouse LV and lung leukocyte infiltration. 4’, 6’-diamidino-2-phenylindole (DAPI) was from Life Technologies. CD45 antibody staining was visualized by using a secondary Alexa Fluor 555-conjugated antibody (Life Technologies). The slides were pictured using a ZEISS
microscope (FluoView 1000 Olympus). Foxp3 and CD45 staining on the LV was quantified from 5 random fields per slide. The summarized results were expressed as staining positive cells per mm$^2$. LV and lung fibrosis was stained using Sirius red and Fast green Stain Kit from Chondrex, Inc. Relative tissue fibrosis was calculated by dividing the area of red staining by the total measured section area using digitized images. Samples from 5 mice were analyzed per group.

**Quantitative real-time PCR:** Quantitative PCR was processed as described before.$^1$ Primer pairs used in this study are listed in Table S2. The relative amount of each gene in each sample was estimated by the $\Delta\Delta C_T$ method. Results were normalized to 18S rRNA level.

**Flow cytometric analysis:** Cells were isolated from spleens, lungs and LVs.$^3$ Individual LV was excised, cutted into small pieces, and enzymatically digested in 5 ml of collagenase digestion buffer (HBSS without Ca+/Mg+ (Life technologies), 1.5 mg/ml collagenase type II (Worthington Laboratories)) at 37$^\circ$C for 30 min with agitating, subsequently transferred into 5 ml of collagenase/dispase digestion buffer (HBSS without Ca+/Mg+, 1 mg/ml collagenase/dispase (Roche)) for another 20 min at 37$^\circ$C with agitating, and then pressed against the bottom of a 100 µm strainer with the plunger of a 3 ml syringe. Individual lung was excised, cutted into small pieces, and enzymatically digested in 5 ml of digestion buffer (HBSS without Ca+/Mg+ (Life technologies), 1 mg/ml collagenase (Roche)) at 37$^\circ$C for 30 min with agitating, and then passed through a 100 µm strainer. Individual spleen was excised, cutted into small pieces, and passed through a 100 µm strainer. Single cells from tissues were washed through the strainer with 10 ml cold buffer (PBS + 0.5%BSA + 2 mM EDTA). After erythrocyte lysis using Red Blood Cell Lysing Buffer (Sigma), cells were counted using a hemocytometer. After erythrocyte lysis using Red Blood Cell Lysing Buffer (Sigma), cells were counted using a hemocytometer. Dead cells were stained using LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Life technologies). And then single cell suspensions were pre-incubated with anti-mouse CD16/32 antibody to prevent non-specific binding of antibodies to FcRγ, followed by multi-staining with fluorescence directly conjugated anti-membrane antigen primary antibodies (Table S3), accordingly. For some tests, cells were fixed and permeabilized using Mouse Foxp3 Buffer Set (BD Biosciences), followed by staining with Alexa Fluor 488-conjugated anti-Foxp3 and subjected to FACS Aria II analysis (BD Biosciences). Data were analyzed by FlowJo_V10 (FlowJo, OR) software. Gating strategies were present in Supplementary Figure S1-S3. Values represent the mean of five independent experiments.

**Statistics:** A normality test (Shapiro-Wilk) provided by SigmaPlot was used to determine whether data were normally distributed. If data were normally distributed, the data were presented as mean ± SEM. A Student’s t-test was used to test for differences between 2 groups. A two-way ANOVA followed by a Bonferroni correction post-hoc test was used to test for differences among more than 2 groups. If mouse physiological data were not normally distributed or the sample size in one of the experimental groups was less than 10, a non-parametric test (Mann-Whitney or Kruskal-Wallis) followed by a Bonferroni post hoc correction was performed. All pairwise p-values are two-sided. The null hypothesis was rejected at P < 0.05.
Supplemental References


Supplemental Tables, Figures and Figure Legends

Table S1. Anatomic data of mice with existing LV failure treated with IL2/JES6-1 or PBS

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CHF-PBS</th>
<th>CHF-IL2/JES6-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Bodyweight (g)</td>
<td>23.4 (23.2-23.5)</td>
<td>19.7 (18.6-20.8)</td>
<td>22.7 (22.1-23.4)</td>
</tr>
<tr>
<td>Left ventricular (LV) weight (mg)</td>
<td>77.5 (77.3-79.2)</td>
<td>105.4 (98.0-110.6)*</td>
<td>95.7 (92.8-102.6)</td>
</tr>
<tr>
<td>Left atria (LA) weight (mg)</td>
<td>2.8 (2.7-2.8)</td>
<td>42.2 (26.1-56.4)*</td>
<td>18.0 (6.5-40.3)†</td>
</tr>
<tr>
<td>Lung mass (mg)</td>
<td>169.1 (165.3-175.6)</td>
<td>354.7 (304.8-466.5)*</td>
<td>207.2 (190.8-293.4)†</td>
</tr>
<tr>
<td>Right ventricular (RV) weight (mg)</td>
<td>20.7 (20.3-22.2)</td>
<td>27.5 (26.2-30.0)*</td>
<td>22.2 (21.1-27.3)†</td>
</tr>
<tr>
<td>Ratio of LV weight to body weight (mg/g)</td>
<td>3.33 (3.10-3.37)</td>
<td>5.29 (4.91-5.88)*</td>
<td>4.32 (4.13-4.41)</td>
</tr>
<tr>
<td>Ratio of LA weight to body weight (mg/g)</td>
<td>0.12 (0.12-0.12)</td>
<td>2.13 (1.45-3.05)*</td>
<td>0.77 (0.29-2.37)</td>
</tr>
<tr>
<td>Ratio of lung weight to body weight (mg/g)</td>
<td>7.29 (7.11-7.57)</td>
<td>20.1 (14.2-25.9)*</td>
<td>8.82 (8.56-12.3)†</td>
</tr>
<tr>
<td>Ratio of RV weight to body weight (mg/g)</td>
<td>0.89 (0.82-1.01)</td>
<td>1.37 (1.22-1.59)*</td>
<td>1.10 (0.95-1.22)†</td>
</tr>
<tr>
<td>Tibial length (mm)</td>
<td>16.9 (16.6-17.0)</td>
<td>16.8 (16.6-17.1)</td>
<td>16.8 (16.6-17.0)</td>
</tr>
<tr>
<td>Ratio of LV weight to tibial length (mg/mm)</td>
<td>4.59 (4.42-4.65)</td>
<td>6.21 (5.89-6.58)*</td>
<td>5.84 (5.58-6.11)</td>
</tr>
<tr>
<td>Ratio of LA weight to tibial length (mg/mm)</td>
<td>0.16 (0.16-0.16)</td>
<td>2.48 (1.57-3.33)*</td>
<td>1.06 (0.39-2.47)</td>
</tr>
<tr>
<td>Ratio of lung weight to tibial length (mg/mm)</td>
<td>9.78 (9.71-10.5)</td>
<td>21.1 (18.2-27.4)*</td>
<td>12.2 (11.4-17.2)†</td>
</tr>
<tr>
<td>Ratio of RV weight to tibial length (mg/mm)</td>
<td>1.23 (1.23-1.34)</td>
<td>1.61 (1.57-1.76)*</td>
<td>1.34 (1.26-1.62)†</td>
</tr>
<tr>
<td>Survival rate</td>
<td>100%</td>
<td>60.4%</td>
<td>63.2%</td>
</tr>
</tbody>
</table>

Data are presented as median (quartile1-quartile3). *p<0.05 as compared with corresponding control conditions. †p<0.05 as compared with corresponding CHF-PBS conditions.
<table>
<thead>
<tr>
<th>Mouse Gene</th>
<th>sense</th>
<th>antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxp3</td>
<td>5’-TGG ACT ACT TCA AGT ACC ACA ATA TGC-3’</td>
<td>5’-GCG AAC ATG CGA GTA AAC CAA T-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5’-CTT CTG TCT ACTGAA CTT CGG G-3’</td>
<td>5’-CAG GCT TGT CAC TCG AAT TTT G-3’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5’-TCC TGT GTA ATG AAA GAC GGC-3’</td>
<td>5’-ACT CCA CTT TGC TCT TGA CTT C-3’</td>
</tr>
<tr>
<td>TGF-β</td>
<td>5’-CCT GAG TGG CTG TCT TTT GA-3’</td>
<td>5’-CGT GGA GTT TGT TAT CTT TGC TG-3’</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5’-GCA TCC ACG TGT TGG CTC A-3’</td>
<td>5’-CTC CAG CCT ACT CAT TGG GAT CA-3’</td>
</tr>
<tr>
<td>IL-10</td>
<td>5’-GAT GCC CCA GGC AGA GAA-3’</td>
<td>5’-CAC CCA GGG AAT TCA AAT GC-3’</td>
</tr>
<tr>
<td>18S</td>
<td>5’-TCG AGG CCC TGT AAT TGG AA-3’</td>
<td>5’-CCC TCC AAT GGA TCC TCG TT-3’</td>
</tr>
</tbody>
</table>
Table S3. Primary antibodies used in flow cytometric analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Name of the company</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-mouse CD16/32</td>
<td>2.4G2</td>
<td>BD Biosciences</td>
<td>553142</td>
</tr>
<tr>
<td>FITC-conjugated anti-CD45</td>
<td>30-F11</td>
<td>BD Biosciences</td>
<td>553080</td>
</tr>
<tr>
<td>PE/Cy7-conjugated anti-CD45</td>
<td>30-F11</td>
<td>BD Biosciences</td>
<td>552848</td>
</tr>
<tr>
<td>APC/Cy7-conjugated anti-CD3</td>
<td>145-2C11</td>
<td>BD Biosciences</td>
<td>557596</td>
</tr>
<tr>
<td>APC-conjugated anti-CD3</td>
<td>145-2C11</td>
<td>BD Biosciences</td>
<td>553066</td>
</tr>
<tr>
<td>PerCP-conjugated anti-CD4</td>
<td>RM4-5</td>
<td>BD Biosciences</td>
<td>553052</td>
</tr>
<tr>
<td>APC-conjugated anti-CD25</td>
<td>PC61</td>
<td>BD Biosciences</td>
<td>557192</td>
</tr>
<tr>
<td>PE-conjugated anti-CD8</td>
<td>53-6.7</td>
<td>BD Biosciences</td>
<td>553033</td>
</tr>
<tr>
<td>APC/Cy7-conjugated anti-CD44</td>
<td>IM7</td>
<td>BD Biosciences</td>
<td>580588</td>
</tr>
<tr>
<td>PE-Cy7-conjugated anti-CD62L</td>
<td>MEL-14</td>
<td>BD Biosciences</td>
<td>560516</td>
</tr>
<tr>
<td>PE-conjugated anti-F4/80</td>
<td>BM8</td>
<td>eBioscience</td>
<td>12-4801-82</td>
</tr>
<tr>
<td>Alexa Fluor 488-conjugated anti-Foxp3</td>
<td>MF23</td>
<td>BD Biosciences</td>
<td>560403</td>
</tr>
</tbody>
</table>
**Figure S1.** For flow cytometric analysis, single cells from the digested LV were stained with directly conjugated primary antibodies according to the manufacturer’s instructions, followed by staining with PI and then subjected to FACS Aria II analysis. Cells that were negative for PI staining were identified as living cells and analyzed.
Figure S2. For flow cytometric analysis, single cells from the digested lung were stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit, followed by directly conjugated anti-membrane antigen primary antibodies according to the manufacturer's instructions. And then cells were fixed and permeabilized using Mouse Foxp3 Buffer Set (BD Biosciences), followed by staining with directly conjugated anti-Foxp3 and then subjected to FACS Aria II analysis. Cells that were negative for Near-IR staining were identified as living cells and analyzed.
Figure S3. For flow cytometric analysis, single cells from the spleen were stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit, followed by directly conjugated anti-membrane antigen primary antibodies according to the manufacturer’s instructions. And then cells were fixed and permeabilized using Mouse Foxp3 Buffer Set (BD Biosciences), followed by staining with directly conjugated anti-Foxp3 and then subjected to FACS Aria II analysis. Cells that were negative for Near-IR staining were identified as living cells and analyzed.
Figure S4. IL2/JES6-1 treatment selectively expands CD4+CD25+Foxp3+ regulatory T cells (Tregs) in mice. Mice were treated with Interleukin-2/anti-Interleukin-2 monoclonal antibody (JES6-1) complex (IL2/JES6-1) or vehicle (PBS) for 3 consecutive days and examined on day 5 or week 12. A and D, Flow cytometry plots and quantitative data represent the percentage of Tregs (CD25+Foxp3+) within the CD4+ population of spleens and lungs 5 days after treatment. B, C, E and F, Quantitative data represent total numbers of CD4+ and CD8+ T cells in spleens and lungs 5 days after treatment. G and H, Representative immunostaining images and quantitative data of Foxp3 in the LV and lung 5 days after treatment. I, Flow cytometry quantitative data represent the percentage of Tregs within the CD4+ population of spleens 12 weeks after treatment. J and K, Flow cytometry quantitative data represent total numbers of CD4+ and CD8+ T cells in spleens. 12 weeks after treatment. n=5 per group.
Figure S5. IL2/JES6-1 treatment suppresses macrophage infiltration and fibrosis in the lung of mice with existing left ventricle (LV) failure. Data were collected from mice under basal conditions (Ctr), or treated with IL2/JES6-1 or PBS under TAC conditions (CHF-IL2/JES6-1 or CHF-PBS). A, Representative images of Mac2 immunostaining in the lung. B, Representative images and quantitative data of Sirius red/Fast green staining for detection of fibrosis in lungs.
Figure S6. Diagram of the proposed underlying mechanism.