IL-33 Independently Induces Eosinophilic Pericarditis and Cardiac Dilation: ST2 Improves Cardiac Function

Abston et al: IL-33 Induces Cardiac Dysfunction

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Abstract

Background—Interleukin (IL)-33 via its receptor ST2 protects the heart from myocardial infarct and hypertrophy in animal models, but paradoxically increases autoimmune disease. In this study we examined the effect of IL-33 or ST2 administration on autoimmune heart disease.

Methods and Results—We used pressure volume relationships and isoproterenol challenge to assess the effect of recombinant (r)IL-33 or rST2 (e.g. soluble ST2) administration on the development of autoimmune coxsackievirus (CVB3) myocarditis and dilated cardiomyopathy (DCM) in male BALB/c mice. rIL-33 treatment significantly increased acute perimyocarditis \( (p=0.006) \) and eosinophilia \( (p=1.3\times10^{-5}) \), impaired cardiac function (maximum ventricular power \( p=0.0002 \)), and increased ventricular dilation (end diastolic volume \( p=0.01 \)). rST2 treatment prevented eosinophilia and improved heart function compared to rIL-33 treatment (ejection fraction, \( p=0.009 \)). Neither treatment altered viral replication. rIL-33 increased IL-4, IL-33, IL-1\( \beta \) and IL-6 levels in the heart during acute myocarditis. To determine whether IL-33 altered cardiac function on its own, we administered rIL-33 to undiseased mice and found that rIL-33 induced eosinophilic pericarditis and adversely affected heart function. We used cytokine knockout mice to determine that this effect was due to IL-33-mediated signaling but not IL-1\( \beta \) or IL-6.

Conclusions—We show for the first time that IL-33 induces eosinophilic pericarditis while sST2 prevents eosinophilia and improves systolic function, and that IL-33 independently adversely affects heart function via the IL-33 receptor.

Key Words: myocarditis, cytokines, inflammation, dilation, eosinophils
Myocarditis, or inflammation of the myocardium, leads to around 46% of dilated cardiomyopathy (DCM) cases in the United States, while DCM is the most common form of cardiomyopathy responsible for heart failure.\(^1\)\(^-\)\(^3\) Coxsackie virus B3 (CVB3) is a major cause of myocarditis and DCM in humans,\(^2\) and infection of mice with heart-passaged CVB3 (containing infectious virus and cardiac tissue) induces autoimmune myocarditis leading to DCM.\(^4\) Only mice that respond to infection with a T helper (Th)2 immune response progress from acute myocarditis to chronic myocarditis/DCM.\(^4\)\(^-\)\(^6\) In patients with heart failure, elevated serum levels of proinflammatory cytokines such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin (IL)-6 independently predict decreased heart function and poor survival.\(^7\) IL-1\(\beta\) and TNF-\(\alpha\) worsen heart disease during myocarditis by increasing inflammation and inducing myocyte hypertrophy, contractile dysfunction, and extracellular matrix remodeling.\(^6\)\(^,\)\(^8\)\(^,\)\(^9\)

IL-33 is a cytokine in the IL-1\(\beta\) family that promotes a Th2 response.\(^10\) IL-1 receptor-like 1 protein, most often abbreviated ST2, exists in two major isoforms; a membrane bound form (ST2L) that is the receptor for IL-33, and a truncated soluble form (sST2) generated by alternative splicing that binds IL-33 and is thought to act as a decoy receptor. Recently, elevated serum and synovial fluid levels of IL-33 were associated with rheumatoid arthritis in patients, and administration of recombinant IL-33 (rIL-33) increased collagen or antibody-induced arthritis in mice.\(^11\)\(^-\)\(^13\) Paradoxically, IL-33 has been shown to be cardioprotective in murine models of myocardial infarct and hypertrophy, where rIL-33 administration improves heart function and diminishes inflammation and fibrosis.\(^14\)\(^,\)\(^15\)

In this study, we were interested in determining the effect of rIL-33 administration on an autoimmune model of CVB3 myocarditis that progresses to DCM in susceptible strains of mice.
We found that rIL-33 impaired cardiac function during myocarditis and in undiseased mice. Although IL-1β and IL-6 were increased in the heart by rIL-33 treatment, and are cytokines capable of causing cardiac dysfunction on their own, we showed that the negative effects of IL-33 on cardiac function occur via the IL-33 receptor and are not due to IL-1β or IL-6.

Methods

Experimental Model

BALB/cJ, C57BL/6J, B6.129SF2/J, type I IL-1R, IL-6, and IL1RAcP deficient male mice (Jackson Laboratory) were maintained under pathogen-free conditions in the animal facility at Johns Hopkins School of Medicine, and approval obtained from the Johns Hopkins University Animal Care and Use Committee for all procedures. Mice were inoculated intraperitoneally (ip) with heart-passaged CVB3 on day 0 and hearts examined on day 10 post infection (pi) during acute myocarditis or day 35 pi during DCM.4 rIL-33 (1μg/0.1mL), rST2 (5μg/0.1mL) or sterile PBS were injected ip on day 1, 3, 5, 7 and 9 in undiseased or CVB3 infected mice. Hearts were fixed in 10% buffered formalin and stained with H&E to assess inflammation, as previously described.6

Cardiac Function

Cardiac function was assessed by pressure-volume catheter (1.2F Scisense Inc) placed in the left ventricle via the apex in open-chest mice anesthetized with 3% isoflurane, as previously described.16-18 To assess β-adrenergic sensitivity an isoproterenol dose-response protocol was adapted from previous protocols.19,20
Plaque Assay and Cytokine Measurements

Individual cardiac supernatants or sera were used in ELISA to measure cytokines or in plaque assays to determine the level of infectious virus, as previously described.4,6

Flow Cytometry

Hearts were digested with 600μ/mL collagenase II (Worthington) +60μ/mL DNAse I (Sigma) according to the manufacturer’s instructions for the GentleMACSTM isolation of cardiac cells (Miltenyi).21-23 Immune cells were stained with fluorochrome-conjugated antibodies against CD45, CD3, CD4, CD19, FceR1α, CD117, CD11b, F4/80, Ly6G, or SiglecF (BD Pharmingen/eBiosciences).

Statistical Analysis

The Mann-Whitney rank sum test was used to evaluate two groups (p<0.05). Comparisons involving three groups were analyzed using Kruskal-Wallis tests. When three groups were significant (p<0.05) then pairwise comparisons were made using Mann-Whitney rank tests with a Bonferroni correction (p<0.013). Repeated measures data (i.e. isoproterenol treatments) were evaluated using generalized estimating equations (GEE) and linear mixed effects models with dose as a covariate. An omnibus test for group differences was used at the first stage (p<0.05) and if significant, then pairwise comparisons were assessed (p<0.013).
Results

IL-33 increases eosinophilic perimyocarditis without affecting virus levels

We recently reported that IL-33 mRNA levels are significantly elevated in the heart during acute and chronic CVB3 myocarditis. To examine the effects of rIL-33 administration in an autoimmune model of viral-induced heart disease, we treated BALB/c mice with rIL-33, rST2 (to block IL-33 released during myocarditis) or PBS ip every other day from day 1-9 pi and examined myocarditis at day 10 pi. We found that rIL-33 treatment significantly increased CVB3 myocarditis (Figure 1A and 1C, \( p=0.006 \)) and pericarditis (Figure 1D) compared to PBS controls, while rST2 did not (\( p=0.19 \)). Neither rIL-33 nor rST2 treatment significantly altered viral replication compared to PBS controls (PBS vs. rIL-33 \( p=0.16 \); PBS vs. rST2 \( p=0.66 \)) (Figure 1B). rIL-33 significantly increased eosinophils in the heart by histology (Figure 1E) and Facs analysis (Figure 1F and 1G absolute numbers \( p=0.001 \) and percent \( p=1.3\times10^{-5} \)). No other major cell populations such as lymphocytes, monocytes, macrophages, neutrophils or mast cells were increased by rIL-33 treatment by Facs analysis (data not shown). rST2-treated mice did not develop pericardial or myocardial eosinophilia (Figure 1D).

rIL-33 exacerbates cardiac dysfunction during CVB3 myocarditis/DCM but rST2 improves cardiac function

To determine the effect of IL-33 or ST2 administration on cardiac function, we gave male BALB/c mice rIL-33, rST2 or PBS ip every other day from day 1-9 pi and examined cardiac function at day 10 or 35 pi using pressure-volume relationships. We found that rIL-33 significantly decreased cardiac function while rST2 improved function (Figure 2, Table 1 and 2). In this myocarditis model, susceptible strains of mice like BALB/c recover from acute
myocarditis but develop DCM by day 35 pi. At day 35 pi mice that received rIL-33 from day 1-9 pi developed worse dilation and reduced EF (p=0.01) compared to PBS controls (Figure 2, Table 2). rST2 improved cardiac function during chronic myocarditis in mice that had received rST2 from day 1-9 pi (Figure 2, Table 2). See Supplemental Results section for a more detailed description of cardiac function.

**rIL-33 induces acute cardiac dysfunction and pericarditis in undiseased mice**

Administration of certain cytokines, like TNF-α or IL-1β, is capable of causing acute cardiac dysfunction in normal mice. To assess whether rIL-33 induced cardiac dysfunction in the absence of myocarditis, uninfected BALB/c mice were injected ip with PBS, rIL-33 or rST2 every other day for a total 5 injections and cardiac function assessed the day after the final treatment (the equivalent of day 10) using pressure-volume relationships or at day 35 by echocardiography. rIL-33 treatment impaired cardiac function in the absence of myocarditis compared to PBS controls at day 10 (Table 3) but not at day 35 (Supplemental Table 1). In contrast, rST2 treatment from day 1-9 had no significant effect on heart function compared to PBS controls at day 10 (Table 3). See Supplemental Results section for a more detailed description of cardiac function in undiseased mice. rIL-33 induced pericarditis in undiseased mice (Supplemental Figure 1) similar in appearance to that observed during acute CVB3 myocarditis (Figure 1D). Eosinophils were abundant in the pericardial infiltrate of rIL-33 (Supplemental Figure 1B), but absent in rST2-treated mice (Supplemental Figure 1C).
rIL-33 induces β-adrenergic insensitivity in diseased and undiseased mice

β-adrenergic insensitivity is a hallmark feature of heart failure. Isoproterenol is a sympathomimetic agonist of beta receptors in the heart that is used to test adrenergic responsiveness in vivo. Certain cytokines, including TNF-α, IL-1β and IL-6, are able to inhibit myocyte function by interfering with calcium handling and by reducing the response to beta receptor stimulation. Because administration of rIL-33 impaired systolic and diastolic function at day 10 pi (Table 1) and in undiseased mice (Table 3), we tested the ability of rIL-33 to affect β-adrenergic responsiveness to isoproterenol. We found that rIL-33 treatment inhibited the characteristic increases in heart rate (HR \( p<0.0001 \)), dP/dT Max (peak rate of pressure rise \( p=0.0001 \)), cardiac output (CO \( p=0.001 \)), and maximum ventricular power (PMX \( p=0.001 \)) in response to isoproterenol for mice with acute myocarditis and undiseased mice (Figure 3, Supplemental Table 2). rST2 treatment did not significantly alter the response to isoproterenol for any of these parameters in diseased or undiseased mice (Figure 3, Supplemental Table 2). Neither rIL-33 nor rST2 affected β-adrenergic responsiveness to isoproterenol during the chronic phase of CVB3 myocarditis at day 35 pi (Supplemental Figure 2, Supplemental Table 2).

rIL-33 increases cardiac IL-33, IL-4, IL-1β, IL-6 and serum sST2, and alters remodeling during acute CVB3 myocarditis

IL-33 has been demonstrated to promote Th2 responses. For this reason we examined the effect of rIL-33 or rST2 treatment on Th2-associated cytokines in the heart (e.g. IL-4, IL-33, ST2) or sera (soluble sST2) during acute CVB3 myocarditis by ELISA. IL-33 also promotes Th1-type proinflammatory responses, and so we also examined proinflammatory/profibrotic
cytokines (e.g. IFN-γ, TNF-α, IL-1β, IL-6). sST2 is a serum biomarker that predicts progression to heart failure in patients.24,32

We found that rIL-33 treatment increased IL-33 (p=0.01), IL-4 (p=0.002), IL-1β (p=0.0009) and IL-6 (p=0.003) in the heart compared to PBS controls during acute myocarditis (Figure 4A), but had no effect on TNF-α (p=0.76) (Figure 4A), IFN-γ (p=0.10), or sST2 (p=0.41) in the heart (data not shown). rIL-33 and rST2 significantly increased serum levels of sST2 during acute myocarditis compared to PBS controls (p=0.0001 and p=0.0004, respectively) (Figure 4A). Importantly, rST2 treatment significantly decreased cardiac IL-33 compared to PBS controls (p=0.001) (Figure 4A), indicating that rST2 treatment partially decreases cardiac IL-33 levels during acute myocarditis. rST2 treatment had no effect on cardiac levels of IL-4 (p=0.06), TNF-α (p=0.10), IL-6 (p=0.17) (Figure 4A) or sST2 (p=0.65) and IFN-γ (p=0.20) (data not shown) compared to PBS controls during myocarditis. rIL-33 also significantly altered matrix metalloproteinase (Mmp) levels in the heart compared to PBS by RT-PCR (see Supplemental Results).

rIL-33 increases cardiac IL-1β and IL-6 in undiseased mice

Because rIL-33 treatment increased cardiac IL-1β and IL-6 levels at day 10 pi (Figure 4A), we asked whether rIL-33 treatment of undiseased mice would also increase these cytokines in the heart. To test this we administered PBS, rIL-33 or rST2 every other day as previously and examined cytokines after the 5th treatment (equivalent to day 10) by ELISA. We found that rIL-33 significantly increased cardiac IL-6 levels (p=0.01) that was prevented by rST2 treatment (p=0.004) in undiseased mice compared to PBS controls, but had no significant effect on TNF-α (Kruskal-Wallis p=0.18, rank test p=0.08) or IL-1β (Kruskal-Wallis p=0.13, rank test p=0.04)
(Figure 4B). A comparison of cytokines in undiseased vs. mice with acute CVB3 myocarditis revealed that rIL-33 treatment specifically increased cardiac IL-1β ($p=0.006$) (rIL-33 group), while TNF-α ($p=0.002$) and IL-6 ($p=0.008$) were increased with disease (PBS group) (Supplemental Figure 3).

**rIL-33 treatment directly decreases heart function**

Because rIL-33 treatment induced cardiac dysfunction and β-adrenergic insensitivity in diseased and undiseased mice at day 10, we wanted to determine whether IL-33 could be independently responsible for these effects.$^9$,$^{26}$ However, since rIL-33 treatment increased IL-1β and IL-6 levels in the heart (Figure 4 and Supplemental Figure 3) it was possible that IL-33 worked indirectly to alter heart function via these cytokines. To test this possibility we treated undiseased male IL-1R, IL-6 or IL-1RAcP (receptor required for IL-1R and IL-33R-mediated signaling)$^{10}$ deficient mice with PBS or rIL-33, using the same protocol as before, and examined heart function by pressure-volume relationships. We found that rIL-33 treatment significantly decreased most cardiac functional parameters compared to PBS-treated IL-1R or IL-6 deficient mice (Figure 5A and 5B), indicating that IL-1β and IL-6 were not responsible for the effect of rIL-33. Cardiac dysfunction following rIL-33 treatment was not significantly different from PBS controls for any parameter examined in IL-1RAcP deficient mice (Figure 5C), demonstrating that the IL-33R is required for the cardiac dysfunction observed in rIL-33 treated mice.

Because IL-1β has been shown to be critical for myocarditis,$^8$,$^{33}$ we examined whether rIL-33 increased CVB3 myocarditis indirectly via IL-1β by treating IL-1R deficient mice with rIL-33 from day 1-9 pi. We found that although rIL-33 significantly increased myocarditis compared to PBS controls, there was no significant difference in myocarditis between WT and
IL-1R deficient mice in response to rIL-33 (Supplemental Figure 4). Thus, rIL-33 does not increase acute CVB3 myocarditis via IL-1β.

Discussion

In this study, we demonstrate that rIL-33 administration induces eosinophilic pericardial inflammation resulting in dilation during acute CVB3 myocarditis and in undiseased hearts. We are the first to report that IL-33 induces eosinophilic myocarditis and pericarditis. Previously we showed that IFN-γ deficient mice develop a Th2-type immune response that leads to eosinophilic pericarditis, dilation and heart failure.6,34 IL-33 had not yet been discovered and so was not examined in those studies. Recently we showed that TRIF deficient mice have elevated IL-33 levels in the heart that is associated with dilation, fibrosis and heart failure.18 Eosinophilic cardiovascular diseases like Churg Strauss syndrome, Kounis syndrome, hypereosinophilic syndrome (HES), eosinophilic myocarditis and giant cell myocarditis are associated with poor heart function and dilation similar to our findings.2,35,36 Recently, rIL-33 administration has been found to promote lung eosinophilia in an animal model of asthma.29,37 The induction of eosinophilic pericarditis by rIL-33 in our model provides a possible explanation for the divergent results we obtained compared to myocardial infarct and aortic constriction animal models as reported by Sanada et al. and Seki et al.14,15 Eosinophils release cationic proteins such as major basic protein, Mmps and cytokines that contribute to cardiac remodeling and dilation.35,36 We report here that rIL-33 treatment increased the profibrotic cytokines IL-4 and IL-1β as well as altering Mmp levels in the heart during myocarditis that contribute to cardiac remodeling. Although the precise reasons that our results differ from other investigators remain unclear, a possible explanation is that we used a different dose and murine rather than rat rIL-33 in these
studies. Additionally, we observe sex differences in IL-33/ST2 levels and function during myocarditis, and we used male mice in these experiments but the sex used in the other studies were not described. Interestingly, HES occurs more frequently in men than women (9:1).

Importantly, the effects of rIL-33 administration on chronic CVB3 myocarditis/DCM were detected at day 35 pi, 25 days after rIL-33 treatment finished (recombinant administered from day 1-9 pi) indicating that early effects of IL-33 (i.e. innate or during acute myocarditis) were responsible for increasing DCM. Others have reported effects of cytokines on heart function that continue up to 10 days after cytokine administration finishes. However, the effect of rIL-33 on dilation was only short-term in undiseased hearts. Additionally, we demonstrate here that IL-33 induces cardiac dysfunction independently from IL-1β or IL-6 but requires the IL-33R. This observation is novel. The similarity of IL-33/ST2L signaling to IL-1β/IL-1R signaling suggests a possible mechanism for the observed effect. The IL-33R, ST2L, and IL-1R share the common IL-1RAcP component, which activates MyD88 and NFκB. Administration of IL-1β to rodents induces systolic and diastolic dysfunction as well as β-adrenergic insensitivity. Our observation that rIL-33 administration independently affects cardiac dilation, however, is an effect that has been observed for TNF-α but not for IL-1β. This suggests that important differences exist between IL-1β/IL-1R and IL-33/ST2L signaling pathways in the heart.

We report here for the first time that rST2 effectively protects the heart from cardiac dysfunction during acute and chronic CVB3 myocarditis/DCM. Administration of rST2 (e.g. sST2) in animal models of myocardial infarct and pressure overload is believed to function as a decoy receptor to counteract IL-33 released during heart damage. In this study rST2 treatment significantly reduced cardiac IL-33 levels in the heart during acute CVB3 myocarditis compared
to PBS controls, but the reduction was small perhaps because the R&D ELISA kit does not distinguish between IL-33 that is bound or unbound to ST2. Thus, some of the IL-33 that we detected in the heart could be inactivated accounting for the strongly protective effect of rST2 treatment. That heart function was conserved essentially to normal undiseased levels by rST2 treatment indicates the importance of sST2 in protecting the heart from inflammatory heart disease and its role in preventing eosinophilia. Additionally, rST2 administration from day 1 to 9 pi effectively prevented progression to DCM, causing susceptible BALB/c mice to resemble resistant mouse strains like C57BL/6.4

In summary, this study demonstrates that IL-33 induces eosinophilic pericarditis and increases CVB3 perimyocarditis and DCM, while ST2 is protective. Although this is a promising pathway to investigate novel therapeutic interventions for cardiovascular disease, and heart failure in particular, therapies targeting the IL-33/ST2 pathway will need to take into account the ability of IL-33 to induce cardiac eosinophilia and pericarditis.

Sources of Funding

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Disclosures

None.
References


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Table 1. *In vivo* hemodynamics of rIL-33 and rST2-treated BALB/c mice during acute CVB3 myocarditis (day 10 pi) based on pressure-volume analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PBS Median [Q1 to Q3]</th>
<th>rIL-33 Median [Q1 to Q3]</th>
<th>rST2 Median [Q1 to Q3]</th>
<th>Kruskal-Wallis p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, bpm</td>
<td>519 [508 to 552]</td>
<td>559 [510 to 577]</td>
<td>554 [510 to 577]</td>
<td>0.26</td>
</tr>
<tr>
<td>ESP, mmHg</td>
<td>91 [86 to 100]</td>
<td>82 [78 to 89] (*0.008)</td>
<td>102 [78 to 89] (†0.07,‡0.0004)</td>
<td>0.0005</td>
</tr>
<tr>
<td>EDP, mmHg</td>
<td>4.9 [3.4 to 6.1]</td>
<td>5.0 [3.7 to 9.6]</td>
<td>4.9 [4.2 to 6.1]</td>
<td>0.76</td>
</tr>
<tr>
<td>dP/dT Max</td>
<td>9557 [7649 to 10090]</td>
<td>7234 [6470 to 8071] (*0.01)</td>
<td>11572 [10213 to 12191] (†0.002,‡0.0002)</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>dP/dT Min</td>
<td>-8042 [-8794 to -7731]</td>
<td>-6141 [-7621 to -4788] (*0.003)</td>
<td>-9428 [-7641 to -4788] (†0.02,‡0.0002)</td>
<td>0.0001</td>
</tr>
<tr>
<td>EF, %</td>
<td>63 [56 to 67]</td>
<td>47 [37 to 63] (*0.04)</td>
<td>66 [61 to 75] (†0.26,‡0.009)</td>
<td>0.02</td>
</tr>
<tr>
<td>ESV, μL</td>
<td>5.6 [4.9 to 6.7]</td>
<td>10 [5.6 to 14] (*0.06)</td>
<td>5.0 [3.8 to 6.7] (†0.36,‡0.02)</td>
<td>0.04</td>
</tr>
<tr>
<td>EDV, μL</td>
<td>15 [14 to 17]</td>
<td>21 [17 to 27] (*0.01)</td>
<td>16 [13 to 27] (†0.88,‡0.02)</td>
<td>0.02</td>
</tr>
<tr>
<td>SV, μL</td>
<td>9.4 [8 to 11]</td>
<td>8.9 [7.3 to 15]</td>
<td>9.6 [8.4 to 12]</td>
<td>0.87</td>
</tr>
<tr>
<td>CO, mL/min</td>
<td>5375 [4131 to 5973]</td>
<td>5162 [4073 to 7722]</td>
<td>5343 [4424 to 6443]</td>
<td>0.84</td>
</tr>
<tr>
<td>SW, mmHg x μL</td>
<td>870 [719 to 919]</td>
<td>822 [556 to 1028]</td>
<td>898 [802 to 1011]</td>
<td>0.62</td>
</tr>
<tr>
<td>PRSW, mmHg</td>
<td>76 [64 to 87]</td>
<td>66 [51 to 71]</td>
<td>75 [64 to 88]</td>
<td>0.07</td>
</tr>
<tr>
<td>PMX, mW</td>
<td>9.2 [8.4 to 11]</td>
<td>6.6 [5.4 to 7.7] (0.0002)</td>
<td>14 [12 to 15] (†0.003,‡0.0002)</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Ees, mmHg/μL</td>
<td>8.8 [6.9 to 11]</td>
<td>5.6 [4.4 to 6.6] (0.02)</td>
<td>10 [7.7 to 12] (†0.81,‡0.006)</td>
<td>0.01</td>
</tr>
<tr>
<td>Ea/Ees</td>
<td>1.1 [0.8 to 1.4]</td>
<td>1.7 [1.3 to 2] (*0.02)</td>
<td>0.99 [0.85 to 1.1] (†0.46,‡0.007)</td>
<td>0.01</td>
</tr>
<tr>
<td>V₀, μL</td>
<td>-7.3 [-12 to -3.2]</td>
<td>-9.5 [-13 to -7]</td>
<td>-7.8 [-10 to -6.9]</td>
<td>0.41</td>
</tr>
<tr>
<td>Tau, ms</td>
<td>5.4 [4.8 to 6.6]</td>
<td>7.0 [5.3 to 9.2] (*0.06)</td>
<td>5.1 [4.9 to 5.8] (†0.65,‡0.02)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

CO, cardiac output; dP/dT Max, peak rate of pressure rise (mmHg/s); dP/dT Min, peak rate of pressure decline (mmHg/s); Ea/Ees, arterial elastance normalized to Ees; EDP, end diastolic pressure; EDV, end diastolic volume; Ees, LV end systolic elastance; EF, ejection fraction; ESP, end systolic pressure; ESV, end systolic volume; HR, heart rate; PMX, maximum ventricular power; PRSW, preload recruitable stroke work; SV, stroke volume; SW, stroke work; Tau, time constant of diastolic relaxation; V₀, X-intercept of the ESP-volume relationship. Male BALB/c mice received CVB3 at day 0 and either PBS, rIL-33 or rST2 ip every other day and data collected at day 10 pi. Data show median with first (Q1) and third (Q3) quartiles for 8 to 10 mice/group. *PBS vs. rIL-33, †PBS vs. rST2, or ‡rIL-33 vs. rST2 using Mann-Whitney rank test.
Table 2. *In vivo* hemodynamics of rIL-33 and rST2-treated mice during chronic CVB3 myocarditis (day 35 pi) based on pressure-volume analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PBS Median [Q1 to Q3]</th>
<th>rIL-33 Median [Q1 to Q3]</th>
<th>rST2 Median [Q1 to Q3]</th>
<th>Kruskal-Wallis p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>567 [555 to 591]</td>
<td>560 [546 to 589]</td>
<td>574 [560 to 603]</td>
<td>0.70</td>
</tr>
<tr>
<td>ESP, mmHg</td>
<td>86 [83 to 91]</td>
<td>78 [77 to 91] (*0.22)</td>
<td>96 [88 to 99] (†0.02,‡0.01)</td>
<td>0.01</td>
</tr>
<tr>
<td>EDP, mmHg</td>
<td>5.9 [5.3 to 8.6]</td>
<td>7.5 [5.5 to 8.7]</td>
<td>5.5 [3.6 to 7.6]</td>
<td>0.25</td>
</tr>
<tr>
<td>dP/dT Max</td>
<td>8009 [6486 to 8580]</td>
<td>7354 [6682 to 7873] (*0.26)</td>
<td>8787 [7922 to 9426] (†0.06,‡0.003)</td>
<td>0.01</td>
</tr>
<tr>
<td>dP/dT Min</td>
<td>-7545 [-8627 to -6002]</td>
<td>-6942 [-7764 to -6388] (*0.29)</td>
<td>-8254 [-9066 to -7423] (†0.13,‡0.02)</td>
<td>0.05</td>
</tr>
<tr>
<td>EF, %</td>
<td>49 [43 to 63]</td>
<td>38 [34 to 48] (*0.01)</td>
<td>71 [55 to 80] (†0.04,‡0.001)</td>
<td>0.001</td>
</tr>
<tr>
<td>ESV, µL</td>
<td>8.2 [5.2 to 12]</td>
<td>12 [11 to 15] (*0.03)</td>
<td>4.8 [3.3 to 7.5] (†0.04,‡0.0004)</td>
<td>0.0008</td>
</tr>
<tr>
<td>EDV, µL</td>
<td>20 [17 to 21]</td>
<td>22 [21 to 26] (*0.04)</td>
<td>17 [15 to 19] (†0.08,‡0.005)</td>
<td>0.007</td>
</tr>
<tr>
<td>SV, µL</td>
<td>10 [9.1 to 12]</td>
<td>9.8 [6.9 to 11]</td>
<td>11 [8.1 to 15]</td>
<td>0.48</td>
</tr>
<tr>
<td>CO, mL/min</td>
<td>6.5 [5.5 to 7.4]</td>
<td>5.5 [4.4 to 6.5]</td>
<td>6.3 [4.9 to 8.5]</td>
<td>0.37</td>
</tr>
<tr>
<td>SW, mmHg x µL</td>
<td>787 [699 to 926]</td>
<td>648 [531 to 880]</td>
<td>935 [822 to 1060]</td>
<td>0.07</td>
</tr>
<tr>
<td>PRSW, mmHg</td>
<td>74 [63 to 89]</td>
<td>59 [51 to 74]</td>
<td>75 [67 to 83]</td>
<td>0.09</td>
</tr>
<tr>
<td>PMX, mW</td>
<td>7.3 [6.1 to 8.9]</td>
<td>6.2 [5.7 to 880] (*0.22)</td>
<td>9 [8.4 to 10] (†0.03,‡0.01)</td>
<td>0.01</td>
</tr>
<tr>
<td>Ees, mmHg/µL</td>
<td>5.9 [0.2 to 8]</td>
<td>5 [4.5 to 5.8]</td>
<td>7.1 [6.5 to 10]</td>
<td>0.15</td>
</tr>
<tr>
<td>Ea/Ees</td>
<td>0.86 [0.2 to 1.2]</td>
<td>1.7 [1.6 to 1.8] (*0.007)</td>
<td>1.0 [0.7 to 1.5] (†0.30,‡0.03)</td>
<td>0.01</td>
</tr>
<tr>
<td>V₀, µL</td>
<td>-0.6 [-3.6 to 2.3]</td>
<td>-6.1 [-6.4 to 0.6]</td>
<td>-8.1 [-8.5 to -4.1]</td>
<td>0.10</td>
</tr>
<tr>
<td>Tau, ms</td>
<td>5.3 [5 to 7]</td>
<td>6.1 [5.6 to 7]</td>
<td>5.4 [4.7 to 6.6]</td>
<td>0.30</td>
</tr>
</tbody>
</table>

CO, cardiac output; dP/dT Max, peak rate of pressure rise (mmHg/s); dP/dT Min, peak rate of pressure decline (mmHg/s); Ea/Ees, arterial elastance normalized to Ees; EDP, end diastolic pressure; EDV, end diastolic volume; Ees, LV end systolic elastance; EF, ejection fraction; ESP, end systolic pressure; ESV, end systolic volume; PMX, maximum
ventricular power; PRSW, preload recruitable stroke work; SV, stroke volume; SW, stroke work; Tau, time constant of diastolic relaxation; V₀, X-intercept of the ESP-volume relationship. Male BALB/c mice received CVB3 at day 0 and either PBS, rIL-33 or rST2 ip every other day from day 1 to 9 pi and data collected at day 35 pi. Data show median with first (Q1) and third (Q3) quartiles for 8 to 10 mice/group. *PBS vs. rIL-33, †PBS vs. rST2, or ‡rIL-33 vs. rST2 using Mann-Whitney rank test.
Table 3. *In vivo* hemodynamics of rIL-33 and rST2-treated uninfected mice at day 10 post inoculation based on pressure-volume analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PBS Median [Q1 to Q3]</th>
<th>rIL-33 Median [Q1 to Q3]</th>
<th>rST2 Median [Q1 to Q3]</th>
<th>Kruskal-Wallis p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>562 [546 to 602]</td>
<td>532 [525 to 537]</td>
<td>571 [548 to 591]</td>
<td>0.01</td>
</tr>
<tr>
<td>ESP, mmHg</td>
<td>97 [95 to 101]</td>
<td>107 [93 to 109]</td>
<td>107 [98 to 110]</td>
<td>0.23</td>
</tr>
<tr>
<td>EDP, mmHg</td>
<td>5.3 [4.4 to 7.1]</td>
<td>5.3 [4.3 to 7]</td>
<td>5.1 [4.8 to 6.3]</td>
<td>0.99</td>
</tr>
<tr>
<td>dP/dT Max</td>
<td>10035 [9246 to 10876]</td>
<td>8063 [7409 to 8252] (*0.002)</td>
<td>10117 [9740 to 10584] (†1.0,‡0.003)</td>
<td>0.003</td>
</tr>
<tr>
<td>dP/dT Min</td>
<td>-9517 [-9640 to -9249]</td>
<td>-6927 [-8090 to -5810] (*0.001)</td>
<td>-10159 [-10484 to -9464] (†0.05,‡0.003)</td>
<td>0.0007</td>
</tr>
<tr>
<td>EF, %</td>
<td>67 [59 to 82]</td>
<td>40 [28 to 60] (*0.02)</td>
<td>67 [62 to 72] (†0.65,‡0.01)</td>
<td>0.02</td>
</tr>
<tr>
<td>ESV, µL</td>
<td>4.7 [2.7 to 8.5]</td>
<td>14 [10 to 20] (*0.005)</td>
<td>4.7 [4.1 to 6.6] (†0.92,‡0.002)</td>
<td>0.003</td>
</tr>
<tr>
<td>EDV, µL</td>
<td>15 [13 to 19]</td>
<td>23 [18 to 27] (*0.02)</td>
<td>16 [13 to 18] (†0.96,‡0.005)</td>
<td>0.01</td>
</tr>
<tr>
<td>SV, µL</td>
<td>12 [8.5 to 13]</td>
<td>8.1 [7.5 to 9.3] (*0.04)</td>
<td>11 [9.8 to 11] (†0.48,‡0.01)</td>
<td>0.04</td>
</tr>
<tr>
<td>CO, mL/min</td>
<td>6708 [4735 to 7417]</td>
<td>4346 [4013 to 5015] (*0.02)</td>
<td>6087 [5722 to 6432] (†0.53,‡0.002)</td>
<td>0.006</td>
</tr>
<tr>
<td>SW, mmHg x µL</td>
<td>1034 [860 to 1054]</td>
<td>720 [620 to 775] (*0.008)</td>
<td>930 [841 to 1024] (†0.48,‡0.003)</td>
<td>0.005</td>
</tr>
<tr>
<td>PRSW, mmHg</td>
<td>82 [74 to 101]</td>
<td>52 [49 to 72] (*0.02)</td>
<td>87 [67 to 93] (†0.85,‡0.01)</td>
<td>0.01</td>
</tr>
<tr>
<td>PMX, mW</td>
<td>12 [11 to 12]</td>
<td>8.7 [7 to 9.4] (*0.003)</td>
<td>12 [10 to 13] (†0.48,‡0.003)</td>
<td>0.003</td>
</tr>
<tr>
<td>Ees, mmHg/µL</td>
<td>9.2 [6.9 to 12]</td>
<td>4.9 [4.7 to 6] (*0.02)</td>
<td>9.6 [8.8 to 11] (†0.80,‡0.008)</td>
<td>0.01</td>
</tr>
<tr>
<td>Ea/Ees</td>
<td>0.9 [0.6 to 1.3]</td>
<td>2.4 [1.4 to 3] (*0.007)</td>
<td>1 [0.8 to 1.2] (†0.70,‡0.002)</td>
<td>0.003</td>
</tr>
<tr>
<td>V₀, µL</td>
<td>-5.7 [-9.5 to -2.9]</td>
<td>-8.8 [-12 to -3.5]</td>
<td>-7.4 [-8.4 to -5.7]</td>
<td>0.56</td>
</tr>
<tr>
<td>Tau, ms</td>
<td>4.6 [4.4 to 5.1]</td>
<td>6.7 [5.6 to 8.6] (*0.01)</td>
<td>4.9 [4.7 to 5] (†0.33,‡0.02)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

CO, cardiac output; dP/dT Max, peak rate of pressure rise (mmHg/s); dP/dT Min, peak rate of pressure decline (mmHg/s); Ea/Ees, arterial elastance normalized to Ees; EDP, end
diastolic pressure; EDV, end diastolic volume; Ees, LV end systolic elastance; EF, ejection fraction; ESP, end systolic pressure; ESV, end systolic volume; PMX, maximum ventricular power; PRSW, preload recruitable stroke work; SV, stroke volume; SW, stroke work; Tau, time constant of diastolic relaxation; V₀, X-intercept of the ESP-volume relationship. Male BALB/c mice received either PBS, rIL-33 or rST2 ip every other day and data collected 10 days later. Data show median with first (Q1) and third (Q3) quartiles for 8 to 10 mice/group. *PBS vs. rIL-33, †PBS vs. rST2, or ‡rIL-33 vs. rST2 using Mann-Whitney rank test.
Figure Legends

**Figure 1.** Recombinant (r)IL-33 treatment induces eosinophilic perimyocarditis at day 10 pi (A) without increasing cardiac viral replication (PBS vs. rIL-33 $p=0.16$; PBS vs. rST2 $p=0.66$) (B). Kruskal-Wallis test $p=0.01$, Mann-Whitney rank test $a$, $p=0.006$; $b$, $p=0.19$; $c$, $p=0.06$ (A). Data represent the mean±SEM of three separate experiments using 10 mice/group (A,B). Representative histology sections of myocarditis (C) and pericarditis (D) for PBS, rIL-33 or rST2-treated BALB/c mice at day 10 pi, magnification x40 (C) and x260 (D) respectively. Histology of eosinophilic myocarditis following rIL-33 treatment, eosinophil (arrow), magnification x520 (E). Representative Facs of PBS 1% (top) vs. rIL-33 17% (bottom), x-axis Siglec F+, y-axis Ly6G+ (F). Absolute (left) and proportional (right) number of eosinophils in the heart by Facs analysis of live CD45+ cells, ***absolute number (left) $p=0.001$ and proportional/percent CD45+ (right) $p=1.3x10^{-5}$ (G). Data representative of 2 separate experiments using 5-8 mice/group (G).

**Figure 2.** Cardiac function in rIL-33 or rST2 treated mice during myocarditis. BALB/c mice were infected with CVB3 at day 0 and rIL-33, rST2 or PBS injected every other day from day 1-9 pi and pressure-volume relationships assessed at day 0, 10 or 35 pi (A). Data show the mean ±SEM of 10-12 mice/group (A). Kruskal-Wallis test compares three treatment groups at day 10 or day 35 pi, *$p<0.05$ (A). Percent change in the average end systolic pressure (ESP), ejection fraction (EF) or end diastolic volume (EDV) at day 10 or 35 pi from day 0 for each treatment group (B).
**Figure 3.** rIL-33 induces β-adrenergic insensitivity during CVB3 myocarditis at day 10 pi (A) and in undiseased mice at day 10 (B). BALB/c mice received either CVB3 at day 0 and PBS, rIL-33 or rST2 every other day from day 1 to 9 pi (A) or PBS, rIL-33 or rST2 every other day from day 1 to 9 but no virus (B). *compares rIL-33 to PBS-treated mice (p<0.013). There were no significant differences in rST2 treated mice compared to PBS controls. Three groups were analyzed using generalized estimating equations and linear mixed effects models, †p<0.05. Data show the mean ±SEM of 10-12 mice/group. HR, heart rate; dP/dT Max, peak rate of pressure rise; CO, cardiac output; PMX, maximum ventricular power.

**Figure 4.** rIL-33 increases proinflammatory cytokines during acute CVB3 myocarditis at day 10 pi (A) and in undiseased mice at day 10 (B) by ELISA. BALB/c mice received either CVB3 at day 0 and PBS, rIL-33 or rST2 every other day from day 1-9 pi (A) or PBS, rIL-33 or rST2 every other day from day 1-9 but no virus (B). Kruskal-Wallis tests: IL-33 p=0.001, sera sST2 p=<0.0001, IL-4 p=0.004, TNF p=0.14, IL-1β p=0.0009, IL-6 p=0.006 (A) TNF p=0.18, IL-1β p=0.13, IL-6 p=0.004 (B). *compares PBS to rIL-33 or rST2 groups, #compares rIL-33 to rST2 by the Mann-Whitney rank test with a Bonferroni correction, * or # p<0.013, ** or ## p < 0.01, *** or ### p<0.001. Data represent the mean ±SEM of three separate experiments using 10 mice/group.

**Figure 5.** IL-33 directly reduces cardiac function in undiseased mice. Undiseased mice deficient (-/-) in the IL-1R (A), IL-6 (B) or IL-1RAcP (chain common to IL-1R and IL-33R/ST2L) (C) were treated with PBS, rIL-33 or rST2 every other day from day 1-9 and cardiac function
assessed at day 10 (no virus) using pressure-volume relationships. Data represent the mean ±SEM of 10 mice/group. *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney rank test.
IL-33 Independently Induces Eosinophilic Pericarditis and Cardiac Dilation: ST2 Improves Cardiac Function

Eric D. Abston, Jobert G. Barin, Daniela Cihakova, Adriana Bucek, Michael J. Coronado, Jessica E. Brandt, Djahida Bedja, Joseph B. Kim, Dimitrios Georgakopoulos, Kathleen L. Gabrielson, Wayne Mitzner and DeLisa Fairweather

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SUPPLEMENTARY MATERIAL

Supplementary Methods

Experimental Model

BALB/cJ (BALB/c) (stock#000651), C57BL/6J (stock#000664), B6.129SF2/J (stock#101045), type I IL-1R deficient (B6.129S7-Il1r1tm1Imx/J, stock#003245), IL-6 deficient (B6;129S2-Il6tmKopf/J, stock#002254) and IL1RAcP deficient (B6;129S1-Il1raptm1Romlj/J, stock# 003284) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained under pathogen-free conditions in the animal facility at Johns Hopkins School of Medicine, and approval was obtained from the Animal Care and Use Committee of the Johns Hopkins University for all procedures. Eight to ten week old male mice were inoculated intraperitoneally (ip) with 10^3 plaque forming units (PFU) of heart-passaged CVB3 containing infectious virus and heart tissue diluted in sterile saline. Recombinant (r)IL-33 (1µg/0.1mL/mouse, Cat#3626-ML), rST2 (5µg/0.1mL/mouse, Cat#1004MR-050) from R&D Systems (Minneapolis, MN), or sterile PBS were injected ip on day 1, 3, 5, 7 and 9 pi following CVB3 injection on day 0. The dose and time-course for rIL-33 and rST2 administration were based on previously published studies that found significantly increased or decreased inflammation using similar doses and time-course. Mice were harvested on day 10 pi during acute myocarditis or day 35 pi during DCM. rIL-33, rST2 or PBS was injected ip using the same protocol as above for healthy undiseased experiments and mice were examined on the equivalent of day 10 (one day after the 5th injection) or day 35 post inoculation.
Histology

Hearts were fixed in 10% buffered formalin and stained with H&E to assess inflammation. Sections were examined by two independent investigators and myocarditis assessed as the percentage of the heart section with inflammation compared to the overall size of the heart section using an eyepiece grid, as previously.\textsuperscript{5-7} The development of DCM was assessed by gross observation of histology sections at low magnification and by echocardiography or pressure-volume relationships.\textsuperscript{5-7}

Cardiac Function

Cardiac function was assessed by pressure-volume catheter (1.2F Scisense Inc., London, Ontario) placed in the left ventricle via the apex in open-chest mice anesthetized with 3\% isoflurane, as previously described.\textsuperscript{7-9} Previously we demonstrated that cardiac dysfunction observed during CVB3 myocarditis produces similar results using open-chest ventricular catheterization and closed-chest echocardiography methods.\textsuperscript{7} Ventricular dilation was assessed by trans-thoracic echocardiography (Acuson Sequoia C256, 15 MHz linear transducer; Siemens, Malvern, PA) in conscious mice, as previously.\textsuperscript{6,7} M-mode left ventricular end-systolic or diastolic cross-sectional diameters (LVESD or LVEDD) were determined from an average of 3-5 cardiac cycles. To assess β-adrenergic sensitivity, an isoproterenol dose-response protocol was adapted from previously described protocols.\textsuperscript{10,11} Left ventricular pressure-volume measurements were recorded continuously. Baseline hemodynamic values were obtained followed by 10μL intravenous bolus doses of vehicle, 0.2, 0.6, 2, 6, or 20 ng of isoproterenol in 3 minute
increments. The response to isoproterenol was determined by subtracting the maximum response obtained within 1 min following injection from values obtained immediately preceding that dose.

**Plaque Assay**

Hearts were homogenized at 10% weight/volume in 2% minimal essential medium (MEM) and individual supernatants used in plaque assays to determine the level of infectious virus (a measure of viral replication). The plaque assay method has been described in detail previously.\(^1\) Briefly, homogenates were incubated for 1 h on Vero cells (ATCC) at 37°C for viral attachment and covered with 2% MEM in methyl cellulose for three days to allow plaque formation. Viral plaques were quantitated using a light microscope and normalized to the size of the heart according to tissue wet weight. Viral replication is shown as the number of PFU/g of heart. The limit of detection for the assay was 10 PFU/g of tissue.

**Cytokine Measurements**

Hearts were homogenized at 10% weight/volume in 2% MEM and individual supernatants or sera used in ELISA kits (R&D Systems) to measure cytokines according to the manufacturer’s instructions and as previously described.\(^5,7\) The limits of detection for the ELISA were as follows: IL-33, 6.9 pg/mL; sST2, 140 pg/mL; IL-4, 2.0 pg/mL; TNF-α, 5.1 pg/mL; IL-1β, 2.3 pg/mL and IL-6, 1.6 pg/mL. To control for differences in heart size individual samples were converted to pg/g of heart tissue.
Flow Cytometry

Mice were anesthetized with avertin and the aorta cannulated to perfuse the hearts, which were then digested with 600 μ/mL collagenase II (Worthington) + 60 μ/mL DNase I (Sigma) according to the manufacturer’s instructions for the GentleMACS™ isolation of cardiac cells (Miltenyi), as previously described. Isolated immune cells were washed and FcγRII/III blocked with anti-CD16/32 (eBiosciences) then stained with fluorochrome-conjugated antibodies against CD45, CD3, CD4, CD19, FceR1α, CD117, CD11b, F4/80, Ly6G or SiglecF (BD Pharmingen or eBiosciences). Samples were acquired on a four-color dual-laser FACScalibur cytometer running CellQuest or the LSR II quadlaser cytometer running FACSDiva (BD Immunocytometry). Data were analyzed with FlowJo 7 (Treestar).

RT-PCR

Trizol Reagent and the PureLink Micro-to-Midi system (Invitrogen, Carlsbad, CA) were used for extraction and purification of RNA, as previously. The hearts were homogenized in 2 mL Trizol and 1 mL of the homogenate was processed according to the manufacturer’s protocol (Invitrogen). Following elution of purified RNA, quantification was performed using a NanoDrop spectrophotometer. For each treatment group, processing and analysis were performed in triplicate. cDNA was generated using a Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA). Gene expression was measured using assay-on-demand probe sets and the ABI 7000 Taqman system according to the manufacturer’s instructions (Applied Biosystems), as previously. Hypoxanthine phosphoribosyltransferase (HPRT) was used for normalization.
Statistical Analysis

The Mann-Whitney rank sum test was used to evaluate two groups ($p<0.05$). Comparisons involving three groups were analyzed using Kruskal-Wallis tests. When three groups were significant ($p<0.05$) then pairwise comparisons were made using Mann-Whitney rank tests with a Bonferroni correction ($p<0.013$). Repeated measures data (i.e. isoproterenol treatments) were evaluated using generalized estimating equations (GEE) and linear mixed effects models with dose as a covariate. An omnibus test for group differences was used at the first stage ($p<0.05$) and if significant then pairwise comparisons were assessed ($p<0.013$). Dose was converted to a logarithmic scale for linear mixed model analysis because dose-response curves were log-linear during preliminary analysis. A small number (equivalent to the next lower dilution, 0.06) was added to allow for log transformation of a 0 dose. For sensitivity analyses, we modeled dose non-linearity with a quadratic non-linear term to assess group differences.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Supplementary Results

rIL-33 exacerbates cardiac dysfunction during CVB3 myocarditis/DCM but rST2 improves cardiac function

To determine the effect of IL-33 or ST2 administration on cardiac function, we treated male BALB/c mice with rIL-33, rST2 (to block IL-33 released during the disease process) or PBS ip.
every other day from day 1 to 9 pi and examined acute myocarditis at day 10 pi and progression to DCM at day 35 pi using pressure volume relationships. Evaluating several cardiac functional parameters over time revealed that rIL-33 significantly decreased cardiac function while rST2 improved function (Figure 2).

During acute CVB3 myocarditis at day 10 pi, mice treated with rIL-33 developed impaired systolic function (Table 1). In rIL-33 treated mice end systolic pressure (ESP) was significantly depressed when compared to PBS treated controls (83±2 mmHg vs. 94±3 mmHg, \( p = 0.008 \)) (Figure 2). dP/dT Max in IL-33 treated mice was 7141 ±312 mmHg/s which was 21% lower than PBS treated mice, 9071 ±546 (\( p = 0.01 \)). Ejection fraction (EF) was also depressed to 49±5% in IL-33 treated vs. 63±3% in PBS treated mice (Table 1). Cardiac geometry was significantly different in IL-33 treated mice compared to PBS controls. End diastolic volume (EDV) increased by 32% (rIL-33 22 ±7μL, PBS 15 ±0.5L, \( p = 0.01 \)) (Figure 2). IL-33 treated mice also demonstrated impaired diastolic function. dP/dT Min was 27% lower in IL-33 treated mice than in PBS treated mice (IL-33 -6224 ±477 mmHg/s vs. PBS -8502 ±424mmHg/s, \( p = 0.003 \)).

In contrast, rST2 treatment was either similar to PBS treated mice or displayed significantly improved systolic/ diastolic ventricular function during acute CVB3 myocarditis (day 10 pi) compared to PBS controls (Table 1). dP/dT Max in ST2 treated mice was 11570 ±350 mmHg/s vs. 9071 ±546 mmHg/s in PBS treated mice (\( p = 0.002 \)). Maximum ventricular power (PMX) was 40% higher in rST2 treated mice as compared to PBS treated controls (\( p =
0.003) and the power output in the left ventricles of rST2 treated mice was double that of rIL-33 treated mice (IL-33 7±0.4 mW, ST2 14±0.6 mW, PBS 10±0.7 mW, p < 0.00001).

In this model of myocarditis, susceptible strains of mice like BALB/c recover from acute myocarditis but develop DCM by day 35 pi. During chronic myocarditis/DCM (day 35 pi) mice that were treated with rIL-33 from day 1-9 pi developed worse dilation and reduced EF (p = 0.01) compared to PBS treated controls (Figure 2, Table 2). Left ventricular dimensions (i.e. dilation) were increased in rIL-33 treated mice compared to PBS treated mice, where ESV was 60% larger than PBS controls (IL-33 13 ±2 μL vs. PBS 5 ±0.8) and EDV was 17% larger with rIL-33 treatment (IL-33 day 35 pi, 24 ±1.4 μL) compared to PBS controls (PBS day 35 pi, 20 ±1.0 μL) that were already dilated at day 35 pi compared to PBS controls at day 10 pi that were not dilated (PBS day 10 pi, 15 ±0.5 μL). Ea/Ees was increased by 53% with rIL-33 treatment vs. PBS (IL-33 1.7 ±0.14 vs. PBS 0.8 ±0.20, p = 0.007). Markers of diastolic function were not significantly different between rIL-33 and PBS treated groups at day 35 pi.

Again, rST2 treatment improved cardiac function compared to rIL-33 treated mice during chronic myocarditis at day 35 pi in mice that had only received rST2 from day 1-9 pi (Figure 2, Table 2). ESP in rST2 treated mice was higher than rIL-33 mice (ST2 96 ±3 mmHg vs. IL-33 96 ±3 mmHg, p = 0.01) (Figure 2). EF was 68±5% in rST2 treated mice and 41±3% in IL-33 treated mice (p = 0.001) (Figure 2). PMX in rST2 treated mice was 9±0.4 mW and 7 ±0.5 mW in IL-33 treated mice (p = 0.01).

rIL-33 induces acute cardiac dysfunction and pericarditis in undiseased mice
Administration of certain cytokines, like TNF-α or IL-1β, is capable of causing acute cardiac dysfunction in normal mice.\textsuperscript{15,16} To assess whether rIL-33 administration could induce cardiac dysfunction in the absence of myocarditis, normal uninfected male BALB/c mice were injected ip with PBS, rIL-33 or rST2 every other day for a total 5 injections (day 1, 3, 5, 7 and 9) and cardiac function assessed the day after the final treatment (the equivalent of day 10) using pressure-volume relationships or at day 35 by echocardiography. We found that rIL-33 treatment was able to impair cardiac function in the absence of myocarditis compared to PBS treated mice at day 10 (Table 3) but not at day 35 (Supplemental Table 1). Although LVEDD and LVESD were significantly increased in rIL-33-treated mice compared to PBS controls at day 35, the increases were within the normal physiologic range and EF and FS were not different between groups indicating that the hearts were not dilated (Supplemental Table 1). In contrast, rST2 treatment from day 1-9 had no significant overall effect on heart function compared to PBS controls at day 10 (Table 3). For this reason, rST2 was not examined at day 35 in undiseased mice.

Specifically, rIL-33 treatment impaired systolic ventricular function and induced bradycardia (IL-33 533 ±3 bpm vs. PBS 570 ±10 bpm, \( p = 0.003 \)) (Table 3). ESP was unchanged by rIL-33 administration. dP/dT Max was decreased by 20\% in rIL-33 treated mice (IL-33 8055 ±395 mmHg/s vs. PBS 10156 ±346 mmHg/s, \( p = 0.002 \)). rIL-33 treatment placed these mice at a risk for heart failure by reducing EF by 44\% from 68±5\% in PBS treated mice to 38±7\% in IL-33 treated mice (Table 3). With rIL-33 treatment ESV significantly increased to 14 ±2.1 µL from 6 ±1.1 µL in PBS treated mice (\( p = 0.005 \)). rIL-33 treatment induced dilation by changing EDV
from 16 ±1.1 μL in PBS treated mice to 23 ±1.8 μL in rIL-33 treated mice. Ventricular-arterial coupling was significantly impacted by rIL-33 treatment (IL-33 2.31 ±0.29, PBS 0.96 ±0.09, p = 0.007). Diastolic function was also impaired by rIL-33 treatment (Table 3). dP/dT Min was -7138 ±551 mmHg/s in rIL-33 treated mice and -9460 ±83 mmHg/s in PBS treated mice (p = 0.001). Tau was 7 ±0.7 ms with rIL-33 treatment and 5 ±0.2 ms with PBS (p = 0.01). There were no significant differences between PBS and ST2 treated mice for any hemodynamic parameter (Table 3).

Histological examination revealed that rIL-33-treatment induced pericarditis in undiseased mice (Supplemental Figure 1) similar in appearance to that observed during acute CVB3 myocarditis (Figure 1D). The pericardium is a single cell layer in normal mice (Supplemental Figure 1A) but was severely inflamed with numerous eosinophils following rIL-33 administration (Supplemental Figure 1B). However, a single injection of rIL-33 at day 0 was not capable of inducing pericarditis, myocarditis or eosinophilia at day 10 in undiseased mice (n = 10/group) (data not shown). In contrast, pericardial damage in rST2-treated mice appeared “lacey” with few inflammatory cells and no eosinophilia (Supplemental Figure 1C). Myocardial inflammation was not present in PBS, rIL-33 or rST2-treated mice that had not received CVB3.

**rIL-33 increases cardiac IL-33, IL-4, IL-1β, IL-6 and serum sST2, and alters remodeling during acute CVB3 myocarditis**
To examine whether rIL-33 treatment affected cardiac remodeling, we conducted RT-PCR during acute myocarditis on mice that had been treated with rIL-33 from day 1-9, as previously, or PBS controls and examined whether changes occurred in the level of the remodeling genes Mmp3 or Mmp9 in the heart. We, and others, have found that reduced levels of Mmp3 and Mmp9 during CVB3 myocarditis are associated with cardiac remodeling and fibrosis.\textsuperscript{17-19} In this study we found that rIL-33 reduced Mmp3 (PBS 11.2±1.3 vs. rIL-33 7.4±1.6, \( p = 0.04 \)) and Mmp9 (PBS 11.0±1.3 vs. rIL-33 6.8±1.6, \( p = 0.04 \)) expression in the heart compared to PBS controls.

**rIL-33 treatment directly decreases heart function**

Because rIL-33 treatment induced cardiac dysfunction and \( \beta \)-adrenergic insensitivity in diseased and undiseased mice at day 10, we wanted to determine whether IL-33 could be independently responsible for these effects.\textsuperscript{15,16} However, since rIL-33 treatment increased IL-1\( \beta \) and IL-6 levels in the heart it was possible that IL-33 worked indirectly to alter heart function via these cytokines. To test this possibility we treated undiseased male IL-1R, IL-6 or IL-1RAcP (this receptor is required for IL-1R and IL-33R-mediated signaling)\textsuperscript{20} deficient mice with PBS or rIL-33, using the same protocol as before, and examined heart function by pressure-volume relationships. We found that rIL-33 treatment significantly decreased most cardiac functional parameters compared to PBS treated IL-1R or IL-6 deficient mice (IL-1R/- ESP \( p = 2.63 \times 10^{-5} \), CO \( p = 0.02 \), SW \( p = 0.02 \), PMX \( p = 2.63 \times 10^{-5} \); IL-6/- ESP \( p = 2.74 \times 10^{-5} \), CO \( p = 0.003 \), SW \( p = 0.0003 \), PMX \( p = 2.74 \times 10^{-5} \)) using the Mann-Whitney rank test (Figure 5A and 5B). These
data indicate that IL-1β and IL-6 were not responsible for the effect of rIL-33. In contrast, cardiac dysfunction following rIL-33 treatment was not significantly different from PBS controls for any parameter examined in IL-1RαCp deficient mice (ESP $p = 0.37$, CO $p = 0.38$, SW $p = 0.30$, PMX $p = 0.39$) (Figure 5C), demonstrating that the IL-33R is required for the cardiac dysfunction observed in rIL-33 treated mice.

Because IL-1β has been shown to be critical for acute CVB3 myocarditis, we examined whether rIL-33 could increase myocarditis indirectly via IL-1β by treating IL-1R deficient mice with rIL-33 from day 1-9 pi. We found that although rIL-33 significantly increased myocarditis compared to PBS control C57BL/6 mice ($p = 0.01$), there was no significant difference in myocarditis between WT and IL-1R deficient mice in response to rIL-33 ($p = 0.42$) (Supplemental Figure 4). These results indicate that rIL-33 does not increase acute CVB3 myocarditis via IL-1β.

Supplementary References


CIRCHF/201/963769-R3


**Supplemental Table 1.** *In vivo* hemodynamics of undiseased rIL-33-treated BALB/c mice at day 35 post inoculation based on echocardiography

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PBS</th>
<th>rIL-33</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>682±10</td>
<td>670±12</td>
<td>0.61</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>2.91±0.04</td>
<td>3.14±0.02</td>
<td>5.46x10^{-5}</td>
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<tr>
<td>LVESD, mm</td>
<td>1.09±0.01</td>
<td>1.17±1.0</td>
<td>0.003</td>
</tr>
<tr>
<td>IVSD, mm</td>
<td>0.82±0.02</td>
<td>0.82±0.02</td>
<td>1.0</td>
</tr>
<tr>
<td>LV PWTED, mm</td>
<td>0.78±0.01</td>
<td>0.78±0.01</td>
<td>1.0</td>
</tr>
<tr>
<td>EF, %</td>
<td>85.42±0.47</td>
<td>86.10±0.34</td>
<td>0.28</td>
</tr>
<tr>
<td>FS, %</td>
<td>61.85±0.61</td>
<td>62.75±.46</td>
<td>0.26</td>
</tr>
<tr>
<td>RWT</td>
<td>0.54±0.01</td>
<td>0.49±0.01</td>
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<tr>
<td>LVmass, mg</td>
<td>72.32±2.44</td>
<td>81.51±1.92</td>
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</table>

LVEDD, left ventricular end diastolic dimension; LVESD, left ventricular end systolic dimension; IVSD, interventricular septal thickness at diastole; LV PWTED, left ventricular posterior wall thickness at end diastole; EF, ejection fraction; FS, fractional shortening; RWT, relative wall thickness. Male BALB/c mice received rIL-33 or PBS ip every other day from day 1 to 9 post inoculation and echocardiography was performed on day 35 post inoculation. Data show mean ±SEM for 10 mice/group. PBS vs. rIL-33 treatment groups were assessed using the Mann-Whitney rank test.
**Supplemental Table 2.** Generalized estimating equation (GEE) analysis of isoproterenol treatments for three groups followed by Mann-Whitney rank tests of two groups.*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group p value</th>
<th>PBS vs. rIL-33</th>
<th>PBS vs. rST2</th>
<th>rIL-33 vs. rST2</th>
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</thead>
<tbody>
<tr>
<td><strong>Day 10 pi CVB3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate</td>
<td>&lt;0.0001</td>
<td>0.0004</td>
<td>0.25</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>dP/dT Max</td>
<td>0.0001</td>
<td>0.003</td>
<td>0.20</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CO</td>
<td>0.0001</td>
<td>0.0003</td>
<td>0.90</td>
<td>0.0003</td>
</tr>
<tr>
<td>PMX</td>
<td>0.002</td>
<td>0.03</td>
<td>0.24</td>
<td>0.0006</td>
</tr>
<tr>
<td><strong>Day 10 Undisease</strong></td>
<td></td>
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<tr>
<td>Heart rate</td>
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<tr>
<td>dP/dT Max</td>
<td>0.0004</td>
<td>0.0001</td>
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<tr>
<td>CO</td>
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<tr>
<td>PMX</td>
<td>0.006</td>
<td>0.001</td>
<td>0.17</td>
<td>0.06</td>
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<tr>
<td><strong>Day 35 pi CVB3</strong></td>
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<tr>
<td>Heart rate</td>
<td>0.64</td>
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<tr>
<td>dP/dT Max</td>
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<td>0.47</td>
<td>0.32</td>
</tr>
<tr>
<td>CO</td>
<td>0.003</td>
<td>0.0007</td>
<td>0.18</td>
<td>0.07</td>
</tr>
<tr>
<td>PMX</td>
<td>0.13</td>
<td>0.05</td>
<td>0.52</td>
<td>0.22</td>
</tr>
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</table>

*Data was log$_{10}$ transformed, with a small number corresponding to the next dilution (0.06) added to the zero dose. Sensitivity analyses without this addition, GEE models without transformation but with nonlinear quadratic dose-response relationship, were also evaluated and remained significant for all groups that were significant in previous analysis. CO, cardiac output (mL/min); Heart rate (bpm); dP/dT Max, peak rate of pressure rise (mmHg/s); PMX, maximum ventricular power (mW).
Supplemental Figure 1. rIL-33 and rST2 induce pericarditis in undisease mice. Male BALB/c mice received PBS (A), rIL-33 (B) or rST2 (C) every other day from day 1 to 9 and myocardial ...
inflammation/ pericarditis was assessed at day 10 post inoculation ($n = 10/\text{group}$). None of the mice developed myocardial inflammation, but rIL-33 ($B$) and rST2 ($C$) treated mice developed pericarditis ($B$) or pericardial damage ($C$). Representative histology sections, magnification x260. The infiltrate of rIL-33 treated mice had abundant eosinophils (arrow and insert) ($B$), which were absent in rST2-treated mice ($C$).
Supplemental Figure 2. rIL-33 treatment from day 1-9 pi does not induce β-adrenergic insensitivity during chronic CVB3 myocarditis at day 35 pi. Male BALB/c mice received CVB3 at day 0 and PBS, rIL-33 or rST2 every other day from day 1 to 9 pi and heart function was assessed at day 35 pi. There were no significant differences between groups. Data show the mean ±SEM of 10 to 12 mice per group. HR, heart rate; dP/dT Max, peak rate of pressure rise; CO, cardiac output; PMX, maximum ventricular power.
Supplemental Figure 3. Comparison of cytokine levels in the heart in normal mice (no myocarditis) and during acute CVB3 myocarditis. Male BALB/c mice received either PBS or CVB3 ip at day 0 and PBS, rIL-33 or rST2 every other day from day 1 to 9 pi. Cytokine levels in the heart were assessed by ELISA one day after the final injection (day 10). Mice with or without myocarditis were compared for each treatment (e.g. PBS, rIL-33 or rST2) using the Mann-
Whitney rank test. Data represent the mean ±SEM of three separate experiments using 10 mice/group.
**Supplemental Figure 4.** IL-1β via the IL-1R is not responsible for elevated CVB3 myocarditis following rIL-33 treatment. Male C57BL/6 mice received CVB3 ip at day 0 and PBS or rIL-33 every other day from day 1 to 9 pi and acute myocarditis was assessed histologically at day 10 pi. a, BL/6 PBS vs. BL/6 rIL-33 $p = 0.01$; b, no significant difference between BL/6 rIL-33 vs. IL-1R/-/ rIL-33, $p = 0.42$; $n = 10$ mice/group. Data analyzed using Kruskal-Wallis test and Mann-Whitney rank test.