IL-33 Independently Induces Eosinophilic Pericarditis and Cardiac Dilation

ST2 Improves Cardiac Function

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

Background—IL-33 through 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 (eg, soluble ST2) administration on the development of autoimmune coxsackievirus B3 myocarditis and dilated cardiomyopathy in male BALB/c mice. The 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, $P=0.01$). The rST2 treatment prevented eosinophilia and improved heart function compared with rIL-33 treatment (ejection fraction, $P=0.009$). Neither treatment altered viral replication. The rIL-33 treatment increased IL-4, IL-33, IL-1β, 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 to IL-1β or IL-6.

Conclusions—We show for the first time to our knowledge that IL-33 induces eosinophilic pericarditis, whereas soluble ST2 prevents eosinophilia and improves systolic function, and that IL-33 independently adversely affects heart function through the IL-33 receptor. (Circ Heart Fail. 2012;5:366-375.)

Key Words: myocarditis ■ cytokines ■ inflammation ■ idiopathic dilated cardiomyopathy ■ eosinophils

M yocarditis, or inflammation of the myocardium, leads to ~46% of the dilated cardiomyopathy (DCM) cases in the United States, whereas DCM is the most common form of cardiomyopathy responsible for heart failure. Coxackievirus B3 (CVB3) is a major cause of myocarditis and DCM in humans, and infection of mice with heart-passaged CVB3 (containing infectious virus and cardiac tissue) induces autoimmune myocarditis leading to DCM. Only mice that respond to infection with a T helper (Th) 2 immune response progress from acute myocarditis to chronic myocarditis and DCM. In patients with heart failure, elevated serum levels of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and IL-6, independently predict decreased heart function and poor survival. IL-1β and TNF-α worsen heart disease during myocarditis by increasing inflammation and inducing myocyte hypertrophy, contractile dysfunction, and extracellular matrix remodeling.

Clinical Perspective on p 375

IL-33 is a cytokine in the IL-1β family that promotes a Th2 response. IL-1 receptor-like 1 protein, most often abbreviated ST2, exists in 2 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 believed 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 (r) IL-33 increased collagen or antibody-induced arthritis in mice. Paradoxically, IL-33 has been shown to be cardioprotective in murine models of idiopathic dilated cardiomyopathy and associated with improved survival.
myocardial infarct and hypertrophy, where rIL-33 administration improves heart function and diminishes inflammation and fibrosis.\textsuperscript{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-1\textsubscript{a} 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 through the IL-33 receptor and are not due to IL-1\textsubscript{a} 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 the Johns Hopkins School of Medicine, and approval was obtained from the Johns Hopkins University Animal Care and Use Committee for all procedures. Mice were inoculated intraperitoneally with heart-passaged CVB3 on day 0, and hearts were examined on day 10 postinfection during acute myocarditis or day 35 postinfection during DCM.\textsuperscript{4} rIL-33 (1 \textmu g/0.1 mL), rST2 (5 \textmu g/0.1 mL), or sterile PBS were injected intraperitoneally on days 1, 3, 5, 7, and 9 in undiseased or CVB3-infected mice. Hearts were fixed in 10% buffered formalin and stained with hematoxylin eosin to assess inflammation, as previously described.\textsuperscript{6}

**Cardiac Function**

Cardiac function was assessed by a 1.2F pressure-volume catheter (Scisense Inc) placed in the left ventricle through the apex in open-chest mice anesthetized with 3% isoflurane, as previously described.\textsuperscript{16–18} To assess \textalpha\,-adrenergic sensitivity, an isoproterenol dose-response protocol was adapted from previous protocols.\textsuperscript{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.\textsuperscript{4,6}

**Flow Cytometry**

Hearts were digested with 600 \textmu g/mL collagenase II (Worthington) plus 60 \textmu g/mL DNAse I (Sigma) according to the manufacturer’s instructions for the GentleMACS (Miltenyi) isolation of cardiac cells.\textsuperscript{11–23} Immune cells were stained with fluorochrome-conjugated antibodies against CD45, CD3, CD4, CD19, FceR1a, CD117, CD11b, F4/80, Ly6G, or SiglecF (BD Pharmingen/eBiosciences).
Statistical Analysis
The Mann–Whitney rank sum test was used to evaluate 2 groups (P<0.05). Comparisons involving 3 groups were analyzed using the Kruskal-Wallis test. When 3 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 (ie, isoproterenol treatments) were evaluated using generalized estimating equations 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, 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.24 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),25 or PBS intraperitoneally every other day from days 1 to 9 postinfection and examined myocarditis at day 10 postinfection. We found that rIL-33 treatment significantly increased CVB3 myocarditis (P=0.006) (Figure 1A and 1C) and pericarditis (Figure 1D) compared with PBS controls, whereas rST2 did not (P=0.19). Neither rIL-33 nor rST2 treatment significantly altered viral replication compared with PBS controls (PBS versus rIL-33, P=0.16; PBS versus rST2, P=0.66) (Figure 1B). The rIL-33 treatment significantly increased the number of eosinophils in the heart by histology (Figure 1E) and fluorescence-activated cell sorting analysis (absolute numbers, P=0.001; percentages, P=1.3×10^{-5}) (Figure 1F and 1G). No other major cell populations, such as lymphocytes, monocytes, macrophages, neutrophils, or mast cells, were increased by rIL-33 treatment by fluorescence-activated cell sorting analysis (data not shown). The rST2-treated mice did not develop pericardial or myocardial eosinophilia (Figure 1D).

rIL-33 Exacerbates Cardiac Dysfunction During CVB3 Myocarditis and 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 intraperitoneally every other day from days 1 to 9 postinfection and examined cardiac function at day 10 or 35 postinfection using pressure-volume relationships. We found that rIL-33 significantly decreased cardiac function, whereas rST2 improved function (Figure 2, Tables 1 and 2). In this myocarditis model, susceptible strains of mice, like BALB/c, recover from acute myocarditis but develop DCM by day 35 postinfection.4,6 At day 35 postinfection, mice that received rIL-33 from days 1 to 9 postinfection developed worse dilation and reduced ejection fraction (P=0.01) compared with PBS controls (Figure 2, Table 2). The rST2 treatment improved cardiac function during chronic myocarditis in mice that received rST2 from days 1 to 9 postinfection (Figure 2, Table 2). See the online-only Data Supplement 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.9,26 To assess whether rIL-33 induced cardiac dysfunc-
Table 1. In Vivo Hemodynamics of rIL-33- and rST2-Treated BALB/c Mice During Acute CVB3 Myocarditis (Day 10 Postinfection) Based on Pressure-Volume Analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PBS</th>
<th>rIL-33</th>
<th>Mann–Whitney P Value†</th>
<th>rST2</th>
<th>Mann–Whitney P Value†</th>
<th>Mann–Whitney P Value‡</th>
<th>Kruskal-Wallis P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>519 (508 to 552)</td>
<td>558 (510 to 577)</td>
<td>0.008</td>
<td>554 (510 to 577)</td>
<td>0.07</td>
<td>0.0004</td>
<td>0.26</td>
</tr>
<tr>
<td>ESP, mm Hg</td>
<td>91 (86 to 100)</td>
<td>82 (78 to 89)</td>
<td>0.008</td>
<td>102 (78 to 89)</td>
<td>0.07</td>
<td>0.0004</td>
<td>0.0005</td>
</tr>
<tr>
<td>EDP, mm Hg</td>
<td>4.9 (3.4 to 6.1)</td>
<td>5.0 (3.7 to 9.6)</td>
<td>0.008</td>
<td>4.9 (4.2 to 6.1)</td>
<td>0.26</td>
<td>0.009</td>
<td>0.02</td>
</tr>
<tr>
<td>dP/dT max, mm Hg/s</td>
<td>9557 (7649 to 10 090)</td>
<td>7234 (6470 to 8071)</td>
<td>0.01</td>
<td>11 572 (10 213 to 12 191)</td>
<td>0.02</td>
<td>0.0002</td>
<td>0.00001</td>
</tr>
<tr>
<td>dP/dT min, mm Hg/s</td>
<td>−6842 (−8794 to −7731)</td>
<td>−6141 (−7621 to −6788)</td>
<td>0.003</td>
<td>−9428 (−7641 to −4788)</td>
<td>0.02</td>
<td>0.0002</td>
<td>0.0001</td>
</tr>
<tr>
<td>EF, %</td>
<td>63 (56 to 67)</td>
<td>47 (37 to 63)</td>
<td>0.04</td>
<td>66 (61 to 75)</td>
<td>0.26</td>
<td>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)</td>
<td>0.06</td>
<td>5.0 (3.8 to 6.7)</td>
<td>0.36</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>EDV, μL</td>
<td>15 (14 to 17)</td>
<td>21 (17 to 27)</td>
<td>0.01</td>
<td>16 (13 to 27)</td>
<td>0.88</td>
<td>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>0.96</td>
<td>9.6 (8.4 to 12)</td>
<td>0.87</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>CO, mL/min</td>
<td>5375 (4131 to 5973)</td>
<td>5162 (4073 to 7722)</td>
<td>0.0002</td>
<td>5343 (4422 to 6443)</td>
<td>0.03</td>
<td>0.0002</td>
<td>0.0001</td>
</tr>
<tr>
<td>SW, mm Hg×μL</td>
<td>870 (719 to 919)</td>
<td>822 (556 to 1028)</td>
<td>0.02</td>
<td>898 (802 to 1011)</td>
<td>0.81</td>
<td>0.006</td>
<td>0.01</td>
</tr>
<tr>
<td>PRSW, mm Hg</td>
<td>76 (64 to 87)</td>
<td>66 (51 to 71)</td>
<td>0.06</td>
<td>75 (64 to 88)</td>
<td>0.07</td>
<td></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)</td>
<td>0.00002</td>
<td>14 (12 to 15)</td>
<td>0.003</td>
<td>0.0002</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
| Ees, left ventricular end-systolic elastance; Ea/Ees, arterial elastance normalized to Ees; V0, intercept of the ESP-volume relationship; τ, time constant of diastolic relaxation.

Data are presented as median (interquartile range) for 8 to 10 mice per group. Male BALB/c mice received CVB3 at day 0 and either PBS, rIL-33, or rST2 intraperitoneally every other day for a total of 5 injections, and cardiac function was assessed the day after the final treatment (the equivalent of day 10) using pressure-volume relationships or at day 35 by echocardiography. The rIL-33 treatment impaired cardiac function in the absence of myocarditis compared with PBS controls at day 10 (Table 3) but not at day 35.

Table 2. In Vivo Hemodynamics of rIL-33- and rST2-Treated Mice During Chronic CVB3 Myocarditis (Day 35 Postinfection) Based on Pressure-Volume Analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PBS</th>
<th>rIL-33</th>
<th>Mann–Whitney P Value†</th>
<th>rST2</th>
<th>Mann–Whitney P Value†</th>
<th>Mann–Whitney P Value‡</th>
<th>Kruskal-Wallis P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>567 (555 to 591)</td>
<td>560 (546 to 589)</td>
<td>0.22</td>
<td>574 (560 to 603)</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>ESP, mm Hg</td>
<td>86 (83 to 91)</td>
<td>78 (77 to 91)</td>
<td>0.22</td>
<td>96 (88 to 99)</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>EDP, mm Hg</td>
<td>5.9 (5.3 to 6.8)</td>
<td>7.5 (5.5 to 8.7)</td>
<td>0.26</td>
<td>5.5 (3.6 to 7.6)</td>
<td>0.46</td>
<td>0.007</td>
<td>0.01</td>
</tr>
<tr>
<td>dP/dT max, mm Hg/s</td>
<td>8009 (6466 to 8580)</td>
<td>7354 (6682 to 8763)</td>
<td>0.26</td>
<td>8787 (7922 to 9426)</td>
<td>0.06</td>
<td>0.003</td>
<td>0.01</td>
</tr>
<tr>
<td>dP/dT min, mm Hg/s</td>
<td>−7545 (−8627 to −6002)</td>
<td>−6942 (−7764 to −6388)</td>
<td>0.29</td>
<td>−8254 (−9066 to −7423)</td>
<td>0.13</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>EF, %</td>
<td>49 (43 to 63)</td>
<td>38 (34 to 48)</td>
<td>0.01</td>
<td>71 (55 to 80)</td>
<td>0.04</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>ESV, μL</td>
<td>8.2 (5.2 to 12.2)</td>
<td>12 (11 to 15)</td>
<td>0.03</td>
<td>4.8 (3.3 to 7.5)</td>
<td>0.04</td>
<td>0.0004</td>
<td>0.0008</td>
</tr>
<tr>
<td>EDV, μL</td>
<td>20 (17 to 21)</td>
<td>22 (21 to 26)</td>
<td>0.04</td>
<td>17 (15 to 19)</td>
<td>0.08</td>
<td>0.005</td>
<td>0.007</td>
</tr>
<tr>
<td>SV, μL</td>
<td>10 (9.1 to 12)</td>
<td>9.8 (8.9 to 11)</td>
<td>0.11</td>
<td>11 (8.1 to 15)</td>
<td>0.48</td>
<td></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>0.26</td>
<td>6.3 (4.9 to 8.5)</td>
<td>0.37</td>
<td></td>
<td>0.37</td>
</tr>
<tr>
<td>SW, mm Hg×μL</td>
<td>787 (699 to 929)</td>
<td>648 (531 to 860)</td>
<td>0.26</td>
<td>935 (822 to 1060)</td>
<td>0.07</td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>PRSW, mm Hg</td>
<td>74 (63 to 89)</td>
<td>59 (51 to 74)</td>
<td>0.04</td>
<td>75 (67 to 83)</td>
<td>0.09</td>
<td></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)</td>
<td>0.22</td>
<td>9 (6.4 to 10)</td>
<td>0.03</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>
| Ees, left ventricular end-systolic elastance; Ea/Ees, arterial elastance normalized to Ees; V0, x intercept of the ESP-volume relationship; τ, time constant of diastolic relaxation.

Data are presented as median (interquartile range) for 8 to 10 mice per group. Male BALB/c mice received CVB3 at day 0 and either PBS, rIL-33, or rST2 intraperitoneally every other day from days 1 to 9 postinfection, and data were collected at day 35 postinfection. r indicates recombinant; CVB3, coxsackievirus B3; HR, heart rate; ESP, end-systolic pressure; EDP, end-diastolic pressure; dP/dT max, peak rate of pressure rise; dP/dT min, peak rate of pressure decline; EF, ejection fraction; ESV, end-systolic volume; EDV, end-diastolic volume; SW, stroke volume; CO, cardiac output; SW, stroke work; PRSW, preload recruitable stroke work; PMX, maximum ventricular power; Ees, left ventricular end-systolic elastance; Ea/Ees, arterial elastance normalized to Ees; V0, x intercept of the ESP-volume relationship; τ, time constant of diastolic relaxation.

‡rIL-33 vs rST2.
†PBS vs rST2.
Table 3. In Vivo Hemodynamics of rIL-33- and rST2-Treated Uninfected Mice at Day 10 Postinoculation Based on Pressure-Volume Analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PBS</th>
<th>rIL-33</th>
<th>Mann–Whitney P Value*</th>
<th>rST2</th>
<th>Mann–Whitney P Value†</th>
<th>Mann–Whitney P Value‡</th>
<th>Kruskal–Wallis P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>562 (546 to 602)</td>
<td>532 (525 to 537)</td>
<td>571 (548 to 591)</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESP, mm Hg</td>
<td>97 (95 to 101)</td>
<td>107 (93 to 109)</td>
<td>107 (86 to 110)</td>
<td>0.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDP, mm Hg</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>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dP/dt max, mm Hg/s</td>
<td>10 035 (9246 to 10 876)</td>
<td>8063 (7409 to 8252)</td>
<td>0.002</td>
<td>10 117 (8740 to 10 584)</td>
<td>1.0</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>dP/dt min, mm Hg/s</td>
<td>-9517 (−9640 to −9249)</td>
<td>-6927 (−8090 to −5810)</td>
<td>0.001</td>
<td>-10 159 (−10 484 to −9 464)</td>
<td>0.05</td>
<td>0.003</td>
<td>0.0007</td>
</tr>
<tr>
<td>EF, %</td>
<td>67 (59 to 82)</td>
<td>40 (28 to 60)</td>
<td>0.02</td>
<td>67 (82 to 72)</td>
<td>0.65</td>
<td>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)</td>
<td>0.005</td>
<td>4.7 (4.1 to 6.6)</td>
<td>0.92</td>
<td>0.002</td>
<td>0.003</td>
</tr>
<tr>
<td>EDV, µL</td>
<td>15 (13 to 19)</td>
<td>23 (18 to 27)</td>
<td>0.02</td>
<td>16 (13 to 18)</td>
<td>0.96</td>
<td>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)</td>
<td>0.04</td>
<td>11 (9.8 to 11)</td>
<td>0.48</td>
<td>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)</td>
<td>0.008</td>
<td>6087 (5722 to 6432)</td>
<td>0.53</td>
<td>0.002</td>
<td>0.006</td>
</tr>
<tr>
<td>SW, mm Hg×µL</td>
<td>1034 (860 to 1054)</td>
<td>720 (620 to 775)</td>
<td>0.008</td>
<td>930 (841 to 1024)</td>
<td>0.48</td>
<td>0.003</td>
<td>0.005</td>
</tr>
<tr>
<td>PRSW, mm Hg</td>
<td>82 (74 to 91)</td>
<td>52 (49 to 72)</td>
<td>0.02</td>
<td>87 (67 to 93)</td>
<td>0.85</td>
<td>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)</td>
<td>0.003</td>
<td>12 (10 to 13)</td>
<td>0.48</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>Ees, mm Hg/µL</td>
<td>9.2 (6.9 to 12)</td>
<td>4.9 (4.7 to 6)</td>
<td>0.07</td>
<td>9.6 (8.8 to 11)</td>
<td>0.8</td>
<td>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)</td>
<td>0.07</td>
<td>1 (0.8 to 1.2)</td>
<td>0.7</td>
<td>0.002</td>
<td>0.003</td>
</tr>
<tr>
<td>VR, 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>
<td></td>
<td></td>
<td></td>
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<td>τ, ms</td>
<td>4.4 (6.5 to 5)</td>
<td>6.7 (5.6 to 8)</td>
<td>0.01</td>
<td>4.9 (4.7 to 5)</td>
<td>0.33</td>
<td>0.02</td>
<td>0.01</td>
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</table>

Data are presented as median (interquartile range) for 8 to 10 mice per group. Male BALB/c mice received either PBS, rIL-33, or rST2 intraperitoneally every other day, and data collected 10 d later. r indicates recombinant; HR, heart rate; ESP, end-systolic pressure; EDP, end-diastolic pressure; dP/dt max, peak rate of pressure rise; dP/dt min, peak rate of pressure decline; EF, ejection fraction; ESV, end-systolic volume; EDV, end-diastolic volume; SV, stroke volume; CO, cardiac output; SW, stroke work; PRSW, preload recruitable stroke work; PMX, maximum ventricular power; Ees, left ventricular end-systolic elastance; Ea/Ees, arterial elastance normalized to Ees; VR, x intercept of the ESP-volume relationship; τ, time constant of diastolic relaxation.

*PBS vs rIL-33.
†PBS vs rST2.
‡rIL-33 vs rST2.

(online-only Data Supplement Table I). In contrast, rST2 treatment from days 1 to 9 had no significant effect on heart function compared with PBS controls at day 10 (Table III). See the online-only Data Supplement for a more detailed description of cardiac function in undiseased mice. The rIL-33 treatment induced pericarditis in undiseased mice (online-only Data Supplement Figure I) that was similar in appearance to that observed during acute CVB3 myocarditis (Figure 1D). Eosinophils were abundant in the pericardial infiltrate of rIL-33 (online-only Data Supplement Figure IB) but absent in rST2-treated mice (online-only Data Supplement Figure IC).

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.12,25,29 For this reason, we examined the effect of rIL-33 or rST2 treatment on Th2-associated cytokines in the heart (eg, IL-4, IL-33, ST2) or sera (sST2) during acute CVB3 myocarditis (Figure 1D). IL-33 has been shown to promote Th1-type proinflammatory responses,30,31 and so, we also examined proinflammatory/profibrotic cytokines (eg, interferon-γ [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 with 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). The rIL-33 and rST2 treatments significantly increased serum levels of sST2 during acute myocarditis compared with PBS controls (P = 0.0001 and P = 0.0004, respectively) (Figure 4A). Importantly, rST2 treatment significantly decreased cardiac IL-33 compared with PBS con-
controls (P=0.001) (Figure 4A), indicating that rST2 treatment partially decreases cardiac IL-33 levels during acute myocarditis. The 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 on IFN-γ level (P=0.20) (data not shown) compared with PBS controls during myocarditis. The rIL-33 treatment also significantly altered matrix metalloproteinase levels in the heart compared with PBS by real-time polymerase chain reaction (online-only Data Supplement).

**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 postinfection (Figure 4A), we asked whether rIL-33 treatment of undiseased mice would also increase these cytokine levels in the heart. To test this, we administered PBS, rIL-33, or rST2 every other day as previously described 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 were prevented by rST2 treatment (P=0.004) in undiseased mice compared with PBS controls but had no significant effect on TNF-α levels (Kruskal-Wallis P=0.18, Mann–Whitney rank test P=0.08) or IL-1β (Kruskal-Wallis P=0.13, Mann–Whitney rank test P=0.04) (Figure 4B). A comparison of cytokines in undiseased mice versus mice with acute CVB3 myocarditis revealed that rIL-33 treatment specifically increased cardiac IL-1β levels (P=0.006) (rIL-33 group), whereas TNF-α (P=0.002) and IL-6 (P=0.008) levels were increased with disease (PBS group) (online-only Data Supplement Figure III).

**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, because rIL-33 treatment increased IL-1β and IL-6 levels in the heart (Figure 4, online-only Data Supplement Figure III), it was possible that IL-33 worked indirectly to alter heart function through 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
dilation, and heart failure. IL-33 had not yet been discovered, we examined whether rIL-33 increased CVB3 myocarditis. We showed that IFN-γ deficiency decreases most cardiac functional parameters compared with 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 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, we examined whether rIL-33 increased CVB3 myocarditis indirectly through IL-1β by treating IL-1R-deficient mice with rIL-33 from days 1 to 9 postinfection. We found that although rIL-33 significantly increased myocarditis compared with PBS controls, there was no significant difference in myocarditis between wild-type and IL-1R-deficient mice in response to rIL-33 (online-only Data Supplement Figure IV). Thus, rIL-33 does not increase acute CVB3 myocarditis through 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. To our knowledge, 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. IL-33 had not yet been discovered, and, thus, was not examined in those studies. Recently, we showed that TIR-domain-containing adapter-inducing IFN-β-deficient mice have elevated IL-33 levels in the heart that is associated with dilation, fibrosis, and heart failure.

Eosinophilic cardiovascular diseases, such as Churg-Strauss syndrome, Kounis syndrome, hypereosinophilic syndrome, eosinophilic myocarditis, and giant cell myocarditis, are associated with poor heart function and dilation similar to the present findings. Recently, rIL-33 administration has been found to promote lung eosinophilia in an animal model of asthma. The induction of eosinophilic pericarditis by rIL-33 in our model provides a possible explanation for the divergent results we obtained compared with myocardial infarct and aortic constriction animal models as reported by Sanada et al and Seki et al. Eosinophils release cationic proteins, such as major basic protein, metalloproteinases, and cytokines, that contribute to cardiac remodeling and dilation. We report here that rIL-33 treatment increases the profibrotic cytokines IL-4 and IL-1β as well as altered the metalloproteinase levels in the heart during myocarditis that contribute to cardiac remodeling. Although the precise reasons for this 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 and ST2 levels and function during myocarditis, and we used male mice in these experiments, but the sex used in the other studies was not described. Interestingly, hypereosinophilic syndrome occurs more frequently in men than in women.

Importantly, the effects of rIL-33 administration on chronic CVB3 myocarditis and DCM were detected at day 35 postinfection, 25 days after rIL-33 treatment finished (recombinant administered from days 1 to 9 postinfection), indicating that early effects of IL-33 (ie, innate, during acute myocarditis) were responsible for increasing DCM. Others have
reported effects of cytokines on heart function that continued 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 demonstrated in the present study 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. 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. The present 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, suggesting that important differences exist between IL-1β/IL-1R and IL-33/ST2L signaling pathways in the heart.

To our knowledge, we report for the first time that rST2 effectively protects the heart from cardiac dysfunction during acute and chronic CVB3 myocarditis and DCM. Administration of rST2 (eg, 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 with 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 days 1 to 9 postinfection effectively prevented progression to DCM, causing susceptible BALB/c mice to resemble resistant mouse strains such as C57BL/6.

In summary, the present study demonstrates that IL-33 induces eosinophilic pericarditis and increases CVB3 myocarditis and DCM, whereas 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

This study was supported by grants from the National Institutes of Health (NIH) (R01 HL087033) to Dr Fairweather and the National Center for Research Resources (UL1 RR 025005), a component of NIH and the NIH Roadmap for Medical Research.
Disclosures

Dr Cihakova and Ms Bedja have received a Grant In Aid from the American Heart Association for the study of the role of NK cells in myocarditis. Dr Cihakova has received NIH grant funding (R56HL077611) for the study of genetic contributions to myocarditis. Ms Bedja has received NIH grant funding (RO1 HL087033) for the study of innate immunity in myocarditis.

References


**CLINICAL PERSPECTIVE**

IL-33 and its receptor ST2 have recently been found to prevent remodeling in animal models of myocardial infarction. Myocardial remodeling is the primary mechanism responsible for the progression to dilated cardiomyopathy and heart failure following myocarditis, yet the role of IL-33 and ST2 has not been investigated. Here, we examined the role of IL-33 and ST2 in the progression of myocarditis to dilated cardiomyopathy in an animal model of myocarditis. Administration of recombinant IL-33 increased dilation and cardiac dysfunction in undiseased hearts as well as during myocarditis and dilated cardiomyopathy, which was reversed by soluble ST2. Negative effects on heart function by recombinant IL-33 were shown to be due to IL-33R signaling rather than to indirect induction of other signaling pathways, such as the IL-1R. The primary effect of recombinant IL-33 administration was to induce eosinophilia and pericarditis in the hearts of diseased and undiseased mice. Eosinophilic forms of cardiovascular disease are known to have a poor prognosis. This study is the first to our knowledge to report IL-33 as a mediator of eosinophilic myocarditis. Therapies targeting the IL-33/ST2 pathway will need to take into account the ability of IL-33 to induce cardiac eosinophilia and pericarditis.
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

_Circ Heart Fail._ 2012;5:366-375; originally published online March 27, 2012; doi: 10.1161/CIRCHEARTFAILURE.111.963769

_Circulation: Heart Failure_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circheartfailure.ahajournals.org/content/5/3/366

Data Supplement (unedited) at:
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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-Il1raptm1Roml/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.\(^5\) The development of DCM was assessed by gross observation of histology sections at low magnification and by echocardiography or pressure-volume relationships.\(^5\)\(^7\)

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.\(^7\)\(^9\) Previously we demonstrated that cardiac dysfunction observed during CVB3 myocarditis produces similar results using open-chest ventricular catheterization and closed-chest echocardiography methods.\(^7\) Ventricular dilation was assessed by trans-thoracic echocardiography (Acuson Sequoia C256, 15 MHz linear transducer; Siemens, Malvern, PA) in conscious mice, as previously.\(^6\)\(^7\) 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.\(^10\)\(^11\) 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.\(^1,5\) Briefly, homogenates were incubated for 1h 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-\(\alpha\), 5.1 pg/mL; IL-1\(\beta\), 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.12-14 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, FcεR1α, 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.6,7 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.6,7 Hypoxanthine phosphoribosyltransferase (HPRT) was used for normalization.

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**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 ±7\( \mu \)L, PBS 15 ±0.5\( \mu \)L, \( p = 0.01 \)) (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**

CIRCHF/201/963769-R3
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. \( \frac{dP}{dT} \) 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-1R\( \alpha \)C.P (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 \( p = 2.63\times10^{-5} \), CO \( p = 0.02 \), SW \( p = 0.02 \), PMX \( p = 2.63\times10^{-5} \); IL-6/- ESP \( p = 2.74\times10^{-5} \), CO \( p = 0.003 \), SW \( p = 0.0003 \), PMX \( p = 2.74\times10^{-5} \)) 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-1RACp 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,\textsuperscript{21-23} 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**


5. Fairweather D, Frisancho-Kiss S, Yusung SA, Barrett MA, Gatewood SJL, Davis SE, Njoku DB, Rose NR. IFN-γ protects against chronic viral myocarditis by reducing mast cell degranulation, fibrosis, and the profibrotic cytokines TGF-β1, IL-1β, and IL-4 in the heart. Am J Pathol. 2004; 165:1883-1894.


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>
</tr>
<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±0.46</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>
<tr>
<td>LVmass, mg</td>
<td>72.32±2.44</td>
<td>81.51±1.92</td>
<td>0.008</td>
</tr>
</tbody>
</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>
</tr>
</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 Undiseased</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Heart rate</td>
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<td>&lt;0.0001</td>
<td>0.08</td>
<td>0.0001</td>
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<tr>
<td>dP/dT Max</td>
<td>0.0004</td>
<td>0.0001</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>CO</td>
<td>0.005</td>
<td>0.001</td>
<td>0.17</td>
<td>0.05</td>
</tr>
<tr>
<td>PMX</td>
<td>0.006</td>
<td>0.001</td>
<td>0.17</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Day 35 pi CVB3</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate</td>
<td>0.64</td>
<td>0.43</td>
<td>0.92</td>
<td>0.40</td>
</tr>
<tr>
<td>dP/dT Max</td>
<td>0.18</td>
<td>0.06</td>
<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>
</tbody>
</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 undiseased 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.