Interleukin-33 Prevents Apoptosis and Improves Survival After Experimental Myocardial Infarction through ST2 Signaling

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ABSTRACT

**Background**- ST2 is an interleukin-1 receptor family member with membrane-bound (ST2L) and soluble (sST2) isoforms, and, sST2 is a biomarker for poor outcome in patients with myocardial infarction (MI). IL-33, the recently-discovered ligand for ST2, activates NF-kB and thus may regulate apoptotic cell death. We tested the hypothesis that IL-33 is cardioprotective after MI through ST2 signaling.

**Methods and Results**- IL-33 protected cultured cardiomyocytes from hypoxia-induced apoptosis, and this cardioprotection was partially inhibited by sST2. IL-33 induced expression of the anti-apoptotic factors XIAP, cIAP1 and survivin. To define the cardioprotective role of IL-33 in vivo, we performed a blinded and randomized study of ischemia/reperfusion in rats. IL-33 reduced cardiomyocyte apoptosis, suppressed caspase-3 activity and increased expression of IAP family member proteins. IL-33 decreased both infarct and fibrosis volumes at 15 days; furthermore, both echocardiographic and hemodynamic studies revealed that IL-33 improved ventricular function. To determine if cardioprotection by IL-33 is mediated through ST2 signaling, a randomized and blinded study of ST2-/- vs. wild type (WT) littermate mice was performed in 98 mice subjected to MI. At 4 weeks following MI, IL-33 reduced ventricular dilation and improved contractile function in WT mice, but not in ST2-/- mice. Finally, IL-33 improved survival after MI in WT, but not in ST2-/- mice.

**Conclusion**- IL-33 prevents cardiomyocyte apoptosis and improves cardiac function and survival after MI through ST2 signaling.

**Key Words:** Myocardial Infarction, Cytokine, Apoptosis
Introduction

The interleukin-1 receptor family includes the IL-1, IL-18, and ST2 receptors. The ST2 receptor was identified two decades ago, but remained an orphan receptor until the recent discovery of interleukin-33 (IL-33) as its endogenous ligand. IL-1 family members participate in diverse processes, including inflammation, fibrosis, and autoimmune disorders, and IL-33 is a therapeutic target as a pro-inflammatory mediator of diverse diseases. However, IL-33 reduces atherosclerosis in animal models, and IL-33 can also protect against cardiac dysfunction in mechanically overloaded hearts. Thus, IL-33 signaling could possibly have both pro-inflammatory properties as well as cardioprotective properties.

In humans, numerous large clinical trials have demonstrated that the soluble form of ST2 (sST2) is an important biomarker for poor outcomes in heart failure and myocardial infarction (MI), but the mechanisms by which IL-33 signaling may affect the heart are not defined. IL-33 binds to the transmembrane form of ST2 (ST2L), activates NF-kB signaling and may regulate cell survival. Apoptotic cell death occurs in cardiomyocytes following myocardial infarction, and prevention of myocardial apoptosis may improve cardiac contractile function. Therefore, we hypothesized that IL-33 regulates cardiac dysfunction after MI. In this study, we demonstrate that IL-33 inhibits cardiomyocyte apoptosis both in vitro and in vivo. IL-33 improved cardiac contractile function after ischemia-reperfusion myocardial injury in rats. The cardioprotective effects of IL-33 were abolished in ST2 null mice, demonstrating that IL-33 is cardioprotective through ST2 signaling. These data highlight the diverse effects of IL-33 and reveal a potential therapeutic role for IL-33 in acute cardiac injury.
Furthermore, these data suggest that anti-inflammatory strategies that chronically target IL-33/ST2 signaling should consider potential adverse cardiovascular consequences.

MATERIALS AND METHODS

Myocardial cell culture. Rat neonatal cardiomyocytes and fibroblasts (1–2 days old) were isolated from Sprague-Dawley rats (Charles River Laboratories) as previously described.

Production of recombinant rat IL-33 protein and polyclonal anti-rat IL-33 antibody. Recombinant rat IL-33 protein was expressed and purified, and rabbit polyclonal antibody was raised as described previously. Exclusion of endotoxin contamination (<0.03 EU/μg protein) was determined as previously described.

Cardiomyocyte apoptosis assays. Apoptosis assays were performed as described previously. After culture in low glucose DMEM for 48 hours and serum-free DMEM for an additional 24 hours, rat neonatal cardiomyocytes were subjected to 0.1 mM H$_2$O$_2$ for 24 hours with or without IL-33 and/or sST2. In hypoxic experiments, cardiomyocytes were cultured in low glucose DMEM for 24 hours, followed by serum-free DMEM for 24 hours, and then serum-free M199 with or without IL-33 and/or sST2. Cardiomyocytes were then exposed to 1% oxygen for 72 hours and collected for flow cytometric analysis, fixed with 4% paraformaldehyde for in situ TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) staining, or lysed with RIPA buffer for Western analysis. For flow cytometry experiments, both adherent and floating cells
were collected, washed with PBS, fixed with 2% PFA for 1 hour, and permeabilized (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice prior to TUNEL staining (Roche Applied Systems, 11684795910). Fluorescein nucleotide labeling solution lacking terminal deoxynucleotidyl transferase was used as a negative staining control in subsequent flow cytometric analyses.

Analysis of cardiomyocyte specific DNA fragmentation in cell culture was performed by TUNEL staining and immunofluorescent staining with alpha-sarcomeric actinin antibodies (EA-53, 1:100, Sigma, 1:100). TUNEL-positive and total alpha-sarcomeric actinin-positive cardiomyocytes were counted using Image J (Version 1.38). A minimum of 3,000 total nuclei from five separate wells were analyzed. Only nuclei clearly located within cardiomyocytes were scored.

**Western analysis.** Western analyses were performed as described previously. Membranes (PVDF, Perkin Elmer) were incubated with primary antibodies (anti-cIAP1, anti-cIAP2, anti-survivin, Bcl-2, anti-Bcl-xL, anti-cleaved caspase 3 diluted 1:1000, from Cell Signaling Technology, anti-XIAP diluted 1:2000, from BD Bioscience, anti-α-actin diluted 1:1000 from Sigma), detected with horseradish peroxidase-conjugated antibodies (1:1000, from Bio-Rad) and enhanced chemiluminescence (Perkin Elmer).

**ST2-/- mice.** ST2 -/- mice were maintained on a 129 X C57BL/6 background as described previously. All animal procedures were conducted in accordance with guidelines described in the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, DC, 1996) and approved by
the Harvard Medical School Standing Committee on Animals.

Randomization, blinding, and IL-33 treatment. Sprague-Dawley rats (Charles River Laboratories) or mice, 8-10 week of age, were randomized to receive subcutaneous injections of IL-33 (0.1mg/kg) dissolved in phosphate buffered saline (PBS) with 0.2% bovine serum albumin (BSA) (IL-33 treatment group) or PBS with 0.2% BSA without IL-33 (Control group) daily for the first three days after operation and once every three days. Treatment groups were coded so that investigators performing all analyses were blinded, and coding was unblinded upon completion of sample processing and analyses.

Coronary artery ligation. All operative procedures were performed by a single operator with over 20 years of rodent cardiac surgery experience, as previously described 17.

Echocardiography and Hemodynamics. Echocardiography and hemodynamics were performed as previously described 18.

Infarct volume. Determination of left ventricular (LV) infarct volume was performed as previously described 19. Rats were anesthetized with pentobarbital, and the heart was excised and mounted on an isolated heart perfusion apparatus. The isolated heart was perfused at 37°C and 60 mmHg retrograde through the aorta with Krebs buffer followed by 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution for 10 min to stain viable myocardium. The coronary artery was re-occluded with the suture that was left in place.
at the time of reperfusion. The heart was then perfused with filtered 1.0% phthalocyanine blue in phosphate-buffered saline to define the LV volume at risk. The LV was sliced in cross section into 6 sections and fixed in 4% buffered paraformaldehyde. Each section was weighed and photographed. Non-jeopardized LV tissue was identified by deep blue staining, ischemic but viable myocardium was identified by deep red staining, and necrotic LV tissue was identified by white coloration. The infarcted and non-infarcted areas were measured by Image-Pro Plus (Version 4.5). The areas of all slices were summed to calculate their respective volumes. Infarct volume was expressed as percentage of LV volume at risk. Averaged area from both sides of each slice were calculated for each color. Volume at risk was calculated by adding 6 area at risk (AAR) volumes, where AAR volume is defined as \( \frac{\text{LV Red + White areas}}{\text{total LV area}} \times \frac{\text{Weight of each LV section}}{\text{total weight of 6 LV sections}} \times 100\% \). Infarct volume was calculated by adding 6 infarct section volumes, where infarct volume is defined as \( \frac{\text{White area}}{\text{White + Red areas}} \times \frac{\text{Weight of each LV section}}{\text{total weight of 6 LV sections}} \times 100\% \). All measurements were performed by an investigator blinded to treatment group.

**Histological analysis.** Fibrosis volume was quantified with Masson’s trichrome staining as an area stained positive for collagen. Fibrotic and non-fibrotic areas were calculated by automated computer imaging analysis (MATLAB R2007a, version 7.4) as previously described. Fibrosis volume was calculated by integrating 6 fibrosis section volumes, where fibrosis volume is defined as \( \frac{\text{blue area}}{\text{total area}} \times \frac{\text{Weight of each section}}{\text{total weight of 6 sections}} \times 100\% \). Triple staining with TUNEL, anti-\( \alpha \)-sarcomeric actin
antibody (1:100, Sigma, A2172), and DAPI was performed with ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, S7165). The number of both TUNEL-positive myocytes and cardiomyocyte nuclei were counted by Image J (version 1.38). Triple staining with anti-mouse IL1R/ST2L (R&D Systems, Minneapolis), anti-α-sarcomeric actin, and DAPI was performed to detect cardiomyocyte specific ST2L expression. CD4+ lymphocytes were stained using anti-mouse CD4 (Abcam, Cambridge, MA, #ab8167), 1:50 dilution. CD8+ lymphocytes were stained using anti-mouse CD8 (BD Pharmingen, San Diego, CA, #550298), 1:50 dilution. Macrophages/monocytes were stained using anti-mouse CD68 (AbD Serotec, Raleigh, NC, #MCA341GA), 1:50 dilution. Mast cells were stained using anti-AA4 (BD Pharmingen, San Diego, CA, #551770), 1:50 dilution.

Statistics. Data are expressed as mean ± SEM. Statistical significance was performed with paired t test, unpaired Student's t-test, and two-way ANOVA with Fisher post hoc test and Bonferroni inequality test when appropriate. For survival analyses, the K-M curves were further analyzed by Cox hazard analysis. Differences were considered statistically significant at p<0.05.

RESULTS

IL-33 prevents cardiomyocyte apoptosis. Both high concentrations of H$_2$O$_2$ and hypoxia are known to induce cardiomyocyte apoptosis in vitro$^{21,22}$. Both methods were employed to evaluate the effect of IL-33 on apoptosis, which was quantified with two independent assays: flow cytometric TUNEL analysis and in situ TUNEL staining.
followed by immunofluorescent analysis. Rat neonatal cardiomyocyte apoptosis was induced by 72-hour exposure to 1% oxygen. Hypoxia increased cardiomyocyte apoptosis as shown by TUNEL staining and flow cytometry (Figure 1A). IL-33 reduced apoptosis in a dose dependent manner. Addition of sST2 decreased the effect of IL-33. To exclude the possibility that IL-33 induced a concomitant increase in necrosis, cell death, representing a combination of necrosis and late-phase apoptosis, was assessed by flow cytometric analysis of 7-AAD uptake by unfixed cells after hypoxia. IL-33 reduced the total fraction of dead cells in a dose dependent manner, and this was neutralized by sST2 (data not shown). Thus, the IL-33 mediated reduction in apoptosis was not accompanied by an increase in necrosis.

Apoptotic cardiomyocytes cultured in hypoxia were also quantitated after in situ TUNEL staining and concomittant immunofluorescent identification by alpha-sarcomeric actinin staining. Using this methodology, the pro-apoptotic effect of hypoxia was ameliorated by IL-33 treatment in a dose dependent manner (Control: 26.2±1.9, IL-33 10ng/ml: 14.4±2.0, p<0.05, IL-33 100ng/ml: 7.0±2.1, p<0.01, Figure 1B). Co-application of sST2 attenuated the effect of IL-33 (sST2+IL-33 10ng/ml; 32.5±6.7, vs IL-33 10ng/ml p<0.05, sST2+IL-33 100ng/ml; 24.5±2.7, vs IL-33 100 ng/ml p<0.01). Similar results were obtained when apoptosis was induced with H_{2}O_{2}, as IL-33 administration decreased apoptosis in a dose dependent fashion (H2O2 control: 47.76±5%, 10 ng/ml of IL-33: 26±4%, p=0.08 and 100 ng/ml of IL-33: 18±3%, p<0.05, Figure 1C). Co-application of sST2 decreased the anti-apoptotic effect of IL-33 (44±14% for 10 ng/ml of IL-33 + sST2, p<0.01 vs 25.7±4.4% for IL-33 10 ng/ml; 37±2% for 100ng/ml of IL-33 + sST2, p =0.095 vs 17.9±3.4 for IL-33 100 ng/ml, Figure 1C). Regardless of the apoptotic stimulus, sST2
alone did not induce apoptosis nor affect H₂O₂ or hypoxia induced apoptosis. These results indicate that IL-33 has a dose-dependent anti-apoptotic cardioprotective effect that can be at least partially blocked by sST2, consistent with a decoy receptor effect of sST2.

**IL-33 regulates anti-apoptotic protein expression.** Caspases comprise a family of cysteine proteases with critical roles in mammalian apoptosis and in the proteolytic activation of cytokines. To explore the role of caspase-3 activation, an important step in apoptosis, in the cardioprotective effect of IL-33, cleaved caspase-3 was measured in four independent experiments. Hypoxia increased cleaved caspase-3, and IL-33 decreased cleaved caspase-3 in a dose-dependent manner (Figure 2B). Furthermore, the effect of IL-33 on activation of caspase-3 was attenuated by sST2. We also investigated the effect of IL-33 on anti-apoptotic factors. IL-33 can regulate the activation of NF-kB, which can control apoptosis through regulation of anti-apoptotic genes; XIAP, cIAP1, cIAP2, survivin, Bcl-2 and Bcl-xL are NF-kB regulated proteins that can promote cell survival. Western analysis revealed that IL-33 increased the expression of the anti-apoptotic proteins cIAP1, XIAP, survivin, Bcl-2, and Bcl-xL in rat neonatal cardiomyocytes exposed to hypoxia (Figure 2C). Addition of sST2 decreased the IL-33 effect (Figure 2C). These results imply that IL-33 can promote cardiomyocyte survival, at least in part, through the regulation of IAP family proteins and reduction of cleaved caspase-3.

**IL-33 reduces infarct size, fibrosis, and apoptosis after ischemia reperfusion in**
rats. Apoptosis plays a prominent role in ischemia/reperfusion injury. Therefore, we hypothesized that IL-33 can prevent apoptosis after ischemia/reperfusion. A total of 31 male rats underwent surgery with randomization to IL-33 or control, with all procedures blinded to treatment. The IL-33 treatment protocol was subcutaneous injection of 0.1 mg/kg IL-33 once per day for three days after operation and then once every three days thereafter. Eight rats (25%) died within 24 h of surgery, and there were no differences in perioperative mortality between groups, yielding two groups of surviving rats after surgery: IL-33 (n=12) and control (n=11). At 15 days after reperfusion, infarct volume was significantly reduced by IL-33 (10±2% for IL-33 vs. 24±5% for control, p=0.003; Figure 3A, B, and C). The volume at risk was similar in control (n=11) and IL-33 (n=12) treated rats (43±4% and 45±5%, respectively, p=N.S., Figure 3D), indicating that ligature placement was similar between groups. Fibrosis volume in the IL-33 treated group was significantly reduced compared with the control group (27±2% for IL-33 vs. 35±2% for control, p=0.013, Figures 3E and F).

To examine whether IL-33 reduces cardiomyocyte apoptosis in vivo, triple staining with TUNEL, anti-α sarcomeric actin antibody, and DAPI was performed on myocardial sections harvested 1 day after reperfusion, and images were analyzed in a blinded manner. IL-33 significantly reduced cardiomyocyte apoptosis in the infarct area (5.9±3.2% for IL-33 vs. 27.8±3.9% for control, p=0.001, Figure 4A). The observed percentage of TUNEL positive cardiomyocytes in the post-infarct area (5.9±3.2%) was within the previously reported range of apoptotic rates in post-infarct areas after 30-60 min ischemia and 24-hour reperfusion (0.7-29.8%) . Furthermore, the level of cleaved caspase-3 was decreased by IL-33 at day 1 (Figure 4B). Consistent with in vitro
data described above, western analysis revealed increased XIAP, ciAP1 and Survivin in the infarct area, whereas Bcl-2 was unaffected by IL-33 (Figure 4B). While ST2 was detectable on cultured cardiomyocytes by immunostaining and ST2L gene expression was detectable by real-time PCR in infarcted tissue, expression was not changed by IL-33 treatment (Supplement Figure 1). These results suggest that IL-33 may suppress apoptosis through induction of anti-apoptotic proteins after ischemia/reperfusion injury.

**IL-33 improves cardiac function after ischemia/reperfusion injury in rats.** We evaluated cardiac function by performing treatment-blinded echocardiography and invasive hemodynamic studies. Echocardiographic fractional shortening at 1 day and 7 days was not different between groups, but IL-33 improved fractional shortening at 15 days after reperfusion (50±2% vs. 44±1%, respectively, p=0.028). IL-33 also improved most invasively-measured hemodynamic parameters, including cardiac index, systolic and diastolic dp/dt max, maximum elastance, Stroke Volume, Ejection Fraction, Stroke Work, Arterial Elastance, time constant of exponential decay, Maximal Power, and Preload adjusted maximal power (Table 1). Pressure volume loops in IL-33 treated group shifted to lower volumes compared to control (Supplement Figure 2). These data suggest that IL-33 not only reduces apoptosis and fibrosis after ischemia/reperfusion injury, but contractile function is also improved.

**IL-33 and ST2 signaling after myocardial infarction in mice.** To further characterize the mechanism of the cardioprotective effect of IL-33, a blinded and randomized experiment using 128 mice with targeted deletion of ST2 or littermate wild type controls
was performed. This study was performed with coronary ligation to determine if the cardioprotective effect of IL-33 was also apparent without reperfusion.

Echocardiography revealed that fractional shortening of WT mice after MI was significantly improved by IL-33 treatment at 4 weeks (49.16 ± 2.4% for IL-33, n = 4, vs. 38.3 ± 2.9% for control, n = 7, p < 0.038, Figure 5A), but no significant effect of IL-33 was observed in ST2-/- mice (32.1 ± 3.9% for IL-33, n= 4 and 36.6 ± 3.4 % for control, n= 7, p< 0.35, Figure 5A). Furthermore, heart weight to tibia length ratio, a post-mortem measure of cardiac size, was reduced by IL-33 in WT (7.0r 0.5, n=13 for IL-33 vs. 8.0r 0.8, n=13 for control), but not ST2-/- mice (8.0r 2.2, n=16 for control).

We further tested the effect of IL-33 on survival after MI in mice. A total of 149 mice were randomly assigned to treatment groups, including a pre-specified number of 32 sham-operated animals. Perioperative mortality in WT mice was 9/55 (16%) compared with 10/62 (16%) in ST2-/- mice indicating no difference in operative mortality based on genotype; thus, 98 mice in four groups (IL-33 vs. control, WT vs. ST2-/-) were followed for survival. There were 15 (60%) male and 10 (40%) female mice from WT, and 17 male (55%) and 14 (45%) female KO mice.

All deaths were spontaneous, and IL-33 treatment did not appear to cause delayed cardiac rupture, as none of the wild type mice treated with IL-33 died of cardiac rupture. Survival in IL-33 treated WT-MI mice (n=18) was significantly improved compared with control treated WT-MI mice (n=28) (p<0.05, Figure 5B). By Cox analysis, the relative risk of WT + IL-33 vs. WT alone was 0.39, 95%CI =0.15 to 0.99, p<0.05. However, no survival benefit from IL-33 treatment was observed compared to placebo.
treatment in ST2/- MI mice. These data demonstrate that IL-33 improves survival following MI through ST2 signaling.

**IL-33 and Inflammatory responses in the ischemic-injured heart.**

Since IL-33 is known to induce inflammatory responses \(^2\), we assessed lymphocyte and, macrophage/monocyte infiltration of ischemic-injured hearts by immunohistochemistry.

An analysis of CD4+ lymphocytes, CD8+ lymphocytes, and CD68+ leukocytes in the infarct and non-infarct areas revealed no significant differences between IL-33 treated and control groups (Supplement Figure 3). We also stained for mast cells, demonstrating a decrease in mast cell density in the infarct area after IL-33 treatment (Supplement Figure 4). Because mast cells may participate in cardiac injury \(^3\), this could represent an additional mechanism of cardioprotection by IL-33. To address Th1 and Th2 cytokine gene expression, we performed real-time PCR on tissues from animals treated with IL-33. These experiments (n=3 rats per time point) showed an IL-33 induced shift towards a Th2 response with modest, but statistically significant, increases in the expression of the Th2 genes IL-10, IL-4, and GATA3 and corresponding reductions in the expression of the Th1 genes interferon-gamma and T-Bet (Supplement Figure 5).

**DISCUSSION**

In this study, we demonstrate that the cytokine IL-33 can prevent cardiomyocyte apoptosis and improve cardiac function and survival after myocardial infarction in both mice and rats. Because this benefit was absent in mice with deletion of the ST2 gene, these data indicate that IL-33 is cardioprotective through the ST2 receptor. Our data
also support the concept that sST2 functions as a decoy receptor. These experimental data are relevant to extensive clinical data demonstrating that sST2 is a powerful biomarker for poor outcome in patients with cardiovascular disease \(^7\text{-}^9,^32\). It is possible that sST2 functions as a decoy receptor in vivo, decreasing endogenous IL-33 signaling and thus worsening cardiac function. In this scenario, stimulating IL-33/ST2 signaling in patients with high levels of sST2 could be beneficial, provided that the pro-inflammatory effects of IL-33 can be avoided with short-term treatment.

In this study of hypoxia and ischemic/reperfusion, the anti-apoptotic effect of IL-33 was clear both in vitro and in vivo, while our previous study of pressure overload did not show an anti-apoptotic effect. This may represent fundamentally different physiology between the two stimuli, as apoptosis is not a prominent feature of pressure overload compared to ischemia. While apoptosis is an important feature of ischemia/reperfusion, necrosis and autophagy are important mechanisms of cellular death \(^34,^35\). We have not detected an increase in necrosis with IL-33 treatment in vitro, but it is possible that IL-33 affects other pathways and future research is warranted. In this study, we focused on apoptosis because IL-33 activates NF-kappaB pathways that can regulate apoptosis. It is quite plausible that IL-33 decreases other mechanisms of cell death as well. It is also plausible that IL-33 affects regenerative processes. IL-33 activates NF-kB \(^2,^6\), which can play multiple roles in cell survival and apoptosis. NF-kB signaling in the heart regulates hypertrophy \(^36\) as well as hypoxia-induced mitochondrial dysfunction and cell death \(^37\). In this study, we report that some of the anti-apoptotic IAP family proteins are increased by IL-33 in vitro and in vivo. These IAPs may also be regulated by NF-kB and promote cell survival \(^24,^25\). However, our experiments do not exclude other
cardioprotective mechanisms, such as through MAP kinases, which can be activated by IL-33\(^2\) and are known to protect against cardiomyocyte apoptosis\(^38\).

IL-33 significantly reduced cardiac fibrosis volume after ischemia/reperfusion injury. Cardiac fibroblasts are activated in response to injury, and cardiac fibrosis can cause cardiac dysfunction and lethal rhythm disturbances\(^39\). Perivascular fibrosis surrounding intracoronary arterioles can also impair myocyte oxygen availability, reduce coronary reserve, and exacerbate myocyte ischemia\(^40\). Efforts to develop novel therapies that specifically target the cardiac fibroblast are at a relatively early stage, but anti-fibrotic strategies have potential for prevention of heart failure\(^41\). In a previous study of pressure overloaded myocardium, cardiac fibroblasts appeared to be a prominent source of endogenous IL-33\(^6\). Understanding the interplay of cardiac fibroblasts and cardiac myocytes at the molecular level may provide new insight into prevention of fibrosis and protection of cardiac myocytes.

IL-33 can be pro-inflammatory and is a target in numerous diseases of inflammation\(^4\). These data, together with recent data suggesting that IL-33 can inhibit atherosclerosis\(^5\), suggest that anti-inflammatory strategies that inhibit IL-33 chronically may cause adverse cardiovascular effects. Similarly, activation of IL-33/ST2 signaling in cardiovascular diseases may have unwanted pro-inflammatory adverse effects. In this study, we saw no excess pulmonary inflammation in the IL-33 treated animals (data not shown), possibly due to the dosing regimen when compared with prior studies\(^2,6\). Further studies may determine if limiting IL-33 treatment to the earliest period post-injury could allow cardioprotection without pro-inflammatory systemic effects.
Acknowledgments

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Disclosures

Brigham and Women’s Hospital has filed provisional patents based on IL-33, listing Dr. Richard Lee as an inventor.

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Table 1. Hemodynamic parameters at 15 days after ischemia/reperfusion.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>control (n=11)</th>
<th>IL-33 (n=12)</th>
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<tbody>
<tr>
<td>Heart rate (per minute)</td>
<td>392.6 ± 13.9</td>
<td>423.8 ± 13.6</td>
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<tr>
<td>End-systolic Volume (uL)</td>
<td>376.1 ± 35.7</td>
<td>343.3 ± 25.1</td>
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<tr>
<td>End-diastolic Volume (uL)</td>
<td>453.8 ± 41.3</td>
<td>493.2 ± 31.9</td>
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<tr>
<td>End-systolic Pressure (mmHg)</td>
<td>114.1 ± 6.0</td>
<td>120.3 ± 6.60</td>
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<tr>
<td>End-diastolic Pressure (mmHg)</td>
<td>6.4 ± 0.9</td>
<td>7.4 ± 1.8</td>
</tr>
<tr>
<td>Stroke Volume (uL)</td>
<td>114.5 ± 9.6</td>
<td>171.7 ± 16.9 **</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>24.5 ± 1.0</td>
<td>34.1 ± 2.6 **</td>
</tr>
<tr>
<td>Cardiac Index (ml/min*100g)</td>
<td>13.6 ± 1.3</td>
<td>22.3 ± 2.30 **</td>
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<tr>
<td>Stroke Work (mmHg*uL)</td>
<td>8487 ± 924</td>
<td>14003 ± 1714 **</td>
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<tr>
<td>Arterial Elastance (mmHg/uL)</td>
<td>1.1 ± 0.1</td>
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<tr>
<td>+dPdt max (mmHg/sec)</td>
<td>7681 ± 595</td>
<td>9727.1 ± 741.3 *</td>
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<td>-dPdt, min (mmHg/sec)</td>
<td>6699 ± 619</td>
<td>8601.3 ± 645.1 *</td>
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<td>Tau (msec)</td>
<td>15.2 ± 0.6</td>
<td>13.3 ± 0.5 *</td>
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<td>Maximal Power (mWatts)</td>
<td>73.2 ± 9.8</td>
<td>128.9 ± 17.1 **</td>
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<td>54.2 ± 23.0</td>
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<tr>
<td>Emax (mmHg/ul)</td>
<td>0.9 ± 0.1</td>
<td>1.5 ± 0.2 **</td>
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PSRW = Preload Recruitable Stroke Work. Values are mean ± SEM. * p < 0.05, ** p < 0.01 vs. control.
FIGURE LEGENDS

Figure 1. IL-33 promotes cardiomyocyte survival. (A) Neonatal rat cardiomyocyte apoptosis using TUNEL assayed by flow cytometry in normal vs. 1% oxygen for 72 h. Hypoxia induced apoptosis (red curve) and IL-33 reduced apoptosis dose-dependently (green and blue curves). § sST2 inhibited the effect of IL-33 (lower panels). § Fluorescein nucleotide labeling solution without the terminal deoxynucleotidyl transferase enzyme (B) TUNEL positive rat neonatal cardiomyocytes were co-stained with α-sarcomeric actin and DAPI to identify cardiomyocytes. IL-33 reduced apoptosis from hypoxia in a dose-dependent manner and sST2 inhibited IL-33. Data are from 3 independent experiments. (C) TUNEL positive cells were co-stained with α-sarcomeric actinin and DAPI to identify cardiomyocytes. Data are from 5 independent experiments. IL-33 protected against hydrogen peroxide-induced apoptosis in a dose-dependent manner. *p<0.05; **p<0.01

Figure 2. IL-33 regulates cleaved caspase-3 and anti-apoptotic proteins. (A) Time course of cleaved caspase-3 expression in rat neonatal cardiomyocytes after exposure to 1% oxygen. After 72 hours, cleaved caspase3 was detected by Western analysis. (B) IL-33 dose-dependently decreased activated Caspase-3 induced by hypoxia. Cleaved Caspase-3 detected by Western analysis. Data are representative of 3 independent experiments. (C) Western analysis revealed that IL-33 increased the expression of proteins in the Inhibitor of Apoptotic Proteins family (cIAP1, cIAP2, XIAP and survivin) as well as Bcl-2 and Bcl-xL in hypoxic conditions.
Figure 3. IL-33 decreases infarct and fibrosis volumes after ischemia/reperfusion injury. (A and B) Heart samples from Control (A) and IL-33 (B) treated rats. Blue indicates the non risk area. Red indicates the area at risk. White indicates the infarct area. Infarct volume (C) and Volume At Risk (D) in IL-33 and control treated rats with transient (45 min) myocardial ischemia followed by reperfusion. IL-33 decreased infarct volume compared with control treated rats. The Volume at Risk of IL-33 treated rats was not significantly different between groups, as expected. (E) Ventricular myocardium subjected to Masson’s trichrome staining. Red indicates cardiomyocytes and blue indicates fibrosis. Scale bar = 20 um. (F) IL-33 significantly decreased fibrosis volume compared with control treated rats. *p < 0.05.

Figure 4. IL-33 reduces cardiomyocyte apoptosis and induces anti-apoptotic factors in vivo. (A) TUNEL positive cardiomyocytes were measured by triple staining with TUNEL (red), anti-alpha-sarcomeric actin antibody (green), and DAPI (blue). The number of TUNEL positive cardiomyocytes (CM) at the infarct area at 1 day after reperfusion in IL-33 treated rats was significantly decreased compared with that of control treated rats (control: 27.8±3.9%, n=9, IL-33: 5.9±3.2%, n=7, **p<0.01). TUNEL positive cardiomyocytes (light purple) are indicated by arrows in the representative images. Scale bar represents 10 um. (B) Expression level of cleaved caspase-3 (Cl-Cas 3), XIAP, cIAP1, cIAP2, Survivin and Bcl-2 by Western analysis at 1 day after ischemia/reperfusion in vivo. IL-33 decreased the level of cleaved caspase-3 and increased expression of XIAP, cIAP1, cIAP2 and Survivin compared to control (Con).

Figure 5. IL-33 improves survival and cardiac function after myocardial infarction
in wild type but not in ST2-/- mice. Experimental MI was performed on WT and ST2-/- littermates. (A) Echocardiographic analysis was performed at 1 day, 1 week, and 4 weeks after operation. IL-33 significantly improved fractional shortening in WT mice (49.2 ± 2.4%, n = 4) compared with control mice (38.3 ± 2.9%, n=7). However, fractional shortening in ST2-/- mice was not improved by IL-33 treatment (32.1 ± 3.9%, n = 4) compared with control treatment (36.6±3.4, n=7). (B) Echocardiographic measurement of end diastolic dimensions revealed a trend towards reduced ventricular dilation by IL-33 in WT mice but not in ST2-/- mice. (C) Kaplan-Meier survival analysis revealed that survival of WT mice (n=18) was significantly improved by IL-33 treatment compared with control WT mice (n=28). However, IL-33 treatment did not benefit ST2-/- mice (n=24) compared with ST2-/- without IL-33 treatment (n=28). *p< 0.05; **p< 0.01 vs. the same treatment in WT.
Figure 1

A

B

C

TUNEL positive cardiomyocytes

(n=3)

cells/100 cardiomyocyte nuclei

Normoxia

Hypoxia

TUNEL positive cardiomyocytes

(n=5)
cells/100 cardiomyocyte nuclei

Control

IL-33 10 ng/ml

IL-33 100 ng/ml

sST2 10 ug/ml

Control

IL-33 10 ng/ml

IL-33 100 ng/ml

sST2 10 ug/ml

Control

IL-33 10 ng/ml

IL-33 100 ng/ml

sST2 10 ug/ml

H2O2 100 μM

H2O2 + IL-33 (10 ng/ml)

H2O2 + IL-33 (100 ng/ml)

H2O2 + sST2

H2O2 + sST2 + IL-33 (10 ng/ml)

H2O2 + sST2 + IL-33 (100 ng/ml)

IL-33 (10 ng/ml)

IL-33 (100 ng/ml)

sST2 (10 μg/ml)

sST2 (100 μg/ml)

sST2 + IL-33 (10 ng/ml)

sST2 + IL-33 (100 ng/ml)

Merge (Control, IL-33 10 ng/ml + 100 μg/ml)

Merge (Control, IL-33 10 ng/ml + 1000 μg/ml)

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Figure 2

A

Hypoxia

Cleaved Caspase3

0 24 48 72 hr

19 kD

17 kD

B

Control

IL-33 100 ng/ml

ST2 10 ug/ml

Control

IL-33 (10 ng/ml)

IL-33 (100 ng/ml)

ST2 (10 ug/ml)

ST2 (10 ug/ml) + IL-33 (100 ng/ml)

Cleaved Caspase3

Actin

19 kD

17 kD

C

Normoxia

Hypoxia

Control

IL-33 100 ng/ml

ST2 10 ug/ml

Control

IL-33 (10 ng/ml)

IL-33 (100 ng/ml)

ST2 (10 ug/ml)

ST2 (10 ug/ml) + IL-33 (100 ng/ml)

cIAP1

cIAP2

XIAP

Survivin

Bcl-2

Bcl-xL

Actin

Normoxia

Hypoxia
Figure 3
Figure 4

A

Control

IL-33

B

Cleaved Caspase3

Border zone

Infarct area

IL-33

Con

IL-33

Con

IL-33

Con

IL-33

Con

XIAP

Survivin

clAP1

Bcl-2

Actin

Actin

n=7

n=9

CM Tunel positive cells/1000 CM nuclei

**

n=9

n=7

19 kD

17 kD
Figure 5
Interleukin-33 Prevents Apoptosis and Improves Survival After Experimental Myocardial Infarction through ST2 Signaling
Kenjiro Seki, Shoji Sanada, Anastacia Yurievna Kudinova, Matthew L. Steinhauser, Vandna Handa, Joseph Gannon and Richard T. Lee

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SUPPLEMENTAL MATERIAL

SUPPLEMENT METHODS

Real Time PCR.

Gene expression was analyzed by real time PCR (SYBR GREEN PCR Master Mix, Roche Applied Science) using specific oligonucleotides. Rat ST2L forward primer: 5’-AGTTGTGCATTTCGCCGAGA-3’ and reverse primer 5’-
GGATACTGCTTTCCACCACAG-3’, rat IL-4 forward primer: 5’-
ACAGGAGAAGGGACGCCAT-3’ and reverse primer: 5’-
GAAGCCCTACAGACGAGCTCA-3’, rat IL-10 forward primer: 5’-
GTTGCCAAGCCTTGTCAAGAAA-3’, 5’- TTTCTGGGCCCATGGTCTCTCT-3’, rat
GATA3 forward primer: 5’- GCCTGCGGATGTGTACTG-3’, reverse primer: 5’-
TGTGCCCATTTATAGTAGGTAGA-3’, rat IFN-gamma forward primer: 5’ –
CACGCCGCGTCTTGGT-3’, reverse primer: 5’-
TCTAGGCTTTTCAATGAGTGCGCC-3’, rat T-Bet forward primer: 5’-
TCCTGTCTCCAGCCGTTTTCT-3’, reverse primer 5’-
CGCTCAGTGCTCGGAAA C-3’ and rat GAPDH forward primer: 5’-
GGCAAGTTCAATGGCAGAGT-3’ and reverse primer: 5’-
TGTTAAGAGACGCCAGTAGACT-3’
SUPPLEMENT FIGURES

FIGURE LEGENDS

Supplement Figure 1. Hypoxic injury does not alter the expression level of ST2 on cardiomyocytes in vitro. (A) Rat neonatal cardiomyocytes were cultured in normoxia or 1% oxygen for 72 hours. Cells were stained for alpha sarcomeric actin (green) and ST2 (red). The number of ST2 positive cardiomyocytes in hypoxia was not significantly different from normoxia. Scale bar indicates 20 um. (B) Gene expression for ST2L measured by quantitative Real Time PCR at 7 days after ischemia reperfusion injury in vivo. Expression of ST2L was normalized by GAPDH. ST2L gene expression was not changed by ischemia/reperfusion or by IL-33 treatment after 7 days.

Supplement Figure 2. Sample Pressure-Volume loops from rats at 15 days after ischemia reperfusion injury. IL-33 shifted loops to the left. (A) Control, (B) IL-33 treated.

Supplement Figure 3. IL-33 does not affect the inflammatory infiltrate after ischemia reperfusion injury. (A) CD4+ lymphocytes, (B) CD8+ lymphocytes, and (C) CD68+ cells (macrophages and monocytes) were analyzed by immunohistochemistry. Although CD4+, CD8+, and CD68+ cells were
significantly increased within the infarct area 15 days after ischemia reperfusion injury compared to the non-infarct area, IL-33 did not affect the number of infiltrating CD4+, CD8+, and CD68+ cells. n=7-11 rats for each measurement. Scale bar indicates 10 um.

Supplement Figure 4. IL-33 reduces the density of mast cells in the infarct area at 15 days after ischemia reperfusion injury. The density of mast cells in both IL-33 treated (n=6) and control (n=7) rats was significantly increased in the infarct area compared to non-infarcted areas. However, the density of mast cells in the infarct area of the IL-33 group was significantly decreased compared to controls * p<0.05). Scale bar indicates 10 um.

Supplement Figure 5. Analysis of Th1 and Th2 related gene expression after ischemia-reperfusion injury. We analyzed the mRNA levels of IL-4, IL-10, and GATA3 (Th2 related), as well as IFN-gamma and T-Bet (Th1 related) using quantitative Real Time PCR 7 days after ischemia reperfusion injury in vivo (n=3 rats for each measurement). mRNA levels of IL-4 and IL-10 in the infarct area were slightly reduced by ischemia reperfusion injury (p<0.05), while the mRNA level of GATA3 was unaffected by ischemia reperfusion injury. Il-33 therapy modestly increased IL-4, IL-10, and GATA3 mRNA expression (P<0.05). mRNA levels of both IFN-gamma and T-Bet were increased in the infarct area 7 days post-ischemia reperfusion injury * p<0.05, but the expression of both genes was reduced by IL-33 ** p<0.01.
Supplement Figure 1

**A**

**Normoxia ST2**

**Hypoxia ST2**

ST2 positive cells /100 cardiomyocyte nuclei (n=3)

P = 0.29 (N.S.)

**B**

**ST2L/GAPDH**

- Control Sham
- IL-33 Sham
- Control I/R
- IL-33 I/R

Ratio

**Supplement Figure 1**
Supplement Figure 2
Supplement Figure 3

A

CD 4

Control (infarct area)  IL-33 (infarct area)

B

CD 8

Control (infarct area)  IL-33 (infarct area)

C

CD 68

Control (infarct area)  IL-33 (infarct area)
Supplement Figure 4