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

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Background—ST2 is an interleukin (IL)-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 nuclear factor κB 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 antiapoptotic 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 whether cardioprotection by IL-33 is mediated through ST2 signaling, a randomized and blinded study of ST2+/− versus wild-type littermate mice was performed in 98 mice subjected to MI. At 4 weeks after MI, IL-33 reduced ventricular dilation and improved contractile function in wild-type mice but not in ST2−/− mice. Finally, IL-33 improved survival after MI in wild-type but not in ST2−/− mice.

Conclusion—IL-33 prevents cardiomyocyte apoptosis and improves cardiac function and survival after MI through ST2 signaling. (Circ Heart Fail. 2009;2:684-691.)

Key Words: myocardial infarction ■ cytokine ■ apoptosis

The interleukin (IL)-1 receptor family includes the IL-1, IL-18, and ST2 receptors.1 The ST2 receptor was identified two decades ago but remained an orphan receptor until the recent discovery of IL-33 as its endogenous ligand.2 IL-1 family members participate in diverse processes, including inflammation, fibrosis, and autoimmune disorders,3 and IL-33 is a therapeutic target as a proinflammatory mediator of diverse diseases.4 However, IL-33 reduces atherosclerosis in animal models,5 and it can also protect against cardiac dysfunction in mechanically overloaded hearts.6 Thus, IL-33 signaling could possibly have both proinflammatory and cardioprotective properties.

Clinical Perspective on p 691

In humans, numerous large clinical trials have demonstrated that the soluble form of ST2 (sST2) is an important biomarker for poor outcomes in heart failure7 and myocardial infarction (MI).8,9 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 nuclear factor (NF)-κB signaling,2 and may regulate cell survival.10 Apoptotic cell death occurs in cardiomyocytes after MI,11 and prevention of myocardial apoptosis may improve cardiac contractile function.11–13 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 to 2 days old) were isolated from Sprague-Dawley rats (Charles River Laboratories) as previously described.2

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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 culturing in low-glucose DMEM for 48 hours and serum-free DMEM for an additional 24 hours, rat neonatal cardiomyocytes were subjected to 0.1 mmol/L H₂O₂ 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 staining, or lysed with radiomuno-precipitation assay buffer for Western blot analysis. For flow cytometry experiments, both adherent and floating cells were collected, washed with PBS, fixed with 2% paraformaldehyde for 1 hour, and permeabilized (0.1% Triton X-100 in 0.1% sodium citrate) for 2 minutes on ice before TUNEL staining (Roche Applied Science, 116847059101). 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 α-sarcogenic actin antibodies (EA-53, 1:100; Sigma). TUNEL-positive and total α-sarcogenic actin-positive cardiomyocytes were counted using ImageJ (version 1.38). A minimum of 3000 total nuclei from 5 separate wells were analyzed. Only nuclei clearly located within cardiomyocytes were scored.

Western Blot Analysis

Western blot analyses were performed as described previously. Membranes (polyvinylidene fluoride, PerkinElmer Life Sciences) were incubated with primary antibodies (anti-cIAP1, anti-cIAP2, anti-survivin, Bcl-2, anti-Bcl-xL, anticleaved caspase-3 diluted 1:1000, from Cell Signaling Technology; anti-XIAP diluted 1:2000, from BD Bioscience; and anti-α-actin diluted 1:1000 from Sigma) and detected with horseradish peroxidase-conjugated antibodies (1:1000, from Bio-Rad) and enhanced chemiluminescence (PerkinElmer Life Sciences).

ST2⁻/⁻ Mice

ST2⁻/⁻ mice were maintained on a 129 × 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 were 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 to 10 weeks of age, were randomized to receive subcutaneous injections of IL-33 (0.1 mg/kg) dissolved in PBS with 0.2% BSA (IL-33 treatment group) or PBS with 0.2% BSA without IL-33 (control group) daily for the first 3 days after operation and once every 3 days. Treatment groups were coded so that investigators performing all analyses were blinded, and coding was unblinded on completion of sample processing and analyses.

Coronary Artery Ligation

All operative procedures were performed by a single operator with >20 years of rodent cardiac surgery experience, as described previously.

Echocardiography and Hemodynamics

Echocardiography and hemodynamics were performed as described previously.

Infarct Volume

Determination of left ventricular (LV) infarct volume was performed as described previously. 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 mm Hg retrograde through the aorta with Krebs buffer, followed by 1% 2,3,5-triphenyl tetrazolium chloride solution for 10 minutes to stain viable myocardium. The coronary artery was reocluded with the suture that was left in place at the time of reperfusion. The heart was then perfused with filtered 1.0% phthaloerythrin blue in PBS to define the LV volume at risk. The LV was sliced in cross-section into 6 sections and fixed in 4% buffered parafomaldehyde. Each section was weighed and photographed. Nonjeopardized 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 noninfarcted 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 was calculated for each color. Volume at risk was calculated by adding 6 areas at risk volumes, where area at risk volume is defined as (LV red + white areas/total LV area)×(weight of each LV section/total weight of 6 LV sections)×100%. Infarct volume was calculated by adding 6 infarct section volumes, where infarct volume is defined as (white area/total white + red areas)×(weight of each LV section/total weight of 6 LV sections)×100%. All measurements were performed by an investigator who was blinded to treatment group.

Histological Analysis

Fibrosis volume was quantified with Masson’s trichrome staining as an area stained positive for collagen. Fibrotic and nonfibrotic areas were calculated by automated computer imaging analysis (MATLAB R2007a, version 7.4) as described previously. Fibrosis volume was calculated by integrating 6 fibrosis section volumes, where fibrosis volume is defined as (blue area/total area)×(weight of each section/total weight of 6 sections)×100%. Triple staining with TUNEL, anti-α-sarcogenic actin antibody (1:100, Sigma, A2172), and 4′,6-diamidino-2-phenylindole was performed with ApopTag Fluoroescin In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, S7165). The number of both TUNEL-positive myocytes and cardiomyocyte nuclei was counted by ImageJ (version 1.38). Triple staining with anti-mouse IL-1R/ST2L (R&D Systems, Minneapolis, Minn), anti-α-sarcogenic actin, and 4′,6-diamidino-2-phenylindole was performed to detect cardiomyocyte-specific ST2L expression. CD4⁺ lymphocytes were stained using anti-mouse CD4 (ab8167, 1:50 dilution; Abcam, Cambridge, Mass). CD8⁺ lymphocytes were stained using anti-mouse CD8 (550298, 1:50 dilution; BD Pharmingen, San Diego, CA). Macrophages/monocytes were stained using anti-mouse CD68 (MCA431GA, 1:50 dilution; AbD Serotec, Raleigh, NC). Mast cells were stained using anti-AA4 (551770, 1:50 dilution; BD Pharmingen, San Diego, CA).

Statistics

Data are expressed as mean±SEM. Statistical significance was performed with paired t test, unpaired Student t test, and 2-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 H₂O₂ and hypoxia are known to induce cardiomyocyte apoptosis in vitro. Both methods were
used to evaluate the effect of IL-33 on apoptosis, which was quantified with 2 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-amino-actinomycinD 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 concomitant

Figure 1. IL-33 promotes cardiomyocyte survival. A, Neonatal rat cardiomyocyte apoptosis using TUNEL assayed by flow cytometry in normal versus 1% oxygen for 72 hours. 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 costained with α-sarcomeric actin and 4’,6-diamidino-2-phenylindole 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 costained with α-sarcomeric actin and 4’,6-diamidino-2-phenylindole 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.
immunofluorescent identification by α-sarcomeric actin staining. By using this methodology, the proapoptotic effect of hypoxia was ameliorated by IL-33 treatment in a dose-dependent manner (control: 26.2±1.9, IL-33 10 ng/mL: 14.4±2.0, P<0.05, IL-33 100 ng/mL: 7.0±2.1, P<0.01; Figure 1B). Coapplication of sST2 attenuated the effect of IL-33 (sST2+IL-33 10 ng/mL: 32.5±6.7, versus IL-33 10 ng/mL P<0.05, sST2+IL-33 100 ng/mL: 24.5±2.7, versus 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 (H$_2$O$_2$ 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). Coapplication of sST2 decreased the antiapoptotic effect of IL-33 (44±14% for 10 ng/mL of IL-33+sST2, P<0.01 versus 25.7±4.4% for IL-33 10 ng/mL; 37±2% for 100 ng/mL of IL-33+sST2, P=0.095 versus 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 H$_2$O$_2$ or hypoxia-induced apoptosis. These results indicate that IL-33 has a dose-dependent antiapoptotic cardioprotective effect that can be at least partially blocked by sST2, consistent with a decoy receptor effect of sST2.

**IL-33 Regulates Antiapoptotic 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 4 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 antiapoptotic factors. IL-33 can regulate the activation of NF-κB, which can control apoptosis through regulation of antiapoptotic genes; XIAP, cIAP1, cIAP2, survivin, Bcl-2, and Bcl-xL are NF-κB–regulated proteins that can promote cell survival. Western blot analysis revealed that IL-33 increased the expression of the antiapoptotic 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 3 days after the operation and then once every 3 days thereafter. Eight rats (25%) died within 24 hours of surgery, and there were no differences in perioperative mortality between groups, yielding 2 groups of surviving rats after surgery: IL-33 (n=12) and control (n=11). At 15 days after reperfusion, infarct volume was reduced significantly by IL-33 (10±2% for IL-33 versus 24±5% for control, P=0.003; Figure 3A through 3C). The volume at risk was similar in control (n=11) and IL-33–treated (n=12) rats (43±4% and 45±5%, respectively, P=NS; 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 versus 35±2% for control, P=0.013; Figure 3E and 3F).

To examine whether IL-33 reduces cardiomyocyte apoptosis in vivo, triple staining with TUNEL, anti-α-sarcomeric actin antibody, and 4′,6-diamidino-2-phenylindole 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 versus 27.8±3.9% for control, P=0.001; Figure 4A). The observed percentage of TUNEL-positive cardiomyocytes was significantly reduced by IL-33 (10±2% for IL-33 versus 24±5% for control, P=0.003; Figure 3A through 3C). The volume at risk was similar in control (n=11) and IL-33–treated (n=12) rats (43±4% and 45±5%, respectively, P=NS; 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 versus 35±2% for control, P=0.013; Figure 3E and 3F).

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positive cardiomyocytes in the postinfarct area (5.9±3.2%) was within the previously reported range of apoptotic rates in postinfarct areas after 30 to 60 minutes ischemia and 24-hour reperfusion (0.7 to 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 blot analysis revealed increased XIAP, cIAP1, cIAP2, and survivin in the infarct area, whereas Bcl-2 was unaffected by IL-33 treatment (Figure 4B). Although ST2 was detectable on cultured cardiomyocytes by immunostaining and ST2L gene expression was detectable by real-time polymerase chain reaction in infarcted tissue, expression was not changed by IL-33 treatment (Supplemental Figure I). These results suggest that IL-33 may suppress apoptosis through induction of antiapoptotic 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% versus 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). Pressure volume loops in IL-33–treated group shifted to lower volumes compared with control (Supplemental Figure II). These data suggest that IL-33 not only reduces apoptosis and fibrosis after ischemia/reperfusion but also improves contractile function.

IL-33 and ST2 Signaling After MI in Mice

To characterize further the mechanism of the cardioprotective effect of IL-33, a blinded and randomized experiment using 128 mice with targeted deletion of ST2 or littermate WT controls was performed. This study was performed with coronary ligation to determine whether 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, versus 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 postmortem measure of cardiac size, was reduced by IL-33 in WT (7.0±0.5, n=13 for IL-33 versus 8.0±0.8, n=13 for control) but not in ST2−/− mice (8.0±0.8, n=13 for IL-33 versus 8.0±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 prespecified number of 32 sham-operated animals. Perioperative mortality in WT mice was 9 of 55 (16%) compared with 10 of 62 (16%) in ST2−/− mice, indicating no difference in operative mortality based on genotype; thus, 98 mice in 4 groups (IL-33 versus control, WT versus ST2−/−) were followed up for survival. There were 15 (60%) male and 10 (40%) female mice from WT, and 17 male (55%) and 14 (45%) female knockout mice.

All deaths were spontaneous, and IL-33 treatment did not seem to cause delayed cardiac rupture because none of the WT 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 versus 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 with placebo treatment in ST2−/− MI mice. These data demonstrate that IL-33 improves survival after MI through ST2 signaling.

IL-33 and Inflammatory Responses in the Ischemic-Injured Heart

Because 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 noninfarct areas revealed no significant differences between IL-33–treated and control groups (Supplemental Figure III). We also stained for mast cells, demonstrating a decrease in mast cell density in the infarct area after IL-33 treatment (Supplemental Figure IV). Because mast cells may participate in cardiac injury,31 this could represent an additional mechanism of cardioprotection by IL-33. To address Th1 and Th2 cytokine gene expression, we performed real-time polymerase chain reaction on tissues from animals treated with IL-33. These experiments (n=3 rats per time point) showed an IL-33–

Table. 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>
</tr>
</thead>
<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, μL</td>
<td>376.1±35.7</td>
<td>343.3±25.1</td>
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<tr>
<td>End-diastolic volume, μL</td>
<td>453.8±41.3</td>
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<tr>
<td>End-systolic pressure, mmHg</td>
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<td>120.3±6.60</td>
</tr>
<tr>
<td>End-diastolic pressure, mmHg</td>
<td>6.4±0.9</td>
<td>7.4±1.8</td>
</tr>
<tr>
<td>Stroke volume, μL</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 per 100 g</td>
<td>13.6±1.3</td>
<td>22.3±2.30†</td>
</tr>
<tr>
<td>Stroke work, mmHg/μL</td>
<td>8487±924</td>
<td>14003±1714†</td>
</tr>
<tr>
<td>Arterial elastance, mmHg/μL</td>
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</tr>
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<td>9727.1±741.3*</td>
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<tr>
<td>−dP/dt min, mmHg/s</td>
<td>6699±619</td>
<td>8601.3±645.1*</td>
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<tr>
<td>τ, ms</td>
<td>15.2±0.6</td>
<td>13.3±0.5*</td>
</tr>
<tr>
<td>Maximal power, mW</td>
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<td>128.9±17.1†</td>
</tr>
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<td>PRSW, mmHg</td>
<td>54.2±23.0</td>
<td>62.7±11.7</td>
</tr>
<tr>
<td>Emax, mmHg/μL</td>
<td>0.9±0.1</td>
<td>1.5±0.2†</td>
</tr>
</tbody>
</table>

Values are mean±SEM. PSRW indicates preload recruitable stroke work. *P<0.05. †P=0.01 vs. control.

Figure 5. IL-33 improves survival and cardiac function after myocardial infarction in WT, but not in ST2−/− mice. Experimental MI was performed on WT and ST2−/− litters. 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 toward 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 versus the same treatment in WT.
induced shift toward 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-γ and T-Bet (Supplemental Figure V).

Discussion

In this study, we demonstrate that the cytokine IL-33 can prevent cardiomyocyte apoptosis and improve cardiac function and survival after MI 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.7–9,32 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 proinflammatory effects of IL-33 can be avoided with short-term treatment.

In this study of hypoxia and ischemia/reperfusion, the antiapoptotic effect of IL-33 was clear both in vitro and in vivo, whereas our previous study of pressure overload did not show an antiapoptotic effect. This may represent fundamentally different physiology between the two stimuli because apoptosis is not a prominent feature of pressure overload compared with ischemia. Although apoptosis is an important feature of ischemia/reperfusion,33 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-κB pathways that can regulate apoptosis. It is 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-κB,2,6 which can play multiple roles in cell survival and apoptosis. NF-κB signaling in the heart regulates hypertrophy36 as well as hypoxia-induced mitochondrial dysfunction and cell death.37 In this study, we report that some of the antiapoptotic IAP family proteins are increased by IL-33 in vitro and in vivo. These IAPs may also be regulated by NF-κB and promote cell survival.28,25 However, our experiments do not exclude other cardioprotective mechanisms, such as through mitogen-activated protein kinases, which can be activated by IL-332 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 antifibrotic strategies have potential for prevention of heart failure.41 In a previous study of pressure-overloaded myocardium, cardiac fibroblasts seemed to be a prominent source of endogenous IL-33.6 Understand-

IL-33 can be proinflammatory 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 proinflammatory 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 previous studies.7,6 Further studies may determine whether limiting IL-33 treatment to the earliest period postinjury could allow cardioprotection without proinflammatory systemic effects.

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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.

References

The primary therapeutic focus during an acute myocardial infarction is to restore blood flow with the goal of minimizing cardiomyocyte death and the development of ventricular dysfunction and heart failure. However, even after optimal reperfusion, patients experience ongoing cardiomyocyte loss caused by already triggered apoptotic pathways or secondary reperfusion injury. No currently available pharmacotherapies interrupt cardiomyocyte death pathways during either an acute ischemic insult or a subsequent reperfusion injury. In this study, we demonstrate that treatment with interleukin (IL)-33, a member of the cytokine family of signaling proteins, can achieve cardioprotection in vitro and in a rodent model of ischemia/reperfusion injury. In addition to protecting cultured cardiomyocytes from hypoxia-induced apoptosis, the subcutaneous administration of IL-33 to mice in a randomized, blinded fashion after ischemia-reperfusion injury resulted in reduced cardiomyocyte apoptosis, which translated into diminished infarct size, an improvement in echocardiographic parameters of left ventricular function, and increased survival compared with vehicle control. The therapeutic effect was dependent on the interaction between IL-33 and its receptor, ST2, because cardioprotective benefits were neutralized when IL-33 was administered to ST2-null mice after injury. These results suggest that the administration of IL-33 after myocardial infarction may represent a clinically viable cardioprotective therapy, particularly if administered during an acute ischemic event or coincident with reperfusion therapy.

**CLINICAL PERSPECTIVE**

The primary therapeutic focus during an acute myocardial infarction is to restore blood flow with the goal of minimizing cardiomyocyte death and the development of ventricular dysfunction and heart failure. However, even after optimal reperfusion, patients experience ongoing cardiomyocyte loss caused by already triggered apoptotic pathways or secondary reperfusion injury. No currently available pharmacotherapies interrupt cardiomyocyte death pathways during either an acute ischemic insult or a subsequent reperfusion injury. In this study, we demonstrate that treatment with interleukin (IL)-33, a member of the cytokine family of signaling proteins, can achieve cardioprotection in vitro and in a rodent model of ischemia/reperfusion injury. In addition to protecting cultured cardiomyocytes from hypoxia-induced apoptosis, the subcutaneous administration of IL-33 to mice in a randomized, blinded fashion after ischemia/reperfusion injury resulted in reduced cardiomyocyte apoptosis, which translated into diminished infarct size, an improvement in echocardiographic parameters of left ventricular function, and increased survival compared with vehicle control. The therapeutic effect was dependent on the interaction between IL-33 and its receptor, ST2, because cardioprotective benefits were neutralized when IL-33 was administered to ST2-null mice after injury. These results suggest that the administration of IL-33 after myocardial infarction may represent a clinically viable cardioprotective therapy, particularly if administered during an acute ischemic event or coincident with reperfusion therapy.
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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’-AGTTGTGCATTTCGGAGAGAG-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’-GGTGCAAGCTTGTAGAA-3’, 5’-TTTCTGGCCATGGATTTCTCT-3’, rat GATA3 forward primer: 5’-GGCGGGAGATGGTACTG-3’, reverse primer: 5’-TCTGCCCATTCATTTTATGGTAGA-3’, rat IFN-gamma forward primer: 5’-CACGCCCGTCTTTGGT-3’, reverse primer: 5’-TCTAGGCTTTCAATGAGTGGCC-3’, rat T-Bet forward primer: 5’-TCCTGTCTCCAGCCGTTTCT-3’, reverse primer 5’-CGCTCACTGCTCGGGACTC-3’ and rat GAPDH forward primer: 5’-GGCAAGTTCAATGGCACAGT-3’ and reverse primer: 5’-TGGTGAAGAGCCCAGTAGACTC-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
  - Normoxia ST2
  - Hypoxia ST2
  - P=0.29 (N.S.)
  - (n=3)

B  ST2L/GAPDH

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

Supplement Figure 1
Supplement Figure 2
Supplement Figure 3

A. CD4

B. CD8

C. CD68

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

Control NIA  
IL-33 NIA

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

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

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