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ORIGINAL RESEARCH

Interleukin-22 Directly Activates Myocardial STAT3 (Signal Transducer and Activator of Transcription-3) Signaling Pathway and Prevents Myocardial Ischemia Reperfusion Injury

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BACKGROUND: Interleukin (IL)-22, a member of the IL-10 cytokine family, is the only known cytokine that is secreted by immune cells but does not target immune cells; it mainly targets epithelial cells. In this study, we aimed to determine whether IL-22 administration could activate the myocardial STAT3 (signal transducer and activator of transcription-3) signaling pathway, and thus prevent myocardial injury, in a mouse model of ischemia reperfusion injury.

METHODS AND RESULTS: We evaluated the STAT3 activation after IL-22 injection by Western blot analysis and immunostaining for phosphorylated STAT3 in the heart and found that STAT3 activation in heart tissue rapidly peaked after IL-22 injection. Coimmunostaining of phosphorylated STAT3 and α-actinin revealed that STAT3 activation occurred in cardiomyocytes after IL-22 administration. In heart tissue from intact mice, real-time PCR demonstrated significant expression of IL-22 receptor subunit 1, and coimmunostaining of IL-22 receptor subunit 1 and α-actinin showed IL-22 receptor subunit 1 expression in cardiomyocytes. In cultured cardiomyocytes, IL-22 activated STAT3, and we detected IL-22 receptor subunit 1 expression. Overall, these results indicated that IL-22 directly activated the myocardial IL-22-receptor subunit 1–STAT3 signaling pathway. Following ischemia reperfusion, compared with PBS-treated mice, IL-22-treated mice exhibited a significantly reduced infarct size, significantly reduced myocardial apoptosis, and significantly enhanced phosphorylated STAT3 expression. Moreover, heart tissue from IL-22-treated mice exhibited a significantly reduced expression ratio of phosphorylated p53 to p53.

CONCLUSIONS: Our present findings suggest that IL-22 directly activated the myocardial STAT3 signaling pathway and acted as a cardioprotective cytokine to ameliorate acute myocardial infarction after ischemia reperfusion.

Key Words: apoptosis ■ cytokine ■ ischemia reperfusion injury ■ signal transduction

Following acute myocardial infarction (MI), early reperfusion therapy via primary percutaneous coronary intervention is the most effective way to minimize infarct size.[1–3] However, although reperfusion therapy can salvage the ischemic myocardium, paradoxically, reperfusion itself induces further myocardial injury—generally known as myocardial ischemia reperfusion (I/R) injury.[4–7]

Evidence from animal studies suggests that the myocardial STAT3 (signal transducer and activator of transcription-3) pathway is a potent prosurvival signaling pathway during I/R injury.[8–14] In animals,

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CLINICAL PERSPECTIVE

What Is New?

- We demonstrate expression of the interleukin-22 (IL-22) receptor subunit 1 in cardiomyocytes and find that IL-22 administration rapidly activates the myocardial STAT3 (signal transducer and activator of transcription-3) signaling pathway in vitro and in vivo.
- IL-22 administration significantly reduces the myocardial infarct size, improves cardiac function, and inhibits cardiomyocyte apoptosis during ischemia and reperfusion.
- We detect significantly augmented STAT3 activation and reduced expression ratio of phosphorylated-p53 to p53 in injured heart tissue after ischemia and reperfusion.

What Are the Clinical Implications?

- Our findings suggest that the IL-22-receptor subunit 1–STAT3–p53 axis may contribute to inhibiting myocardial injury and apoptosis after ischemia and reperfusion.
- We propose that IL-22 is a unique cardioprotective cytokine against myocardial injury during ischemia and reperfusion, which may serve as a novel therapeutic target for preventing ischemic injury in patients with acute myocardial infarction.

Nonstandard Abbreviations and Acronyms

AAR	area at risk
G-CSF	granulocyte-colony stimulating factor
I/R	ischemia reperfusion
IL	interleukin
IL-22BP	IL-22 binding protein
IL-22R1	IL-22 receptor subunit 1
LV	left ventricle
MI	myocardial infarction
STAT3	signal transducer and activator of transcription-3

administration of STAT3-activating cytokines, such as erythropoietin or granulocyte-colony stimulating factor (G-CSF), prevents myocardial I/R injury.[15–20] However, despite promising results from experimental and preclinical studies, clinical trials and meta-analysis have shown that cytokine therapy with erythropoietin or G-CSF does not improve cardiac function, reduce infarct size, or decrease all-cause of mortality.[21–24] Moreover, erythropoietin and G-CSF are hematopoietic factors that mainly act on bone marrow cells rather than cardiomyocytes.[21–24] Thus, concerns have been raised that these cytokines may induce polycythemia, and leukocytes and platelets activation can lead to thrombosis and inflammation, potentially exacerbating the pathophysiology of acute MI.[21–24]

Interleukin (IL)-22, a member of the IL-10 cytokine family, plays important roles in preventing inflammation and tissue injury.[25–27] IL-22 signaling is transduced through the cell surface receptors IL-22 receptor subunit 1 (IL-22R1) and IL-10 receptor 2 (IL-10R2) followed by activation of the STAT3 signaling pathway activation.[25–27] Importantly, unlike other cytokines, IL-22R1 is absent on the immune cells but expressed on the cells of nonhematopoietic origin, such as epithelial, renal tubular, and pancreatic ductal cells.[25–27] IL-22 is the only known cytokine that is produced by immune cells but does not directly target immune cells.[25–27]

Accumulating evidence reveals both pathogenic and protective properties of IL-22 and STAT3 interaction related to a number of conditions, including tissue injury and inflammation.[25–27] IL-22 dysregulation can lead to proinflammatory conditions, such as psoriasis or inflammatory bowel disease.[25–27] On the other hand, in solid organs, such as the liver and kidney, IL-22 appears to play roles in preventing cellular apoptosis and promoting cell survival.[25–27] Several recent reports describe the IL-22 involvement in cardiovascular pathophysiology—such as cardiac hypertrophy, myocarditis, and hypertension—suggesting that IL-22 plays an important role in the cardiovascular system.[28–31] Thus, it is plausible that IL-22 contributes to the development of myocardial injury after I/R.

In the present study, we assessed the direct effects of IL-22 on cardiomyocytes in vivo. We also investigated whether exogenous IL-22 administration prevented acute ischemic injury in the mouse model of myocardial I/R injury.

METHODS

The data, analytical methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure as long as the situation allows.

In Vivo Mouse Model of Myocardial I/R Injury

Male 8- to 12-week-old C57BL/6 mice were purchased from Charles River Laboratories Japan. Animals were anesthetized using inhaled isoflurane administered via an endotracheal tube and provided with positive-pressure ventilation using a constant-volume ventilator operating on the Starling principle (HSE MiniVent;

Harvard Apparatus GmbH). The thoracic cavity was opened by left thoracotomy, and then an 8-0 prolene suture was passed under the left anterior descending coronary artery at the inferior edge of the left atrium and tied to produce an occlusion.[32,33] Ischemia was confirmed based on blanching downstream of the ligation, and persistent ST segment elevation on the ECG. Body temperature was maintained at 37°C using a heating pad, and temperature was monitored using a rectal thermometer. After 60 minutes of ischemia, the ligature was released. Reperfusion of the left anterior descending coronary artery was confirmed based on color restoration in the ischemic myocardium, and T-wave inversion on ECG.[32,33] The chest was closed using continuous 6-0 prolene sutures, and the endotracheal tube was removed following resumption of respiration. At 30 minutes before reperfusion, the mice were intraperitoneally injected with recombinant mouse IL-22 (1000 ng/g; Peprotech EC Ltd., Rocky Hill, NJ). This time point was chosen based on findings that STAT3 activation in heart tissue of intact mice peaked at 30 minutes after IL-22 injection (Figure 1A). The study protocol was approved by the Institutional Animal Care and Use Committee of Kurume University School of Medicine.

Evans Blue Dye and Triphenyltetrazolium Chloride Staining

At 24 hours postreperfusion, each mouse was anesthetized as described previously, the chest was reopened, and the left anterior descending coronary artery was reoccluded. The heart was perfused with 5% Evans blue dye, which stained the normally perfused area, such that an absence of staining indicated the ischemic area—that is, the area at risk (AAR). Next the heart was excised, and the left ventricle (LV) cut into 5 transverse slices from the apex to the base. These slices were incubated in 1% triphenyltetrazolium chloride solution at 37°C for 10 minutes, photographed with a digital camera (Leica, M165; Wetzlar, Germany), and weighed. On each image, we measured the infarct area (ie, the area lacking triphenyltetrazolium chloride staining) and the AAR and LV areas using a planimeter with Image-Pro PLUS software (version 7.0J). For each slice, we determined the ratios of infarct to LV area and AAR to LV area and multiplied these ratios by the slice weight to calculate net infarct area and AAR weights, respectively. We then summed these values for all slices. Total infarct area weight was divided by the total AAR weight (infarct area/AAR) to obtain infarct size, and total AAR weight was divided by the total LV weight (AAR/LV) to obtain ischemic size.[32,33] We compared the infarct size in the LV at 24 hours postreperfusion between the PBS-treated (n=7) and IL-22-treated (n=6) groups.

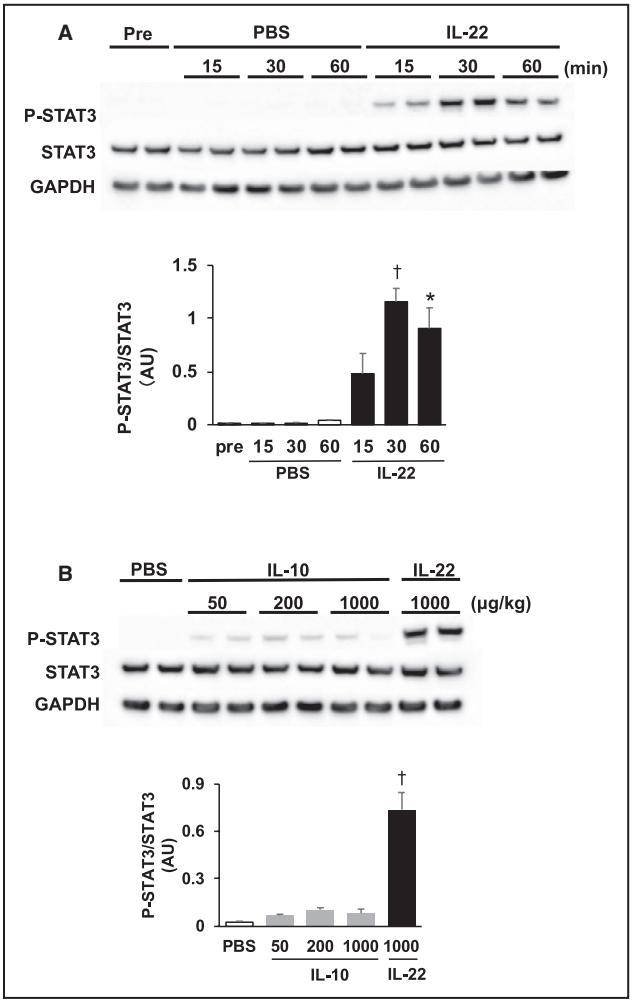

Figure 1. IL-22 activates the STAT3 signaling pathway in heart tissue.
A, Total cell lysates were prepared from mouse hearts at the indicated times after injection of IL-22 or PBS. Blots were probed using antibodies against phosphorylated STAT3 (P-STAT3), STAT3, and GAPDH. Graphs represent quantitative differences in the expression between the ratio of P-STAT3 to STAT3 (n=3 per group). *P<0.05 vs pre-injection, †P<0.01 vs pre-injection (Dunn's test). **B**, Total cell lysates were prepared from mouse hearts at 30 minutes after injection of IL-10 or IL-22. Blots were probed using antibodies against P-STAT3, STAT3, and GAPDH. Graphs represent quantitative differences in the expression ratio of P-STAT3 to STAT3 (n=3 per group). *P<0.05 vs PBS injection (Dunn's test). AU indicates arbitrary units; IL, interleukin; Pre, pre-injection; P-STAT3, phosphorylated STAT3; STAT3, signal transducer and activator of transcription-3.

Western Blot Analysis

At specific time points after cytokine injection or after I/R, tissues were collected and homogenized in lysis buffer containing 25 mmol/L Hepes (pH 7.5), 1% Triton X100, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, and protease inhibitor cocktail (Sigma Aldrich, St Louis, MO). Equal amounts of proteins were separated by denaturing

SDS-PAGE and then transferred onto nitrocellulose membrane (Thermo Fisher Scientific, Waltham, MA). Membranes were probed with the primary antibody and then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody. Protein signals were detected using the enhanced chemiluminescence (ECL) plus system (GE Healthcare, Chicago, IL). Expression levels were determined from band intensities using Image J software, and values were expressed relative to total STAT3 or GAPDH signals.[34–36] We purchased antibodies against phosphorylated STAT3 (P-STAT3; No. 9145, D3A7, rabbit monoclonal, 1:200 dilution), STAT3 (No. 9132, rabbit polyclonal, 1:200 dilution), Caspase 6 (No. 9762, rabbit polyclonal, 1:1000 dilution), Cleaved Caspase 3 (No. 9664, rabbit monoclonal, 1:1000 dilution), Mcl-1 (myeloid cell leukemia sequence 1) (No. 5453, rabbit monoclonal, 1:500 dilution), Bax (No. 2772, rabbit polyclonal, 1:1000 dilution), Bcl-2 (B-cell CLL/lymphoma 2) (No. 3498, rabbit monoclonal, 1:1000 dilution), Bcl-xL (B-cell lymphoma-extra large) (No. 2764, rabbit monoclonal, 1:500), and phosphorylated p53 (P-p53; No. 9284, rabbit polyclonal, 1:1000 dilution) from Cell Signaling Technology and antibodies against IL-22R1 (No. ab5984, rabbit polyclonal, 1:1000 dilution), BNIP3L (BCL2/adenovirus E1B interacting protein 3-like) (No. ab8399, rabbit polyclonal, 1:1000 dilution), Bok (BCL2-related ovarian killer protein) (No. ab233072, rabbit monoclonal, 1:200), CIDEA (cell death-inducing DNA fragmentation factor, α subunit-like effector A) (No. ab8402, rabbit polyclonal, 1:1000 dilution), NOD1 (nucleotide-binding oligomerization domain containing 1) (No. ab217798, rabbit polyclonal, 1:1000 dilution), DIABLO (Drosophila Diablo homolog) (No. ab8115, rabbit polyclonal, 1:200), and p53 (No. ab13144, rabbit polyclonal, 1:500 dilution) from Abcam.

Cardiomyocyte Culture

Mouse ventricular cardiomyocytes were purchased from COSMO Bio Co. Ltd.(Tokyo, Japan). These cells were plated in cardiomyocyte culture medium (COSMO Bio) containing 10% fetal calf serum in 6-well plates precoated with fibronectin. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were cultured overnight under serum-free conditions and then treated with PBS or IL-22.

Double Immunofluorescence Staining

A total of 5 micron frozen sections were collected on glass slides and fixed in 4% paraformaldehyde for 10 minutes. Slides were washed in PBS between each step. Then endogenous peroxidase activity was inhibited by treatment with 3% hydrogen peroxide for 60 minutes. Slides were incubated for 60 minutes at room temperature with 10% goat serum, followed by overnight incubation at 4°C with the antibodies against P-STAT3 (No. 9145, D3A7, rabbit monoclonal, 1:50 dilution), IL-22R1 (No. ab5984, rabbit polyclonal, 1:50 dilution), and α-actinin (No. ab9465, mouse monoclonal, 1:50 dilution). P-STAT3 and IL-22R1 were amplified using Alexa Fluor 488 Tyramide SuperBoost kit (No. B40922, Thermo Fisher Scientific). The α-actinin was visualized using Cy3 conjugated anti-mouse immunoglobulin G (No. 115-165-003, Jackson ImmunoResearch Laboratories, 1:50 dilution). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) solution (No. 340–07971; Dojindo Laboratories).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay

To detect apoptotic cells in the heart after I/R, we performed a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using an in situ apoptosis detection kit (Takara Bio Inc, Kusatsu, Japan), following the manufacturer's protocol.[34,35] Apoptotic cells were quantified from digital photographs taken at ×2 magnification. We compared the percentage of TUNEL–positive cells at 3 hours post-treperfusion between the PBS-treated (n=6) and IL-22-treated (n=5) groups.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from LV tissue using TRIzol (Thermo Fisher Scientific), as previously described,[32,34] and 1 μg of total RNA was converted into cDNA. We obtained apoptosis expression profiles using the RT2 Profiler polymerase chain reaction (PCR) array for murine apoptosis (Qiagen, Hilden, Germany), following the manufacturer's instructions. PCR was performed using the StepOne real-time PCR system (Thermo Fisher Scientific), and the ΔΔCt method was applied to analyze gene expression levels of each gene. We evaluated the dissociation curve for each gene and excluded genes with nonspecific amplification or undetectable expression. The gene expression profiles were displayed as a heat map created using the Qiagen web portal at Gene Globe.[32,34] We also analyzed the heart, peripheral leukocytes, spleen, and thymus by performing real-time PCR assays to assess the gene expression of mouse IL-22, L-22R1, IL-10R2, IL-22BP (IL-22 binding protein), and GAPDH using the corresponding primer pairs (No. Mm01226722_g1, No. Mm01192943_m1, No. Mm00434157_m1, No. Mm01192969_m1, and No. Mm99999915_g1, respectively; Thermo Fisher Scientific) and the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific).[32,34]

Echocardiogram

At 24 hours postreperfusion, mice were placed under light anesthesia with isoflurane and subjected to echocardiography as previously described.[33–35] Transthoracic echocardiography was performed using a Vevo3100 ultrasound machine (VisualSonics Inc, Toronto, Canada) equipped with a 30-MHz probe. Recording was performed as previously described.[32,34,35]

We compared the percentage of fractional shortening and LV end-systolic dimension at 24 hours postreperfusion between the PBS-treated (n=5) and IL-22-treated (n=5) groups.

Bio-Plex Analysis

Serum samples were centrifuged, frozen, and stored at −80°C until use. IL-22 serum levels before ischemia and at specific time point after reperfusion using a Bio-Plex system (Bio-Rad Laboratories, Hercules, CA), as previously described.[32]

Statistical Analysis

Data are expressed as the mean±SE. Statistical analyses were performed using JMPpro12. For comparisons among multiple groups, we applied the Kruskal–Wallis test followed by the Dunn's test. Comparisons between 2 groups were performed using the Wilcoxon rank-sum test. The PCR array results were statistically analyzed using the Qiagen web portal at Gene Globe. Comparisons between the PBS-treated (n=3) and IL-22-treated (n=3) groups were performed using the Wilcoxon rank-sum test. A $P<0.05$ was considered to indicate statistical significance.

RESULTS

IL-22 Activates Myocardial STAT3 Signaling Pathway

We performed Western blot analysis to determine whether IL-22 injection activated the STAT3 signaling pathway in mouse heart tissue. We did not detect STAT3 phosphorylation before IL-22 injection or after PBS injection. After IL-22 injection, we detected faint STAT3 phosphorylation at 15 minutes, and peak STAT3 phosphorylation at 30 minutes (Figure 1A). Because IL-10 ameliorates post-I/R myocardial injury,[24–26] we compared the degree of STAT3 phosphorylation in heart tissue between IL-10-treated mice and IL-22-treated mice. STAT3 phosphorylation was much greater in IL-22-treated hearts compared with IL-10-treated hearts (Figure 1B). Next, to confirm STAT3 activation in cardiomyocytes, we performed coimmunostaining of heart sections with antibodies against P-STAT3 and α-actinin. Nuclear staining of P-STAT3 was detected in cardiomyocytes in IL-22-treated heart tissues, but not in PBS-treated hearts (Figure 2A). We further examined STAT3 activation by IL-22 stimulation in cultured cardiomyocytes. Western blot analysis revealed significantly enhanced STAT3 activation in IL-22-treated cardiomyocytes compared with in PBS-treated cardiomyocytes (Figure 2B). Thus, IL-22 effectively activated the myocardial STAT3 signaling pathway both in vivo and in vitro.

Expression of IL-22 Receptors in the Heart

We next measured the expression of the IL-22 receptor IL-22R1 using real-time PCR analysis, Western blotting, and immunostaining. In real-time PCR, peripheral blood leukocytes from intact mice were used as the negative control. Compared with leukocytes, heart tissue from intact mice exhibited significantly higher IL-22R1 expression, similar IL-10R2 expression, and significantly lower expression of the soluble inhibitory IL-22 receptor IL-22BP (Figure 3A). Western blot analysis revealed protein expression of IL-22R1 (molecular weight, ~64 kDa) in heart tissue from intact mice (Figure 3B), with liver tissue from intact mice used as a positive control. To confirm IL-22R1 expression in cardiomyocytes, heart sections were coimmunostained with antibodies against IL-22R1 and α-actinin. The myocardium was positively stained with the anti-IL-22 antibody, but not with the isotype IgG (Figure 3C). We further examined IL-22R1 expression in cultured cardiomyocytes by Western blot analysis, which revealed IL-22R1 protein expression in cardiomyocytes. The liver cancer cell line hepatoma G2 (HepG2) was used as a positive control (Figure 3D).

Expression of IL-22 and Its Receptors in the Hearts After Ischemia Reperfusion

We further evaluated the expressions of IL-22 and its receptors in the heart tissue during I/R. Real-time PCR revealed that the expressions of IL-22R1 and IL-10R2 did not increase during I/R, but were significantly decreased at 3 and 24 hours after reperfusion, respectively. IL-22 expression peaked at 1 hour postreperfusion, and IL-22BP expression was significantly increased at 24 hours postreperfusion (Figure 4A). In contrast to its mRNA expression, IL-22R1 protein expression was significantly increased at 3 hours after reperfusion (Figure 4B). Moreover, Bio-Plex analysis also revealed that the IL-22 serum level was significantly increased at 3 hours postreperfusion (Figure 4C). To investigate the source of IL-22, we measured the IL-22 expression levels in heart tissue, peripheral leukocytes, and lymphatic

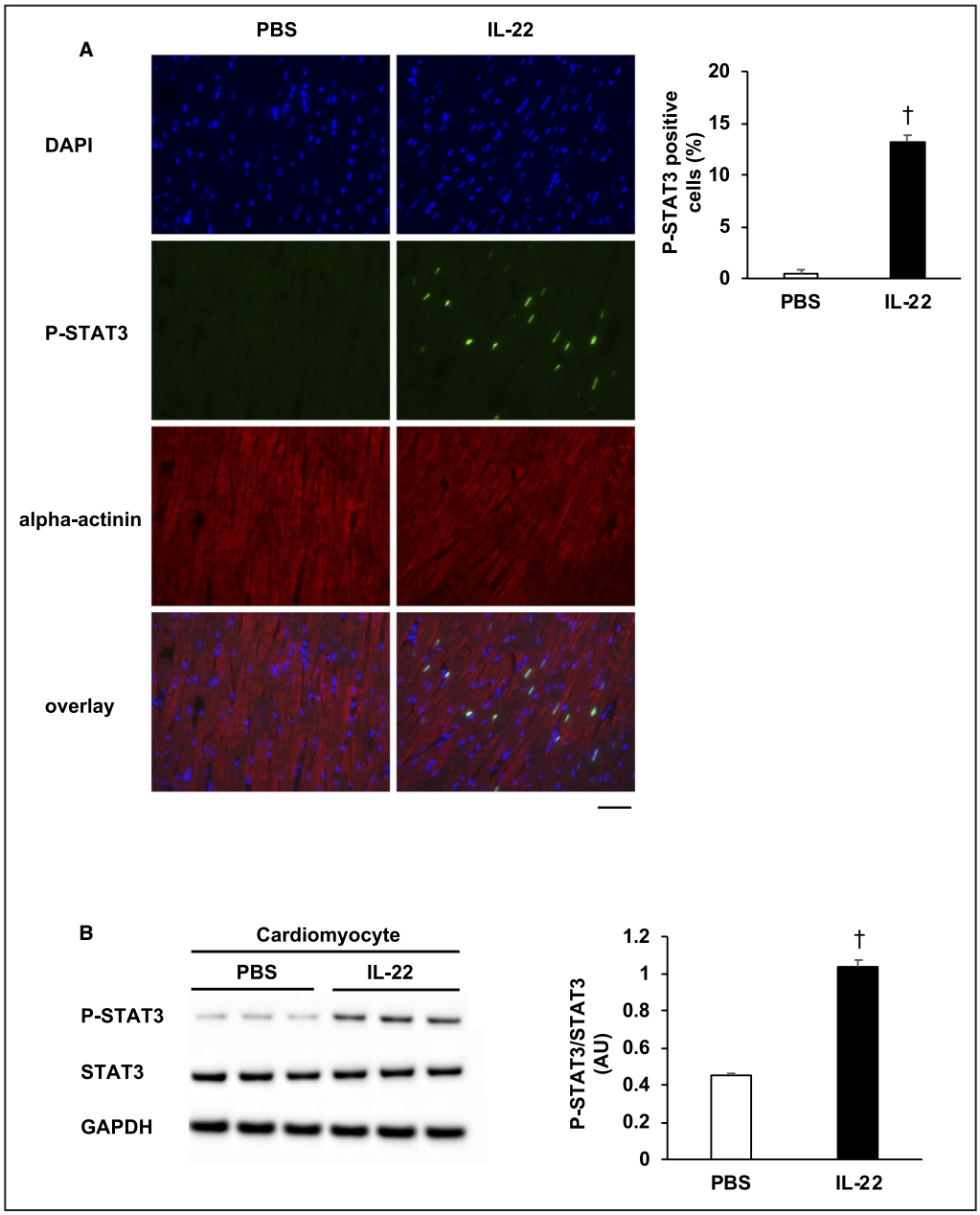

Figure 2. IL-22 activates the myocardial STAT3 both in vivo and in vitro.
A, Double immunofluorescence staining of P-STAT3 (green) and α-actinin (red) in mouse heart tissue at 30 minutes after IL-22 or PBS injection (n=3 for each group). Scale bar=100 μm. Representative photographs of hearts from each group are shown. Values indicate the percentage of P-STAT3–positive cells in the hearts. †P<0.01 vs PBS injection (Wilcoxon rank-sum test). **B**, IL-22 enhances STAT3 activation in mouse ventricular cardiomyocytes. After overnight serum depletion, myocytes were stimulated with PBS or 10 ng/mL IL-22 for 15 minutes, and total cell lysates were prepared. Blots were probed using antibodies against P-STAT3, STAT3, and GAPDH. Graphs represent quantitative differences in the expression between the ratio of P-STAT3 to STAT3 (n=3 per group). †P<0.01 vs PBS injection (Wilcoxon rank-sum test). AU indicates arbitrary units; IL, interleukin; P-STAT3, phosphorylated STAT3; STAT3, signal transducer and activator of transcription-3.

tissues including (the spleen and thymus) prereperfusion and 3 hours postreperfusion. Real-time PCR revealed that IL-22 expression levels in the spleen, thymus, and leukocytes were significantly increased after I/R injury compared with preinjury (Figure 5). The IL-22 expression levels in the spleen and thymus were much higher than in peripheral leukocytes or heart tissue.

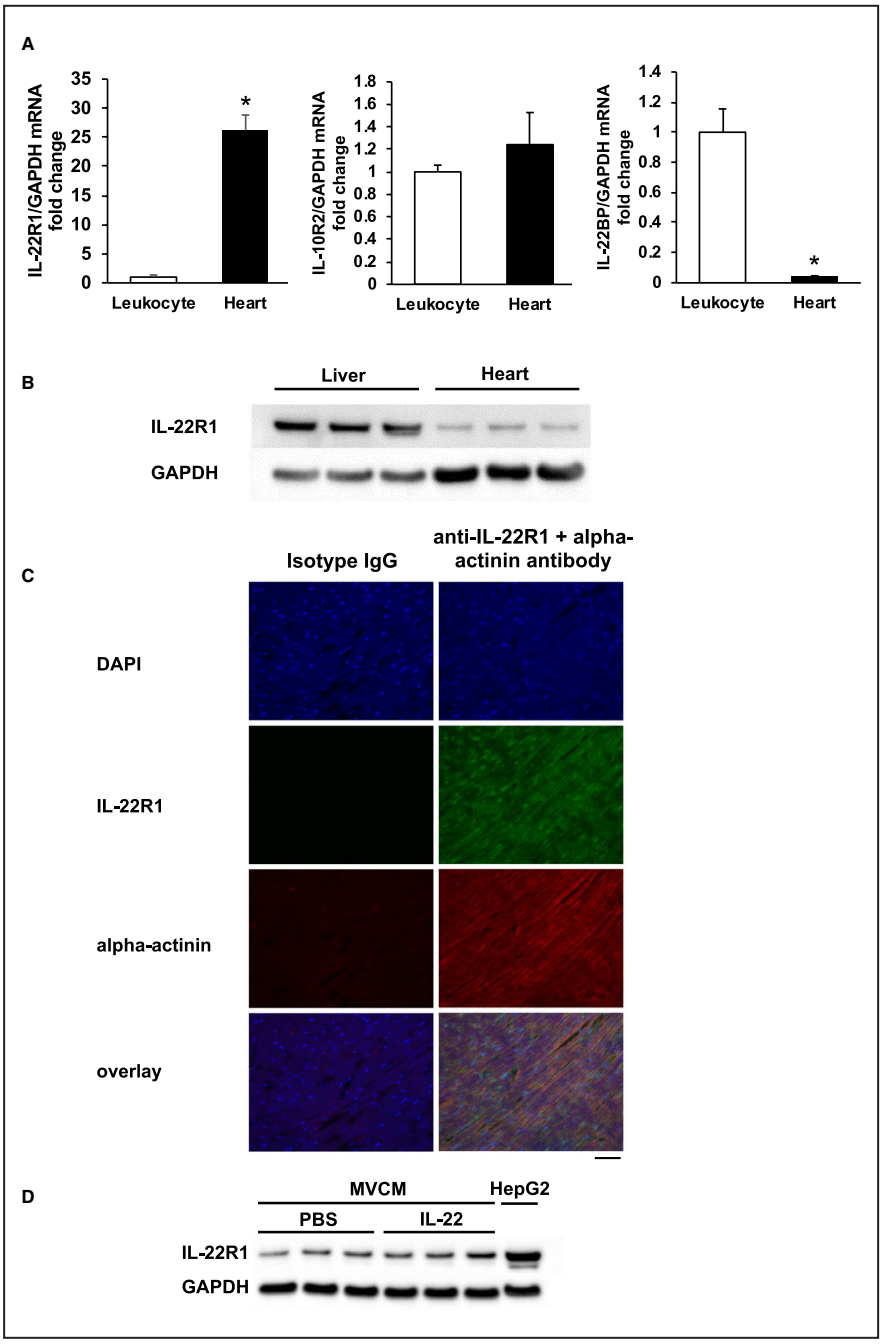

Figure 3. IL-22 receptor expression in the heart tissue of intact mice.
A, From peripheral leukocytes and hearts of intact mice, mRNA was prepared and subjected to real-time polymerase chain reaction analyses for the IL-22 receptors, including IL-22R1 and IL-10R2, and IL-22BP. Presented values are normalized to GAPDH expression (n=3 per group). *P<0.05 vs leukocytes (Wilcoxon rank-sum test). **B**, Total cell lysates were prepared from the livers and hearts of intact mice. Blots were probed using antibodies against IL-22R1 and GAPDH (n=3 per group). **C**, Double immunofluorescence staining of IL-22R1 and α-actinin in heart tissue from intact mice. Frozen sections were stained with antibodies against IL-22R1 (green) and anti-α-actinin (red). Isotype IgG was used as a negative control for anti-IL-22R1 antibody. Scale bar=100 μm. **D**, IL-22R1 expression in mouse ventricular cardiomyocytes. After overnight serum depletion, myocytes were stimulated with PBS or 10 ng/mL IL-22 for 15 minutes, and total cell lysates were prepared. Blots were probed using antibodies against IL-22R1 and GAPDH (n=3 per group). DAPI indicates 4′,6-diamidino-2-phenylindole; HepG2, hepatoma G2; IgG, immunoglobulin G; IL, interleukin; IL-10R2, IL-10 receptor 2; IL-22BP, IL-22 binding protein; IL-22R1, IL-22 receptor subunit 1; MVCM, mouse ventricular cardiomyocytes.

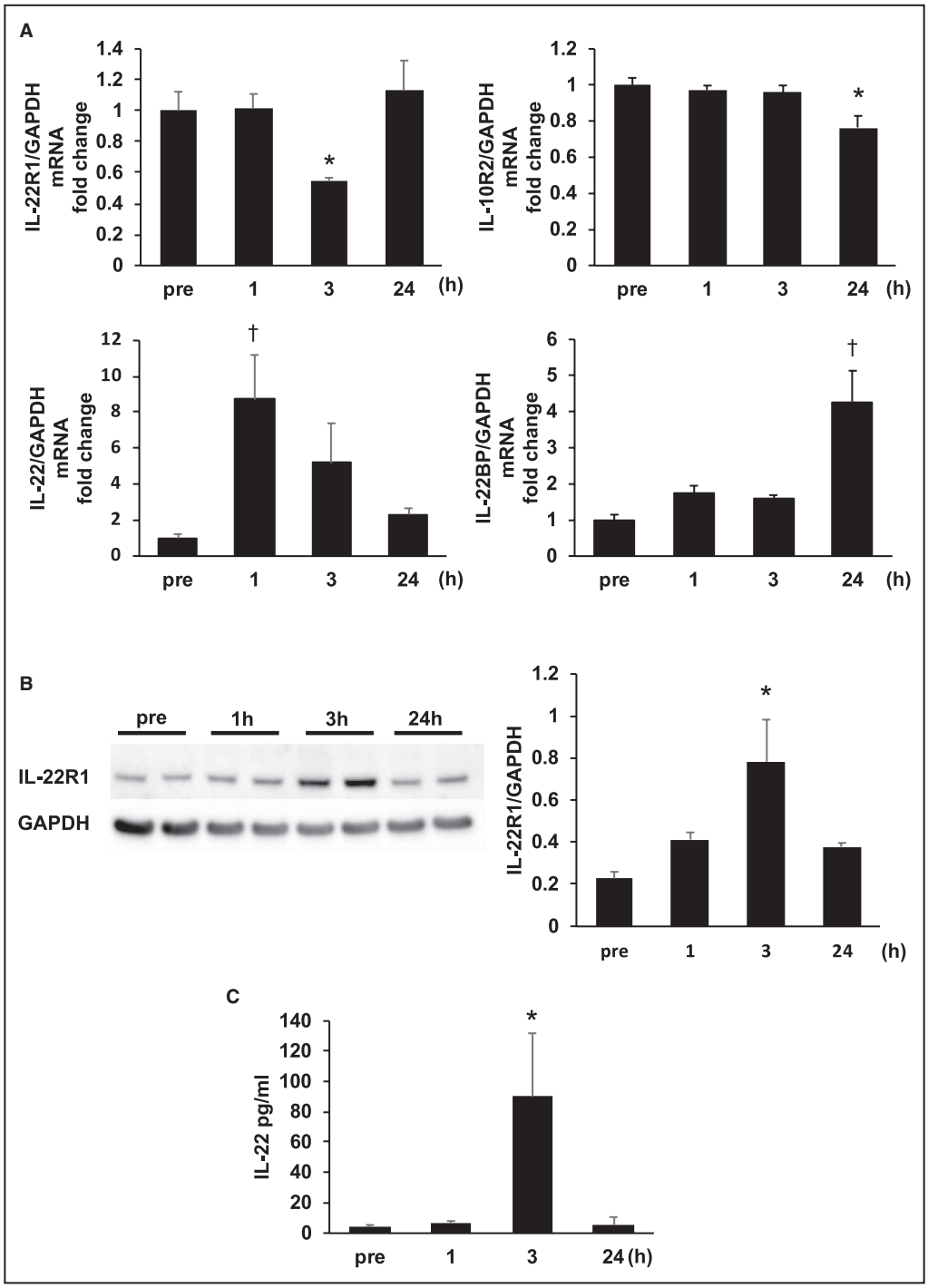

Figure 4. Expression of IL-22 and its receptors in heart tissue after ischemia reperfusion.
A, From mouse hearts at the indicated times after ischemia reperfusion, mRNA was prepared and subjected to real-time polymerase chain reaction analyses for IL-22R1, IL-10R2, IL-22, and IL-22BP. Presented values are normalized to GAPDH and expressed as the fold-change from pre-ischemia values (n=4–5 per group). **P*<0.05 vs pre-ischemia. †*P*<0.01 vs pre-ischemia (Dunn's test). **B**, Total cell lysates were prepared from mouse hearts of at the indicated times after ischemia reperfusion. Blots were probed using antibodies against IL-22R1 and GAPDH. Graphs represent quantitative differences in the expression between the ratio of IL-22R1 to GAPDH (n=4–5 per group). **P*<0.05 vs pre-ischemia (Dunn's test). **C**, Serum IL-22 levels at the indicated times after ischemia reperfusion, determined by Bio-Plex (n=3). **P*<0.05 vs pre-ischemia (Dunn's test). IL indicates interleukin; IL-10R2, IL-10 receptor 2; IL-22BP, IL-22 binding protein; IL-22R1, IL-22 receptor subunit 1; pre, pre-ischemia.

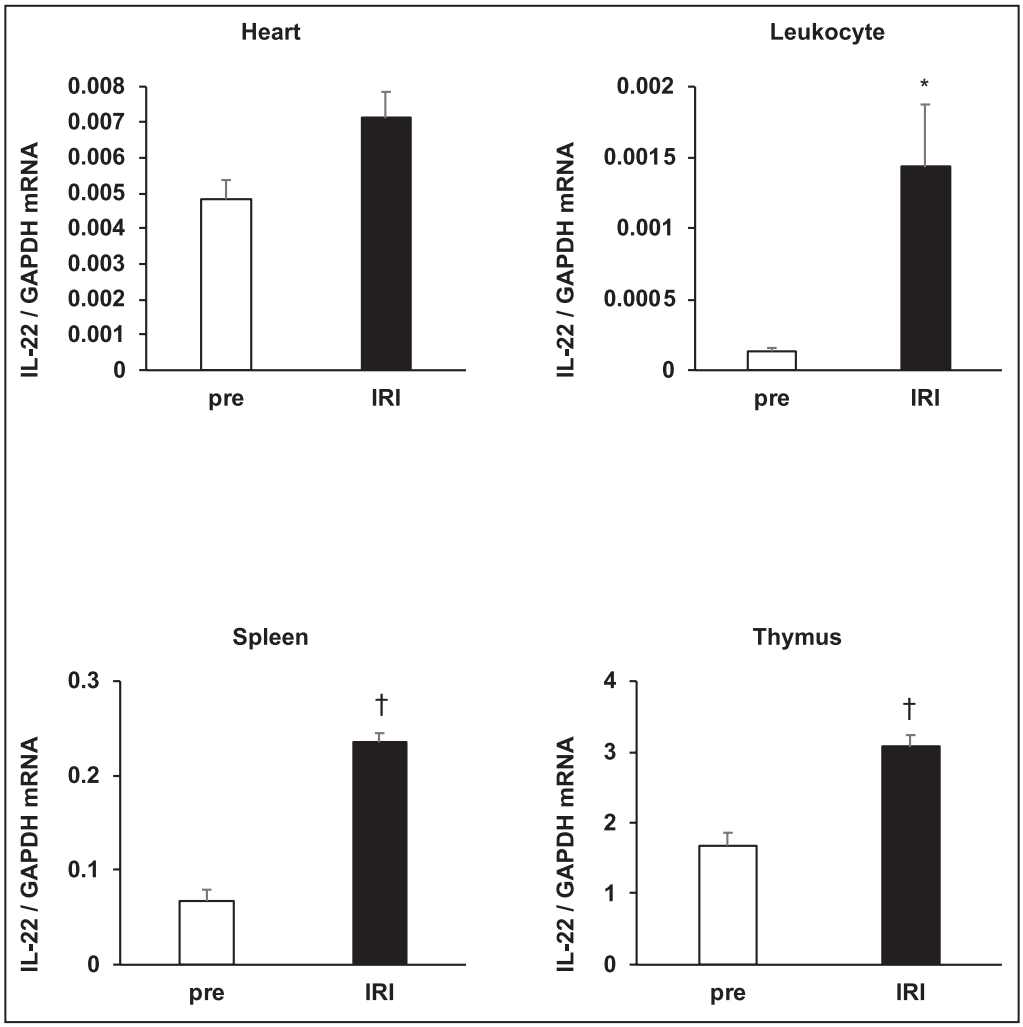

Figure 5. IL-22 expression in the heart, peripheral leukocytes, spleen, and thymus after ischemia reperfusion.
Real-time polymerase chain reaction analyses for IL-22 was performed using mRNA from each tissue, pre-ischemia, or at 3 hours after ischemia reperfusion. The presented values are normalized to GAPDH (n=3 per group). *P<0.05 vs pre-ischemia. †P<0.01 vs pre-ischemia (Wilcoxon rank-sum test). IL indicates interleukin; IRI, ischemia reperfusion injury; pre, pre-ischemia.

Reduced Infarct Size and Preserved Cardiac Contraction After Ischemia Reperfusion in IL-22-Treated Mice

Next, we evaluated how IL-22 administration affected post-I/R infarct size in mice. Myocardial injury was induced by 1-hour ligation of the left anterior descending coronary artery, and mice were injected with recombinant murine IL-22 at 30 minutes before reperfusion. At 24 hours postreperfusion, we double stained the heart tissue with Evans blue and triphenyltetrazolium chloride to determine the infarct area, AAR (indicating the ischemic area), and normally perfused area of the LV. Infarct size was defined as the weight ratio of infarct area to AAR, and ischemic size as the weight ratio of AAR to LV. Evans blue and triphenyltetrazolium chloride staining revealed that the postreperfusion myocardial infarct size was significantly reduced in IL-22-treated mice compared with PBS-treated mice, although the 2 groups showed comparable ischemic sizes (Figure 6A). Echocardiographic assessment further showed a greater percent of fractional shortening and smaller LV end-systolic dimension after I/R in IL-22-treated mice compared with PBS-treated mice (Figure 6B).

IL-22 Prevents Myocardial Apoptosis After Ischemia and Reperfusion

Because cardiomyocyte apoptosis plays a central role in the development of acute myocardial injury after reperfusion,[8,11] we performed a TUNEL assay to measure the number of apoptotic cells. Compared with PBS-treated mice, IL-22-treated mice showed a significantly

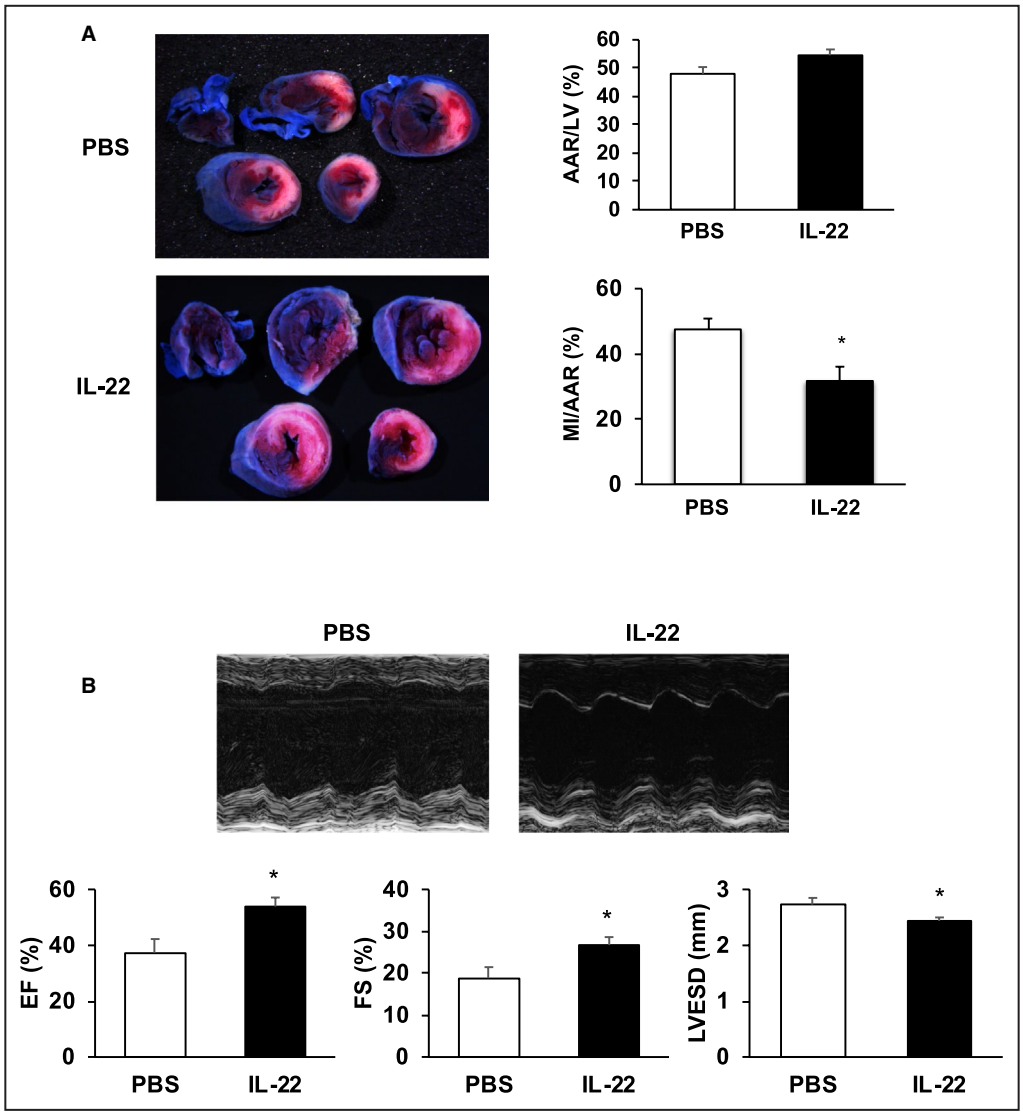

Figure 6. IL-22 prevents myocardial injury after ischemia reperfusion.
A, Representative images of Evans blue and triphenyltetrazolium chloride staining in PBS-treated or IL-22-treated mice at 24 hours postreperfusion (n=6–7 per group). The infarct size of the LV is expressed as a percentage of the AAR for each group. Graphs show quantification of AAR/LV and infarct area/AAR. *P<0.05 vs PBS-treated mice (Wilcoxon rank-sum test). **B**, Echocardiography was performed in PBS-treated or IL-22-treated mice at 24 hour postreperfusion (n=4–7 per group). *P<0.05 vs PBS-treated mice (Wilcoxon rank-sum test). AAR indicates area at risk; EF, ejection fraction; FS, fractional shortening; IL, interleukin; LV, left ventricle; LVESD, left ventricular end systolic dimension; MI, myocardial infarction.

reduced number of TUNEL–positive cells at 3 hours after reperfusion (Figure 7A). In addition, Western blot analysis revealed greater STAT3 activation at 30 minutes after I/R in IL-22-treated mice compared with PBS-treated mice (Figure 7B).

Expression of Apoptosis-Related Molecules in IL-22-Treated Hearts

We further evaluated the in vivo effects of IL-22 administration on apoptosis in heart tissue by performing real-time PCR array analysis for apoptosis-related genes in the hearts of intact mice. Compared with PBS-treated mice, IL-22-treated mice showed significantly reduced expressions of several proapoptotic genes (Table), including BCL2/adenovirus E1B interacting protein-3-like (*Bnip3l*), BCL2-related ovarian killer protein (*Bok*), caspase-6, cell death-inducing DNA fragmentation factor, α subunit-like effector A (*Cidea*), diablo homolog (*Diablo*), and nucleotide-binding oligomerization domain containing 1 (*Nod1*). We also performed Western blot analysis to examine the protein expression levels of these proapoptotic molecules. However, the

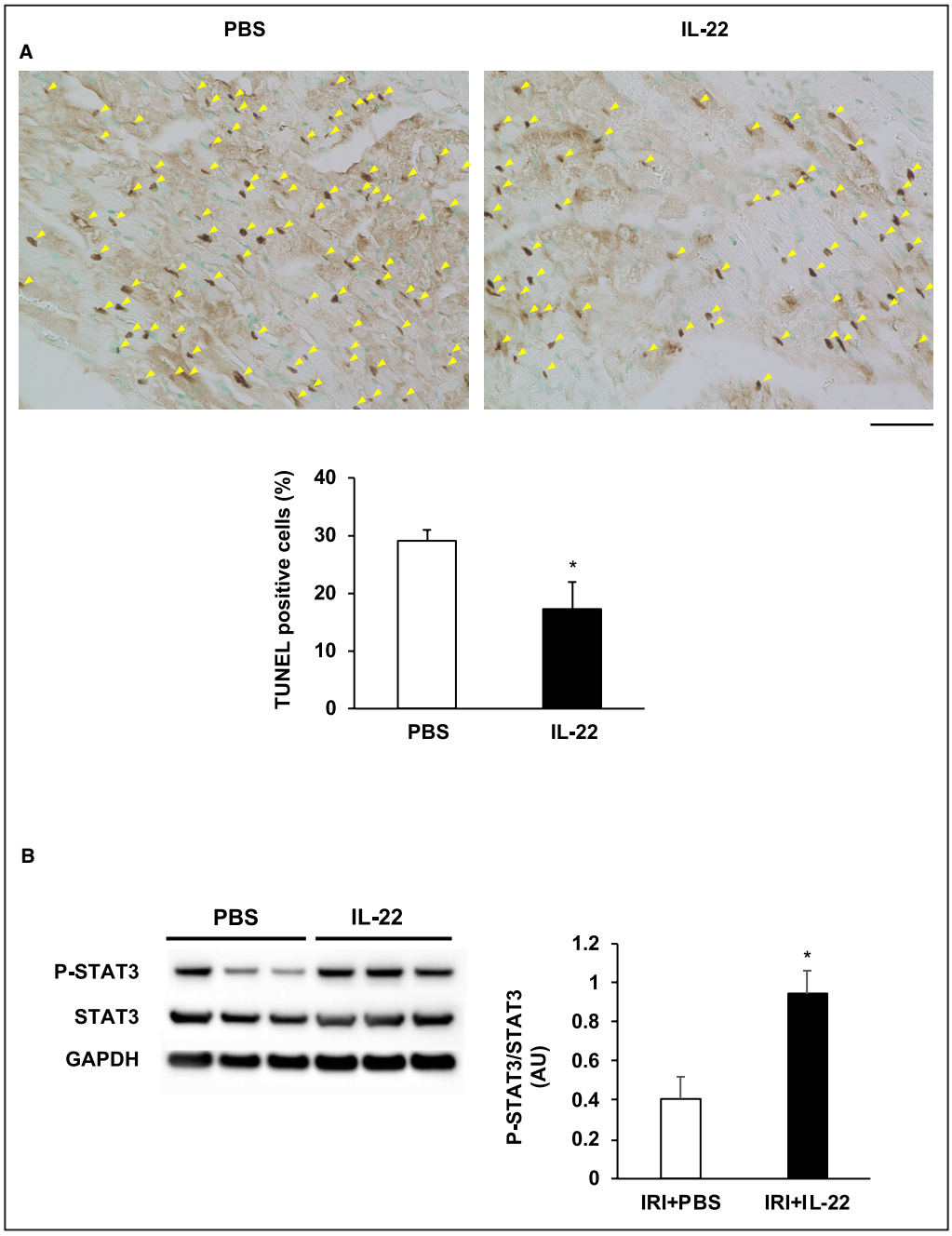

Figure 7. IL-22 inhibits myocardial apoptosis after ischemic reperfusion injury.
A, TUNEL assays of heart tissue from PBS-treated or IL-22-treated mice at 3 hours postreperfusion (n=5–6 per group). Representative images of the ischemic areas of hearts from each group. Graph shows the percentage of TUNEL-positive cells. *P<0.05 vs PBS-treated mice (Wilcoxon rank-sum test). **B**, Total cell lysates were prepared from the left ventricle of PBS-treated or IL-22-treated mice at 30 minutes postreperfusion. Blots were probed using antibodies against P-STAT3, STAT3, and GAPDH. Graphs represent quantitative differences in the expression between the ratio of P-STAT3 to STAT3 (n=3 per group). *P<0.05 vs PBS-treated mice (Wilcoxon rank-sum test). AU indicates arbitrary units; IRI, ischemia reperfusion injury; IL, interleukin; P-STAT3, phosphorylated STAT3; STAT3, signal transducer and activator of transcription-3; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

protein expression levels in heart tissue were comparable between PBS-treated and IL-22-treated intact mice (Figure 8A). These 2 groups also exhibited comparable expressions of other major proapoptotic molecules, including cleaved caspase 3 and Bax, and antiapoptotic molecules, such as Bcl-xL and Bcl-2 (Figure 8A). Among these apoptosis-related molecules, the expression ratio of P-p53 to p53 was markedly reduced in the

Table. Proapoptotic Genes With Reduced Expression in the Heart Tissue From Intact Mice After IL-22 Administration

Symbol	Description	Fold Change (Comparing to Control Group)	*P* Value
Bnip3l	BCL2/adenovirus E1B interacting protein 3-like	0.8484	<0.05
Bok	BCL2-related ovarian killer protein	0.555	<0.05
Casp6	Caspase 6	0.8827	<0.05
Cidea	Cell death-inducing DNA fragmentation factor, α subunit-like effector A	0.796	<0.05
Diablo	Diablo homolog (Drosophila)	0.8684	<0.05
Nod1	Nucleotide-binding oligomerization domain containing 1	0.8229	<0.05

BCL2 indicates B-cell CLL/lymphoma 2; and IL, interleukin.

hearts from the IL-22-treated intact mice compared with the PBS-treated intact mice (Figure 8A). Furthermore, 3 hours after ischemia and reperfusion, the expression ratio of P-p53 to p53 was significantly reduced in the hearts from the IL-22-treated mice compared with the PBS-treated mice (Figure 8B).

DISCUSSION

In the present study, we investigated the role of IL-22 in the mechanism of cardioprotection during myocardial I/R injury in mice. Our results showed that IL-22 injection rapidly activated the myocardial STAT3 signaling pathway in intact mice. The IL-22 receptor IL-22R1 was expressed both in cultured cardiomyocytes and in heart tissue, and its protein expression was upregulated after I/R. IL-22 administration prevented post-I/R myocardial injury and apoptosis. Moreover, IL-22 suppressed the expression ratio of P-p53 to p53 in the heart tissue from intact mice as well as in injured heart tissue post-I/R. Overall, these findings suggested that IL-22 directly activates the myocardial STAT3 signaling pathway and acts as a cardioprotective cytokine, attenuating MI during I/R.

IL-22 Targets Cardiomyocytes During Myocardial I/R Injury

Although most cytokines target hematopoietic cells, IL-22 predominantly impacts nonhematopoietic epithelial cells and fibroblasts in a wide range of tissues, including lung, liver, kidney, thymus, pancreas, gut, skin, and the synovium.[25–27] Several recent reports have demonstrated IL-22 involvement in the cardiovascular pathophysiology, including cardiac hypertrophy and myocarditis,[25–27] suggesting that IL-22 has direct actions on myocardial cells. Correspondingly, our present results revealed that in vivo IL-22 administration activated STAT3 within cardiomyocytes, triggering a key downstream signaling pathway of the IL-22 receptor. Moreover, we demonstrated IL-22R1 expression in cardiomyocytes.

Interestingly, at 3 hours postreperfusion, we detected increased protein expression of IL-22R1, enhanced STAT3 activation following IL-22 administration, and increased circulating IL-22. Thus, it is likely that the IL-22-IL-22R1-STAT3 axis was fully activated at 3 hours after I/R injury. On the other hand, the IL-22R1 mRNA level was significantly reduced at this time point. The negative regulatory mechanism of IL-22R1 has scarcely been reported. However, the increasing activation of the IL-22-IL-22R1-STAT3 axis suggests the possible action of a negative feedback mechanism. Our present results suggest that IL-22 targeted cardiomyocytes and that the IL-22-IL-22R1-STAT3 axis may play an important role in the preventing post-I/R myocardial injury.

IL-22 Prevents Myocardial Injury and Apoptosis via STAT3 Activation

Many lines of evidence indicate that STAT3 is a transcriptional factor with cardioprotective effects against ischemic injury.[8–14] Cardiomyocyte-specific, STAT3-deficient mice exhibit larger infarct size after I/R, which is associated with enhanced cardiomyocyte apoptosis.[8,11] In our present study, IL-22 administration reduced myocardial injury and apoptosis by augmenting STAT3 activation, suggesting that the inhibition of myocardial apoptosis may be a main mechanism underlying the IL-22-induced prevention of myocardial injury after I/R. However, Western blot analysis revealed that IL-22-treated mice and PBS-treated mice showed comparable expression levels of apoptosis-related molecules, including Bcl-xL and Bcl-2 (data not shown). This was likely attributable to the massive tissue damage in infarct myocardium, with corresponding secretion of many cytokines and activation of various intracellular signal transduction pathways, which could make it difficult to detect the effects of IL-22 administration.

IL-22 treatment suppressed the mRNA expression of several proapoptotic molecules in heart tissue from intact mice (Table); however, the protein levels of these molecules were comparable to in the PBS-treated mice (Figure 8A). Among major apoptosis-related molecules, only the ratio of P-p53 to p53

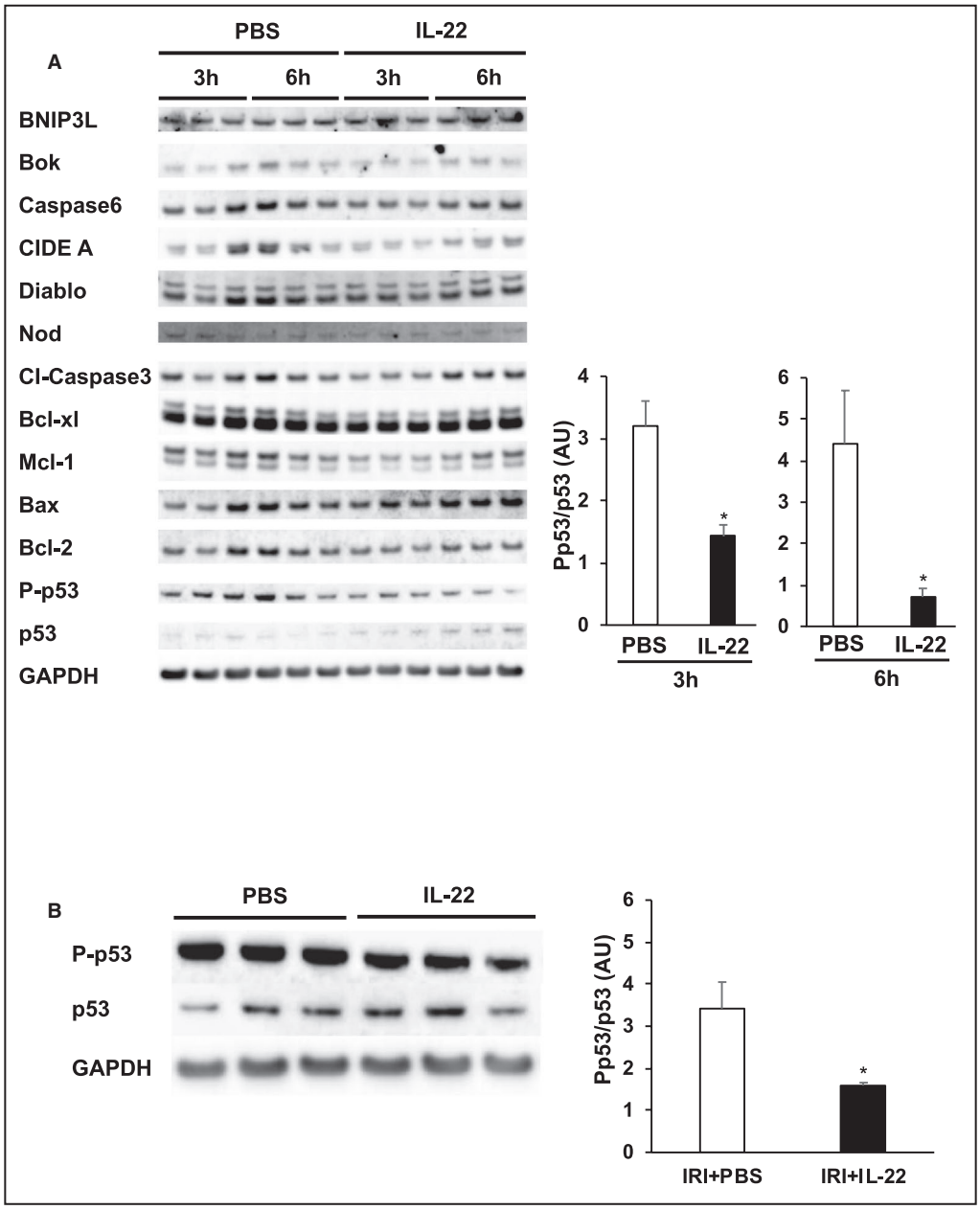

Figure 8. Expression of apoptosis-related molecules in hearts of IL-22-treated mice.
A, Total cell lysates were prepared from hearts of intact mice at 3 or 6 hours after injection of IL-22 or PBS. Blots were probed using antibodies against BNIP3L, Bok, caspase 6, CIDEA, Diablo, Nod1, cleaved-caspase 3, Bcl-xL, Mcl-1, Bax, Bcl-2, P-p53, p53, and GAPDH. Graphs represent quantitative differences in the expression ratio of P-p53 to p53 (n=3 per group). *P<0.05 vs PBS injection (Wilcoxon rank-sum test). **B**, Total cell lysates were prepared from the left ventricle of PBS-treated or IL-22-treated mice at 3 hours after ischemia reperfusion. Blots were probed using antibodies against P-p53, p53, and GAPDH. Graphs represent quantitative differences in the expression ratio of P-p53 to p53 (n=3 per group). *P<0.05 vs PBS injection (Wilcoxon rank-sum test). AU indicates arbitrary units; Bax, Bcl-2-associated X protein; Bcl-2, B-cell CLL/lymphoma 2; Bcl-xL, B-cell lymphoma-extra large; BNIP3L, BCL2/adenovirus E1B interacting protein 3-like; Bok, BCL2-related ovarian killer protein; CIDEA, Cell death-inducing DNA fragmentation factor, α subunit-like effector A; IL, interleukin; Nod1, nucleotide-binding oligomerization domain containing 1; Mcl-1, myeloid cell leukemia sequence 1; P-p53, phosphorylated p53.

was markedly reduced after IL-22 administration. Phosphorylation of p53 stabilizes p53, consequently inducing apoptosis in cells.[37] Naito et al[38] reported that decreased p53 expression prevents myocardial apoptosis after MI in mice. Therefore, inhibition of p53 phosphorylation by IL-22 may contribute to

myocardial apoptosis after I/R. The p53 promoter lesion reportedly includes a STAT3 DNA binding site, and STAT3 activation represses p53 expression in cells.[39] Taken together, previous findings and our present results suggest that the IL-22R1-STAT3-p53 axis may contribute to inhibiting myocardial apoptosis after I/R. However, further investigations are needed to identify potential mechanisms downstream of the IL-22R1-STAT3 signaling pathway.

Comparison of Cardioprotective Mechanisms by IL-10 and IL-22 During Myocardial I/R Injury

The IL-10 family cytokines IL-10 and IL-22 both activate the STAT3 signaling pathway and exert anti-inflammatory effects,[40] and IL-10 reportedly prevents myocardial injury after I/R in animal models.[41–43] In this study, we demonstrated that exogenous IL-22 administration ameliorated myocardial injury after I/R in mice. However, IL-10 and IL-22 may have different cardioprotective mechanisms against post-I/R myocardial injury. Several reports indicate that IL-10 prevents myocardial injury after I/R by inhibiting leukocyte infiltration and the expression of inflammatory cytokines, such as tumor necrosis factor-α or IL-1.[41–43] In contrast, here we demonstrated that IL-22 administration prevented post-I/R myocardial injury by inhibiting myocardial apoptosis and the expression ratio of P-p53 to p53. We also detected greater STAT3 phosphorylation in heart tissue of IL-22-treated mice compared with in IL-10-treated mice (Figure 1B) and found low IL-22R1 expression in peripheral leukocytes (Figure 3). Thus, although IL-10 prevents post-I/R myocardial injury by acting on immune cells, it appears likely that IL-22 prevents post-I/R myocardial injury through direct actions on cardiomyocytes.

IL-22 is a Potential Therapeutic Target for Myocardial Injury After I/R

STAT3-activating cytokines reportedly prevent ischemia-induced cardiac injury in animal models.[8–14] Notably, erythropoietin and G-CSF seemed promising for clinical application in acute MI treatment. However, clinical trials have yielded negative or controversial results regarding the efficacy of erythropoietin or G-CSF for reducing myocardial injury in patients with acute MI.[21–24] Erythropoietin and G-CSF are hematopoietic factors, raising concerns about cytokine therapy-induced serious adverse effects, such as inflammation and thrombosis.[21–24] In contrast, IL-22 does not target hematopoietic cells, which may be an advantage for cytokine therapy against acute ischemic injury after reperfusion. Thus, IL-22 may have therapeutic potential for preventing ischemic injury in patients with acute MI.

LIMITATIONS AND FUTURE DIRECTIONS

The present study has several limitations. To examine the release of IL-22 during myocardial I/R injury, we measured mRNA expression of IL-22 in tissues including the spleen and thymus and found significant increases of IL-22 in the spleen and thymus 3 hours after I/R (Figure 5). Primarily cells of the lymphoid lineage produce IL-22, including αβ T cells, γδ T cells, natural killer T (NKT) cells, and innate lymphoid cells.[25–27] Thus, it remains necessary to further investigate the release of IL-22 from these specific immune cells using flowcytometric analysis.

Our present findings suggest that exogenous IL-22 administration prevented MI and apoptosis after I/R, suggesting that IL-22 may be an attractive therapeutic target for post-I/R myocardial injury. However, IL-22 also has the potential to induce inflammation, and issues may be encountered relating to strong endogenous suppressors, such as IL-22BP. Therefore, further studies are required to examine the possibility of using IL-22 as a therapeutic target.

CONCLUSIONS

In the present study, we demonstrated expression of the IL-22 receptor IL-22R1 in cardiomyocytes and found that IL-22 administration activated the myocardial STAT3 signaling pathway in vivo. IL-22 administration prevented myocardial injury and apoptosis and augmented STAT3 activation after I/R. We also detected significantly reduced expression ratio of P-p53 to p53 in heart tissue from intact mice as well as in injured heart tissue post-I/R. Based on these findings, we propose that IL-22 is a unique cardioprotective cytokine against myocardial injury after I/R, which may serve as a novel therapeutic target for preventing post-myocardial injury.

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Disclosures

None.

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