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ORIGINAL RESEARCH - PRECLINICAL

Recurrent Myocardial Injury Leads to Disease Tolerance in a Murine Model of Stress-Induced Cardiomyopathy

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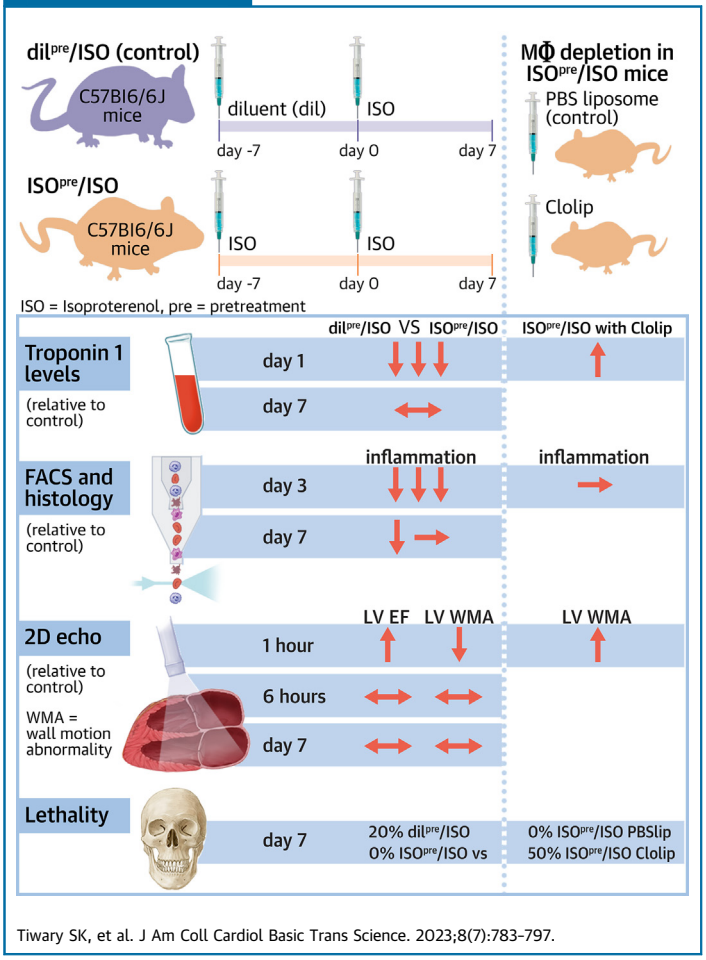
VISUAL ABSTRACT



Tiwary SK, et al. J Am Coll Cardiol Basic Trans Science. 2023;8(7):783-797.

HIGHLIGHTS

- **A single intraperitoneal dose of ISO provoked an acute inflammatory response in the heart that was accompanied by reversible changes in LV structure and function, whereas a second injection of ISO 7 days later had no effect on cardiac myocyte cell death, inflammation, or changes in LV structure and function, demonstrating that prior ISO injury and inflammation confers a cytoprotective effect in the heart.**
- **The cytoprotective effects of ISO-induced injury were not secondary to decreased β1-adrenergic responsiveness, were maintained for 2 weeks, and were partially diminished after 5 weeks**
- **Depletion of cardiac macrophages partially attenuated the cytoprotective effects of ISO-induced injury, suggesting that preconditioning stimuli stimulate macrophage populations to release mediators that up-regulate cytoprotective programs in the heart following tissue injury.**

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ABBREVIATIONS AND ACRONYMS

DAMP = damage-associated molecular patterns

dil = diluent

dil^{pre}/dil = mice pretreated with diluent on day −7 and injected with diluent on day 0

dil^{pre}/ISO = mice pretreated with diluent on day −7 and injected with isoproterenol on day 0

IL = interleukin

ISO = isoproterenol

ISO^{pre}/dil = mice pretreated with isoproterenol on day −7 and injected with diluent on day 0

ISO^{pre}/ISO = mice pretreated with isoproterenol on day −7 and injected with isoproterenol on day 0

LV = left ventricle/ventricular

SUMMARY

Whereas the innate immune response to an initial episode of cardiac injury has been studied extensively, the response of the immune system to recurrent cardiac tissue injury is not well understood. Specifically, it is not known whether the immune system adapts to the initial episode of cardiac injury and whether any adaptations that occur lead to immune cell hypo-responsiveness or, alternatively, immune cell hyper-responsiveness. Here, we studied the role of adrenergic-mediated stress using a simple model of reversible stress-induced cardiomyopathy, and show that isoproterenol-induced tissue injury and inflammation are sufficient to protect the heart from the myopathic effects of a subsequent exposure to isoproterenol. Remarkably, pharmacological depletion of macrophages partially attenuated the isoproterenol-induced cytoprotective response, suggesting that immune-mediated tissue repair mechanisms confer tolerance to subsequent tissue damage.

The immune system is essential for coordinating host responses to environmental danger, most notably through the detection and elimination of intruding pathogens.[1] It is equally clear that the coordinated recruitment of immune cells to sites of tissue injury is a prerequisite for the initiation of effective tissue repair responses, which are essential for maintaining host homeostasis and fitness.[2-4] The ability of the host immune system to simultaneously protect against both invading pathogens and orchestrate tissue repair processes is the result of evolutionary pressures that led to the selection of a diverse family of germ line encoded innate immune receptors, which recognize shared molecular motifs that are common to both invading pathogens (referred to as pathogen-associated molecular patterns) and to the molecular signals released by dying or dead/necrotic cells (referred to as damage-associated molecular patterns [DAMPs]). Once activated, these innate immune pattern recognition receptors initiate a sequence of highly optimized immune responses that preserve host survival and maintain host fitness.[1,3,5]

Whereas the release of DAMPs by dying and necrotic myocytes and the ensuing innate immune response following an initial episode of cardiac injury have been studied extensively (reviewed by Frangogiannis,[6] Epelman et al,[7] and Swirski and Nahrendorf[8]), the response of the immune system to recurrent cardiac tissue injury is vastly understudied. For example, it is not known whether the innate immune system adapts in response to tissue injury by becoming more activated (ie, hyperresponsive) or less activated (ie, hyporesponsive) in the setting of recurrent myocardial injury. Germane to this discussion, we and others have shown that cytokines released by cardiac myocytes and cardiac resident immune cells are sufficient to confer cytoprotective responses in the heart following the initial myocardial injury.[9-14] To understand whether the immune response to a single episode of myocardial injury is adaptive or maladaptive in the setting or recurrent tissue injury, we studied the role of adrenergic-mediated stress using a simple model of reversible stress-induced cardiomyopathy. Here we show that isoproterenol (ISO)-induced tissue injury and inflammation are sufficient to protect the heart from the myopathic effects of a subsequent exposure to ISO. We further demonstrate that pharmacological depletion of macrophages partially attenuates the ISO-induced cytoprotective response, suggesting that immune mediated tissue repair mechanisms confer tolerance to subsequent tissue damage.

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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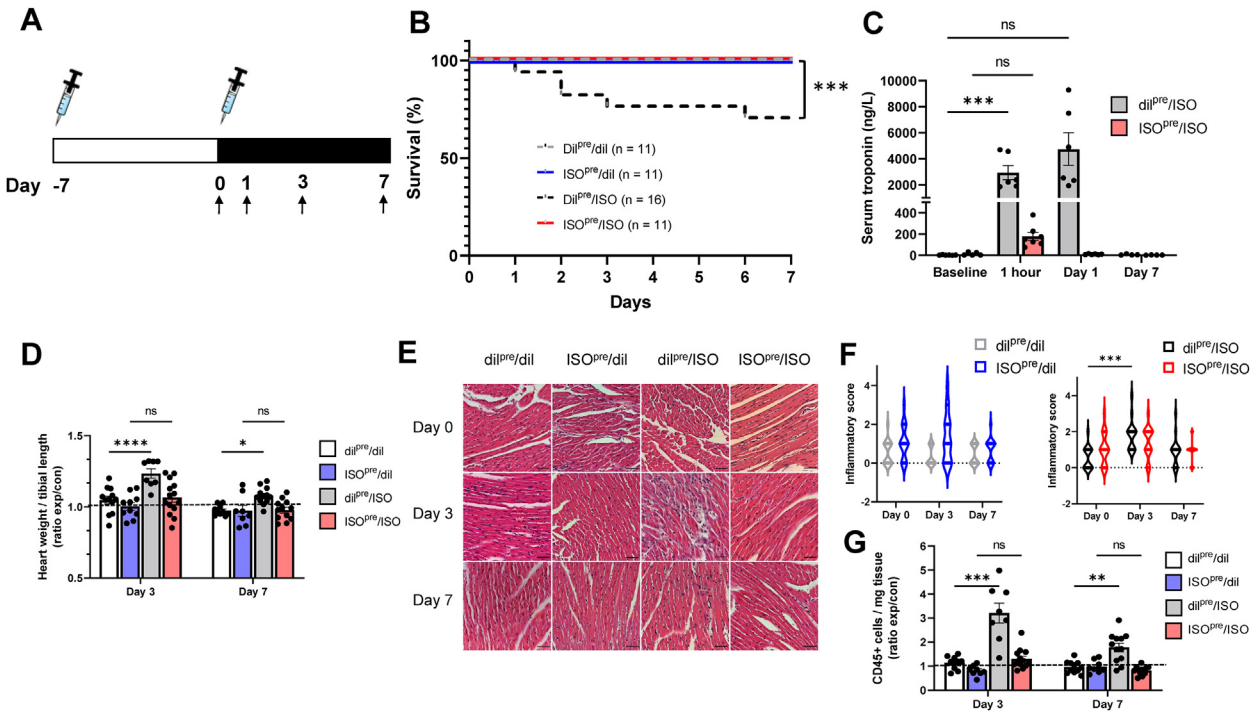
FIGURE 1 Characterization of ISO-Induced Model of Preconditioning



(A) Study protocol and time course of evaluations for isoproterenol (ISO)-induced preconditioning model. Mice were injected i.p. with ISO (300 mg/kg) or an equal volume of diluent (phosphate-buffered saline) on day −7. On day 0 (baseline), the mice receive a second i.p. injection with ISO (300 mg/kg) or diluent, and are then evaluated at baseline and days 1, 3, and 7 (**arrows** indicate the timing of evaluations). **(B)** Kaplan-Meier survival curves for diluent (dil)^{pre}/dil, ISO^{pre}/dil, dil^{pre}/ISO, and ISO^{pre}/ISO mice (n = 11-16 per group). Analyzed by log-rank Mantel Cox test. **(C)** Serum troponin I levels at baseline, 1 hour, 1 day, and 7 days following ISO injection in dil^{pre}/ISO and ISO^{pre}/ISO mice (n = 4-6 per group/time). **(D)** Heart weight-to-tibial length ratios on day 3 and 7 (data are normalized to the respective baseline values [see Supplemental Figure 3]) (n = 8-14 hearts/group/time). **(E)** Representative photomicrographs of hematoxylin and eosin-stained hearts from dil^{pre}/dil, ISO^{pre}/dil, dil^{pre}/ISO, and ISO^{pre}/ISO mice at baseline, day 3, and day 7 (20× magnification; scale bars = 50 μmol/L). **(F)** Inflammatory score index (see Methods section for details) at baseline, day 3, and day 7 (n = 18-24 fields/group/time). **Dashed line** indicates a value of zero (no inflammation). **(G)** Flow cytometry analysis of CD45+ leukocytes in the hearts of dil^{pre}/dil, ISO^{pre}/dil, dil^{pre}/ISO, and ISO^{pre}/ISO mice studied on days 3 and 7 (n = 8-14 hearts/group/time). For ease of comparison, the data are expressed as the ratio of the number of cells relative to the respective number of cells in each group at baseline (see Supplemental Figure 3). Values above the **dashed horizontal line** indicate an increase in cell number relative to baseline values. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data show mean ± SEM. **C, D, F, and G** analyzed by 2-way analysis of variance with Dunnett's correction for multiple comparisons. Outliers were excluded.

METHODS

MICE. Ten-week-old female C57BL/6J (WT; stock number 000664) and Vert-X mice (C57BL/6J; B6(Cg)-Il10$^{tm1.1Karp}$/J; stock number 014530) were purchased from The Jackson Laboratory. The mouse colonies were maintained in a pathogen-free environment at the Washington University School of Medicine and were fed standard chow diet pellets and water ad libitum.

STUDY APPROVAL. All experimental procedures were performed in accordance with approved animal protocols from the Institutional Animal Care and Use Committee at Washington University School of Medicine. These investigations conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

ISO-INDUCED PRECONDITIONING. The protocol for the ISO-induced preconditioning is illustrated in **Figure 1A**. Mice were injected intraperitoneally (i.p.) with 300 mg/kg of (−) isoproterenol hydrochloride (ISO) (Sigma-Aldrich). ISO was dissolved in endotoxin-free phosphate-buffer saline (PBS) (Millipore, 0.05 mg/μL) that was prepared on ice for immediate use, as described.[15] Control mice were injected i.p. with an equal volume of endotoxin-free PBS. ISO-pretreated (primed) and control mice were allowed to recover for 7 days to permit resolution of tissue injury and inflammation, and then they were restimulated with a second dose of ISO (300 mg/kg,

i.p.) or PBS and followed for an additional 7 days. The heart and blood samples were harvested at baseline (day 0), 1, 3, and 7 days (Figure 1A). Four groups of mice were studied: mice pretreated with an i.p. injection of diluent (dil) on day −7 and a second injection of diluent (dil^{pre}/dil) on day 0 (baseline); mice pretreated with an i.p. injection of ISO (300 mg/kg) on day −7 and diluent on day 0 (ISO^{pre}/dil); mice pretreated with an i.p. injection of diluent on day −7 and ISO (300 mg/kg) i.p. on day 0 (dil^{pre}/ISO); and mice pretreated with an i.p. injection of ISO (300 mg/kg) on day −7 and ISO (300 mg/kg) i.p. on day 0 (ISO^{pre}/ISO).

To determine whether the ISO-induced cytoprotective response was maintained over time, we exposed ISO-pretreated mice to a second ISO injection 21 and 42 days after the initial ISO injection on day −7. To remain consistent with the nomenclature that was used to designate the timing of the studies in the ISO^{pre}/ISO mice, wherein the initial ISO injection occurred on day −7 and baseline measurements were performed on day 0, for the mice that were injected 21 and 42 days after their initial ISO injection on day −7, their respective baseline measurements were performed on weeks 2 (referred to as ISO^{pre}/ISO^{2W} mice) and 5 (referred to as ISO^{pre}/ISO^{5W} mice). The heart and blood samples were harvested at baseline and 1 and 3 days later.

SERUM TROPONIN I RELEASE. Serum troponin was measured using the ARCHITECT i2000 analyzer (Abbot Laboratories). Blood was collected by mandibular bleeding in Becton Dickinson Microtainer tubes at the time of terminal sacrifice. The serum was diluted 1:4 in PBS (80 μL serum + 240 μL PBS).

EVANS BLUE DYE UPTAKE. As a second measurement of cardiac myocyte injury, we measured Evans blue dye uptake in the hearts of dil^{pre}/ISO and ISO^{pre}/ISO hearts. Briefly, 5 μL/g body weight of Evans Blue dye (Sigma) in PBS was injected i.p. on day 2 after ISO injection. Hearts were harvested 24 hours after injection of Evans blue dye, and the inferoapical region of the heart was excised and minced, weighed, and incubated at 55 °C in 1 mL formamide for 2 hours, as described by Heydemann et al.[16] The amount of Evans blue dye uptake in the samples was quantified by determining the spectrophotometric absorbance of the samples measured at 620 nm. Results are reported as arbitrary OD units/mg tissue.

GRAVIMETRIC AND HISTOLOGICAL ANALYSIS. Mice were sacrificed at baseline or at days 3 and 7 following the second injection with ISO or diluent. Hearts were removed and weighed to determine the heart weight/tibia length ratio as described.[17] Hearts were processed, paraffin-embedded, and stained with hematoxylin and eosin, as described previously.[17,18] Myocardial inflammation was assessed in sections stained with hematoxylin and eosin. The degree of myocardial inflammation was scored semi-quantitatively in the following manner: 0 = no infiltrate; 1+ = infiltrates involving <25% of the ventricular myocardium; 2+ = infiltrates involving 25% to 50% of the myocardium; 3+ = infiltrates involving 50% to 75% of the myocardium; and 4+ = infiltrates involving 75% to 100% of the myocardium, as described.[18]

2-DIMENSIONAL ECHOCARDIOGRAPHIC STUDIES.

Image acquisition. Ultrasound examination of the cardiovascular system was performed using a speckle-based strain analysis implemented in the Vevo 2100 system (VisualSonics), as described previously.[17]

Imaging protocol. Mice were imaged by echocardiography at baseline, 1 hour, 6 hours, and 7 days after second ISO injection to evaluate LV regional and global structure and function, as described.[15] Avertin (0.005 mL/g) was used for sedation for all imaging studies.

Assessment of LV regional wall motion. LV regional wall motion was assessed using a bull's eye plot that was configured to display 84 segments (7 myocardial cross-sections × 12 radial sections within each cross-section), where the inner ring represents the apex of the LV, the middle ring represents the segments of the mid-LV, and the outer ring represents the basal segments of the LV, as described.[15] LV wall motion within each segment was assessed visually as normal, hypokinetic, or akinetic, by an experienced operator (A.K.) blinded to the experimental protocol at the time of analysis. The sum of all abnormal LV segments was determined as the segmental wall motion score index (SWMSI), which represents the sum of the abnormal segments of the LV wall motion obtained from serial short-axis views divided by the total number of segments of the LV wall (values >1 indicate abnormal segmental wall motion), as described.[15]

ASSESSMENT OF LEFT VENTRICULAR CONTRACTILITY BY LANGENDORFF PERFUSION. Hearts were isolated 7 days after the mice were pretreated with diluent (dil^{pre}) or ISO (ISO^{pre}) and were perfused ex vivo at a constant pressure of 70 mm Hg at 37 °C, using a modified Krebs-Henseleit buffer (KHB) (MilliporeSigma), exactly as described.[19] All hearts were paced at 420 bpm with pacing electrodes placed on the right atrium. After a 5-minute stabilization period, the buffer was switched to KHB buffer supplemented with 1 μmol/L ISO solution. Functional

data were recorded at 1 kHz at baseline and after ISO perfusion on a data acquisition system (PowerLab, AD Instruments). LV-developed pressure was calculated as the difference between peak systolic pressure and LV end-diastolic pressure, and the resulting LV functional recovery data were expressed as the percentage of LV-developed pressure at baseline.

FLOW CYTOMETRY. Isolation of leukocytes from the heart. Single-cell suspensions of hearts were prepared and generated immediately before analysis by flow cytometry (FACS), exactly as previously described.[15] Red blood cells were lysed using ACK lysing buffer (Gibco) for 15 minutes on ice, and the remaining cells were resuspended in 450 μL of FACS buffer (PBS with 2% FBS and 2 mmol/L EDTA) and stained with conjugated antibodies (see Supplemental Table 1) for 30 minutes at 4 °C and washed with FACS buffer before analysis. Data were acquired using the Becton Dickinson LSRFortessa at the Washington University Department of Pathology Flow Cytometry and Sorting Core facility. Compensation controls were generated using UltraComp eBeads (Invitrogen) and verified on single-color control samples obtained by staining primary splenocytes. The gating strategy is summarized in Supplemental Figure 1. Cell cytometric data were analyzed by FlowJo software (Tree Star). Raw data are expressed as the number of cells per mg of heart tissue, and normalized data are expressed as the number of cells per mg of tissue at the time point of interest divided by the average number of cells per milligram of tissue for the respective baseline group.

MACROPHAGE DEPLETION. Macrophages were depleted in the hearts of ISO-preconditioned mice using clodronate-containing liposomes (Liposoma BV).[20] Control mice were injected with an equal volume of PBS liposomes (Liposoma BV). In preliminary control experiments, we determined that i.p. clodronate (400 μL) reliably depleted tissue resident cardiac macrophages within 3 days using 2 sequential doses of clodronate liposomes given 2 days apart (Supplemental Figure 2). Mice displaying abnormal behavior, rapid weight loss, clots within the left atrium, ischemic bowel or liver, or evidence for paresis or ataxia after clodronate injection were excluded from analysis.

STATISTICAL ANALYSIS. All data are presented as mean ± SEM. The Shapiro-Wilks test was applied to all data to ensure normality before parametric statistical testing. Statistical comparisons between 2 experimental groups were performed using 2-tailed Student's *t* test. Standard 1- or 2-way analysis of variance with Dunnett's (multiple comparisons to a control), Tukey's (all pairwise comparisons), or Sidak's correction for multiple post hoc comparisons were performed, where appropriate. Longitudinal analyses were not applied as individual mice were not always followed across multiple time points. The Kaplan-Meier survival curves were compared by the log-rank (Mantel-Cox) test. All data were analyzed using GraphPad Prism version 9. A P value <0.05 was considered statistically significant.

RESULTS

ISO-MEDIATED PRECONDITIONING. We have shown previously that a single intraperitoneal (i.p.) injection of 300 mg of ISO induces a brisk inflammatory response in the heart that is accompanied by LV dysfunction and LV dilation that is completely reversible following resolution of inflammation.[15] To determine whether a brief episode of ISO-induced tissue injury and inflammation confers cytoprotection in the heart, we restimulated mice with a second injection of ISO (300 mg/kg, i.p.) 7 days after the initial ISO injection, at a time when tissue injury and inflammation were resolved, and there was normalization of LV structure and function. Control mice were injected i.p. with an equal volume of diluent (PBS). As shown in **Figure 1B**, there were no deaths in the dil^{pre}/dil or ISO^{pre}/dil mice. In contrast, there was a statistically significant ($P = 0.001$) decrease in survival in the dil^{pre}/ISO treated mice, consistent with our prior observations,[15] as well as others.[21,22] However, the salient finding shown by **Figure 1B** is that there were no deaths in the ISO^{pre}/ISO mice. Troponin I levels were increased significantly ($P = 0.001$) as early as 1 hour after ISO injection and remained significantly ($P = 0.001$) elevated at 1 day following ISO injection in the dil^{pre}/ISO mice, whereas there was no significant ($P = 0.99$) increase in troponin I levels at 1 hour or at 1 day ($P = 0.99$) after ISO injection in the ISO^{pre}/ISO mice. Troponin I levels were not different from baseline values on day 7 after ISO-injection in the dil^{pre}/ISO and ISO^{pre}/ISO treatment groups, in accordance with our prior observations in this experimental model.[15] As a second measurement of cardiac myocyte injury, we measured Evans Blue dye uptake in the hearts of dilpre/ISO and ISO^{pre}/ISO mice. As shown in Supplemental Figure 3C there was a significant ($P < 0.001$) decrease in Evans blue dye uptake in the hearts of the ISO^{pre}/ISO mice, consistent with the decrease in troponin I release in the ISO^{pre}/ISO mice (**Figure 1C**). Consistent with the increase in ISO-induced myocyte injury in the dil^{pre}/ISO mice, we observed a significant ($P = 0.001$) increase in the ratio

of heart weight-to-tibia length on the third day after ISO injection (relative to baseline), which returned to near baseline levels by day 7 ($P = 0.040$) (Figure 1D). In contrast, there were no significant changes in the heart weight-to-tibia length ratios on day 3 or day 7 in the ISO^{pre}/ISO mice.

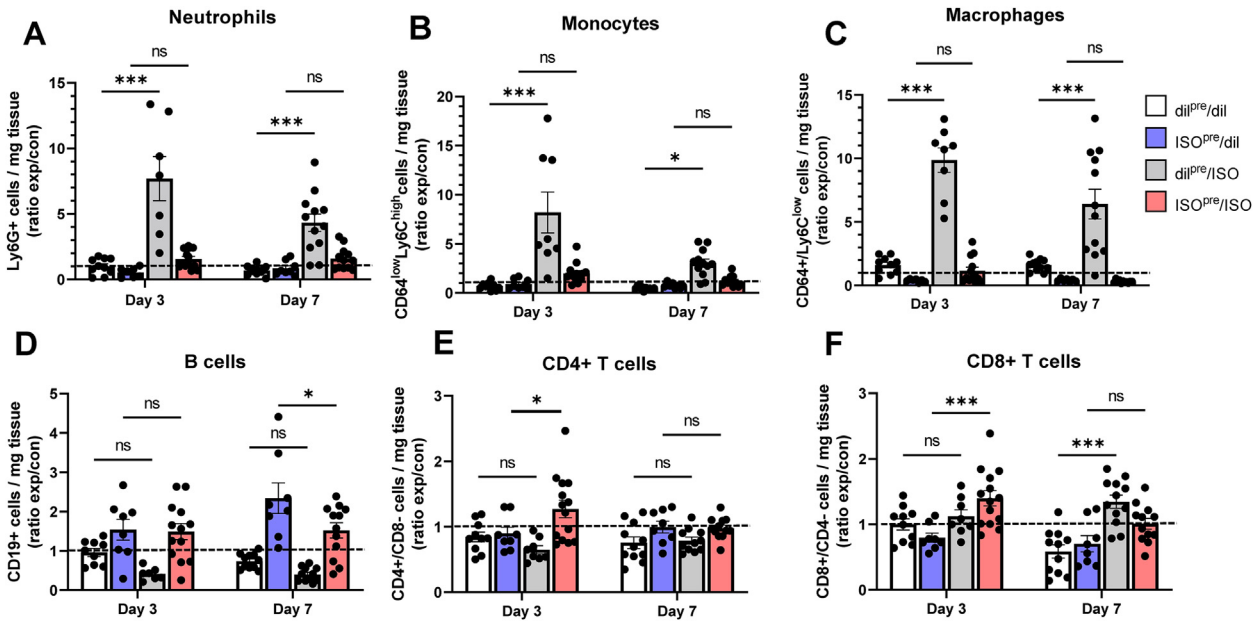
We performed tissue immunohistochemistry and FACS on dil^{pre}/dil, dil^{pre}/ISO, ISO^{pre}/dil, and ISO^{pre}/ISO mouse hearts to assess whether ISO preconditioning had an effect on the myocardial inflammatory response to tissue injury. Figure 1E displays representative hematoxylin and eosin staining of leukocyte infiltrates at baseline, day 3, and day 7 after diluent or ISO treatment, whereas the results of group data are summarized in Figure 1F. There were no significant inflammatory infiltrates in the dil^{pre}/dil, dil^{pre}/ISO, ISO^{pre}/dil, and ISO^{pre}/ISO mouse hearts at baseline, whereas there was a significant ($P = 0.001$) increase in the inflammatory infiltrates on day 3 in the dil^{pre}/ISO mouse hearts that returned to baseline levels by day 7 ($P = 0.34$ compared with baseline). Importantly, when compared with baseline levels, there was no increase in the inflammatory infiltrate score above baseline values on day 3 ($P = 0.34$) or day 7 ($P = 0.76$) in the ISO^{pre}/ISO mouse hearts. Consistent with these findings, FACS (Figure 1G) showed that relative to dil^{pre}/dil control mice, the number of CD45+ leukocytes in the dil^{pre}/ISO mouse hearts increased significantly from baseline values on day 3 after ISO-injection ($P = 0.0001$), whereas there was no significant increase in the relative number of myocardial leukocytes on day 3 ($P = 0.15$) or day 7 ($P = 0.90$) in the ISO^{pre}/ISO mouse hearts relative to ISO^{pre}/dil control subjects. Although the number of the CD45+ leukocytes decreased toward baseline values in the dil^{pre}/ISO mouse hearts by day 7, they remained significantly elevated ($P = 0.001$) when compared with baseline values in the dil^{pre}/dil mouse hearts (Figure 1G).

CHARACTERIZATION OF THE MYOCARDIAL IMMUNE CELL PROFILE FOLLOWING ISO PRETREATMENT. We next examined immune cell subsets in the dil^{pre}/ISO and ISO^{pre}/ISO mouse hearts 3 and 7 days after ISO injection. Dil^{pre}/dil and ISO^{pre}/dil mice, studied at comparable time points, served as the appropriate control groups. The gating strategy for the FACS analyses is provided in Supplemental Figure 1. To facilitate comparisons among the different groups of mice at the different time points, the FACS analyses for the different immune cell subsets are expressed as the fold-change in the number of cells relative to the number of cells at baseline (see Supplemental Figure 4 for absolute cell numbers at baseline).

The relative number of Ly6G^{+} neutrophils, Ly6C^{high}CD64^{low} monocytes, and CD64+Ly6C^{low} macrophages increased significantly on days 3 ($P = 0.001$) and 7 ($P = 0.018$ to $P = 0.001$) after ISO injection in the dil^{pre}/ISO mouse hearts relative to dil^{pre}/dil control mouse hearts, whereas the number of CD8+ T lymphocytes was increased significantly ($P = 0.001$) on day 7 (Figure 2). In sharp contrast, there was no significant increase in the number of Ly6G^{+} neutrophils, Ly6C^{high}CD64^{low} monocytes, and CD64+Ly6C^{low} macrophages in the ISO^{pre}/ISO mouse hearts relative to the ISO^{pre}/dil control mouse hearts at any time point examined. Relative to the ISO^{pre}/dil treated mouse hearts, there was a significant increase in the CD4+ T cells ($P = 0.026$) and CD8+ ($P = 0.001$) on day 3 and a significant decrease in the number of CD19+ B lymphocytes ($P = 0.019$) on day 7. Viewed together, these findings suggest that prior ISO-induced tissue injury and inflammation allows the heart to adapt (ie, become hyporesponsive) to a second exposure to ISO, with attenuated cardiac myocyte cell death and a dampened inflammatory response to tissue injury.

FUNCTIONAL SIGNIFICANCE OF ISO-INDUCED PRECONDITIONING. We have shown that a single dose of ISO provokes transient LV dilation, LV dysfunction, and LV regional wall motion abnormalities that are linked temporally with the onset and resolution of tissue inflammation.[15] To explore whether ISO preconditioning prevented ISO-induced abnormalities in LV structure and function, we performed 2-dimensional (2D) echocardiography in dil^{pre}/ISO and ISO^{pre}/ISO mouse hearts at baseline and at 1 hour, 6 hours, and 7 days after a repeat ISO injection. Figure 3A shows representative 2D echocardiographic images of the LV long-axes obtained at baseline and 1 hour, 6 hours, and 7 days after ISO injection (see also Videos 1 and 2), whereas the group data for these respective time points are summarized in Figures 3B to 3D. Consistent with our prior observations in this model,[15] ISO injection in dil^{pre}/ISO mice resulted in a transient significant increase in left ventricular end-diastolic volume (LVEDV) at 1 ($P = 0.001$) and 6 hours ($P = 0.024$), with a return to baseline values by day 7. The left ventricular ejection fraction (LVEF) was significantly ($P = 0.001$) decreased 1-hour post-ISO injection in the dil^{pre}/ISO mice, but returned to baseline values by 6 hours. Remarkably, ISO injection had no effect on LVEDV or LVEF in the ISO^{pre}/ISO mice at any of the time points that were assessed. To determine whether ISO preconditioning also protected against the development of regional wall motion abnormalities, we performed

FIGURE 2 Immunophenotyping of CD45+ Cells in the Heart Following ISO Preconditioning



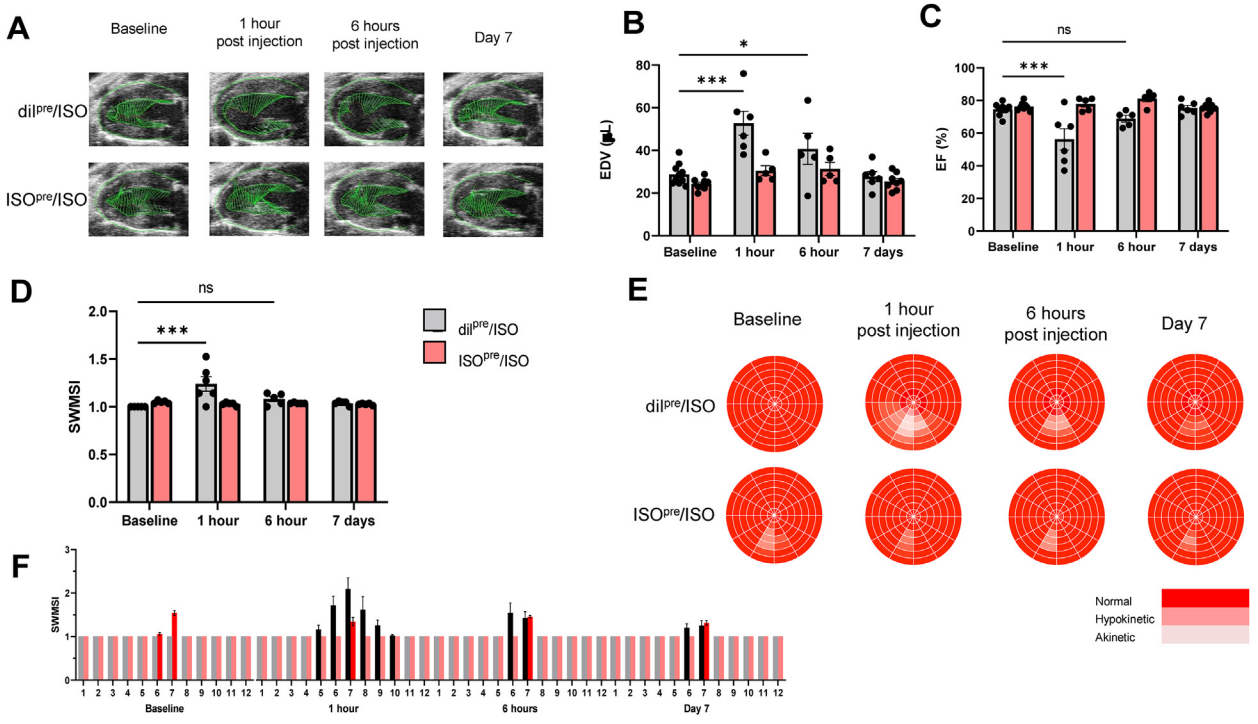
Flow cytometric analyses of myocardial CD45+ cells isolated from the hearts of dil^{pre}/dil, ISO^{pre}/dil, dil^{pre}/ISO, and ISO^{pre}/ISO mice studied on days 3 and 7 (n = 8-14 hearts/group/time). The data are expressed as the ratio of the number of cells relative to the respective number of cells in each group at baseline (see Supplemental Figure 4). **(A)** Ly6G^{+} neutrophils. **(B)** Ly6C^{high}CD64^{low} monocytes. **(C)** CD64+Ly6C^{low} macrophages. **(D)** CD19+ B lymphocytes. **(E)** CD4+ T lymphocytes. **(F)** CD8+ T lymphocytes. Values above the **dashed horizontal line** indicate an increase in cell number relative to baseline values. *P < 0.05, **P < 0.01, ***P < 0.001.Data show mean ± SEM. Analyzed by 2-way analysis of variance with Dunnett's correction for multiple comparisons. ns = not significant; other abbreviations as in **Figure 1**.

a semiquantitative analysis of LV segmental wall motion after ISO injection. **Figures 3D to 3F** show that injection of ISO in the dil^{pre}/ISO mice provoked segmental wall motion abnormalities in the mid to apical inferior LV wall segments that were detectable as early as 1 hour after ISO injection, consistent with our prior observations.[15] Although segmental wall motion abnormalities were still detectable in the mid to apical inferior wall of LV segments 6 hours after ISO injection in the dil^{pre}/ISO mice, the SWMSI was not significantly (P = 0.24) different from baseline values. There were 3 inferoapical hypokinetic segments detected at baseline in 1 of the 6 ISO-pretreated mice, consistent with prior ISO-induced tissue injury. However, the important finding shown by **Figures 3D to 3F** is that there was no significant increase in the SWMSI in the ISO^{pre}/ISO mice from baseline at 1 (P = 0.052) or 6 hours (P = 0.38), or 7 days after the second ISO-injection.

ASSESSMENT OF LEFT VENTRICULAR CONTRACTILITY BY LANGENDORFF PERFUSION. To explore the possibility that ISO-induced desensitization of myocardial β_1-adrenergic receptors could explain the decreased troponin I levels observed in the ISO^{pre}/ISO mice, we isolated mouse hearts 7 days after the mice were pretreated with diluent (dil^{pre}) or ISO (ISO^{pre}). LV contractility was assessed ex vivo in a Langendorff apparatus by measuring the change in LV developed pressure during KHB buffer perfusion and after switching the perfusate to KHB supplemented with 1 μmol/L isoproterenol. As shown in Supplemental Figure 5, the change in LV developed pressure in the isoproterenol perfused dil^{pre} and ISO^{pre} mouse hearts was not significantly (P = 0.93) different, suggesting that that ISO pretreatment did not blunt β_1-adrenergic responsivity in the ISO^{pre}/ISO hearts.

MAINTENANCE OF ISO-INDUCED PRECONDITIONING. To gain a broader understanding of whether the ISO-induced cytoprotective response was maintained over time, we restimulated ISO-pretreated mice with a second ISO injection at weeks 2 (ISO^{pre}/ISO^{2W}) and 5 (ISO^{pre}/ISO^{5W}), and then repeated assessments of tissue injury, myocardial inflammation, and LV structure and function on the seventh day after the second ISO injection (see **Figure 4A** for outline of experimental protocol). Intriguingly, there was no increase in troponin I levels 1 day after ISO injection in the ISO^{pre}/ISO^{2W} mice (**Figure 4B**). In contrast, there

FIGURE 3 Left Ventricular Structure, Function, and Regional Wall Motion in Model of ISO-Preconditioning Model

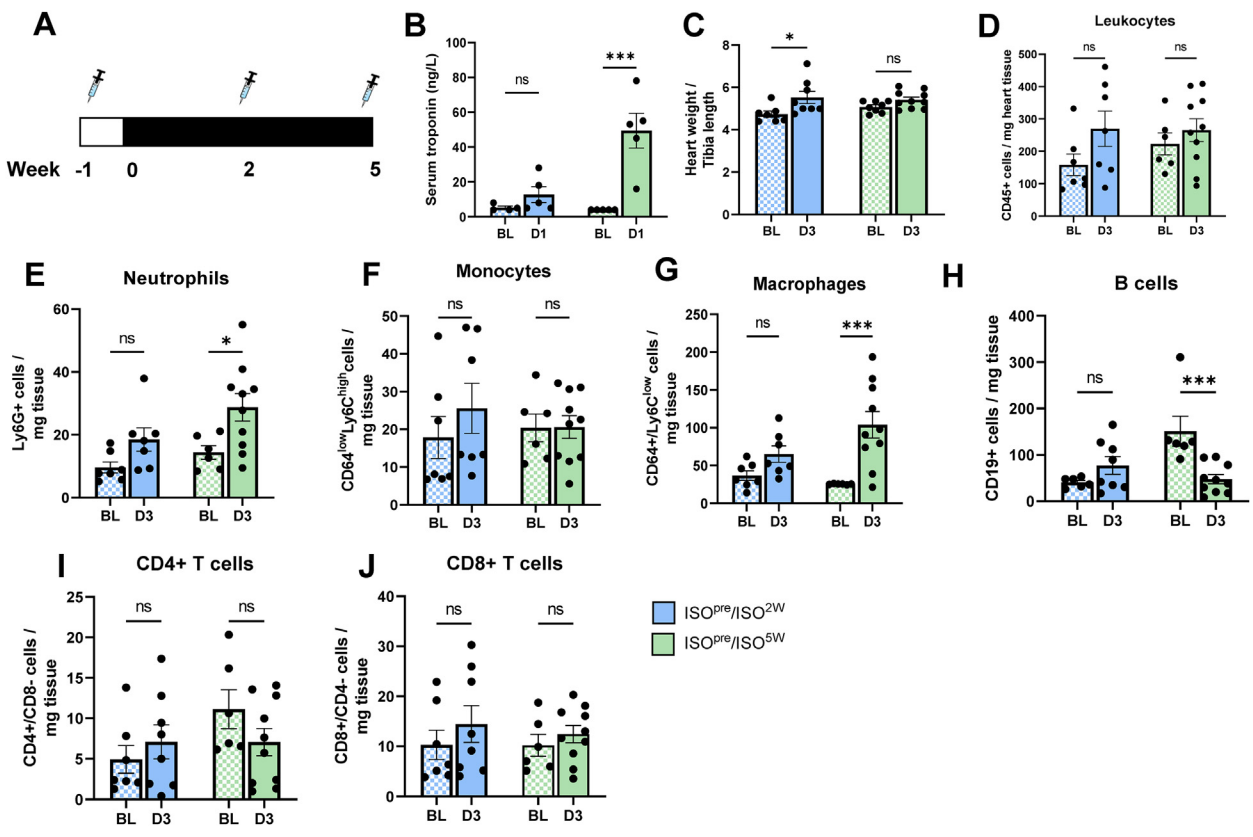


(A) Representative left ventricular long-axis 2-dimensional echocardiographic images at midsystole from dil^{pre}/ISO **(top)** and ISO^{pre}/ISO **(bottom)** mouse groups at baseline (day −1), 1 hour after ISO injection (day 0), 6 hours after ISO injection (day 0), and day 7 (see also Videos 1 and 2). Superimposed **green lines** represent speckle-tracking analysis of endocardial and epicardial contours; radially oriented vectors represent the direction and amplitude of segmental myocardial deformation. **(B)** Left ventricular end-diastolic volume (EDV), **(C)** left ventricular ejection fraction (EF), and **(D)** global segmental wall motion score index (SWMSI). € composite bulls eye plot of LV regional wall motion with 7 myocardial cross-sections (each containing 12 radial segments) from base to apex. **(F)** LV regional segmental wall motion within radial segments at baseline, 1 hour, 6 hours, and 7 days after ISO injection in dil^{pre}/ISO and ISO^{pre}/ISO mice (n = 5-7 mice/group/time). The global SWMSI was determined as the average of 84 regional LV segments, where 1 = normal wall thickening, 2 = hypokinesis, and 3 = akinesis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data show mean ± SEM. Analyzed by 2-way analysis of variance with Dunnett's correction for multiple comparisons. Abbreviations as in Figures 1 and 2.

was a small but significant ($P = 0.001$) increase in troponin I release in the ISO^{pre}/ISO^{5W} mice, suggesting a partial loss of cardioprotection at 5 weeks. Of note, the release of troponin I from the ISO^{pre}/ISO^{5W} mouse hearts was ~ 100-fold less relative to the troponin I release in the dil^{pre}/ISO mice (Figure 1C) studied at a comparable time point after ISO injection, suggesting that there was not a complete loss of the cytoprotection in the ISO^{pre}/ISO^{5W} mice. There was no difference in the heart-weight-to-tibia length ratios in the ISO^{pre}/ISO^{2W} and ISO^{pre}/ISO^{5W} mouse hearts (Figure 4C). FACS (Figure 1D) analyses revealed that relative to baseline values, the number of CD45+ leukocytes was numerically but not significantly increased from baseline values in the ISO^{pre}/ISO^{2W} ($P = 0.14$) or ISO^{pre}/ISO^{5W} ($P = 0.71$) mouse hearts.

We next examined immune cell subsets in the ISO^{pre}/ISO^{2W} and ISO^{pre}/ISO^{5W} mouse hearts at baseline and 3 days after the second ISO injection. Figures 4E to 4J show that relative to baseline values, there was no increase in the number of LY6G+ neutrophils ($P = 0.20$), Ly6C^{high}CD64^{low} monocytes ($P = 0.47$), CD64+LY6C^{low} macrophages ($P = 0.28$), CD4+ T cells ($P = 0.69$), or CD8+ T cells ($P = 0.50$) in the hearts of the ISO^{pre}/ISO^{2W} mice on day 3 after ISO injection. Compared with the findings in the ISO^{pre}/ISO^{2W} mice, there was a significant increase in the number of LY6G+ neutrophils ($P = 0.020$) and CD64+LY6C^{low} macrophages ($P = 0.001$) and a significant decrease in the number of CD19+ B lymphocytes ($P = 0.001$) in the ISO^{pre}/ISO^{5W} mouse hearts on day 3 after ISO injection relative to baseline values. Viewed together, these data suggest that the cytoprotection conferred by ISO preconditioning was persistent through 2 weeks, whereas there was a loss of some, but not all, aspects of the cytoprotective response by 5 weeks, as evidenced by the small

FIGURE 4 Maintenance of ISO-Induced Preconditioning

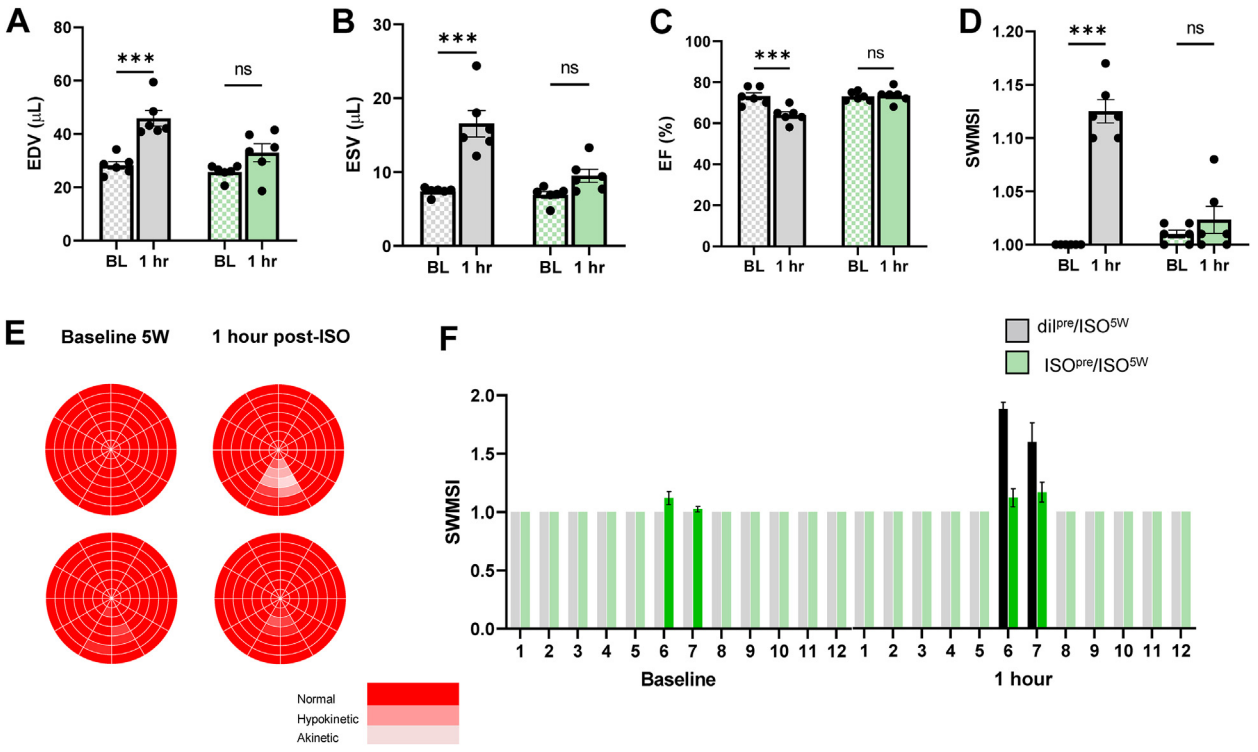


(A) Study protocol. Mice were pretreated with ISO or diluent on day −7 and then given a second injection of ISO at 2 weeks (ISO^{pre}/ISO^{2W}) or 5 weeks (ISO^{pre}/ISO^{5W}). **(B)** Serum troponin levels, **(C)** heart weight-to-tibia length ratio, **(D)** CD45+ leukocytes, **(E)** Ly6G+ neutrophils, **(F)** $Ly6C^{high}CD64^{low}$ monocytes, **(G)** CD64+$Ly6C^{low}$ macrophages, **(H)** CD 19+ B lymphocytes, **(I)** CD4+ T lymphocytes, and **(J)** CD8+ T lymphocytes in ISO^{pre}/ISO^{2W} mice at baseline (2 weeks) and day 1 after ISO injection, and ISO^{pre}/ISO^{5W} mice at baseline (5 weeks) and day 1 after ISO injection (n = 6-10 hearts/group/time). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Data show mean ± SEM. Analyzed by 2-way analysis of variance with Tukey's correction for multiple comparisons. BL = baseline; D = day; other abbreviations as in Figures 1 and 2.

increase in troponin release and the increase in Ly6G+ neutrophils and CD64+ macrophages in the ISO^{pre}/ISO^{5W} mouse hearts.

To establish whether the partial loss of cardioprotection in the ISO^{pre}/ISO^{5W} mouse hearts was accompanied by a concomitant loss of myocardial homeostasis, we assessed LV structure and function in the ISO^{pre}/ISO^{5W} mice after the second ISO injection. Dil^{pre}/ISO^{5W} mouse hearts served as the control group for this analysis. Figure 5 shows there was a significant increase in LVEDV ($P = 0.001$) and LVESV ($P = 0.001$) and a significant decrease in LVEF ($P = 0.001$) in the dil^{pre}/ISO^{5W} mouse hearts at 1 hour, consistent with our findings in the dil^{pre}/ISO mouse hearts (Figure 2). However, the important finding shown by Figure 5 is that there was a numerically small but not statistically significant increase in LVEDV ($P = 0.090$) and LVESV ($P = 0.16$) and no change in LVEF ($P = 0.96$) in the ISO^{pre}/ISO^{5W} mouse hearts at 1 hour after ISO injection. When we examined LV regional wall motion abnormalities in the $dil^{pre}/ISO^{5W}$and ISO^{pre}/ISO^{5W} hearts, we observed a significant increase in the SWMSI index in the dil^{pre}/ISO^{5W} mice at 1 hour, confirming our prior findings (Figure 3). A total of 1 of 6 of the ISO^{pre}/ISO^{5W} mice had baseline regional wall motion abnormalities in 2 segments, consistent with prior ISO-induced myocardial injury. Importantly, however, relative to baseline values there was no significant ($P = 0.49$) increase in the SWMSI index in the ISO^{pre}/ISO^{5W} group 1 hour after ISO injection. Collectively, these data indicate that despite partial loss of cytoprotection, the effects conferred by ISO preconditioning were sufficient to preserve myocardial homeostasis,

FIGURE 5 Left Ventricular Structure, Function, and Regional Wall Motion in dil^{pre}/ISO^{5W} and ISO^{pre}/ISO^{5W} Mouse Hearts



Mice were pretreated with ISO or diluent on day −7 and then given a second injection of ISO at 5 weeks (ISO^{pre}/ISO^{5W}). **(A)** Left ventricular EDV, **(B)** left ventricular end-systolic volume (ESV), **(C)** left ventricular EF, and **(D)** global SWMSI. **(E)** Composite bulls eye plot of left ventricular regional wall motion with 7 myocardial cross-sections (each containing 12 radial segments) from base to apex. **(F)** Left ventricular regional segmental wall motion within radial segments at baseline after ISO injection in dil^{pre}/ISO^{5W} and ISO^{pre}/ISO^{5W} mice (n = 5-6 mice/group/time). *P < 0.05, **P < 0.01, ***P < 0.001. Data show mean ± SEM. Analyzed by standard 2-way analysis of variance with Tukey's correction for multiple comparisons. BL = baseline; other abbreviations as in Figures 1 to 3.

insofar as LV structure and function were not significantly different from baseline in the ISO^{pre}/ISO^{5W} mouse hearts.

To explore the possibility that the diminished response to the second injection of ISO in the ISO^{pre}/ISO^{5W} mice was age-related rather than secondary to ISO-mediated preconditioning, we pretreated 16-week-old mice with ISO or diluent on day −7 and then evaluated the response to a second ISO injection 1 week later. As shown in Supplemental Figure 6, the qualitative and quantitative inflammatory response in the 16-week-old dil^{pre}/ISO overlapped the magnitude of the inflammatory response in 10-week-old mice on day 3 after ISO injection (**Figure 3**), indicating that the cytoprotective responses observed in the ISO^{pre}/ISO^{5W} were not secondary to age-related decreased responsiveness in the 16-week-old mice.

ISO-INDUCED UP-REGULATION OF LEUKOCYTE INTERLEUKIN-10. Prior studies have shown that the sympathetic nervous system can suppress inflammation through engagement and activation of beta-adrenergic receptors residing on immune cells, leading to increased secretion of interleukin (IL)-10, with a concomitant decrease in the release of IL-12, tumor necrosis factor, and other proinflammatory cytokines.[23] To determine whether the ISO-induced cardioprotection was secondary, at least in part, to up-regulation of IL-10 in myocardial immune cells, we performed a FACs analysis of leukocytes harvested from the hearts of female IL-10 Vert-X mice at baseline and 2 and 7 days after ISO injection (the gating strategies for these studies in shown in Supplemental Figure 7). When compared with baseline values there was a numerically small, statistically significant increase in the number of IL-10+ CD45+ leukocytes (P = 0.001), CD64+ macrophages (P = 0.001), CD4+ T cells (P = 0.006), or CD 8+ T cells (P = 0.009) in the hearts of the ISO^{pre} mouse hearts on day 7 when compared with the respective number of cells enumerated at baseline (Supplemental Figure 8). Although these changes were statistically significant,

the percentage of IL-10+ cells within each immune cell subset was small, and therefore was unlikely to be important biologically.

MACROPHAGE DEPLETION ATTENUATES ISO-INDUCED PRECONDITIONING. We have shown that ISO-induced tissue injury provokes the expansion of LYVE1− macrophages that had increased expression of gene modules that are enriched for genes involved in increased efferocytosis and antigen presentation,[15] which are essential for effective myocardial repair in the neonatal and adult heart.[24-26] When we examined gene lists that were related to negative regulation of an inflammatory response (GO term: 0050728), there were several gene clusters that were enriched for genes that negatively regulate innate inflammatory responses (Supplemental Figure 9C, Supplemental File). We identified 4 different subclusters of cells within the pool of LYVE1− macrophages (Supplemental Figure 10A), and noted the emergence of a new sub cluster of ApoE^{+} MS4A7^{+} macrophages (subcluster 2) on day 7 that exhibited increased expression of gene ontogenies that were associated with suppression of innate immune responses and T-cell activation on day 7 after ISO injury (Supplemental Figure 10G). Further studies will be required to determine whether the pool of ApoE^{+} MS4A7^{+} macrophages play an important role in mediating cytoprotective effects after tissue injury.

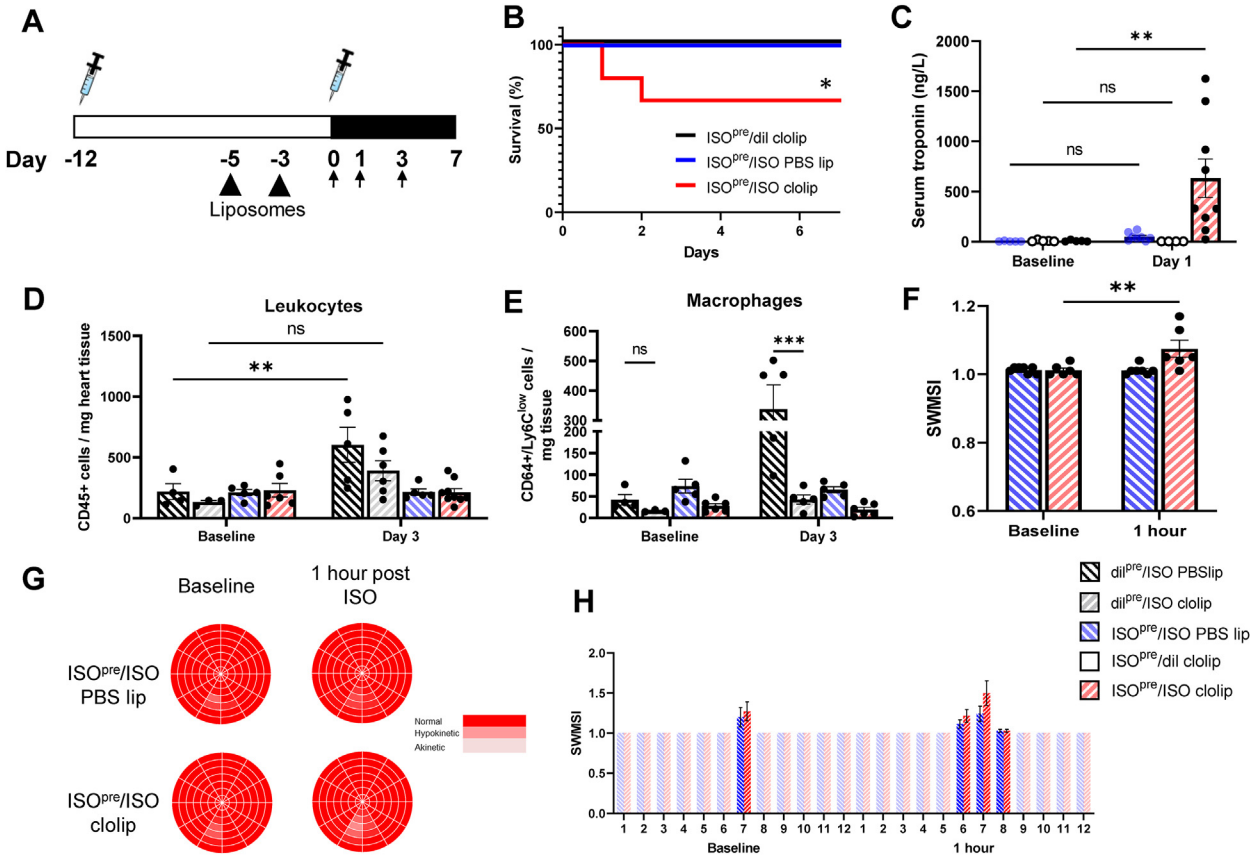
To explore the possibility that tissue-resident macrophages were responsible, at least in part, for the ISO-induced cytoprotection, we depleted macrophages using clodronate liposomes. Mice treated with an equal volume of liposome encapsulated aqueous PBS served as the appropriate control subjects. In preliminary control studies, we determined that i.p. clodronate liposomes (400 μL) reliably depleted tissue resident cardiac macrophages within 3 days after giving 2 sequential doses 2 days apart (Supplemental Figure 2). For the macrophage depletion studies presented herein, mice were pretreated with ISO or diluent on day −12 (see **Figure 6A** for protocol), and then were injected with clodronate liposomes (400 μL) or PBS liposomes (400 μL) on days −5 and day −3, followed by a second i.p. injection of ISO or diluent on day 0. As shown in **Figure 6B**, there was a significant ($P = 0.015$) increase in lethality in ISO^{pre}/ISO mice treated with clodronate liposomes, whereas there was no increase in lethality in the ISO^{pre}/ISO mice treated with PBS liposomes. Importantly, there was no increase in lethality in the ISO^{pre}/dil clodronate liposome treated mice, suggesting that the second exposure to ISO was causing the increase in lethality in the ISO^{pre}/ISO mice. Consistent with the increased lethality in the clodronate liposome-treated ISO^{pre}/ISO mice, we also observed a significant increase ($P = 0.002$) in troponin I levels on day 1 (**Figure 6C**) and a significant ($P = 0.012$) increase in Evans Blue dye uptake in the heart (Supplemental Figure 3D) in the clodronate liposome-treated ISO^{pre}/ISO mice, whereas there was no detectable release of troponin in the PBS liposome-treated ISO^{pre}/ISO mice.

With the exception of macrophages, which were significantly depleted by the clodronate liposomes, the numbers of immune cells in the other subsets were not affected by treatment with clodronate when compared with the numbers in PBS liposome-treated control mouse hearts (**Figure 6**, Supplemental Figure 11). The kinetics of the ISO-induced myocardial inflammatory response on day 3 were not different between PBS liposome and clodronate-treated dil^{pre}/ISO mouse hearts with respect to the timing of influx of LY6G+ neutrophils, CD4+ and CD8+ T lymphocytes, and CD19+ B cells (**Figures 6**, Supplemental Figure 11), and were similar to the kinetics observed in the dil^{pre}/ISO hearts (**Figure 3**), suggesting that the PBS and clodronate liposomes did not influence the immune response to tissue injury. As expected, there was a significant ($P = 0.001$) 8-fold decrease in CD64+ macrophages on day 3 in the clodronate-treated dil^{pre}/ISO mouse hearts relative to PBS liposome-treated dil^{pre}/ISO mouse hearts (**Figure 6E**), consistent with the known effects of clodronate in depleting tissue resident macrophages. 2D echocardiographic assessment of LV function showed that LV regional wall motion abnormalities were significantly ($P = 0.004$) increased 1 hour after ISO injection in the clodronate treated ISO^{pre}/ISO hearts, whereas there was no significant ($P = 0.99$) change in the SWMSI in the PBS liposome-treated ISO^{pre}/ISO hearts (**Figures 6F to 6H**). Because treatment with clodronate liposomes had a significant effect on hemodynamics in the clodronate-treated ISO^{pre}/ISO mice,[27] we did not perform 2D echocardiographic measurements of LV systolic function in these mice. Viewed together, these data suggest that clodronate liposome depletion of cardiac macrophages results in a partial attenuation of the ISO-induced cytoprotective response.

DISCUSSION

The ability of cardiac muscle to protect itself against injury has been termed *cardioprotection*.[28] The seminal studies by Murry, Jennings, and Reimer[29]

FIGURE 6 Effect of Macrophage Depletion on ISO-Induced Preconditioning



(A) Study protocol and time course of evaluations (**arrows** indicate the timing of evaluations). Mice are injected with ISO (300 mg/kg) on day −12, injected i.p. with 400 μL clodronate (clolip) or PBS liposomes on days −5 and −3, given a second injection of ISO (300 mg/kg) or diluent on day 0 (baseline), and then studied on days 1, 3, and 7. **(B)** Kaplan-Meier survival curves (n = 6-15 mice/group) and **(C)** serum troponin I levels (n = 4-9) in ISO^{pre}/dil-clolip, ISO^{pre}/ISO-clolip, and ISO^{pre}/ISO-PBS liposome mice; **(D)** CD45+ leukocytes (cells/mg tissue); and **(E)** CD64+$Ly6C^{low}$ macrophages (cells/mg tissue) in ISO^{pre}/dil-clolip, dil^{pre}/ISO-PBS liposome, ISO^{pre}/ISO-clolip, and ISO^{pre}/ISO-PBS liposome mouse hearts at baseline and day 3 after ISO injection (n = 4-9 hearts/group/time). **(F)** Global SWMSI. **(G)** Composite bulls eye plot of left ventricular regional wall motion with 7 myocardial cross-sections (each containing 12 radial segments) from base to apex **(F)** Left ventricular regional segmental wall motion within radial segments at baseline after 1 hour after ISO injection (n = 6-7 hearts/group/time). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data show mean ± SEM. Analyzed by 2-way analysis of variance with Tukey's correction for multiple comparisons. PBS = phosphate-buffered saline; PBSlip = phosphate-buffered saline liposomes baseline; other abbreviations as in Figures 1 to 3.

revealed that brief episodes of repetitive ischemia protected the heart against a subsequent bout of prolonged ischemia, whereas ensuing studies demonstrated that a variety of diverse stimuli are sufficient to activate signaling pathways that protect the heart against a future ischemic event (reviewed by Jovanovic[28] and Tsai et al[30]). Here, we extend and expand upon the paradigm of preconditioning by demonstrating that ISO-induced tissue injury and inflammation protects the heart from the myopathic effects of a subsequent exposure to ISO, including decreased ISO-induced cardiac myocyte cell death (Figure 1), reduced inflammation (Figures 1 and 2), preserved LV structure and function (Figure 3), and increased survival (Figure 1). The ISO-induced preconditioning was not secondary to desensitization of myocardial β_1-adrenergic receptors, insofar as prior exposure to ISO did not blunt the inotropic effects of ISO on LV contractility (Supplemental Figure 5). The cytoprotective responses conferred by ISO-induced preconditioning were durable and were maintained through 2 weeks, whereas there was a loss of some, but not all aspects of the cytoprotective response by 5 weeks, as evidenced by the small increase in

troponin release and the increase in Ly6G+ neutrophils and CD64+ macrophages in the hearts of mice that were re-exposed to ISO (**Figure 4**), as well as the development of increased LV regional wall motion abnormalities (**Figure 5**). However, despite the partial loss of cytoprotection at 5 weeks, the salutary effects conferred by ISO-induced preconditioning were still sufficient to preserve overall myocardial homeostasis, insofar as a second exposure to ISO did not provoke significant LV dysfunction and LV dilation (**Figure 5**).

Given the prominent role of the immune system in resolving inflammation and restoring myocardial homeostasis following tissue injury,[25,24,28] we first asked whether ISO-induced up-regulation of IL-10+ in immune cells infiltrating the heart after tissue injury contributed to the diminished inflammatory response after the second ISO injection. However, analysis of the immune cell subsets in the hearts of Vert-X IL-10 gene reporter mice on day 7 after ISO injury revealed that although there was a numerically small and statistically significant increase in IL-10+ CD45+ cells, CD64+ macrophages, CD4+ and CD8+ T cells in the heart after ISO injection, the relative number of IL-10+ cells within each immune cell subset was <14%, and was therefore unlikely to fully explain the effects of ISO-induced preconditioning (Supplemental Figure 8). Previously we demonstrated that ISO-induced tissue injury resulted in the expansion of clusters of LYVE1- macrophages, IFN-inducible macrophages and dendritic cells in the heart (Supplemental Figures 9A to 9B).[15] To explore the possibility that ISO-induced expansion of immune cell clusters may have contributed to ISO-induced preconditioning, we used clodronate liposomes to deplete tissue resident macrophages prior to the second ISO injection. Remarkably, clodronate liposome depletion of tissue resident macrophages resulted in a partial loss of cytoprotection, with increased ISO-induced cardiac myocyte cell death (**Figure 6C**), increased LV regional wall motion abnormalities (**Figures 6F to 6H**), and increased ISO-induced lethality (**Figure 6B**). Viewed together, these latter data suggest that prior tissue injury stimulate myocardial macrophage populations to release mediators that up-regulate cytoprotective programs in the heart following issue injury.

DISEASE TOLERANCE IN THE HEART. The observation that ischemic tissue injury up-regulates portfolios of cytoprotective signal transduction cascades, genes, and proteins that precondition the heart to better withstand a second ischemic injury has focused downstream research efforts in the field on identifying preconditioning stimuli that can be leveraged therapeutically to reduce ischemic injury in the clinical setting.[30-32] At the time of this writing, this therapeutic approach has not translated into improved outcomes when tested in clinical trials.[31,33] A broader teleological consideration of preconditioning suggests that this phenomenon is analogous to the concept of *disease tolerance*, which is essential for the immune responses in plants,[34] insects,[35] and mammals, including humans.[5,36,37] Disease tolerance refers to the activation of a repertoire of biological mechanisms that limit the severity of infectious diseases by protecting the host from the direct tissue damage that is caused directly by the invading pathogen and by minimizing the collateral tissue damage that occurs indirectly secondary to the activation of the innate and adaptive immune systems that are activated in order to destroy the invading pathogen.[1,5,38,39] Disease tolerance is distinct from disease resistance, which is a host defense strategy that limits disease severity by reducing pathogen load through the activation of the innate and adaptive immune systems that either destroy or neutralize the invading pathogen.[5,40]

At present, little is known about the full spectrum of tolerance mechanisms. Moreover, these concepts have, to date, focused almost exclusively on understanding the immune response to pathogen-associated molecular patterns that are associated with various infectious,[5,38,39] and have not as yet been extended to the much broader consideration of how the immune response to DAMPs released by dead or dying cells may confer cytoprotective responses that decrease/minimize direct and indirect immune-mediated collateral tissue damage in the setting of recurrent tissue injury. The results of the present study suggest that ISO-induced tissue injury and the subsequent immune response to that tissue injury reduce the susceptibility of the heart to subsequent tissue damage from a second exposure to ISO, by limiting myocyte cell death and by preserving myocardial homeostasis and LV function. The observation that clodronate liposome depletion of tissue resident macrophages attenuates ISO-induced cytoprotective response suggests that biological signals emanating from tissue resident innate immune cells confer cytoprotective responses in the heart. One of the limitations of our approach with clodronate is that it does not allow us to dissect out the individual contributions that can be ascribed to tissue resident and bone marrow–derived macrophages and dendritic

cells, because all of the cell populations are depleted with clodronate liposomes. With that said, it is likely that there is considerable redundancy of the biological functions of tissue-resident and bone marrow-derived macrophage function in the setting of acute tissue injury. And, indeed, we noted that there was up-regulation of gene clusters that suppress inflammation in multiple macrophage populations after ISO injury (Supplemental Figure 9C). Prior studies from this and other laboratories have shown that cytokines that are known to be released by macrophages and dendritic cells are sufficient to up-regulate cytoprotective responses in the heart.[10,19,41,42] With that said, it is likely that disease tolerance in the heart will not be restricted to a single cytokine, single signaling pathway, or a single mechanism, given the importance of preserving tissue homeostasis in response to the multitude of different forms of environmental injury and hemodynamic stresses that the heart encounters. Moreover, the current study did not address whether the ISO-induced cytoprotective effects are secondary to the release of cytokines/chemokines by macrophages, or by direct interactions between macrophages and cardiac myocytes. Additional studies will be necessary to address this important question.

CONCLUSIONS

This study shows that ISO-induced tissue injury and inflammation protects the heart from the myopathic effects of a subsequent exposure to ISO. The observation that the ISO-induced cytoprotective responses were partially attenuated by depleting macrophages and dendritic cells suggests that components of the innate immune response play an important role in reducing the susceptibility of the heart to subsequent tissue damage from a second exposure to ISO, and raise the intriguing possibility that immune-mediated tissue repair mechanisms may confer tolerance to tissue damage regardless of the cause of tissue damage. Although speculative, this may provide a potential explanation for why cytoprotective responses in the heart and other organs (eg, liver and kidney) are up-regulated following physical injury (eg, ischemia), environmental injury (eg, hypothermia), as well as by various toxic molecules (eg, reactive oxygen species).[28,30] Finally, the results of this study suggest that future efforts in the field of preconditioning may benefit from focusing more on the specific mechanisms that allow the immune system to restore tissue homeostasis while minimizing the collateral tissue damage from an exuberant immune response, as opposed to continued attempts to identifying specific new preconditioning stimuli.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Despite the increasing recognition that the immune system resolves inflammation and restores myocardial homeostasis following tissue injury, very little is known with respect to the role the immune system plays in recurrent myocardial injury. The present study highlights the cytoprotective role of the immune system in cardiac preconditioning following tissue injury, and discusses these findings in light of the emerging concept of immune disease tolerance.

TRANSLATIONAL OUTLOOK: The observation that ischemic tissue injury up-regulates portfolios of cytoprotective signal transduction cascades, genes, and proteins that precondition the heart to better withstand a second ischemic injury has focused downstream research efforts in the field on identifying preconditioning stimuli that can be leveraged therapeutically to reduce ischemic injury in the clinical setting. However, at the time of this writing clinical trials that have used this therapeutic approach have not translated into improved outcomes. The results of the present study show that prior isoproterenol-induced tissue injury and the resulting inflammatory response protect the heart against recurrent isoproterenol-induced tissue injury, which extends and expands upon the paradigm of preconditioning by highlighting the importance of the immune response to this process. The results of this study suggest that future efforts in the field of preconditioning may benefit from focusing more on the specific mechanisms that allow the immune system to restore tissue homeostasis, as opposed to continued attempts to identifying specific new preconditioning stimuli.

REFERENCES

1. Soares MP, Gozzelino R, Weis S. Tissue damage control in disease tolerance. *Trends Immunol*. 2014;35:483–494.

2. McDonald B, Pittman K, Menezes GB, et al. Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science*. 2010;330:362–366.

3. Zindel J, Kubes P. DAMPs, PAMPs, and LAMPs in immunity and sterile inflammation. *Annu Rev Pathol*. 2020;15:493–518.

4. Gong T, Liu L, Jiang W, Zhou R. DAMP-sensing receptors in sterile inflammation and inflammatory diseases. *Nat Rev Immunol*. 2020;20:95–112.

5. Medzhitov R, Schneider DS, Soares MP. Disease tolerance as a defense strategy. *Science*. 2012;335:936–941.

6. Frangogiannis NG. The inflammatory response in myocardial injury, repair, and remodelling. *Nat Rev Cardiol*. 2014;11:255–265.

7. Epelman S, Liu PP, Mann DL. Role of innate and adaptive immune mechanisms in cardiac injury and repair. *Nat Rev Immunol*. 2015;15:117–129.

8. Swirski FK, Nahrendorf M. Cardioimmunology: the immune system in cardiac homeostasis and disease. *Nat Rev Immunol*. 2018;18:733–744.

9. Mann DL. Stress-activated cytokines and the heart: from adaptation to maladaptation. *Annu Rev Physiol*. 2003;65:81–101.

10. Kurrelmeyer K, Michael L, Baumgarten G, et al. Endogenous myocardial tumor necrosis factor protects the adult cardiac myocyte against ischemic-induced apoptosis in a murine model of acute myocardial infarction. *Proc Natl Acad Sci U S A*. 2000;290:5456–5461.

11. Lecour S, Suleman N, Deuchar GA, et al. Pharmacological preconditioning with tumor necrosis factor-alpha activates signal transducer and activator of transcription-3 at reperfusion without involving classic prosurvival kinases (Akt and extracellular signal-regulated kinase). *Circulation*. 2005;112:3911–3918.

12. Deuchar GA, Opie LH, Lecour S. TNFalpha is required to confer protection in an in vivo model of classical ischaemic preconditioning. *Life Sci*. 2007;80:1686–1691.

13. Boengler K, Hilfiker-Kleiner D, Drexler H, Heusch G, Schulz R. The myocardial JAK/STAT pathway: from protection to failure. *Pharmacol Ther*. 2008;120:172–185.

14. Penna C, Granata R, Tocchetti CG, Gallo MP, Alloatti G, Pagliaro P. Endogenous cardioprotective agents: role in pre and post-conditioning. *Curr Drug Targets*. 2015;16:843–867.

15. Hayashi T, Tiwary SK, Lavine KJ, et al. The programmed death 1 signaling axis modulates inflammation and left ventricular structure and function in a stress-induced cardiomyopathy model. *J Am Coll Cardiol Basic Trans Science*. 2022;7(11):1120–1139.

16. Heydemann A, Ceco E, Lim JE, et al. Latent TGF-beta-binding protein 4 modifies muscular dystrophy in mice. *J Clin Invest*. 2009;119:3703–3712.

17. Adamo L, Staloch LJ, Rocha-Resende C, et al. Modulation of subsets of cardiac B lymphocytes improves cardiac function after acute injury. *JCI Insight*. 2018;3:e120137.

18. Rocha-Resende C, Weinheimer C, Bajpai G, et al. Immunomodulatory role of non-neuronal cholinergic signaling in myocardial injury. *JCI Insight*. 2019;4:e128961.

19. Evans S, Tzeng HP, Veis DJ, et al. TNF receptor-activated factor 2 mediates cardiac protection through noncanonical NF-kappaB signaling. *JCI Insight*. 2018;3:e98278.

20. Weisser SB, van Rooijen N, Sly LM. Depletion and reconstitution of macrophages in mice. *J Vis Exp*. 2012:4105.

21. Sachdeva J, Dai W, Kloner RA. Functional and histological assessment of an experimental model of takotsubo's cardiomyopathy. *J Am Heart Assoc*. 2014;3:e000921.

22. Wilson HM, Cheyne L, Brown PAJ, et al. Characterization of the myocardial inflammatory response in acute stress-induced (takotsubo) cardiomyopathy. *J Am Coll Cardiol Basic Trans Science*. 2018;3:766–778.

23. Sharma D, Farrar JD. Adrenergic regulation of immune cell function and inflammation. *Semin Immunopathol*. 2020;42:709–717.

24. Epelman S, Lavine KJ, Beaudin AE, et al. Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during Inflammation. *Immunity*. 2014;40:91–104.

25. Lavine KJ, Epelman S, Uchida K, et al. Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart. *Proc Natl Acad Sci U S A*. 2014;111:16029–16034.

26. Aurora AB, Porrello ER, Tan W, et al. Macrophages are required for neonatal heart regeneration. *J Clin Invest*. 2014;124:1382–1392.

27. Wenstedt EFE, van Croonenburg TJ, van den Born B-JH, Van den Bossche J, Hooijmans CR, Vogt L. The effect of macrophage-targeted interventions on blood pressure – a systematic review and meta-analysis of preclinical studies. *Transl Res*. 2021;230:123–138.

28. Jovanovic A. Cardioprotective signalling: past, present and future. *Eur J Pharmacol*. 2018;833:314–319.

29. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986;74:1124–1136.

30. Tsai BM, Wang M, March KL, Turrentine MW, Brown JW, Meldrum DR. Preconditioning: evolution of basic mechanisms to potential therapeutic strategies. *Shock*. 2004;21:195–209.

31. Kloner RA, Shi J, Dai W, Carreno J, Zhao L. Remote ischemic conditioning in acute myocardial infarction and shock states. *J Cardiovasc Pharmacol Ther*. 2020;25:103–109.

32. van Hoeven KH, Factor SM. Pathology of the cardiac collagen matrix: mechanical and functional effects. In: Hori M, Suga H, Baan J, Yellin EL, eds. *Cardiac Mechanics and Function in the Normal and Diseased Heart*. Springer; 1989.

33. Purroy F, Garcia C, Mauri G, et al. Induced neuroprotection by remote ischemic perconditioning as a new paradigm in ischemic stroke at the acute phase, a systematic review. *BMC Neurol*. 2020;20:266.

34. Schafer JF. Tolerance to plant disease. *Ann Rev Phytopathol*. 1971;9:235–252.

35. Ayres JS, Freitag N, Schneider DS. Identification of Drosophila mutants altering defense of and endurance to Listeria monocytogenes infection. *Genetics*. 2008;178:1807–1815.

36. Raberg L, Sim D, Read AF. Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science*. 2007;318:812–814.

37. Gozzelino R, Andrade BB, Larsen R, et al. Metabolic adaptation to tissue iron overload confers tolerance to malaria. *Cell Host Microbe*. 2012;12:693–704.

38. McCarville JL, Ayres JS. Disease tolerance: concept and mechanisms. *Curr Opin Immunol*. 2018;50:88–93.

39. Martins R, Carlos AR, Braza F, et al. Disease tolerance as an inherent component of immunity. *Annu Rev Immunol*. 2019;37:405–437.

40. de Preux Charles AS, Bise T, Baier F, Sallin P, Jaźwińska A. Preconditioning boosts regenerative programmes in the adult zebrafish heart. *Open Biol*. 2016;6.

41. Eddy LJ, Goeddel DV, Wong GHW. Tumor necrosis factor-a pretreatment is protective in a rat model of myocardial ischemia-reperfusion injury. *Biochem Biophys Res Commun*. 1992;184:1056–1059.

42. Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN. Identification of a novel role for sphingolipid signaling in TNF alpha and ischemic preconditioning mediated cardioprotection. *J Mol Cell Cardiol*. 2002;34:509–518.

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APPENDIX For supplemental videos, figures, and tables, as well as a supplemental file, please see the online version of this paper.