### Title

Selectin-targeting glycosaminoglycan-peptide conjugate limits neutrophil-mediated cardiac reperfusion injury




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# Selectin-Targeting Glycosaminoglycan-Peptide Conjugate Limits Neutrophil Mediated Cardiac Reperfusion Injury

--Manuscript Draft--

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| **Abstract:** | Aims: One of the hallmarks of myocardial infarction (MI) is excessive inflammation. During an inflammatory insult, damaged endothelial cells shed their glycocalyx, a carbohydrate-rich layer on the cell surface which provides a regulatory interface to immune cell adhesion. Selectin-mediated neutrophilia occurs as a result of endothelial injury and inflammation. We recently designed a novel selectin-targeting glycocalyx mimetic (termed DS-IkL) capable of binding inflamed endothelial cells. This study examines the capacity of DS-IkL to limit neutrophil binding and platelet activation on inflamed endothelial cells, as well as the cardio-protective effects of DS-IkL after acute myocardial infarction.<br>Methods and Results : In vitro , DS-IkL diminished neutrophil interactions with both recombinant selectin and inflamed endothelial cells , and limited platelet activation on inflamed endothelial cells. Our data demonstrated that DS-IkL localized to regions of vascular inflammation in vivo after 45 minutes of left anterior descending coronary |

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| | artery ligation induced MI. Further, findings from this study show DS-IkL treatment was cardioprotective by diminishing neutrophil and platelet-mediated injury. Mice treated with DS-IkL immediately after ischemia/reperfusion and 24 hours later exhibited reduced neutrophil extravasation, macrophage accumulation, fibroblast and endothelial cell proliferation, and fibrosis compared to saline controls.<br>Conclusions: Our findings suggest that DS-IkL has great therapeutic potential after MI by limiting reperfusion injury induced by the immune response.<br>Translational Perspective: Cardiovascular disease remains the leading cause of mortality worldwide. Acute inflammation from myocardial infarction (MI) results in damaged endothelial cells, leading to the loss of glycocalyx. Here, we designed a novel selectin-targeting glycocalyx mimetic (termed DS-IkL) capable of binding inflamed endothelial cells. DS-IkL limits neutrophil binding and platelet activation on inflamed endothelial cells. Treatment with DS-IkL in a preclinical model of MI reduces infarct size by preventing neutrophil extravasation, macrophage accumulation, fibroblast and endothelial cell proliferation, and fibrosis. Our findings suggest that DS-IkL has great therapeutic potential after MI by limiting reperfusion injury induced by the immune response. |

# ABSTRACT

**Aims:** One of the hallmarks of myocardial infarction (MI) is excessive inflammation. During an inflammatory insult, damaged endothelial cells shed their glycocalyx, a carbohydrate-rich layer on the cell surface which provides a regulatory interface to immune cell adhesion. Selectin-mediated neutrophilia occurs as a result of endothelial injury and inflammation. We recently designed a novel selectin-targeting glycocalyx mimetic (termed DS-IkL) capable of binding inflamed endothelial cells. This study examines the capacity of DS-IkL to limit neutrophil binding and platelet activation on inflamed endothelial cells, as well as the cardio-protective effects of DS-IkL after acute myocardial infarction.

**Methods and Results:** *In vitro*, DS-IkL diminished neutrophil interactions with both recombinant selectin and inflamed endothelial cells, and limited platelet activation on inflamed endothelial cells. Our data demonstrated that DS-IkL localized to regions of vascular inflammation *in vivo* after 45 minutes of left anterior descending coronary artery ligation induced MI. Further, findings from this study show DS-IkL treatment had short- and long-term cardioprotective effects after ischemia/reperfusion at the left anterior descending coronary artery. Mice treated with DS-IkL immediately after ischemia/reperfusion and 24 hours later exhibited reduced neutrophil extravasation, macrophage accumulation, fibroblast and endothelial cell proliferation, and fibrosis compared to saline controls.

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**Keywords:** inflammation, myocardial infarction, endothelial cell dysfunction, fibrosis, glycocalyx

## Translational Perspective

Cardiovascular disease remains the leading cause of mortality worldwide. Acute inflammation from myocardial infarction (MI) results in damaged endothelial cells, leading to the loss of glycocalyx. Here, we designed a novel selectin-targeting glycocalyx mimetic (termed DS-IkL) capable of binding inflamed endothelial cells. DS-IkL limits neutrophil binding and platelet activation on inflamed endothelial cells. Treatment with DS-IkL in a preclinical model of MI reduces infarct size by preventing neutrophil extravasation, macrophage accumulation, fibroblast and endothelial cell proliferation, and fibrosis. Our findings suggest that DS-IkL has great therapeutic potential after MI by limiting reperfusion injury induced by the immune response.

Cover Letter/Declaration

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October 1, 2020

Professor Tomasz J. Guzik, MD, PhD, FRCP, FACP
Editor-in-Chief, *Cardiovascular Research*
Institute of Cardiovascular and Medical Sciences
University of Glaskow, UK

Dear Editor Guzik:

Thank you for the opportunity to provide a revised manuscript entitled "Selectin-Targeting Glycosaminoglycan-Peptide Conjugate Limits Cardiac Injury After Myocardial Infarction by Abating Neutrophil Recruitment" for consideration for publication *Cardiovascular Research*. We have carefully considered the reviewer's concerns and modified the manuscript accordingly. The change to the manuscript is done in blue font for ease of identification within the manscuprt. It is also described in our response to reviewers.

On behalf of all authors, I attest that this work has not been previously published or submitted elsewhere for publication, nor have any discussions with journal editors occurred regarding the work.

Sincerely,

Alyssa Panitch
Edward Teller Professor
Department of Biomedical Engineering
University of California, Davis

We would like to again thank the editors and reviewers for the time they have taken to review our manuscript and to provide critical feedback that has helped improve to manuscript. We have fully addressed each concern below and within the manscipt.

Reviewers' comments:

Reviewer #1: The manuscript was well revised.
Thank you

Reviewer #3: No further comments.
Thank you

Reviewer #4: The authors have not adjusted their facs experiments. immunofluorescence images quantifying Ly6G+ cells and F4/80+ cells separately does reinforce the facs data that hinted that some combination of CD11b+, LyC/Ly6G+ (ie mix of myeloid cells) cell numbers are changing. The endothelial and fibroblast facs is very difficult to interpret without CD45 to gate out the hematopoietic cells that CD31 (monocytes, macrophages, T cells) and Thy1+ (all the T cells and some myeloid cells)---this is not a major point in the papers, so if the authors can not do further experiments, they need to acknowledge the caveats of this interpretation in the text.

We understand and agree with your concern. We have acknowledged the limitations of the study in the discussion be adding the following statement (lines 441-444).

It must be noted that CD45 was not used to gate out hematopoietic cells that may also be positive for CD31 or Thy1.2+. Thus, while the changes in endothelial and fibroblast cell numbers are suggestive, future work is needed to definitively show these changes.

# Selectin-Targeting Glycosaminoglycan-Peptide Conjugate Limits Neutrophil Mediated Cardiac Reperfusion Injury

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Short Title: Glycocalyx mimic limits cardiac reperfusion injury

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# ABSTRACT

**Aims:** One of the hallmarks of myocardial infarction (MI) is excessive inflammation. During an inflammatory insult, damaged endothelial cells shed their glycocalyx, a carbohydrate-rich layer on the cell surface which provides a regulatory interface to immune cell adhesion. Selectin-mediated neutrophilia occurs as a result of endothelial injury and inflammation. We recently designed a novel selectin-targeting glycocalyx mimetic (termed DS-IkL) capable of binding inflamed endothelial cells. This study examines the capacity of DS-IkL to limit neutrophil binding and platelet activation on inflamed endothelial cells, as well as the cardio-protective effects of DS-IkL after acute myocardial infarction.

**Methods and Results:** *In vitro*, DS-IkL diminished neutrophil interactions with both recombinant selectin and inflamed endothelial cells, and limited platelet activation on inflamed endothelial cells. Our data demonstrated that DS-IkL localized to regions of vascular inflammation *in vivo* after 45 minutes of left anterior descending coronary artery ligation induced MI. Further, findings from this study show DS-IkL treatment had short- and long-term cardioprotective effects after ischemia/reperfusion at the left anterior descending coronary artery. Mice treated with DS-IkL immediately after ischemia/reperfusion and 24 hours later exhibited reduced neutrophil extravasation, macrophage accumulation, fibroblast and endothelial cell proliferation, and fibrosis compared to saline controls.

**Conclusions:** Our findings suggest that DS-IkL has great therapeutic potential after MI by limiting reperfusion injury induced by the immune response.



**Keywords:** inflammation, myocardial infarction, endothelial cell dysfunction, fibrosis, glycocalyx

## Translational Perspective

Cardiovascular disease remains the leading cause of mortality worldwide. Acute inflammation from myocardial infarction (MI) results in damaged endothelial cells, leading to the loss of glycocalyx. Here, we designed a novel selectin-targeting glycocalyx mimetic (termed DS-IkL) capable of binding inflamed endothelial cells. DS-IkL limits neutrophil binding and platelet activation on inflamed endothelial cells. Treatment with DS-IkL in a preclinical model of MI reduces infarct size by preventing neutrophil extravasation, macrophage accumulation, fibroblast and endothelial cell proliferation, and fibrosis. Our findings suggest that DS-IkL has great therapeutic potential after MI by limiting reperfusion injury induced by the immune response.

# INTRODUCTION

Cardiovascular disease remains the leading cause of mortality in the United States. Approximately 805,000 Americans will be diagnosed with coronary artery disease (CAD), the most common heart disease, this year[1]. Approximately half of those diagnosed with CAD will be susceptible to acute myocardial infarction (MI), as well as complications that can lead to heart failure and sudden cardiac death[2]. Although initial damage occurs during acute MI due to oxygen deprivation during the ischemic phase, it is well documented that further injury can result from reperfusion[3]. The effects of reperfusion injury (RI) are complex. In the absence of oxygen, ischemic tissues experience severe disruptions to their homeostasis. A necessary shift from aerobic to anaerobic metabolism results in the accumulation of lactic acid, which contributes to a decline in intracellular pH, an impairment of ion exchange, and an increase in reactive oxygen species (ROS) production[3, 4], subsequently damaging cells within the vicinity. Paradoxically, the restoration of blood flow further contributes to cellular damage, partly due to inefficient oxidative

phosphorylation that results in further production of ROS, reductions in nitric oxide (NO), and the recruitment of immune cells[5, 6].

Ischemia/reperfusion induced damage to vascular endothelial cells (EC) initiates an inflammatory cascade that recruits neutrophils and platelets to the sites of damage[5]. Increased permeability of the vasculature potentiates RI by allowing the infiltration of immune cells into the tissue. Reperfused ECs experience disruptions to their $Ca^{2+}$ homeostasis and begin to contract[7], giving rise to endothelial gaps that facilitate the extravasation of leukocytes being recruited by ROS and cytokines. Though these cells have vital cardioprotective functions, overaccumulation of leukocytes and activated platelets exacerbates myocardial damage, contributing to the overall infarct size.

During RI, ECs shed their glycocalyx, a carbohydrate-rich protective layer that resides on the surface of healthy endothelium[8, 9]. The glycocalyx is known to play a role in many cellular processes, including mechanotransduction[10], microvascular permeability[11], and modulation of inflammatory mediators[12]. Diminution of the endothelial glycocalyx has been shown to contribute to vascular edema as well as neutrophil and platelet adhesion[13, 14]. While the endothelial glycocalyx has been shown to recover from enzyme-induced damage, this recovery was inversely proportional to white blood cell count[15]. The inflammatory response hence plays a critical role in RI with respect to the glycocalyx, with neutrophil influx[16, 17] and increased enzyme production[8, 18] likely delaying glycocalyx regeneration. Therefore, a molecule that provides a stable, stealthy interface with the capacity to down-regulate platelet activation and neutrophil capture could prevent inflammation-mediated RI.

To address this critical problem, we have designed a novel multivalent selectin-targeting carbohydrate conjugate (termed DS-IkL) that effectively abates neutrophil interactions within

damaged regions of the vasculature. Given the elevated levels of matrix metalloproteinases and other proteolytic enzymes within the inflammatory zone, the glycosaminoglycan-derived backbone is conjugated to several selectin binding peptides consisting of D- or unnatural amino acids in order to increase the enzymatic stability of the molecule in the harsh inflammatory environment. We directly test the hypothesis that DS-IkL will significantly interfere with neutrophil capture and platelet activation on ECs *in vitro,* and provide beneficial effects *in vivo* in a preclinical model of MI by reducing neutrophil extravasation, macrophage accumulation, fibroblast and EC proliferation, and fibrosis.

Our study demonstrates that DS-IkL interfered with neutrophil capture and adhesion on E-selectin substrates and cytokine stimulated cardiac-derived ECs. DS-IkL provided a protective effect against platelet activation on ECs *in vitro*, maintaining a similar activation state as on unstimulated ECs. In corroboration with these findings, we observed cardioprotection after a left anterior descending coronary artery (LAD) ligation-induced MI in mice *in vivo*. Treatment with DS-IkL resulted in improved cardiac function, reduced fibrosis, and a significant decrease in neutrophil, macrophage, proliferative fibroblasts, and proliferative ECs in the infarcted region. Taken together, these findings establish a therapeutic role of DS-IkL after MI by reducing reperfusion injury mediated by the immune response.

## MATERIALS AND METHODS

For more detailed methods, please see **Supplementary Materials**.

### Animal Model / IR Surgery

We used 10-16 week-old male and female C57Bl/6J mice for this study. All animal handling and laboratory procedures were performed in accordance with the approved protocols of

the Institutional Animal Care and Use Committee of the University of California, Davis, which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th Edition, 2011). Mice were selected to undergo either sham-operation, or ischemia followed by 24 hours [triphenyltetrazolium chloride (TTC) staining and cardiac troponin measurments], 36 hours [in vivo imaging (IVIS) and fluorescence assisted cell sorting (FACS) analysis], or 2 weeks (electrophysiology, histology, immunohistochemistry) of reperfusion (I/R). Mice were anesthetised with 80 mg/kg ketamine and 5 mg/kg xylazine, intraperitoneal, prior to surgery. After toe pinch and corneal reflexes were lost, ischemia was induced by ligating the proximal LAD coronary artery for 45 minutes. Anaesthesia was maintained by supplementing 1% isoflurane throughout the I/R procedure. Mice were injected with 100 µl of either 0.9% saline or 30 µM DS-IkL in saline via the tail vein immediately after reperfusion and at t=24 hours. Mice were randomly selected to receive either DS-IkL or saline. Mice were given buprenorphine twice daily for 48-72 hours post-operation (0.1 mg/kg subcutaneously). For euthanasia, mice were injected with 80 mg/kg ketamine and 5 mg/kg xylazine to achieve a surgical plane of anaesthesia followed by exsanguination upon removal of the heart. Cardiac structure and function, and neutrophil and macrophage aggregation, were assessed 2 weeks after surgery. For the *in vivo* imaging system and flow cytometry experiments, mice were allowed to recover for 36 hours after surgery. Animals were imaged on an IVIS Spectrum in vivo imaging system (Perkin Elmer) under 1% isoflurane 1, 12, 24, and 36 hours after reperfusion prior to euthanasia. For TTC staining and troponin analysis, mice were euthanized 24 hours following reperfusion.

**Peptide Library**

A combinatorial peptide library biased toward a known 7-mer selectin-binding peptide sequence, Ile-Glu-Leu-Leu-Asp-Ala-Arg[19, 20], was created using the one-bead-one-peptide split synthesis method[21] on Tentagel S resin. The library was split a total of 7 times, yielding $31^7$ unique sequences (**Figure 1)**. Protecting groups were cleaved (82.5% trifluoroacetic acid, 5% phenol, 5% water, 5% thioanisole, 2.5% triisopropylsilane) and resin screened for binding to recombinant human E-selectin Fc chimera. One sequence, Ile-(D)Lys-Leu-Leu-(D)Pro-Hydroxyproline-Arg (IkL) was selected for use in the experiments described herein.

**DS-IkL Synthesis**

Dermatan sulphate (DS, 41816 Da, Celsus Laboratories) was dissolved in phosphate buffer (pH 4.54) and reacted with 45 equivalents of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM) for 5 minutes. HyNic-GRGsIkLLpHypR (IkL) (InnoPep) was dissolved in phosphate buffer and added to the reaction. The reaction was left to complete for 48-60 hours at room temperature with constant shaking, quenched with water, then filtered using tangential flow filtration. Purified molecules were frozen and lyophilized for future use. The number of peptides bound was quantified by reading absorbance at 280 nm on a NanoDrop Microvolume Spectrophotometer (ThermoFisher Scientific) and comparing it to a standard curve of free peptide. Unless otherwise stated, molecule with an average of 14-16 peptides bound per DS was used for the present studies. For fluorescent molecules, CF594 Dye Hydrazide (Biotum) was conjugated to DS using 2 equivalents of DMTMM and the complex purified before conjugation to IkL.

**Cell Culture**

Human cardiac microvascular endothelial cells (HCMEC, PromoCell) p4-6 were used. Stimulation media was prepared by diluting to 0.4 ng/ml tumour necrosis factor alpha (TNF-α) and 0.3 ng/ml interleukin 1 beta (IL-1β) in complete endothelial growth medium MV (PromoCell).

**Neutrophil and Platelet Isolation**

Human whole blood was collected into EDTA (neutrophil experiments) or sodium citrate (platelet experiments) tubes in accordance with approved protocols of the Institutional Review Board Administration at UC Davis, which conform to the principles outlined in the Declaration of Helsinki. All participants gave informed consent prior to participation in the study. Neutrophils were isolated using an EasyStep Direct Human Neutrophil Isolation Kit (Stem Cell Technologies) and used at 0.3-2x$10^6$ cells/ml in Hank's Balanced Salt Solution with calcium and magnesium ($HBSS^{+/+}$) supplemented with 0.1% human serum albumin (HSA). For HCMEC binding experiments, neutrophils were stained with 1.5 nM Calcein-AM (BioLegend) for 30 minutes at 37˚C before use. For microsphere experiments, neutrophils were stained with AF488 anti-human CD11a/CD18 (clone m24) and PE anti-human CD15 (clone HI98) antibodies (BioLegend) for 20 minutes on ice. For platelet experiments, blood was separated by centrifugation for 20 minutes at 200xg; the resultant platelet rich plasma (PRP) layer was collected and used.

**Platelet Activation**

HCMECs were grown to confluence on 96-well CellBind plates then treated with 30 µM DS-IkL, 450 µM IkL peptide, or 30 µM DS in stimulation media for 4 hours. Wells were rinsed 2x with $HBSS^{+/+}$ then treated with 100 µL of PRP at 37˚C. After 1 hour, 45 µL of PRP was removed from each well and added to tubes containing 5 µL of ETP [107 mM

Ethylenediaminetetraacetic acid, disodium salt (EDTA), 12 mM Theophylline, and 2.8 µM Prostaglandin E1 in water]. Samples were briefly centrifuged and flash frozen.

**NAP-2 and PF-4 ELISA**

Unless otherwise stated, all incubations occurred at room temperature with shaking (700 RPM) and wells were rinsed 3x with PBS+0.05% Tween 20 (PBST) or 1% BSA in PBS between steps. Mouse monoclonal anti-hNAP-2 IgG and anti-hPF4 $IgG_{2B}$ capture antibodies (R&D Systems) were coated on 96-well EIA/RIA high binding plates (Corning) at 2 µg/mL in 1x PBS and incubated at 4˚C overnight. Wells were then blocked for 1 hour with 1% BSA. Samples were thawed and centrifuged at 2000 x g for 20 minutes and supernatant diluted 1:5000 in 1% BSA. Blocking buffer was removed (no rinse) and samples added for 2 hours. Biotinylated polyclonal goat anti-hNAP-2 IgG and anti-hPF4 IgG detection antibodies (R&D systems) were added at 0.2 µg/ml in 1% BSA for 2 hours. Streptavidin-HRP (diluted 1:200 in PBS) was then added for 20 minutes. Colorimetric change was induced with 1:1 hydrogen perodide:tetramethylbenzidine solution for 20 minutes. The reaction was stopped by addition of 2N sulfuric acid and absorbance measured at 450 nm and 540 nm. Signals were subtracted to obtain a final absorbance.

**Neutrophil Binding to E-selectin Coated Microspheres**

Fluoresbrite 641 1.75 µm carboxylate microspheres were coated with 10 µg/ml E-selectin using a PolyLink coupling kit (PolySciences, Inc.). Functionalized microspheres were treated with $HBSS^{+/+}$ (vehicle), EC-SEAL (30 µM), DS-$IkL_{10}$ (30 µM), or DS-$IkL_{15}$ (30 µM) for one hour. Microspheres were pelleted at 1000xg and resuspended in stained isolated human neutrophils, then incubated for 30 minutes at room temperature with rotation. Samples were fixed in 4%

paraformaldehyde (PFA) and read on an Attune NxT Flow Cytometer (Invitrogen). Data was analysed using FlowJo software.

**Neutrophil Binding to HCMECs**

HCMECs were grown to confluence in a tissue culture treated 96-well plate, stimulated for 4 hours, then treated with DS-IkL (30 µM), DS (30 µM), IkL alone (450 µM), or $HBSS^{+/+}$ containing 0.2% HSA (vehicle) for 1 hour. Cells were washed twice with $HBSS^{+/+}$ then treated with 50,000 Calcein-AM labelled neutrophils per well with rotation (150 RPM, 37˚C). After 30 minutes, cells were washed to remove non-adherent neutrophils and fluorescence read on a SpectraMax M5 plate reader (Molecular Devices).

**CF594-DS-IkL Binding to Selectin Surfaces**

Piranha etched glass coverslips were coated with 0.2 mg/ml protein A/G and 25 mM (bis(sulfosuccinimidyl)suberate) (BS3) overnight before being adhered to sticky-Slides VI 0.4-untreated (Ibidi). Channels were coated with E-selectin-FC or P-selectin-FC at 20 µg/ml (R&D Systems) for 1.5 hours with rocking, blocked with 0.2% HSA for 1 hour, then treated with serially diluted CF594-DS-IkL in $HBSS^{+/+}$ for 1 hour with rocking. Channels were rinsed and stored in $HBSS^{+/+}$ at 4˚C overnight before imaging to remove non-specifically bound molecule, then imaged on a Nikon TE2000 inverted microscope (Nikon, Minato, Tokyo, Japan). For each channel, five images were acquired in random locations and average fluorescence (mean grey value) within a two-dimensional region of interest (ROI) quantified using ImageJ.

**Echocardiography**

Cardiac structure and function were monitored using 2D echocardiography (VisualSonic Vevo 2100 with a MS 550D probe). All mice underwent echocardiography 2 weeks after either sham-operation or I/R surgery. Systolic function was recorded under conscious conditions, while diastolic function was recorded with isoflurane (~0.5-1%). Systolic function was acquired from 2-D M-mode images and B-mode videos at the short axis, and diastolic function was obtained from pulse-wave Doppler images.

**Fibrosis Measurements**

After 2 weeks, hearts from all four groups were fixed in 4% PFA solution in PBS, followed by paraffin embedding. Five micrometre sections were cut at the transverse plane of the heart, at the point of ligation. Sections were then deparaffinized in a series of xylene/alcohol (xylene, xylene, 1:1 xylene:ethanol, 100% ethanol, 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol; 3 minutes in each solution). These sections were stained for both Picrosirius Red and Masson's Trichrome, which provide information on the collagen level. Images were analysed in a blinded fashion.

**Cell Isolation**

Cells were isolated using a Langendorff perfusion system as previously described [22]. After isolation, cells were fixed in 0.4% PFA, and then subjected to flow cytometry.

**Flow Cytometric Analysis of Non-Myocyte Cells**

Single cell suspension was obtained from 10- to 12-week-old C57BL/6 mice as described above. The cells were re-suspended in $Ca^{2+}$ and $Mg^{2+}$ free PBS, treated with phytoerythrin-

conjugated anti-Thy1.2 (BD Bioscience, San Diego, CA), anti-CD11b, anti-Ly-6C, anti-Ly-6G (BD Bioscience), anti-CD31 (BD Bioscience), anti-MyHC (Developmental Studies Hybridoma Bank, created by NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA), and proliferation-specific Ki67 antibodies (BD Bioscience). Cells were also stained with 40 µg/ml 7-amino- actinomycin D (7AAD, BD Bioscience, San Jose, CA) to measure the DNA content. Data was collected using a standard FACScan cytometer (BD Biosciences, San Jose, CA) upgraded to a dual laser system with the addition of a blue laser (15 mW at 488 nm) and a red laser (25 mW at 637 nm Cytek Development, Inc, Fremont, CA) or Becton Dickinson LSR-II Flow Cytometer. Data was acquired using CellQuest and DIVA 6.2 software (BD Bioscience). Cells stained with isotype-matched IgG antibodies were used as controls to determine the positive cell population. Data was analysed using FlowJo software (ver9.4 Treestar Inc., San Carlos).

**IVIS Imaging**

Mice were injected with 30 µM CF594-DS-IkL or free fluorophore in 0.9% saline after surgery and after 24 hours (100 µl via the tail vein). Prior to imaging, mice were anesthetized by isoflurane inhalation. Mice (n=6 per group) were imaged in an IVIS Spectrum Imaging System (PerkinElmer). Fluorescent images were taken 1, 12, and 24 hours after the initial injection, and 12 hours after the second injection (36 hours after the initial injection). Images were analysed using Living Image system software (PerkinElmer, version 4.3.1). Two-dimensional ROI circles were drawn around the heart to quantify fluorescence and a control ROI was drawn on the flank to quantify background. Fluorescence values were normalized to background by dividing average radiant efficiency of heart by that of background.

**Immunohistochemistry (IHC)**

Hearts were fixed in 4% PFA overnight then washed with PBS the following day. Hearts were paraffin embedded and sliced into 5 µm sections. The sections were deparaffinized with xylene and rehydrated in a descending grade of ethanol. Antigen retrieval was performed in Citrate Buffer (pH 6.0, 15 mins at 95°C). Sections were permeabilized with 0.25% Triton X-100 in PBS for 30 minutes at room temperature, and then blocked with 5% donkey serum diluted by 0.25% Triton X-100. For macrophage staining, sections were incubated with rat anti-F4/80 (BioLegend, monoclonal, 1:200) and mouse anti-α-Actinin (Sigma, monoclonal, 1:200) primary antibodies. For neutrophil staining, primary antibodies used were rat anti-Ly6G (BioLegend, monoclonal, 1:200) and mouse anti-α-Actinin (Sigma, monoclonal, 1:200). Primary antibodies were diluted in blocking solution and sections incubated overnight at 4°C. Secondary antibodies used were donkey anti-mouse Alexa Fluor 488 (Invitrogen, 1:500) or donkey anti-rat Alexa Fluor 554 (Abcam, 1:500). Secondary antibodies were diluted in blocking solution and incubated for 1 hour at room temperature. Slides were mounted and then imaged using a Zeiss confocal LSM 700 microscope. Images were analysed by ImageJ.

**TTC Staining**

Hearts were embedded in 2% agarose gel in a heart matrix, then subsequently sliced into 1-1.5 mm sections. Sections were immersed in 1% TTC solution for 30 mins at 37°C. They were then fixed in 4% PFA for 20 minutes before images were taken under a dissection microscope connected to a Dino-Lite USB microscope.

### Troponin I Measurements

Troponin I (CNTI) was measured using a commercial kit (Life Diagnostics) according to the manufacturer instructions. Sample concentrations were calculated from a standard curve generated in GraphPad Prism (San Diego, CA) using 4-parameter logistic regression. Sample concentrations were multiplied by their respective dilution factors to obtain final concentration values.

### Statistics

All *in vitro* experiments were repeated at least 3 times for an $n \geq 3$. Each experiment was performed with 3-4 technical replicates. Data were analysed using one-way or two-way ANOVA with post-hoc Tukey multi-comparisons analysis. Data are represented as means ± SEM.

## RESULTS

### *Combinatorial peptide library screening generated the selectin-binding sequence, IkLLpHypR*

Selectins mediate the initial capture of leukocytes to the vascular surface[16] and are therefore a promising target for modulating leukocyte recruitment. We first set out to create a combinatorial peptide library containing unnatural amino acids, biased toward the selectin binding sequence of EC-SEAL, a molecule we previously described[19, 20]. We used the one-bead-one-compound combinatorial library method, which allows for the unrestricted incorporation of natural and unnatural amino acids (**Figure 1**)[21], to discover the sequence Ile-(D)Lys-Leu-Leu-(D)Pro-Hydroxyproline-Arg.

***DS-IkL exhibited binding to E- and P-selectin coated surfaces and reduced neutrophil binding to E-selectin in solution***

To confirm that DS-IkL binds selectins, P- and E-selectin coated surfaces were treated with increasing concentrations of DS-IkL or DS alone. Dermatan sulphate (DS) is a negatively charged proteoglycan that is known to interact with P-selectin[23]. We directly determined if the addition of IkL would improve binding to P- or E-selectin compared to DS alone. **Figure 2** shows that DS-IkL exhibited significantly enhanced binding to both selectins with increasing concentrations of molecule, which is not observed in the DS group. The ability of DS-IkL to interfere with E-selectin binding to neutrophils in suspension was further confirmed in **Supplementary Figure 1**. Treatment with DS-IkL inhibited neutrophil binding to E-selectin, but not P-selectin, at 5-30 μM concentrations by up to 35%. These reductions were similar to those seen on endothelial cell surfaces (**Figure 3D**). Higher concentrations were needed to reduce P-selectin binding. Similarly, in the presence of DS-IkL, stimulated neutrophils bound less E-selectin, but not intercellular adhesion molecule-1 (ICAM-1) (**Supplementary Figure 1**), suggesting DS-IkL preferentially binds E-selectin.

***Treatment with DS-IkL significantly reduced platelet activation on stimulated endothelial cells***

Enhanced platelet activation and binding have been implicated in a multitude of chronic inflammatory diseases, including acute coronary and pulmonary syndromes[24]. Activated platelets release cytokines such as platelet factor 4 (PF-4) and neutrophil activating peptide 2 (NAP-2) that can in turn activate neutrophils[25]. Given their contributions to neutrophil recruitment to sites of inflammation, we aimed to test if treatment of inflamed ECs with DS-IkL could reduce PF-4 and NAP-2 activation of platelets. Platelets were allowed to interact with stimulated HCMECs under

standard culture conditions for 1 hour before supernatant was collected. Levels of PF-4 and NAP-2 that accumulated in the supernatant during this time were significantly diminished in DS-IkL treated cells ($p<0.05$), as shown in **Figure 3A-B**. Notably, DS-IkL reduced NAP-2 activation on stimulated HCMECs to levels similar to unstimulated controls, implying our molecule has the potential to act as a barrier to platelet-induced neutrophil activation.

***DS-IkL reduced neutrophil arrest on E-selectin coated microspheres and stimulated endothelial cells***

The neutrophil adhesion cascade begins with initial capture and slow rolling along selectins that are upregulated on inflamed endothelium and activated platelets; therefore, interfering with this initial capture could prove therapeutically beneficial. To test our molecule's ability to block neutrophil-selectin interactions, E-selectin coated microspheres were treated with DS (30 μM), IkL peptide (450 μM), DS-IkL (30 μM), DS-$IkL_{10}$ (30 μM), or vehicle prior to incubation with isolated human neutrophils. Only IkL peptide and DS-IkL were able to reduce neutrophil adhesion to microspheres to approximately 75% of control ($p<0.05$, **Figure 3C**), with DS and DS-$IkL_{10}$ exhibiting similar neutrophil-microsphere colocalization as control. Given the importance of tightly regulated neutrophil capture and activation in proper wound healing[26], this slight reduction could be the tipping point back toward a restorative state.

We next sought to investigate if the effect of DS-IkL on neutrophil-selectin interactions was retained on ECs, which are known to upregulate their selectin expression upon stimulation with inflammatory cytokines such as TNF-α and IL-1β[27]. HCMECs were treated with DS-IkL under stimulatory conditions prior to incubation with neutrophils. Neutrophils exhibited reduced adhesion to treated ECs as compared with vehicle controls ($p<0.01$, **Figure 3D**), to a similar extent

as on selectin-coated microbeads. These results support our hypothesis that DS-IkL reduces selectin-mediated neutrophil capture at sites of inflammation.

***DS-IkL targeted to the heart after I/R***

Since DS-IkL suppressed neutrophil adhesion *in vitro*, we investigated whether this would occur *in vivo* following a myocardial infarction, induced by 45 minutes of ischemia at the LAD with subsequent reperfusion. To verify proper occlusion of the LAD, we monitored ECG recordings during occlusion (**Supplementary Figure 1**). Only I/R mice with ST-segment elevations were included in the I/R group. Immediately after sham or LAD operation, we injected either 30 μM DS-IkL conjugated to a fluorophore (CF594-DS-IkL) or saline with equal concentration of the fluorophore, and monitored *in vivo* fluorescent intensity at 1, 12, 24, and 36 hours after surgery. Representative images of all four groups are shown in **Figure 4A**. As evidenced by these images, fluorescence signals were primarily localized to the cardiac region only in the I/R group after the CF594-DS-IkL injection. We observed some targeting to throat regions that sustained damage during tracheotomy, as well as background fluorescence in the abdomen, likely from autofluorescent components of mouse chow[28]. Quantitatively, signal intensity was significantly higher an hour after surgery in mice treated with DS-IkL after I/R, relative to mice treated with the fluorophore dissolved in saline ($p<0.001$, **Figure 4B**). There was no difference in fluorescent intensity in the cardiac region in saline sham mice vs saline I/R mice, suggesting that the molecule just circulated in the blood, but did not localize to the heart. In addition, fluorescent signal persisted for at least 24 hours in the cardiac region after I/R surgery in mice treated with DS-IkL. After a subsequent dosage at the 24-hour mark, no noticeable difference was observed in this group relative to the other groups at the 36-hour time point. Taken together,

our *in vivo* imaging suggested that DS-IkL targeted to the heart, and the binding of DS-IkL lasted for at least 24 hours.

***Mice treated with DS-IkL exhibited reduced infarct size and improved cardiac function after I/R***

To examine the *in vivo* effects of DS-IkL on cardiac structure and function, we performed either sham or 45 mins I/R. To assess the short-term effects of DS-IkL on cardiac injury, we administered one injection of either saline or DS-IkL immediately after surgery and examined the degree of myocardial infarction and extent of cardiac injury after 24 hours of reperfusion. Representative sections stained with TTC are displayed in **Figure 5A**. As evident from the sections, I/R induced a more pronounced increase in damaged, non-viable cardiac regions, relative to DS-IkL treated mice. Sham-operated sections are displayed in **Supplementary Figure 4**. Quantification, percentage of infarcted region relative to left ventricular area, demonstrated a significant reduction in infarct size in DS-IkL hearts (**Figure 5B**). Cardiac injury was assessed by measuring cardiac Troponin I. Although there was not a significant difference, there was a trend towards a reduction in injury at 24 hours post injury (**Figure 5C**).

To determine the long-term effects of DS-IkL, we performed the same procedure, but mice were subjected to 2 weeks of reperfusion. To ensure robust activity of DS-IkL, we administered an additional dose of DS-IkL 24 hours after surgery on top of the dose given immediately after surgery. Cardiac structure and function were assessed 2 weeks post-operation. Representative M-mode tracings at the parasternal short-axis are shown for all four groups and display the beat-to-beat changes in wall thickness and diameter of the left ventricle (**Figure 5D**). Recordings were performed in conscious mice; heart rate in all groups were therefore similar (**Figure 5E**).

Structurally, I/R resulted in an increase in the left ventricular mass in groups treated with either saline or DS-IkL, relative to their respective sham-operated groups ($p<0.05$, **Figure 5F**). Similar to our echocardiography findings, I/R induced an increase in heart weight to tibial length ratio in mice treated with either saline or DS-IkL, relative to their sham-operated controls (**Supplementary Figure 2)**. Even though I/R resulted in a reduction in fractional shortening (**Figure 5G**) and ejection fraction (**Supplementary Table 1**) in both groups, there was a significant improvement in fractional shortening in the DS-IkL treated mice compared to saline alone ($p<0.05$, **Figure 5G**). Furthermore, strain analysis revealed a significant reduction in global radial strain (**Figure 5H**) in both I/R groups relative to their respective sham groups. However, the decrease was significantly less in DS-IkL mice treated groups. Although the local radial strain was not significant (**Figure 5I**), the small improvement in all the different regions of the heart contributed to an overall and global increase in global radial strain in DS-IkL mice. Global circumferential strain was significantly reduced in saline treated mice relative to their control sham (**Figure 5J**). However, this was not observed in DS-IkL treated mice.

Diastolic function was assessed using the blood flow velocity through the mitral valve during the cardiac cycle. Representative tracings using pulse-wave Doppler show two distinct waveforms, which correspond to left ventricular filling during early diastole (E wave) and left ventricular filling during late diastole (A wave, **Figure 5K**). The ratio of the two provides an indication of diastolic function[29]. Mice treated with DS-IkL did not exhibit a change in diastolic function, as compared to their sham control; whereas, mice treated with saline showed a significant reduction in the E/A ratio, indicating an impairment in diastolic function (**Figure 5L**). Indeed, the E/A ratio of mice treated with DS-IkL was significantly higher than mice treated with saline, after I/R ($p<0.05$). Although the isovolumetric relaxation time (IVRT, **Figure 5M**) did not change

significantly, the MV deceleration time was significantly elevated in the I/R group treated with saline, relative to the saline sham group ($p<0.05$, **Figure 5N**). This was not seen when the mice were treated with DS-IkL. Together, these data suggest that both systolic and diastolic function was significantly improved with the treatment with DS-IkL.

***DS-IkL limited fibrosis after I/R***

MI induces loss of cardiomyocytes with concomitant increase in fibrosis[30]. Histological analyses of cardiac sections were performed 2 weeks after I/R. Representative whole heart images of all four groups, and short-axis sections stained with Masson's Trichrome (MT) and Picrosirius Red (PSR) are shown (**Figure 6A**). Percentages of fibrotic area relative to the total left ventricular area were quantified in a blinded fashion and were low and not significantly different in the sham-operated groups. In contrast, there was a significant elevation in collagen deposition in both I/R groups, relative to their respective sham-operated controls ($p<0.01$, **Figure 6B**). However, I/R mice treated with DS-IkL showed significantly less fibrosis than mice treated with saline (**Figure 6D-E**, $p<0.05$), consistent with TTC data observed 24 hours after I/R (**Figure 5A-B**). Together, the data show that DS-IkL limited fibrosis, which in part contributed to the improved cardiac function as assessed by echocardiography.

***DS-IkL prevented neutrophil and macrophage aggregation, fibroblast proliferation, and endothelial cell proliferation after I/R***

I/R causes cardiac endothelial cell dysfunction, leading to an amplified inflammatory state and an increase in vascular permeability[31]. If not tightly regulated, immune cells recruited to the damaged regions accentuate damage and contribute to tissue fibrosis. To determine the effect of

DS-IkL on neutrophil and fibroblast accumulation, we isolated non-myocyte cell populations from the hearts from all four groups 36 hours after sham or I/R surgeries. Flow cytometric analysis showed a significant increase in $CD11b^+/Ly6\text{-}C/G^+$ neutrophils in the saline treated I/R group compared to sham controls ($p<0.05$), but no significant increase in the DS-IkL treated I/R group **(Figures 7A-B)**, suggesting that treatment with DS-IkL limited neutrophil accumulation after I/R. Furthermore, there was a significant increase in both total ($CD31^+$) and proliferative ECs ($Ki67^+/CD31^+$) in the saline I/R group, but not in the DS-IkL treated I/R groups (**Figures 7C-E**), suggesting a significant decrease in adverse vascular remodelling by DS-IkL. Moreover, the percentage of proliferative fibroblasts ($Thy1.2^+$) in the saline I/R group was significantly higher than in the DS-IkL treated I/R group (**Figures 7F-H**), consistent with the significant increase in collagen deposition observed 2 weeks after reperfusion. It must be noted that CD45 was not used to gate out hematopoietic cells that may also be positive for CD31 or Thy1.2. Thus, while the changes in endothelial and fibroblast cell numbers are suggestive, future work is needed to definitively show these changes.

Although the immune response is heightened shortly after MI, the effects can manifest long-term as the body tries to re-establish homeostasis. To determine the long-term effects of DS-IkL on neutrophil and macrophage accumulation, we performed immunohistochemistry on all four groups after 2 weeks of I/R. Representative images of IHC are shown in **Figure 7I**. Tissue sections were stained with α-actinin to stain for cardiomyocytes, F4/80 to stain for macrophages, and Ly6G to stain for neutrophils. DS-IkL treatment significantly reduced the number of macrophages per area (**Figures 7I and 7J**) and neutrophils per area (**Figures 7I** and **7K**) at both 36 hours (Figure 7I) and 2 week (Figures 7J and 7K). Together, our data suggest that DS-IkL limited neutrophil and macrophage aggregation, as well as fibroblast and EC proliferation. Mechanistically, DS-IkL

treatment prevented multiple aspects of the immune response by acting as a molecular bandage on the damaged endothelial surface, which ultimately resulted in less fibrosis and improved cardiac function (**Figure 7L**).

# DISCUSSION

Inflammatory responses significantly contribute to further injury after the initial insult of an MI. Ischemic coronary vessels undergo drastic alterations to their microstructure and homeostasis upon reperfusion, conducive to immune cell infiltration. Ion imbalance disrupts cell-cell junction integrity along the endothelium[32], creating migration points for neutrophils and macrophages that are recruited to these cytokine-rich environments [31]. Furthermore, ECs exhibit signs of endothelial cell dysfunction, namely enhanced cell contractility, upregulated cell adhesion molecules such as E- and P-selectin, and the loss of the glycocalyx[10, 31]. The dysfunctional EC phenotype facilitates neutrophil recruitment and extravasation. Neutrophils that have been recruited to these sites activate and secrete a milieu of pro-inflammatory cytokines that in turn recruit additional leukocytes[33], shifting the inflammatory process from restorative to destructive. Therapeutics that limit the initial binding and activation of circulating immune cells could tip the balance back to a restorative state.

## Design of the novel DS-IkL

We previously described a molecule termed "EC-SEAL" composed of oxidized dermatan sulphate (DS), to which known selectin-binding peptides were conjugated via a heterobifunctional crosslinker[19]. Although EC-SEAL mitigated the immune response *in vitro*, it is not an acceptable drug candidate for the clinic. The chemistry used to functionalize the DS uses glycol splitting by

periodate oxidation, a mechanism which cleaves carbohydrate rings at vicinal diols to create a reactive intermediate[34]. This chemistry renders the molecule unstable; thus, it cannot be stored long-term. Furthermore, the chain conformation changes dramatically, resulting in a chain coiled polymer chain rather than an extended linear structure seen with natural glycosaminoglycans.

In this study, we designed an improved molecule termed DS-IkL. We demonstrated that DS could be modified with peptides without cleaving the ring structure and still maintain the ability to bind to the selectin receptors to interfere with platelet and neutrophil binding. Importantly, this new chemistry, which better maintains DS structure, eliminates the reactive intermediates that reduced the stability of EC-SEAL. Additionally, the selectin binding sequence in EC-SEAL consisted solely of L-amino acids, which are readily degraded by proteolytic enzymes present at sites of inflammation. Though not directly addressed here, we believe that the incorporation of D-amino acids within the selectin binding sequence delays DS-IkL's degradation and clearance, supporting its presence on ECs for at least 24 hours.

To design DS-IkL, we first created a combinatorial peptide library containing unnatural amino acids, biased toward the E-selectin binding sequence of EC-SEAL[19, 20]. We chose to use the one-bead-one-compound combinatorial library method, which allows for the unrestricted incorporation of natural and unnatural amino acids[21], to discover the sequence I-k-L-L-p-Hyp-R. Our preliminary efforts focused on the ability of IkL peptide and our DS-IkL glycoconjugate to bind selectins (**Figure 2**) and interfere with neutrophil binding to E-selectin coated microspheres (**Figure 3A**). Selectins mediate the initial capture of leukocytes to the vascular surface[16], and are therefore a promising target for modulating leukocyte recruitment. We hypothesized that selectins blanketed with our glycoconjugate would be masked from circulating leukocytes, therefore limiting their capture. In line with this hypothesis, we observed significant reductions in neutrophil

binding to both selectin coated microspheres and stimulated ECs upon treatment with DS-IkL$_{15}$, suggesting DS-IkL$_{15}$ interacted with upregulated selectins on the cell surface.

**DS-IkL reduced platelet activation on endothelial cells**

Given the destructive role platelets play in myocardial I/R injury[17, 35], we were interested in DS-IkL's effect on the activation state of platelets incubated on stimulated ECs. Interestingly, we saw significant reductions in NAP-2 levels and a trend toward reduction in PF-4 in cultures that had been pre-treated with DS-IkL as compared to HBSS treated controls, suggesting ECs treated with DS-IkL presented fewer available platelet activating ligands such as endothelial P-selectin. Though we did not observe significant reductions in PF-4, a chemokine which induces firm adhesion of neutrophils in the presence of TNF-α[25, 36], we believe our modest results may be an artefact of the experiment. PF-4 has an affinity for GAGs[36] and therefore may have been sequestered by DS-IkL or heparin on the cell surface or within the media.

In addition to direct damage caused by activated platelets, these bioactive blood components have been shown to play a pivotal role in neutrophil capture along inflamed endothelium, offering an anchor point via platelet P-selectin and facilitating endothelial transmigration[24, 35]. Although E- and P-selectin are very similar molecules, both in structure and function, we observed slight differences in binding to E- and P-selectin coated surfaces (**Figure 2**), with DS-IkL exhibiting more consistent binding to E-selectin at higher concentrations. Our results suggest that IkL peptide plays a more influential role in E- and P-selectin binding when DS-IkL is administered at higher concentrations, whereas at lower concentrations, selectin binding may be influenced more by DS. By interacting with both P- and E-selectin, DS-IkL may simultaneously protect from neutrophil interactions, platelet aggregation, and the formation of

platelet-neutrophil complexes. Therefore, DS-IkL presents a two-fold cardioprotective potential by limiting damage from both activated platelets and neutrophils.

**DS-IkL improved cardiac function by disrupting components of the immune response**

Since one of the hallmarks of reperfusion injury is overactivation of the immune system due to leukocyte accumulation and platelet activation, we assessed the potential therapeutic benefits of DS-IkL in a clinically relevant murine model of I/R. Our data show that DS-IkL localized to the damaged cardiac region (**Figure 4**) and limited neutrophil and fibroblast accumulation after I/R, which resulted in less tissue fibrosis (**Figure 6**), reduced myocardial infarction, and improved cardiac function (**Figure 5**). Interestingly, DS-IkL treated I/R mice did not exhibit the increase in total and proliferative ECs that was observed in the saline I/R group. In some cases, a heightened presence of proliferative ECs may indicate tissue regeneration[37]; however, the saline treated I/R group exhibited poor cardiac function and enhanced fibrosis. Our results therefore suggest that rather than regeneration, early remodelling and myocardial hypertrophy was beginning to occur, necessitating an increase in cardiac angiogenesis[38].

**Proposed Mechanism of DS-IkL**

Other attempts have been made to limit I/R induced injury after MI[39], including ischemic pre- and postconditioning, pharmacologic interventions targeting metabolic pathways that contribute to RI[40, 41], and the use of monoclonal antibodies to limit platelet and neutrophil adhesion[39]. In particular, antibody therapy to P-selectin[42, 43] and ICAM-1 have been investigated as cardioprotective therapies against neutrophil and platelet mediated RI[44]. However, as evidenced by the SELECT-ACS trial evaluating the effect of Inclacumab[45], a recombinant monoclonal

antibody against P-selectin, a major drawback of antibody therapy is the need for high doses for therapeutic effect[46], lending to high production costs[47]. It is worth noting that monoclonal P-selectin antibody crizanluzumab[48] (awarded Breakthrough Therapy designation) and the pan-selectin inhibitor Rivipansel[49] (failed at Phase 3) have shown promise in vaso-occlusive crises in sickle cell disease. However, neither of these have been tested in RI.

Our strategy is unique from antibody therapeutics. DS-IkL is generally smaller than antibody therapeutics (~62 kDa) and has been designed to interrupt multiple parts of the immune pathway simultaneously by masking adhesion molecules on the endothelial surface. We designed a *molecular bandage* that targets DS – a native component of the glycocalyx – to inflamed endothelium. By blanketing DS over the dysfunctional endothelial surface, we aimed to directly reduce selectin availability while indirectly interfering with immune cell interactions with other adhesion molecules on the endothelial surface. We demonstrated that DS-IkL binds to E- and P-selectin, limited neutrophil and platelet adhesion to inflamed ECs, reduced neutrophil, macrophage, and fibroblast accumulation after MI (**Figure 7**), and improved cardiac function after MI. We conducted our current study at a treatment concentration of 30 μM. **Figure 2** suggests DS-IkL may have a greater therapeutic benefit at higher concentrations; therefore, future studies will be required to determine the concentration of DS-IkL that produces the optimum therapeutic effect, as well as to further elucidate the mechanism by which DS-IkL works.

## Conclusions

We created DS-IkL using the one-bead-one-peptide synthesis approach. *In vitro*, DS-IkL bound to both P- and E-selectin coated surfaces and prevented neutrophil binding and platelet activation. *In vivo,* DS-IkL localized to the cardiac region in mice after MI and limited neutrophil

and fibroblast accumulation. Mice treated with DS-IkL exhibited improved cardiac function and less fibrosis. Taken together, our data suggest that our recently developed, novel DS-IkL molecule reduced the inflammatory response induced by MI, which resulted in cardioprotection.

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## Authors' Contributions

TD and PNT designed the research study, conducted experiments, acquired data, analysed data, and wrote the manuscript. HS, LR, and PS conducted experiments, acquired data, analysed data. CN and VT conducted experiments. JLO analysed data. XL and KL helped conceptualize and design the peptide library. NC and AP designed the research study, provided monetary support, and wrote the manuscript.

## Data Availability Statement

The data underlying this article are available in the Dryad Digital Repository, https://doi.org/10.25338/B8M32V.

## Conflict of Interest

The authors claim no conflict of interest.

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# FIGURES

**Figure 1. One-bead-one-peptide library synthesis. A**) A combinatorial peptide library was designed using the split synthesis method of Lam et al. biased toward a known E-selectin binding sequence. (1) Resin was split in half; (2) half was coupled to the first amino acid of the selectin binding sequence and the other half divided evenly into 30 tubes, each containing a different amino acid. (3) After coupling was complete, resin was combined and deprotected. (4) The cycle was repeated for each amino acid in the selectin-binding sequence to create a library with $31^7$ unique peptide sequences. **B**) In each step, resin is divided in two and coupled to a different amino acid, $b_x$ or $a_x$. The cycle is repeated three times to create a library with $2^3$ unique sequences. **C**) An example of the screening result is shown. Resin with peptide that bound E-selectin can be seen in brown (red arrows). This figure was created with BioRender.

**Figure 2. Improved binding to immobilized selectin surfaces with DS-IkL.** P- and E-selectin coated surfaces were treated with CF594-DS-IkL (30 µM, red) or CF594-DS (30 µM, black). CF594-DS-IkL binding increased with increasing concentration, as assessed by mean fluorescence intensity (MFI) normalized to vehicle (HBSS). Data are represented as means ± SEM of 3 biological replicates. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by two-way ANOVA with post-hoc Tukey test.

**Figure 3. Treatment with DS-IkL reduced neutrophil binding and platelet activation.** Platelet activation on stimulated HCMECs as assessed by **A)** NAP-2 and **B)** PF-4 was reduced after treatment with DS-IkL (30 µM) and/or IkL peptide (450 µM), but not DS (30 µM). n=3 biological replicates. **C)** E-selectin coated microspheres were treated with HBSS, DS-IkL, IkL peptide, or DS prior to incubation with isolated human neutrophils (PMN). Data represent PMN binding to microspheres as assessed by flow cytometry. n=3-6 biological replicates. **D)** DS-IkL reduced PMN binding to stimulated HCMECs toward the level of unstimulated controls ($p=0.09$). n=3-4 biological replicates. All data are represented as means ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by one-way ANOVA with post-hoc Tukey test.

**Figure 4. DS-IkL targeted to the heart after I/R.** Animals were injected with either CF594 dissolved in saline or CF594-DS-IkL after surgery and 24 hours later. **A**) Representative images taken 1 hour after each injection, and every 12 hours after reperfusion until the 36-hour time point. **B)** Data summary of CF594 (black bars) and CF594-DS-IkL (red bars) targeting to heart at 1, 12, 24, and 36 hours after surgery. Black arrows depict injections. Data are represented as means ± SEM of n=6 mice per group. * $p<0.05$, ** $p<0.01$; *** $p<0.001$ by two-way ANOVA with post-hoc Tukey test.

**Figure 5. Mice treated with DS-IkL exhibited reduced infarct size and improved cardiac function after I/R. A)** After 24 hours of reperfusion, we assessed size of myocardial infarction using TTC staining. Representative sections are shown. **B**) Quantification revealed an increase in infarct size with saline treated mice. **C**) Cardiac injury was also assessed using cardiac troponin I.

After 2 weeks of reperfusion, mice were subjected to conscious echocardiography to examine the cardiac structure and function. **D**) Representative M-mode images at the parasternal short-axis for all four groups are depicted. **E**) Heart rate was not significantly different in these conscious mice. **F**) I/R induced an increase in left ventricular (LV) mass, and **G**) reduced fractional shortening and **H**) global radial strain. **I**) Summary data of region-specific radial strain and **J**) circumferential strain are displayed. **K**) Diastolic function was assessed by the blood flow velocity through the mitral valve, as shown in representative images in all four groups. **L**) The E/A ratio was significantly decreased in both groups but was more obvious in the saline I/R group. **M**) Although the isovolumetric relaxation time (IVRT) was not significantly different in all four groups, **N**) there was a difference in the mitral valve (MV) deceleration time in the saline I/R group vs the saline sham group. In each graph, data are represented as means ± SEM of n=5-7 mice per group. * $p<0.05$, ** $p<0.01$ by one-way ANOVA with post-hoc Tukey test.

**Figure 6. DS-IkL limited fibrosis after I/R. A)** Representative images of whole hearts (top), Masson's Trichome (middle), and Picrosirius Red (bottom) stained sections. Collagen deposition by Masson's Trichrome (**B and C**) and Picrosirius Red (**D and E**) was significantly reduced in DS-IkL treated mice after I/R. Data are represented as means ± SEM of n=5-7 mice per group; datapoints display 2-4 sections analysed per mouse. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by one-way ANOVA with post-hoc Tukey test.

**Figure 7. DS-IkL prevented neutrophil and macrophage aggregation, fibroblast proliferation, and endothelial cell proliferation after I/R.** We investigated the short- and long-term effects of DS-IkL on the immune response. **A**) Flow cytometric analysis of isolated cardiac cells. Nucleated cells were selected from debris based on the incorporation of 7-ADD and the separation of the myocytes from the non-myocyte cells (NMC) using a cardiac myosin heavy chain (MF20)-specific antibody. Neutrophils and monocytes were separated based on the presence of CD11b/Lys6-C/G from all four groups as depicted. Neutrophils were further distinguished by gating on the SSC-high and FSC-low cells from $CD11b^{+}/Ly6C/G^{+}$ population. **B**) Summary data shows that there was a significant reduction in neutrophils in the DS-IkL I/R group, compared to the saline I/R group. 3 technical replicates per mouse are shown. **C**) Flow cytometric analysis of $CD31^{+}/Thy1.2^{-}$ endothelial cells in all four groups. **D**) Summary data showing a significant increase in endothelial cells in saline I/R group relative to saline sham. DS-IkL prevented the increase. Data show 2 technical replicates per mouse. **E**) Assessment of proliferation using the Ki67 proliferative marker showed a similar trend. Data represent averages of 2 technical replicates per mouse. **F**) Fibroblasts ($Thy1.2^{+}/CD11b^{-}/Ly6^{-}C/G^{-}$) were detected using flow cytometric analysis. **G**) Summary data of Thy1.2 positive cells (2 technical replicates per mouse) and **H**) proliferative Thy1.2 positive cells using Ki67 (averages of 2 technical replicates per mouse) are shown. X and Y axes represent arbitrary units. **I**) We examined the accumulation of neutrophils (Ly6G) and macrophages (F4/80) after 2 weeks of I/R using IHC. Representative images are shown for all four groups. DS-IkL prevented accumulation of both **J**) macrophages and **K**) neutrophils. n=3-5 mice per group for flow cytometric analysis. n=3 mice per group, 2-3 sections per mouse for IHC. Data are represented as means ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by one-way ANOVA with post-hoc Tukey test.

Figure 1

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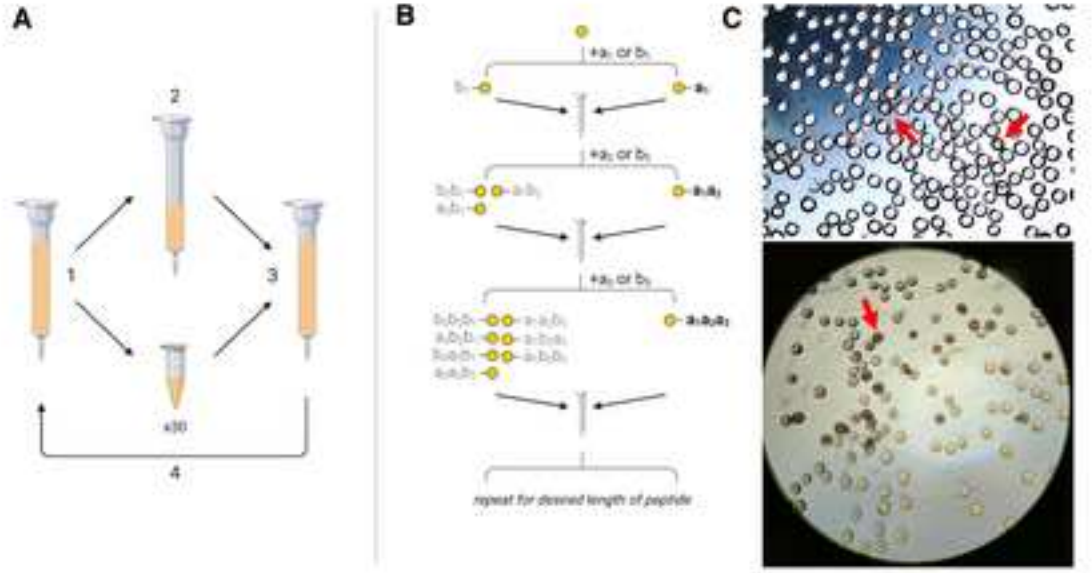

Figure 2



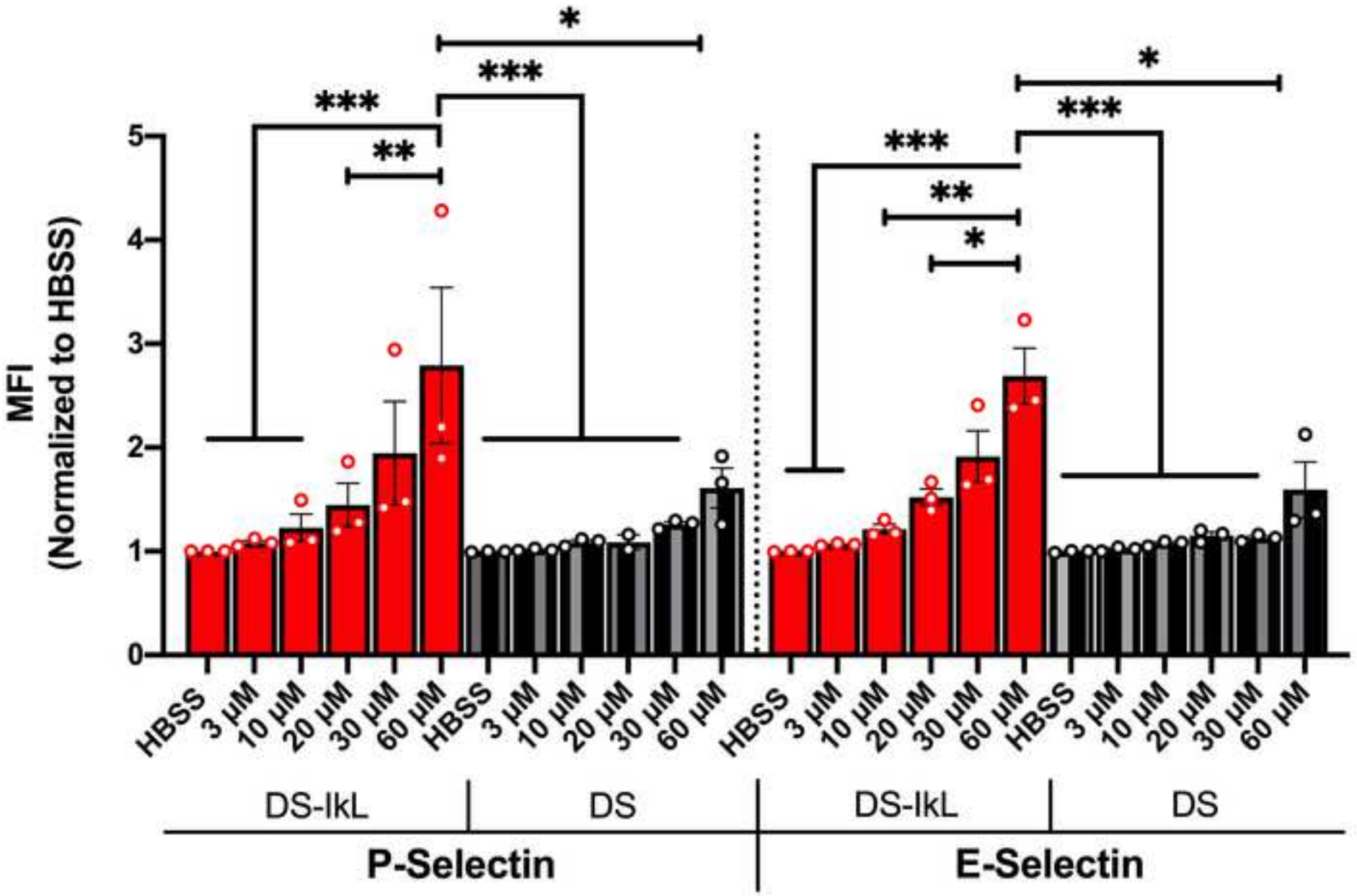

Figure 3



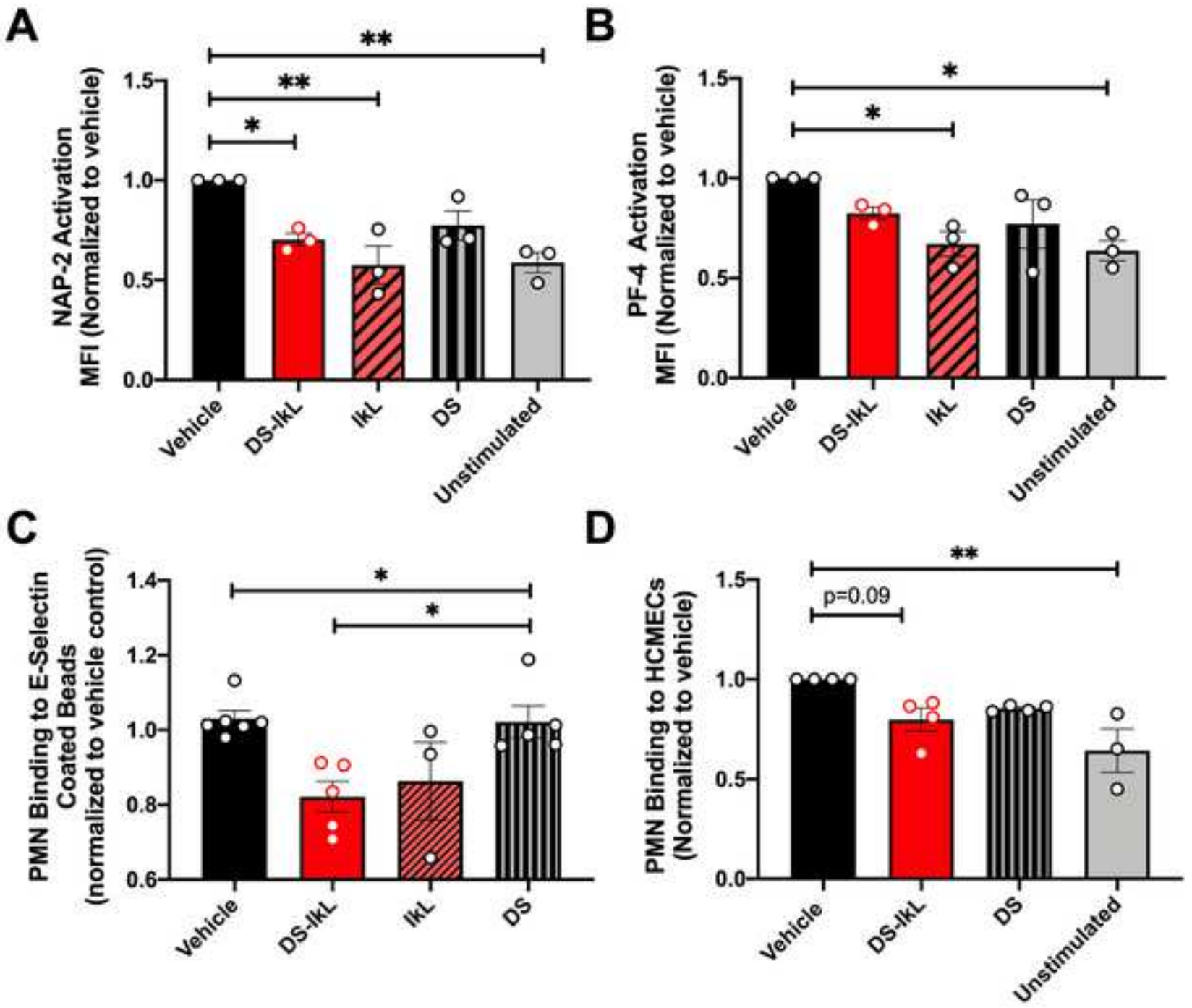

Figure 4

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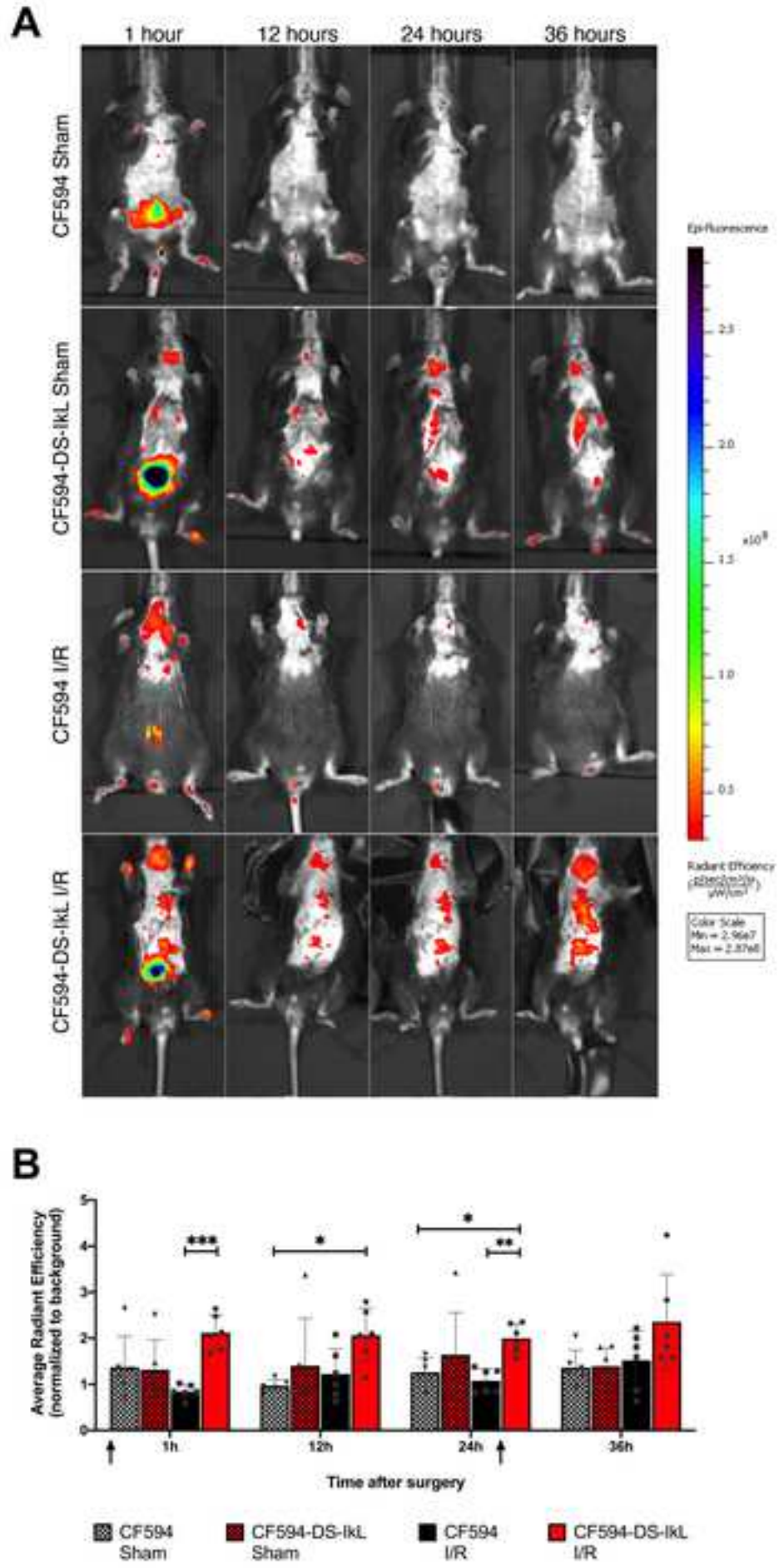

Figure 5

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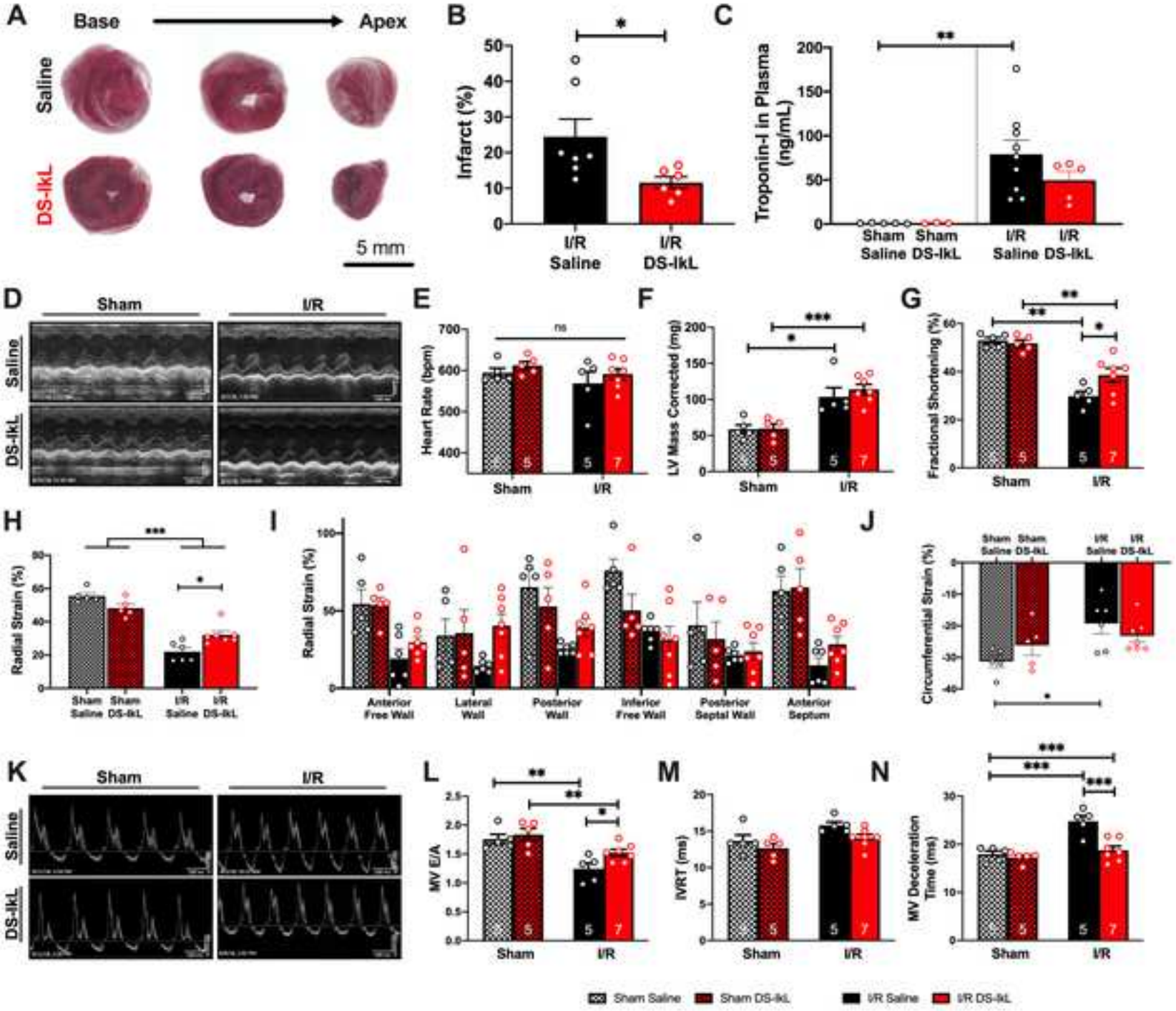


Figure 6



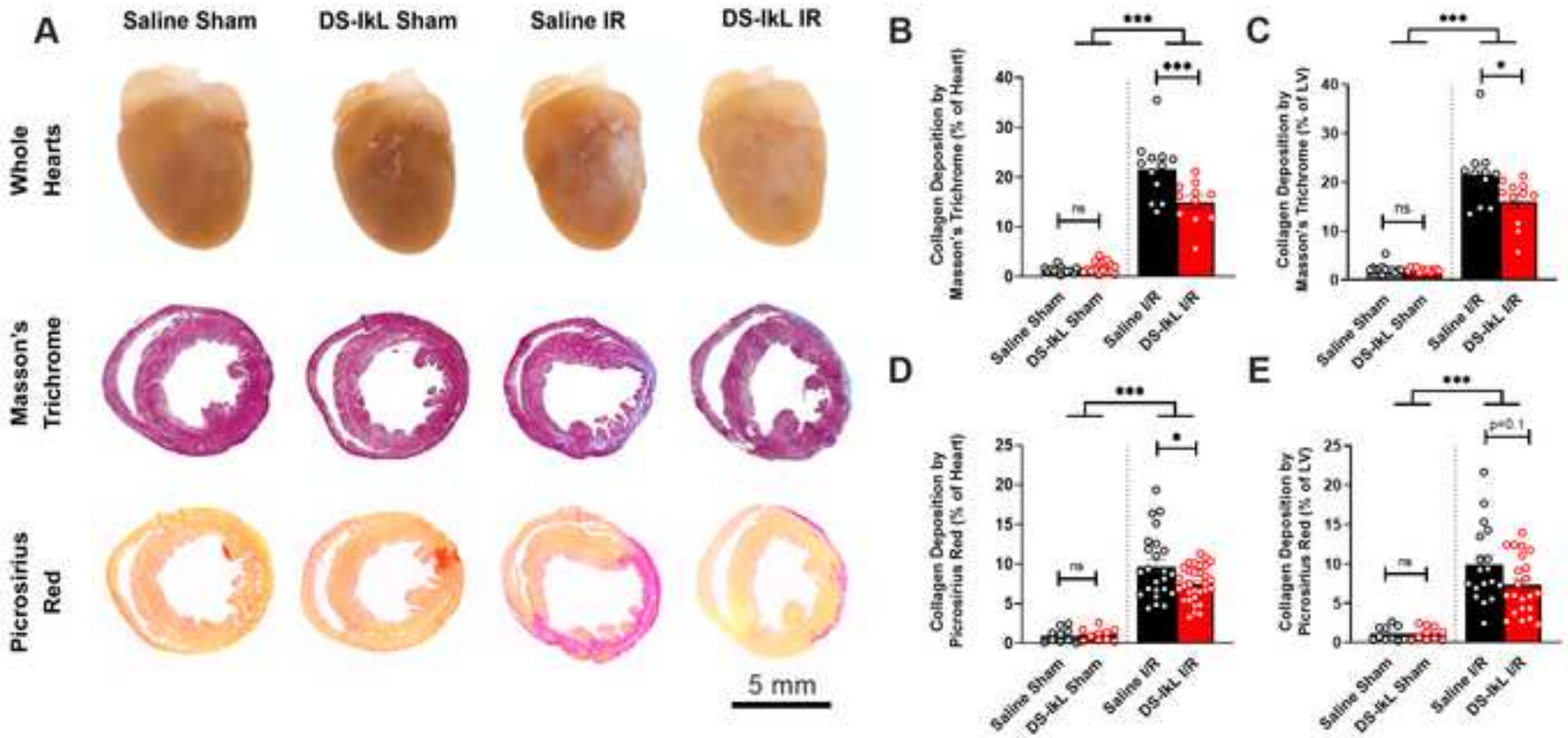
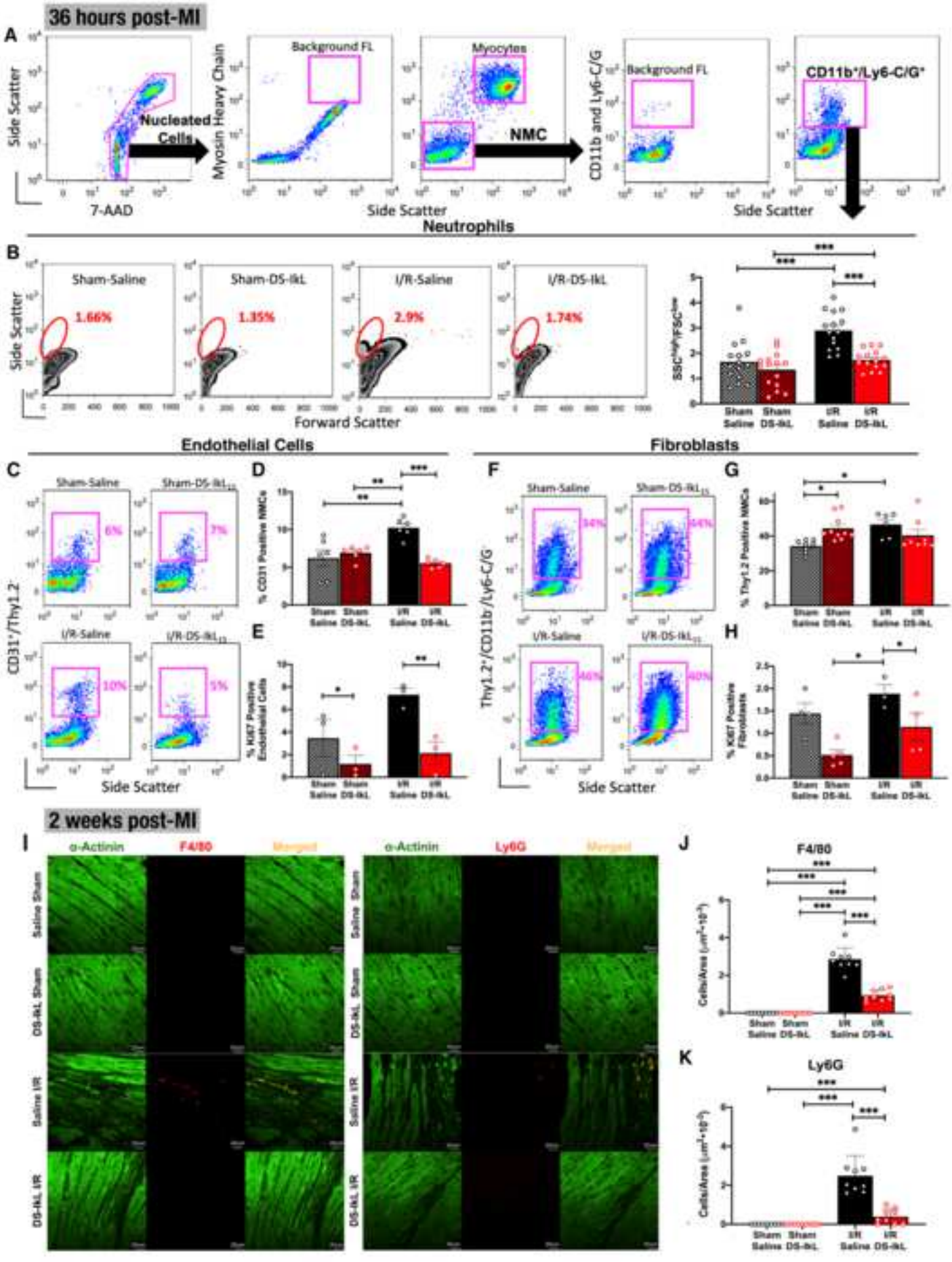
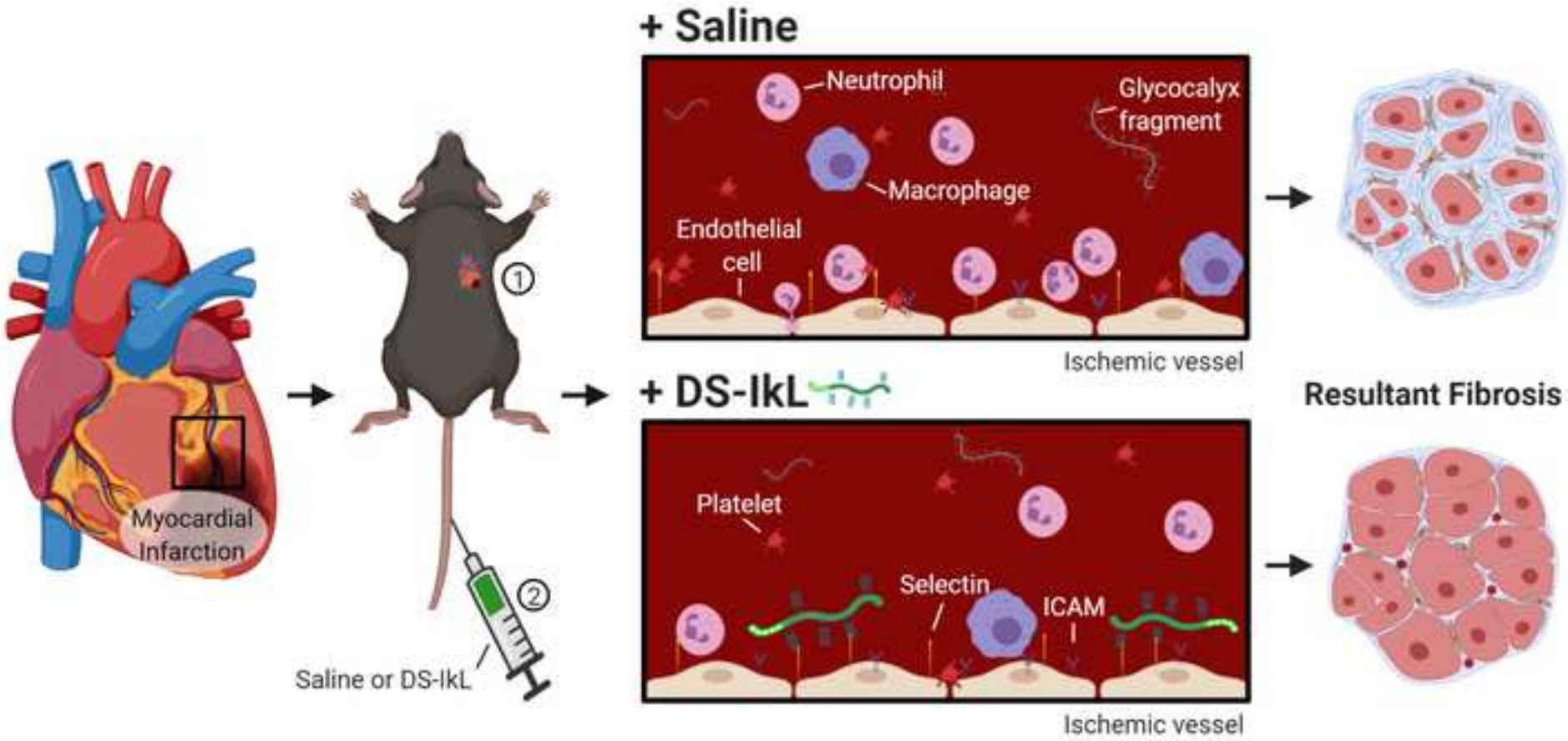


Figure 7





Graphical Abstract



# Selectin-Targeting Glycosaminoglycan-Peptide Conjugate Limits Neutrophil Mediated Cardiac Reperfusion Injury

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*Authors contributed equally to this project

Short Title: Glycocalyx mimic limits cardiac reperfusion injury

# Supplementary Materials

## Methods

### Peptide Library

1 g of resin was swollen in dimethylformamide (DMF) overnight and divided in half. Half of the resin was coupled to the first amino acid in the known sequence; the other half was split equally into 30 polypropylene columns, each containing a different amino acid (10 equivalents in DMF). Ten equivalents of N-N'-diisopropylcarbodiimide (DIC) and hydroxybenzotriazole (HOBt) were added. Complete coupling was confirmed by a colorless Kaiser test. The resin was then combined into a single column and deprotected with 20-25% piperidine. The process was repeated for the next amino acid in the known sequence.

### Library Screening for E-Selectin Binding Peptide

Peptide-conjugated Tentagel S resin was immobilized with 90% DMF to form a confluent monolayer on a 12-well polystyrene plate. Resin was blocked with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) then pre-screened for binding to the HRP-anti-His secondary antibody (1:400 in PBS, 1 hour at room temperature with rocking). Resin which stained positive was removed prior to screening. Resin was then blocked with a human FC fragment (1mg/ml in 1% BSA) for 1 hour at room temperature with rocking. FC-chimera human recombinant E-selectin with a 6-histidine tag (R&D Systems) was added to each well and incubated overnight at 4°C with rocking, washed 3x with PBS, then treated with HRP-anti-His for 1 hour at room temperature. Resin was washed and visualized using a 3,3' diaminobenzidine (DAB) solution. Resin which stained positive for E-selectin binding was removed and sequenced. This procedure was repeated until 30 unique sequences were identified. Sequences were compared

for homology and multiple selected for further analysis. Sequences were remade on Tentagel S resin and binding to E-selectin compared.

**DS-IkL inhibiting selectin and ICAM binding to neutrophils**

Isolated neutrophils were resuspended in $HBSS^{+/+}$ with 0.4% HSA and added at 165k cells per tube. DS-IkL was dissolved in $HBSS^{+/+}$ at 120 uM, sterile filtered through a 0.2 um filter, and serially diluted to 60 uM, 30 uM, and 10 uM. Fifty microlitres of DS-IkL was added to a 1.5 mL Eppendorf tube containing 40 uL of the neutrophil suspension, followed by 10 uL of 100 ug/mL recombinant human ICAM, P-selectin, or E-selectin. This yielded final DS-IkL concentrations of 60 uM, 30 uM, 15 uM, and 5 uM and 10 ug/mL selectin. For stimulated groups, TNF-$\alpha$ was added to samples at a final concentration of 5 ng/mL. Tubes were gently mixed then incubated on ice with rotation (120 RPM). After 30 minutes samples were washed twice with $HBSS^{+/+}$ then blocked with 5% BSA in $HBSS^{+/+}$ + 5mM HEPES for 60 minutes. Samples were stained with PE anti-human CD62E, PE anti-human CD62P, or PE anti-human CD54 antibodies (BioLegend, San Diego, CA) for 30 minutes (4 uL antibody per 100 uL solution), washed twice, then immediately ran on an Attune NxT Flow Cytometer. Data was analysed in FlowJo by plotting SSC-A versus FSC-A and selecting the granulocyte population, excluding doublets by plotting FSC-A versus FSC-H, then gating for PE+ populations compared to unstained controls.

**Troponin I Measurements**

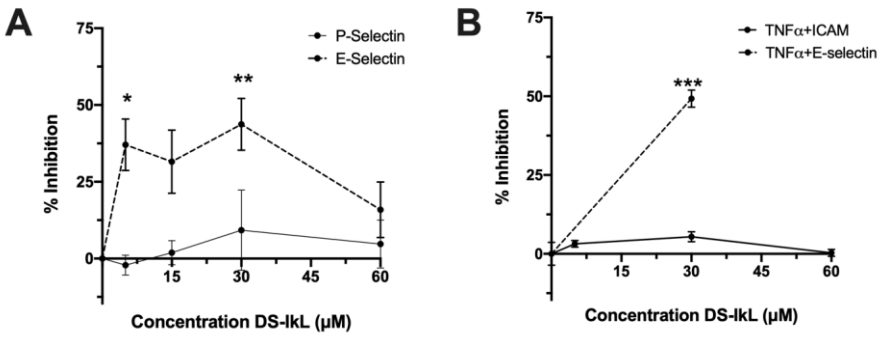
Mice were injected with heparin before they were sacrificed. After removal of hearts, blood was collected into EDTA-coated blood tubes and spun down at 2000xg for 30 mins. Separated plasma was transferred into a new tube and flash frozen. Troponin I (CNTI) was measured using a commercial kit (Life Diagnostics). Plasma was thawed then centrifuged at 2000xg for 20 minutes

at 7°C. Supernatant was diluted 1:4 in plasma diluent, then further diluted in standard diluent as needed (final dilution 1:8 or 1:12). Standards were prepared by serially diluting a CNTI stock solution to 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.156 ng/mL. HRP conjugate (100 µL) was added to anti-CTNI coated wells followed by 100 µL of standards or diluted plasma, each ran in duplicate. Wells were incubated on an orbital micro-plate shaker at 150 rpm and room temperature for one hour. Wells were emptied and washed 6x with 1x wash solution. TMB chromogen (100 µL) was added and incubated for 15 minutes at 150 rpm and room temperature. Stop solution (100 µL) was added to each well and absorbance measured immediately at 450 nm. Sample concentrations were extrapolated from a standard curve generated in GraphPad Prism (San Diego, CA) using 4-parameter logistic regression. Sample concentrations were multiplied by their respective dilution factors to obtain final concentration values.

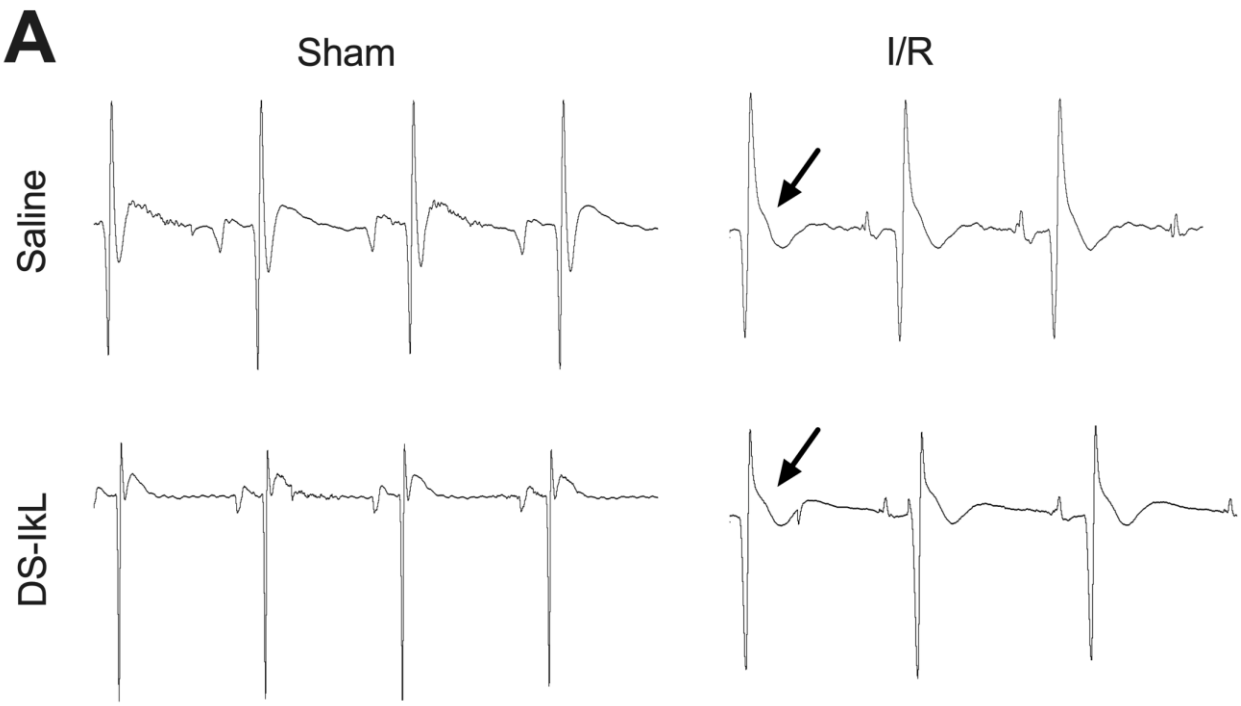
**Cell Isolation**

Mice were injected with 0.1 ml heparin (1000 units/ml) 10 mins prior to heart extraction. Mice were anesthetized with ketamine/xylazine (80 mg/kg / 5 mg/kg). Hearts were removed and placed in Tyrode's solution containing in mM: 140 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), and 5 glucose at a pH 7.4. The aorta was cannulated, mounted on a Langendorff setup, and retrogradely perfused with Tyrode's solution containing $O_2$ at 37°C for 3 mins at a flow rate of approximately 3 ml/min. The perfusion pressure was monitored, and the flow rate was adjusted to maintain perfusion pressure at ~80 mmHg. The solution was then switched to Tyrode's solution containing collagenase type 2 (1 mg/ml, 330 units/mg, Worthington Biochemical Corporation). After about 12 mins of enzyme perfusion, hearts were removed from the perfusion apparatus and gently separated in high $K^+$

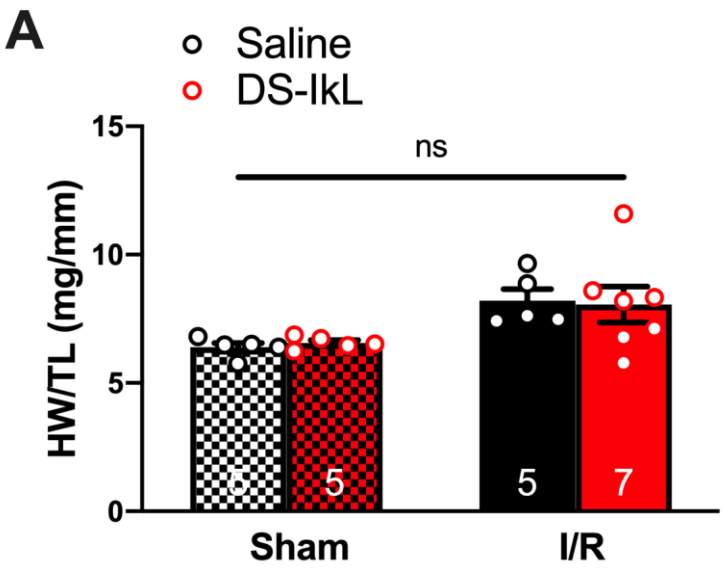
solution containing in mM: 120 potassium glutamate, 20 KCl, 1 $MgCl_2$, 0.3 EGTA, 10 glucose, and 10 HEPES, pH 7.4 with KOH. All chemicals were acquired from Sigma Chemicals (St. Louis, MO) unless stated otherwise.



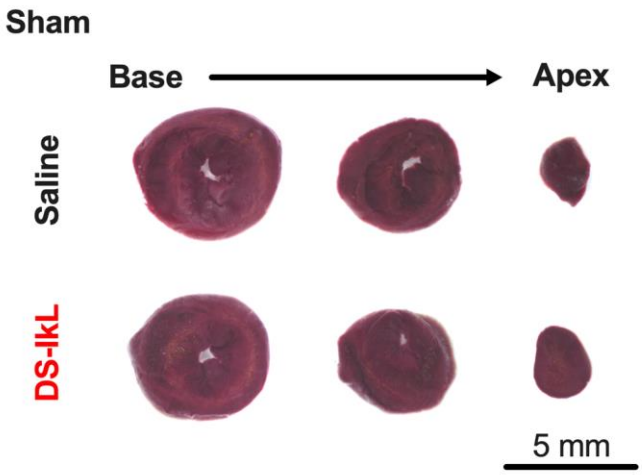

**Supplementary Figure 1. A)** Neutrophils bound less E-selectin, but not P-selectin, in the presence of 5, 15, and 30 uM DS-IkL. **B)** Under stimulatory conditions (5ng/mL TNF-α), 30 uM of DS-IkL had no effect on neutrophil binding to ICAM. Data are represented as means ± SEM of 3 biological replicates. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by one-way ANOVA with post-hoc Tukey test.




**Supplementary Figure 2. A)** Representative ECG recordings during surgical procedure. For I/R mice, confirmation of LAD ligation was done visually (blanching of the heart) and also by the presence of ST-elevation.




**Supplementary Figure 3. A)** Heart weight normalized to tibial length of all four groups. n=5-7 mice per group.




**Supplementary Figure 4.** Representative heart sections from sham-operated mice after TTC staining. Sections were taken from base to apex. n=5 mice per group.

**Supplementary Table 1. Echocardiography Table**

| | Saline Sham (n=5) | DS-IkL Sham (n=5) | Saline I/R (n=6) | DS-IkL I/R (n=7) | p-value: Saline I/R vs DS-IkL I/R |
|---|---|---|---|---|---|
| Heart Rate (bpm) | 580±3 | 612±10 | 569±28 | 591±13 | NS |
| LV Mass Corr (mg) | 58.6±6.0 | 59.8±6.4 | 103.6±12.6 | 113.9±7.1 | NS |
| LVAW;d (mm) | 0.77±0.02 | 0.78±0.07 | 0.90±0.06 | 0.86±0.02 | NS |
| LVAW;s (mm) | 1.43±0.05 | 1.44±0.09 | 1.39±0.10 | 1.54±0.04 | NS |
| LVID;d (mm) | 3.12±0.07 | 3.17±0.13 | 3.91±0.28 | 3.85±0.20 | NS |
| LVID;s (mm) | 1.41±0.05 | 1.53±0.09 | 2.76±0.24 | 2.41±0.25 | NS |
| LVPW;d (mm) | 0.76±0.04 | 0.78±0.05 | 0.85±0.06 | 0.87±0.04 | NS |
| LVPW;s (mm) | 1.38±0.08 | 1.45±0.11 | 1.21±0.11 | 1.48±0.09 | NS |
| CO (ml/min) | 20.1±1.1 | 20.1±2.3 | 22.1±4.4 | 25.4±1.8 | NS |
| Ejection Fraction (%) | 86.5±1.1 | 83.5±1.4 | 57.4±3.1 | 68.2±4.4 | <0.05 |
| Fractional Shortening (%) | 54.8±1.3 | 51.8±1.4 | 29.8±2.0 | 38.1±3.2 | <0.05 |
| LV Vol;d (µl) | 38.7±1.9 | 40.5±4.3 | 68.6±12.7 | 65.5±8.5 | NS |
| LV Vol;s (µl) | 5.8±0.4 | 6.5±0.9 | 29.9±6.6 | 22.8±6.4 | NS |
| Stroke Volume (µl) | 34.3±1.8 | 32.7±3.4 | 38.4±6.7 | 42.9±2.9 | NS |
| Radial Strain (%) | 55.5±1.83 | 48.25±2.4 | 22.0±2.4 | 32.19±2.2 | <0.05 |
| Circumferential Strain (%) | -31.3±1.9 | -26.2±3.2 | -19.3±3.2 | -23.2±2.0 | NS |
| IVRT | 13.67±0.8 | 12.7±0.6 | 15.8±0.5 | 14.1±0.5 | NS |
| MV E/A | 1.77±0.08 | 1.84±0.1 | 1.24±0.1 | 1.52±0.06 | <0.05 |
| MV Deceleration Time (s) | 17.89±0.7 | 17.2±1.2 | 24.8±1.6 | 18.7±0.9 | <0.001 |

**Supplementary Table 2: Commercial Reagent List**

| Reagent/Antibody | Vendor | Location | Catalog Number |
|---|---|---|---|
| PE anti-human CD62E antibody, clone HCD62E | BioLegend | San Diego, CA, USA | 322606 |
| PE anti-human CD62P antibody, clone AK4 | BioLegend | San Diego, CA, USA | 304906 |
| Dermatan Sulfate | Celsus Labs | Cincinnati, OH, USA | |
| Recombinant human E-selectin-FC chimera protein | R&D Systems | Minneapolis, MN, USA | 724-ES-100 |
| Recombinant human P-selectin-FC chimera protein | R&D Systems | Minneapolis, MN, USA | 137-PS-050 |
| EasySep™ Direct Human Neutrophil Isolation Kit | StemCell Technologies | Cambridge, MA, USA | 19666 |
| PE anti-human CD54 antibody, close HA58 | BioLegend | San Diego, CA, USA | 353106 |
| Human Cardiac Microvascular Endothelial Cells | PromoCell | Heidelberg Germany | C-12285 |
| Endothelial growth medium MV | PromoCell | Heidelberg Germany | C-22120 |
| Calcein-AM | BioLegend | San Diego, CA, USA | 425201 |
| Human Serum Albumin Solution – 25% | Gemini Bio | | 800-120 |
| AF488 anti-human CD11a/CD18, clone m24 | BioLegend | San Diego, CA, USA | 363404 |
| PE anti-human CD15, clone HI98 | BioLegend | San Diego, CA, USA | 301906 |
| CF594 Dye Hydrazide | Biotium | Fremont, CA, USA | 92158 |
| Prostaglandin E1 | Enzo Life Sciences | Farmingdale, NY, USA | BML-PG006-0010 |
| Solid state peptide synthesis products | CEM and AAPPTec | | Various |
| 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride | Sigma-Aldrich | St. Louis, MO, USA | 74104-1G-F |
| Human CXCL7/NAP-2 Antibody | R&D Systems | Minneapolis, MN, USA | MAB393 |
| Human CXCL7/NAP-2 Biotinylated Antibody | R&D Systems | Minneapolis, MN, USA | BAF393 |
| Human CXCL4/PF4 Antibody | R&D Systems | Minneapolis, MN, USA | AF795 |

| | | | |
|---|---|---|---|
| Human CXCL4/PF4 Biotinylated Antibody | R&D Systems | Minneapolis, MN, USA | BAF795 |
| EDTA Solution | Promega | Madison, WI, USA | V4231 |
| Theophylline | Sigma-Aldrich | St. Louis, MO, USA | T1633-100G |
| PolyLink Protein Coupling Kit | PolySciences, Inc. | Warrington, PA, USA | 24350-1 |
| Fluoresbrite® 641 Carboxylate Microspheres 1.75µm | PolySciences, Inc. | Warrington, PA, USA | 17797 |
| Pierce™ Recombinant Protein A/G | ThermoFisher | Waltham, Massachusetts, USA | 21186 |
| BS3 (bis(sulfosuccinimidyl)suberate) | ThermoFisher | Waltham, Massachusetts, USA | 21580 |
| Thy1.2-PE antibody | e-Bioscience | San Diego, CA, UCSA | 12-0902-81 |
| Rat Anti-Mouse CD11b antibody | BD Bioscience | Franklin Lakes, NJ, USA | 557397 |
| Rat Anti-Mouse Ly-6C/G antibody | BD Bioscience | Franklin Lakes, NJ, USA | 553128 |
| MyHC antibody | Developmental Studies Hybridoma Bank | Iowa City, IA, USA | |
| Rat Anti-Mouse CD31 antibody | BD Bioscience | Franklin Lakes, NJ, USA | 558737 |
| Rat Anti-Mouse Ki-67 antibody | BD Bioscience | Franklin Lakes, NJ, USA | 558615 |
| Mouse Cardiac Troponin-I ELISA | Life Technologies | West Chester, PA, USA | CTNI-1 HSP |