SUPPLEMENTAL MATERIAL

DUAL INHIBITION OF CATHEPSIN G AND CHYMASE REDUCES MYOCYTE DEATH AND IMPROVES CARDIAC REMODELING AFTER MYOCARDIAL ISCHEMIA REPERFUSION INJURY

Authors: BAHMAN HOOSHDARAN, MIKHAIL A KOLPAKOV, XINJI GUO, SONNI A MILLER, TAO WANG, DOUGLAS G TILLEY, KHADIJA RAFIQ, ABDELKARIM SABRI

Supplemental Materials and Methods

Materials: Dual cathepsin G and chymase inhibitor (DCCI) was from EMD Millipore (219372). Antibodies for Immunohistochemistry of α-SMA (A5228) and tropomyosin (T9283) were from Sigma Aldrich, cathepsin G (219358) was from Millipore. Antibodies for myeloperoxidase (MPO, RB-373-A0) and mac-3 (BD-550292) were from Thermo Scientific and BD Biosciences, respectively. Antibodies for immunoblotting against p-NFKB (3033), NF-kB (8242), Stat3 (9131), p-Paxillin (2541), p-Stat3 (9131), Bax (2772), XIAP (2042) were from Cell Signaling, those against TNF-α (sc-1350), FAK (sc-558), TGF-β1 (sc-146) were from Santa Cruz. Antibody against IL-1β (AF401) was from R&D, p-FAK397 (44-624G) was from Invitrogen and paxillin (610051) was from BD Bioscience. Active enzymes to investigate the effect of cathepsin G or chymase on myocytes and fibroblasts were from MP Biomedical (0219134480) and Sigma Aldrich (C8118), respectively.

Heart function: Echocardiographic measurements were taken before surgery and at 1 or 7 days after IR to determine the baseline heart function and ventricular dimensions in the experimental groups. Briefly, following light sedation with 1% Isoflurane, the mice were placed on a heated platform in the left lateral decubitus position for imaging. A Visualsonic Ultrasound System (Vevo770) containing a 40 Mhz variable frequency probe was used to capture the echocardiogram. Standard long and short axis M-Mode views were recorded when the mouse possessed a target heart rate between 440 and 500 beats per minute. Posterior wall thickness (LVPWTd, LVPWTs) and left ventricular internal diameters (LVEDd, LVESs) were calculated and averaged from 4 consecutive contractions using manufacturer's software. Percent fractional shortening was

calculated using: % FS= [(LVEDD-LVESD)/LVEDD] x 100. LVEF was calculated by the cubed method as follows: % EF = $[(LVEDD)^3 – (LVESD)^3]/(LVEDD)^3$ x 100

Histology and immunohistochemistry: Tissues were fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μm intervals. Picro-sirius red, trichrome and toluidine blue staining were performed using standard procedures. For immunostaining, paraffin heart sections were deparaffinized in xylene and re-hydrated. Antigen retrieval was achieved by boiling the slides in citrate solution for 12-15 minutes and slides were then washed with phosphate-buffered saline (PBS). After quenching endogenous tissue peroxidase activity with 3% H2O2 for 20 minutes, slides were then washed in PBS and samples blocked in PBS containing 5% bovine serum albumin (BSA) at room temperature for 30 minutes. Primary antibodies were applied overnight at 4°C in PBS containing 2% BSA. The next day, samples were washed in PBS and then sequentially incubated with Vectastain Elite ABC Kit (Avidin/Biotin/Horseradish Peroxidase-System (Vector Laboratories) or with fluorophore-secondary antibody conjugates (Molecular Probes). The peroxidase reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) and slides were counterstained with Hematoxylin.

Assessment of area at risk and infarct size: After 24 h of reperfusion, the slipknot was retied and the right carotid artery was cannulated to allow injection of KCL (40 mEq/L) followed by 1% Evan's blue dye for identification of the area at risk (AR). The hearts were excised, rinsed briefly in PBS, and sliced transversely. Slices were then incubated with 1% triphenyltretrazolium chloride (TTC, pH 7.4 in phosphate buffer) at 37°C for 30 minutes, fixed in 10% formalin, photographed, and the images were used to quantify IR-induced myocardial infarction using Bioquant software. The Evan's blue stained area defined the perfused area, whereas the Evan's blue unstained area defined the area at risk (AAR). The area lacking the red TTC staining within the AAR was

considered as the infarct area (IA). Both the surgeon and the evaluator of infarct size were blinded to mouse treatment groups.

For chronic infarct size analysis, paraffin-embedded short-axis heart sections were stained with trichrome staining. Bright field photographs were acquired on a dissecting microscope using a DS-Fi1 color camera and NIS Elements software (all from Nikon Inc.; Melville, NY). Pathologically infarcted regions of the myocardium were identified and their surface area was quantified using NIH Image J software. Infarct area was calculated as a percentage of total ventricular surface area for 3-4 cross-sections.

Terminal deoxynucleotidyl transferase (TdT) and tropomyosin immunolabeling: Three sections from each LV cut perpendicularly to the major axis of the heart were sampled. TdT assay was performed using kit from Promega. TUNEL positive cells were counted throughout the LV and were expressed as percentage of the total number of nuclei as determined by DAPI (Molecular probes). TUNEL co-staining with tropomyosin was used to assess the percentage of apoptotic cardiomyocytes.

Immunoblot analysis: Extraction of proteins from heart tissue samples was performed as described previously.(1) Briefly, lysates were cleared by centrifugation at 12,000 rpm and the supernatants were subjected to immunoblot analysis according to methods published previously or to the manufacturer's instructions. Each panel in each figure represents results from a single gel exposed for a uniform duration, with bands detected by enhanced chemiluminescence and quantified by laser scanning densitometry.

Cardiac troponin-I ELISA assay: Mice were exsanguinated 1 day after IR and the plasma levels of the cardiac-specific isoform of troponin-I were assessed using ELISA assay (Life Diagnostics, PA).

Neonatal rat cardiomyocyte isolation: Myocytes were isolated from the ventricles of neonatal Sprague-Dawley rats by collagenase digestion as previously described.(2) After 30 minutes of preplating (to eliminate non-myocyte cell contamination), myocytes were plated in collagen precoated dishes or in fibronectin (BD bioscience) precoated glass coverslips at a density of 160,000/cm2 in 10% fetal bovine serum DMEM supplemented with 1 mmol/liter L-glutamine, antibiotic/anti-mycotic solution, and 100 µmol/L 5-bromo-2-deoxyuridine (BrdU). Under these high density conditions, the myocytes form cell-cell contacts and display spontaneous contractile activity within 24 hours of plating.

Neonatal and adult rat cardiac fibroblast isolation: Neonatal fibroblasts were isolated from the ventricles of neonatal Sprague-Dawley rats by collagenase digestion as previously described.(3) Briefly fibroblasts from preplated dishes were cultured in 10% fetal bovine serum DMEM supplemented with 1 mmol/liter L-glutamine, antibiotic/antimycotic solution. Media was renewed each 2 days until density reaches 70% confluent. Cells were starved for 2 days before treatments. For isolation of adult cardiac fibroblasts, hearts from 10 week-old C57BL6 mice were digested with collagenase/dispase solution (Roche) and plated for 1 hr. Adherent cells (mainly fibroblasts) were incubated in DMEM containing 10% fetal bovine serum for 7 days, switched to serum free media and then treated with cathepsin G or chymase for 36 h.

Migration wound assay: Cardiac fibroblasts were plated on day 0 and cultured until 70% confluence before treatment. Migration assays started 48 h after treatment by scratching the monolayer with a pipette tip (1 mm width). The time point where the scratch was made was taken as 0 h. The number of migrating fibroblasts was measured after 36 h.

Assessment of MMP activity: Snap frozen left ventricular tissue was homogenized in lysis buffer containing 4% SDS and 25% glycerol, followed by incubation at 37°C for 20 minutes. Equal

amounts of protein (30 μg) were separated by SDS-PAGE gels containing 1 mg/ml gelatin (Bio-Rad 161–1167). The gels were then incubated in renaturing buffer (2.5% X-100) twice for 20 minutes and washed after each incubation period with ddH2O, then incubated in the developing buffer overnight at 37ºC in a shaking water bath. After being rinsed with ddH2O, gels were stained with Coomassie brilliant blue for 30 minutes to 1 hour then partially de-stained using a 10% acetic acid/20% methanol/70% ddH2O solution for 30-45 minutes. Gels were scanned and bands quantified using UnScan-It Image software. Total gelatinase activities were measured using fluorescence-based activity assays from EnzChek (Molecular Probes).

Cathepsin G and chymase activity assay: Snap frozen LV tissues were homogenized in ice-cold buffer containing 100 mmol/L HEPES, pH 7.5, 1 M NaOH, 50 mM CaCl2, and 0.01% Igepal CA-630 in presence or absence of cathepsin G inhibitor. After centrifugation, supernatants containing 100 μg proteins were used for cathepsin G and chymase activity assay by measurement of the rate of cleavage of fluorogenic conjugated substrate Suc-Ala2-Pro-Phe-Amc (R&D Systems) and Suc-Leu-Leu-Val-Tyr-AMC (Boston Biochem) respectively.

Myeloperoxidase (MPO) Assay: Left ventricular tissue samples were homogenized in buffer containing 0.1 M Tris (pH 7.6), 0.15 M NaCl, and 0.5% hexadecyl trimethyl ammonium bromide. MPO activity in the cleared supernatant was measured by measurement of the rate of cleavage of fluorogenic-conjugated substrate 10-aceytyl-3,7-dihydroxyphenoxazine (ADHP). Protein concentrations were determined by using the BCA protein assay kit (Pierce, Rockford, IL) with BSA as a standard.

Caspase-3 activity assay: Caspase-3 activity was measured with CaspACE assay system (Promega, Madison, WI). In brief, LV lysates were prepared by dounce homogenization in lysis buffer provided with the kit. The lysates were centrifuged at 12,000 rpm for 20 minutes at 4 °C,

and the supernatants containing 100 μg protein were used for caspase-3 assay. Caspase-3 activity was examined by measurement of the rate of cleavage of fluorogenic conjugated substrate MCA-Val-Asp-Gln-Met-Asp-Gly-Trp-Lys-(DNP)-NH2. The specificity of the assay was confirmed by addition of the specific caspase-3 inhibitor Z-DQMD-FMK in the reaction mixture at a concentration of 50 μM during the incubation.

Supplemental References

1. Rafiq K, Kolpakov MA, Seqqat R, Guo J, Guo X, Qi Z, Yu D, Mohapatra B, Zutshi N, An W, Band H, Sanjay A, Houser SR, Sabri A. c-Cbl inhibition improves cardiac function and survival in response to myocardial ischemia. Circulation. 2014; 129(20):2031.

2. Rafiq K, Hanscom M, Valerie K, Steinberg S, Sabri A. Novel mode for neutrophil protease cathepsin G-mediated signaling: Membrane shedding of epidermal grwoth factor is required for cardiomyocyte anoikis. Circ Res. 2008;102(1):32-41.

3. Sabri A, Short J, Guo J, Steinberg SF. Protease-Activated Receptor-1-Mediated DNA Synthesis in Cardiac Fibroblast Is via Epidermal Growth Factor Receptor Transactivation: Distinct PAR-1 Signaling Pathways in Cardiac Fibroblasts and Cardiomyocytes. Circ Res. 2002;91(6):532-9.

Supplemental Table 1: Summary of heart weight, body weight and echocardiographic measurements in 24 h post reperfusion in sham and IR mice treated or not with DCCI.

	Sham		**1d IR**	
	-	**DCCI**	**-**	**DCCI**
	(n=6)	**(n=6)**	**(n=8)**	**(n=8)**
HR (bpm)	446 ±15	466 ±16	487 ±11	490 ±27
HW (mg)	115 ±2	115 ±3	118 ±4	118 ±6
BW (g)	23 ±1	25 ±2	23 ±2	24 ±2
HW/BW (mg/g)	5.1 ±0.2	4.7 ±0.3	5.5 ±0.1	5.1 ±0.1
LV Vold (µl)	69 ±8	59 ±4	88 ±6*	72 ±4*†
LV Vols (µl)	29 ±5	28 ±4	57 ±5*	41 ±4*†
LVPWTd (mm)	0.72 ±0.02	0.71 ±0.02	0.61 ±0.03*	0.59 ±0.03*
LVPWTs (mm)	1.05 ±0.04	1.04 ±0.04	0.77 ±0.02*	0.76 ±0.03*

HR indicates heart rate; HW, heart weight; BW, body weight; LV Vold, LV volume during diastole; LV Vols, LV volume during systole; LVPWTd, LV posterior wall thickness diastole; and LVPWTs, LV posterior wall thickness systole. *P<0.05 vs. shams, †P<0.05 vs. non treated IR.

Supplemental Table 2 Summary of heart weight, body weight and echocardiographic measurements in 7 days post reperfusion in sham and IR mice treated or not with 10mg/kg/d of DCCI.

	Sham		**7d IR**	
	-	**DCCI**	**-**	**DCCI**
	(n=6)	**(n=6)**	**(n=8)**	**(n=8)**
HR (bpm)	470 ±14	451 ±24	446 ±22	456 ±14
HW (mg)	124 ±2	126 ±3	132 ±10*	153 ±7*†
BW (g)	25 ±2	26 ±2	24 ±1	26 ±2
HW/BW (mg/g)	5.0 ±0.2	4.8 ±0.3	5.9 ±0.4*	6.1 ±0.3*
LV Vold (µl)	70 ±7	77 ±10	112 ±4*	95 ±3*†
LV Vols (µl)	28 ±3	33 ±6	74 ±3*	59 ±3*†
LVPWTd (mm)	0.63 ±0.01	0.61 ±0.01	0.56 ±0.02*	0.54 ±0.02*
LVPWTs (mm)	0.85 ± 0.06	0.87 ± 0.03	0.69 ± 0.04*	0.66 ±0.03*

HR indicates heart rate; HW, heart weight; BW, body weight; LV Vold, LV volume during diastole; LV VOLs, LV volume during systole; LVPWTd, LV posterior wall thickness diastole; and LVPWTs, LV posterior wall thickness systole. *$P<0.05$ vs. shams, †$P<0.05$ vs. non treated IR.

Supplemental Figure Legend

Figure S1: DCCI treatment attenuates T cells and macrophages infiltration in the reperfused heart. (A) Representative immunolabeling of paraffin-embedded heart sections from sham or mice subjected to ischemia reperfusion (IR) were stained for CD3 (T cells) and Mac3 (macrophages) and counterstained with hematoxylin. Bar: 40 μM. (B and C) Quantification of CD3- (B) or Mac3-positive cells (C) in mice treated with either vehicle or DCCI. Values are presented as mean ± SEM (n=5 each group), $^{*}P < 0.05$ vs. shams, $^{\dagger}P < 0.05$ vs. vehicle-treated IR.

Figure S2: Effect of DCCI treatment on fibronectin accumulation in the reperfused heart. (Top) Representative immunoblot of fibronectin (Fn) accumulation in hearts from sham or mice subjected to ischemia reperfusion (IR) injury treated with vehicle or DCCI for 24 h. GAPDH was included as a loading control. (Bottom) Quantification of experiments represented as fold change compared to WT sham animals treated with vehicle (n = 5 for each group). $^{*}P < 0.05$ vs. WT shams.

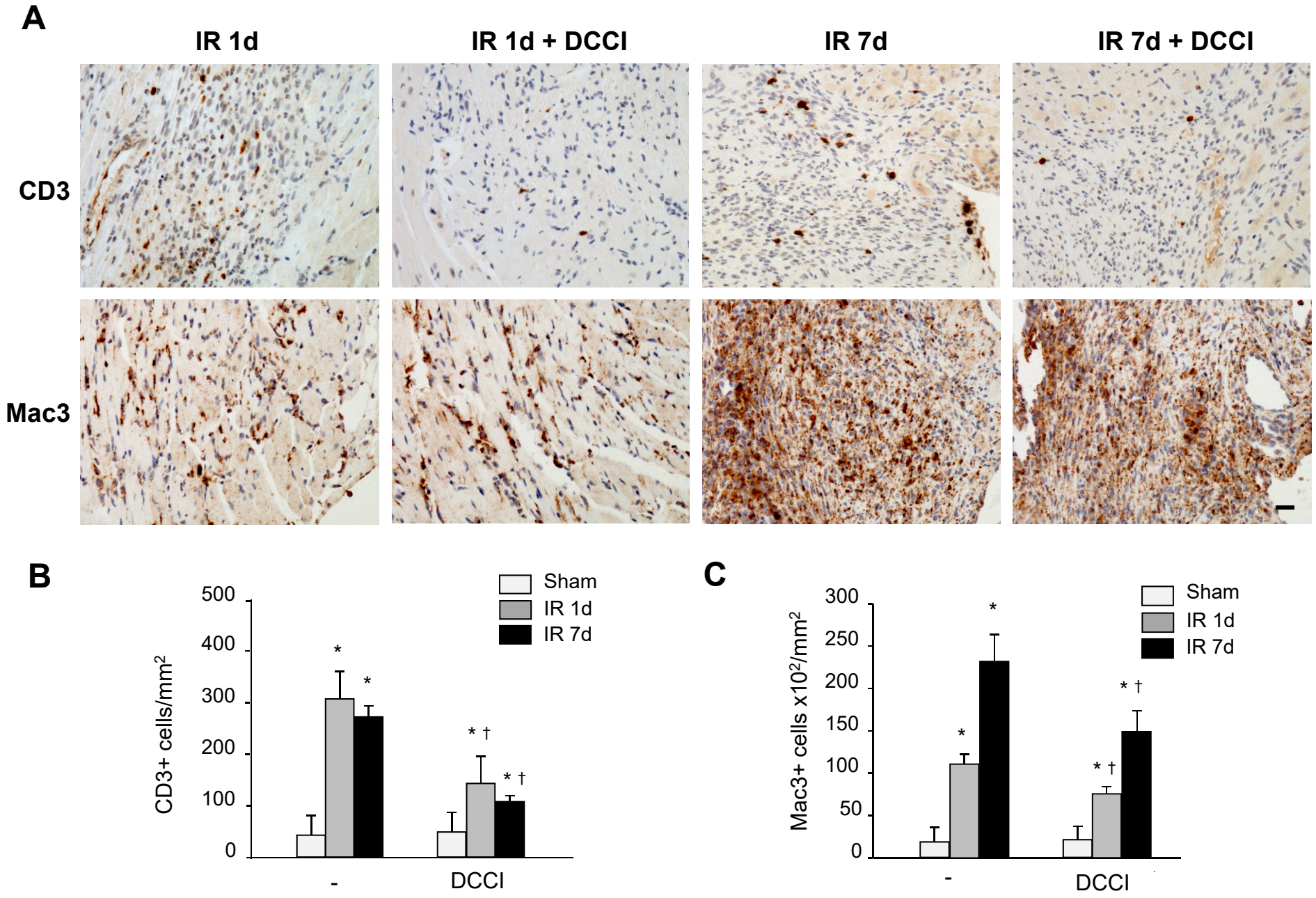
Figure S3: DCCI prevents focal adhesion protein degradation induced by Cat.G. Neonatal rat cardiac myocytes were pretreated with DCCI (5 μM) or vehicle for 15 minutes and then treated with cathepsin G (Cat.G, 0.02 U/ml) for 4 h and cell lysates were processed for immunoblot analysis. (A) Representative immunoblots with GAPDH included as a loading control. (B) Quantification of experiments expressed as mean ± SEM from 3 separate cultures. $^{*}P < 0.05$ vs. control, $^{\dagger}P < 0.05$ vs. vehicle-treated cells.

Figure S4: Cathepsin G and chymase induce adult cardiac fibroblast differentiation to myofibroblasts. Adult cardiac fibroblasts were treated with cathepsin G (Cat.G, 0.02 U/ml) or

chymase (100 nM) for 36 h and then immunostained with vimentin and smooth muscle actin (SMA) and counterstained with DAPI. Scale bar: 40 µm.

Figure S5: Effect of DCCI treatment on MMP2 activity. Conditioned media from cultured cardiac myocytes (A) or fibroblasts (B) treated with cathepsin G (Cat.G, 0.02 U/ml) for 10 minutes in absence or presence of DCCI (5 µM) were processed for in-gel zymography. Top: Representative in-gel zymography. Bottom: Quantification of experiments expressed as mean ± SEM from 3 separate cultures. $^{*}P$ <0.05 vs. control, $^{\dagger}P < 0.05$ vs. vehicle-treated cells.

Figure S6: DCCI attenuates adverse cardiac remodeling post-IR. Schematic overview of cathepsin G and chymase effects on cardiac myocytes and fibroblasts and their consequences on cardiac remodeling and function post-IR.


A
IR 1d
IR 1d + DCCI
IR 7d
IR 7d + DCCI
CD3
Mac3
B
CD3+ cells/mm2
500
400
300
200
100
0
Sham
IR 1d
IR 7d
-
DCCI
C
Mac3+ cells x10²/mm²
300
250
200
150
100
50
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Sham
IR 1d
IR 7d
-
DCCI

Fig. S1

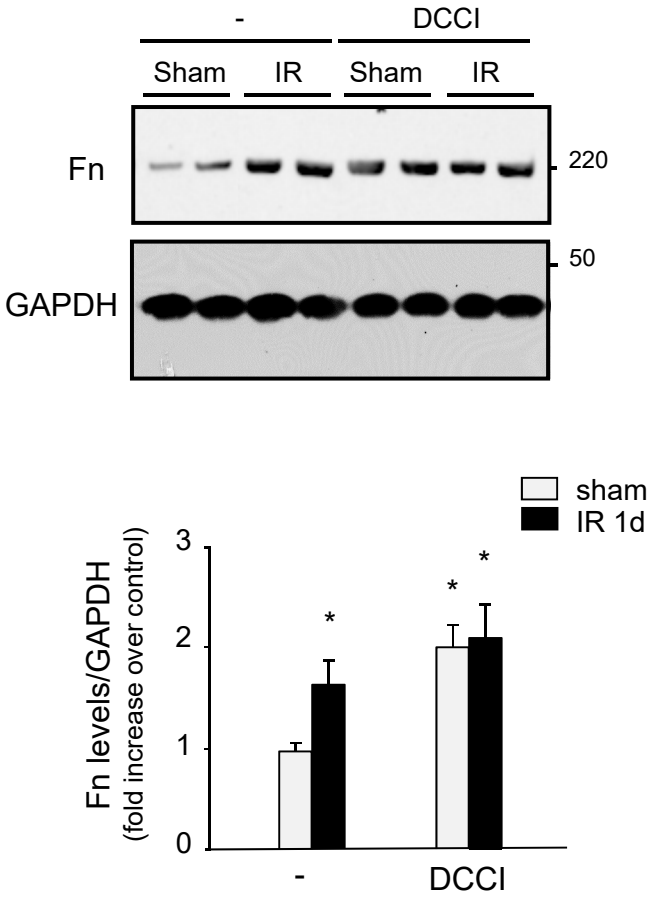

-
DCCI
Sham
IR
Sham
IR
Fn
220
GAPDH
50
sham
IR 1d
Fn levels/GAPDH
(fold increase over control)
3
2
1
0
*
*
*
-
DCCI

Fig. S2

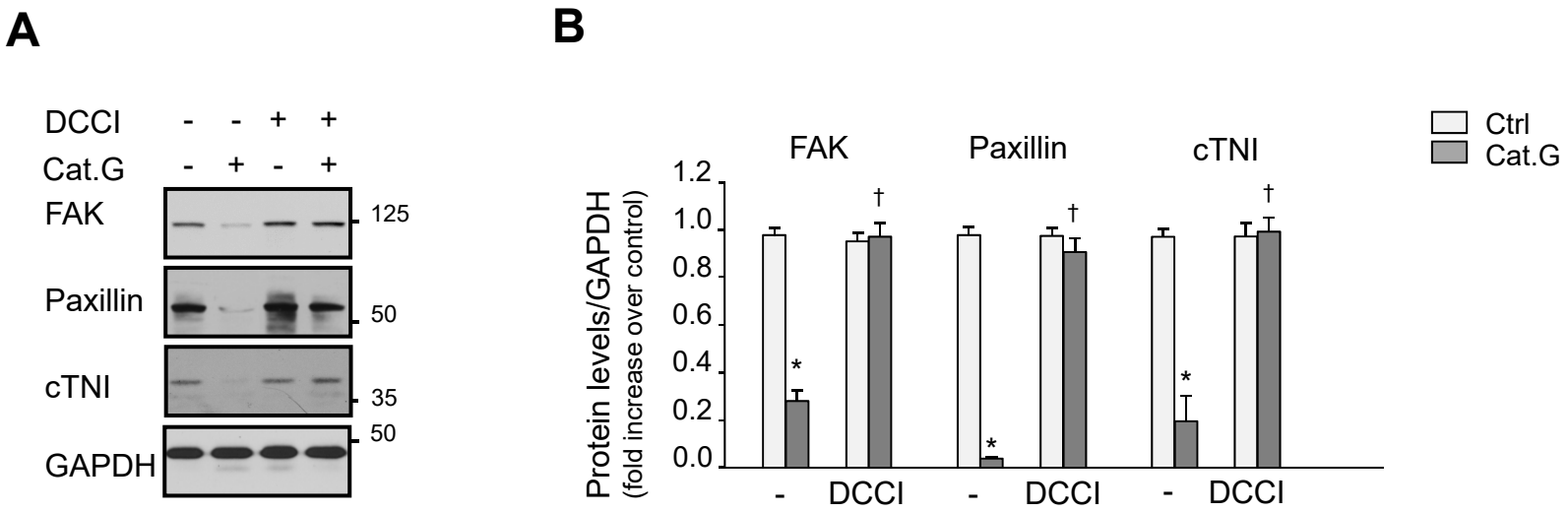

A
DCCI - - + +
Cat.G - + - +
FAK
Paxillin
cTNI
GAPDH
125
50
35
50
B
Ctrl
Cat.G
FAK
Paxillin
cTNI
Protein levels/GAPDH
(fold increase over control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
†
- DCCI - DCCI - DCCI

Fig. S3

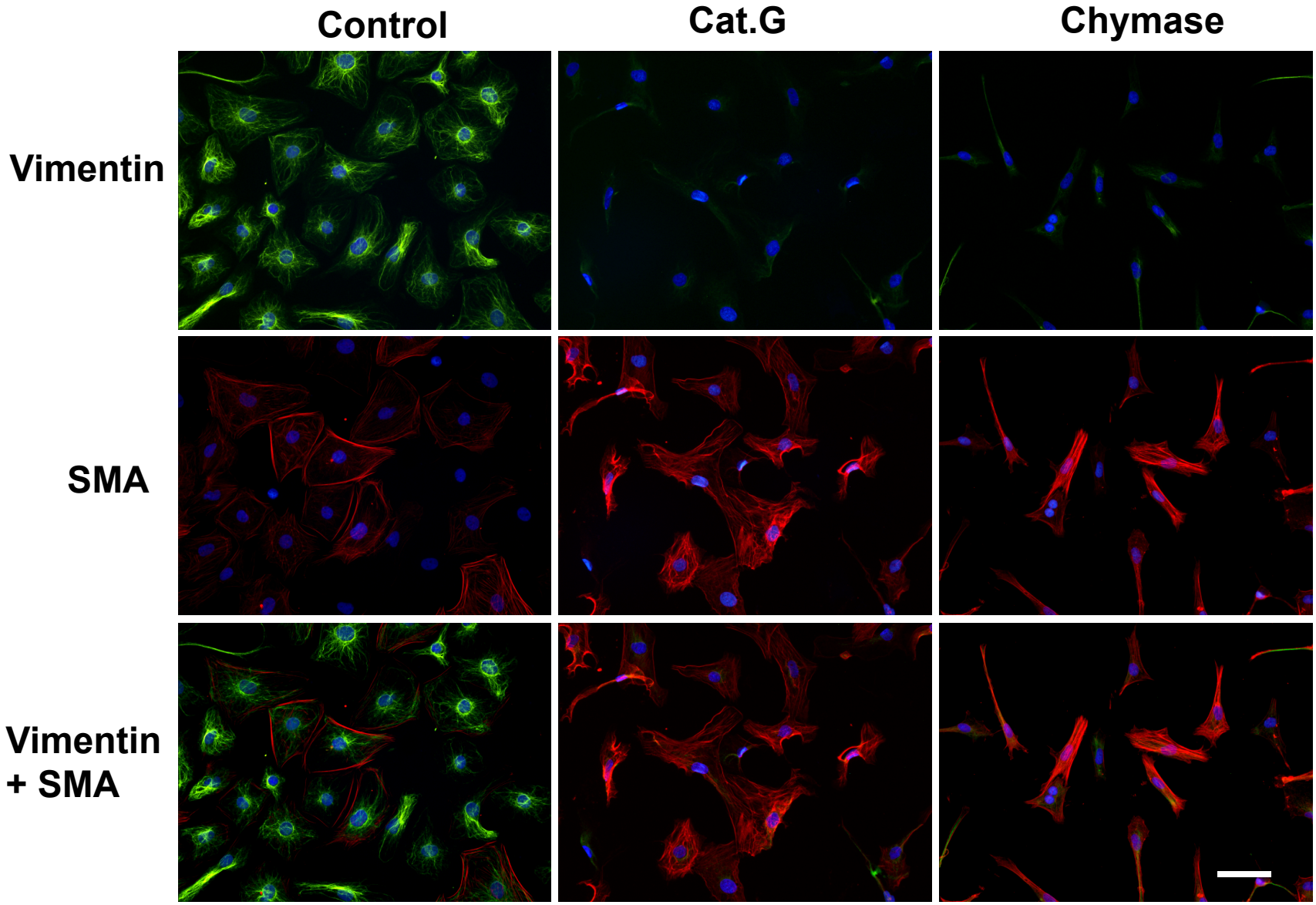
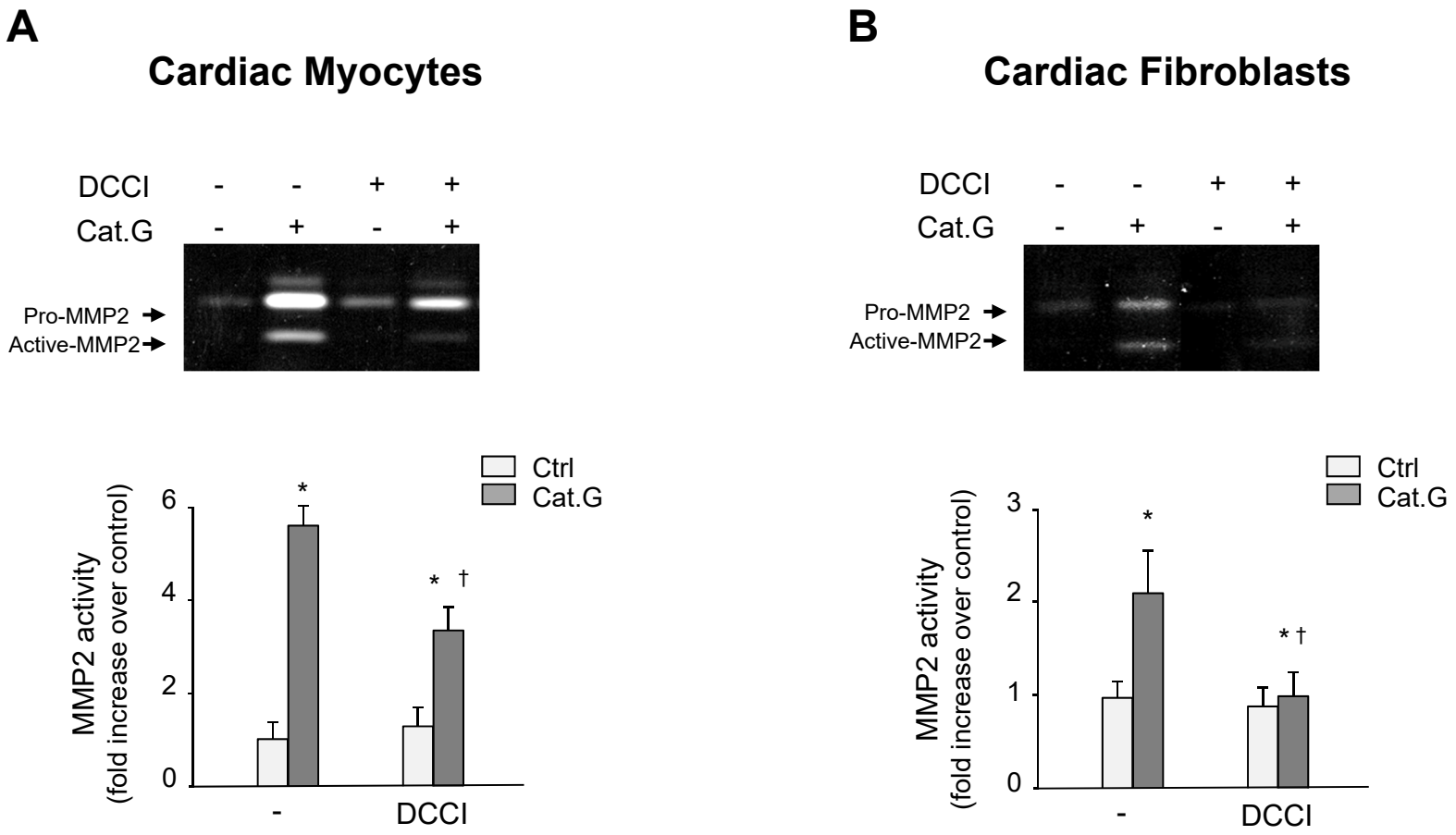


Fig. S4


A
Cardiac Myocytes
DCCI - - + +
Cat.G - + - +
Pro-MMP2
Active-MMP2
MMP2 activity
(fold increase over control)
Ctrl
Cat.G
0
2
4
6
*
* †
-
DCCI
B
Cardiac Fibroblasts
DCCI - - + +
Cat.G - + - +
Pro-MMP2
Active-MMP2
MMP2 activity
(fold increase over control)
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Cat.G
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1
2
3
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* †
-
DCCI

Fig. S5

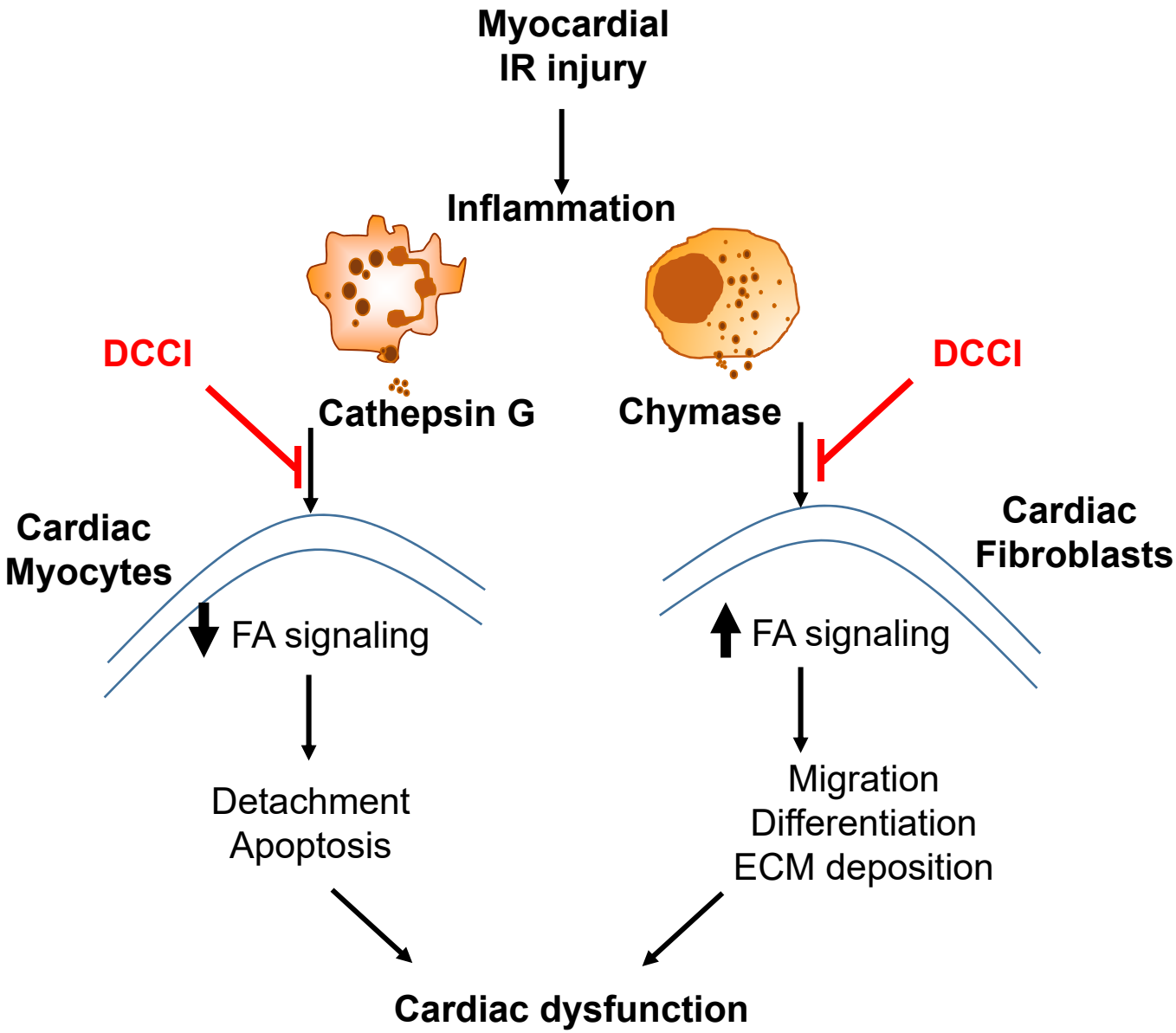
Myocardial
IR injury
Inflammation
DCCI
Cathepsin G
Chymase
DCCI
Cardiac
Myocytes
Cardiac
Fibroblasts
FA signaling
FA signaling
Detachment
Apoptosis
Migration
Differentiation
ECM deposition
Cardiac dysfunction

Fig. S6