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ORIGINAL RESEARCH

Plasmacytoid Dendritic Cells Mediate Myocardial Ischemia/Reperfusion Injury by Secreting Type I Interferons

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BACKGROUND: We previously demonstrated that ischemically injured cardiomyocytes release cell-free DNA and HMGB1 (high mobility group box 1 protein) into circulation during reperfusion, activating proinflammatory responses and ultimately exacerbating reperfusion injury. We hypothesize that cell-free DNA and HMGB1 mediate myocardial ischemia-reperfusion injury by stimulating plasmacytoid dendritic cells (pDCs) to secrete type I interferon (IFN-I).

METHODS AND RESULTS: C57BL/6 and interferon alpha receptor-1 knockout mice underwent 40 minutes of left coronary artery occlusion followed by 60 minutes of reperfusion (40′/60′ IR) before infarct size was evaluated by 2,3,5-Triphenyltetrazolium chloride–Blue staining. Cardiac perfusate was acquired in ischemic hearts without reperfusion by antegrade perfusion of the isolated heart. Flow cytometry in pDC-depleted mice treated with multiple doses of plasmacytoid dendritic cell antigen-1 antibody via intraperitoneal injection demonstrated plasmacytoid dendritic cell antigen-1 antibody treatment had no effect on conventional splenic dendritic cells but significantly reduced splenic pDCs by 60%. pDC-depleted mice had significantly smaller infarct size and decreased plasma interferon-α and interferon-β compared with control. Blockade of the type I interferon signaling pathway with cyclic GMP-AMP synthase inhibitor, stimulator of interferon genes antibody, or interferon regulatory factor 3 antibody upon reperfusion similarly significantly attenuated infarct size by 45%. Plasma levels of interferon-α and interferon-β were significantly reduced in cyclic GMP-AMP synthase inhibitor-treated mice. Infarct size was significantly reduced by >30% in type I interferon receptor monoclonal antibody–treated mice and interferon alpha receptor-1 knockout mice. In splenocyte culture, 40′/0′ cardiac perfusate treatment stimulated interferon-α and interferon-β production; however, this effect disappeared in the presence of cyclic GMP-AMP synthase inhibitor.

CONCLUSIONS: Type I interferon production is stimulated following myocardial ischemia by cardiogenic cell-free DNA/HMGB1 in a pDC-dependent manner, and subsequently activates type I interferon receptors to exacerbate reperfusion injury. These results identify new potential therapeutic targets to attenuate myocardial ischemia-reperfusion injury.

Key Words: ischemia reperfusion injury ■ infarct size ■ cell signaling

Ischemic heart disease remains the single leading cause of death in the United States, accounting for 1 of every 5 deaths. Myocardial infarction and heart failure account for the vast majority of the morbidity and mortality associated with ischemic heart disease. The means to attenuate acute myocardial infarction is 2-fold; both early restoration of the blood flow to the ischemic heart and early inhibition of the inflammatory response during reperfusion are key.[1,2] The inflammatory response during reperfusion is triggered by damage-associated molecular patterns (DAMPs), specifically cell-free DNA (cfDNA) and HMGB1 (high mobility group box protein 1), released from ischemic myocardium into circulation during reperfusion. HMGB1 and cfDNA subsequently activate $CD4^+$ T cells via a cfDNA-HMGB1-RAGE-TLR9 pathway to further

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CLINICAL PERSPECTIVE

What Is New?

- A subset of splenic leukocytes, plasmacytoid dendritic cells, is stimulated by myocardial ischemia to mount a detrimental inflammatory response mediated by type I interferons that exacerbates myocardial infarct size after reperfusion.
- Depletion of plasmacytoid dendritic cells, blockade of type I interferon production, or loss of interferon-α/β receptor function all attenuate myocardial infarct size in a murine coronary occlusion model of ischemia/reperfusion injury.

What Are the Clinical Implications?

- Plasmacytoid dendritic cells and the type I interferon pathway are novel potential therapeutic targets to reduce infarct size following myocardial infarction.
- Future studies are needed to further define the mechanistic pathways contributing to myocardial infarct exacerbation by inflammatory response activation and identify translatable therapeutic interventions to attenuate this process.

Nonstandard Abbreviations and Acronyms

cfDNA	cell-free DNA
cGAS	cyclic GMP-AMP synthase
CP	cardiac perfusate
cTnT	cardiac troponin T
DAMP	damage-associated molecular pattern
DC	dendritic cell
HMGB1	high mobility group box protein 1
IFN-I	type I interferon
IFNAR	type I interferon receptor
IRF3	interferon regulatory factor 3
IRI	ischemia/reperfusion injury
IS	infarct size
mAb	monoclonal antibody
pDC	plasmacytoid dendritic cell
PDCA-1	plasmacytoid dendritic cell antigen-1
RR	ischemic risk region
STING	stimulator of interferon genes
TLR	toll-like receptor
WT	wild-type

mediate reperfusion injury.[2] Given that toll-like receptor (TLR) expression is substantively low in T cells, this pathway likely occurs upstream of $CD4^+$ T cells activation.[2–4]

Dendritic cells (DCs) serve as the sentinels of the immune system and activate the immune response to peripheral pathogens via TLRs.[5–8] Compared with the classic DC (cDC), plasmacytoid DCs (pDCs) represent a small subset of DCs that differ in life cycle and accumulate primarily in the blood and lymphoid tissues.[6,9] It is well established that circulating and residential pDCs sense pathogens and are activated by producing type-I interferon (IFN-I). Naive T cells are subsequently activated by either IFN-I or the antigen-presenting pathway. Recently, several endogenously derived molecules, referred to as DAMPs, have also been shown to interact with pDCs and promote production of IFN-I, including HMGB1[10,11] and endogenous cfDNA.[12,13] We have demonstrated that these DAMPs are released from ischemic myocardium during reperfusion and promote an inflammatory response and induce reperfusion injury.[2,14] pDCs, as well as IFN-I, have been found to play a critical role in organ ischemic/reperfusion injury (IRI).[15–17]

We hypothesize that pDCs are activated during postischemia reperfusion and mediate the proinflammatory response by production of IFN-I and subsequent activation of type I interferon receptor (IFNAR). In this study, we tested this hypothesis anticipating that inhibition of pDCs or the IFN-I pathway would attenuate myocardial IRI.

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. This study complied with the 2011 *Guide for the Care and Use of Laboratory Animals,* 8th edition, as recommended by the US National Institutes of Health, ensuring that all animals received humane care. The University of Virginia Animal Care and Use Committee reviewed and approved the study protocol.

Animals and Materials

C57BL/6 wild-type (WT) mice and interferon alpha receptor-1 knockout ($IFNAR1^{-/-}$) mice (male, aged 9–12 weeks, purchased from the Jackson Laboratory, Bar Harbor, ME) were used in the study. PDCA-1 antibody (CD317 antibody, Miltenyl Biotec, Gaithersburg, MD) was used to deplete pDCs with rat IgG2b was used as isotype control. IFNAR1 monoclonal antibody (MAR1-5A3, ThermoFisher Scientific, Grand Island, NY) was given as an 2 μg/g IV bolus 5 minutes before reperfusion to inhibit IFNAR1 activity. The signaling pathway leading to IFN-I production was blocked using cyclic GMP-AMP synthase (cGAS) inhibitor (RU.521 0.2 μg/g, InvivoGen), anti-STING (stimulator of interferon genes) antibody (1 μg/g, ThermoFisher), anti-IRF3 (interferon regulatory factor 3) antibody (1 μg/g,

ThermoFisher) respectively given as intravenous boluses 5 minutes before reperfusion.

Myocardial IRI and Determination of Infarct Size

Myocardial infarction was induced in intact mice as previously described.[3,4,18] Briefly, anesthetized mice (Avertin 250 mg/kg with additional 125 mg/kg IP dose every 30 minutes) were placed in a supine position on a heating pad, orally intubated with a PE-60 tube, and mechanically ventilated at a tidal volume of 10 µL/g and rate of 130 stroke/min (MiniVent Ventilator, Harvard Apparatus, Holliston, MA). A left thoracotomy was performed by dividing the left third and fourth ribs and intervening intercostal muscle to expose the heart. An 8-0 Prolene suture was passed underneath the left coronary artery (LCA) at the level of the lower edge of the left atrium and tied over a piece of PE-50 tubing to occlude the LCA for 40 minutes. Successful LCA occlusion was confirmed by color change in the region at risk. Reperfusion was achieved by removing the tubing. A volume of 1 to 1.5 mL IP 5% dextrose was given to replace insensible losses during the operation. Core body temperature was monitored throughout the operation with a rectal thermocouple interfaced to a digital thermometer (Barnant Co, Barrington, IL) and maintained between 36.5 and 37.5°C.

Following 60 minutes of reperfusion, mice were euthanized under deep anesthesia, and the heart was isolated and cannulated through the ascending aorta with a blunt 23-gauge needle and sequentially perfused with 3 mL 37°C PBS (pH=7.4) and 3 mL 37°C 1% 2,3,5-Triphenyltetrazolium chloride in PBS. The LCA was then reoccluded by retying the encircling suture, and the heart was then perfused with 0.5 to 1.0 mL 10% Phthalo Blue (Heubach Ltd, Fairless Hills, PA) to delineate the nonischemic region. The heart was then frozen and trimmed of the right ventricle and atria. The left ventricle was cut into 5 to 7 slices, which were fixed in 10% neutral buffered formalin solution. Each slice was weighed and photographed. The sizes of the nonischemic area, the ischemic risk region and the infarct area were calculated as a percentage of corresponding area multiplied by the weight of the slice as previously reported (Figure 1C).[3,4,18–21] The ischemic risk region (RR, expressed as percentage of left ventricular mass) is representative of the area of myocardium supplied by the LCA and becomes ischemic with LCA occlusion, and the infarct size (IS) was expressed as a percentage of the RR.

Depletion of pDCs and Flow Cytometry

pDCs were depleted in WT mice by serial intraperitoneal injection of PDCA-1 antibodies with 250 µg PDCA-1 antibody (Miltenyi Biotec) every 2 days for a total of 4 doses. Control mice were injected with an equivalent amount of isotype IgG (Miltenyi Biotec). Four days after the final injection of antibodies, spleens were harvested from each group (n=4) for flow cytometry. The remaining mice underwent myocardial IRI for infarct size determination.

Flow Cytometry

The spleen was dissociated into a single-cell suspension using PBS supplemented with 10% fetal bovine serum in GentleMACs tubes (Miltenyi Biotec). Red blood cells were lysed by resuspending splenic cells in ammonium chloride-Tris buffer and incubation at room temperature for 8 minutes. Leukocytes were collected following centrifugation and washed twice in PBS. The splenic leukocytes were resuspended in PBS and enriched to 2×10^6/µL. LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoFisher) was used to check the viability of splenic leukocytes. Specimens (100 µL each) were treated with CD16/32 blocking antibodies for 10 minutes followed by antibody mixtures (APC/Cy7-CD45, FITC-CD3, PerCP/Cy5.5-B220, APC-IA/IE, PE-PDCA1, BV421-CD11c) (BioLegend, San Diego CA) for 30 minutes. Corresponding fluorescence minus one controls were also prepared. Then, all specimens were fixed by adding 500 µL 4% paraformaldehyde (Sigma-Aldrich). Flow cytometry was performed using Attune NxT Flow Cytometer (ThermoFisher), and data were analyzed using FlowJo software (BD Company, Ashland, OR). The pDCs were defined by $CD45^+$, CD3-/IA/IE^+ and B220+/$PDCA1^+$, and cDCs by $CD45^+$ and CD11c+/IA/IE^+.

Cell Culture Study With Splenocytes Treated With Cardiac Perfusate

Acquisition of Cardiac Perfusate

Wild type mice were anesthetized and orally intubated before undergoing a period of LCA occlusion (5, 10, 20 or 40 minutes of ischemia) without reperfusion. Then hearts were harvested and cannulated via the ascending aorta with a 23-gauge needle and perfused with 500 µL 37°C phosphate buffered saline (PBS, pH=7.4) for three cycles. The cardiac perfusate (CP) was collected and centrifuged at 0.9*g* for 20 minutes to discard cellular sediments. CP levels of cfDNA, HMGB1, and cardiac troponin T (cTnT) were evaluated using Nanodrop and western blot (anti-HMGB1 and cTnT antibodies purchased from Abcam).[2,14]

Splenocyte Culture With Stimulation by CP

Splenic leukocytes were collected from WT mice (as described in flow cytometry methods) and incubated in 6-well plates (BD Company) at a concentration

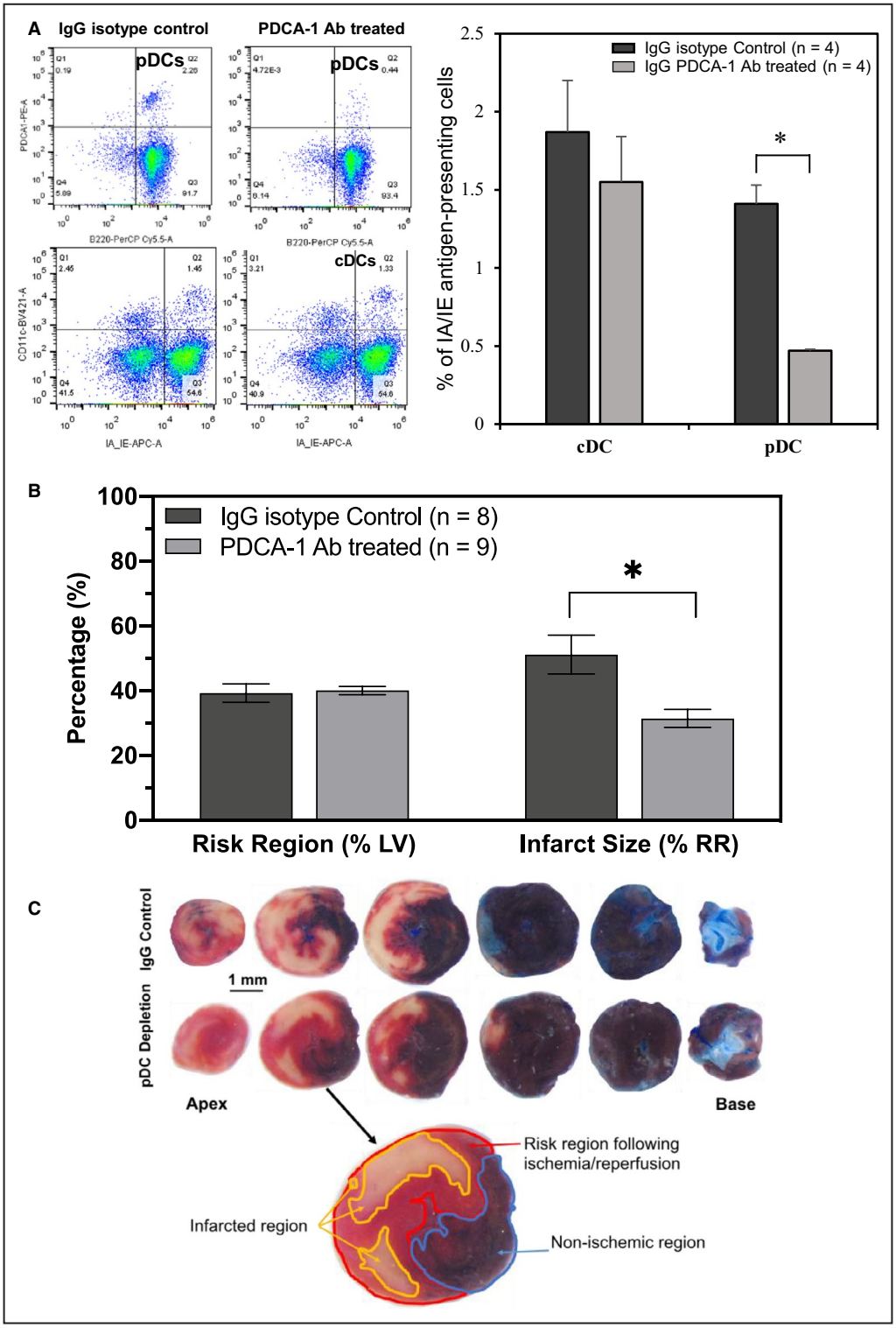

Figure 1. Plasmacytoid dendritic cell (pDC) depletion and effect of infarct size after ischemia/reperfusion injury (IRI).

A, Serial intraperitoneal (IP) injection of PDCA-1 antibodies significantly depleted splenic pDCs without affecting classic dendritic cells (cDCs). The numbers of cDCs and pDCs are presented as percentage of splenic IA/IE positive antigen presenting cells (APC). **B**, Ischemic region at risk (RR; percentage of left ventricle, as defined by the area supplied by the left coronary artery) and infarct size (IS; percentage of RR) after 40′/60′ IRI in pDC-depleted and isotype IgG-treated control mice. *P<0.05. **C**, Representative 2,3,5-Triphenyltetrazolium chloride–Phthalo blue staining left ventricle sections following 40′/60′ IRI from pDC-depleted and control mice. IA/IE, MHC class II antigen.

of 8.2×10^6 cells in 1.2 mL of culture media per well. Then 200 µL of 5′/0′ CP or 40′/0′ CP, respectively, were added to 4-well (n=4/group) cell culture plates and incubated for 4 hours. Another group had splenocytes pretreated with RU.521 at a dose of 8 µg/mL (19 µmol/L) administered 30 minutes before addition of 40′/0′ CP and incubation for 4 hours. Following the incubation period, culture supernatants were collected, and levels of interferon-α and interferon-β were measured by western blot. Live counts of splenic leukocytes were measured using a fluorescence automated cell counter (Cellometer K2, Nexcelom. Lawrence, MA).

Measurement of Interferon-α and Interferon-β

Plasma and culture supernatant levels of interferon-α and interferon-β were determined using an ELISA kit (Bio-Rad Laboratories). Levels of interferon-α and interferon-β in the supernatant of splenic leukocyte cultures were also evaluated using western blot (ThermoFisher).

Statistical Analysis

Comparisons between groups were performed with 1-way ANOVA with Bonferroni's correction for multiple comparisons and unpaired Student's *t* test. Prism 9 (GraphPad Software Inc., La Jolla, CA) was used to perform statistical calculations. Flow cytometry data was analyzed using FlowJo. Data are presented as mean±SEM, with a *P* value of <0.05 indicating statistical significance.

RESULTS

Depletion of pDCs Attenuates Myocardial IRI

Four days after the final injection of the antibodies (PDCA-1 for pDC depletion or isotype IgG control), spleens were harvested from each group (n=4) for flow cytometry, which demonstrated that PDCA-1 antibody treatment had no effect on the number of splenic cDCs (expressed as percentage of MHC class II antigen [IA/IE] positive antigen presenting cells, 1.87±0.33 versus 1.55±0.12 in IgG control mice, *P*>0.05), but significantly reduced splenic pDCs by 60% (0.47±0.01 versus 1.41±0.29 in IgG control mice, *P*<0.05; Figure 1A). In pDC-depleted mice undergoing 40′/60′ IRI, there was no difference in ischemic risk region (RR, expressed as percentage of left ventricular mass) compared with the isotype IgG control group (39±3 versus 40±1, *P*>0.05), but the pDC-depleted mice had significantly smaller IS (expressed as percentage of RR) of 31±3 when compared with IgG control mice (49±3, *P*<0.05; Figure 1B and 1C).

Blockade of IFN-I Production Attenuates Myocardial IRI

Production of IFN-I requires sequential activation of a signaling pathway which includes cGAS, STING, and IRF3. The role of cGAS, STING, and IRF3 in mediating myocardial IRI was thus evaluated using selective inhibitor of cGAS (RU.521 at a dose of 0.2 µg/g body weight), anti-STING antibody (1 µg/g), or anti-IRF3 antibody (1 µg/g), respectively, in C57BL/6 WT mice undergoing 40′/60′ IRI. These compound or antibodies were administered 5 minutes before onset of reperfusion. There was no difference in RR among all groups (*P*>0.05). IgG control mice had an IS of 53±4. Individual blockade of cGAS, STING, or IRF3 similarly attenuated IS by ≈45% to 30.4±4, 31.9±5, and 33.9±5, respectively (*P*<0.05 versus IgG control; Figure 2).

Blockade of pDC – IFN-1 Pathway Reduces Production of IFN-I

Following 40′/60′ myocardial IRI in WT control mice, pDC-depleted mice, and RU.521 treated mice, or 100 minutes of maintenance under anesthesia and mechanical ventilation after thoracotomy in sham-operated mice, the plasma levels of interferon-α and interferon-β were measured by ELISA. Compared with sham, both interferon-α and interferon-β were found to be significantly elevated in following 40′/60′ ischemia/reperfusion (50.3±4.3 versus 22.5±1.2 pg/mL, and 31.4±2.4±18.3±0.6 pg/mL, respectively; *P*<0.05 for both; Figure 3). However, this increase interferon-α and interferon-β was significantly attenuated in RU.521-treated (31.4±2.2 pg/mL and 22.1±0.5 pg/mL, *P*<0.05 versus control for both) and pDC-depleted mice

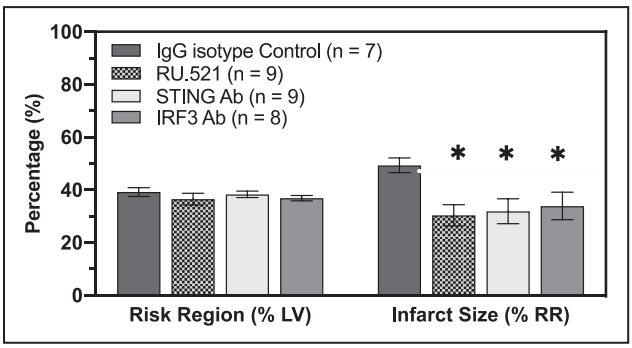

Figure 2. Infarct size analysis after blockade of components of the pathway leading to type I interferon (IFN-I) production. Ischemic region at risk (RR; percentage of left ventricle) and infarct size (IS; percentage of RR) after 40′/60′ ischemia/reperfusion injury (IRI) with individual blockade of cGAS, STING, and IRF3 with selective inhibitors RU.521, anti-STING, or anti-IRF3 mAb, respectively, were compared with a control group that underwent treatment with PBS only. **P*<0.05 vs IgG isotype control group. cGAS indicates cyclic GMP-AMP synthase; IRF3, interferon regulatory factor 3; mAb, monoclonal antibody; and STING, stimulator of interferon genes.

(25.9±1.2 pg/mL and 19.0±0.2 pg/mL; *P*<0.05 versus control for both).

IFN-I Mediates Myocardial IRI by Activating IFNAR1

WT mice treated with PBS (control), control isotype IgG monoclonal antibody (mAb) at a dose of 2 μg/g, or IFNAR1 mAb (MAR1-5A3) at a dose of 2 μg/g as an intravenous bolus 5 minutes before reperfusion, and congenic IFNAR1$^{-/-}$ mice underwent 40′/60′ IRI. RRs were comparable among these groups (*P*>0.05). WT control and IgG control mice had similar IS (53±4% versus 49±3%; *P*>0.05). IS was significantly reduced by >30% in IFNAR1 mAb-treated and IFNAR1$^{-/-}$ mice (29.5±4% and 34.3±5% respectively, *P*<0.05 versus PBS control and *P*<0.05 versus isotype IgG control; Figure 4).

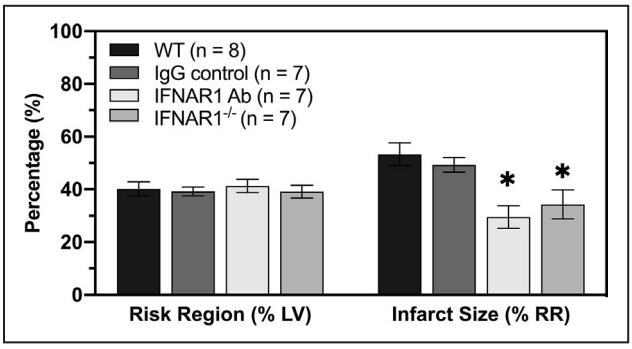

Figure 4. Infarct size analysis with loss of interferon-α/β receptor (IFNAR) function.
Ischemic region at risk (RR, percentage of left ventricle) and infarct size (IS, percentage of RR) after 40′/60′ ischemia/reperfusion injury were compared in interferon-α receptor-1 knockout (IFNAR1) null mice and mice that underwent blockade of IFNAR with IRNAR1 antibody to a wild-type (WT) control group that underwent treatment with PBS only, and an IgG control group that received isotype IgG monoclonal antibody. **P*<0.05 vs WT and IgG control groups.

Cardiac-Derived cfDNA and HMGB1 Stimulate Splenic Leukocytes to Release IFN-I

CP was collected from WT mice undergoing varying durations of myocardial ischemia without reperfusion, and levels of cfDNA, HMGB1, and cTnT were measured. There was no difference between sham and 5′/0′ CP levels of cfDNA or HMGB1 (*P*>0.05), but cTnT was increased in 5′/0′ compared with sham (*P*<0.05; Figure 5A and 5B). CP levels of cfDNA, HMGB1, and cTnT were exponentially more elevated in 40′/0′ compared with 20′/0′ and 5′/0′ (*P*<0.05), and overall increased as a function of ischemia time.

Splenic leukocytes were treated with short ischemia time CP (5′/0′, which served as control), and long ischemia time CP (40′/0′) with or without RU.521 pretreatment of the splenocytes (n=4 culture wells/group). All treatments reduced live splenocyte count by ≈7% after 4 hours of culture with no significant difference among the three groups (*P*>0.05, Figure 6A). While the 5′/0′ CP, containing 22±3 μg/mL cfDNA (Figure 5A), increased production of interferon-α and interferon-β, the 40′/0′ CP, containing 160±19 μg/mL cardiogenic cfDNA, stimulated splenocytes to produce significantly more interferon-α and interferon-β, by >3-fold and 7-fold, respectively (*P*<0.05 versus 5′/0′ CP, Figure 6B). RU.521 (at a dose of 19 μmol/L) significantly reduced the effect of 40′/0′ CP on splenocyte production of interferon-α and interferon-β (*P*<0.05 versus 40′/0′ CP; Figure 6B).

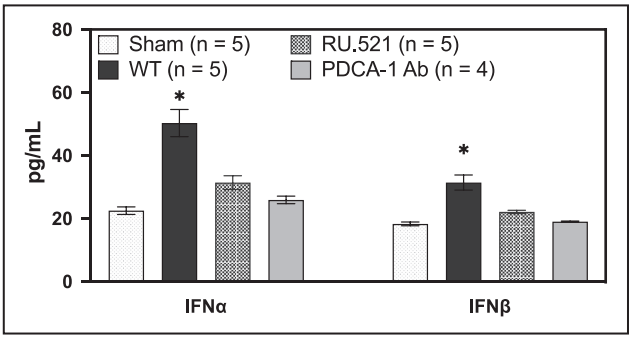

Figure 3. Circulating plasma levels of interferon-α and -β immediately following 40′/60′ ischemia/reperfusion injury were compared after depletion of plasmacytoid dendritic cells by plasmacytoid dendritic cell antigen-1 antibody and inhibition of cyclic GMP-AMP synthase by RU.521 to those in a control group that underwent treatment with PBS only and a sham operated group that underwent thoracotomy only. **P*<0.05 vs all other groups.

DISCUSSION

Our previous studies have demonstrated that necrotic cardiomyocytes release DAMPs, cfDNA, and HMGB1, that enter into the circulation during reperfusion, stimulate inflammatory responses, and ultimately exacerbate myocardial infarct size.[2,14] The present study further demonstrates that DAMPs released from ischemic myocardium upon reperfusion stimulate production of IFN-I by splenic pDCs. IFN-I then activates IFNARs on downstream leukocytes to amplify the detrimental inflammatory responses that exacerbate myocardial infarct size. Blockade of any portion of this pDC–IFN-I–IFNAR pathway suffices to significantly attenuate myocardial IRI.

Interferons have long been associated with regulating the immune response to infection, inflammation, and tumorigenesis. The IFN-1 family consists of

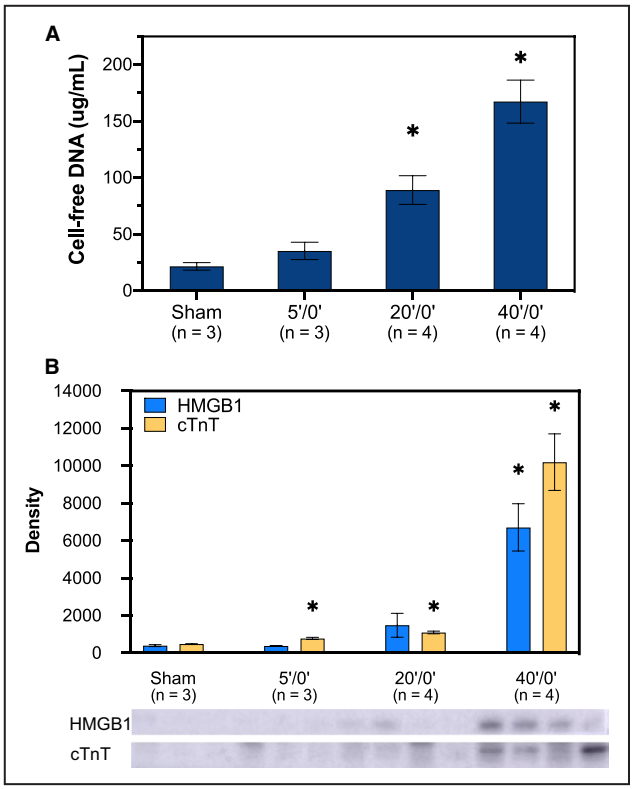

Figure 5. Markers of myocyte injury and damage-associated molecular patterns (DAMPs) released after ischemic injury.
A, Levels of cell-free DNA (cfDNA) and **B**, HMGB1 (high mobility group boxy protein 1) and cardiac troponin T (cTnT) in cardiac perfusate (CP) obtained following coronary artery occlusion without reperfusion. The production of cfDNA and HMGB1 is a function of myocardial ischemic duration and myocardial injury as defined by cTnT. *P<0.05 vs all other groups.

a number of related cytokines that all act through the interferon-α/β receptor (IFNAR), which is composed of 2 subunits, IFNAR1 and IFNAR2. Interferon-α, -β, -ε, -κ, -ω, -δ, and -τ, as well as limitin, all signal through the IFNAR; however, only the first 2 to be discovered, interferon--α and -β, have been studied in depth.[22–24] Type I interferons are pleiotropic cytokines that influence immune responses through their effects on myeloid cells,[25,26] T cells,[27,28] and B cells,[27] as well as promote the acquisition of cytotoxic activity by both T and natural killer cells.[23,24,28,29] IFN-I is expressed rapidly in response to infection and plays a key role in mediating innate host defenses and modulating the adaptive immune response against pathogens.[30,31] Although most cells in the body are able to produce IFN-I, pDCs have been termed the natural "IFN-producing cells" because of their unique molecular adaptations for nucleic acid sensing and ability to produce enormous amounts of IFN-I.[32–34] Dendritic cells (DCs) serve as the sentinels of the immune system and instruct the adaptive immune response to peripheral pathogens.[5,6] Compared with the classic DC (cDC), pDCs represent a small subset of DCs that accumulate mainly in the blood and lymphoid tissues.[6,9] It has been well established that circulating and residential pDCs sense pathogens and, upon activation, produce IFN-I. Naive T cells are subsequently activated by either IFN-I or the antigen-presenting pathway. The significant role of IFN-I in post–myocardial infarction remodeling has been well defined.[35] Our study suggests that pDC-derived IFN-I, whose production is stimulated by cardiogenic DAMPs cfDNA and HMGB1 (Figure 5 and 6), is responsible for mediating postischemia reperfusion injury (Figure 1).

We have shown that DAMPs cfDNA and HMGB1 are released from ischemic cardiomyocytes during reperfusion to promote inflammatory responses and induce reperfusion injury; both cfDNA and HMGB1 are required in this detrimental process as HMGB1 facilitates transportation of cfDNA into the cytosol to activate downstream immune responses.[2,14] By collecting cardiac perfusate at different durations of LCA occlusion in this study, we reaffirmed that myocardial excretion of DAMPs cfDNA and HMGB1 and degree of myocardial necrosis as quantified by CP cTnT level is a function of ischemic duration (Figure 5). We have previously reported that myocardial IRI is minimal if ischemic duration is <10′,[2,18] and showed here that at 5 minutes of LCA occlusion there is no increased release of cfDNA or HMBG1 and negligible ischemia-induced cardiomyocyte necrosis, in comparison to the multifold increase of cTnT with longer durations of LCA occlusion. At 40 minutes of LCA occlusion, there was a dramatic increase in myocardial injury as represented by CP levels of cfDNA and HMGB1, and cTnT (Figure 5). Using CP to treat splenic leukocytes, we found that 5′/0′ CP had minimal effect in stimulating splenic leukocyte production of interferon-α or interferon while 40′/0′ CP significantly increased interferon-α and -β production. Pretreatment of the splenocytes with RU.521, an inhibitor of the signaling pathway leading to IFN-I production, significantly attenuated this stimulatory effect of 40′/0′ CP (Figure 6B). These results demonstrate that cfDNA and HMGB1 released from ischemic cardiomyocytes are capable of stimulating an IFN-I–producing inflammatory response.

Facilitated by HMGB1 and leukocyte-expressed pattern recognition receptor RAGE, cfDNA enters leukocytes and mediates an $CD4^+$ T-cell inflammatory response via a cytosolic TLR9-dependent pathway.[2,4,14] We have shown that the ischemic myocardium is the sole source of circulating DNA immune complex that exacerbate inflammatory responses during reperfusion by activating TLR9.[2,18] TLR9 has been reported to sense and detect endogenous DNA derived from dead cells[36,37] and mediate IRI.[2,36] However, expression of TLRs is substantively low in T cells.[38] Thus, this cfDNA-HMGB1-RAGE-TLR9 pathway must occur in another immune cell subtype upstream of T-cell activation.

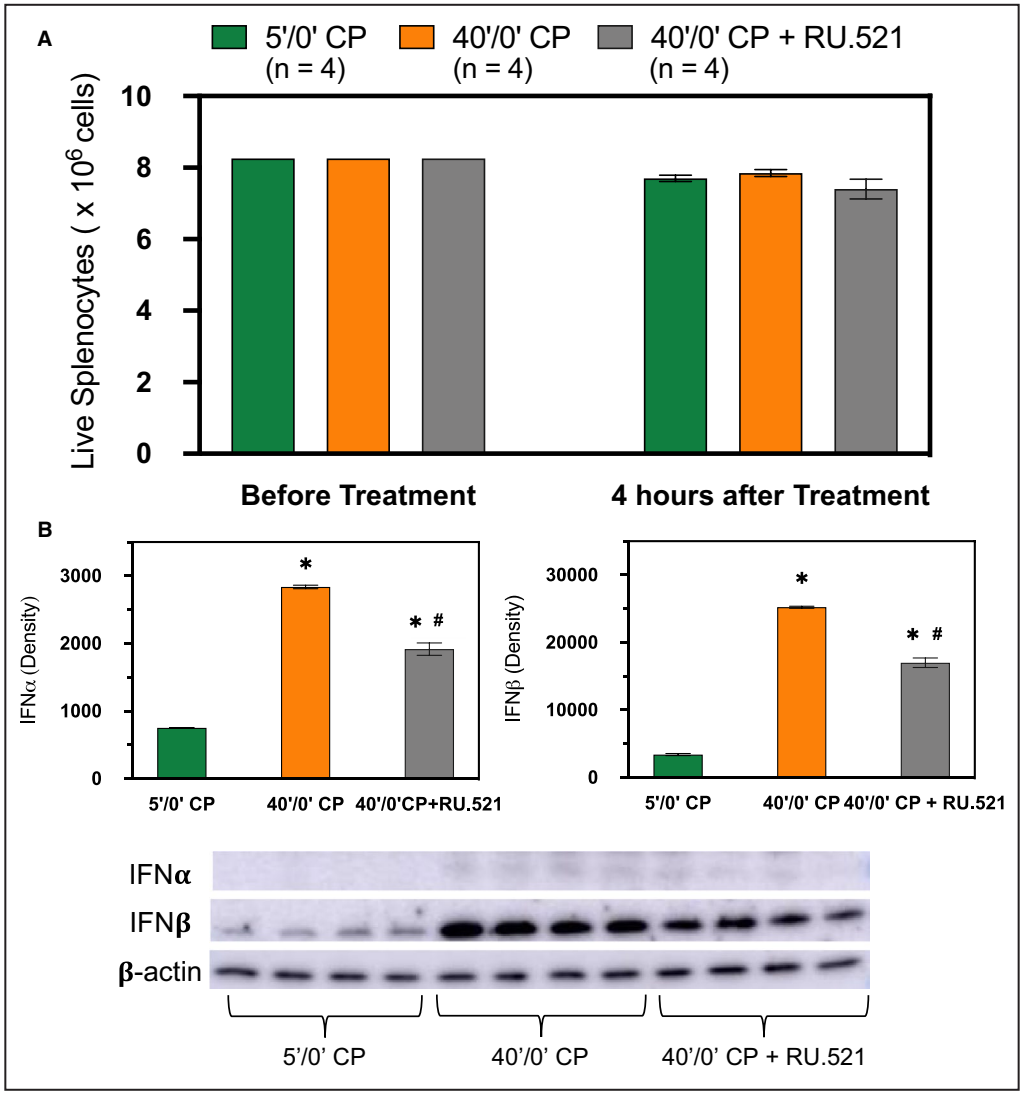

Figure 6. Effect of postischemia cardiac perfusate (CP) on splenocyte production of type I interferons.
Splenocyte cultures were treated CP obtained after 5 minutes of ischemia without a reperfusion period (5′/0′) and CP obtained after 40 minutes of ischemia without reperfusion (40′/0′) both with and without additional pretreatment with RU.521. **A**, Live splenocytes before and at 4 hours following CP treatment. **B**, Levels of interferon-α and -β in splenocyte culture supernatant as measured by western blot after treatment with 5′/0′ CP, 40′/0′ CP, and 40′/0′ CP + RU.521. *P<0.05 vs 5′/0′ CP; #P<0.05 vs 40′/0′ CP.

TLR9 is particular potent in activating pDCs, which subsequently activate T cells by producing IFN-I,[29,39,40] and DAMPs, including HMGB1[10,11] and endogenous cfDNA,[12,13] have been shown to interact with pDCs to promote production of IFN-I. In the current study, pDC depletion using PDCA-1 mAb had an isolated effect in reducing pDCs without any significant effect on cDC numbers (Figure 1A). The pDC-depleted mice had significantly smaller IS and lower plasma interferon-α and -β levels following ischemia and reperfusion (Figure 1B and Figure 3), providing direct evidence that pDCs are activated in this setting to produce IFN-I and contribute importantly to myocardial IRI.

The intracellular cGAS-STING-IRF3 signaling pathway is a component of the immune system that leads to production of IFN-I in response to the presence of cytosolic cfDNA. Upon cfDNA detection, cGAS reacts to create cGAMP, which binds to stimulator of interferon genes (STING), triggering phosphorylation of transcription factor interferon regulatory factor 3 (IRF3). IRF3 then translocates to the nucleus to promote transcription of inflammatory genes including IFN-I.[35,41] To evaluate their role in myocardial IRI, cGAS, STING, and IRF3 were individually inhibited with cGAS inhibitor RU.521, anti-STING antibody, or anti-IRF3 antibody, respectively, before left coronary artery occlusion and subsequent

reperfusion. We found blockade of any 1 of these 3 key components of the IFN-1 pathway attenuated myocardial infarct size equally (Figure 2). Loss of IFNAR function by either antibody inhibition or genetic knock out was similarly effective in significantly attenuating myocardial IRI (Figure 4). These results indicate that IFN-I production contributes importantly to myocardial IRI, and elucidate another step in the pathway that mediates myocardial IRI by demonstrating the important role of IFNARs. This suggests that IFN-I mediates the exacerbation of myocardial injury by binding and activating IFNARs on downstream inflammatory cells, potentially $CD4^+$ T cells which we previously demonstrated to have a central role in this detrimental process.[3,4]

Though it has been reported that most cells in the body are able to produce IFN-1, we posit that the signaling pathway and resultant IFN-I production that primarily mediate myocardial IRI is located inside pDCs. pDCs have been shown to be stimulated by DAMPs, including HMGB1[10,11] endogenous cfDNA,[12,13] to produce IFN-I, and activated pDCs are known to rapidly produce enormous amounts of IFN-I.[32–34] Our previous study demonstrated that myocardial infarct size following IRI is finalized by the first hour after reperfusion.[1] Therefore, the inflammatory response during this period of time is critical to producing reperfusion injury.[2,14] As pDCs are the first line immune response to cfDNA, the surge of cfDNA released from ischemia-injured myocardium (Figure 5) activates pDCs to likely be the major source of IFN-I during this immediate period of time after onset of reperfusion. This is supported by our results finding that pDC depletion completely abrogates the acute increase in circulating interferon-α and -β stimulated by myocardial ischemia and reperfusion, an effect that was equal to or exceeded that of IFN-I production inhibition by RU.521. Additionally, we demonstrated that blockade of the pathway leading to IFN-I production imparted similar cardio-protection as pDC depletion (Figures 1–2). Thus, inhibition of pDCs or the production of IFN-I likely within pDCs suffices to diminish the ischemic injury-induced inflammatory response and attenuate postischemic reperfusion injury.

Study Limitations

We have previously demonstrated that cardiogenic cfDNA mediates myocardial IRI by activating TLR9, and the current study further defines that pDCs have an essential role in the pathway downstream of TLR9 activation. However, we did not provide direct evidence to show that cfDNA binds and activates TLR9 inside pDCs and the possible involvement of other immune cells cannot be excluded. Additionally, researchers were not blinded when performing myocardial infarct measurements, which is a potential source of bias. Overall, though, the present study provides data necessary for designing further studies that will delineate the mechanisms involved in inflammatory response–mediated IRI. Our future directions include further evaluation of the direct interaction between cardiogenic DAMPs and isolated pDCs. Additionally, we plan to focus on the downstream activation of inflammatory cells by IFN-I. This may be broad as IFNAR is expressed on many inflammatory cells.[8,42] In particular, though, we plan to investigate the interaction of $CD4^+$ T cell activation with the IFN-I signaling pathway as we have previously demonstrated that $CD4^+$ T cells play a central role in mediating myocardial IRI,[3,4] and IFN-I is a potent activator of $CD4^+$ T cells via IFNAR.[27,28,43–45] Furthermore, the signaling pathways activated inside $CD4^+$ T cells and following IFN-I binding to IFNAR remain to be defined.

In summary, we demonstrated that production of IFN-I is activated during myocardial IRI by cardiogenic DAMPs released following ischemic injury in a pDC-dependent manner. Blockade of this process, either by depletion of pDCs, inhibition of the cGAS-STING-IRF3 signaling pathway leading to IFN-I production, or downstream loss of IFNAR function attenuates myocardial IRI and infarct size following acute coronary occlusion. These results have important implications because they present new potential therapeutic targets in the pDC–IFN-I–IFNAR pathway to reduce infarct size following MI, and newly identify a specific splenic leukocyte population (pDCs) that play an important role in mediating myocardial IRI.

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Disclosures

None.

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