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Differential tissue-specific function of the Adora2b in cardio-protection

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Abstract

The adenosine A2b-receptor (Adora2b) has been implicated in cardio-protection from myocardial ischemia. As such the Adora2b was found to be critical in ischemic preconditioning (IP) or ischemia reperfusion (IR) injury of the heart. While the Adora2b is present on various cells types, the tissue specific role of the Adora2b in cardio-protection is still unknown.

To study the tissue specific role of Adora2b signaling on inflammatory cells, endothelia or myocytes during myocardial ischemia *in vivo*, we intercrossed floxed *Adora2b* mice with Lyz2-Cre+, VE-Cadherin-Cre+ or Myosin-Cre+ transgenic mice, respectively. Mice were exposed to 60 minutes of myocardial ischemia with or without IP (4×5min) followed by 120 minutes of reperfusion.

Cardio-protection by IP was abolished in *Adora2b*$^{f/f}$-VE-Cadherin-Cre+ or *Adora2b*$^{f/f}$-Myosin-Cre+, indicating that Adora2bs signaling on endothelia or myocytes mediates IP. In contrast, primarily Adora2b signaling on inflammatory cells was necessary to provide cardio-protection in IR injury, indicated by significantly larger infarcts and higher troponin levels in *Adora2b*$^{f/f}$-Lyz2-Cre+ mice only. Cytokine profiling of IR injury in *Adora2b*$^{f/f}$-Lyz2-Cre+ mice pointed towards PMNs. Analysis of PMNs from *Adora2b*$^{f/f}$-Lyz2-Cre+ confirmed PMNs as one source of identified tissue cytokines. Finally, adoptive transfer of *Ador2b*$^{-/-}$ PMNs revealed a critical role of the Adorab2 on PMNs in cardio-protection from IR-injury.

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Adora2b signaling mediates different types of cardio-protection in a tissue specific manner. These findings have implications for the use of Adora2b agonists in the treatment or prevention of myocardial injury by ischemia.

INTRODUCTION

Myocardial infarction (MI) is the leading cause of death worldwide and according to the World Health Organization responsible for 7.25 million deaths each year. In the USA, about every 44 seconds somebody will have a new heart attack. About 34 percent of the people who experience a coronary attack in a given year will die from it (1). Despite standard therapies such as early coronary artery reperfusion for the treatment of acute ST-elevation myocardial infarction, morbidity and mortality from MI remain significant. Based on this, the incidence of congestive heart failure continues to increase, and there is a need to provide better therapy that reduces the amount of necrosis that may be coupled with better clinical outcome in the setting of MI (2, 3).

Substantial research efforts have been dedicated to identify agents modulating the inflammatory response after MI which represent one mechanism in myocardial injury by reperfusion (IR-injury). Multiple studies have suggested that adenosine is critical for protection against IR-injury (4). The mechanism of adenosine dependent cardio-protection involves most likely a shift in parenchymal cells metabolism, vasodilatation of coronary arteries or inhibition of leukocyte-mediated inflammatory responses (4, 5).

Adenosine elicits protective effects through four adenosine receptors [ARs; Adora1, Adora2a, Adora2b and Adora3 (6–10)]. All ARs have been associated with cardiac tissue protection in different settings. In particular, the Adora2b has been implicated in ischemic preconditioning (IP) (11, 12) and post-conditioning (13) effects of the heart. Both represent powerful cardio-protective mechanisms where the heart tissue at risk is exposed to short repeated ischemic periods either prior to the onset of ischemia or at the onset of reperfusion (14–16).

Although *in vivo* experiments have shown the cardio-protective effect of the Adora2b (11, 13, 17–20), these experiments have not dissected the major cellular targets (myocytes, endothelium, or bone-marrow derived cells) responsible for the salutary effect of Adora2b activation in different settings such as cardiac IP or IR-injury of the heart.

In the present study, we used *state-of-the-art* Cre-lox mouse models to generate tissue specific Adora2b deletion on bone marrow derived inflammatory cells (*Adora2b*$^{f/f}$-Lyz2-Cre+), endothelia (*Adora2b*$^{f/f}$-VE-Cadherin-Cre+) or myocytes (*Adora2b*$^{f/f}$-Myosin-Cre+). Exposing those mice to a murine *in-situ* model for IP or IR-injury indicated that the Adora2b has a differential tissue specific function in different settings. These findings implicate that tissue specific targeting of the Adora2b seems to be desirable when using Adora2b agonists to prevent or treat myocardial ischemia.

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MATERIALS & METHODS

Mice

All animal procedures were performed in an AAALAC-accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the University of Colorado Denver Institutional Animal Care and Use Committee. For all studies we used male mice 8–16 weeks old. Studies were in accordance with the NIH guidelines for use of live animals. C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, USA). Adora2b-floxed (*Adora2b*$^{f/f}$) mice were generated by Ozgene (Pert, Australia). Lyz2-Cre+ (B6.129P2-Lyz2tm1(cre)Ifo/J) (21)], VE-Cadherin-Cre+ (B6.Cg-Tg(Cdh5-cre)7Mlia/J) (22) and tamoxifen inducible Myosin-Cre+ (Tg(Myh6-cre/Esr1*)1Jmk/J) (23) mice were purchased from Jackson laboratories. To obtain tissue specific *Adora2b*$^{-/-}$ mice, we crossbred *Adora2b*$^{f/f}$ (*Ozgene*) mice with the appropriate tissue specific Cre recombinase mouse. For studies using Myosin-Cre+ mice, all mice were induced by treatment with tamoxifen 1 mg/day i.p, dissolved in peanut oil for five days. Experiments were performed after additional 14 days following tamoxifen administration. Genotyping PCRs for tissue specific animals were performed by GeneTyper, NY, USA.

Isolation of adult cardiomyocytes

8 – 12 weeks old Myosin Cre+ or *Adora2b*$^{f/f}$ -Myosin-Cre+ mice were anesthetized and the heart was quickly removed from the chest cavity and immediately placed in ice-cold KHB buffer. After weighing, the aorta was cannulated and the heart were perfused with Ca2+-free KHB for 3 min followed by 8–12 min perfusion with Ca2+-free KHB containing collagenase and elastase. After perfusion, ventricles were removed, minced and incubated with the collagenase/elastase solution for an additional 3–7 min. The cells were filtered through a nylon mesh (300 μm) and collected in a 15 ml sterile tube. Myocytes were washed and calcium was slowly re-introduced in a stepwise fashion. Finally, cells were resuspended in MEM and plated on laminin. Cell were harvested the next day and immediately resuspenden in Trizol for mRNA analysis (19).

Isolation of myeloid cells

Heart tissue from Lyz2Cre+ or *Adora2b*$^{f/f}$ -Lyz2-Cre+ mice was minced and digested for 45 minutes using collagenase solution. Myeloid cells were isolated using EasySep CD11b-PE positive selection (StemCell Technologies), according to the manufacturer's instructions. After Trypan Blue staining to confirm cell viability, cells were immediately resuspenden in Trizol for mRNA analysis.

Isolation of endothelia cells

Heart tissue from VE-Cadherin-Cre+ or *Adora2b*$^{f/f}$ -VE-Cadherin-Cre+ mice was minced and digested for 45 minutes using collagenase solution. Endothelial cells were isolated using EasySep CD31-Biotin positive selection (StemCell Technologies), according to the manufacturer's instructions. After Trypan Blue staining to confirm cell viability, cells were immediately resuspenden in Trizol for mRNA analysis.

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Adora2b mRNA analysis from heart tissue cells

Total RNA was isolated from cells using the Trizol Reagent according to the manufacturer's instructions (Invitrogen, 15596–018). For this purpose, cells were homogenized in the presence of Trizol Reagent and chloroform was added. After spinning at 12,000xg for 15 minutes, the aqueous phase was removed and the RNA was precipitated with isopropanol. RNA was pelleted, washed with ethanol, treated with DNAse, and the concentration was quantified. The PCR reactions contained a mix of forward and reverse oligonucleotides with SYBR Green (Bio-Rad, 170–8880). Each target sequence was amplified using increasing numbers of cycles of 94°C for 1 min, 58°C for 0.5 min, 72°C for 1 min. Quantification of transcript levels was determined by real-time RT-PCR (iCycler; Bio-Rad Laboratories Inc.). The primers were Quantitect from Qiagen (Mm_Adora2b, QT01543444).

Murine Model for cardiac IP in IR-injury

Anesthesia was induced (70 mg/kg i.p.) and maintained (10 mg/kg/h i.p.) with sodium pentobarbital. Mice were placed on a temperature-controlled heated table (RT, Effenberg, Munich, Germany) with a rectal thermometer probe to maintain body temperature at 37°C. The tracheal tube was connected to a ventilator (Servo 900C, DRE, USA). Animals were ventilated with a pressure controlled ventilation mode (pressure of 10 mbar, frequency 110 breaths/min, positive end-expiratory pressure of 5 mbar, $FiO_2 = 0.4$). After induction of anesthesia, animals were monitored with a surface electrocardiogram (ECG, Hewlett Packard, DRE, USA). Fluid replacement was performed with normal saline, 0.2ml/h i.a. until the onset of reperfusion, and with 1 ml/h i.a. during reperfusion. Operations were performed under an upright dissecting microscope (Olympus SZX12). Following left anterior thoracotomy, exposure of the heart and dissection of the pericardium, the left coronary artery (LCA) was visually identified and an 8.0 nylon suture (Prolene, Ethicon, USA) was placed around the vessel. Atraumatic LCA occlusion for ischemia and IP studies was performed using a hanging weight system (24, 25). Successful LCA occlusion was confirmed by an immediate color change of the vessel from light red to dark violet, and of the myocardium supplied by the vessel from bright red to white (pale), as well as the immediate occurrence of ST-elevations in the ECG. During reperfusion, the changes of color immediately disappeared when the hanging weights were lifted and the LCA was perfused again. Infarct sizes were determined by calculating the percentage of myocardium that underwent infarction compared to the area at risk (AAR) using a previously described double staining technique with Evan's blue and triphenyltetrazolium chloride (TTC). AAR and the infarct size were determined via planimetry using the NIH software Image 1.0 and the degree of myocardial damage was calculated as percent of infarcted myocardium from the AAR (12, 18).

Heart Enzyme Measurement

Blood was collected by central venous puncture for troponin I (cTnI) measurements using a quantitative rapid cTnI assay (Life Diagnostics, Inc., West Chester, PA) (18–20, 25, 26).

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Analysis of β-gal and F4/80 expression in hearts of Adora2b reporter mice

To localize the Adora2b in heart tissue, we analyzed β-galactosidase (β-gal) expression in Adora2b-KO/β-gal–knock-in mice (27). Hearts were harvested following perfusion fixation in 4% PFA/0.1 M PB (phosphate buffer without saline) and post fixed in the same fixative for 3 hours, followed by cryoprotection in 20% sucrose in 0.1 M PB pH 7.2 overnight at 4°C. Using a cryostat, 16-μm sections were collected onto Superfrost Plus slides (Fisher Scientific). The slides underwent three 10-minute washes in 0.1 M PBS before incubation in blocking solution (2% normal goat serum, 1% BSA, 0.3% Triton in PBS) for 1 hour at room temperature. The samples were then incubated overnight at 4°C in guinea pig anti–β-gal (1:1000, AB was a generous gift from Professor Thomas E. Finger at UC Denver) diluted in blocking solution. To stain for macrophages and determine whether they co-localize with β-gal staining the goat anti-rat F4/80 (1:100) (Serotec, Oxford, UK) was co-incubated with the anti-β-gal antibody diluted in blocking solution. Following three washes in PBS, samples were incubated for 2 hours at room temperature with Alexa Fluor 594 goat anti-guinea pig (1:400)(Invitrogen) and Alexa Flour 488 goat anti-rat (1:400) (Invitrogen) diluted in blocking buffer. Slides were then washed twice in PBS, followed by a 10-minute wash in 0.1 M PB and cover slipped using Vectashield containing DAPI (Vector Laboratories). Immunofluorescent images were taken using a Zeiss 780 LSM.

Cytokine multiplex ELISA

To measure cytokine tissue levels after 60 minutes of ischemia, Lyz2Cre+ or *Adora2*$^{f/f}$-Lyz2Cre+ mice were euthanized following 120 minutes of reperfusion. Remaining blood was removed, the myocardial tissue (area at risk) was excised after delineation with Evans' blue and immediately frozen with liquid nitrogen and stored at −80°C. Tissues were homogenized on ice using a Tissue Master 125 (OMNI International) in T-PER (Thermo Scientific, 78510) containing Pierce Protease Inhibitors according to the manufacturer's recommendations (Thermo Scientific, 88665). After spinning at 10,000 RPM for 5 minutes the supernatant was removed and diluted to a final concentration of 10ug/100uL per well. The cytokine ELISA array (Signosis, EA-4005) was performed according to the manufacturer's instructions.

IL6 and TNFa mRNA analysis from in PMNs

PMNs were harvested from bone marrow according to the product manual for preparing a single cell suspension (STEMCELL Technology, 19762A). The PMNs were subsequently isolated from the bone marrow according to the EasySep protocol (STEMCELL Technology, 19762A) using the purple EasySep magnet (STEMCELL Technology, 18000). The isolated PMNs were transferred immediately to ice to avoid activation. Total RNA was isolated from PMNs using the Trizol Reagent according to the manufacturer's instructions (Invitrogen, 15596–018). For this purpose, liquid nitrogen frozen cells were homogenized in the presence of Trizol Reagent and chloroform was added. After spinning at 12,000xg for 15 minutes, the aqueous phase was removed and the RNA was precipitated with isopropanol. RNA was pelleted, washed with ethanol, treated with DNAse, and the concentration was quantified. The PCR reactions contained a mix of forward and reverse oligonucleotides with SYBR Green (Bio-Rad, 170–8880). Each target sequence was amplified using increasing

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numbers of cycles of 94°C for 1 min, 58°C for 0.5 min, 72°C for 1 min. Quantification of transcript levels was determined by real-time RT-PCR (iCycler; Bio-Rad Laboratories Inc.). The primers were Quantitect from Qiagen (TNFα, QT00104006; IL-6, QT00098875).

ROS assay

Cardiac protein carbonyl was measured using OxiSelect protein carbonyl ELISA kit (Cell Biolabs, San Diego, CA).

Neutrophil depletion and adoptive transfer

Mice were treated with a Ly6G-specific mAb 1A8 [Bio X Cell (28)]. This depletes circulating neutrophils but does not affect circulating GR-1 positive monocytes, as described previously (28). In a subset of experiments mice were treated with an anti-GCSF antibody (PeproTech) that prevents recruitment of endogenous PMNs (29). To perform an adoptive transfer of PMNs into PMN depleted animals, we first euthanized donor wildtype mice (BL6/C57, 6–8 weeks) and harvested the bone marrow by flushing the femoral bones. PMNs we separated by negative selection using the EasySep™ Mouse Neutrophil Enrichment Kit (StemCell Technologies Inc) and counted by a hemocytometer. Then 1×10^6 cells were injected into the neutrophil-depleted mice via an arterial catheter over 10 min. After a waiting period for 60 min, the mice underwent myocardial ischemia and reperfusion injury as described above.

Statistics

Data were compared by Student's t test where appropriate. Values are expressed as mean ± SD from 3–6 animals per condition. The chosen numbers of animals per group was based on findings in previous studies and a subsequent samples size analysis. The studies are designed to be able to reject the null hypothesis that the population means of the experimental and control groups are equal with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05. Data are expressed as mean ± SD. $P < 0.05$ was considered statistically significant. For all statistical analysis GraphPad Prism 5.0 software for Windows was used.

RESULTS

Experimental setup and animal models to investigate the tissue specific role of Adora2b-dependent cardio-protection

The experimental protocols are displayed in Figure 1a. For myocardial ischemia and reperfusion injury we used 60 minutes of ischemia followed by 120 minutes of reperfusion (Figure 1a, *Model 1*). For ischemic precondition (IP) we performed four cycles of 5 minutes ischemia and 5 minutes of reperfusion prior to 60 min of ischemia and 120 min reperfusion (Figure 1a, *Model 2*). After reperfusion we visualized the infarcted area using Evan's Blue and triphenyltetrazolium chloride (TCC). In addition, we determined troponin I from serum samples.

To understand the tissue specific contribution of the Adora2b in cardio-protection we used *state-of-the-art* Cre-lox mouse models. Figure 1b displays the different Adora2b tissue

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specific mouse models that were used in the current studies. For Adora2b deletion on cardiomyocytes we generated *Adora2b*$^{f/f}$-Myosin-Cre+ mice, for deletion on endothelial cells we used *Adora2b*$^{f/f}$-VE-Cadherin-Cre+ mice, and for a bone-marrow derived cell deletion we used *Adora2b*$^{f/f}$-Lys2Cre+ mice (Figure 1b). The Myosin-Cre+ mice have an inducible Cre-recombinase (Figure 1b, upper panel) and therefore the *Adora2b*$^{f/f}$-Myosin-Cre+ and Myosin-Cre+ mice received 1 mg tamoxifen intraperitoneal for 5 days in order to induce Cre-recombinase activity. The other Cre-strains (VE-Cadherin-Cre+, Lyz2-Cre+) express constitutively Cre-recombinase and therefore no induction was necessary.

As shown in Figure 1c, the tissue-specific deletion of the Adora2b in the different tissue specific mouse models was confirmed using a genotyping PCR-analysis from hearts and aortas to understand how the cardiovascular system was affected. *Adora2b* floxed (lox), Cre recombinase (Cre), *Adora2b* knockout (KO) or *Adora2b* wildtype (WT) signal is depicted in each case. PCR-analysis confirmed that all tissue specific strains were positive for Cre recombinase and the floxed *Adora2b* gene. In *Adora2b*$^{f/f}$-Myosin-Cre+ mice we found – as expected - a KO signal in the heart but a WT signal in the aorta. In *Adora2b*$^{f/f}$-VE-Cadherin-Cre+ mice we found KO signal in the heart and the aorta, as both tissues are abundant in endothelial cells. *Adora2b*$^{f/f}$-Lys2Cre+ mice had a heterozygous signal in the heart and the aorta (KO/WT signal in heart and aorta), probably as a result of resident inflammatory cells in these tissues. To analyze the efficiency of *Adora2b* gene deletion in the respective tissues of the tissue specific mice we isolated mRNA from the cells in question and determined Adorab2 transcript levels using real time RT-PCR. Cardiomyocytes were isolated and cultured over night from *Adora2b*$^{f/f}$-Myosin-Cre+ or Myosin-Cre+ mice. Endothelial cells were isolated from *Adora2b*$^{f/f}$-VE-Cadherin-Cre+ or VE-Cadherin-Cre+ hearts. Myeloid cells or bone marrow cells were isolated from *Adora2b*$^{f/f}$-Lyz2Cre+ or Lyz2-Cre+ hearts or femurs, respectively. As shown in Figure 1d–f, *Adora2b* mRNA was significantly depleted in the different cell compartments from our tissue specific- *Adora2b* deleted mice. In summary, we generated three *state-of-the-art* Cre-lox mouse models in order to assess the tissue-specific contribution of Adora2b to IP or myocardial IR injury.

Adora2b signaling on bone marrow derived cells during ischemic preconditioning of the heart

Based on earlier studies using germline *Adora2b*$^{-/-}$ mice, the Adora2b is crucial for the cardio-protective effects of IP (12). To get further insight into a tissue specific function of the Adora2b, we recapitulated what cell types express Adora2b in the heart. To obtain a very specific expression profile of the Adora2b receptor in the mouse heart tissue, we used an *Adora2b*-knockout-β-gal knock-in reporter mouse (27). As shown in Figure 2a–c, β-gal staining (green) of representative heart sections from an Adora2b-β-gal-reporter mouse revealed dominantly Adora2b positive cells in the vessel walls at baseline and a strong and significant up-regulation of the Adora2b on endothelia and cardiomyocytes upon IP or ischemia-reperfusion (IR) injury. While some resident macrophages were detected in the heart (red staining, Figure 2d, e), none co-expressed the Adora2b (Figure 2f). Indeed, expression could be different in circulating vs. tissue resident macrophages (27). In addition, only IR treatment slightly increased the number of resident macrophages (Figure 2f). Based on the fact that IP consists of short non-lethal repeated ischemic periods prior to ischemia

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and based on our expression profile of cardiac Adora2b receptors, we hypothesized that bone marrow derived inflammatory cells do not play an important role during IP of the heart. To test this hypothesis we used *Adora2b*$^{f/f}$-Lyz2-Cre+ mice and their respective controls (Lyz2Cre+) and exposed them to 60 min of ischemia with ischemic preconditioning (+IP; 4 cycles of 5 min of ischemia followed my 5 minutes of reperfusion) or without IP (−IP) followed by 120 minutes of reperfusion. Infarct sizes were measured by double staining using Evan's blue and triphenyl-tetrazolium chloride, while serum troponin I was determined using ELISA. As shown in Figure 3a, c, IP significantly decreased infarct sizes from 39.3±5.5% (n=5) to 22.4±7.5% (n=4) and troponin I serum levels from 179.6±65.1 ng/ml to 68.7±72.7 ng/ml (n=6 per group) in controls (Lyz2-Cre+) mice. However, studies *Adora2b*$^{f/f}$-Lyz2-Cre+ mice (Figure 3b, c) also showed significantly decreased infarct sizes from 73.1±3.0% to 48.8±10.2% (n=5 per group) and troponin I serum levels from 396.0 ± 154.4 ng/ml to 133.9 ± 86.5 ng/ml (n=6 per group) when hearts had been pretreated with IP prior to ischemia.

Taken together, these data are in support of our initial hypothesis that Adora2b receptors on bone marrow derived inflammatory cells do not play an important role in mediating cardio-protection by IP. Since within the heart, the Adora2b is mainly expressed in the vessel wall and to some extent also in cardio-myocytes, those tissues might be important for cardio-protection by IP.

Adora2b signaling on endothelia and myocytes during ischemic preconditioning of the heart

Given that there is no apparent role for Adora2b on bone marrow derived inflammatory cells during cardiac IP, we next investigated the role of the endothelia or myocytes. First, we used our endothelial specific Adora2b deficient mice (*Adora2b*$^{f/f}$-VE-CadherinCre+) and appropriate controls (VE-Cadherin-Cre+). As shown in Figure 3d, f, IP significantly decreased infarct sizes from 45.9±8.4% to 26.5±9.6% (n=5 per group) and troponin I serum levels from 178.6±92.4 ng/ml to 58.2±63.6 ng/ml (n=6 per group) in VE-Cadherin-Cre+ mice. In contrast, IP had no significant effect on infarct sizes (−IP: 54.5±8.9%; n=6) vs. +IP: 46.7±12.7%; n=6) or troponin I serum levels (−IP: 219.9±162.1 ng/ml vs. +IP: 139.6±36.3 ng/ml; n=6 per group) in *Adora2b*$^{f/f}$-VE-Cadherin-Cre+, as shown in Figure 3e, f.

Next we analyzed the contribution of the Adora2b on cardio-myocytes as shown in Figure 4a–d. Here we found, that while IP significantly decreased infarct sizes from 45.9±3.1% (n=5) to 20.4±5.1% (n=4) and troponin I serum levels from 223.1±103.9 ng/ml to 34.9±17.7 ng/ml (n=6 per group) in control mice (Myosin-Cre+), IP in cardio-myocyte-specific Adora2b-deficient mice (*Adora2b*$^{f/f}$-Myosin-Cre+) significantly increased infarct sizes from 47.5±6.8% to 62.1±4.7% (n=5 per group) or troponin I serum levels from 188.2±55.3 ng/ml to 327.5±109.3 ng/ml (n=6 per group). As Myosin-Cre+ controls had been pretreated with tamoxifen and this could have anti-inflammatory effects (30), we performed infarct size studies in Myosin-Cre+ mice without tamoxifen treatment. As shown in Figure 4e, no significant differences in infarct sizes were observed between tamoxifen treated and untreated Myosin-Cre+ mice.

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Taken together these data show an important role of the Adora2b on endothelial cells and myocytes in mediating cardio-protection by IP.

IR injury in cardio-myocyte or endothelial specific Adora2b deletion

After we found an important role of the Adora2b on myocytes and endothelia in mediating cardio-protection from ischemia by IP we next analyzed their role in IR injury. Surprisingly, as shown in Figure 5a and Figure 5b, infarct sizes and troponin I serum values were unchanged between control mice and mice with a tissue specific Adora2b deletion on endothelia or myocytes (VE-Cadherin-Cre+*:* Infarct sizes 54.4±8.8%, cTnI 219.9±162.1 ng/m vs. *Adora2b*$^{f/f}$-VE-Cadherin-Cre+*:* infarct sizes 45.9±8.4%, cTnI 178.60±92.4 ng/ml; n=6 per group or Myosin-Cre+: Infarct sizes 45.9±3.1%, cTnI 223.1±103.9 ng/ml vs. *Adora2b*$^{f/f}$-Myosin-Cre+*:* Infarct sizes 47.5±6.8%, cTnI 188.2±55.3 ng/ml; n=6 per group). Taken together, these data indicate that Adora2b expressing cardiac cells like endothelia or myocytes do not play a major role in cardiac IR injury.

IR injury in bone marrow derived inflammatory cell specific Adora2b deletion

After we discovered that mice with a tissue specific deletion of the Adora2b on endothelia or myocytes do not have increased IR injury when compared to their respective controls, we next analyzed mice with an Adora2b deletion on bone marrow derived inflammatory cells which include macrophages, monocytes and PMNs. As shown in Figure 6a, *Adora2b*$^{f/f}$-Lyz2-Cre+ mice have increased infarct sizes when compared to Lyz2-Cre+ mice (Lyz2-Cre +*:* 39.2±5.5% vs. *Adora2b*$^{f/f}$-Lyz2-Cre+*:* 73.1 ± 3.0%) and troponin I serum levels (Lyz2-Cre+ mice: 179.6±65.1 ng/ml vs. *Adora2b*$^{f/f}$-Lyz2-Cre+*:* 396.0±154.4 ng/ml; n=6 per group). To identify potential cell specific cytokines responsible for the increased damage within the heart tissue during IR injury we performed a cytokine screen by using a multiplex ELISA. We found that *Adora2b*$^{f/f}$-Lyz2-Cre+ had higher levels of tumor necrosis factor alpha (TNFα) or Interleukin-6 (IL-6) in the area at risk after 60 minutes of ischemia and 120 minutes of reperfusion. In addition, lower levels of G-CSF, stem cell factor, IL-10 and resistin were found in *Adora2b*$^{f/f}$-Lyz2-Cre+ when comparted to controls (n=2 per group; Figure 6b). Based on the cytokine profile, we hypothesized that PMNs could be the major source of these cytokine changes (17). While Lyz2-Cre+ mice affect other bone marrow derived cells such as macrophages, they have been found to be particularly effective in deleting gene targets on PMNs in a tissue specific manner (31). We therefore isolated PMNs from Lyz2-Cre+ or *Adora2b*$^{f/f}$-Lyz2-Cre+ mice and determined transcript levels of IL6 or TNFα at baseline (Figure 6c). RT PCR studies revealed that IL6 baseline transcript values were increased by 3.8±0.3, and TNFα baseline transcript values were increased by 4.2±0.2-fold in *Adora2b*$^{f/f}$-Lyz2-Cre+ mice when compared to controls (Lyz2-Cre+). Values were normalized to the housekeeping gene β-actin (n=6). Based on the pro-inflammatory phenotype in *Adora2b*$^{f/f}$-Lyz2-Cre+ we next investigated if *Adora2b*$^{f/f}$-Lyz2-Cre+ mice could also have more superoxide production (ROS) during IR. As shown in Figure 6d, IR significantly increased ROS levels in the ischemic area of control mice. Interestingly, *Adora2b*$^{f/f}$-Lyz2-Cre+ had already increased ROS levels at baseline when compared to control animals. This is consistent with earlier studies showing an augmented pro-inflammatory phenotype in *Adora2b*$^{-/-}$ mice in conjunction with enhanced leucocyte rolling

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at baseline (27). Taken together, these data indicate an important anti-inflammatory role of the Adora2b in IR injury.

Adoptive transfer of *Adora2b*$^{-/-}$ or wildtype PMNs in IR injury

To further understand the role for Adora2bs on PMN cells in cardio-protection from IR injury, we next tested if adoptive transfer *of Adora2b*$^{-/-}$ PMNs into PMN depleted animals would increase infarct sizes when compared to a transfer of wildtype PMNs (see details of experimentally setup Figure 7a). First we treated wildtype animals with 1A8 Ly6G-specific antibodies 24h prior to the experiment. This treatment leads to a significant reduction of peripheral PMNs but not circulation monocytes in mice (28). On the day of experiment we isolated PMNs from wildtype or *Adora2b*$^{-/-}$ bone marrow and re-infused these cells via a carotid-catheter prior to the onset of myocardial ischemia. As seen in Figure 7b, *Adora2b*$^{-/-}$ PMN infusion significantly increased infarct sizes when compared to the wildtype PMN infusion (41.2± 6.1% (wildtype) vs. 57.5 ± 7.2% (*Adora2b*$^{-/-}$); n=4 per group). To control for possible effects of the 1A8 Ly6G-specific antibody on transferred PMNs we repeated the experiment with an anti-GCSF antibody (Figure 7c). This antibody prevents the recruitment of endogenous PMNs but does not interfere with transferred PMNs (29). As shown in Figure 7d, a similar infarct size was observed with transferred *Adora2b*$^{-/-}$ PMNs. Therefore, in contrast to ischemic preconditioning, the Adora2b on bone marrow derived inflammatory cells, seemingly PMNs, represents an important therapeutic target during IR injury.

DISCUSSION

Adora2b signaling has been shown to effectively protect the myocardium from ischemia in various settings such as IP or IR injury (11–13). In the present study, we investigated the cellular source of Adora2b-dependent cardio-protection. Studies using *state-of-the-art* Cre-lox mouse models for the Adora2b revealed an important role of myocytes or endothelial expressed Adora2b in IP of the heart as it was abolished in *Adora2b*$^{f/f}$-VE-Cadherin-Cre+ or *Adora2b*$^{f/f}$-Myosin-Cre+ mice. In contrast, protection from IR injury was primarily mediated by Adora2b signaling on bone marrow derived inflammatory cells. Characterization of the post-ischemic inflammatory response in *Adora2b*$^{f/f}$-Lyz2-Cre+ mice revealed a PMN driven cytokine profile. In proof of principle studies, an adoptive cell transfer of *Adora2b*$^{-/-}$ PMNs confirmed the hypothesis that Adora2b signaling on PMNs had dominantly been responsible for the observed phenotype in *Adora2b*$^{f/f}$-Lyz2-Cre+ mice. Taken together, these studies suggest that activation of Adora2bs on different tissues represent different therapeutic strategies.

The extent of myocardial cell death determines patient outcome after myocardial ischemia (32). Thus, it is not surprising that protective strategies to make the heart more resistant to ischemia or limit the damage during IR are an area of intense investigations (6, 16, 17, 19, 33–38). It is accepted that a number of G protein-coupled receptors can activate cardio-protective mechanisms (32). These receptors include the adenosine, opioid and bradykinin families (39). Over the past 20 years, substantial evidence indicates that adenosine, administered either prior to ischemia or during reperfusion, reduces myocardial injury (32). These effects are mediated via the activation of one or more of the four known adenosine

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receptor (AR) subtypes (Adora1, Adora2a, Adora2b and Adora3). All four ARs have been associated with cardiac tissue protection in different settings (12, 40, 41). Experimental studies in different species and models implicated that activation of the Adora1 or Adora3 prior to ischemia is cardio-protective (42, 43). Other studies revealed that the administration of Adora2a or Adora2b agonists during reperfusion can reduce MI (41, 44). However, while all ARs have been found to mediate cardio-protection from ischemia, the Adora2b might be the only one that was found to play a role in almost all known cardio-protective settings. As such, the Adora2b has been implicated in post-conditioning, which protects the re-perfused heart from infarction (44). Other studies have shown the importance of Adora2b signaling for cardio-protection mediated by IP (12) which seems to be associated with the initiation of a metabolic program to make heart more oxygenic efficient (19). Further mechanistic studies on Adora2b mediated cardio-protection revealed the involvement of hypoxia inducible factor 1 (HIF1), an important transcription factor implicated in tissue adaptation to hypoxia (18, 19). Other studies indicated reduction of superoxide generation from mitochondria through ERK, PI3K, and NOS as Adora2b mediated - all of which have been implicated in protection from ischemia (45). A very elegant study recently discovered that the Adora2b is present in or near mitochondria suggesting that Adora2b signaling results in inhibition of mitochondrial transition pores (46). Since it is believed that cardio-protective signaling pathways converge on the mitochondria, inhibition of mitochondrial transition pores is thought to be ‘the holy grail of cardio-protection’ (47). However, it needs to be pointed out that the Adora2b is the only one of the four-adenosine receptors whose cardiac expression was found to be induced by ischemia in both mice and humans and whose function is implicated in ischemic preconditioning and post conditioning of the heart (19).

Even though there is rising evidence for Adora2b signaling in mediating cardio-protection, no study has evaluated the tissue specific contribution yet. IP has been consistently demonstrated to be a potent protective mechanism in freshly isolated and cultured cardiomyocytes across multiple species, indicating that much of the innate protection of IP resides in cardiomyocytes (48). However, studies using *state-of-the-art* Cre-lox mouse models have not been performed yet. Based on earlier studies in germline *Adora2b*$^{-/-}$ mice (12, 17), we now generated tissue specific Adora2b knockout mice. Comparing an Adora2b tissue specific deletion in myocytes, endothelia or bone marrow derived inflammatory cells, indeed confirmed studies hypothesizing that cardio-myocytes are crucial for IP of the heart. In addition, Adora2b on the endothelium was also found to be important for the mechanism of IP. In fact, hypoxic preconditioning to model ischemic preconditioning *in vitro* has repeatedly been found to protect endothelial cells from subsequent long term hypoxia (19, 49, 50). In contrast, Adora2b signaling on inflammatory cells seems less important during IP. However, this is not surprising as IP consists of short nonlethal ischemic periods that are most likely not able to attract a significant amount of inflammatory cells (17). In addition, while we found some resident macrophages in the heart, Adora2bs were dominantly expressed on cardiomyocytes and endothelial cells, supporting our findings in IP of the heart.

Interestingly, the findings that Adora2b-elicited cardioprotection by IP involves vascular endothelial cells is also reflected in a recent study examining the role of HIF1A in

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cardioprotection. This study demonstrates convincingly, by using tissue specific HIF1A deficient mice, that vascular endothelial HIF1A is required for mediating the cardio-protective effects of IP. The authors conclude that HIF1A is functioning as a transcriptional activator, despite the acute nature of the response (51). As discussed above, HIF1A is a critical transcriptional enhancer of Adora2b signaling during IP (52). Therefore, it is conceivable that the transcriptional induction of the Adora2b via HIF1A is a critical component of cardioprotection elicited by IP.

While IP seems to be mainly linked to endothelial cells and cardio-myocytes, compelling evidence from both animal and clinical studies has indicated that leukocytes are the principal effector cells of IR injury (53). Reperfusion induces a vigorous inflammatory response and a dramatic increase in neutrophil adherence to the re-perfused endothelium (17, 54). As Adora2bs are widely distributed in hematopoietic cells (27, 37, 55, 56), studies using *in vivo* animal models have shown that Adora2b deficiency is associated with enhanced inflammation (20, 27, 37, 57). Other studies indicated that Adora2b mediated protection from vascular injury is based on anti-inflammatory processes (37, 58).

Based on its anti-inflammatory role, it is convincing that Adora2b signaling could dampen IR injury by interaction with bone marrow derived inflammatory cells. In fact, in the current study, we have established an important role for the Adora2b on bone marrow–derived cells in mediating cardio-protection against IR injury. Earlier studies using germline *Adora2b*$^{-/-}$ mice, showed that plasma levels of the pro-inflammatory cytokine TNFα was elevated at baseline (27) and in mice subjected to femoral artery or myocardial IR injury (17, 58). Thus, our findings on a TNFα guided pro-inflammatory phenotype in tissue specific deletion of the *Adora2b* on bone marrow derived cells (*Adora2b*$^{f/f}$-Lyz2-Cre+) during cardiac IR are consistent with these previous findings.

In this study, as a proof of concept, we isolated *Adora2b*$^{-/-}$ PMNs from germline *Adora2b*$^{-/-}$ mice and transferred them into neutropenic mice. Following ischemia, we found significantly increased infarct sizes when compared to mice that were transferred with wildtype PMNs. These studies support our findings that only *Adora2b*$^{f/f}$-Lyz2-Cre+ had larger infarcts when compared to controls. As bone marrow derived cells include neutrophils and macrophages, it seems compelling that PMNs play the dominant role as they are the dominant cell type during the first hours of reperfusion (17). However, it also indicates that our studies cannot completely rule out a role of the Adora2b on macrophages in IR injury. Future studies using tissue specific mice for different inflammatory cell types will be necessary to further elucidate the detailed mechanisms.

Interestingly, apart from the Adora2b dependent regulation of pro-inflammatory TNFα, we also found significantly lower levels of Stem cell factor (SCF) in *Adora2b*$^{f/f}$-Lyz2-Cre+ mice. SCF is a cytokine that improves myocardial function (59) and enhances cardiac healing after myocardial injury (60). In addition, it has been found that adenosine can enhance SCF signaling *in vitro* (61). Thus, apart from a reduction of the post-ischemic inflammation, Adora2b signaling might act as a switch to start cardiac repair early in reperfusion. Indeed, pharmacological studies have indicated an important role of Adora2b in cardiac healing after IR injury (62).

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Taken together, using a novel tissue specific approach for Adora2b signaling during IP or IR, we found different functions for the Adora2bs in different tissues. While Adora2b signaling in vascular endothelial cells and cardiac myocytes was critical for mediating IP-elicited cardioprotection, the extent of IR injury was determined by Adora2b signaling on inflammatory cells. These findings indicate that when using the Adora2b as therapeutic target it might be best when done in a tissue specific manner. One possible approach could be the administration of an Adora2b agonist into a coronary artery prior to a high risk intervention to “precondition” the heart, whereas systematic administration of an Adora2b agonist may be preferable during reperfusion. Future studies using specific Adora2b agonists in clinical trials will be necessary to get insight into tissue specific therapies for myocardial ischemia.

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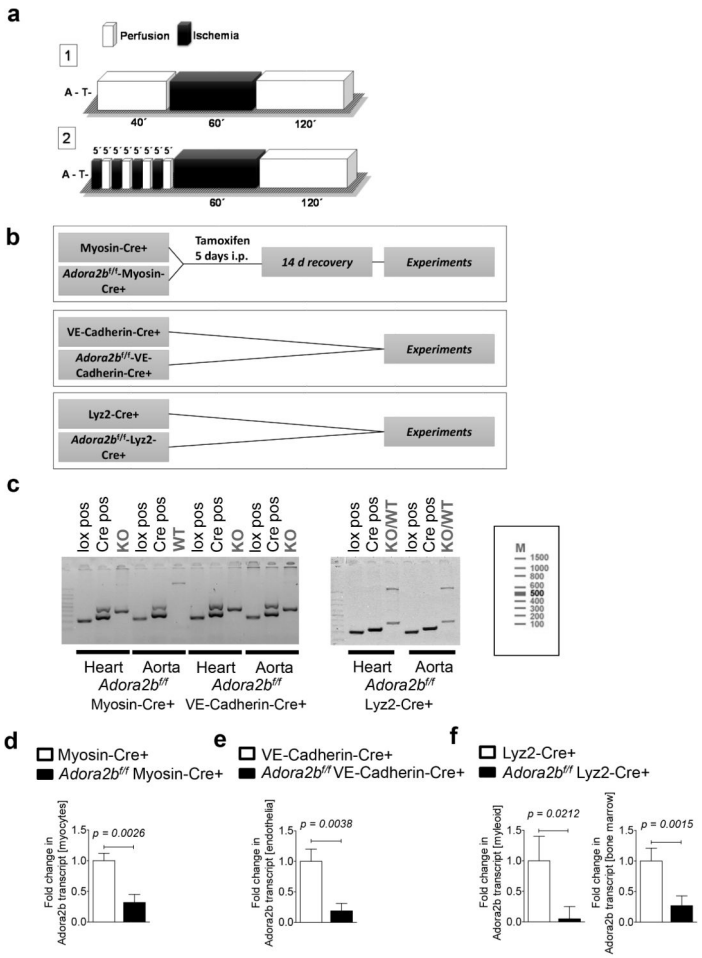

Figure 1. Overview of the experimental protocol and tissue-specific Adora2b-deficient mice

(a) Experimental protocols used. *Model 1*. Myocardial ischemia consisted of 60 minutes of ischemia followed by 2 h of reperfusion. *Model 2*. For ischemic preconditioning studies, 4 cycles of 5 min ischemia and 5 min reperfusion prior to the onset of myocardial ischemia was performed. **(b)** Overview of tissue-specific Adora2b deletion using a Cre-lox-system. **(c)** Genotyping data. Tissue was collected from the organs as labeled and genotyped using conventional PCR (GeneTyper, NY, USA). Genotyping analyzed for *Adora2b* floxed (lox), Cre-recombinase (Cre), *Adora2b* wildtype (WT) and for the *Adora2b* knockout (KO) signal. ***Left***, representative PCR results from heart or aortic tissue from *Adora2b*$^{f/f}$ -Myosin-Cre+ mice (cardio-myocyte specific); *note: the positive KO band in cardiac tissue, in contrast to only WT band present in the aortic tissue.* ***Middle***, representative PCR result from *Adora2b*$^{f/f}$-VE-Cadherin-Cre+ mice (endothelial specific); *note: the positive KO signal in heart and aorta.* ***Right***, representative PCR results from *Adora2b*$^{f/f}$-Lyz2-Cre+ mice (bone-marrow derived cell specific); *note: positive KO and WT band in heart and aorta.* The Cre signal for Myosin-Cre+ or VE-Cadherin-Cre+ was different than the signal from Lyz2-Cre+ as a different PCR strategy was used (according to Jackson Lab protocols); Adora2b flox signal: 200 base pairs (bp); Adora2b KO signal: 366 bp, Adora2b WT signal: 1600 bp, Myosin-Cre+ and VE-Cadherin-Cre+ signal: double band at 270 and 497 bp, Lyz2-Cre+

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signal: 260 bp; ladder used is depicted to the right. **(d–f)** RT-PCR data showing Adora2b mRNA transcript levels from the respective tissues of the tissue specific- *Adora2b* deleted mice. (**d**) Cardiomyocytes were isolated from Myosin Cre+ and *Adora2b*$^{f/f}$ -Myosin-Cre+ mice and cultured overnight, (**e**) endothelia and (**f**) myeloid cells were isolated via positive selection (CD31+ for endothelia from VE-Cadherin-Cre+/ *Adora2b*$^{f/f}$ -VE-Cadherin-Cre+ hearts and CD11b+ for myeloid cells from Lyz2-Cre+/ *Adora2b*$^{f/f}$ -Lyz2-Cre+ hearts) using magnetic beads (EasySep) and bone marrow cells were isolated from the Lyz2-Cre and *Adora2b*$^{f/f}$ -Lyz2-Cre femurs.

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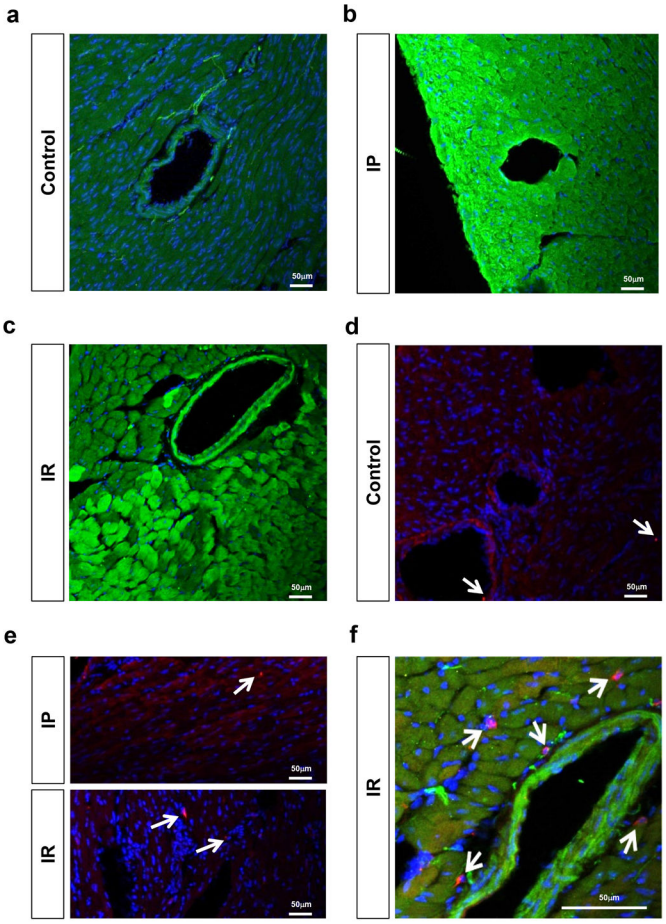

Figure 2. Effect of ischemic preconditioning or ischemia reperfusion injury on Adora2b and F4/80 positive cell expression

(a–c) Beta-gal staining of representative sections of an Adora2b-beta-gal-reporter mouse at baseline (**a**), after ischemic preconditioning (**b**) or ischemia reperfusion injury (**c**). *Green stain:* beta-gal-positivity indicating Adora2b promoter activation; *Note:* Adora2b positive cells are dominantly located in the vessel wall at baseline, while ischemic preconditioning or ischemia reperfusion injury significantly upregulates Adora2b gene promoter activity on endothelia but also on myocardial cells. (**d–f**) F4/80 staining (red) at baseline (**d**), after ischemic preconditioning (**e**) or ischemia reperfusion injury (**e,f**). *Note:* while F4/80 positive cells are rarely found at baseline or after ischemic preconditioning, a slight increase of F4/80 cells was found after ischemia reperfusion injury (**f**). *Note:* No beta gal staining was observed on residential macrophages (**f**); *Green stain*: Adora2b; *Red stain:* Macrophages; *Blue stain:* DAPI; blue stain representing nuclei.

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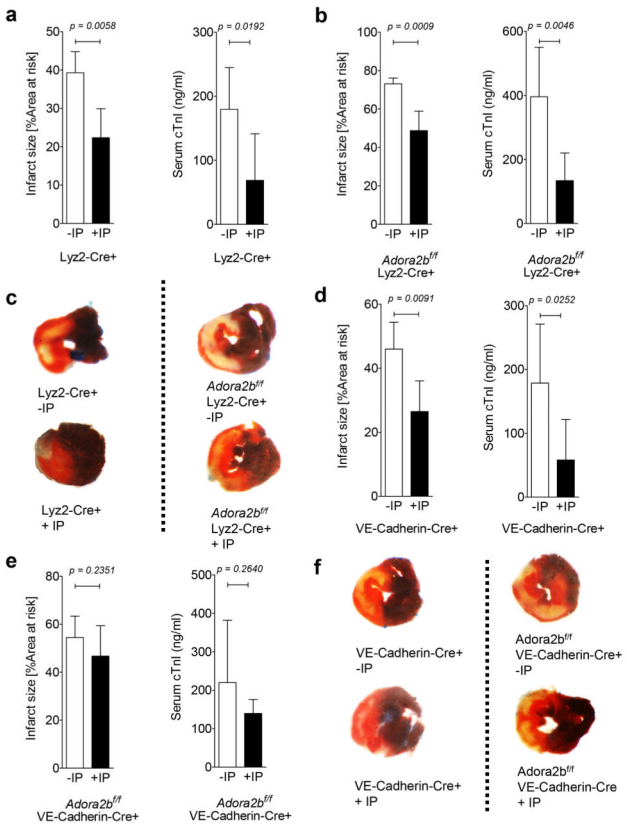

Figure 3. Effect of ischemic preconditioning on myocardial injury in bone-marrow derived cell and endothelial-specific Adora2b-deficient mice

(a–f) Mice underwent 60 min of ischemia with ischemic preconditioning (+IP; 4 cycles of 5 min of ischemia followed my 5 minutes of reperfusion) or without IP (−IP) followed by 120 minutes of reperfusion. Infarct sizes were measured by double staining with Evan's blue and triphenyl-tetrazolium chloride. Infarct sizes are expressed as the percent of the area at risk (AAR) that underwent infarction. Serum troponin I concentrations were measured by enzyme-linked immunosorbent assay (ELISA). **(a, b)** Infarct sizes and serum troponin I levels in Lyz2-Cre+ (controls) or *Adora2b*$^{f/f}$-Lyz2-Cre+ with and without IP. (**c**) Representative infarct staining from Lyz2Cre (controls) or *Adora2b*$^{f/f}$-Lyz2-Cre+ mice; (n=5–6; ±SD).

(**d, e**) Infarct sizes and serum troponin I levels in VE-Cadherin-Cre+ (controls) and *Adora2b*$^{f/f}$-VE-Cadherin-Cre+ with and without IP. (**f**) Representative infarct staining from VE-Cadherin-Cre+ (controls) and *Adora2b*$^{f/f}$-VE-Cadherin-Cre+ (n=5–6, ±SD).

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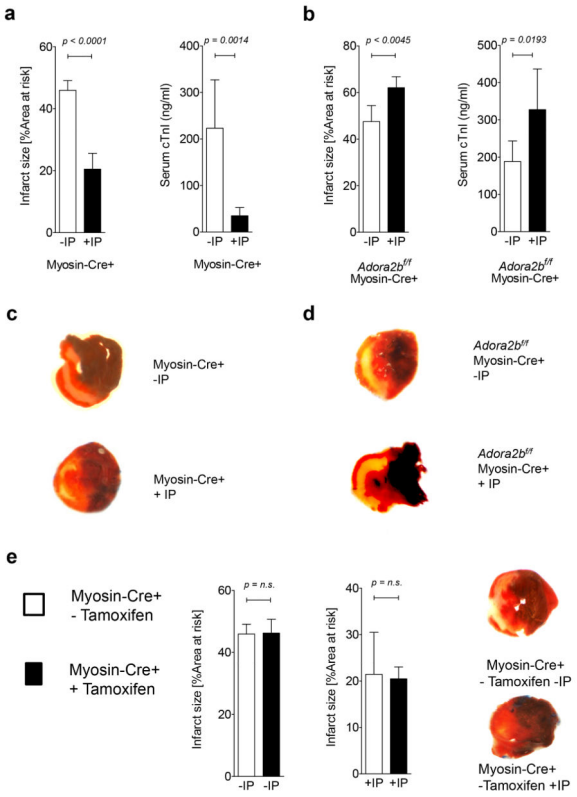

Figure 4. Effect of ischemic preconditioning on myocardial injury in cardiomyocyte-specific Adora2b-deficient mice

(**a–e**) Mice underwent 60 min of ischemia with ischemic preconditioning (+IP; 4 cycles of 5 min of ischemia followed my 5 minutes of reperfusion) or without IP (−IP) followed by 120 minutes of reperfusion. Infarct sizes were measured by double staining with Evan's blue and triphenyl-tetrazolium chloride. Infarct sizes are expressed as the percent of the area at risk (AAR) that underwent infarction. Serum troponin I concentrations were measured by enzyme-linked immunosorbent assay (ELISA). (**a, b**) Infarct sizes and serum troponin I levels in Myosin-Cre+ (controls) and *Adora2b*$^{f/f}$-MyosinCre+ with and without IP. (**c, d**) Representative infarct staining from Myosin-Cre+ (controls) and *Adora2b*$^{f/f}$-MyosinCre+. (**e**) Infarct sizes in Myosin-Cre+ (controls) with and without tamoxifen pretreatment. (n=3–6, ±SD).

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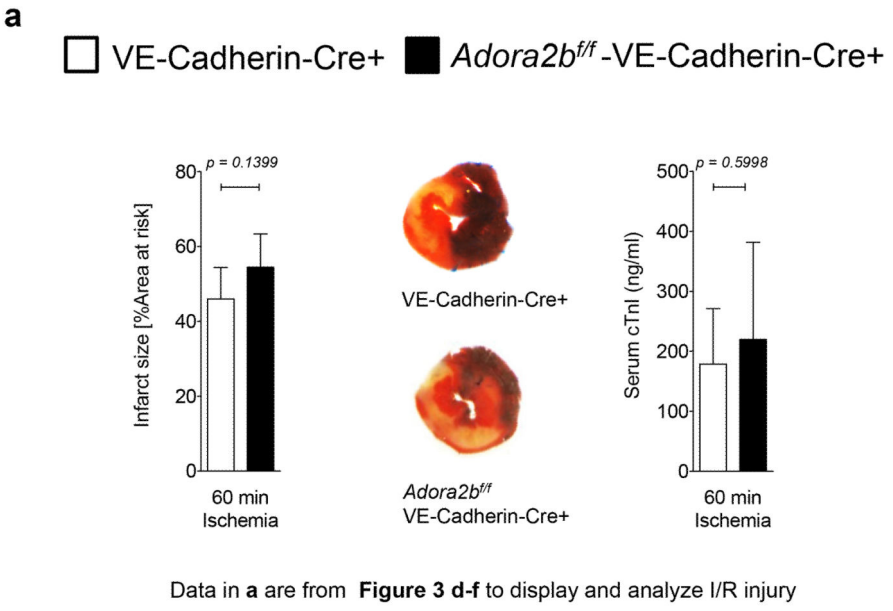
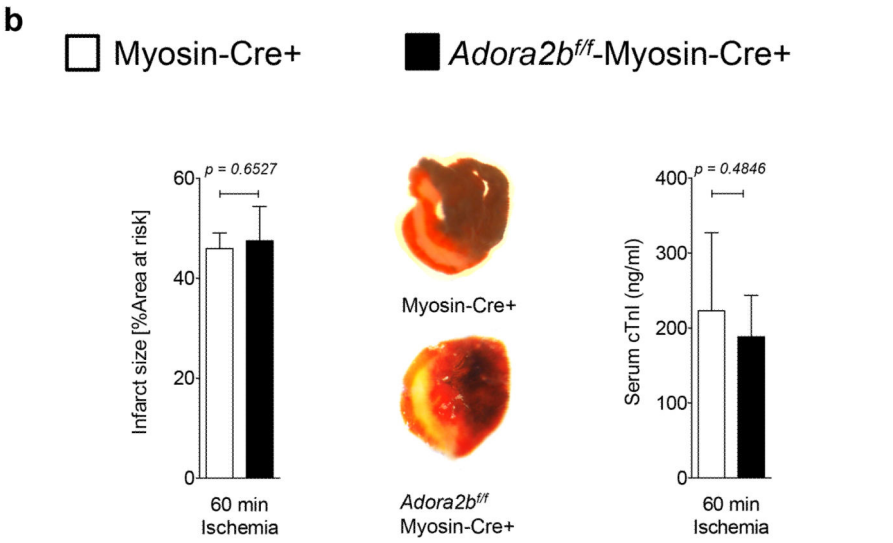


Data in **a** are from **Figure 3 d-f** to display and analyze I/R injury



Data in **b** are from **Figure 4 a-d** to display and analyze I/R injury

Figure 5. Effect of myocardial ischemia-reperfusion injury on myocardial damage in endothelial or cardio-myocytes specific Adora2b deficient mice

(a,b) Mice underwent 60 min ischemia and 120 min reperfusion. Infarct sizes were measured by double staining with Evan's blue and triphenyl-tetrazolium chloride. Infarct sizes are expressed as the percent of the AAR that underwent infarction. Serum troponin I concentrations were measured by enzyme-linked immunosorbent assay (ELISA) **(a)** Infarct sizes and serum troponin I levels in VE-Cadherin-Cre+ (controls) and *Adora2b*$^{f/f}$-VE-Cadherin-Cre+ with representative infarct staining [*Note*: Data in **a** are from Figure **3 d–f** to display and analyze I/R injury]. (**b**) Infarct sizes and serum troponin I levels in Myosin-Cre+ (controls) and *Adora2b*$^{f/f}$-Myosin-Cre+ with representative infarct staining (middle); (n=6, ±SD); [*Note*: Data in **b** are from Figure **4 a–d** to display and analyze I/R injury].

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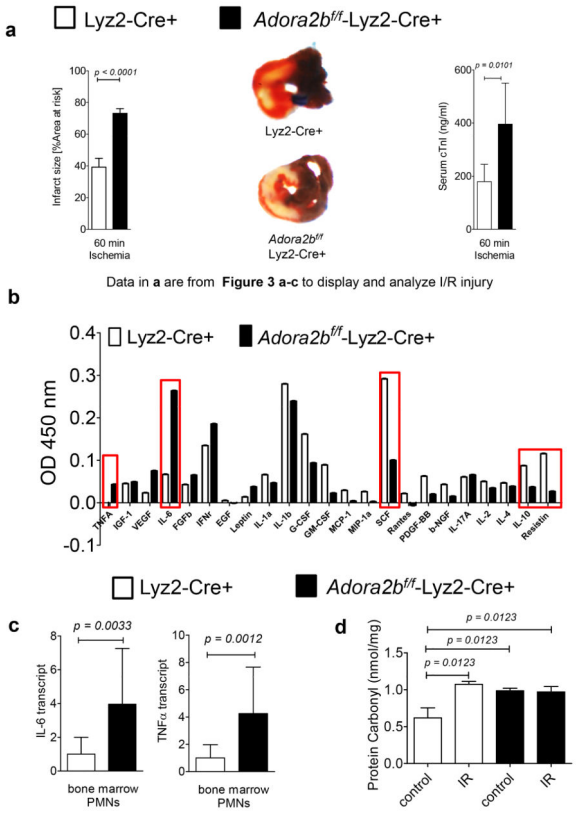

Figure 6. Myocardial IR injury in bone marrow-specific Adora2b-deficient mice

(a) Mice underwent 60 min ischemia and 120 min reperfusion. Infarct sizes were measured by double staining with Evan's blue and triphenyltetrazolium chloride. Infarct sizes are expressed as the percent of the AAR that underwent infarction. Infarct sizes in Lyz2-Cre+ (controls) or *Adora2b*$^{f/f}$-Lyz2-Cre+ with representative infarct staining. Serum troponin I concentrations were measured by enzyme-linked immunosorbent assay (ELISA); [*Note*: Data in **a** are from Figure 3a–c re-arranged to display I/R injury]; (**b**) Multiplex Elisa from the are at risk (AAR) after 60 minutes of ischemia and 120 minutes of reperfusion comparing Lyz2-Cre+ (controls) and *Adora2b*$^{f/f}$-Lyz2-Cre+. **(c)** Isolated PMNs from Lyz2-Cre+ (controls) or *Adora2b*$^{f/f}$-Lyz2-Cre+ were analyzed for IL-6 or TNFα transcript levels; (n=5–6; ±SD). (**d**) Cardiac protein carbonyl, as indicator of ROS production, was measured using OxiSelect protein carbonyl ELISA kit in control or ischemic heart tissue from Lyz2-Cre+ (controls) or *Adora2b*$^{f/f}$-Lyz2-Cre+ mice.

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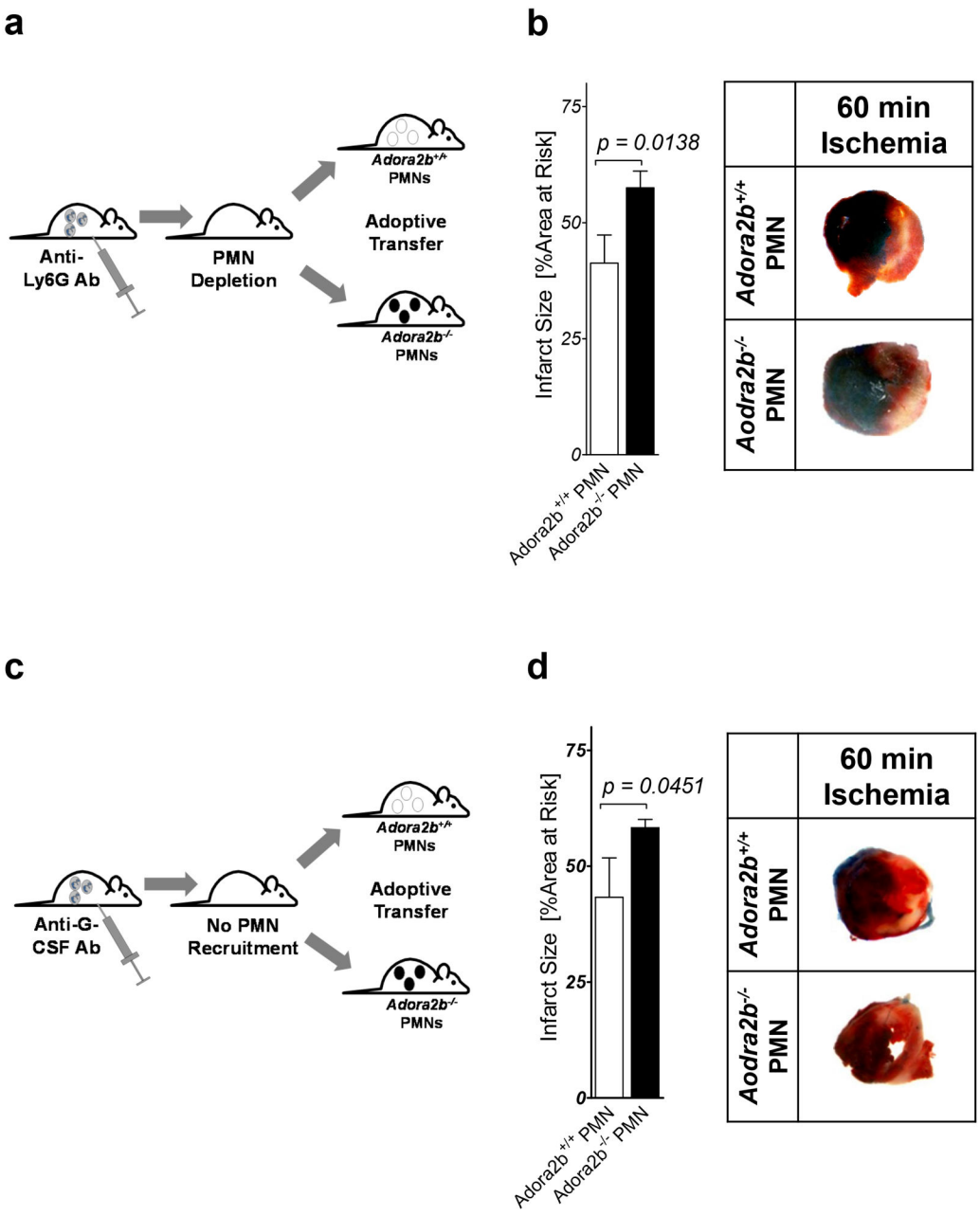

Figure 7. Effects of adoptive transfer of PMNs from Adora2b$^{-/-}$ into wildtype mice in IR injury

(**a,c**) Model. Wildtype PMNs or *Adora2b*$^{-/-}$ PMNs were isolated and transferred into PMN depleted (1A8 Ly6G-specific antibody treatment 24 h prior to ischemia) or anti-GCSF (24 h prior to ischemia) treated animals. **(b, d**) Mice underwent 60 min ischemia and 120 min reperfusion. Infarct sizes were measured by double staining with Evan's blue and triphenyltetrazolium chloride. Infarct sizes are expressed as the percent of the AAR that underwent infarction. Infarct sizes in PMN depleted wildtype mice that received either wildtpe PMNs (controls) or *Adora2b*$^{-/-}$ PMNs with representative infarct staining are shown; (n=3–4; ±SD)