Adora2b Signaling on Bone Marrow Derived Cells Dampens Myocardial Ischemia-Reperfusion Injury

Michael Koeppen, M.D.,* Patrick N. Harter, M.D.,† Stephanie Bonney, B.S.,‡ Megan Bonney, B.S.,‡ Susan Reithel, B.S.,‡ Cornelia Zachskorn, B.S.,§ Michel Mittelbronn, M.D.,‖ Tobias Eckle, M.D., Ph.D.#

**ABSTRACT**

**Background:** Cardiac ischemia-reperfusion (I-R) injury represents a major cause of cardiac tissue injury. Adenosine signaling dampens inflammation during cardiac I-R. The authors investigated the role of the adenosine A2b-receptor (Adora2b) on inflammatory cells during cardiac I-R.

**Methods:** To study Adora2b signaling on inflammatory cells, the authors transplanted wild-type (WT) bone marrow (BM) into Adora2b<sup>−/−</sup> mice or Adora2b<sup>−/−</sup> BM into WT mice. To study the role of polymorphonuclear leukocytes (PMNs), neutrophil-depleted WT mice were treated with an Adora2b agonist. After treatments, mice were exposed to 60 min of myocardial ischemia and 120 min of reperfusion. Infarct sizes and troponin I concentrations were determined by triphenyltetrazolium chloride staining and enzyme-linked immunosorbent assay, respectively.

**Results:** Transplantation of WT BM into Adora2b<sup>−/−</sup> mice decreased infarct sizes by 19 ± 4% and troponin I by 87.5 ± 25.3 ng/ml (mean ± SD, n = 6). Transplantation of Adora2b<sup>−/−</sup> BM into WT mice increased infarct sizes by 20 ± 3% and troponin I concentrations by 69.7 ± 17.9 ng/ml (mean ± SD, n = 6). Studies on the reperfused myocardium revealed PMNs as the dominant cell type. PMN depletion or Adora2b agonist treatment reduced infarct sizes by 30 ± 11% or 26 ± 13% (mean ± SD, n = 4); however, the combination of both did not produce additional cardioprotection. Cytokine profiling showed significantly higher cardiac tumor necrosis factor α concentrations in Adora2b<sup>−/−</sup> compared with WT mice (39.3 ± 5.3 vs. 7.5 ± 1.0 pg/mg protein, mean ± SD, n = 4). Pharmacologic studies on human-activated PMNs revealed an Adora2b-dependent tumor necrosis factor α release.

**Conclusion:** Adora2b signaling on BM-derived cells such as PMNs represents an endogenous cardioprotective mechanism during cardiac I-R. The authors’ findings suggest that Adora2b agonist treatment during cardiac I-R reduces tumor necrosis factor α release of PMNs, thereby dampening tissue injury.

**What We Already Know about This Topic**
- Adenosine receptor (Adora)2b has been shown to be protective during myocardial ischemia-reperfusion (I-R) injury
- Based on the notion that adenosine elicits cardioprotection through inhibition of inflammation, the current study investigated whether Adora2b activation on bone marrow derived cells such as polymorphonuclear leukocytes (PMNs) inhibits proinflammatory cytokine release after myocardial I-R

**What This Article Tells Us That Is New**
- Adora2b activation on PMNs is cardioprotective during myocardial I-R injury by limiting the release of the proinflammatory cytokine tumor necrosis factor α

---

**ALTHOUGH early reperfusion is the only way to prevent additional tissue injury after myocardial ischemia (MI), it initiates reversible and irreversible organ damage, which is referred to as ischemia-reperfusion (I-R) injury.** Postischemic myocardial tissue is a strong stimulus for an...
inflammatory response that initiates repair processes but also can damage myocardial tissue, thereby increasing the size of the infarct. Thus, attenuation of the inflammatory response may diminish reperfusion damage and maximize the benefit of revascularization.2,3 Numerous exogenous and endogenous agents have been identified to modulate the inflammatory response after MI. In particular, adenosine has been shown to be cardioprotective because of its antiinflammatory properties.4

Adenosine mediates its protective effects through four adenosine receptors (Adora1, Adora2a, Adora2b, and Adora3).5 Although all adenosine receptors have been associated with cardiac tissue protection, Adora1, Adora2a, and Adora3 are the most widely studied.6–8 Recently, adenosine receptors have been associated with cardiac tissue protection, Adora1, Adora2a, and Adora3 are the most widely studied.6–8 Recently, Adora2b was reported to elicit strong cardioprotection in I-R injury.9–11 It is expressed on cardiomyocytes, endothelial cells, and on all main bone marrow derived inflammatory cells, such as polymorphonuclear leukocytes (PMNs), macrophages, and lymphocytes.12 However, it is unknown on which of these cells Adora2b needs to be activated to be cardioprotective. Based on the notion that adenosine elicits cardioprotection through inhibition of inflammation, Adora2b activation on inflammatory cells could represent a possible cardioprotective mechanism. It has been shown that Adora2b−/− mice have higher concentrations of tumor necrosis factor α (TNFα).13 TNFα plays a major role in I-R-induced apoptosis of cardiomyocytes during reperfusion.14 Bone marrow derived inflammatory cells such as PMNs could represent a source of TNFα release, so we hypothesized that Adora2b activation on bone marrow derived cells is responsible for its cardioprotective effect through inhibition of proinflammatory cytokine release in the myocardium upon reperfusion.

To test this hypothesis, we first submitted wild-type (WT) or Adora2b−/− mice to an in situ model for MI and reperfusion. Next, we used bone marrow transplantation from WT mice into Adora2b−/− and vice versa (chimeric mice) to identify the contribution of inflammatory cells for Adora2b-mediated cardioprotection. This way, Adora2b was either absent on cardiac cells (endothelia, cardiomyocytes) or on bone marrow derived inflammatory cells (PMNs, lymphocytes, or macrophages). Subsequent analysis of cells attracted to the posts ischemic myocardium in WT or Adora2b−/− mice identified PMNs as the dominant cell type. To understand Adora2b signaling on PMNs, WT mice with or without PMNs were exposed to in situ MI and treated with a specific Adora2b agonist (BAY 60-6583) upon reperfusion. Finally, to study the effects of Adora2b signaling on PMNs, in vivo studies using Adora2b−/− mice or in vitro studies using isolated human PMNs with pharmacologic inhibition or activation of the Adora2b were performed.

Using the above described multimodal approach, we found a critical role for Adora2b on bone marrow derived inflammatory cells in eliciting the cardioprotective effect. Consistent with these findings, we observed that Adora2b signaling is cardioprotective only when activated on PMNs. Finally, using in vivo and in vitro studies, we identified an Adora2b-dependent TNFα release via PMNs.

Materials and Methods

Mice

Experimental protocols were approved by the Institutional Review Board at the University of Tübingen, Tübingen, Baden-Wuertemberg, Germany, or the University of Colorado Anschutz Medical Campus, Aurora, Colorado. They were in accordance with the German Law on the Protection of Animals and the National Institutes of Health guidelines for use of live animals. C57BL/6j mice were obtained from Charles River Laboratories (Sulzfeld, Germany) or Jackson Laboratories (Bar Harbor, ME). Adora2b-deficient mice were provided by Deltagen, Inc. (San Carlos, CA).10

Murine Model for Cardiac Ischemia

Anesthesia was induced (70 mg/kg body weight intraperitoneal) and maintained (10 mg · kg−1 · h−1) with sodium pentobarbital. Mice were placed on a temperature-controlled heated table (RT; Effenberg, Munich, Germany) with a rectal thermometer probe attached to a thermal feedback controller to maintain body temperature at 37°C. The endotracheal tube was connected to a mechanical ventilator (Servo 900C; Siemens, Munich, Germany) with pediatric tubing, and the animals were ventilated with a pressure-controlled ventilation mode (peak inspiratory pressure of 10 mbar, frequency 110 breaths/min, positive end-expiratory pressure of 3 mbar, PETCO2 = 0.3). Using this ventilator regimen, blood gas analysis revealed normal PAO2 (115 ± 15 mmHg) and PACO2 (38 ± 6 mmHg) during establishment of the method. Therefore this was used as standard setting in the current study. After anesthesia was induced, animals were monitored with a surface electrocardiogram (Hewlett Packard, Bühligen, Germany). Fluid replacement was performed with 0.2 ml/h intravenous normal saline until the end of ischemia. To avoid cardiovascular collapse and to establish a stable reperfusion after ischemia, the infusion rate was increased to 0.6 ml/h during the first hour of reperfusion. To avoid volume overload or dilution of the hematocrit, the rate was reduced to 0.2 ml/h in the second hour of reperfusion. The carotid artery was catheterized for continuous recording of blood pressure with a statham element (WK 280; WKK, Kaltbrunn, Switzerland). Operations were performed under an upright dissecting microscope (SZX12; Olympus, Center Valley, PA). After left anterior thoracotomy, exposure of the heart and dissection of the pericardium, the left coronary artery was identified visually and an 8.0 nylon suture (Prolene; Ethicon, Norderstedt, Germany) was placed around the vessel. Atraumatic left coronary artery occlusion for ischemia studies was performed using a hanging weight system.15 Successful left coronary artery occlusion was confirmed visually by an immediate color change of the vessel...
from light red to dark violet. The myocardium supplied by the vessel turned from bright red to pale, and an immediate onset of ST elevations in the electrocardiogram was noted. During reperfusion, the change of tissue color was reversed immediately when the hanging weights were lifted and electrocardiogram changes were reversed. To study cardioprotective effects, it is ideal to use an ischemia time associated with infarct sizes (ISs) of approximately 30–40% of the area at risk (AAR). Thus, it is possible to demonstrate changes in both directions (e.g., smaller or larger IS) with an experimental therapeutic or a specific gene deletion. An ischemia time of 60 min resulted in a mean IS of approximately 45% of the AAR as shown previously. Thus, a 60-min ischemia period was chosen in the current study. ISs were determined by calculating the percentage of infarcted myocardium to the AAR using a double-staining technique with Evans blue and triphenyltetrazolium chloride. Evans blue is excluded from the area of the heart perfused by the left coronary artery and thus allows one to identify the AAR. Triphenyltetrazolium chloride stains tissue red except parts that are depleted in nicotinamide adenine dinucleotide phosphate. This allows one to visualize infarcted tissue because of its white color. Using planimetry via the National Institutes of Health software Image 1.0 (National Institutes of Health, Bethesda, MA), the AAR and the IS were determined. To measure the reliability of IS analysis, interobserver variability was tested. ISs of animals were assessed by two independent investigators both blinded to the experimental protocol. Moreover, IS was measured twice on two separate days by the same investigator to reveal intraobserver variability.

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). Thus, a 60-min ischemia period was chosen in the current study. ISs were determined by calculating the percentage of infarcted myocardium to the AAR using a double-staining technique with Evans blue and triphenyltetrazolium chloride. Evans blue is excluded from the area of the heart perfused by the left coronary artery and thus allows one to identify the AAR. Triphenyltetrazolium chloride stains tissue red except parts that are depleted in nicotinamide adenine dinucleotide phosphate. This allows one to visualize infarcted tissue because of its white color. Using planimetry via the National Institutes of Health software Image 1.0 (National Institutes of Health, Bethesda, MA), the AAR and the IS were determined. To measure the reliability of IS analysis, interobserver variability was tested. ISs of animals were assessed by two independent investigators both blinded to the experimental protocol. Moreover, IS was measured twice on two separate days by the same investigator to reveal intraobserver variability.

Generation of Adora2b Bone Marrow Chimeric Mice

Bone marrow chimeric mice were generated by irradiation of WT (C57BL/6J) or Adora2b−/− mice followed by reconstitution with bone marrow derived from WT or Adora2b−/− mice. Briefly, male donor mice (6–8 week old, 20–22 g) were euthanized, and the marrow from the tibia and femur were harvested by flushing the marrow cavity with sterile isotonic NaCl solution. The bone marrow cells were then centrifuged at 400g for 5 min, resuspended, and counted. Recipient mice (8–10 week of age, 20–25 g) were irradiated with a total dose of 12 Gy from a 137Cs source. Immediately after irradiation, 107 bone marrow cells/recipient in 0.3 ml NaCl (0.9%) were injected into the jugular vein. The resulting chimeric mice were housed in microisolators for at least 8 weeks before experimentation and fed with water containing tetracycline (100 mg/l) in the first 2 weeks after bone marrow transplantation. Transplantation efficiencies were determined in preliminary experiments using the same conditioning regimen and transplanting CD45.1-positive bone marrow into irradiated CD45.1-negative mice. In short, to confirm efficiency of reconstitution, a mutated mouse strain, B6.SJL-Ptprc+Pep3b/BoyJ, was used as the source of donor bone marrow. The CD45.1 epitope, absent in cells of recipient mice, was detected by immunofluorescent cell analysis 8 weeks after bone marrow transplantation. The percentage of cells expressing CD45.1 was determined in each population of cells. For this purpose, blood was taken from transplanted recipients; FACSTM lysing solution (BD Pharmingen, San Diego, CA) was added to lysed erythrocytes. After centrifugation, cells were resuspended in 200 μl phosphate buffered saline containing 1% bovine serum albumin. Peripheral blood stem cells were incubated with R-phycocerythrin conjugated antimouse CD45.1 monoclonal antibody (clone A20, BD Pharmingen) and allophycocyanin-conjugated rat antimouse CD11b (Mac-1 α-chain, monocytic cells) monoclonal antibody (BD Pharmingen), rat antimouse Ly6G monoclonal antibody (neutrophils, BD Pharmingen), rat antimouse B220 monoclonal antibody (B cells, BD Pharmingen), fluorescein isothiocyanate-conjugated rat antimouse CD8α monoclonal antibody (CD8+ T lymphocytes, BD Pharmingen), or allophycocyanin-conjugated rat antimouse CD4 monoclonal antibody (CD4+ T lymphocytes, BD Pharmingen) on ice for 30 min. CD11b−, CD8a−, and CD4−, B220−, and Ly6G-positive cells were sorted by flow cytometry, and the percentage of cells expressing CD45.1 was determined in each population of cells (FACScan; CellQuest, Becton Dickinson, San Jose, CA).

Immunohistochemistry

Hearts from untreated controls or from mice subjected to 60 min of ischemia were removed at indicated time points during reperfusion (15, 30, 60, 120 min ischemia) and fixed in 4% buffered formalin (pH 7.4) for 24 h and paraffin embedded afterward. The samples were cut in 5-μm slices, placed on SuperFrost Plus slides (Microm International, Walldorf, Germany), and deparaffinized. Histologic and enzyme-histochemical evaluation consisted of hematoxylin-eosin staining and chloroacetaet esterase staining for visualization of neutrophils. Immunohistochemical staining was performed with rat antimouse antibodies against F4–80 (Serotec, Oxford, United Kingdom), CD-3 (BD Biosciences, Franklin Lakes, NJ), Ly6G (BD Biosciences), and myeloperoxidase (Dako, Hamburg, Germany). In addition, an Adora2b antibody (Millipore, Billerica, MA) was used to confirm Adora2b deficiency at the protein level. Immunohistochemistry was performed using the DiscoveryXT immunohistochemistry system (Ventana, Tucson, AZ). All histologic and immunohistochemical staining was performed on serial sections. Evaluation of the histologic and immunohistochemical staining and photographic documentation were performed using an Olympus BX-50 light microscope (Hamburg, Germany). Pictures were taken with a digital camera (DP72; Olympus, Hamburg, Germany).
Neutrophil Depletion
In selected experiments, neutrophil depletion was achieved with an anti-GR-1 monoclonal antibody (BD Pharmingen) as described previously. C57BL/6 mice were treated with GR-1 monoclonal antibody 24 h before the experimental procedure, and neutrophil depletion was confirmed by differential blood counts.

Myeloperoxidase and Cytokine Multiplex Enzyme-linked Immunosorbtent Assay (ELISA)
To measure myeloperoxidase and cytokine tissue (TNFα, interferon γ, interleukin-1β, interleukin-2, interleukin-4, interleukin-5, keratinocyte-derived cytokine, interleukin-10, interleukin-12) after 60 min of ischemia, C57BL/6 or Adora2b−/− mice were euthanized after 120 min of reperfusion. Remaining blood was removed; the myocardial tissue (AAR) was excised after delineation with Evans blue and immediately frozen at −80°C. Tissues were homogenized and a myeloperoxidase ELISA (Signosis, Inc., Sunnyvale, CA) or a cytokine multiplex ELISA (MSD Cytokine Assays; Meso Scale Discovery, Gaithersburg, MA) was performed according to the manufacturer’s recommendations.

Isolation of Human PMNs
After approval was given by the Institutional Review Board and written informed consent was provided from each individual, PMNs were freshly isolated as described previously. Five healthy males (32 yr old, age range: 28–35 yr) were included. The isolation of human neutrophils from whole blood was performed by the gradient density centrifugation method. Briefly, 50 ml blood was collected with 10 ml acid citrate dextrose. This was then mixed with 3% Dextran-500 and allowed to sit for sedimentation. The top layer, approximately 25 ml clear leukocyte-rich plasma, was layered carefully on top of 10 ml Histopaque-1077 (Sigma Aldrich, St. Louis, MO). The tube was centrifuged at 2,700 rpm for 30 min at 20°C. Once the centrifugation was complete, the final layer containing erythrocytes and neutrophils. The pellet was resuspended and washed with erythrocyte lysis buffer. The sample was centrifuged at 1,000 rpm for 10 min at 20°C, and a white pellet was observed. The neutrophil pellet was resuspended and washed with Hank’s balanced salt solution without Ca2+ and Mg2+. The suspension was then centrifuged at 1,000 rpm for 10 min at 20°C, after which the supernatant was decanted and the neutrophil pellet was resuspended in Hank’s balanced salt solution with Ca2+ and Mg2+. The time from venipuncture to experimentation with agonist or antagonist was the same in all experiments (2 h). Isolated neutrophils were kept in ice until use. The neutrophils were used from one volunteer per experiment.

Preparation of Activated PMN Supernatants and Measurement of TNFα
Supernatants from PMNs were harvested 5, 30, 60, and 120 min after activation with formyl-Met-Leu-Phe (10 nM). TNFα was determined in the supernatant using a human TNFα ELISA (Thermo Fisher Scientific Inc., Waltham, MA).

Pharmacologic Agents
As a specific inhibitor of the Adora2b, PSB1115 (Tocris, Minneapolis, MN) was used at a concentration of 100 μM. A highly specific agonist for the Adora2b, BAY 60-6583, developed by Bayer HealthCare AG (Wuppertal, Germany) was used at a concentration of 1 μM.

Statistics
An a priori sample size analysis for IS and cTnI measurement was based on previous studies suggesting a SD for IS and cTnI serum concentration of 5% and 21 ng/ml, respectively. A biologic relevant difference of at least 10% for IS and 40 ng/ml for cTnI between control and experimental groups resulted in four animals per group being needed to obtain statistically significant results with a probability of 0.8. A one-factor ANOVA was used when cell numbers or IS or cTnI values from different bone-marrow transplanted mice were compared. The dependent variable was the time in regard to bone marrow ablation, and the independent variable was the cell numbers or ISs or cTnI values. When different treatment regimens were combined, data were compared by two-factor ANOVA. Once a significant interactive effect was established, a one-way ANOVA was performed using the Bonferroni correction. Where appropriate, an independent Student t test was applied. For all comparisons in the current study, two-tailed testing was performed and P < 0.05 was considered to be significant. In the current study, no “missing data” occurred. Data are expressed as mean ± SD. For statistical analysis, GraphPad Prism 5.0 software for Windows or Bias for Windows® (Epsilon-Verlag, Frankfurt, Germany) was used. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Adora2b Activation Decreases IS In Vivo
To determine whether Adora2b needs to be activated on cardiac cells (endothelial and cardiomyocytes) or on inflammatory cells, we used different in vivo experimental procedures. An overview of the different experimental designs and timelines is given in figure 1A. We first confirmed that Adora2b protein was absent in commercially available Adora2b−/− mice using real-time polymerase chain reaction analysis (data not shown) or immunohistochemistry. No Adora2b transcript or protein was detected compared with WT controls (fig. 1B). To confirm the cardioprotective effects of Adora2b activation, Adora2b−/− mice or WT controls were subjected to 60 min of ischemia followed by 120 min of reperfusion (experiment 1, fig. 1A). IS staining using Evans blue and triphenyltetrazolium chloride or serum cTnI...
analysis showed significantly increased IS or cTnI concentrations in Adora2b−/− compared with WT mice (fig. 1C; IS\textsubscript{WT}: 48 ± 4% and cTnI\textsubscript{WT}: 36.5 ± 4.7 ng/ml vs. IS\textsubscript{Adora2b−/−}: 64 ± 3.5% and cTnI\textsubscript{Adora2b−/−}: 110.5 ± 12.7 ng/ml). Blood pressure and heart rate at baseline (107.3 ± 5 mmHg; 492 ± 60 beats/min) or after ischemia (71.3 ± 5 mmHg; 485 ± 60 beats/min) were not different between experimental groups. These results show that the absence of Adora2b increases myocardial injury during I-R.

We next investigated whether pharmacologic activation of Adora2b also reduces myocardial injury in response to I-R. To answer this question, we submitted WT mice to I-R injury (experiment 1, fig. 1A). A single bolus of the Adora2b agonist BAY 60-6583 (10 μg/kg body weight) was given
Bone Marrow Transplantation Changes the Genotype of Leukocytes

To study the contribution of cardiac cells (endothelial and cardiomyocytes) to Adora2b-mediated cardioprotection, we generated bone marrow chimeric mice for the Adora2b (fig. 2, A and B). This was achieved by whole body irradiation with subsequent bone marrow transplantation. After irradiation treatment, a recovery period of 56 days followed. Then, mice underwent cardiac I-R (fig. 2B; see also experiment 2, fig. 1A). The erythrocyte count was unchanged by irradiation. In contrast to that, leukocyte numbers significantly decreased on days 3 and 4 after irradiation. However, at the recovery phase, numbers of leukocytes were not significantly different compared with controls (fig. 2C; before radiation 10.1 ± 0.9 × 10³ cells/µl blood; 56 days after radiation 9.3 ± 0.9 × 10³ cells/µl blood). Next, we tested the efficiency of leukocyte reconstitution after irradiation. For this purpose, a mutated mouse strain (B6.SJL-Ptprca Pep3b/Boj) carrying the CD45.1 alloantigen was used as the source of donor bone marrow. The percentage of cells expressing CD45.1 was determined in different leukocyte populations using fluorescence-activated cell sorting analysis as done previously. The percentage of cells expressing CD45.1 was between 95 and 98% (fig. 2D). Thus, bone marrow transplantation changes the genotype of the leukocyte population.

Bone-marrow Derived Cells Require Adora2b to Protect from Myocardial I-R Injury

After having generated bone marrow chimeric mice, we next questioned on which cell type Adora2b needs to be activated to be cardioprotective: In chimeric mice, Adora2b protein is present either only on cardiac cells and other tissues (Adora2b⁻/⁻ → WT_BM) or only on inflammatory cells (WT_BM → Adora2b⁻/⁻). These chimeric mice underwent 60 min of ischemia and 120 min of reperfusion (experiment 2, fig. 1A), followed by IS and cTnI measurements. When WT mice received Adora2b⁻/⁻ bone marrow, they developed a phenotype similar to that of Adora2b⁻/⁻ mice and myocardial injury increased significantly (IS: from 42 to 62%; cTnI: 21.9–121.6 ng/ml; fig. 3, A and B). When Adora2b⁻/⁻ mice were transplanted with WT bone marrow, they developed a phenotype similar to that of WT mice and myocardial injury was significantly decreased (IS: from 60 to 48%; cTnI: from 91.6 to 34.1 ng/ml; fig. 3, A and B). Representative infarct staining is shown in figure 3C. These results show that Adora2b needs to be present on bone marrow derived cells to elicit its cardioprotective effect.

PMNs Are the Dominant Inflammatory Cell Type in Myocardial I-R

To understand the pathologic and cellular changes during reperfusion, we performed hematoxylin and eosin staining of myocardial tissue from WT mice exposed to 60 min of ischemia followed by 5, 15, 30, 60, or 120 min of reperfusion (experiment 3, fig. 1A). Leukocytes adhered sporadically to the vessel wall in the early phase of reperfusion (15 min). The myocardium showed no structural changes (fig. 4A). Morphologically these leukocytes appeared to be PMNs. With longer reperfusion (120 min), significantly more leukocytes adhered to and crossed the vessel wall (fig. 4B). Myocardial cross striations blurred with slight edema of the cardiac tissue as a sign of severe myocardial damage. We next asked which leukocyte population was primarily present at 120 min of reperfusion. To answer this question, we performed immunohistochemistry using myeloperoxidase as marker for PMNs, F4–80 as marker for monocytes, and CD3 as marker for T cells. We found that most leukocytes adhering to or crossing cardiac vessel walls stained positive for myeloperoxidase (fig. 4, C–E). To confirm this finding, we performed additional immunohistochemistry using the other highly specific PMN-marker Ly6G. Again, adherent leukocytes after 120 min of reperfusion stained positive for Ly6G (fig. 4F). Based on this, our results show that PMNs are the dominant bone marrow derived cell population during early and late reperfusion.

Adora2b-dependent Cardioprotection Relies on the Presence of PMNs

Next, we asked whether the presence of PMNs increases myocardial injury in reperfusion. PMNs release different cytokines that have been shown to damage myocardial tissue even further in reperfusion. To deplete WT mice of PMNs, they received anti-GR1 antibody intraperitoneally 24 h before 60 min of MI and 120 min of reperfusion (experiment 4, fig. 1A). This antibody specifically targets PMNs, and it reduced the PMN numbers in the peripheral blood by 98% (fig. 5A). PMN depletion significantly decreased myocardial damage in response to I-R injury (IS_PMN depletion: 28 ± 8% and cTnI_PMN depletion: 12.1 ± 3.6 ng/ml, fig. 5, B–D). In fact, PMN depletion was just as effective as Adora2b agonist treatment (fig. 5, B–D). Thus, PMNs further enhance myocardial damage in the reperfusion after MI.

Next, we asked whether cardiac cells (endothelia and cardiomyocytes) also contribute to Adora2b-dependent cardioprotection in I-R injury. To answer this question, we injected the Adora2b agonist BAY 60-6583 into PMN-depleted WT mice upon reperfusion and again determined the extent of myocardial injury. Although these animals received a combination of two cardioprotective treatments, the ad-
dition of Adora2b agonist to PMN depletion had no additional effect on IS or cTnI (ISPMN depletion: 28 ± 6.3% vs. ISPMN depletion + BAY60-6583: 36 ± 4.7 ng/ml; fig. 5, B–D). These results indicate that Adora2b signaling is only cardioprotective when activated on PMNs.

**Adora2b Suppresses TNFα Release in Myocardial Tissue after I-R Injury**

Next, we wanted to determine whether Adora2b activation has an influence in the recruitment of PMNs into the AAR. We used myeloperoxidase as a surrogate parameter to quantify...
PMN numbers in the tissue. WT or Adora2b−/− mice underwent 60 min ischemia followed by 120 min of reperfusion. Next, we harvested the AAR and measured the myeloperoxidase protein content by ELISA (experiment 3, fig. 1A). The myeloperoxidase content in Adora2b−/− mice was not increased compared with that in WT mice (fig. 6A). Thus, Adora2b activation has no influence on the number of PMNs infiltrating the myocardial tissue after I-R injury.

To understand why Adora2b signaling protects the myocardium from I-R despite that it has no influence on...
PMN numbers, we quantified the protein concentrations of several proinflammatory cytokines in the AAR of Adora2b−/−. These proinflammatory cytokines contribute to the damage of the myocardium by enhancing the inflammatory response after MI. We measured the TNFα, interferon-γ, interleukin-1β, interleukin-2, interleukin-4, interleukin-5, keratinocyte-derived cytokine, interleukin-10, and interleukin-12 protein content in the AAR using a multiplex ELISA (Meso Scale; fig. 6, B–D). This experiment revealed that interferon-γ, interleukin-1β, interleukin-2, interleukin-4, interleukin-5, keratinocyte-derived cytokine, interleukin-10, and interleukin-12 concentrations were not different between WT and Adora2b−/− mice in the AAR (fig. 6, B and C). However, TNFα protein content was five times

**Fig. 5.** Adora2b signaling in polymorphonuclear leukocyte (PMN)-depleted mice. Wild-type mice were treated with a monoclonal antibody directed against the granulocyte receptor 1 (GR-1) exclusively expressed on polymorphonuclear leukocytes (PMNs) 24 h before the experiment. GR-1 treatment decreased PMNs in peripheral blood from 2,500 ± 150 PMNs/μl (control) to 150 ± 75 PMNs (mean ± SD, n = 4 per group) (A). Infarct sizes (ISs) are presented as percentage of the area at risk (AAR). The pretreatment with a PMN-depleting antibody alone led to ISs of 28 ± 8%. Additional administration of BAY 60-6583 during reperfusion in the presence of PMNs led to ISs of 24 ± 5%, and to 36 ± 2% in the absence of PMNs (B). Measurement of serum troponin I concentrations by enzyme-linked immunosorbent assay (ELISA). Treatment with BAY 60-6583 during reperfusion resulted in troponin I values of 10 ± 4 ng/ml. GR-1 antibody treatment resulted in troponin I values of 12 ± 6 ng/ml. PMN depletion and subsequent BAY 60-6583 administration in the reperfusion resulted in troponin I values of 9 ± 4 ng/ml (C). ISs were measured by double staining with Evans blue and triphenyltetrazolium chloride. ISs are expressed as the percentage of the AAR that underwent infarction. Representative images of ISs from the experiments in B and C are displayed (mean ± SD; n = 5 per group) (D).
higher in the AAR from Adora2b⁻/⁻ mice than from that of WT mice (39.3 ± 2.7 vs. 7.5 ± 0.5 pg/mg protein, P < 0.05; fig. 6D).

These findings provide evidence that Adora2b activation inhibits TNFα release, but it has no influence on absolute PMN numbers in the myocardial tissue after I-R injury.

**Adora2b Agonist (BAY 60-6583) Dampens TNFα Release from Activated Human PMNs**

We next asked whether Adora2b directly inhibits the release of TNFα from PMNs. To this end, we isolated PMNs and activated the cells with formyl-Met-Leu-Phe (10 nM). This was done in the presence of an Adora2b antagonist (PSB 1115) or agonist (BAY 60-6583). To eventually translate such findings into a clinical setting, we used human PMNs. Then we measured the content of TNFα in the supernatant over time by ELISA. Activated PMNs increased their TNFα release, 2.3 ± 0.4 and 3.2 ± 0.3 (P < 0.05, n = 5) at 30 and 60 min, respectively. Pretreatment with a specific Adora2b antagonist PSB 1115 (100 μM) significantly amplified the TNFα release throughout the experiment (fig. 7A).

In contrast to that, pretreatment of human PMNs with a specific Adora2b agonist (BAY 60-6583, 1 μM) abrogated the TNFα release at any time point throughout the experiment (fig. 7B). Moreover, pretreatment with BAY 60-6583 suppressed TNFα release from PMNs below baseline at 120 min (fig. 7B).

Based on our findings, we propose that Adora2b activation suppresses TNFα release by PMNs in I-R injury. Therefore, we suggest Adora2b treatment to reduce PMN-dependent injury and TNFα release, thereby ameliorating I-R injury (fig. 7, C and D).

**Discussion**

The issue addressed in this study was the identification of the contribution of Adora2b on inflammatory cells to cardioprotection in I-R injury. The main finding of our study is that Adora2b-mediated cardioprotection occurs primarily through inflammatory cells, most likely due to an inhibition of TNFα release from invading PMNs.

Adora2b is expressed on cardiac, vascular, hematopoietic cells, and its activation protects the myocardium from I-R injury.21 Theoretically, all these cell types could be responsible for Adora2b-dependent cardioprotection. In this study we provide evidence that Adora2b is cardioprotective through activation of PMNs during reperfusion after MI. Two findings from our experiments support this concept. First, when Adora2b is present on inflammatory cells (but absent on all other cells), ISs are identical to those of WT controls. This effect is reversed when Adora2b is absent on inflammatory cells. In addition, myocardial injury is similar to that of whole-body Adora2b-deficient mice. Second, a highly specific agonist for Adora2b reduces ISs to the same extent as depletion of PMNs. The combination of both treatments (Adora2b agonist and PMN depletion) has no additional protective effect. Therefore, the activation of Adora2b on endothelial cells or cardiomyocytes does not contribute to the cardioprotective effects. Our observation that Adora2b is cardioprotective when activated on PMNs is in line with other studies. Adenosine receptor blockade on PMNs drastically increases I-R injury.21 Stimulation of adenosine receptors with AMP-579 is highly cardioprotective, primarily through the inhibition of PMN activation and adhesion to the vascular wall.22,23 Unfortunately, AMP-579 is an unspecific Adora1, Adora2a, and Adora2b agonist, which makes it difficult to draw conclusions about the adenosine receptor subtype involved.24,25 Studies in gene-targeted mice suggest that primarily signaling through Adora2b is responsible for this effect on PMNs.15

Ischemia-reperfusion injury elicits a strong inflammatory response in the heart, leading to the recruitment of leukocytes to the myocardium. In our study, the vast majority of
leukocytes are PMNs. This is in line with several animal and clinical studies indicating that the principal effector cells of reperfusion injury are blood-borne cells such as PMNs. PMNs mediate myocardial necrosis during ischemia, reperfusion, or both. In our experiments, PMN depletion or treatment with an Adora2b agonist decreases IS to a similar extent. However, the protective effect of the two interventions is not cumulative. When Adora2b is activated, it inhibits neutrophil transmigration and the expression of adhesion molecules. Based on this, we suspected that the number of PMNs in the AAR in Adora2b-deficient mice should be higher than that in WT animals. However, we were surprised to find identical myeloperoxidase concentrations in the postischemic tissue of WT and Adora2b-deficient mice. This means that the number of PMNs in the AAR is identical in the two genotypes. Because Adora2b-deficient mice have an enhanced inflammatory response and activation of Adora2b elicits antiinflammatory responses, we subsequently assessed whether Adora2b activation changes PMNs activity rather than recruitment in I-R injury.

A screening of seven cytokines revealed significantly higher TNFα concentrations in Adora2b-deficient mice compared with WT mice after I-R injury. This is consistent with previous reports, showing that Adora2b−/− mice have higher plasma concentrations of the proinflammatory cytokine TNFα at baseline and after vascular injury. The suppression of TNFα might be the primary mechanism by which Adora2b is protective in myocardial I-R injury. Sev-

**Fig. 7.** Adora2b signaling in human polymorphonuclear leukocytes (PMNs). Human PMNs were isolated from healthy individuals and activated using formyl-Met-Leu-Phe (fMLP 10 nM). Cells were treated with a specific Adora2b antagonist (PSB 1115, 100 µM, A) or an Adora2b agonist (BAY 60-6583, 1 µM, B) or vehicle. Tumor necrosis factor α (TNFα) release into the supernatant was measured by enzyme-linked immunosorbent assay (ELISA). Vehicle treatment of PMNs significantly increased TNFα, with a maximal response 30 and 60 min after activation (22.88 ± 4.0 pg/ml and 31.4 ± 5.4 pg/ml, respectively) (A, B). Pretreatment with an Adora2b antagonist enhances TNFα secretion significantly (24.8 ± 4.8, 42.3 ± 3.2, 48.6 ± 4.0, and 28 ± 4.6 pg/ml at 5, 30, 60, or 120 min, respectively) (A). Pretreatment with an Adora2b agonist significantly dampens TNFα release (7.89 ± 1.3, 10.70 ± 2.6, 15.9 ± 2.5, and 5.5 ± 0.9 pg/ml at 5, 30, 60, and 120 min, respectively; n = 5 per group) (B). Proposed role and potential therapeutic intervention: Cardiac ischemia reperfusion injury leads to PMN adhesion to the endothelial cells in the area at risk (AAR) (C, D). PMNs release TNFα, which further damages the myocardial tissue and enhances reperfusion injury (C). Pharmacologic treatment upon reperfusion using an Adora2b agonist activates A2B adenosine receptors on PMNs, which inhibits TNFα release and thereby protects the myocardium from additional damage (D).
eral findings in this study support that. Adora2b−/− mice have higher concentrations of TNFα. Many cell types, such as mast cells, endothelia, cardiomyocytes, and PMNs, could be the source of this TNFα release during I-R.33 In our model of myocardial I-R injury, Adora2b mainly mediated cardioprotection through activation on PMNs. The pharmacologic blockade of the receptor increases TNFα release from PMNs. Vize versa activation of Adora2b suppresses TNFα release from PMNs. In patients with ischemic heart disease, leukocytes are the primary source of TNFα.32,33 TNFα can damage the myocardium in two ways. First, it directly induces apoptosis of cardiomyocytes in reperfusion.14,34 Second, it enhances the activation and recruitment of neutrophils into the postischemic myocardium that further damages the myocardium.35 A suppression of TNFα is beneficial in acute myocardial I-R injury,36,37 and we suggest Adora2b signaling as a different route to achieve this.

Several considerations have to be kept in mind when interpreting data from our study. First, in adenosine-receptor-deficient mice, compensatory up-regulation of other adenosine receptors has been described.38 However, this cannot explain the observed phenotype in our pharmacologic studies on WT mice treated with the Adora2b agonist BAY 60-6583 upon reperfusion. In addition, previous studies on transcript concentrations of other adenosine receptors (Adora1, Adora2a, Adora3) were unaltered in the Adora2b−/− mouse strain used in the current study.10 Based on this, we believe that all observations made in Adora2b−/− mice are a consequence of the gene deletion. Second, we used a coronary artery ligation model in mice. Collateral blood flow during the coronary occlusion can change the size of an infarction significantly. Certain species (e.g., guinea pigs and dogs) have an extensive collateral flow, and a single-vessel ligation is less effective than in other species.39 In contrast to that, mice and rats have only limited collateral flow.40 However, only mice with the same genetic background should be used because there are differences in collateral flow between different mouse strains.41 All mice used in this study have a BL6/C57J background. Therefore, we believe that a difference in collateral blood flow did not contribute significantly to observed changes in ISs. Third, there are obvious differences between mouse and human physiology, despite that 99% of mouse genes have an equivalent in humans.42 Mice have a heart rate 10 times greater than that of humans. As a consequence, murine cardiomyocyte contraction relies almost completely on calcium release from the sarcoplasmatic reticulum. Thus, caution must be exercised when extrapolating findings from these models to the human setting. Nevertheless, genetically engineered mouse models are the mainstay for the study of cardiac structure–function relationships.

In summary, this study shows that Adora2b is dominantly protective when activated on PMNs in myocardial I-R injury. It decreases the release of the proinflammatory cytokine TNFα from PMNs and limits inflammatory responses after I-R injury. We believe that Adora2b agonist administration may provide a novel therapeutic option in the treatment of myocardial I-R injury.

References