Ecto-5′-Nucleotidase on Immune Cells Protects From Adverse Cardiac Remodeling

Florian Bönner, Nadine Borg, Christoph Jacoby, Sebastian Temme, Zhaoping Ding, Ulrich Flögel, Jürgen Schrader

Rationale: Ecto-5′-nucleotidase (CD73) on immune cells is emerging as a critical pathway and therapeutic target in cardiovascular and autoimmune disorders.

Objective: Here, we investigated the role of CD73 in postinfarction inflammation, cardiac repair, and remodeling in mice after reperfused myocardial infarction (50-minute ischemia).

Methods and Results: We found that compared with control mice (1) cardiac function in CD73−/− mice more severely declined after infarction (systolic failure with enhanced myocardial edema formation) as determined by MRI and was associated with the persistence of cardiac immune cell subsets, (2) cardiac adenosine release was augmented 7 days after ischemia/reperfusion in control mice but reduced by 90% in CD73 mutants, (3) impaired healing involves M1-driven immune response with increased tumor necrosis factor-α and interleukin-17, as well as decreased transforming growth factor-β and interleukin-10, and (4) CD73−/− mice displayed infarct expansion accompanied by an immature replacement scar and diffuse ventricular fibrosis. Studies on mice after bone marrow transplantation revealed that CD73 present on immune cells is a major determinant promoting cardiac healing.

Conclusions: These results, together with the upregulation of CD73 on immune cells after ischemia/reperfusion, demonstrate the crucial role of purinergic signaling during cardiac healing and provide groundwork for novel anti-inflammatory strategies in treating adverse cardiac remodeling. (Circ Res. 2013;113:301-312.)

Key Words: adenosine ■ CD73 ■ inflammation ■ myocardial infarction

Although early reperfusion strategies have dramatically improved survival rates in patients with acute myocardial infarction (MI), an increasing number of patients are at risk of developing heart failure.1 Progressive alterations in cardiac structure (dimensions, mass, and shape) as a result of endogenous repair mechanisms in response to MI are commonly referred to as ventricular remodeling.2

The cardiac repair mechanisms initiated directly after MI are considered an inflammatory condition that ideally leads to rapid elimination of injurious stimuli and initiation of myocardial healing. The recruitment of immune cells depends on the release of damage-associated molecular patterns that are recognized by receptors on leukocytes and subsequently stimulate the production of proinflammatory cytokines.3 In addition, fragments of the extracellular matrix and complement act through specific ATP receptors (P2). ATP can also be directly released from immune cells on activation.7,8 In addition, ATP released from neutrophils promotes recognition of chemotactic gradients that guides these cells to infected and inflamed tissue.9 Although extracellular ATP is rapidly dephosphorylated, the role of its breakdown product adenosine in modulating the kinetics of immune cell infiltration into the injured heart and its influence on cardiac healing and remodeling after ischemia/reperfusion (I/R) have not been explored in detail.

The half-life of extracellular ATP is critically determined by the activity of ectoenzymes, such as various ectonucleoside triphosphatases, including CD39 that hydrolyzes ATP to ADP and AMP.10 AMP is further hydrolyzed by alkaline phosphatase and ecto-5′-nucleotidase (CD73) to adenosine, which acts on P1 receptors mediating both anti- and proinflammatory effects, depending on the receptor subtype.11 Four distinct subtypes of P1 receptors have...
been identified: adenosine A1, A2A, A2B, and A3 receptors (ARs) that are G-protein coupled. Activation of A1R is considered to be proinflammatory, the A2AR triggers potent anti-inflammatory effects, whereas the A2BR can mediate both anti- and proinflammatory responses. Conceptually, it is important to recognize that it is the activity of the ectonucleotide cascade, especially the rate-limiting step of CD73, which determines whether ATP or adenosine dominates via activation of P2 or P1 receptors, respectively.

We have recently reported that the most prominent immune cell population within the unstressed heart are myeloic antigen-presenting cells (APCs: CD11b+CD11c+F4/80+MHCII+) that express high levels of CD39 but lack CD73. We also observed that 3 days after I/R, CD73 was significantly upregulated on invading granulocytes and T cells, which favors the enhanced local formation of anti-inflammatory adenosine. Remarkably, CD73 associated with leukocytes comprised two third of the total cardiac CD73. These findings suggest that accumulation of adenosine at the site of inflammation may be part of an autocrine signaling loop, which limits the uncontrolled distribution of inflammation and thus infarct expansion. This study extends these previous findings to the functional level and explores the mechanisms by which CD73-derived adenosine influences cardiac healing after MI. To this end, we used mice with global deficiency of CD73 and chimeric mice with reconstituted CD73 on immune cells, which were subjected to severe ischemia (50 minutes) and reperfusion. Functional analysis by MRI and kinetics of immune cell infiltration for 3 weeks after MI, together with histology and cytokine measurements, demonstrated important immune cell regulation in myocardial healing by autocrine CD73-mediated purinergic signaling.

Methods

Mouse Models
Animal experiments were performed in accordance with the national guidelines on animal care and were approved by Landesamt für Natur, Umwelt und Verbraucherschutz. Reconstitution of CD73 on leukocytes in CD73−/− mutants (chimera mice) was realized by bone marrow transplantation. For technical details, see Online Data Supplement. Reperfused MI was induced as previously described and explained in detail in the Online Data Supplement.

MRI Experiments
MRI data were recorded on a Bruker Avance III 9.4 T Wide Bore NMR spectrometer (Bruker, Rheinstetten, Germany) operating at frequencies of 400.13 MHz for 1H and 376.46 MHz for 19F measurements. For a detailed description of the MRI setup, acquisition parameters, and quantification procedures, please see Flögel et al and the Online Data Supplement.

Isolation and Flow Cytometry of Cardiac Immune Cells
We have elaborated a method for immune cell extraction from whole murine hearts for quantitative analysis with flow cytometry. For details of the isolation protocol and modifications caused by postisolation quantitative real-time polymerase chain reaction, please see the Online Data Supplement. Flow cytometry experiments were performed with an FACS Canto II (Becton, Dickinson) equipped with 3 lasers and capable of detecting 58 colors. For detailed antibody staining protocol and gating strategy, see Bönner et al and the Online Data Supplement.

Isolation of Macrophages for Real-Time Polymerase Chain Reaction
The modifications of the conventional isolation protocol in terms of macrophage isolation are stated in the Online Data Supplement.

Light Microscopy and Polarized Light Microscopy
Explanted mouse hearts were fixed in Tissue-Tek (Sakura Finetec Inc, CA) and frozen at −20°C. Hearts were cut into 7 µm sections from apex to base. Staining was performed with hematoxylin and eosin and picrosirius red (Sigma Aldrich) according to the manufacturer’s protocols. For image acquisition, an Olympus MX 61 microscope was used. Further details on analysis and polarized light microscopy are presented in the Online Data Supplement.

Cytokine Measurements
Explanted hearts were homogenized, and cytosolic proteins were extracted using NE-Per Nuclear and Cytoplasmic Extraction Reagents (Pierce, Themoscientific, Rockford, IL). Total protein concentration was determined by a BCA protein assay (Pierce). Cytokine levels of interleukin (IL)-6, IL-17, IL-10, and tumor necrosis factor-α (TNF-α) were detected with magnetic Bio-Plex pro mouse assays using a Bio-Plex 200 System. Transforming growth factor-β (TGF-β1) was detected with a quantitative ELISA kit (R&D Systems).

Langendorf Experiments and High-Performance Liquid Chromatography
Hearts were isolated from sham-operated animals and from animals 7 days after I/R and perfused according to the Langendorff technique. Coronary effluent perfusate was collected on ice and analyzed with high-performance liquid chromatography for adenosine. For details, please see the Online Data Supplement.

Statistics
Unless otherwise stated, data are presented as mean±SD. Data were statistically analyzed by Mann–Whitney U Test or the Student t test.

Results

Cardiac Function Deteriorates in CD73+/− Mice After I/R Accompanied by a Sustained Myocardial Edema
Previous findings of our group suggested a role for CD73-derived adenosine present on cardiac immune cells, because of significant upregulation on granulocytes and T-cell subsets 3 days after I/R. Applying the same protocol of I/R, we investigated the functional significance of CD73 in the process of cardiac healing using CD73-null mice. Ventricular function, necrosis, and myocardial edema were analyzed by MRI at days 1, 7, 14, and 27 after MI (Figure 1). The size of necrosis was assessed by contrast-enhanced MRI (late gadolinium enhancement) 1 day after I/R. Late gadolinium enhancement

Nonstandard Abbreviations and Acronyms:

<table>
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<tr>
<th>Acronym</th>
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<tr>
<td>APCs</td>
<td>myeloic antigen-presenting cells</td>
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<tr>
<td>AR</td>
<td>adenosine A1, A2A, A2B, and A3 receptors</td>
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<td>CD73</td>
<td>ecto-5′-nucleotidase</td>
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<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>IL</td>
<td>interleukin</td>
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<td>I/R</td>
<td>ischemia and reperfusion</td>
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<td>MI</td>
<td>myocardial infarction</td>
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<td>TGF-β</td>
<td>transforming growth factor-β</td>
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<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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correlated well with 2,3,5-triphenyltetrazolium chloride staining in a separate experimental series (Online Figure 1). As shown in Figure 2A, the extent of cardiac necrosis of the left ventricular myocardium was not different between wild-type (WT) and CD73−/− mice subjected to 50 minutes of ischemia followed by reperfusion. This is in line with the observation that global ejection fraction was equally reduced in both genotypes (WT: 86% versus CD73−/−: 82%).

To identify the area of myocardium at risk in vivo and to further explore the underlying cause of the observed systolic ventricular impairment at day 14 after I/R, we mapped T2 relaxation times. This MRI approach is known to identify cardiac myocardial edema formation, and in the acute setting, this is equivalent to the myocardial area at risk.17 As shown in Figure 2F and in representative midventricular short-axis MRI slices (Figure 2G), myocardial edema determined 1 day after I/R was not different between CD73-null and WT mice. Thereafter, however, cardiac edema continuously decreased to baseline values within 28 days in the WT animals, whereas the respective values in CD73−/− mutants tended to further increase.

**Impaired Adenosine Formation in CD73−/− Mice**

To evaluate whether disruption of CD73 translates into impaired adenosine formation, we measured the release of adenosine into the effluent perfusate of isolated perfused hearts from WT and CD73−/− mice 7 days after I/R. As shown in Figure 3, lack of CD73 significantly reduced adenosine release from the uninjured heart, which is consistent with previous findings.18 Interestingly, however, we found a 2.5-fold increase in adenosine release in WT hearts injured by I/R, and this effect was abolished (10% of adenosine release compared with respective WT controls) in mice lacking CD73 (Figure 3).

**Lack of CD73-Derived Adenosine During Cardiac Healing Elicits a Th1- and M1-Driven Immune Response**

As the lack of CD73-derived adenosine was associated with myocardial edema formation, we used 19F-MRI and fluorescence-activated cell sorting to investigate whether this phenomenon is associated with enhanced cellular inflammation. Using 19F-MRI, which noninvasively detects monocyte invasion into inflamed tissues in vivo,16 we found that the intramyocardial 19F signal was significantly increased in hearts of CD73−/− mice on day 7 after I/R (Online Data Supplement Figure II). Figure 4 summarizes results from a comprehensive fluorescence-activated cell sorting analysis of leukocytes present in WT and CD73−/− hearts on days 3, 7, and 14 after I/R using a gating strategy outlined in Online Figure III. As shown in Figure 4A, the number of APCs (CD45+, CD11b+, CD11c+, MHCII+, F4/80highlow)—the supposed imaging substrate of 19F-MRI—initially increased in number in both genotypes and decreased to control values in WT animals on day 14 after I/R. In contrast, cardiac APCs continuously increased in CD73−/− mutants. Of note, the kinetics of APCs mirrored the changes observed during edema formation (Figure 4B). At day 14 after I/R, we found 1.85±0.94×10^3 APCs/mg heart tissue in WT hearts and 5.36±1.25×10^3 APCs/mg heart tissue in CD73−/− hearts (P<0.05). To evaluate the macrophage activation state, we analyzed the expression of F4/80 on APCs, because downregulation of the F4/80 antigen is associated with macrophage activation by interferon-γ and an M1-driven phenotype.19 As shown in Figure 4B, F4/80-expressing APCs were significantly lower in CD73−/− mice at all time points after I/R. Mean fluorescence intensity of F4/80-positive macrophages tended to be decreased as well (Figure 4C). In addition, the CD73−/− monocyte population (CD45+, CD11b+, CD11c−, Ly6G−, Ly6Chighlow), serving as macrophage precursors, was dominated by Ly6Chigh-expressing cells even 14 days after I/R (Figure 4D), whereas WT mice monocytes were dominated by Ly6Chow-expressing counterparts.
Fluorescence-activated cell sorting analysis further revealed that the total amount of leukocytes (CD45+) was 3-fold higher at day 14 after I/R in CD73−/− mutant compared with WT hearts (Figure 4E: WT, 2.91±1.18×10^3; CD73−/−, 9.36±3.71×10^3 leukocytes/mg heart tissue; P<0.05). The observed cell kinetics was similar in several leukocyte subtypes. Granulocytes (CD45+, CD11b+, CD11c−, Ly6G+), barely detectable in the uninjured myocardium of both genotypes, increased ~90-fold on day 3 after I/R, but failed to disappear 14 days after I/R in CD73−/− hearts (Figure 4F). Data
CD73−/− Mice Display Infarct Expansion Accompanied by an Immature Replacement Scar and Remote Ventricular Fibrosis

To investigate the structural features of the observed phenotype and impaired tissue remodeling associated with an increased number of infiltrating immune cells in CD73−/− hearts, we next analyzed the extent of scar formation at day 28 after I/R by histology. As shown in Figure 6A, scar tissue in CD73−/− mice was associated with significantly greater ventricular dilatation compared with WT controls. Fibrosis within the adjacent myocardium (peri-infarct fibrosis) again was significantly greater in CD73−/− animals (Figure 6B). This difference was already observed at day 14 after I/R (data not shown). In addition, we found the extent of interstitial fibrosis in the remote area to be significantly increased in CD73−/− hearts (Figure 6C). Qualitative analysis of collagen with circularly polarized illumination microscopy demonstrated predominantly loosely assembled green fibers (assigned to collagen III) in CD73−/− hearts compared with orange and more organized fibers (assigned to collagen I) in WT hearts. Qualitative collagen analysis with circularly polarized illumination microscopy revealed that the ratio of collagen I to collagen III was clearly smaller in CD73−/− hearts compared with WT controls (Figure 6D), suggesting immature scar formation.

Significant differences in the collagen ratio were already detectable at day 14 after I/R (data not shown).

CD73 Present on Leukocytes Is the Major Determinant of Proper Cardiac Healing

To discriminate whether the observed effects on post-I/R inflammatory state and scar formation are because of CD73 present on endothelial cells or infiltrating immune cells or both cell types, bone marrow of WT mice was transplanted to constitutional CD73−/− mice. As appropriate controls, WT and CD73−/− mice were treated accordingly and received autologous bone marrow from the respective genotype. In all cases, bone marrow from male mice was transplanted into female mice, and the effectiveness of transplantation was controlled by fluorescence in situ hybridization. Using the identical experimental protocol (Figure 1), the initial extent of myocardial necrosis was similar in all experimental groups (Figure 7A), and the decrease in ventricular function in CD73−/− mice (Figure 7B) was comparable with data shown in Figure 2B. Transplantation of WT bone marrow into CD73−/− mice fully restored ventricular function to WT controls 2 weeks after I/R (CD73−/−→CD73−/−, 38.65±4.71%; WT→WT, 55.72±8.11%; WT→CD73−/−, 59.23±13.87%; P<0.05). A detailed analysis of ventricular volumes (Figure 7C) shows that restoration of CD73−/− leukocytes fully abrogated the development of cytokines TNF-α and IL-17 and significantly reduced levels of TGF-β on day 7 after I/R, whereas on day 14 the concentration of IL-10 was significantly lower (P<0.05). TGF-β levels were observed to be decreased in CD73 mutants without reaching the level of significance (P=0.06). At this latter point of time, IL-6 was no longer detectable. Cytokine levels were also measured in plasma of peripheral blood at the 2 points in time, but no differences were observed between the 2 genotypes (data not shown).

on the abundance of T-lymphocyte subsets are summarized in Figure 4G–4I. As can be seen, all T lymphocytes (CD45+, CD3+) in WT mice increased until day 7 after I/R and decreased afterward (Figure 4G and 4H). In contrast, this cell fraction continuously increased in CD73−/− hearts, with significantly greater amounts after 14 days compared with WT hearts. Although at this time point the number of CD8+ T-lymphocytes in CD73−/− hearts was 6-fold higher compared with WT controls, CD4+ T-helper cells were more frequent by a factor of 10. Because regulatory T cells play an important role in extracellular purine metabolism, we additionally analyzed the temporal changes in Treg abundance (CD45+, CD3+, CD4+ CD25+, FoxP3+) related to the total CD4+ T cells. As shown in Figure 4I, Tregs were decreased on day 3 after I/R in both genotypes. In general, the Treg numbers tended to be decreased in CD73−/− hearts during the healing process (Figure 4I).

To support the notion that macrophages of CD73−/− mice exhibit a M1-driven phenotype, we have measured the expression of representative M1 (TNF-α, IL-1β, IL-6) and M2 genes (arginase-1, IL-10, TGF-β) in fluorescence-activated cell-sorted cardiac APCs with quantitative real-time polymerase chain reaction. Analysis revealed that CD73−/− macrophages compared with WT counterparts in general show increased expression of genes related to the M1 phenotype (Figure 5A) and a decrease of genes characteristic of the M2 phenotype (Figure 5B). Although all genes attributed to a particular phenotype were collectively changing in the same direction, none of the individual genes reached the level of significance.

To further explore whether the observed differences in immune cell abundance and quality are accompanied by changes in the global cardiac cytokine pattern, we have measured IL-6, IL-17, TNF-α, IL-10, and TGF-β on days 7 and 14 after I/R in cardiac tissue. As shown in Figure 5C, CD73−/− mutants showed significantly elevated levels of the proinflammatory cytokines TNF-α and IL-17 and significantly reduced levels of TGF-β on day 7 after I/R, whereas on day 14 the concentration of IL-10 was significantly lower (P<0.05). TGF-β levels were observed to be decreased in CD73 mutants without reaching the level of significance (P=0.06). At this latter point of time, IL-6 was no longer detectable. Cytokine levels were also measured in plasma of peripheral blood at the 2 points in time, but no differences were observed between the 2 genotypes (data not shown).
Figure 4. Sustained immune cell infiltration with preference of inflammatory cell types as a key feature of CD73−/− hearts in myocardial healing. Cardiac leukocyte subsets were isolated and analyzed ex vivo by flow cytometry in sham-operated (control) wild-type (WT) and CD73−/− mice and 3, 7, and 14 days after ischemia and reperfusion (I/R). Exemplarily, flow cytometry plots illustrate both genotypes 14 days after I/R. A, Myeloid antigen-presenting cells (APCs: CD45+, CD11b+, CD11c+, MHCII+, F4/80high/low) as total number per mg heart tissue. B, Percentage of APCs expressing F4/80 and (C) antigen expression of F4/80 in APCs as mean fluorescence intensity. D, Monocytes (CD45+, CD11b+, CD11c−, Ly6G−) expressing Ly6Chigh as percentage of total monocytes, (E) total leukocyte numbers (CD45+) as percentage of total monocytes, (F) total granulocyte numbers per mg heart tissue (CD45+, CD11b+, CD11c−, Ly6G+), (G) T-cytotoxic lymphocytes (CD45+, CD3+, CD8+), (H) T-helper lymphocytes (CD45+, CD3+, CD4+) as total number per mg heart tissue, and (I) T-regulatory lymphocytes (CD45+, CD3+, CD4+, CD25+, Foxp3+) as percentage of total T-helper lymphocytes. Values are mean±SD of n=4 to 6 experiments for each time point and group; *P<0.05. CD73 indicates ecto-5′-nucleotidase.
increased end-diastolic and end-systolic volumes. Survival of CD73−/− mice receiving CD73−/− bone marrow and subjected to I/R was only 33% compared with 72% when WT controls received WT bone marrow.

Discussion

This study reports that CD73, previously shown by us to be upregulated on granulocytes and T cells, is a major modulator of cardiac remodeling after I/R. Lack of CD73-derived adenosine (1) prevented the timely resolution of inflammation, (2) showed enhanced cardiac edema formation and contractile dysfunction, and (3) caused the formation of an immature replacement scar and enhanced remote ventricular fibrosis, which together resulted in enhanced heart failure. Experiments with chimeric mice demonstrate that CD73 present on infiltrating immune cells can fully account for the observed phenotype. This makes immune cell-derived extra- cellular adenosine to be an important regulator of the resolution of inflammation in the failing heart.

Extracellular adenosine is formed by the sequential dephosphorylation of extracellular ATP by action of an ectonucleo- side triphosphate diphosphohydrolase (CD39) followed by CD73. Although ATP primarily acts on purinergic P2 receptors, its degradation product adenosine signals through P1 purinergic receptors. We have recently reported that CD73 on granulocytes and T cells infiltrating the heart after injury were significantly upregulated 3 days after the identical I/R protocol. In fact, when comparing the cell-associated CD73 in the heart before and after I/R, we found that under control conditions CD73 on coronary endothelial cells contributes ≈90% of total cardiac enzyme activity, whereas after I/R, leukocyte-de- derived CD73 amounted to approximately two third of the entire cardiac CD73. The expression data on CD39 and CD73 suggest a significantly accelerated formation of adenosine. This notion is supported by our finding that normally adenosine formation is significantly enhanced 7 days after I/R (Figure 3) and that loss of CD73 decreased the total adenosine formation by ≈90%. Furthermore, transplantation of WT bone marrow to CD73−/− mice fully salvaged the heart failure phenotype. This provides additional evidence that CD73 on infiltrating granulocytes and T cells, acting as key immune cells in this process, was responsible for the observed phenotype. It also lends support to the notion that CD73 on coronary endothelial cells does not importantly contribute to cardiac dysfunction and edema formation. Most likely, proinflammatory mediators released from immune cells infiltrating the heart in CD73 null mice were responsible for the development of cardiac edema. It is well known from studies in lung and bowel disease models that local inflammation causes local hypoxia with release of hypoxia inducible factor-1α, which is solely capable of regulating vascular barrier function by acting on endothelial cells. Besides this, hypoxia inducible factor-1α is known to upregulate ectonucleotidases and ARs, thereby sensitizing anti-inflammatory pathways to provide containment of inflammation and vascular fluids.

Flow cytometry revealed that immune cell infiltration after I/R is normally dominated by granulocytes and monocytes in the first wave and T cells in the second wave, which is consistent with data from the literature. Loss of CD73, however, was associated with a persistent abundance of granulocytes and inflammatory monocytes after the first wave and elevated levels of T cells and macrophages even at 14 days after I/R (Figure 4). Macrophages are considered to be of central importance in wound healing after MI and can be differentiated into classically activated (M1) and alternatively activated (M2) counterparts. When challenged by adenosine through the A2A and A2B receptors, macrophages undergo alternative activation into the M2 phenotype. Consistent with these observations, we found that macrophages isolated from infarcted hearts of CD73 null mice were characterized by a higher expression of M1 genes (TNF-α, IL-1β, IL-6),
Figure 6. Infarct expansion, remote ventricular fibrosis, and immature replacement scar in CD73−/− hearts 28 days after ischemia and reperfusion (I/R). Representative micrographs of sirius red-stained hearts are given on the left and planimetric analysis on the right. A, Infarct expansion as measured by expansion index (left ventricular cavity area/total left ventricular area)×(septum thickness/scar thickness). B, Peri-infarct fibrosis next to replacement scar as percentage of the analyzed region of interest (ROI). C, Remote fibrosis as percentage of the analyzed ROI. D, Ratio of collagen subtypes I and III within scar area as measured by polarized light microscopy (yellow=collagen I, green=collagen III); Scale bars, (A) 500 µm and (B–D) 100 µm. Values are mean±SD of n=5 (CD73−/−) and n=6 (WT) experiments; *P<0.05. CD73 indicates ecto-5′-nucleotidase.
whereas the expression of M2 genes (arginase-1, IL-10, TGF-β) was decreased (Figure 5). Our flow cytometry data also support the proposed macrophage polarization: macrophages isolated from CD73−/− mutants expressed a higher fraction of F4/80low, indicative for a more immature and inflammatory subtype. In addition, mice lacking CD73 were characterized by the increased appearance of macrophage precursors, expressing Ly6Chigh. Monocyte imbalance toward inflammatory Ly6Chigh-expressing monocytes has already been associated with adverse myocardial healing and ventricular dilatation after MI in both experimental models and clinical settings. Consistent with the observed macrophage M1-driven phenotype of CD73−/− mice, tissue levels of the proinflammatory cytokines, such as TNF-α, were increased and IL-10 was diminished. A direct suppressive effect of adenosine on TNF-α production in monocytes and macrophages has been shown in both human and murine studies. In summary, CD73-derived adenosine seems to regulate the macrophage phenotype importantly in vivo, in that it normally suppresses inflammation through feedback inhibition by adenosine at the cellular level. The TNF-α-rich environment produced by the M1 phenotype switch is most likely responsible for the persistent appearance of granulocytes in CD73−/− hearts, because TNF-α is known to upregulate chemokines for granulocyte influx and promotes granulocyte survival.

Besides macrophages, CD4+ T cells were recently reported to become activated after MI and to facilitate wound healing of the myocardium. Regulatory T cells express CD39, as well as CD73, and adenosine generated by this pathway mediates immune suppression. In this study, we found cytotoxic and helper T cells to be elevated in CD73−/− mice, whereas the number of Tregs tended to be generally lower. This balance of cells was reflected by the cytokine pattern because we found reduced anti-inflammatory IL-10 and TGF-β levels in the heart of CD73−/− mutant 2 weeks after I/R. These results again emphasize the important modulatory role of adenosine. However, it still remains to be investigated to what extent CD73 on T cells influences cardiac remodeling given the high enzymatic activity present on granulocytes within the injured heart.

Current therapeutic strategies in myocardial fibrosis after infarction aim to inhibit proinflammatory cytokine activation. Within this context, TGF-β is considered as the master switch for the transition of the infarct from the inflammatory phase to scar formation. It was, therefore, highly surprising that in CD73 mutant mice we found significantly increased adjacent and remote fibrosis, although the levels of TGF-β were reduced by ≈50%. This effect was associated with a pronounced decrease in the ratio of collagen I/III in the replacement scar. The cardiac extracellular collagen matrix consists

Figure 7. Reconstitution of ecto-5′-nucleotidase (CD73) on immune cells in CD73−/− mice by bone marrow transplantation recovered the depressed cardiac phenotype in CD73−/− mutants. Wild-type (WT) and CD73−/− bone marrow was transplanted in WT and CD73−/− mice and underwent the experimental protocol given in Figure 1. A, Necrotic area measured by late gadolinium enhancement (LGE) in percentage of left ventricular myocardium (LVM). B, Global ejection fraction of bone marrow–transplanted WT and CD73−/− hearts 1 and 14 days after I/R. C, Ventricular volumes of WT and CD73−/− mice 14 days after ischemia and reperfusion (I/R). Cardiac phenotyping was performed using MRI. Values are mean±SD of n=6 (WT+WT), n=4 (CD73−/−+CD73−/−), and n=6 (CD73−/−+WT) experiments; *P<0.05. EDV indicates end-diastolic volume; ESV, end-systolic volume; and SV, stroke volume.
of >80% collagen. In the border zone of the infarct area, myofibroblasts start de novo synthesis of collagen between day 2 and day 3 after MI. Early collagen type III de novo synthesis is followed by collagen type I deposition, which contributes tensile strength to the infarcted tissue. Type I and III collagen have different physical properties, and a decrease of type I/III ratio is indicative of immature scar formation and may have a deleterious impact on myocardial compliance. It has been reported that adenosine inhibits collagen and protein synthesis in cardiac fibroblasts through A2B receptors. Very recently cultivated cardiac fibroblasts were identified to release ATP that activates profibrotic P2Y2 receptors. Our in vivo data clearly indicate that development of cardiac fibrosis after reperfusion injury is dominated by an adenosinergic P1 environment.

As to purinergic signaling during cardiac I/R, we hypothesize that 2 phases must be differentiated. The initial phase immediately after ischemia-induced cell death is characterized by massive ATP release from dying cardiomyocytes. In this phase, before immune cell infiltration, the only cell type within the heart, which can break down ATP to the level of adenosine, is coronary endothelial cells. Thus, ATP released by necrotic or apoptotic cardiomyocytes will preferentially activate P2 receptors causing additional cell death, also functioning as paracrine find-me signal promoting phagocytic clearance of cellular debris. CD73-derived adenosine does not seem to play a role in this initial phase (1 day after I/R), because the ischemia-induced changes in global ejection fraction (Figure 2) and immune cell infiltration into the injured heart (Figure 4) were not different between CD73−/− mice and WT controls. Only after day 7 after I/R, the mutant mice showed a progressive deterioration of cardiac function, which was associated with a continuous increase in the number of APCs of T cells (cytotoxic T cells, helper T cells), as well as inflammatory monocytes and granulocytes 2 weeks after infarction. This second phase seems to be adenosine dominated. Figure 8 schematically summarizes our view of the second phase of purinergic signaling, when granulocytes, as well as T cells, have migrated into the injured heart concomitantly upregulating their CD73. Quantitatively, the most important immune cell fraction migrating into the heart after I/R are granulocytes that express significantly upregulated CD39 and CD73 and release ATP by themselves when activated. Adenosine formed by this upregulated ectonucleotidase pathway can inhibit—in an autacoid fashion—the oxidative burst, chemotaxis, and transmigration of granulocytes. Similarly, we found upregulated CD73 on T cells recovered from the infarcted heart, as well as in CD3/CD28-stimulated T cells, which also have been reported to release ATP. Therefore, both granulocytes and T cells have the ability to release ATP that is degraded to adenosine by an upregulated ectonucleotidase cascade. Quite remarkable is the observation that cardiac APCs and cardiac fibroblasts lack CD73 and, therefore, require granulocytes and T cells for further degradation to adenosine. Thus, adenosinergic signaling on granulocytes and T cells is autocrine, whereas it acts in a paracrine fashion on APCs and cardiac fibroblast in the heart (Figure 8). The functional implications of this interesting metabolic compartmentation are presently not known.

The ARs involved in mediating the actions of adenosine on the individual immune cell subsets within the inflamed myocardium are difficult to discern at present. On granulocytes, most likely all the 4 ARs are involved in mediating the different functional effects of adenosine, whereas the anti-inflammatory action of adenosine in T cells importantly involves the A2A receptor that can be activated by a nonvasoactive A2AR agonist. On APCs and cardiac fibroblasts, the A2B receptor seems to play a dominant role. A common denominator of adenosine action seems to be nuclear factor-κB which is tonically suppressed by adenosine formed by T cells and by vascular endothelium.
inflammatory response (M2 phenotype) and limits the development of fibrosis involving IL-10, interferon-γ, and TGF-β. Thus, adenosine formed by CD73 must be considered as an important regulator in the remodeling process, which suppresses immune cell activation by a concerted action on various immune cell subsets, thereby limiting the inflammatory response and the development of fibrosis. Overexpression of CD73 on immune cells, as well as direct interfering with AR signaling, might be a promising strategy to promote cardiac healing after MI.

Acknowledgments

We thank Bodo Steckel, Barbara Emde, Sabine Bongardt, Stefanie Becker, and Jutta Ziemann for excellent technical support.

Sources of Funding

This study was supported by Sonderforschungsbereich 612-B6.

Disclosures

None.

References


What Is Known?

- The ectoenzyme ecto-5'-nucleotidase (CD73) is the rate-limiting step that promotes a shift from an ATP-driven proinflammatory to an anti-inflammatory milieu induced by adenosine.

What New Information Does This Article Contribute?

- Deletion of CD73 results in more severe decline in cardiac function after infarction and is associated with the persistence of immune cells in mouse heart.
- Impaired healing in CD73-null heart involves M1-driven response with a suppression of anti-inflammatory mediators.
- CD73-derived adenosine on immune cells limits local inflammation after myocardial infarction.

A major finding of this study is that adenosine formed by the ectoenzyme CD73 on infiltrating immune cells is quantitatively sufficient to promote myocardial healing after infarction. If CD73 is lacking, cardiac function after myocardial infarction further deteriorates with systolic failure and enhanced edema formation followed by infarct expansion accompanied by an immature replacement scar and diffuse ventricular fibrosis. This cardiac phenotype was caused by a delayed clearance of cardiac immune cell subsets and a TH1- and M1-driven immune response. Most likely, CD73 present on granulocytes and T cells is the major source for the formation of anti-inflammatory adenosine. Our study demonstrates the crucial role of purinergic signaling on immune cells during cardiac healing. Future delineation of the adenosine receptor subtypes on immune cells responsible for the anti-inflammatory action of endogenously formed adenosine will permit the development of novel anti-inflammatory strategies for treating adverse cardiac remodeling.
Ecto-5′-Nucleotidase on Immune Cells Protects From Adverse Cardiac Remodeling
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Circ Res. 2013;113:301-312; originally published online May 29, 2013;
doi: 10.1161/CIRCRESAHA.113.300180

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/113/3/301

Data Supplement (unedited) at:
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Supplemental Material

Reperfused myocardial infarction
The mice (C57BL/6, 18-23 g body weight, 8-12 weeks of age) used in this study were bred at the Tierversuchsanlage of Heinrich-Heine-Universität, Duesseldorf, Germany. They were fed with a standard chow diet and received tap water ad libitum. Mice were briefly sedated with isofluorane, thereafter rapidly intubated and anesthetized by mechanical ventilation with isoflurane (1.5%) at a rate of 150 strokes/min and a body weight- adapted tidal volume between 225–275 ml. Each animal was placed on a warming plate in a supine position, the chest wall was shaved, and electrocardiogram (ECG) needles were inserted subcutaneously representing lead II to measure ST-segment elevations during myocardial infarction. All operations were performed under an upright dissecting microscope (Leica MS05). The chest was opened with a lateral cut along the left side of the sternum. Subsequently, the pericardium was gently dissected to allow visualization of coronary artery anatomy. The LAD was ligated by a suture (8-0 polypropylene) that was threaded through a small piece of plastic tube (PE-10 tubing) with blunt edges, and two small weights (~1 g) were attached to each end resulting in a hanging weight system. LAD occlusion was assured by ST-Segment elevation in ECG recordings and was terminated at once after 50 min, when the weights were removed. Sham animals were operated the same way without LAD occlusion. Successful reperfusion was assured by the reddening of the previous pale myocardium and termination of ST-elevation. After reperfusion the chest was closed in layers with a 6-0 polypropylene suture and skin wound was treated with Betaisodona for disinfection.

Bone marrow transplantation
Mice subjected to bone marrow transplantsations were 8 week old and were lethally irradiated with 2 times 10 minutes of 8 Gray radiation from an X-ray source 1 day before transplantation. Heparinized male mice (C57BL/6, 18-23 g body weight, 8-12 weeks of age) were sacrificed by cervical dislocation and bone marrow from carefully dissected and opened femur and tibia was flushed out by PBS injection with a syringe. The solution was carefully rinsed through a 70 µm mesh filter and centrifuged with 700 x g for 7 minutes. The supernatant was aspirated and 1 ml RBC lysing buffer was added to the pellet and gently mixed for 1 min. To dilute the lysing buffer, 20 ml PBS were added and centrifuged at 700 g for 7 min, and then the supernatant was discarded. The pellet was resuspended in complete RPMI medium. The number of cells was counted using hematocytometer and 1–2×10^7 cells in 0.5 ml of PBS were injected into recipients intravenously via the tail vein. Successful bone marrow transplantation was verified by FISH (98% success). Until 3 weeks after this procedure mice were given water ad lirbitum with 40 µl/ml of enofloxacine and 80µl/ml vancomycine. Mice were allowed to recover for one week from antibiotic treatment, thereafter all healthy mice were subjected to the respective protocols.

MRI experiments
MRI data were recorded on a Bruker AVANCE III 9.4 T Wide Bore NMR spectrometer (Bruker, Rheinstetten, Germany) operating at frequencies of 400.13 MHz for ^1^H and 376.46 MHz for ^19^F measurements. Mice were anesthetized with 1.5% isoflurane and kept at 37°C as was previously published. For functional cardiac analysis, ^1^H images of murine hearts were acquired with an ECG- and respiratory-triggered gradient echo cine sequence. For calculation of the ejection fraction (EF), contiguous short axis slices covering the complete long axis of the heart were analyzed in end-systole and end-diastole, respectively. For late gadolinium enhancement (LGE) imaging, a PE-10 tube was inserted and fixed in the peritoneal cavity in order to realize contrast agent (CA) application (0.1 mmol Gd-DTPA per kg BW) at a defined time relative to acquisition of the images to be analyzed. A gradient echo sequence with the following parameters was used: echo time (TE) = 2.1 ms, repetition time (TR) = 25 ms, acquisition time (TA) = 1 min (depending on heart and respiration rates), slice thickness (ST) = 1 mm, field of view (FOV) = 3 x 3 cm², matrix size (MS) = 128 x 128. Quantification of ventricular volumes and LGE-positive areas were manually performed with the Bruker Paravision
region of interest (ROI) tool. Serving as a validation study, 8 mice underwent I/R as described above and LGE measurement was performed 1 day thereafter followed by a histological infarct quantification with triphenyl tetrazolium chloride (TTC). The results are presented in Figure I. LGE measurements were used to quantify the area of necrosis and to exclude animals with small subendocardial and big transmural infarcts. T2 maps were calculated from images recorded with a gated multi echo sequence (16 echos, separated by TE = 5 ms, TR = 500 ms, TA = 2 min, ST = 1 mm, FOV = 3 x 3 cm², MS = 128 x 128). During each expiration phase only one early ECG trigger was used in order to provide constant initial conditions before recording of the echo train. To be robust against motion artefacts induced by contraction of the heart, a threshold was defined to assure the presence of tissue oedema. For this, a threshold of 28 ms was chosen which is considerably higher than the average value of 20 ± 2 ms under basal conditions. T2 map calculation and quantification of oedemas were realized by an inhouse developed software tool based on the LabVIEW (National Instruments, Austin, TX) environment, which calculated the area of pixels exceeding the T2 threshold and expressed them as percentage of the whole LVM. ¹⁹F images were recorded using a non-gated multislice turbo spin echo sequence (8 slices, turbo factor = 64, effective TE = 104.4 ms, TR = 4.5 s, ST = 2 mm, FOV = 3 x 3 cm², MS = 64 x 64, NS = 256, TA = 19.2 min). For overlays with ¹⁹F images additional ¹H datasets with an identical ST of 2 mm were recorded. One day before measurements, 500 µl of PFC emulsion (Perfluoro-15-crown-ether) was intravenously injected to label circulating monocytes¹. A detailed scheme of the experimental protocols applied to the different groups is shown in the manuscript Figure I and representative fluorine images are given in online Figure II.

Isolation of cardiac immune cells for flow cytometry and real time polymerase chain reaction (RT-PCR)

Mice (C57/Bl-6) were anesthetized with pentobarbital (1mg/Kg BW) and anticoagulated with heparine (100 IE) whereafter hearts were rapidly explanted and prepared in pre-cooled buffer. Aortas were cannulated and perfused (perfusion pressure of 80 mmHg, 37°C) in a retrograde manner with an oxygenated buffer containing 4 mM NaHCO₃, 10 mM HEPES, 30 mM 2,3-butanedion-monoxime, 11 mM Glucose, 0.3 mM EGTA, 6.6 mM NaCl, 0.22 mM KCl, and 0.1 mM MgCl₂H₂O for 5 minutes according to the Langendorff procedure. Thereafter, oxygenated collagenase II (1050 U/ml, BioChrom AG, Berling, Germany) was delivered for 35 minutes intracoronarily at 37°C for controlled tissue digestion. Subsequently, treated tissue was weighted, mechanically dissociated with a sterile scalpel and a defined pipeting/meshing (100 µm)-scheme. The separation of cardiac tissue in cardiomyocytes and non-cardiomyocytes (endothelial cells, fibroblasts and immune cells) was established by a low g centrifugation in a highly viscous albumin-buffer. In case of rtPCR experiments the procedure was modified as follows: A broad range collagenase solution (NB 8, Serva) with collagenase activity of 1mg/ml was used and the non-cardiomyocyte supernatant was transferred to a cell sorter (MoFlo XDP, Beckmann-Coulter). Here, CD11b+CD11c+ cells were directly sorted in RNAlater solution (Quiagen).

RT-PCR experiments

After FACS sorting, macrophages were directly isolated in RLT buffer (Qiagen) and immediately stored at −20 °C. Total RNA was isolated according to the manufacturer’s protocols using RNeasy Micro Kit (Quiagen). QuantiTect Whole Transcriptome Kit (Qiagen) was used for preamplification and reverse transcription. Quantitative real-time PCR was performed by use of FAST qPCR MasterMix (Eurogentec). Primers and probes were obtained as TaqMan Gene Expression Assay sets (Applied Biosystems) against the following target genes: TNF-α, IL-1β, IL-6, Arginase 1, IL-10 and TGF-β. Experiments were performed on an Applied Biosystems Step One Plus system. Cycling conditions were: 10 min at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each sample was analysed in triplicate. mRNA expression was normalized to RPLP0 as housekeeping gene (Manuscript Figure 5).
Flow Cytometry

After myocardial immune cell isolation as described in the manuscript cells were resuspended in MACS buffer, preincubated with FcR Blocking Reagent (Miltenyi Biotech) and stained with the following antibodies: anti-CD45-PE, 30-F11 (BD Bioscience), -CD11b-APC, M1/70 (BD Bioscience), -CD8a-APC-H7, 53-6.7 (BD Bioscience), -CD25-PE-Cy7, PC61 (BD Bioscience), -Ly-6CFITC, AL-21 (BD Bioscience), TER-119-PE, TER119 (BD Bioscience), -CD45-PE, 30-F11 (Miltenyi Biotech), -CD11b-APC, M1/70.15 (Miltenyi Biotech), -CD3-APC, 145-2C11 (Miltenyi Biotech), CD49b-APC, DX5 (Miltenyi Biotech), NKp46-APC, 29A1.4 (Miltenyi Biotech), -CD39-PE-Cy7, 24DMS1 (eBioscience), -CD4-PerCP-Cy5.5, RM4-5 (eBioscience), -Ly-6G (Gr-1)-PerCP-Cy5.5, RB6-8C5 (eBioscience), -CD45R(B220)-APC-eFluor780, RA3-6B2 (eBioscience), -F4/80-APC-eFluor780, BM8 (eBioscience), -CD11c-PE-Cy7, N418 (eBioscience), -CD73-FITC, 496406 (R&D Systems), -CD39-FITC, 495826 (R&D Systems), -CD3-APC, 390 (Biolegend), FoxP3-PE, MF23 (BD Bioscience), Ly6C-APC-Cy7, AL-21 (BD Bioscience), MHCII-FITC, M5/114.15.2 (Miltenyi Biotech). A representative gating strategy is shown in Online Figure III.

To identify the individual subsets of the total pool of CD45+ immune cells, we used a panel of antibodies against different cell-specific leukocyte markers: cytotoxic T cells (CD3+, CD8+), T-helper cells (CD3+, CD4+, CD49b, FoxP3+), NK cells (CD49b, NKp46+), granulocytes (CD11b+, Ly6G+), monocytes (CD11b+, Ly6G, CD11c, F4/80, Ly6Clow/high) and myeloid antigen-presenting cells (APCs; CD11b+, Ly6G, CD11c+, F4/80+, MHCI-). For intracellular staining of Foxp3, we used the Foxp3-Staining Buffer Set (eBioscience) according to the manufacturer’s protocol. After 5 min of incubation at room temperature, cells were washed and resuspended in 200 ml MACS buffer for flow cytometry. For analysis, the placement of gates was based on fluorescence minus one (FMO) controls. The minimum number of events used to define a cell population was 150. Mean fluorescence intensity (MFI) calculations for antigen expression were conducted by subtracting FMO control from MFI. Analysis was always performed on individual hearts. Data were processed and analysed with FACSDiva (BD Bioscience, Heidelberg) and presented with Kaluza 1.2 (Beckman Coulter) software.

Light Microscopy and Polarized Light Microscopy (PLM)

Myocardial expansion index was calculated according to: Expansion Index = (Left Ventricular Cavity Area/Total Left Ventricular Area) × (Septum Thickness/Scar Thickness). Polarized light microscopy (PLM) was performed using an Olympus BX polarizer to facilitate visualization of interstitial collagen and collagen type discrimination. Calculation of area fractions was done using Image J software (1.44, NIH) and the plugin “color deconvolution” for separation of different collagen subtypes. The analysed regions of interest (ROIs) were defined as follows: Adjacent fibrosis was defined as interstitial fibrosis in areas epicardial or directly lateral to the replacement scar. Remote fibrosis was defined as interstitial fibrosis in the cardiac septum without having any contact to the replacement scar of the antero-lateral wall.

Langendorff Experiments

The experiments according to the Langendorff technique were conducted with modifications according to a previously published protocol. In brief, mice were anesthetized with pentobarbital (1mg/Kg BW) and anticoagulated with heparine (100IE) whereafter hearts were explanted and prepared in pre-cooled modified Krebs buffer (containing (mM): 119 NaCl, 4.56 KCl, 1.2 MgSO4, 1.18 KH2PO4, 24.9 NaHCO3, 8.27 glucose, 2.0 pyruvate, 0.65 EDTA and 3.0 CaCl2; bubbled through with carbogen (95% O2 and 5% CO2 at ambient pressure) under a dissecting microscope. The ascending aorta was cannulated with a stainless steel tubing (1mm o.d.) and perfused with modified Krebs buffer at 37°C in a retrograde manner with perfusion pressure at 1 meter H2O. Steady state values of coronary flow were reached after ~ 25 minutes. Coronary venous effluates were collected on ice in periods of 2 minutes continuously and frozen at -80° afterwards. Effluates, collected for two minutes, were enriched for nucleosides by solid phase extraction (SPE) with HLB 150mg cartridges (OASIS).
High Performance Liquid Chromatography (HPLC)
The samples were analyzed using a WATERS HPLC system equipped with an analytical Hypersil BDS column (4.6x150cm, Thermo Scientific, Waltham, USA). The HPLC system consists of a degaser, W600 pump, W717 injector and a 996 PDA detector. Samples were filtered through 0.2 mm nylon filters and kept at 4°C. The column was equilibrated at ambient temperature with Buffer A (150 mM KCl/150 mM KH$_2$PO$_4$ at pH 6) and a constant flow of 0.9 ml/min. Buffer B was a 15% (v/v) solution of acetonitrile in buffer A. The composition of the mobile phase was controlled by a low-pressure gradient mixing device. The amount of buffer B was changed linearly between the following time points: 0 min, 0% B; 0.1 min, 3% B; 3.5 min, 9% B; 5 min, 100% B; 7 min, 100% B; 7.1 min, 0% B. The reequilibration time was 4.9 min resulting in a cycle time of 12 min between injections. The wavelength scanned was 254 nm. Identification and quantification of adenosine peaks was done offline by comparison to retention times of known standards and peak integration and normalization. The nucleotide measurements of 3 continuously collected effluates in the steady-state were averaged.
Online Figure I. Validation of late gadolinium enhancement at 9.4 Tesla

8 WT mice were subjected to ischemia (50 min) and reperfusion (I/R). MRI measurement was conducted 1 day thereafter. Images were acquired 15 minutes after i.p. application of 0.1 mmol/Kg BW Gadolinium DTPA. Mice were sacrificed thereafter, hearts explanted, sliced (4 slices each) and stained with Triphenyltetrazoliumchloride (TTC). TTC+ and LGE+ areas are expressed as percentage of left ventricular muscle (LVM). The data are correlated with $R^2=0.81$. 
Online Figure II. CD73−/− mutants show enhanced monocyte infiltration during myocardial healing as assessed in vivo by 19F-MRI.

Images were acquired 7 days after I/R, while perfluorocarbon (PFC) injection was done the day before (24 h time of monocyte accumulation). (A) Examples of merged fluorine (colour: hot iron) and proton images in WT and CD73−/− mice. Due to surgery trauma there is also accumulation of fluorine signal in the thoracic wall. (B) Total midwall fluorine intensity normalized to LVM of WT and CD73−/− hearts (24 hours after injection of PFC at day 7 after I/R). Values are means ± SD of n=4 (WT) and n=4 (CD73−/−); * p<0.05.
Online Figure III. Gating strategy of isolated cardiac immune cells. After isolation from cardiac tissue, non-cardiomyocytes were immediately stained and prepared for flow cytometry. Having identified all leukocytes as CD45 positive cells, dead cells were excluded by DAPI staining. Thereafter, cell fractions were identified by a specific marker combination (e.g. CD45⁺ CD11b⁺ CD11c⁺ MHCII⁺ F480 incompetent = APCs) as indicated in the text.
Reference List


