CD73 on Immune Cells Protects from Adverse Cardiac Remodeling

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ABSTRACT

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

Objective: Here we investigated the role of CD73 in post infarction inflammation, cardiac repair and remodelling in mice after reperfused myocardial infarction (50 min ischemia).

Methods and Results: We found that compared to control mice a) cardiac function in CD73−/− mice more severely declined after infarction (systolic failure with enhanced myocardial oedema formation) as determined by MRI and was associated with the persistence of cardiac immune cell subsets, b) cardiac adenosine release was augmented 7 days after I/R in control mice but reduced by 90% in CD73 mutants, c) impaired healing involves M1-driven immune response with increased TNF-α and IL-17 as well as decreased TGF-β and IL-10, d) 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 I/R demonstrate the crucial role of purinergic signaling during cardiac healing and provide groundwork for novel anti-inflammatory strategies in treating adverse cardiac remodeling.

Keywords: Adenosine, CD73, myocardial infarction, immune system, inflammation

Nonstandard Abbreviations and Acronyms:

- DAMPs: damage-associated molecular patterns
- ENTPDases: ectonucleoside triphosphate diphosphohydrolases
- CD73: ecto-5′-nucleotidase
- AR: adenosine A1, A2A, A2B and A3 receptors
- APCs: myeloic antigen-presenting cells
- LGE: late gadolinium enhancement
- I/R: ischemia and reperfusion
- LVM: left ventricular myocardium
- MFI: mean fluorescence intensity
- CPI: circularly polarised illumination microscopy
- BMT: bone marrow transplantation
- PFC: perfluorocarbons
- RT-PCR: real time polymerase chain reaction

INTRODUCTION

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

The cardiac repair mechanisms initiated directly after myocardial infarction are considered as an inflammatory condition which ideally leads to a rapid elimination of the injurious stimuli and initiation of myocardial healing. The recruitment of immune cells depends on the release of damage-associated molecular patterns (DAMPs) which are recognized by receptors on leukocytes and
subsequently stimulate the production of proinflammatory cytokines. In addition, fragments of the extracellular matrix and complement factors trigger the production of proinflammatory cytokines. These mediators then act on local vascular endothelium, resulting in an increased permeability with subsequent invasion of inflammatory cells such as neutrophils and monocytes followed by T-cells.

A hallmark of ischemic cells is the early release of ATP, normally present within cardiomyocytes in millimolar concentrations, into the extracellular space which then acts as endogenous danger signal. ATP is also well recognized as a “find me signal” that guides phagocytes to inflammatory sites, promotes clearance of damaged and apoptotic cells, and acts through specific ATP receptors (P2). ATP can also be directly released from immune cells upon activation. In addition, ATP released from neutrophils promotes recognition of chemotactic gradients that guides these cells to infected and inflamed tissue. 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 I/R has not been explored in detail.

The half-life of extracellular ATP is critically determined by the activity of ectoenzymes such as various ectonucleoside triphosphate diphosphohydrolases (ENTPDases) including CD39 which hydrolyzes ATP to ADP and AMP. 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. Four distinct subtypes of P1 receptors have been identified: adenosine A1, A2A, A2B and A3 receptors (AR) which 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 pro- and antiinflammatory responses. Conceptually it is important to recognize that it is the activity of the ecto-nucleotide 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⁺) which express high levels of CD39 but lack CD73. We also observed that three 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 2/3 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. The present 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 min) and reperfusion. Functional analysis by MRI and kinetics of immune cell infiltration for 3 weeks post 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 the Landesamt für Natur-, Umwelt- und Verbraucherschutz (LANUV). Reconstitution of CD73 on leukocytes in CD73-/- mutants (Chimaera mice) was realized by bone marrow transplantation (BMT). For technical details see Online Data Supplement. Reperfused myocardial infarction 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 \(^1\text{H}\) and 376.46 MHz for \(^{19}\text{F}\) measurements. For a detailed description of the MRI setup, acquisition parameters and quantification procedures please see references\(^{16}\) 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\(^{15}\). For details of the isolation protocol and modifications due to post-isolation quantitative real time PCR, please see the Online Data Supplement. Flow cytometry experiments were performed with a FACS CANTO II (Becton, Dickinson) equipped with 3 lasers and capable of detecting up to 8 colours. For detailed antibody staining protocol and gating strategy, see reference\(^{15}\) and Online Data Supplement.

Isolation of macrophages for real time polymerase chain reaction (RT-PCR).
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 (PLM).
Explanted mouse hearts were fixed in Tissue-Tek (Sakura Finetec Inc., U.S.A.) and frozen at -20°C. Hearts were cut into 7 µm sections from apex to base. Staining was performed with H.E. 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 PLM 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, USA). Total protein concentration was determined by a BCA protein assay (Pierce). Cytokine levels of IL-6, IL-17, IL-10 and TNF-\(\alpha\) were detected with magnetic Bio-Plex pro mouse assays using a Bio-Plex 200 System. TGF-\(\beta\) was detected with a quantakine ELISA kit (R&D Systems).

Langendorff Experiments and High Performance Liquid Chromatography (HPLC).
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 analysed with HPLC for adenosine. For details please see the Online Data Supplement.

Statistics.
Unless otherwise stated, data are presented as mean values ± standard deviation (SD). Data were statistically analysed by Mann-Whitney U Test or the Student’s \(t\)-test.

RESULTS

Cardiac function deteriorates in CD73\(^{-/-}\) mice after I/R accompanied by a sustained myocardial oedema.

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 ischemia reperfusion (I/R)\(^{15}\). 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 oedema were analysed by MRI at days 1, 7, 14, and 27 post MI (Figure 1). The size of necrosis was assessed by contrast enhanced MRI (late gadolinium enhancement, LGE) one day after I/R. LGE correlated well with TTC staining in a separate experimental series (Online Data Supplement).
Supplement Figure I). As shown in Figure 2A, the extent of cardiac necrosis of the left ventricular myocardium (LVM) was not different between WT and CD73−/− mice subjected to 50 min ischemia followed by reperfusion. This is in line with the observation that global ejection fraction (EF) was equally reduced in both genotypes one day after I/R (Figure 2B). Thereafter, however, EF continuously worsened in CD73−/− mice over three weeks, while the EF of WT controls tended to recover (day 28 after I/R: CD73−/− 38.42 ± 7.19%; WT 53.41 ± 2.61%; p<0.01). Figure 2C displays representative end-diastolic and end-systolic images of the two genotypes, and detailed volumetric analyses of the hearts 14 and 28 days after I/R are summarized in Figure 2D and E. As can be seen, 14 days after I/R end-systolic but not end-diastolic volumes were significantly increased in CD73−/− mice resulting in a substantial reduction in stroke volume and cardiac output (Figure 2D). In fact, at a later stage of myocardial healing (day 28), end-diastolic volumes were additionally increased (Figure 2E) indicating unfavourable ventricular remodeling. Overall survival was not significantly different between the genotypes (WT: 86% vs. 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 edema formation and in the acute setting this is equivalent to the myocardial area at risk17. 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 while the respective values in CD73−/− mutants even 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 findings18. 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 to 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.**

Since the lack of CD73-derived adenosine was associated by myocardial edema formation, we used 19F-MRI and FACS to investigate whether this phenomenon is associated with enhanced cellular inflammation. Using 19F MRI, which noninvasively detects monocyte invasion into inflamed tissues in vivo16, 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 FACS 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 Data Supplement Figure III. As shown in Figure 4A, the number of myeloid antigen presenting cells (APCs: CD45+, CD11b+, CD11c+, MHCII+, F4/80high/low) - 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 x 10^3 in WT hearts and 5.36 ± 1.25 x 10^3 APCs / mg heart tissue in CD73−/− hearts, p<0.05. To evaluate the macrophage activation state, we analysed the expression of F4/80 on APCs, since down-regulation of the F4/80 antigen is associated with macrophage activation by IFN-γ and a M1-driven phenotype19. 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 (MFI) of F4/80 positive macrophages tended to be decreased as well (Figure 4C). Additionally, the CD73−/− monocyte population (CD45+, CD11b+, CD11c+, Ly6G+, Ly6Chi/lo), serving as macrophage precursors, was dominated by Ly6Chi expressing cells even 14 days after I/R (Figure 4D), while WT mice monocytes were dominated by Ly6C−-expressing counterparts.
FACS analysis further revealed that the total amount of leukocytes (CD45+) was 3-fold higher at day 14 after I/R in CD73−/− mutant as compared to WT hearts (Figure 4E: WT: 2.91 ± 1.18 x 10^3; CD73−/−: 9.36 ± 3.71 x 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 about 90-fold on day 3 after I/R, but failed to disappear at 14 days after I/R in CD73−/− hearts (Figure 4F). Data on the abundance of T-lymphocyte subsets are summarized in Figure 4G-I. As can be seen all T-lymphocytes (CD45+, CD3+) in WT mice increased till day 7 after I/R and decreased afterwards (Figure 4G, H). In contrast, this cell fraction continuously increased in CD73−/− hearts with significantly greater amounts after 14 days compared to WT hearts. While at this time point the number of CD8+ T-cytotoxic cells of CD73−/− hearts was about 6-fold higher compared with WT controls, CD4+ T-helper were more frequent by a factor of 10. Since regulatory T-cells play an important role in extracellular purine metabolism, we additionally analysed the temporal changes in Treg abundance (CD45+, CD3+, CD4+ CD25+, FoxP3+) related to the total CD4+ T-cells. As shown in Fig 4 I, 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 an M1-driven phenotype, we have measured the expression of representative M1 (TNF-α, IL-1b, IL-6) and M2 genes (Arginase-1, IL-10, TGF-β) in FACS-sorted cardiac APCs with quantitative RT-PCR. Analysis revealed that CD73−/− macrophages compared to WT counterparts in general show increased expression of genes related to the M1 phenotype (Figure 5A) and a decrease of genes characteristic for the M2 phenotype (Figure 5B). While all genes attributed to a particular phenotype were collectively changing into 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 pro-inflammatory cytokines TNF-α and IL-17 and significantly reduced levels of TGF-β on day 7 after I/R, while 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 two points in time but no differences were observed between the two genotypes (data not shown).

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 analysed 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 significant greater ventricular dilatation as compared to 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 the interstitial fibrosis in the remote area to be significantly increased in CD73−/− hearts (Figure 6C). Qualitative analysis of collagen with circularly polarised illumination microscopy (CPI) demonstrated predominantly loosely assembled green fibres (assigned to Collagen III) in CD73−/− hearts compared to orange and more organized fibres (assigned to Collagen I) in WT hearts. Qualitative collagen analysis with CPI revealed that the ratio of collagen I to collagen III was clearly smaller in CD73−/− hearts compared to 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 for proper cardiac healing.

To discriminate whether the observed effects on post I/R inflammatory state and scar formation are due to 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 to female mice and the effectiveness of transplantation was controlled by fluorescence in situ hybridisation (FISH). 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-/- (Figure 7B) was comparable to data shown in Figure 2B. Transplantation of WT bone marrow to CD73-/- mice fully restored ventricular function to WT controls two weeks after I/R (CD73-/- → CD73-/- 38.65 ± 7.1%; 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 increased enddiastolic and endsystolic volumes. Survival of CD73-/- mice receiving CD73-/- bone marrow and subjected to I/R was only 33% as compared to 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 ischemia and reperfusion (I/R). Lack of CD73-derived adenosine (i) prevented the timely resolution of inflammation, (ii) showed enhanced cardiac oedema formation and contractile dysfunction (iii), and 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 extracellular 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 ecto-nucleoside triphosphate diphosphohydrolase (CD39) followed by CD73. While 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 was significantly upregulated three 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 about 90% of total cardiac enzyme activity while after I/R, leukocyte–derived CD73 amounted to about 2/3 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 about 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 oedema formation. Most likely proinflammatory mediators released from immune cells infiltrating the heart in CD73 null mice were responsible for the development of cardiac oedema. It is well known from studies in lung and bowel disease models, that local inflammation causes local hypoxia with release of HIF-1α, which is solely capable of regulating vascular barrier function by acting on endothelial cells. Besides this, HIF-1α is known to upregulate ectonucleotidases and adenosine receptors thereby sensitising 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 and T-cells in the second wave, which is consistent with data from the literature\(^4\). 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 myocardial infarction and can be differentiated into classically activated (M1) and alternatively activated (M2) counterparts\(^5\). When challenged by adenosine through the A2A and A2B receptors, macrophages undergo alternative activation into the M2 phenotype\(^25\). 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-\(\alpha\), IL-1b, IL-6), while the expression of M2 genes (Arginase-1, IL-10, TGF-\(\beta\)) was decreased (Figure 5). Our flow cytometry data also support the proposed macrophage polarization: macrophages isolated from CD73\(^{-/-}\) mutants expressed a higher fraction of F4/80\(^{\text{low}}\), indicative for a more immature and inflammatory subtype\(^26\). Additionally, mice lacking CD73 were characterized by the increased appearance of macrophage precursors, expressing Ly6C\(^{hi}\). Monocyte imbalance towards inflammatory Ly6C\(^{hi}\)-expressing monocytes has already been associated with adverse myocardial healing and ventricular dilatation after myocardial infarction in both experimental models and in clinical settings\(^27\), \(^28\). Consistent with the observed macrophage M1-driven phenotype of CD73\(^{-/-}\) mice, tissue levels of the proinflammatory cytokines like TNF-\(\alpha\) were increased and IL-10 were diminished. A direct suppressive effect of adenosine on TNF-\(\alpha\) production in monocytes and macrophages has been shown in both human and murine studies\(^24\). In summary, CD73-derived adenosine appears to importantly regulate the macrophage phenotype \(\textit{in vivo}\), in that it normally suppresses inflammation through feedback inhibition by adenosine at the cellular level. The TNF-\(\alpha\) rich environment produced by the M1 phenotype switch is most likely responsible for the persistent appearance of granulocytes in CD73\(^{-/-}\) hearts, since TNF-\(\alpha\) is known to upregulate chemokines for granulocyte influx and promotes granulocyte survival\(^29\).

Besides macrophages, CD4\(^+\) T-cells were recently reported to become activated after MI and to facilitate wound healing of the myocardium\(^30\). Regulatory T-cells express CD39 as well as CD73 and adenosine generated by this pathway mediates immune suppression\(^31\). In the present study we found cytotoxic and helper T-cells to be elevated in CD73\(^{-/-}\) mice while the number of T\(_{\text{regs}}\) tended to be generally lower. This balance of cells was reflected by the cytokine pattern since we found reduced anti-inflammatory IL-10 and TGF-\(\beta\) levels in the heart of CD73\(^{-/-}\) mutant two 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 to 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\(^32\). Within this context, TGF-\(\beta\) is considered as “master switch” for the transition of the infarct from the inflammatory phase to scar formation\(^33\). It was therefore highly surprising that in CD73 mutant mice we found significantly increased adjacent and remote fibrosis, although the levels of TGF-\(\beta\) were reduced by about 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 of more than 80% collagen I\(^34\). In the border zone of the infarct area, myofibroblasts start de novo synthesis of collagen between day 2 and day 3 after myocardial infarction\(^35\). Early collagen type III de novo synthesis is followed by collagen type I deposition, which contributes tensile strength to the infarcted tissue\(^21\). Type I and III collagen have different physical properties, and a decrease of type I/III ratio is indicative of immature scar formation\(^36\) 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\(^37\). Very recently cultivated cardiac fibroblasts were identified to release ATP which activates profibrotic P2Y2 receptors\(^38\). Our \(\textit{in vivo}\) 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 two 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, prior to immune cell infiltration, the only cell type within the heart which can break down ATP to the level of adenosine are coronary endothelial cells\(^1\)\(^,\)\(^2\),\(^15\). Thus, ATP released by necrotic or apoptotic cardiomyocytes will preferentially activate P2 receptors causing additional cell death\(^3\)\(^9\) also functioning as paracrine “find me” signal promoting phagocytic clearance of cellular debris\(^6\). CD73-derived adenosine does not appear to play a role in this initial phase (one day after I/R), since 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 following 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 two weeks after infarction. This second phase appears 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\(^1\)\(^5\). Quantitatively the most important immune cell fraction migrating into the heart after I/R are granulocytes expressing significantly upregulated CD39 and CD73\(^1\)\(^5\) and release ATP by themselves when activated\(^1\). Adenosine formed by this upregulated ectonucleotidase pathway can inhibit in an autacoid fashion the oxidative burst, chemotaxis, and transmigration of granulocytes\(^4\)\(^0\). Similarly, we found upregulated CD73 on T-cells recovered from the infarcted heart\(^1\)\(^5\) as well as in CD3/CD28-stimulated T-cells\(^3\)\(^1\) which also have been reported to release ATP\(^8\). Therefore both granulocytes and T-cells have the ability to release ATP which 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, while 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 adenosine receptors 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 four adenosine receptors are involved in mediating the different functional effects of adenosine\(^4\)\(^1\), whereas the anti-inflammatory action of adenosine in T-cells importantly involves the A2A receptor\(^3\)\(^4\) which can be activated by a non-vasoactive A2AR prodrug\(^4\)\(^2\). T\(_{reg}\) have been shown to additionally express the A3 and A2B receptor\(^3\)\(^1\). On APCs and cardiac fibroblasts the A2B receptor appears to play a dominant role\(^4\)\(^3\). A common denominator of adenosine action appears to be NFkB which is tonically suppressed by adenosine formed by T-cells\(^3\)\(^1\) and by vascular endothelium\(^4\)\(^4\).

In summary, the present study provides first experimental evidence that extracellular adenosine most likely formed by CD73 on granulocytes and T-cells during I/R is a key metabolite initiating the healing process. CD73-derived adenosine reduces the inflammatory response (M2-phenotype) and limits the development of fibrosis involving IL10, IFN-\(\gamma\) and TGF-\(\beta\). 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 adenosine receptor signaling might be a promising strategy to promote cardiac healing after MI.

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DISCLOSURES
The authors have no conflict of interest.
REFERENCES


FIGURE LEGENDS

Figure 1. Scheme of experimental protocol. WT and CD73-/- mice were subjected to ischemia (50 min) and reperfusion (I/R) after baseline assessment of myocardial function and T2 values. Thereafter, mice were randomly selected for characterisation by MRI and structural analysis by histology or assessment of immune cells by flow cytometry.

Figure 2. Depression of cardiac function and persistent oedema after I/R in CD73-/- mice. (A) Necrotic area measured by late gadolinium enhanced MRI (LGE) in percentage of LVM in WT and CD73-/- hearts. Insert shows a typical LGE-image (arrow = necrosis). (B) Global ejection fraction (EF %) of WT and CD73-/- hearts under control conditions (basal) and 1, 7, 14 and 28 days after I/R. (C) Representative MR images of WT and CD73-/- hearts 28 days after I/R in end-systole and end-diastole. (D) Ventricular volumes and cardiac output of WT and CD73-/- mice 14 day after I/R and (E) 28 days after I/R. EDV (end-diastolic volume), ESV (endsystolic volume), SV (stroke volume). Values are means ± SD of n=12 in each genotype; * p<0.05. (F) Results from serial measurement of myocardial oedema expressed area fraction of T2 values exceeding a threshold of 28 ms. Values are means ± SD of n=12 in each genotype, (G) Representative T2 maps of both genotypes at indicated time points. Blue= 20 ± 2 ms; Green= 30 ± 2 ms, Red= 37 ± 2ms.

Figure 3. Release of adenosine from the isolated heart is augmented by I/R, but substantially decreased in hearts lacking CD73 compared to WR controls. Hearts of WT and CD73-/- mice were perfused in the Langendorff mode after sham operation and 7 days after I/R. Coronary effluent perfusate was collected and analysed by HPLC. Values are means ± SD; n=5 in the sham operated group for each genotype; n=4 in the I/R group for each genotype; * p<0.05 between genotypes, + p<0.05 between sham and I/R.

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 analysed ex vivo by flow cytometry in sham-operated (control) WT and CD73-/- mice and 3, 7, and 14 days after 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 (MFI). (D) monocytes (CD45+, CD11b+, CD11c+, Ly6G-) expressing Ly6C^hi as percentage of total monocytes, (E) Total leukocyte numbers (CD45+) per mg heart tissue, (F) total granulocyte numbers per mg heart tissue (CD45^+,CD11b^+, CD11c^-, Ly6G^+) (G) T-cytotoxic lymphocytes (CD45^+, CD3^+, CD8^+) as total number per mg heart tissue, and (H) T-regulatory lymphocytes (CD45^+, CD3^+, CD4^+, CD25^+, Foxp3^+) as percentage of total T-helper lymphocytes. Values are means ± SD of n = 4-6 experiments for each time-point and group; p* < 0.05.

Figure 5. CD73 disruption resulted in the uniform upregulation of M1 genes, downregulation of M2 genes, and in a proinflammatory cardiac cytokine pattern 7 and 14 days after I/R. (A, B) Macrophages were isolated from myocardial tissue 7 days after I/R in WT and CD73-/- mice. After cell sorting and RNA isolation, real time PCR was conducted for typical M1 (TNF-α, IL-1β and IL-6; note different scaling in case of the latter gene) and M2 genes (Arginase-1, IL-10 and TGF-β). (C) Cytokines within myocardial tissue was measured 7 days and 14 days after I/R in WT and CD73-/- mice. IL-6, IL-17, TNF-α, IL-10 were analysed by a Bio Plex assay, TGF-β was measured with ELISA. Values are means ± SEM (A, B) means ± SD (C) of n=4 (WT) and n=4 (CD73-) experiments in each group and time point. p* < 0.05.

Figure 6. Infarct expansion, remote ventricular fibrosis and immature replacement scar in CD73-/- hearts 28 days after 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 analysed region of interest (ROI). (C) Remote fibrosis as percentage of the analysed ROI. (D) Ratio of collagen subtypes I and III within
scar area as measured by polarized light microscopy (PLM; yellow=collagen I, green=collagen III); bars: (A) 500µm, (B, C, D) 100 µm. Values are means ± SD of n = 5 (CD73−/−) and n=6 (WT) experiments; * p< 0.05.

**Figure 7.** Reconstitution of CD73 on immune cells in CD73−/− mice by bone marrow transplantation recovered the depressed cardiac phenotype in CD73−/− mutants. 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 LGE in percentage of 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 day after I/R (EDV=Enddistolic volume, ESV=Endsystolic volume, SV=Stroke volume). Cardiac phenotyping was performed using MRI. Values are means ± SD of n = 6 (WT+WT), n = 4 (CD73−/-CD73−/-), and n = 6 (CD73−/-WT) experiments; * p< 0.05.

**Figure 8.** Schematic outline of suggested extracellular purine metabolism on cardiac immune cells after I/R. In the initial phase of I/R, ATP is released predominantly by apoptotic and necrotic cardiomyocytes, while at a later stage is released from infiltrating immune cells. After I/R, CD39 and CD73 become upregulated on neutrophils and T-cells (see red arrow; data from 15). Since cardiomyocytes, myeloid antigen presenting cells (APCs), and cardiac fibroblasts do not express CD73, formation of adenosine is predominantly performed by neutrophils and T-cells. According to the literature, signaling of adenosine on APCs, cardiac fibroblasts, and T-cells is predominantly through A2A and A2B receptors. On neutrophils all adenosine receptors (AR = A1, A2A, A2B, A3) are likely to be involved in mediating the functional effects. Parameters listed in the cell-associated boxes were based on our measurements or data from the literature. For details see text and citations.
Novelty and Significance

What Is Known?

- The ectoenzyme CD73 is the rate liming step which 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 the present study is that adenosine formed by the ectoenzyme CD73 on infiltrating immune cells is quantitative sufficient to promote myocardial healing after infarction. If CD73 is lacking, cardiac function after MI further deteriorates with systolic failure, enhanced oedema 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.
Figure 1

MRI
functional imaging
T2 Mapping

MRI
functional imaging
T2 Mapping
LGE Imaging

MRI
functional imaging
T2 Mapping
19F Imaging

MRI
functional imaging
T2 Mapping

day -1

day +1

day +3

day +7

day +14

day +28

basal | I/R | acute phase of myocardial healing |

FACS

FACS

FACS

FACS

histology

postacute phase