Basic Res Cardiol (2014) 109:425
DOI 10.1007/s00395-014-0425-x

ORIGINAL CONTRIBUTION

The endocannabinoid-CB2 receptor axis protects the ischemic heart at the early stage of cardiomyopathy

Georg D. Duerr · Jan C. Heinemann · Georg Suchan · Elvis Kolobara · Daniela Wenzel · Caroline Geisen · Michaela Matthey · Kristine Passe-Tietjen · Walid Mahmud · Alexander Ghanem · Klaus Tiemann · Judith Alferink · Sven Burgdorf · Rainer Buchalla · Andreas Zimmer · Beat Lutz · Armin Welz · Bernd K. Fleischmann · Oliver Dewald

Received: 24 April 2014 / Revised: 17 June 2014 / Accepted: 23 June 2014

Abstract Ischemic heart disease is associated with inflammation, interstitial fibrosis and ventricular dysfunction prior to the development of heart failure. Endocannabinoids and the cannabinoid receptor CB2 have been claimed to be involved, but their potential role in cardioprotection is not well understood. We therefore explored the role of the cannabinoid receptor CB2 during the initial phase of ischemic cardiomyopathy development prior to the onset of ventricular dysfunction or infarction. Wild type and CB2-deficient mice underwent daily brief, repetitive ischemia and reperfusion (I/R) episodes leading to ischemic cardiomyopathy. The relevance of the endocannabinoid-CB2 receptor axis was underscored by the finding that CB2 was upregulated in ischemic wild type cardiomyocytes and that anandamide level was transiently increased during I/R. CB2-deficient mice showed an increased rate of apoptosis, irreversible loss of cardiomyocytes and persistent left ventricular dysfunction 60 days after the injury, whereas wild type mice presented neither morphological nor functional defects. These defects were due to lack of cardiomyocyte protection mechanisms, as CB2-deficient hearts were in contrast to controls unable to induce switch in myosin heavy chain isoforms, antioxidative enzymes and chemokine CCL2 during repetitive I/R. In addition, a prolonged inflammatory response and adverse myocardial remodeling were found in CB2-deficient hearts because of postponed activation of the M2a macrophage subpopulation. Therefore, the endocannabinoid-CB2 receptor axis plays a key role in cardioprotection during the initial phase of ischemic cardiomyopathy development.

Keywords Endocannabinoids · Cardioprotection · Cardiomyopathy · Macrophages · Myocardial remodeling

Electronic supplementary material The online version of this article (doi:10.1007/s00395-014-0425-x) contains supplementary material, which is available to authorized users.

G. D. Duerr · J. C. Heinemann · G. Suchan · E. Kolobara · K. Passe-Tietjen · W. Mahmud · A. Welz · O. Dewald (✉)
Department of Cardiac Surgery, University Clinical Centre Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany
e-mail: o.dewald@uni-bonn.de

D. Wenzel · C. Geisen · M. Matthey · B. K. Fleischmann
Institute of Physiology I, Life&Brain Centre, University of Bonn, Bonn, Germany

A. Ghanem · K. Tiemann
Department of Medicine II-Cardiology, University Clinical Centre Bonn, Bonn, Germany

J. Alferink · A. Zimmer
Institute of Molecular Psychiatry, Life&Brain Centre, Bonn, Germany

Present Address:
J. Alferink
Department of Psychiatry, University of Münster, Münster, Germany

S. Burgdorf
Life & Medical Sciences Institute, University of Bonn, Bonn, Germany

R. Buchalla · B. Lutz
Institute of Physiological Chemistry, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

Published online: 01 July 2014

Introduction

Ischemic heart disease and coronary artery disease are associated with progressive and mostly subtle morphological and functional changes in myocardium, which untreated lead to deterioration of left ventricular (LV) function and terminal heart failure even without myocardial infarction. In clinical and experimental studies on ischemic heart disease [9, 16], the presence of macrophage-driven inflammatory activity has been shown to be beneficial for functional recovery after revascularization of human hibernating myocardium [10]. The myocardial hibernation is a condition, where viable myocardium is preserved under poor substrate availability as clinically observed after repetitive ischemic episodes [12]. A substantial number of patients do not show this recovery, thus suggesting a possible low-rate loss of cardiomyocytes resulting in ischemic cardiomyopathy and heart failure. The therapeutic options for these patients are very limited, and to better understand the underlying pathomechanisms we developed a murine model of repetitive, brief myocardial ischemia and reperfusion (I/R). This model showed several pathological and functional similarities with human hibernating myocardium: interstitial fibrosis and LV-dysfunction after 7 and 28 days I/R, and reversibility of both after discontinuation of the protocol over 60 days [6]. We revealed a transient inflammatory reaction involving induction of chemokine CCL2 and macrophage infiltration being dependent on reactive oxygen species as the key event for morphological and functional recovery from interstitial fibrosis and LV-dysfunction in absence of infarction [6, 9]. Therefore, we seek to identify factors modulating this inflammatory response for potential pharmacological strategies to treat this severe disorder.

Based on historical use of exogenous cannabinoids, i.e. Δ^9-tetrahydrocannabinol or marijuana, and some evidence of potential clinical benefits, the role of endocannabinoids in homeostasis and pathology of different organ systems emerged with description of endogenous ligands and their receptors [2, 12, 15, 26]. A study suggested an antifibrotic role for cannabinoid receptor 2 (CB2) in liver fibrosis [14], while another pharmacological study using low-flow ischemia on isolated rat hearts suggested cardioprotective effects [18]. While recent studies indicated a potentially cardioprotective role of CB2 receptors after myocardial infarction [5, 19, 22, 30], we have chosen another clinically relevant heart disorder, namely early phase in development of ischemic heart disease prior to prominent loss of cardiomyocytes. Pharmacological intervention at this stage could also prove extremely helpful for patients to avoid the progression of the disease and prevent myocardial infarction [11].

We demonstrate that endocannabinoids enhance the functional recovery of the post-ischemic heart through CB2 receptor-mediated mechanisms in the ischemic myocardium, which also affect the subsequent myocardial remodeling.

Methods

An expanded Methods section is available in the online data supplement.

Study animals

All mouse experiments were performed in accordance with an animal protocol approved by the local governmental authorities on 18–25 g and 8–10 weeks old mice. Beside wild type (WT-) C57BL/6J-mice (Charles River, Sulzfeld, Germany), the homozygote CB2-deficient ($Cnr2^{-/-}$)-mice on C57BL/6J-background were derived and bred from homozygote pairs as published [3]. We used both male and female mice in an equal distribution for all experiments. Generation of bone marrow (BM)-chimeric mice is described in detail in supplementary material [1]. We performed initial surgery on 241 WT-mice with 24.07 % mortality and additional 7.47 % of mice were excluded due to infarction after repetitive I/R protocol. A total of 196 $Cnr2^{-/-}$ mice underwent initial surgery with 22.96 % mortality rate and 4.59 % infarction-related exclusion after I/R. We also generated 57 chimeric mice, which had 14.04 % mortality rate after initial surgery and 7.02 % infarctions post-I/R.

Mouse model of brief repetitive myocardial ischemia and reperfusion (I/R)

Briefly, in an initial surgery, an 8-0 Prolene suture (Ethicon, Norderstedt, Germany) was placed around the left descending coronary artery and stored subcutaneously as previously described [6]. Mice were allowed to recover for 7 days from initial surgery trauma. Brief repetitive I/R was performed using one daily episode of 15 min coronary artery occlusion followed by 24 h reperfusion until the next day. This daily episode was performed over 1, 3, 5 or 7 consecutive days. Echocardiography was performed on selected groups as described in online supplement. Whole hearts were excised and fixated in zinc-paraformaldehyde (Z-fix, 4 %; Anatech, Battle Creek, MI, USA) over 18–22 h at 4 °C for histology, or directly frozen in Tissue Tek (Sakura, Staufen, Germany) to perform superoxide staining. Hearts were dissected free from atria and large vessels and only ventricles were either processed fresh for protein extraction, immediately snap frozen in liquid nitrogen and stored dry on −80 °C for endocannabinoid

measurements, or immediately stored in RNA-later (Qiagen, Hilden, Germany) for mRNA-studies. Whole hearts were used for extraction of adult cardiomyocytes in Langendorff-apparatus, for isolation of myofibroblasts or retrieval of inflammatory cells using MACS sorting.

Hypertrophy measurements

We measured heart weight to body weight and to tibia length ratios on freshly excised hearts and right tibia after I/R. Hearts were cannulated, perfused with ice-cold Krebs Henseleit buffer (KH) and the atria were removed. Hearts were dried on a paper towel for 1 min before their weight measurement.

Histology

Basic histological evaluation and quantitative planimetric analysis of collagen-stained area was performed as already published [6]. Immunohistochemical staining was performed using Vectastain Elite ABC kits and diaminobenzidine (AXXORA, Lörrach, Germany). The list of primary antibodies and the evaluation methods are provided in the online supplement.

Endocannabinoid measurements by LC–MS/MS

Analyses were performed on an LC–MS/MS system (Agilent 1200 LC system; 5500 QTrap; AB SCIEX, Darmstadt, Germany); see supplement for details. Endocannabinoid concentration was normalized to protein and concentration of anandamide and 2-arachidonoyl glycerol, as well as arachidonic acid were measured in ischemic tissue samples.

Statistical analysis

Data are presented as mean ± SEM. Two-way ANOVA with a Student's–Newman–Keuls corrected post hoc analysis was done to compare differences between the groups (Prism 5.0; GraphPad, San Diego, CA, USA). Unpaired *t* test was used for comparison when only two groups were examined. Differences with $P \leq 0.05$ were considered significant.

Results

CB2-deficiency leads to irreversible loss of cardiomyocytes and sustained endocannabinoid release in ischemic heart

Naïve CB2-deficient mice showed comparable myocardial morphology, echocardiography parameters or expression of key genes when compared with respective shams, WT-mice or their shams, thus excluding overt heart dysfunction under control conditions. Repetitive I/R caused persistent CB2 mRNA induction (Fig. 1a) and significantly increased its protein expression after 3 days I/R (Fig. 1b) in the whole heart of WT-mice. To show induction of CB2 on cardiomyocytes despite the lack of specific CB2-antibodies, we isolated cardiomyocytes from WT-hearts after 3 days I/R using Langendorff-apparatus and found a significant CB2 mRNA induction in them (Fig. 1c). Repetitive I/R led to cellular infiltration of the widened interstitial space in WT-mice after 7 days (Fig. 1d). In clear contrast, strong cellular infiltrations with microinfarctions in the ischemic LV-wall were detected in hearts of $Cnr2^{-/-}$-mice (Fig. 1e). The hallmark of this disease model is an evenly distributed interstitial fibrosis in the ischemic area of WT-hearts (Fig. 1f). However, interstitial fibrosis was rare in $Cnr2^{-/-}$-mice, and instead we found replacement fibrosis in microinfarctions due to cardiomyocyte loss (Fig. 1g). This was further supported by a significant increase of microinfarcted areas in $Cnr2^{-/-}$-mice (collagen in microinfarcted area as percentage of total collagen area: $Cnr2^{-/-}$-mice 18.39 ± 3.34 % vs. WT-mice 1.08 ± 0.46 %; $P \leq 0.05$, $n = 8$). Despite no significant difference between WT- and $Cnr2^{-/-}$-hearts in total collagen-stained area in naïve, sham or 7 days I/R groups (Fig. 1h), we observed a complete regression of interstitial fibrosis in WT-mice 60 days after discontinuation of I/R in contrast to only partial regression of fibrosis in $Cnr2^{-/-}$-mice (Fig. 1h). To further corroborate the involvement of endocannabinoids, we measured anandamide concentration in the heart at different time points during I/R protocol. We found that anandamide was only transiently increased in the ischemic area of LV in WT-mice (Fig. 1i), whereas its levels constantly increased during I/R episodes in $Cnr2^{-/-}$-mice. Anandamide levels in the $Cnr2^{-/-}$-hearts were significantly different after 7 days I/R, indicating its involvement in adaptation to repetitive ischemic stress. Concentration of 2-arachidonoylglycerol was unchanged in $Cnr2^{-/-}$-hearts (Fig. 1j) and tissue concentration of arachidonic acid showed a similar concentration pattern to that of anandamide (Fig. 1k).

CB2-mediated protection of cardiomyocytes during the development of left ventricular dysfunction

In the next step, we evaluated the impact of morphological findings on left ventricular function. The echocardiography measurements showed normal values for fractional shortening as a global function parameter (Fig. 2a), anterior wall thickening as a regional function parameter (Fig. 2b) and left ventricular end-diastolic diameter (LVEDD; Fig. 2c) in sham WT- and $Cnr2^{-/-}$-mice. In parallel to

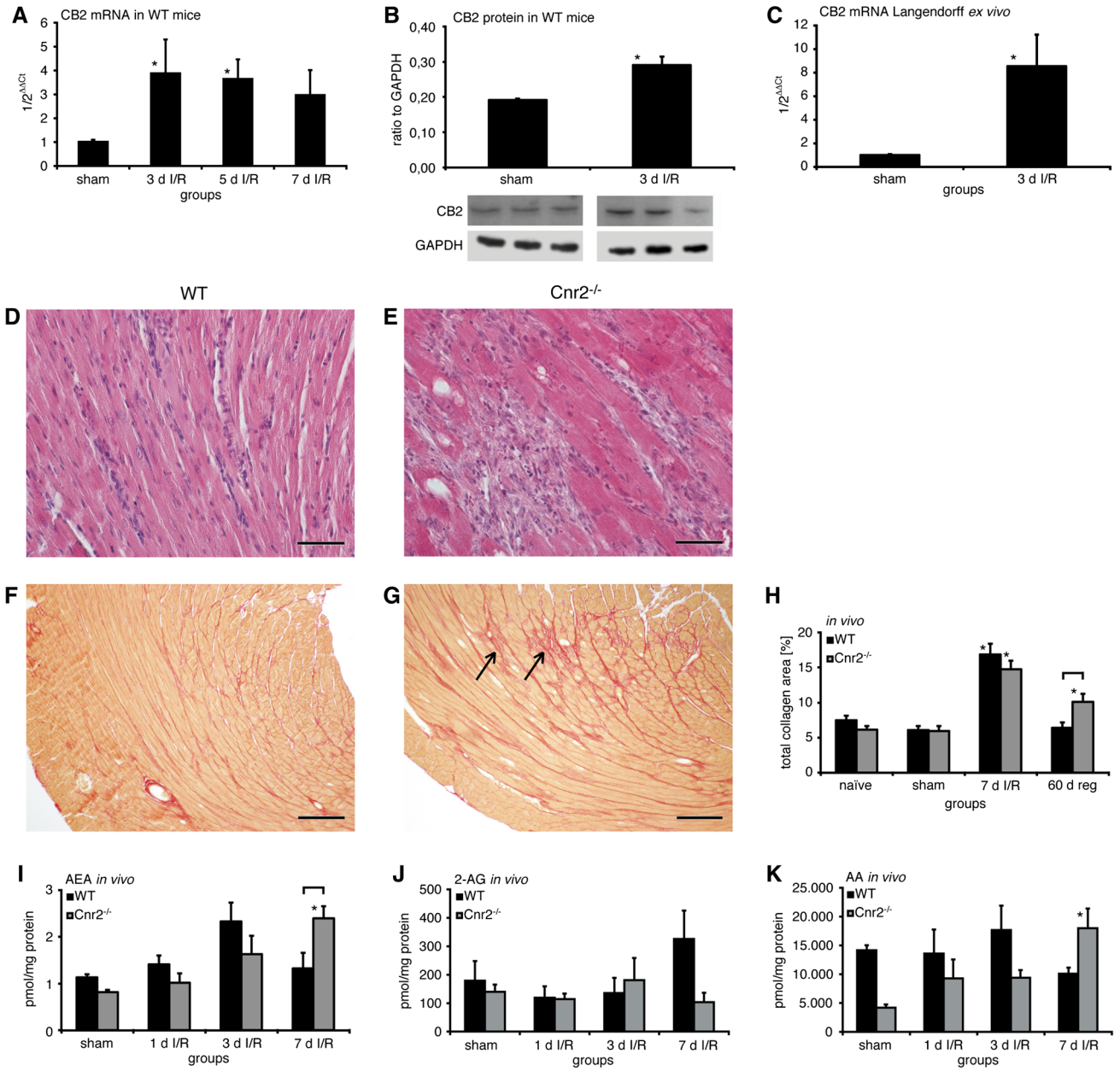


Fig. 1 CB2-deficiency leads to microinfarctions after repetitive I/R. **a** mRNA expression of CB2 receptor in wild type (WT)-mouse hearts during repetitive I/R. **b** Quantitative analysis of CB2 protein expression in relation to GAPDH using Western blot in sham WT-mice and after 3 days I/R. **c** CB2-mRNA expression in Langendorff-isolated adult WT-cardiomyocytes after 3 days I/R. **d** Representative HE-staining section of wild type (WT)-mouse heart after 7 days I/R shows interstitial cellular infiltration in contrast to **e** irreversible loss of cardiomyocytes in microinfarctions of $Cnr2^{-/-}$-mice. **f** Collagen staining using picrosirius red shows only interstitial fibrosis in WT-mice. **g** $Cnr2^{-/-}$-mice present intense collagen deposition in microinfarctions (*arrows*) and less interstitial collagen in remaining ischemic area. **h** Quantitative planimetric evaluation of total collagen-stained area in relation to the left ventricular area in WT- and $Cnr2^{-/-}$-hearts. **i** Tissue concentration of anandamide (AEA), **j** 2-arachidonoylglycerol (2-AG) and **k** arachidonic acid (AA) in ischemic left ventricular wall measured with mass spectrometry. $n = 8$/group; for protein analysis $n = 6$/group; RT-qPCR using Taqman®, expression is related to controls and GAPDH using comparative $\Delta\Delta C_t$-method. *Scale bars* **a**, **b** 50 µm; **d**, **e** 200 µm; *bar* indicates $P \leq 0.05$ between the genotypes; *asterisk* indicates $P \leq 0.05$ vs. respective sham

development of interstitial fibrosis and microinfarctions, we found a significant global and regional left ventricular dysfunction in both strains after 7 days I/R, when compared to their respective shams. The extent of dysfunction was comparable between the strains at this stage, while we observed no significant increase in LVEDD. However, 60 days after discontinuation of I/R, WT-mice showed significantly better function with full recovery

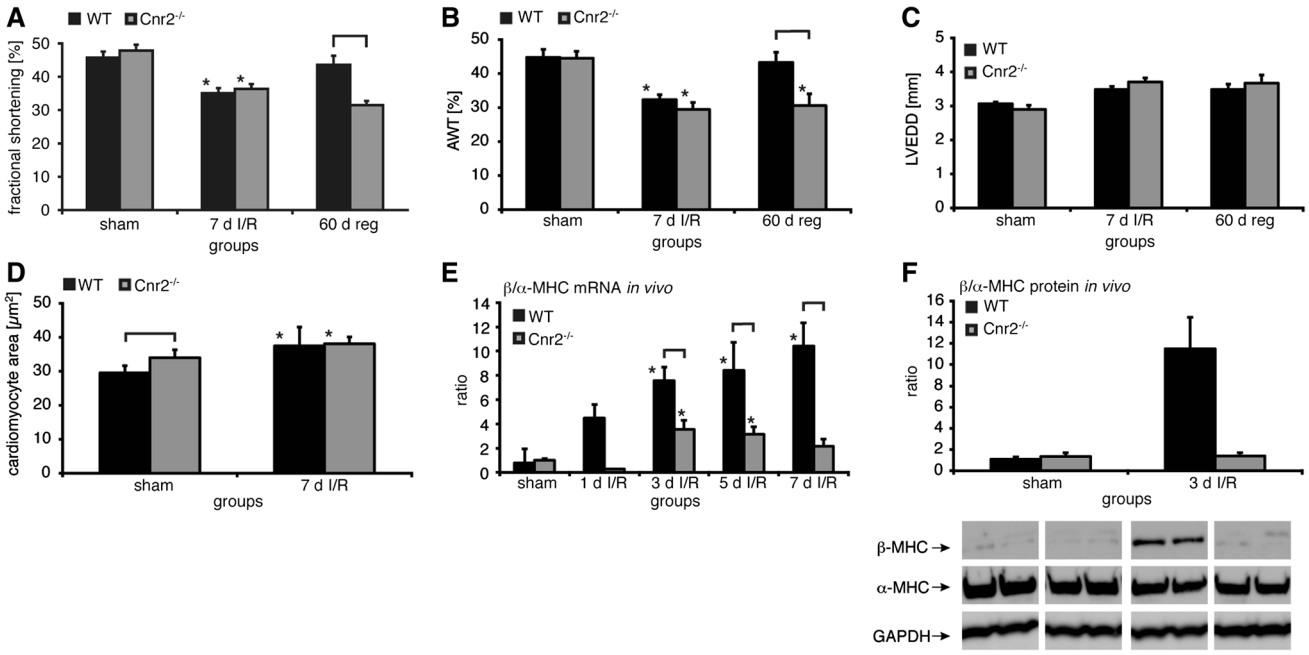
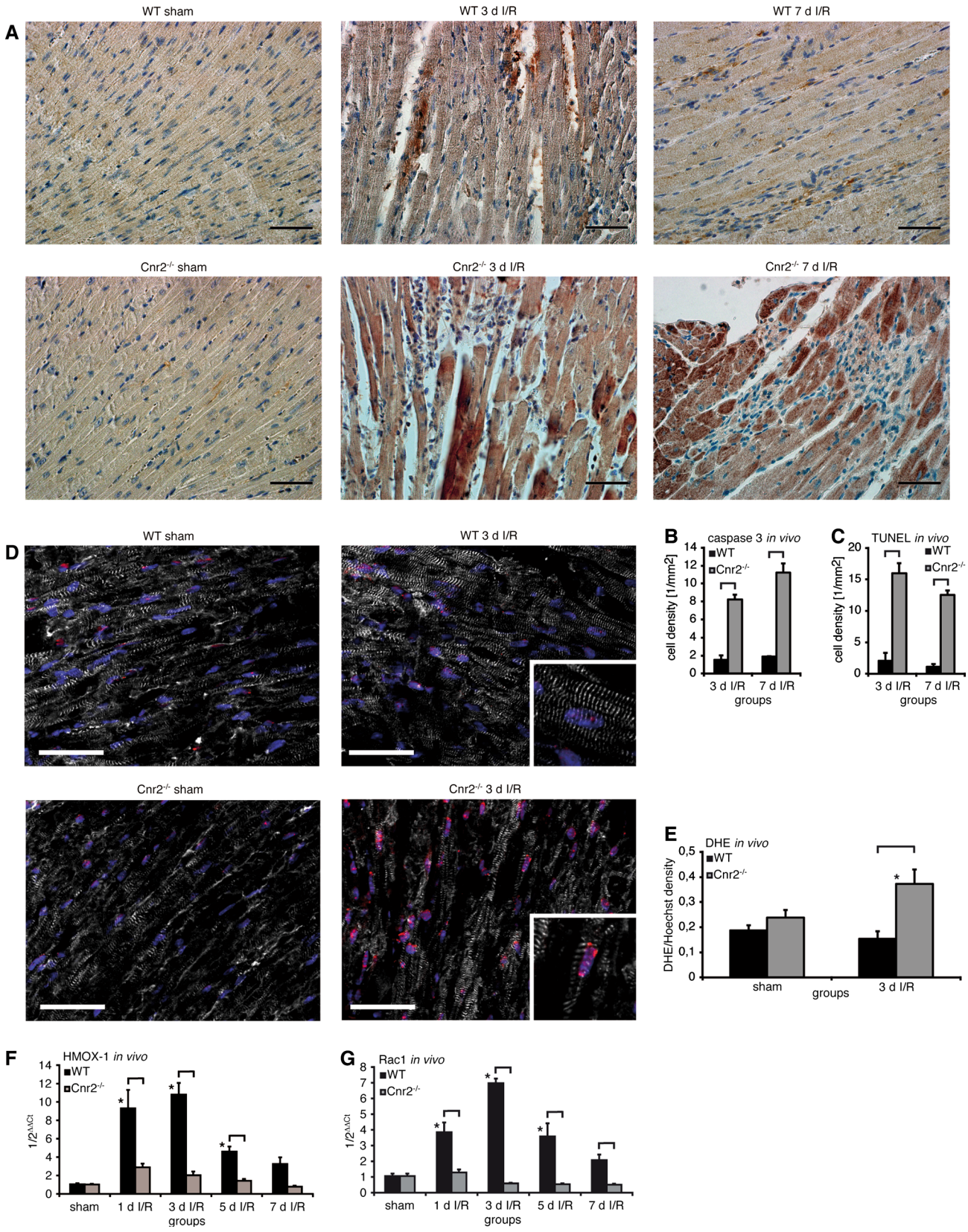


Fig. 2 CB2-deficiency leads to persistent left ventricular dysfunction after repetitive I/R. **a** Fractional shortening as parameter of global left ventricular function, **b** anterior wall thickening (AWT) as parameter of regional function, and **c** left ventricular end-diastolic diameter (LVEDD) in WT- vs. Cnr2$^{-/-}$-hearts after 7 days I/R and 60 days after discontinuation of I/R (60 days reg). **d** Cardiomyocyte area after 7 days I/R in WT- vs. Cnr2$^{-/-}$-hearts. **e** Ratio of β/α-myosin heavy chain (MHC) mRNA expression during repetitive I/R. **f** Ratio of β/α-MHC protein concentration after 3 days I/R. $n = 8$/group, for protein analysis $n = 6$/group; RT-qPCR using Taqman®, expression is related to controls and GAPDH using comparative $\Delta\Delta C_t$-method; *bar* indicates $P \leq 0.05$ between the genotypes; *asterisk* indicates $P \leq 0.05$ vs. respective sham

accompanying the above-mentioned complete regression of fibrosis, whereas Cnr2$^{-/-}$-mice presented with persistent global and regional LV-dysfunction without increase in LVEDD. Since myocardial ischemia may induce compensatory hypertrophy of non-ischemic areas, we measured heart weight/tibia length ratio and found a significant difference after 7 days I/R (WT 66.29 ± 2.40 mg/cm vs. Cnr2$^{-/-}$-mice 77.00 ± 2.46 mg/cm, $P \leq 0.05$, $n = 8$). The heart weight/body weight ratio was comparable between both groups (WT 0.00513 ± 0.00018 vs. Cnr2$^{-/-}$-mice 0.00510 ± 0.00018, n.s., $n = 8$). Also, the cardiomyocyte area was comparable between WT- and Cnr2$^{-/-}$-mice after 7 days I/R, but significantly higher than in respective shams (Fig. 2d). The significant difference in cardiomyocyte area between the shams of both strains may be related to the initial surgery trauma.

To investigate the adaptation mechanisms of cardiomyocytes during development of left ventricular dysfunction, we measured mRNA expression of contractile elements. We found a constant and significantly increased ratio of β to α isoforms of myosin heavy chain (MHC) in WT-mice when compared to their shams. This was caused by a persistent downregulation of the α isoform, indicating adaptation to repetitive ischemic stress using an embryonic expression pattern to survive (Fig. 2e). In contrast, the Cnr2$^{-/-}$-mice presented with a significantly lower ratio of β to α isoforms, when compared to the respective WT-groups. Cnr2$^{-/-}$-mice were unable to downregulate the α isoform, and this difference in adaptation to ischemic stress may have contributed to the loss of cardiomyocytes. The Western blot analysis of MHC isoforms confirmed our findings of the RNA analysis (Fig. 2f). We further investigated this hypothesis using an apoptosis assay for cleaved caspase-3. In WT-hearts only scattered caspase-positive cardiomyocytes in the ischemic area were found, whereas, in Cnr2$^{-/-}$-hearts significantly more caspase-3-positive cardiomyocytes were detected after 3 and 7 days I/R (Fig. 3a, b). We assessed apoptosis also using TUNEL staining (Online Figure 1). Quantitative analysis (Fig. 3c) yielded similar results as obtained with caspase-3 staining. The clear difference in the rate of apoptosis prompted us to explore more in detail CB2-dependent cardioprotective mechanisms.

Based on our previous findings on the crucial role of reactive oxygen species and its related mediators for cardioprotection in the model of repetitive I/R [6, 28], we assumed that the free radical burden may be higher in Cnr2$^{-/-}$-cardiomyocytes and thereby causing apoptosis and cell loss. For this purpose, we measured production of reactive oxygen species in tissue using dihydroethidine


A
WT sham
WT 3 d I/R
WT 7 d I/R
Cnr2-/- sham
Cnr2-/- 3 d I/R
Cnr2-/- 7 d I/R
D
WT sham
WT 3 d I/R
Cnr2-/- sham
Cnr2-/- 3 d I/R
B
caspase 3 in vivo
WT
Cnr2-/-
cell density [1/mm2]
3 d I/R
7 d I/R
groups
C
TUNEL in vivo
WT
Cnr2-/-
cell density [1/mm2]
3 d I/R
7 d I/R
groups
E
DHE in vivo
WT
Cnr2-/-
DHE/Hoechst density
sham
groups
3 d I/R
F
HMOX-1 in vivo
WT
Cnr2-/-
1/2ΔΔCt
sham
1 d I/R
3 d I/R
5 d I/R
7 d I/R
groups
G
Rac1 in vivo
WT
Cnr2-/-
1/2ΔΔCt
sham
1 d I/R
3 d I/R
5 d I/R
7 d I/R
groups

◀**Fig. 3** CB2-deficiency causes increased apoptosis and production of reactive oxygen species during repetitive I/R. **a** Representative slides of cleaved caspase 3 staining for apoptosis in wild type (WT)- and Cnr2$^{-/-}$-mice during repetitive I/R. Cellular density analysis of **b** cleaved caspase 3 staining and **c** TUNEL staining revealed significantly more apoptotic cardiomyocytes in Cnr2$^{-/-}$-hearts. **d** Representative dihydroethidine (DHE; *red*) staining sections show increased reactive oxygen species production in Cnr2$^{-/-}$-hearts after 3 days I/R when compared to the WT or respective shams (nuclei Hoechst blue, cardiomyocytes stained with α-actinin white). **e** Quantitative analysis of the total DHE signal density as a ratio to the total Hoechst signal density. **f** Cnr2$^{-/-}$-hearts had significantly weaker mRNA induction of heme oxygenase-1 (HMOX-1) and no induction of **g** Rac-1 than the WT-hearts during I/R protocol. *Scale bar* 50 μm, for the *insert* 25 μm; $n = 8$/group, except for **a**, **b**: $n = 4$–5/group; RT-qPCR using Taqman®, expression is related to controls and GAPDH using comparative $\Delta\Delta C_t$-method; *bar* indicates $P \leq 0.05$ between the conditions; *asterisk* indicates $P \leq 0.05$ vs. respective sham

(DHE). Representative sections of both genotypes in sham hearts and specimen after 3 days I/R are shown in Fig. 3d. Quantitative analysis of DHE/Hoechst density ratio after 3 days I/R revealed that WT-mice do not show increase in DHE in contrast to a significantly higher ratio in Cnr2$^{-/-}$-mice (Fig. 3e). Since reactive oxygen species are neutralized by different factors under ischemic stress, we measured mRNA expression of antioxidative enzymes. We found a significant, transient increase of heme oxygenase-1 in WT-mice in contrast to only a modest, significantly lower induction in Cnr2$^{-/-}$-mice (Fig. 3f). A comparable difference in expression pattern was found for Rac1, the main regulator of NADPH oxidase (Fig. 3g), glutathione peroxidase-1, superoxide dismutase 3 and metallothionein 1 (Online Figure 1). These data provide evidence that the tissue production of reactive oxygen species seems to be quenched by induction of antioxidative mediators and their related regulators in WT-hearts. This cardioprotective action is weaker in Cnr2$^{-/-}$-hearts and therefore may contribute to the loss of cardiomyocytes. Since cardiomyocytes are not the only cells in the heart producing antioxidative mediators in vivo, we investigated cardiomyocytes in vitro. To this aim embryonic cardiomyocytes (eCM) from WT- and Cnr2$^{-/-}$-mice were isolated and treated with IFN-γ under normoxia or hypoxia in vitro to simulate the in vivo situation of repetitive I/R. No evidence of increased apoptosis (bcl-2, caspase 3 and 8) or cell loss (manual cell count) was found in Cnr2$^{-/-}$-eCMs when compared with WT-cells (Online Figure 2). Comparable to the in vivo data and as a proof of concept, we found no heme oxygenase-1 induction in hypoxic Cnr2$^{-/-}$-eCM under IFN-γ stimulation (Fig. 4a). To unequivocally rule out potential fibroblast contribution, we took advantage of puromycin-purified embryonic stem cell-derived cardiomyocytes (~97 %-pure cardiomyocyte cell culture) [17]. The puromycin-derived WT-cardiomyocytes showed an early upregulation of heme oxygenase-1-mRNA under hypoxia (Fig. 4b) and thus confirmed the specificity to the

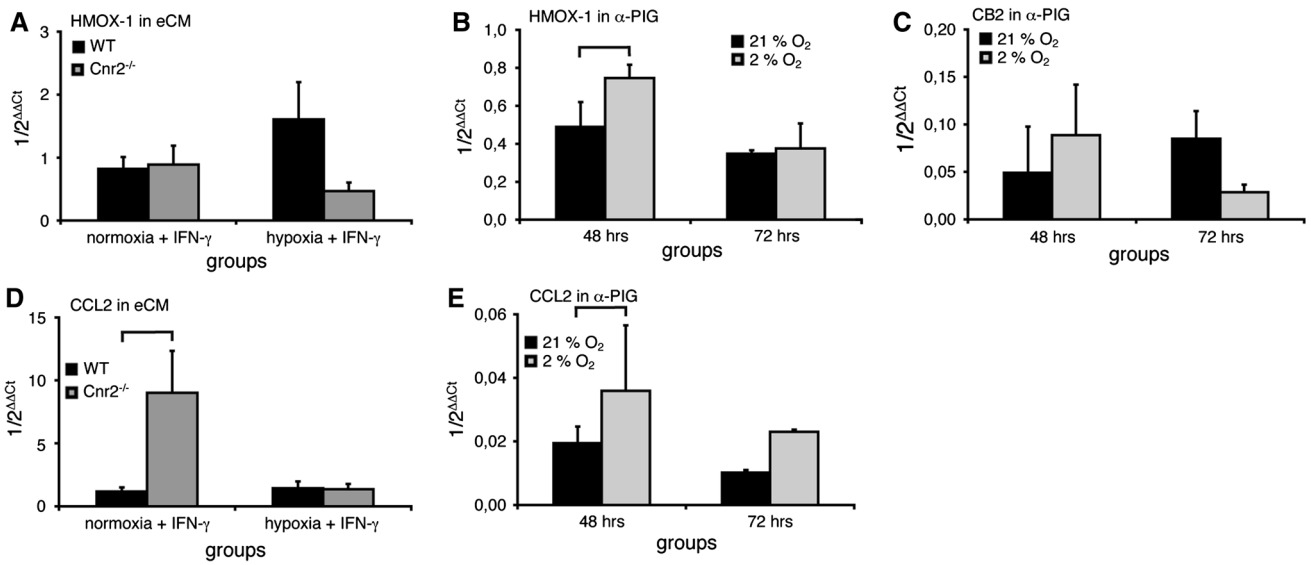


Fig. 4 CB2 receptor-dependent cardioprotection in vitro. **a** Embryonic cardiomyocytes (eCM; E13.5–15.5) from Cnr2$^{-/-}$-mice taken into culture and stimulated with 100 ng IFN-γ are unable to induce heme oxygenase-1 (HMOX-1) mRNA under hypoxic stress in contrast to a strong induction in eCM from wild type (WT)-mice. **b** Puromycin-purified cardiomyocytes (>97 % cell purity) from α-PIG cell line significantly induce HMOX-1 in response to hypoxia and **c** show a tendency toward less induction of CB2-mRNA under hypoxic conditions over 72 h. **d** eCM show an O_2-dependent induction of CCL2-mRNA in Cnr2$^{-/-}$-cells. **e** Puromycin-purified cardiomyocytes show a significant induction of CCL2-mRNA under hypoxic conditions. eCM $n = 5$/group, α-PIG $n = 4$–5/group; RT-qPCR using Taqman®, mRNA expression is related to controls and 18S using comparative $\Delta\Delta C_t$-method. *bar* indicates $P \leq 0.05$ between the genotypes, *asterisk* indicates $P \leq 0.05$ vs. respective controls

cardiomyocytes. Interestingly, we found a dynamic regulation of CB2 receptor under hypoxia in this puromycin-derived system (Fig. 4c). Based on this finding and the role of CB2 in regulation of chemokines and other inflammatory mediators, we then measured mRNA expression of the chemokine CCL2 in cardiomyocyte cell culture. We found a significant induction of CCL2 in Cnr2$^{-/-}$-eCM under normoxic conditions, whereas this was abrogated under hypoxia (Fig. 4d). This could represent a lower capability of Cnr2$^{-/-}$-cardiomyocytes to trigger an inflammatory reaction after ischemic injury. Indeed, the puromycin-derived WT-cardiomyocytes showed a consistent upregulation of CCL2-mRNA under hypoxia, thus showing their preserved potential to trigger the inflammatory cascade as an important part of cardioprotective mechanisms (Fig. 4e).

CB2-related modulation of macrophage function supports cardioprotective mechanisms

Since endocannabinoids exert CB2 receptor-mediated immunosuppressive effects on inflammatory mediators and macrophages [3, 31], we hypothesized that macrophage dysfunction could be the second main reason leading to microinfarctions in Cnr2$^{-/-}$-mice. Mac-2 staining revealed evenly distributed macrophages in ischemic LV-wall in WT-mice in contrast to Cnr2$^{-/-}$-mice, where a strong predominant infiltration of microinfarctions was found (Fig. 5a, b). Total macrophage density in ischemic LV-wall was higher in Cnr2$^{-/-}$-mice (Fig. 5c) with ~4-fold higher cell density in microinfarctions than in the remaining ischemic area after 5 days I/R (data not shown). To test the specific role of CB2 receptor on macrophages and bone marrow derived vs. cardiac-resident cells for immune response, we reconstituted WT-mice with Cnr2$^{-/-}$- or WT-bone marrow cells and performed the 7 days I/R protocol. The chimeric WT/WT-mice recapitulated the morphological findings in HE-staining of WT-mice, and chimeric WT/CB2$^{-/-}$-mice showed extensive microinfarctions in ischemic LV-wall comparable to the findings in Cnr2$^{-/-}$-mice (Fig. 5d, e). Total collagen area and ventricular function parameters were comparable between the chimeric groups and the respective non-reconstituted WT- or Cnr2$^{-/-}$-mice (data not shown). In full agreement with our findings in non-reconstituted mice, total macrophage density was significantly higher in WT/CB2$^{-/-}$-mice than in WT/WT-mice after 7 days I/R (Fig. 5f), and there was a clear affinity of macrophages toward microinfarctions in WT/CB2$^{-/-}$-mice (Fig. 5g–i), as found in non-reconstituted Cnr2$^{-/-}$-mice. Both chimeric groups showed significantly lower total macrophage density than the respective non-chimeric animals, which could be attributable to the reconstitution procedure.

The contribution of other inflammatory cells in vivo and CB2-related actions on macrophages in vitro are presented in supplemental data available online.

Post-infarction remodeling involves different subpopulations of macrophages depending on Th-polarization [24], and a further differentiation into M2a, M2b and M2c subtypes was reported, where M2b subpopulation is known not to be associated with tissue remodeling [21]. We applied a protocol for cellular extraction from whole hearts followed by MACS sorting of CD11b+ cells and RT-qPCR gene analysis to explore potential changes in macrophage subpopulations. Gene analysis showed no difference in M1 or M2c subpopulation between WT- or Cnr2$^{-/-}$-mice (Table 1). However, we could detect lack of expression of the M2a-specific cytokines IL4 and IL-13 in Cnr2$^{-/-}$-mice after 3 days I/R in contrast to comparable expression of these cytokines in both genotypes after 7 days. This revealed a postponed induction of M2a macrophage subtype in CB2-deficient I/R hearts, clearly indicating the cellular mechanism behind the CB2-mediated resolution of inflammatory response.

Our previous work showed that a well-orchestrated, transient inflammatory response is needed for development of interstitial fibrosis in our repetitive I/R model [6, 9]. Therefore, we analyzed the post-ischemic cascade and found an initiation of inflammatory response by mRNA induction of TNF-α after 1 day I/R (Fig. 6a). TNF-α protein concentration was significantly higher only in WT-mice after 3 days I/R when compared to their shams and showed a non-significant increase in Cnr2$^{-/-}$-hearts (Fig. 6b). Also, significantly increased mRNA expression of IL-1β (Fig. 6c) and M-CSF (Fig. 6d) were found in Cnr2$^{-/-}$-hearts after only 1 day I/R, when compared to their shams. In contrast, WT-mice presented with a transient upregulation of several proinflammatory cytokines and chemokines. To our surprise, Cnr2$^{-/-}$-mice had a significantly lower mRNA- and protein-induction of potent macrophage-related chemokine CCL2 (Fig. 6e, f). At the same time, we found no induction of its receptor CCR2 in Cnr2$^{-/-}$-hearts (Fig. 6g). mRNA expression of chemokine CCL4 showed comparable pattern to the CCL2 (Fig. 6h), thus suggesting a specific CB2 receptor-dependent regulation of chemokine expression. The anti-inflammatory cytokine IL-10 was not induced in ischemic Cnr2$^{-/-}$-hearts, thus indicating an impaired resolution of the inflammatory response in these mice (Fig. 6i).

CB2 receptor-dependent mechanisms of myocardial remodeling and fibrosis

We further investigated the mechanisms underlying the differences in fibrotic response between interstitial fibrosis and only few microinfarctions in WT-hearts and less

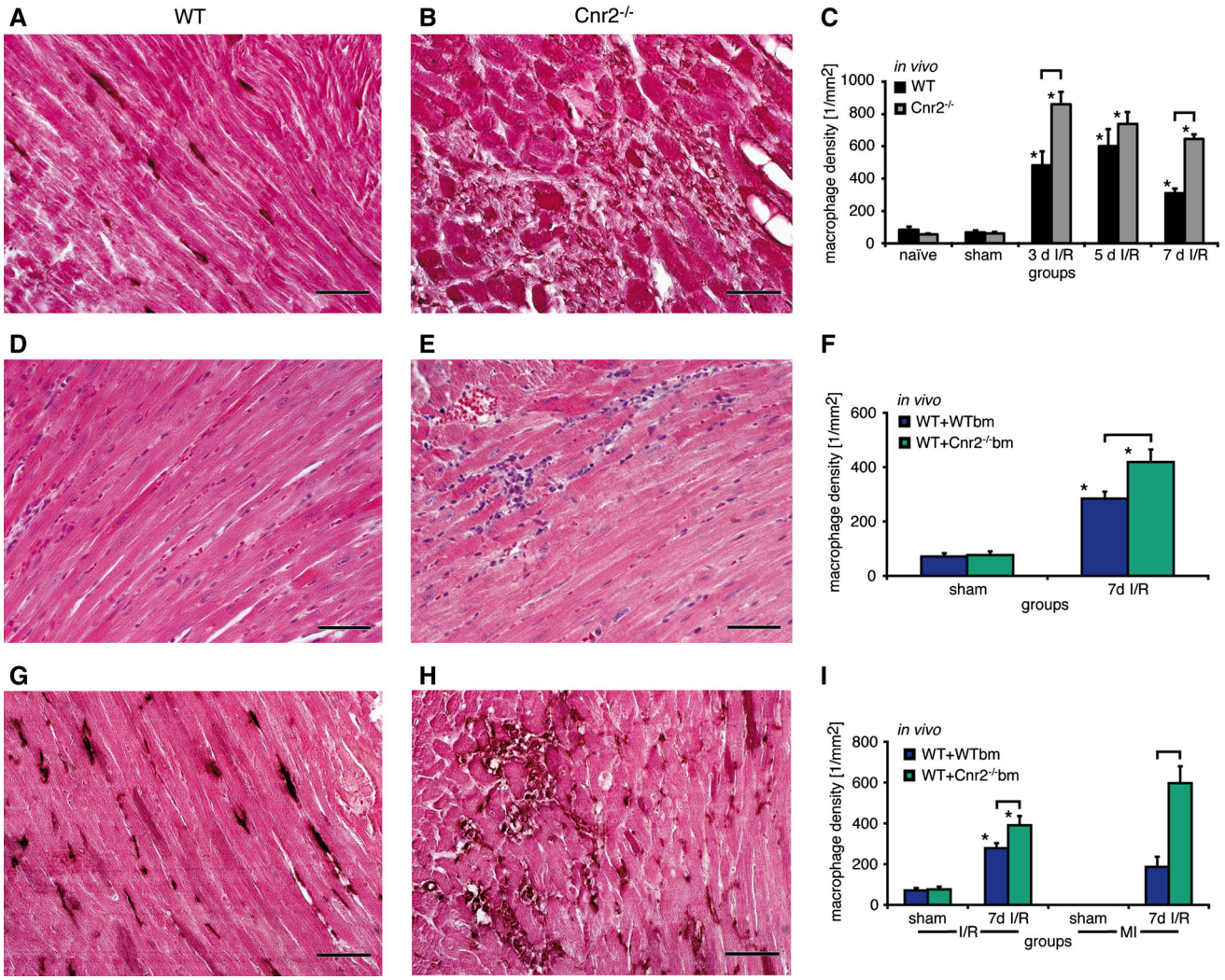


Fig. 5 Critical cardioprotective role of macrophages in repetitive I/R. **a** Representative section of a wild type (WT)-heart shows low interstitial macrophage infiltration after 7 days I/R using Mac-2 antibody. **b** In contrast, Cnr2$^{-/-}$-heart presents with persistent high cellularity in microinfarctions containing macrophages. **c** Total macrophage density (Mac-2+ cells) during repetitive I/R. **d** Chimeric mice after reconstitution with WT-bone marrow (bm) or with **e** Cnr2$^{-/-}$-bone marrow show comparable HE histology after 7 days I/R to the non-reconstituted groups. **f** Macrophage density of chimeric mice after 7 days I/R. **g** Representative heart section stained with Mac-2 antibody of a chimeric wild type (WT)-mouse reconstituted with WT-bone marrow (WTbm) and **h** chimeric WT-heart with Cnr2$^{-/-}$-bone marrow (Cnr2$^{-/-}$bm). **i** Differential evaluation of macrophage distribution between ischemic area (I/R) vs. microinfarctions (MI) in chimeric mice. *Scale bars* **a**, **b**, **g**, **h** 50 μm; **d**, **e** 200 μm. $n = 8$–11/group; *bar* indicates $P \leq 0.05$ between the genotypes; *asterisk* indicates $P \leq 0.05$ vs. respective sham

Table 1 Subpopulation-specific gene expression on macrophages in vivo

	WT 3 days I/R	CB2$^{-/-}$ 3 I/R	WT 7 days I/R	CB2$^{-/-}$ 7 days I/R
IFN-γ (M1)	27.54 ± 0.78	28.13 ± 0.46	25.64 ± 0.67	27.16 ± 0.20
IL-4 (M2a)	27.70 ± 0.54	n.d.	26.89 ± 0.15	26.96 ± 0.10
IL-13 (M2a)	27.04 ± 0.49	n.d.	25.86 ± 0.78	26.54 ± 0.61
IL-10 (M2c)	19.99 ± 0.18	19.91 ± 0.24	20.36 ± 0.30	20.15 ± 0.26

Macrophages were MACS sorted using CD11b from whole hearts (Langendorff perfusion) and their mRNA was processed for RT-qPCR. M2b subpopulation was not found in the hearts. Data ($n = 8$–9/group) are expressed as mean + SEM of ΔC_t. Unpaired *t* test showed no significant differences between the groups

n.d. not determined (gene expression not measurable)

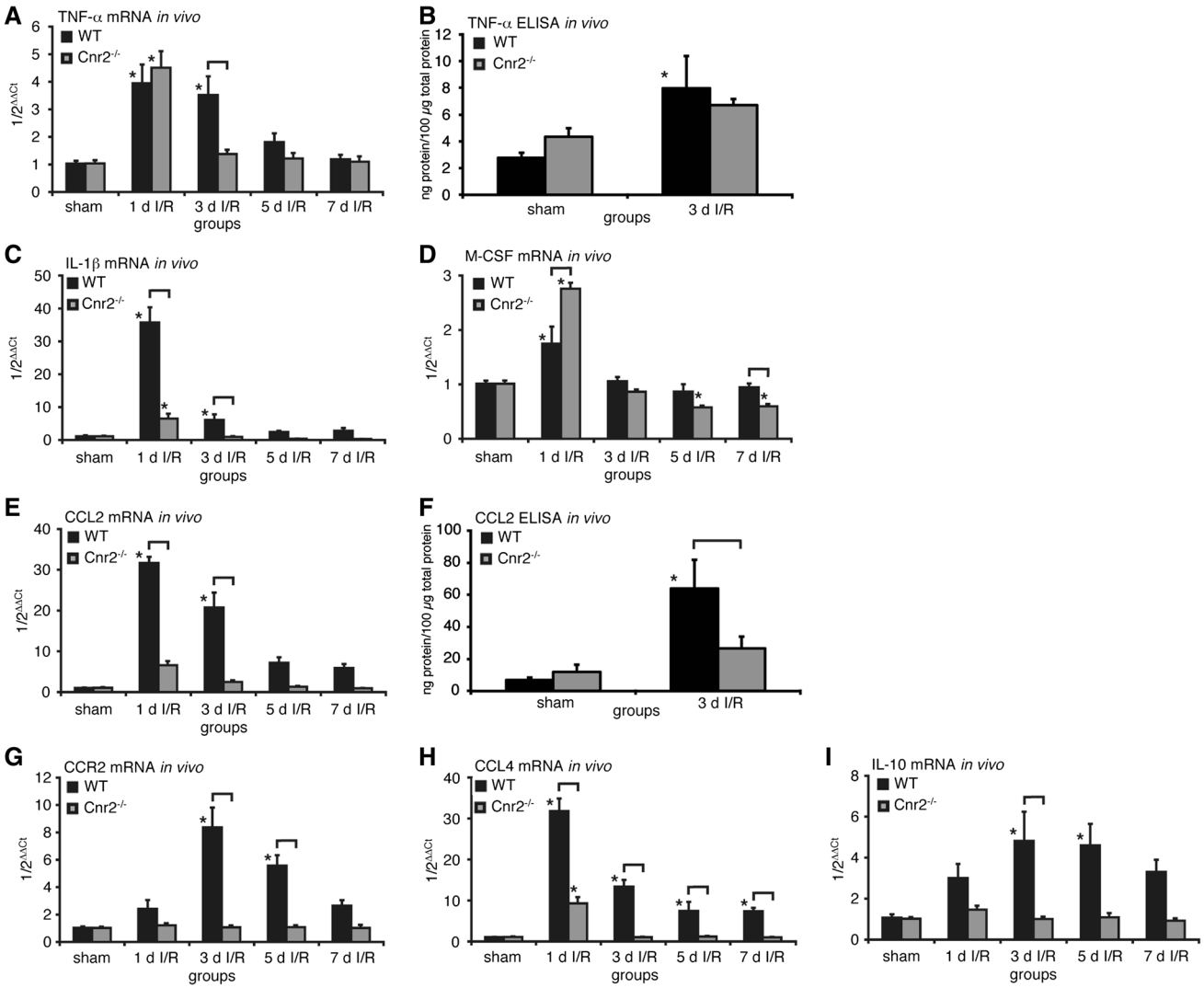


Fig. 6 Inflammatory mediators are differentially regulated through CB2 receptor in vivo. **a** mRNA expression and **b** protein concentration (ELISA) of cytokine TNF-α from whole hearts of wild type (WT)- and Cnr2$^{-/-}$-mice. **c** IL-1β and **d** M-CSF mRNA expression during repetitive I/R. **e** Chemokine CCL2 mRNA expression, **f** its protein concentration (ELISA), **g** the mRNA expression of its receptor CCR2, and **h** the expression of chemokine CCL4 are significantly decreased in Cnr2$^{-/-}$-mice. **i** mRNA expression of anti-inflammatory cytokine IL-10 in not upregulated in Cnr2$^{-/-}$-mice. $n = 8$/group; for protein analysis $n = 6$/group; RT-qPCR using Taqman®, mRNA expression is related to controls and GAPDH using comparative $\Delta\Delta C_t$-method; *bar* indicates $P \leq 0.05$ between the genotypes; *asterisk* indicates $P \leq 0.05$ vs. respective sham

interstitial fibrosis accompanied by non-compacted replacement fibrosis in microinfarctions in Cnr2$^{-/-}$-hearts, which suggested altered remodeling under CB2-deficiency. To obtain information on the potential reversibility of fibrosis, we determined the ratio of the reversible collagen type III and the irreversibly deposited collagen Iα. We found that WT-hearts contained significantly higher amounts of the reversible collagen isoform, whereas the irreversible collagen isoform was dominant in Cnr2$^{-/-}$-hearts after 5 days I/R (Fig. 7a). This finding was fully in line with the observed morphological, irreversible collagen deposition in microinfarctions after 7 days I/R and 60 days recovery in Cnr2$^{-/-}$-hearts, while fibrosis was fully reversible in WT-hearts (Online Figure 3). Since the collagen deposition appeared relatively loose after 7 days I/R in Cnr2$^{-/-}$-hearts, we measured the mRNA expression of the main collagen cross-linking enzyme lysyl oxidase. Interestingly, WT-heart showed a significantly higher induction of lysyl oxidase after 3 and 5 days I/R in contrast to lack of induction in the Cnr2$^{-/-}$-hearts (Fig. 7b). Myofibroblast staining in WT- and Cnr2$^{-/-}$-hearts after 7 days I/R (Fig. 7c, d) was evaluated using planimetry and revealed a very low persistence of myofibroblasts in Cnr2$^{-/-}$-hearts in contrast to WT-mice (Fig. 7e).

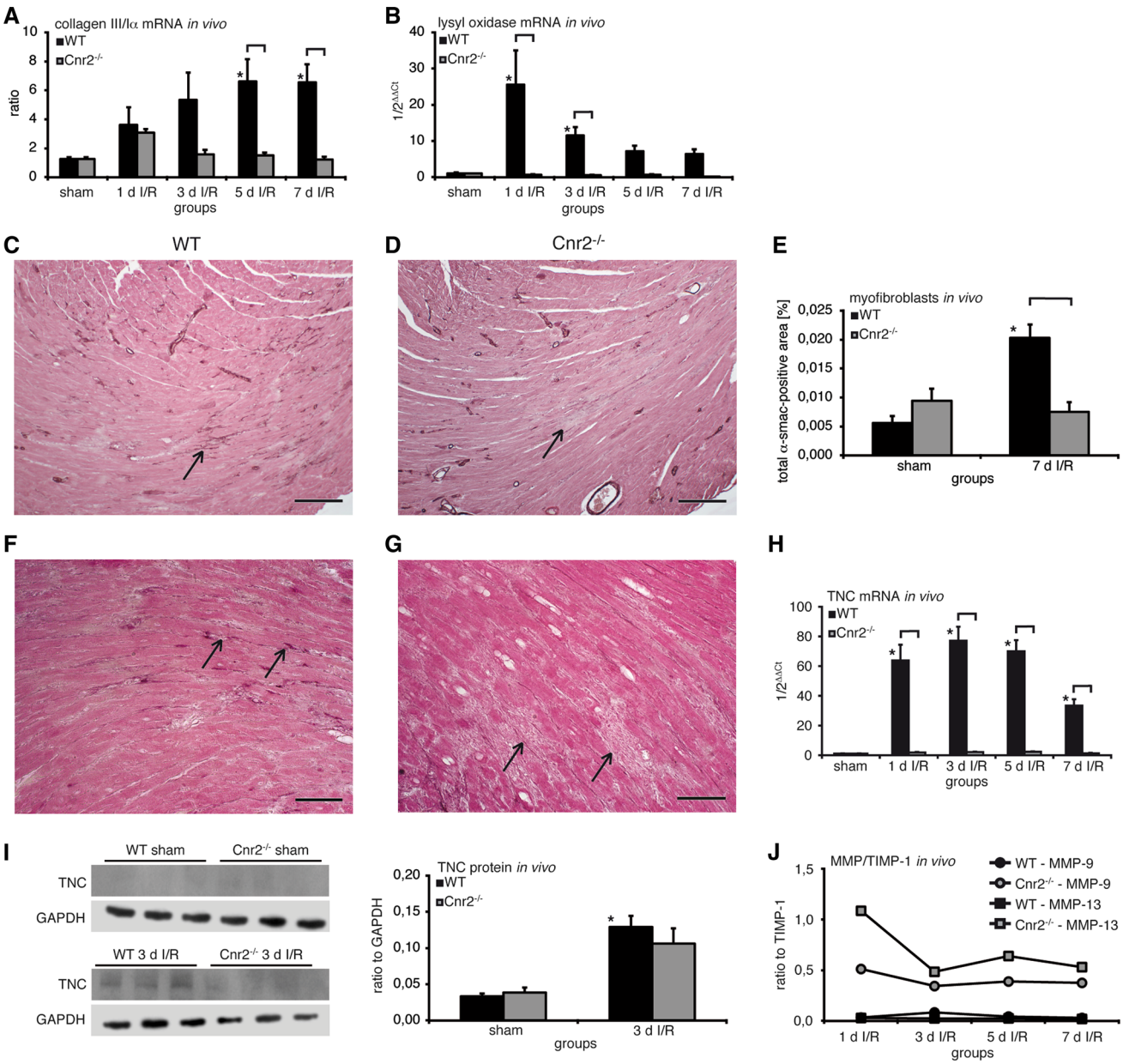


Fig. 7 Modulation of myocardial remodeling via CB2 receptor. **a** Ratio of collagen III/Iα mRNA expression shows upregulation of reversible collagen III expression only in WT-hearts. **b** mRNA expression of collagen cross-linking enzyme lysyl oxidase shows strong upregulation only in WT-hearts. **c** Myofibroblasts stained with α-smac antibody (*arrow*) of a WT-heart after 7 days I/R. **d** Representative Cnr2$^{-/-}$-heart shows a very faint staining of myofibroblasts (*arrow*) in microinfarction. **e** Planimetrical evaluation of myofibroblasts in vivo using the α-smac-stained area as a percentage of the total myocardial area after 7 days I/R. **f** Tenascin C-staining of a representative wild type (WT)-heart after 7 days I/R shows positive signals in interstitial space (*arrows*). **g** Cnr2$^{-/-}$-heart after 7 days I/R shows no tenascin-positive signals in microinfarctions (*arrows*). **h** Tenascin C mRNA induction during repetitive I/R and **i** its representative Western blots with relative protein concentration after 3 days I/R. **j** mRNA-ratios of MMP-9/TIMP-1 and MMP-13/TIMP-1 during repetitive I/R show their unbalanced regulation in Cnr2$^{-/-}$-hearts. *Scale bar* 200 µm; $n = 8$/group; for protein analysis $n = 6$/group; RT-qPCR, mRNA expression is related to controls and GAPDH using comparative $\Delta\Delta C_t$-method; *bar* indicates $P \leq 0.05$ between the genotypes, *asterisk* indicates $P \leq 0.05$ vs. respective sham

Evaluation of tissue sections stained for early remodeling marker tenascin C [8] showed strong signals in WT-mice and a barely detectable staining in Cnr2$^{-/-}$-hearts (Fig. 7f, g). Accordingly, tenascin C-mRNA induction was very strong in WT-hearts, but absent in Cnr2$^{-/-}$-hearts in vivo (Fig. 7h). Quantification of tenascin C protein level (Western blot) after 3 days I/R showed significant increase in WT-mice and only a tendency to a higher level in Cnr2$^{-/-}$-

hearts being partially caused by a lower GAPDH-expression (Fig. 7i). A different pattern in production and degradation of extracellular matrix in Cnr2$^{-/-}$-hearts was shown by higher MMP-9/TIMP-1 and MMP-13/TIMP-1 mRNA-ratios in Cnr2$^{-/-}$-hearts (Fig. 7j), thus suggesting a prolonged, active interstitial remodeling. Additional data on expression of other remodeling mediators in vivo and in vitro are presented in Supplemental data available online.

Discussion

Endocannabinoids and CB2 receptors have been suggested to be involved in cardioprotective mechanisms in an experimental model of myocardial infarction in vivo [20] and other studies in vitro [29]. The myocardial infarction causes detrimental damage to a large portion of the left ventricle, and it is leading to a significant functional impairment and development of terminal heart failure. It is also associated with a strong inflammatory reaction during reperfusion, and the myocardial remodeling thereafter aims to a rapid formation of a stable scar. From the clinical point of view, patients who suffered myocardial infarction have a coronary artery disease as the underlying disorder. Modern therapeutic strategies focus on treating coronary artery disease before extensive myocardial remodeling and/or infarction occur, to improve its poor long-term prognosis. Since current cellular replacement strategies are not that promising, a better understanding of cardioprotective mechanisms becomes even more important. In the light of these facts, we have chosen a murine model of repetitive, brief I/R, which provides temporal and spatial resolution of very early developmental stages of ischemic cardiomyopathy (only a few episodes of short ischemia), before left ventricular dysfunction develops. This model has similar characteristics to the human condition of hibernating myocardium: repetitive I/R episodes as underlying cause and reversibility of interstitial fibrosis and left ventricular dysfunction [6]. Importantly, this injury type does not induce myocardial infarction and is associated with a transient inflammatory response, thereby offering a unique basis for analyzing cardioprotective mechanisms.

Herein we provide novel evidence for specific cardioprotective mechanisms involving the endocannabinoid-CB2 receptor axis on cardiomyocytes and macrophages during development of murine ischemic cardiomyopathy. The anandamide-CB2 receptor axis is assisting cardiomyocytes in their adaptation of contractile protein components and upregulation of antioxidative mediators to prevent apoptosis. In addition, the anadamide-CB2 receptor axis is also modulating the macrophage function via inflammatory mediators providing a balanced inflammatory response and its timely resolution during repetitive I/R. Both of these mechanisms appear to be key determinants for myocardial remodeling resulting in reversible interstitial fibrosis and left ventricular dysfunction.

These findings are novel in respect to the already published studies for several reasons: previous studies indicated induction of CB2 receptor mRNA isolated from a whole mouse heart 24 h after myocardial infarction [22] or from rat cardiomyocytes in cell culture [29]. The cardioprotective role of the CB2 receptor has been postulated in a mouse model of infarction based on upregulation of ERK1/2 after 30 min ischemia and 10 min reperfusion [22]. Another infarction study showed CB2-dependent regulation of caspase 3 after 1-h ischemia and 3 days reperfusion, with almost normal LV function in both WT- and Cnr2$^{-/-}$-hearts 4 weeks after reperfusion of infarction [5]. This later study also postulated CB2 receptor-mediated cardioprotection based on H_2O_2-treatment in vitro leading to an increased Cnr2$^{-/-}$-cardiomyocyte apoptosis and higher differentiation potential of Cnr2$^{-/-}$-myofibroblasts [5]. In addition, a role for CB2 receptor in regulation of macrophage function has only been shown in vitro [25]. In the present study we have identified important mediators acting in CB2-dependent cardioprotection, which are not only expanding our understanding of it, but in part are also contrasting the previously published data. We show that WT-hearts, preferentially cardiomyocytes, constantly express CB2 during repetitive I/R, which was confirmed in our purified cardiomyocyte preparation under hypoxia in vitro. In Cnr2$^{-/-}$-hearts loss of cardiomyocytes already after 3 days of repetitive I/R episodes was observed leading ultimately to persistent fibrosis and irreversible LV-dysfunction after 60 days recovery from I/R. This is clearly different to WT-mice, which experienced full functional recovery and morphological regression of fibrosis at the same time point. This regression of fibrosis is clinically important for recovery of the LV function upon revascularization and associated with persistent low influx of macrophages in the human myocardium [10]. We could also identify CB2 receptor-dependent cardiomyocyte protective pathways, which prevent apoptosis during repetitive I/R. One was regulation of MHC isoform expression. β-MHC is upregulated under hypoxia and pressure overload [27] due to its decreased contractile velocity, while it conserves more ATP per contraction than α-MHC. WT-hearts were able to adapt to repetitive I/R by decrease in α-MHC and increase in β-isoform, thus resulting in increased β/α-ratio without cardiomyocyte loss. Cnr2$^{-/-}$-hearts were unable to induce the energetically more effective β-MHC and to decrease the α-isoform despite morphological signs of hypertrophy, and subsequently the cardiomyocytes were irreversibly damaged. This could be one of the reasons leading to increased apoptosis of Cnr2$^{-/-}$-cardiomyocytes in vivo. Another important point seems to be higher

production of reactive oxygen species in Cnr2$^{-/-}$-hearts, which was associated with lower induction of antioxidative mediators in vivo and in vitro. Here one could argue that hypoxia may induce cell proliferation of embryonic cardiomyocytes and that this cannot be correlated with the in vivo data. On the other hand, the cardiomyocyte loss per se could lead to the prolonged proinflammatory response, but our data from chimeric mice argue against this. Additional support to our interpretation comes from CB2 receptor-dependent regulation of Rac1, the main regulator of NADPH oxidase, associated with induction of antioxidative enzymes, cytokines and chemokines in vivo [4], and metallothionein, which both suggest cardioprotective action of CB2 in kinase-dependent regulation of cascades and in different cell compartments or systems, e.g. zinc-storage.

Several experiments including chimeric mice helped to establish a crucial role for CB2 receptor in modulation of macrophage function in vivo. The data obtained from chimeric mice experiments provided first evidence for a specific role of CB2 receptor on bone marrow derived mononuclear cells in ischemic myocardium. We were able to confirm the transient course of inflammatory reaction in WT-hearts associated with both cytokine and chemokine induction, as previously described [6]. Previous studies showed a strong up-regulation of TNF-α in microinfarctions after microembolization being associated with ventricular dysfunction [7]. In contrast, our ischemic Cnr2$^{-/-}$-hearts revealed that the inflammatory response was predominantly driven by a short upregulation of TNF-α and M-CSF. This discrepancy may be attributed to the CB2 receptor knockout itself, but also to washing-out effects, since our hearts were retrieved 5 h after the last ischemic episode. The proinflammatory milieu in our study was associated with prolonged macrophage persistence being concentrated to the areas of microinfarctions. This was accompanied by a lack of significant chemokine induction, which itself is necessary for development of interstitial fibrosis [9], and may suggest a possible specific regulation of chemokines through CB2 receptor. Also, the lack of IL-10 and TGF-β induction in Cnr2$^{-/-}$-hearts shows their inability for timely resolution of inflammation and Th-polarization toward remodeling. Th-polarization mediated transition of classic to alternative macrophage subpopulation is necessary for timely resolution of the inflammatory response [21], and was recently described during post-infarction remodeling [24]. Our data clearly demonstrate a CB2-dependent regulation of alternative M2a macrophage subpopulation, which is responsible for the postponed resolution of inflammatory response in Cnr2$^{-/-}$-hearts. The observed induction of CCL2 in Cnr2$^{-/-}$-alternative macrophages in vitro could be associated with these findings. We could also exclude effects of the CB2-deficiency on the tissue immigration of other inflammatory cells, e.g. neutrophils, lymphocytes or dendritic cells.

As a result of the inability to timely resolve the inflammatory process during repetitive I/R, Cnr2$^{-/-}$-hearts presented with prolonged, adverse myocardial remodeling, which did not influence replacement fibrosis. The loose interstitial collagen accumulation due to missing induction of cross-linking enzyme lysyl oxidase and microinfarctions in Cnr2$^{-/-}$-hearts further support the already postulated cardioprotective effects of interstitial collagen deposition in ischemic cardiomyopathy [12]. The reversible interstitial deposition of collagen mechanically hinders the cardiomyocytes to "overwork" under poor substrate availability conditions in ischemic heart and thus prevents irreversible loss of cardiac function. Studies provided evidence for involvement of CB2 receptor in liver fibrosis [14], but the available data on post-infarction fibrosis and remodeling are controversial. In contrast to the study reporting an increasing differentiation of Cnr2$^{-/-}$-myofibroblasts after H_2O_2-treatment in vitro [5], we found significantly less α-smac positive myofibroblasts in CB2-deficient myocardium in vivo, when compared to the WT-hearts. We also demonstrate in vivo and in vitro that CB2 receptor regulates tenascin C-expression in cardiac myofibroblasts. The adverse remodeling in Cnr2$^{-/-}$-hearts is further underlined by MMP/TIMP-ratios and our in vitro myofibroblasts data, which is in accordance to the postulated relation between CB2 receptor-dependent MMP activity in liver fibrosis [23].

Our data on endocannabinoid concentrations in tissue cannot be linked to a specific cell type in the heart. We found increasing concentrations of the endocannabinoid anandamide during repetitive I/R in Cnr2$^{-/-}$-hearts, which may reflect the rising burden of ischemia on cardiomyocytes, but it could also be associated with persistent macrophage actions. In contrast, WT-hearts showed only a short increase of anandamide tissue concentration during transient inflammatory response. Therefore, we suspect that the anandamide concentration is rather linked to the inflammatory response, than to the loss of cardiomyocytes. Indeed, in a recent study it was reported that anandamide concentration is increased only after 30 min of murine coronary occlusion and 24 h reperfusion, but not after permanent ligation [30]. This study used pharmacological intervention on CB2 receptor and found reduction in infarct size and CB2-dependent regulation of reactive oxygen species production in serum after 24 h when using CB2 agonist HU-308, thus postulating a cardioprotective effect of the CB2 receptor.

In conclusion, our study revealed a finely regulated and well-interconnected system of endocannabinoid- and CB2 receptor-mediated cardioprotection, mainly affecting cardiomyocytes and macrophages. The underlying

mechanisms on cardiomyocytes involve expression of CB2, modulation of contractile elements expression and reduction of oxidative stress to prevent apoptosis. CB2 receptor-related mechanisms on macrophages include regulation of inflammatory mediators during differentiation of macrophage subpopulations and timely resolution of inflammation, and are thereby affecting the subsequent myocardial remodeling. The data clearly stress the critical role of the endocannabinoid-CB2 receptor axis for cardiac adaptation to repetitive non-lethal ischemia episodes. The similarities of our mouse model with the human hibernating myocardium and ischemic heart disease, and the limited therapeutic options for patients with terminal heart failure [13] could make specific CB2 receptor agonists appealing for clinical applications in future.

Acknowledgments We thank Christine Peigney for expert assistance in immunohistochemistry, Tobias Heuft, Katharina Keppel and Andreas Feißt for performing RT-qPCR experiments, Frank Holst for performing the Langendorff perfusion, Stilla Frede and Markus Velten for performing the ELISA, Alexander von Ruecker for providing RT-qPCR equipment, Martin Breitbach for assistance in preparation and culture of embryonic cardiomyocytes, T. Schwandt for assistance in flow cytometry, Anne Zimmer for assistance in mouse breeding, Meliha Karsak for assistance in cell culture, Karola Poppensieker and Claudia Schwitter for excellent technical support in endocannabinoid measurements, and JoAnn Trial for helpful comments on macrophage experiments.

This work was supported by BONFOR Grants from Medical School, University of Bonn (to G.D.D., T.H. and O.D.) and by the Research Unit FOR926 from the Deutsche Forschungsgemeinschaft (DFG): O.D. and B.K.F. (Subproject 8, DE-801/1-2), B.L. (Central Project 1, Lu 775/4-1), and A.Z. (Central Project 2, Zi 361/5-1).

References

1. Alferink J, Tafuri A, Vestweber D, Hallmann R, Hammerling GJ, Arnold B (1998) Control of neonatal tolerance to tissue antigens by peripheral T cell trafficking. Science 282:1338–1341
2. Arnaud C, Burger F, Steffens S, Veillard NR, Nguyen TH, Trono D, Mach F (2005) Statins reduce interleukin-6-induced C-reactive protein in human hepatocytes: new evidence for direct antiinflammatory effects of statins. Arterioscler Thromb Vasc Biol 25:1231–1236. doi:10.1161/01.ATV.0000163840.63685.0c
3. Buckley NE, McCoy KL, Mezey E, Bonner T, Zimmer A, Felder CC, Glass M, Zimmer A (2000) Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. Eur J Pharmacol 396:141–149
4. Chen XL, Zhang Q, Zhao R, Medford RM (2004) Superoxide, H_2O_2, and iron are required for TNF-alpha-induced MCP-1 gene expression in endothelial cells: role of Rac1 and NADPH oxidase. Am J Physiol Heart Circ Physiol 286:H1001–H1007. doi:10.1152/ajpheart.00716.2003
5. Defer N, Wan J, Souktani R, Escoubet B, Perier M, Caramelle P, Manin S, Deveaux V, Bourin MC, Zimmer A, Lotersztajn S, Pecker F, Pavoine C (2009) The cannabinoid receptor type 2 promotes cardiac myocyte and fibroblast survival and protects against ischemia/reperfusion-induced cardiomyopathy. FASEB J 23:2120–2130. doi:10.1096/fj.09-129478
6. Dewald O, Frangogiannis NG, Zoerlein M, Duerr GD, Klemm C, Knuefermann P, Taffet G, Michael LH, Crapo JD, Welz A, Entman ML (2003) Development of murine ischemic cardiomyopathy is associated with a transient inflammatory reaction and depends on reactive oxygen species. Proc Natl Acad Sci USA 100:2700–2705. doi:10.1073/pnas.0438035100
7. Dorge H, Schulz R, Belosjorow S, Post H, van de Sand A, Konietzka I, Frede S, Hartung T, Vinten-Johansen J, Youker KA, Entman ML, Erbel R, Heusch G (2002) Coronary microembolization: the role of TNF-alpha in contractile dysfunction. J Mol Cell Cardiol 34:51–62. doi:10.1006/jmcc.2001.1489
8. Fluck M, Mund SI, Schittny JC, Klossner S, Durieux AC, Giraud MN (2008) Mechano-regulated tenascin-C orchestrates muscle repair. Proc Natl Acad Sci USA 105:13662–13667. doi:10.1073/pnas.0805365105
9. Frangogiannis NG, Dewald O, Xia Y, Ren G, Haudek S, Leucker T, Kraemer D, Taffet G, Rollins BJ, Entman ML (2007) Critical role of monocyte chemoattractant protein-1/CC chemokine ligand 2 in the pathogenesis of ischemic cardiomyopathy. Circulation 115:584–592. doi:10.1161/CIRCULATIONAHA.106.646091
10. Frangogiannis NG, Shimoni S, Chang SM, Ren G, Shan K, Aggeli C, Reardon MJ, Letsou GV, Espada R, Ramchandani M, Entman ML, Zoghbi WA (2002) Evidence for an active inflammatory process in the hibernating human myocardium. Am J Pathol 160:1425–1433. doi:10.1016/S0002-9440(10)62568-0
11. Heusch G (2013) Cardioprotection: chances and challenges of its translation to the clinic. Lancet 381:166–175. doi:10.1016/S0140-6736(12)60916-7
12. Heusch G (1998) Hibernating myocardium. Physiol Rev 78:1055–1085
13. Hofmann U, Frantz S (2013) How can we cure a heart "in flame"? A translational view on inflammation in heart failure. Basic Res Cardiol 108:356. doi:10.1007/s00395-013-0356-y
14. Julien B, Grenard P, Teixeira-Clerc F, Van Nhieu JT, Li L, Karsak M, Zimmer A, Mallat A, Lotersztajn S (2005) Antifibrogenic role of the cannabinoid receptor CB2 in the liver. Gastroenterology 128:742–755
15. Karsak M, Gaffal E, Date R, Wang-Eckhardt L, Rehnelt J, Petrosino S, Starowicz K, Steuder R, Schlicker E, Cravatt B, Mechoulam R, Buettner R, Werner S, Di Marzo V, Tuting T, Zimmer A (2007) Attenuation of allergic contact dermatitis through the endocannabinoid system. Science 316:1494–1497. doi:10.1126/science.1142265
16. Kloner RA, Bolli R, Marban E, Reinlib L, Braunwald E (1998) Medical and cellular implications of stunning, hibernation, and preconditioning: an NHLBI workshop. Circulation 97:1848–1867
17. Kolossov E, Bostani T, Roell W, Breitbach M, Pillekamp F, Nygren JM, Sasse P, Rubenchik O, Fries JW, Wenzel D, Geisen C, Xia Y, Lu Z, Duan Y, Kettenhofen R, Jovinge S, Bloch W, Bohlen H, Welz A, Hescheler J, Jacobsen SE, Fleischmann BK (2006) Engraftment of engineered ES cell-derived cardiomyocytes but not BM cells restores contractile function to the infarcted myocardium. J Exp Med 203:2315–2327. doi:10.1084/jem.20061469
18. Lepicier P, Bouchard JF, Lagneux C, Lamontagne D (2003) Endocannabinoids protect the rat isolated heart against ischaemia. Br J Pharmacol 139:805–815. doi:10.1038/sj.bjp.0705313
19. Lim SY, Davidson SM, Yellon DM, Smith CC (2009) The cannabinoid CB1 receptor antagonist, rimonabant, protects against acute myocardial infarction. Basic Res Cardiol 104:781–792. doi:10.1007/s00395-009-0034-2
20. Mach F, Montecucco F, Steffens S (2008) Cannabinoid receptors in acute and chronic complications of atherosclerosis. Br J Pharmacol 153:290–298. doi:10.1038/sj.bjp.0707517
21. Martinez FO, Sica A, Mantovani A, Locati M (2008) Macrophage activation and polarization. Front Biosci 13:453–461

22. Montecucco F, Lenglet S, Braunersreuther V, Burger F, Pelli G, Bertolotto M, Mach F, Steffens S (2009) CB(2) cannabinoid receptor activation is cardioprotective in a mouse model of ischemia/reperfusion. J Mol Cell Cardiol 46:612–620. doi:10.1016/j.yjmcc.2008.12.014
23. Munoz-Luque J, Ros J, Fernandez-Varo G, Tugues S, Morales-Ruiz M, Alvarez CE, Friedman SL, Arroyo V, Jimenez W (2008) Regression of fibrosis after chronic stimulation of cannabinoid CB2 receptor in cirrhotic rats. J Pharmacol Exp Ther 324:475–483. doi:10.1124/jpet.107.131896
24. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R, Pittet MJ (2007) The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med 204:3037–3047. doi:10.1084/jem.20070885
25. Pacher P, Ungvari Z (2008) Pleiotropic effects of the CB2 cannabinoid receptor activation on human monocyte migration: implications for atherosclerosis and inflammatory diseases. Am J Physiol Heart Circ Physiol 294:H1133–H1134. doi:10.1152/ajpheart.00020.2008
26. Pertwee RG (2006) Cannabinoid pharmacology: the first 66 years. Br J Pharmacol 147(Suppl 1):S163–S171. doi:10.1038/sj.bjp.0706406
27. Razeghi P, Essop MF, Huss JM, Abbasi S, Manga N, Taegtmeyer H (2003) Hypoxia-induced switches of myosin heavy chain isogene expression in rat heart. Biochem Biophys Res Commun 303:1024–1027
28. Sharma S, Dewald O, Adrogue J, Salazar RL, Razeghi P, Crapo JD, Bowler RP, Entman ML, Taegtmeyer H (2006) Induction of antioxidant gene expression in a mouse model of ischemic cardiomyopathy is dependent on reactive oxygen species. Free Radic Biol Med 40:2223–2231. doi:10.1016/j.freeradbiomed.2006.02.019
29. Shmist YA, Goncharov I, Eichler M, Shneyvays V, Isaac A, Vogel Z, Shainberg A (2006) Delta-9-tetrahydrocannabinol protects cardiac cells from hypoxia via CB2 receptor activation and nitric oxide production. Mol Cell Biochem 283:75–83. doi:10.1007/s11010-006-2346-y
30. Wang PF, Jiang LS, Bu J, Huang XJ, Song W, Du YP, He B (2012) Cannabinoid-2 receptor activation protects against infarct and ischemia–reperfusion heart injury. J Cardiovasc Pharmacol 59:301–307. doi:10.1097/FJC.0b013e3182418997
31. Yuan M, Kiertscher SM, Cheng Q, Zoumalan R, Tashkin DP, Roth MD (2002) Delta 9-tetrahydrocannabinol regulates Th1/Th2 cytokine balance in activated human T cells. J Neuroimmunol 133:124–131