

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2011 June ; 31(6): 1377–1386. doi:10.1161/ATVBAHA.111.225771.

Disrupting the EMMPRIN-Cyclophilin A interaction reduces infarct size and preserves systolic function after myocardial ischemia and reperfusion

Peter Seizer¹, Carmen Ochmann¹, Tanja Schönberger¹, Sebastian Zach¹, Melanie Rose¹, Oliver Borst^{1,6}, Karin Klingel², Reinhard Kandolf², H. Robson MacDonald³, Romana A. Nowak⁴, Stefan Engelhardt⁵, Florian Lang⁶, Meinrad Gawaz¹, and Andreas E. May¹

¹Medizinische Klinik III, Kardiologie und Kreislauferkrankungen, Eberhard Karls-Universität Tübingen, Germany ²Institut für Molekulare Pathologie, Eberhard Karls-Universität Tübingen, Germany ³Ludwig Center for Cancer Research of the University of Lausanne, Epalinges, Switzerland ⁴Department of Animal Sciences, University of Illinois, Urbana, IL 61801, USA ⁵Institut für Pharmakologie und Toxikologie, Technische Universität München, Germany ⁶Institute of Physiology, Eberhard Karls-Universität Tübingen, Germany

Abstract

Introduction—Inflammation and proteolysis crucially contribute to myocardial ischemia and reperfusion injury. The Extracellular Matrix Metalloproteinase Inducer (EMMPRIN, CD147) and its ligand Cyclophilin A (CyPA) may be involved in both processes. The aim of the study was to characterize the role of the CD147 and CyPA interplay in myocardial ischemia/reperfusion (I/R) injury.

Methods and results—Immunohistochemistry showed enhanced expression of CD147 and CyPA in myocardial sections from human autopsies of patients who had died from acute myocardial infarction and from mice at 24 hours after I/R. At 24 hours and 7 days after I/R, the infarct size was reduced in CD147^{+/-} mice versus CD147^{+/+} mice (C57Bl/6), in mice (C57Bl/6) treated with mAb anti-CD147 vs. control mAb, and in CyPA^{-/-} mice vs. CyPA^{+/+} mice (129SvEv), all of which being associated with reduced monocyte and neutrophil recruitment at 24h and with a preserved systolic function at 7 days. Combination of CyPA^{-/-} mice with anti-CD147 treatment did not yield further protection in comparison to either inhibition strategy alone. In vitro, treatment with CyPA induced monocyte chemotaxis in a CD147- and PI3-kinase dependent manner and induced monocyte rolling and adhesion to endothelium (HUVEC) under flow in a CD147-dependent manner.

Conclusion—CD147 and its ligand CyPA are inflammatory mediators after myocardial ischemia and reperfusion and represent potential targets to prevent myocardial I/R injury.

Correspondence to: Andreas E. May, MD, Medizinische Klinik III, Universitätsklinikum Tübingen, Otfried-Müller Str.10, 72076 Tübingen, Germany. Phone: +49-7071-29-83160; Fax: +49-7071-29-4473. andreas.may@med.uni-tuebingen.de.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosures

None.

Keywords

EMMPRIN; Cyclophilin A; MMPs; myocardial infarction

Introduction

The most effective treatment for acute myocardial infarction is the early coronary reperfusion which limits infarct size and preserves cardiac function^{1–3}. Various processes including the mitochondrial permeability transition pore (mPTP), and oxidative burst of reactive oxygen species (ROS) production contribute to myocardial injury^{4–7}. Ischemia and reperfusion (I/R) is accompanied by an extensive inflammatory response with leukocyte recruitment, induction of a complex inflammatory chemokine network as well as generation of matrix metalloproteinases (MMP)^{8–10}. Monocyte infiltration and reorganization of the extracellular matrix by metalloproteinases (MMPs) is critical for repair mechanisms of the infarcted myocardium, however, the exact role of leukocyte infiltration and MMPs in these remodeling processes is not clear yet^{9–12}. While optimal healing requires a balanced MMP-mediated extracellular matrix turnover¹³, inadequate MMP activity is thought to trigger an intensified remodeling process leading to fibrosis and cardiac rupture^{14, 15}.

Cyclophilin A (CyPA) and its receptor, the Extracellular Matrix Metalloproteinase Inducer (synonyms: CD147, EMMPRIN, basigin) represent a ligand/receptor pair that critically regulates both leukocyte recruitment and MMP activity^{16–18}. CD147 is involved in regulating MMPs in various cell types (e.g. tumor cells, monocytes, endothelial cells, smooth muscle cells), growth factors such as VEGF (tumor cells, endothelial cells) and NFκB-regulated cytokines like IL-6 or M-CSF (monocytes, macrophages, foam cells)^{18–23}. We have recently found that CD147 is upregulated on circulating monocytes in patients with acute myocardial infarction and that this regulator modulates MMP-9 and MT1-MMP activity in monocytes²⁰. Only recently Kandam et al. showed, that an inhibitor of MMPs, TIMP-2, reduces myocardial remodeling post infarction primarily via inhibition of MT1-MMP²⁴. CD147 serves as a surface receptor for CyPA^{16, 17}. CyPA is originally known as a chaperone and as an intracellular ligand for the immunosuppressive drug, cyclosporine²⁵. Upon cell activation or cell damage CyPA is released and binds to its extracellular receptor CD147 on various cell types.^{26–28} Binding of CyPA to CD147 results in outside-in signaling and induces NF-κB-regulated inflammatory early response genes including MMP-2 and/or MMP-9 in various cell types including monocytes/macrophages or smooth muscle cells^{20, 29, 30}. However, to the best of our knowledge, a functional in vivo relevance for CyPA/CD147 has not been described in myocardial infarction so far. In this study we show that CyPA/CD147 interaction is a critical mechanism in the pathophysiology of infarcted myocardium, mediates monocyte chemotaxis and represents a promising therapeutic target to preserve myocardial function.

Material and Methods

HE and Immunostaining

Paraffin embedded cardiac sections were stained using mAbs anti-CyPA (Abcam, Cambridge, MA, USA), anti-CD147 (Abcam), anti-Mac-3 (BD Biosciences, San Jose, California, USA), anti-CD3 (Neomarkers, Fremont, CA, USA), anti-PMN (NIMP-R14, GeneTex, Inc., Irvine, CA, USA) and isotype control mAb according to standard protocol. Rabbit anti-rat (Dako, Heverlee, Belgium), goat anti-rabbit (Dako) and donkey anti-rabbit mAbs (Amersham, Amersham, UK) were used as secondary antibodies depending on the respective primary antibody. Tissue sections were stained with a streptavidin-biotin-immunoperoxidase method (StreptABComplex/HRP, liquid DAB, Substrate-Chromogen,

Dako). Staining with hematoxylin and eosin was according to a standard protocol. Human cardiac tissue sections were obtained from autopsies of patients with acute myocardial infarction. The study was approved by the local Ethics Committee (Project-No. 253/2009BO2).

Myocardial ischemia and reperfusion in mice

10 to 12 weeks old mice CyPA^{+/+} and CyPA^{-/-} (129S6/SvEv, from Jackson laboratory) as well as C57Bl/6 and appropriate littermates were used. C57Bl/6 CD147^{+/-} and CD147^{+/+} mice were a kind gift from Dr. Takashi Muramatsu (Nagoya, Japan)³¹. After anesthesia of the mice and preparation of the left anterior descending artery (LAD) the LAD was ligated for 30min. Successful occlusion was confirmed by visual inspection of color in the apex. After 24 hours or 7 days of reperfusion the mice were sacrificed and the ischemic area (area at risk, AAR) was quantified by negative staining with 4% Evans Blue (after re-ligation of the left coronary artery at the level marked by the suture left in place) and the infarcted area (infarct size, IS) was detected by triphenyltetrazolium staining (Sigma Aldrich, St Louis, Mo, USA)³². Areas were digitally quantified by videoplanimetry. For in vivo inhibition experiments, blocking mAb anti-CD147 (RL 73.2) or its isotype control mAb (HB-189) were injected (1µg per g body weight) at the time of LAD ligation, 6 hrs and 18 hrs post ischemia, and then daily. Rat anti-mouse mAb anti-CD147 was purified from the RL73.2 hybridoma³³ by Genovac (Freiburg, Germany). Isotype control mAb obtained from American Type Culture Collection¹⁶ was purified from rat IgG2a hybridoma (HB-189) (Genovac).

Gelatin zymography and immunoblotting

For extraction of heart samples the free wall of the left ventricle was minced into 1-mm³ pieces and incubated with PBS containing 0.5% Triton X-100 (Sigma) and 0.01% sodium azide in 4 °C for 18h. The samples were centrifuged (14000 rpm, 10 min, 4 °C) and the supernatants were harvested. After determining concentration for each sample with the BioRad DC Protein Assay kit, 100µg were subjected to electrophoresis as described¹⁸. For Western blot analysis tissue of the free wall of the left ventricle was homogenized and lysated in lysis buffer containing protease inhibitors (Roche, Freiburg, Germany). We used rabbit anti-CyPA (Abcam) and anti-N-tyrosine (Santa Cruz), followed by a secondary fluorescence labeled Ab. Phospho-Akt (Ser 473) antibody (Cell signaling) was used in 1:1000 dilution with a fluorescence labelled secondary antibody (licor). Gelatin zymography and Western blots were quantified by imagJ software.

Echocardiography

was performed using a VEVO 770 ultrasound machine and a 30 Mhz linear transducer as described recently³⁴.

Chemotaxis and flow chamber assay

Monocytes were isolated as described¹⁸. Cell migration was performed using 48-well-modified Boyden chamber (Neuro Probe Inc) with two compartments separated by a 5 µm polycarbonate membrane. Monocytes were added to the upper compartments, and medium containing cardiac homogenates (1:200) from CyPA^{+/+} mice and CyPA^{-/-} mice or medium alone was added to the lower chamber. A chemotactic index was calculated for each well by dividing the number of migrating cells within each test well by the number of cells migrating to recombinant CyPA (R&D Systems GmbH). Heart homogenates were obtained from hearts of CyPA^{+/+} and CyPA^{-/-} mice by using a glass homogenizer and PBS containing a cocktail of protease inhibitors (Roche). Protein content was equilibrated in every sample (10µg/µl). For blocking studies anti-EMMPRIN (Ancell, clone UM-8D6) and

appropriate isotype control were used. LY294002 (Calbiochem, 25 μ M) and wortmannin (Calbiochem, 100nM) were used for inhibition of PI3-kinase. For flow chamber assay human umbilical vein endothelial cells (HUVEC) were allowed to adhere on glass coverslips until they were confluent and then activated with TNF α (50ng/ml, Peprotech) and INF γ (20ng/ml, Peprotech) over night (18h). Monocytes (2 $\times$ 10⁵/ml) were perfused over glass coverslips using arterial shear rates (2000 s⁻¹) as described³⁵.

Statistical analysis

Data are represented as mean $\pm$ SEM. Difference of means was analyzed by unpaired students t-Test. Values of $p < 0.05$ were considered significant.

An expanded Methods section is available in the Online Data Supplement at <http://atvb.ahajournals.org>.

Results

Expression of CyPA and CD147 is enhanced in infarcted myocardium obtained from humans and in mice

Cardiac sections from human autopsies of patients who had died from acute myocardial infarction (AMI) (n = 5) were immunostained with anti-CyPA and anti-CD147 and compared with specimen from autopsies of three patients with a virtually normal myocardial histology (HE), who had died of non-cardiac causes (figure 1A, upper panel). Both CyPA and its receptor CD147 were found to be highly expressed in myocardium derived from patients with AMI. Whereas CD147 was predominantly expressed on cardiomyocytes, CyPA appeared to be highly expressed on infiltrating leukocytes adjunct to the infarcted area (figure 1A, lower panel).

Similar results were obtained in mice with myocardial ischemia and reperfusion injury (I/R) (figure 1B): Myocardial expression of CyPA and CD147 was substantially enhanced 24h after a transient 30-minute-ligation of the LAD and was sustained for at least 7 days after ischemia and reperfusion (I/R) (figure 1B). Similar to human autopsies (figure 1A) CD147 was predominantly present on cardiomyocytes, whereas CyPA was highly expressed on infiltrating leukocytes consisting mainly of neutrophils (anti-PMN) and macrophages (anti-MAC-3) (figure 1B). At 7 days CyPA was still present in the scar mainly associated with macrophages (figure 1B). Interestingly, MMP-9 expression was primarily found in areas of infiltrating leukocytes both in the acute (d1) and subacute (d7) phase (figure 1B).

These data indicate that both the receptor CD147 and its ligand CyPA are physically present in substantial amounts in acute myocardial infarction allowing functional activities.

CyPA^{-/-} mice are protected from myocardial I/R injury

Based on the findings that CD147 and its ligand CyPA are upregulated in myocardial ischemia we analyzed CyPA-deficient mice (CyPA^{-/-})³⁶ in the model of myocardial ischemia and reperfusion (figure 2). After a 30min ligation of the left coronary artery (LAD) infarct size was determined in triphenyltetrazolium-stained myocardium on day 1 and day 7 of reperfusion. As compared to wild-type mice, the infarct size was significantly attenuated in CyPA-deficient mice at day 1 (figure 2A/B) and at 7 days (figure 2C). These data were confirmed by echocardiography: The systolic left ventricular function was quantified by analysis of the fractional area shortening and was found significantly preserved at day 7 (figure 2D).

CD147^{+/-} mice and mAb anti-CD147-treated WT-mice are largely protected from myocardial I/R injury

These data encouraged us to further evaluate the role of the cell-membrane-associated CyPA-receptor, CD147. We studied two additional animal models: i) CD147^{+/-} mice were analyzed in comparison to CD147^{+/+} mice and ii) wild-type mice were treated with a mAb anti-CD147,³⁷ which is known to solely inhibit the chemotactic activity of CD147³⁸. This antibody has been well characterized and used in various animal models to inhibit the interaction between extracellular CyPA and CD147³⁷⁻³⁹.

Since our breeding did not generate any viable complete CD147^{-/-} mice we decided to combine the analysis of heterozygote CD147^{+/-} mice with antibody blockade of CD147 in WT mice. In fact, CD147^{+/-} mice showed a decreased expression of CD147 in splenocytes (data not shown).

Figure 3B shows that infarct size at day 1 and at day 7 after the I/R is reduced to a similar extent in both animal models – in mice with reduced CD147 expression (CD147^{+/-}) and in WT mice treated with anti-CD147 in comparison to their respective control mice (d1: 24% reduction in CD147^{+/-}, 52% reduction in anti-CD147 treated mice; d7: 36% reduction in CD147^{+/-}, 46% reduction in anti-CD147 treated mice; figure 3 A/B). Moreover, decreased expression of CD147 (CD147^{+/-}) or antibody blockade of CD147 both resulted in a preserved left ventricular systolic function as analyzed by echocardiography (figure 3C). Interestingly, the use of mAb RL73.2 (anti-CD147), which predominantly blocks the chemotactic activity of CD147 rather than MMP-induction³⁸ yielded similar results as achieved in CyPA^{-/-} or CD147^{+/-} mice suggesting that CD147-mediated chemotaxis is an important pathway in this model of ischemia and reperfusion. In addition, these data suggest that a pharmacological inhibitory strategy directed against CD147 may have beneficial effects after myocardial ischemia. In fact, the application of the mAb anti-CD147 directly before opening the LAD also resulted in a significant reduction of infarct size (figure 3D) which was found to persist after a period of 28 days (figure 3E).

Anti-CD147 treatment does not yield further protection from I/R in CyPA^{-/-} mice

To further analyze the respective contribution of CyPA and CD147 in this model of I/R, we have treated CyPA^{-/-} mice with mAb anti-CD147. Interestingly, this additional treatment did not further decrease infarct size in comparison to CyPA^{-/-} treated with control mAb (figure 4). These data suggest that CyPA and CD147 act through a common pathway in mediating I/R injury.

CyPA and CD147 regulate macrophage and neutrophil recruitment in I/R injury

Previous data indicated that CyPA and CD147 regulate monocyte and also neutrophil recruitment in vivo^{37, 40}. Based on our findings that CyPA and CD147 expression is upregulated in myocardial infarction (figure 1) and that inhibition of the chemotactic activity of CD147 by a specific mAb largely protects mice from I/R injury over a 28 days period, we decided to study the influence of CyPA and CD147 on chemotactic activity in more detail.

In fact, the immunohistological analysis of cardiac sections revealed that monocyte and neutrophil recruitment was largely and to a comparable extent inhibited in the three mouse models: Neutrophil recruitment at day 1 into the infarct border zone was reduced by 65% in CyPA^{-/-} mice in comparison to CyPA^{+/+} mice, by 59% in CD147^{+/-} mice in comparison to CD147^{+/+} mice, and by 52% in mice treated with anti-CD147 mAb in comparison to mice treated with control-mAb ($p < 0.01$, figure 5). Similarly, macrophage recruitment was reduced by 58% in CyPA^{-/-} mice in comparison to CyPA^{+/+} mice, by 69% ($p < 0.01$) in

CD147^{+/-} mice in comparison to CD147^{+/+} mice, and by 55% ($p<0.01$) in mice treated with anti-CD147 mAb in comparison to mice treated with control-mAb ($p<0.01$, figure 5).

It has been described that the anti-CD147 antibody RL73.2 does not directly influence MMP-activity^{37, 38}. Interestingly, the analysis of the free wall of the left ventricle by zymography revealed no significant difference in MMP-2 and MMP-9 activity at 24h after I/R in anti-CD147 treated mice in comparison to isotype mAb-treated mice (figure 6A/B).

NADPH oxidase-derived reactive oxygen species play a major role for I/R injury⁴¹. We hypothesized that a reduced presence of monocytes and neutrophils may be accompanied by a reduced oxidative activity. By analyzing the left free wall of the infarcted ventricles after 24h of I/R we found a decreased presence of 3-nitrotyrosine residues as a measure for oxidative stress in anti-CD147 treated mice compared to their respective controls (figure 6C/D).

CyPA is a cardiac chemoattractant for monocytes and can induce monocyte adhesion

CyPA has previously been described as a potent chemokine for monocytes.¹⁷ We now studied the relative contribution and relevance of *cardiac* CyPA for monocyte chemoattraction. Hearts from wild-type (CyPA^{+/+}) and CyPA^{-/-} mice were explanted and homogenized as described in material and methods. In a modified boyden chamber CyPA^{+/+} cardiac homogenates exhibited a strong chemotactic activity for monocytes (figure 7B). In contrast, CyPA^{-/-} cardiac homogenates only exhibited a weak chemotactic activity (figure 7B). We hypothesized, if CyPA is a relevant factor in the wild-type homogenates the migration should be inhibited by the presence of recombinant CyPA in the upper chamber because of an alignment of the CyPA gradient. Indeed, adding 100nM recombinant CyPA into the upper chamber completely abolished monocytic migration towards CyPA^{+/+} homogenates. Similar inhibition was achieved by the presence of anti-CD147 into the upper compartment (figure 7B). These findings suggest that cardiac CyPA can function as a relevant chemokine in a CD147 dependent manner.

It has been reported that CD147 mediates phosphatidylinositol 3-kinase (PI3K) in tumor cells and smooth muscle cells^{42, 43}. Therefore we analyzed the expression of Phospho-Akt expression in monocytes after CyPA-treatment. Indeed CyPA induced Phospho-Akt. Additionally induction of Phospho-Akt as well as monocytic migration were abrogated by PI3-kinase inhibitors wortmannin and LY294002 (figure 7C/D).

Finally we investigated whether CyPA-activated monocytes show enhanced adhesion under flow conditions. We perfused monocytes over activated human umbilical vein endothelial cells (HUVECs) in a flow chamber under arterial shear stress and analyzed rolling and adherent monocytes. CyPA stimulation strongly induced monocyte rolling and adherence which could be inhibited by preincubation with anti-CD147 antibody (Figure 7E).

Discussion

This manuscript provides evidence that EMMPRIN (CD147) and its ligand CyPA play a pathophysiological role in the extent of myocardial injury after I/R in mice. By using various types of mouse models we show that i) expression of CyPA and CD147 is enhanced in infarcted myocardium obtained from humans and upon I/R injury in mice ii) absence of CyPA in CyPA^{-/-} mice or decreased expression of CD147 in CD147^{+/-} mice protects mice from myocardial injury upon I/R with respect to infarct size and systolic function at 7 days, and iii) pharmacological inhibition of the chemotactic function of CD147 by mAb anti-CD147 decreases monocyte/macrophage and neutrophil recruitment and infarct size and preserves left ventricular function. Interestingly, treatment of CyPA^{-/-} mice with mAb anti-

CD147 yields no additional benefit as compared to otherwise untreated CyPA^{-/-} mice or mAb anti-CD147-treated WT mice, which suggests a common pathway. The fact, that the application of the mAb anti-CD147 at the time of reperfusion reduced myocardial injury to a comparable extent as achieved by treatment before induction of ischemia makes a future therapeutic strategy in humans at the time of reperfusion (primary PCI) feasible³. The protective effect of mAb anti-CD147 treatment persisted over at least 28 days.

In all mouse models monocyte/macrophage and neutrophil recruitment were reduced at 24 hours. In addition, oxidative stress as measured by the expression of 3-nitrotyrosine residues was reduced in mice treated with mAb anti-CD147 as compared to control IgG. This study cannot definitely (and was not intended to) clarify the general role of leukocyte recruitment in I/R or of distinct (sub)types of leukocytes. Currently, it is well established that CD147 ligation by its extracellular ligand CyPA activates leukocyte chemotaxis⁴⁴. Constant and coworkers have characterized an anti-mouse CD147 mAb, which specifically reduced leukocyte accumulation and reduced collagen-induced arthritis and bronchial hyperreactivity in two mouse models, respectively^{37, 38}. In our study, the application of this mAb, which specifically inhibits the chemotactic activity of CD147 rather than MMP-induction³⁸ severely reduced monocyte/macrophage and neutrophil infiltration into the infarct border zone, reduced the extent of infarct size and improved left ventricular systolic function. This specific inhibition of the chemotactic function of CD147 yielded similar effects as found in mice with decreased expression of CD147 (CD147^{+/-}). Given that different mouse strains were used, the extent of the inhibition of leukocyte recruitment, infarct size and improvement of systolic function appears to be similar in CyPA^{-/-} mice. Notably we did not find any differences in MMP-activity in myocardial samples of CD147^{+/-} mice vs. CD147^{+/+} (not shown) or in mice treated either with an anti-CD147 mAb or isotype mAb (figure 6A/B). The decreased monocyte and neutrophil recruitment may account at least in part for the observed reduced oxidative stress as assessed by 3-nitrotyrosine expression.

In vitro experiments further support the concept that leukocyte recruitment is a relevant consequence of CyPA/CD147 interaction: i) Monocytic chemotaxis towards homogenates from CyPA^{+/+} hearts was inhibited in the presence of mAb anti-CD147 or by application of CyPA into the upper compartment and was reduced towards CyPA^{-/-} cardiac homogenates (figure 7B). ii) CyPA induces Phospho-Akt in monocytes, and inhibition of PI3-kinase by wortmannin or Ly294002 inhibits CyPA-induced monocyte chemotaxis (figure 7C/D); iii) apart from chemotaxis, CyPA induces monocyte rolling and adhesion to endothelial cells (HUVEC) under arterial flow conditions in a CD147-dependent manner (figure 7E). Notably, an involvement of RISK (reperfusion injury salvage kinases) signalling in cardioprotection was found in rodents but not in pigs⁴⁵.

Our data are supported by the work of Berk and coworkers using CyPA^{-/-} mice in two cardiovascular models: The absence of CyPA was resulted in decreased angiotensin II-induced aneurysm formation⁴⁰ as well as in reduced atherosclerosis in ApoE/CyPA^{-/-} mice⁴⁶. In both models, the benefit was associated with reduced leukocyte recruitment. These two studies did not discriminate between intra- and extracellular effects of CyPA nor investigate the role of CD147.

Here we provide evidence that CyPA and CD147 represent important factors for the recruitment of neutrophils and monocytes and the resulting myocardial injury upon I/R. Although we do not provide clear evidence, the link between CyPA and CD147 is supported by two observations: i) The extent of the effects on leukocyte recruitment, infarct size and systolic function upon I/R was comparable in the two models that interfere with CyPA (CyPA^{-/-} vs. CyPA^{+/+}) and CD147 (mAb anti-CD147 vs. isotype IgG). ii) The combined

inhibitory strategy using mAb anti-CD147 in CyPA^{-/-} mice did not yield any additional benefit suggesting a common pathway for CyPA and CD147.

There exist some established inhibitors of CyPA such as cyclosporine A (CsA) or its analogue NIM811. However, in addition to the blockade of extracellular CyPA interactions with CD147, these drugs interfere with various intracellular CyPA activities such as inhibition of the calcium-activated protein phosphatase calcineurin (CsA), protein folding (PPIase activity), and inhibit intracellular CyPD-mediated processes involving the mitochondrial permeability transition pore (mPTP)^{5, 6}. In specific, CyPD-mediated inhibition of the mPTP provides a powerful antiischemic protection⁴. Treatment with CsA or NIM811 have been shown to effectively protect from myocardial injury in various animal models of I/R^{5, 6, 47} and CsA decreases infarct size in patients with acute myocardial infarction⁴⁸. It has been suggested that the CyPD-mediated inhibition of the mPTP mainly accounted for the protective effects.

The focus of our study was to elucidate the role of the extracellular receptor of CyPA, CD147 (EMMPRIN) by avoiding potential intracellular crossreactions, specifically CyPD-mediated mitochondrial effects. To our knowledge, there is no known direct interaction between CyPA and CyPD, namely no effects of CyPA on e.g. the mPTP. Therefore, we have decided to pharmacologically interfere with the extracellular counterpart of CyPA, CD147 in addition to studying CD147^{+/-} mice and CyPA^{-/-} mice. The experiments using CyPA^{-/-} mice clearly demonstrate the relevance of the entire CyPA system and should not directly influence the CyPD-dependent mPTP. The data using anti-CD147 mAb or the CD147^{+/-} mice provide clear evidence that the CD147-mediated pathway is of relevance.

In conclusion our data demonstrate that CD147 and its ligand CyPA are crucially involved in mediating I/R injury and may represent therapeutic targets for myocardial salvage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The technical assistance of Klaudia Posavec is highly appreciated. We thank Harald Langer for critical reading the manuscript.

Sources of funding

This study was supported by the Deutsche Stiftung für Herzforschung (to P.S.), the Fortüne Program (to P.S.) and the Deutsche Forschungsgesellschaft (DFG, SFB TR19.9 to AEM and MG, DFG Li 683/2-1 to M.G.) and by an NIH SCCPRR U54 grant (#HD40093) to RAN.

References

1. Cannon CP, Gibson CM, Lambrew CT, Shultz DA, Levy D, French WJ, Gore JM, Weaver WD, Rogers WJ, Tiefenbrunn AJ. Relationship of symptom-onset-to-balloon time and door-to-balloon time with mortality in patients undergoing angioplasty for acute myocardial infarction. *JAMA*. 2000 June 14;283:2941–2947. [PubMed: 10865271]
2. Ovize M, Baxter GF, Di LF, Ferdinandy P, Garcia-Dorado D, Hausenloy DJ, Heusch G, Vinten-Johansen J, Yellon DM, Schulz R. Postconditioning and protection from reperfusion injury: where do we stand? Position paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology. *Cardiovasc Res*. 2010 August 1;87:406–423. [PubMed: 20448097]
3. Hausenloy DJ, Baxter G, Bell R, Botker HE, Davidson SM, Downey J, Heusch G, Kitakaze M, Lecour S, Mentzer R, Mocanu MM, Ovize M, Schulz R, Shannon R, Walker M, Walkinshaw G,

- Yellon DM. Translating novel strategies for cardioprotection: the Hatter Workshop Recommendations. *Basic Res Cardiol*. 2010 November.105:677–686. [PubMed: 20865418]
4. Heusch G, Boengler K, Schulz R. Inhibition of mitochondrial permeability transition pore opening: the Holy Grail of cardioprotection. *Basic Res Cardiol*. 2010 March.105:151–154. [PubMed: 20066536]
 5. Argaud L, Gateau-Roesch O, Muntean D, Chalabreysse L, Loufouat J, Robert D, Ovize M. Specific inhibition of the mitochondrial permeability transition prevents lethal reperfusion injury. *J Mol Cell Cardiol*. 2005 February.38:367–374. [PubMed: 15698843]
 6. Argaud L, Gateau-Roesch O, Raissy O, Loufouat J, Robert D, Ovize M. Postconditioning inhibits mitochondrial permeability transition. *Circulation*. 2005 January 18.111:194–197. [PubMed: 15642769]
 7. Heusch G, Boengler K, Schulz R. Cardioprotection: nitric oxide, protein kinases, and mitochondria. *Circulation*. 2008 November 4.118:1915–1919. [PubMed: 18981312]
 8. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc Res*. 2002 January.53:31–47. [PubMed: 11744011]
 9. Liehn EA, Piccinini AM, Koenen RR, Soehnlein O, Adage T, Fatu R, Curaj A, Popescu A, Zernecke A, Kungl AJ, Weber C. A new monocyte chemotactic protein-1/chemokine CC motif ligand-2 competitor limiting neointima formation and myocardial ischemia/reperfusion injury in mice. *J Am Coll Cardiol*. 2010 November 23.56:1847–1857. [PubMed: 21087715]
 10. Kaikita K, Hayasaki T, Okuma T, Kuziel WA, Ogawa H, Takeya M. Targeted deletion of CC chemokine receptor 2 attenuates left ventricular remodeling after experimental myocardial infarction. *Am J Pathol*. 2004 August.165:439–447. [PubMed: 15277218]
 11. Creemers E, Cleutjens J, Smits J, Heymans S, Moons L, Collen D, Daemen M, Carmeliet P. Disruption of the plasminogen gene in mice abolishes wound healing after myocardial infarction. *Am J Pathol*. 2000 June.156:1865–1873. [PubMed: 10854210]
 12. Arslan F, Smeets MB, O'Neill LA, Keogh B, McGuirk P, Timmers L, Tersteeg C, Hoefer IE, Doevendans PA, Pasterkamp G, de Kleijn DP. Myocardial ischemia/reperfusion injury is mediated by leukocytic toll-like receptor-2 and reduced by systemic administration of a novel anti-toll-like receptor-2 antibody. *Circulation*. 2010 January 5.121:80–90. [PubMed: 20026776]
 13. Yarbrough WM, Mukherjee R, Escobar GP, Mingoia JT, Sample JA, Hendrick JW, Dowdy KB, McLean JE, Lowry AS, O'Neill TP, Spinale FG. Selective targeting and timing of matrix metalloproteinase inhibition in post-myocardial infarction remodeling. *Circulation*. 2003 October 7.108:1753–1759. [PubMed: 12975256]
 14. Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, Dyspersin GD, Cleutjens JP, Shipley M, Angellilo A, Levi M, Nube O, Baker A, Keshet E, Lupu F, Herbert JM, Smits JF, Shapiro SD, Baes M, Borgers M, Collen D, Daemen MJ, Carmeliet P. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nat Med*. 1999 October.5:1135–1142. [PubMed: 10502816]
 15. Matsumura S, Iwanaga S, Mochizuki S, Okamoto H, Ogawa S, Okada Y. Targeted deletion or pharmacological inhibition of MMP-2 prevents cardiac rupture after myocardial infarction in mice. *J Clin Invest*. 2005 March.115:599–609. [PubMed: 15711638]
 16. Arora K, Gwinn WM, Bower MA, Watson A, Okwumabua I, MacDonald HR, Bukrinsky MI, Constant SL. Extracellular cyclophilins contribute to the regulation of inflammatory responses. *J Immunol*. 2005 July 1.175:517–522. [PubMed: 15972687]
 17. Yurchenko V, Constant S, Bukrinsky M. Dealing with the family: CD147 interactions with cyclophilins. *Immunology*. 2006 March.117:301–309. [PubMed: 16476049]
 18. Seizer P, Schonberger T, Schott M, Lang MR, Langer HF, Bigalke B, Kramer BF, Borst O, Daub K, Heidenreich O, Schmidt R, Lindemann S, Herouy Y, Gawaz M, May AE. EMMPRIN and its ligand cyclophilin A regulate MT1-MMP, MMP-9 and M-CSF during foam cell formation. *Atherosclerosis*. 2010 March.209:51–57. [PubMed: 19758589]
 19. Schmidt R, Bultmann A, Fischel S, Gillitzer A, Cullen P, Walch A, Jost P, Ungerer M, Tolley ND, Lindemann S, Gawaz M, Schomig A, May AE. Extracellular matrix metalloproteinase inducer (CD147) is a novel receptor on platelets, activates platelets, and augments nuclear factor kappaB-

- dependent inflammation in monocytes. *Circ Res*. 2008 February 15;102:302–309. [PubMed: 18048771]
20. Schmidt R, Bultmann A, Ungerer M, Joghetaei N, Bulbul O, Thieme S, Chavakis T, Toole BP, Gawaz M, Schomig A, May AE. Extracellular matrix metalloproteinase inducer regulates matrix metalloproteinase activity in cardiovascular cells: implications in acute myocardial infarction. *Circulation*. 2006 February 14;113:834–841. [PubMed: 16461815]
 21. Schmidt R, Redecke V, Breitfeld Y, Wantia N, Miethke T, Massberg S, Fischel S, Neumann FJ, Schomig A, May AE. EMMPRIN (CD 147) is a central activator of extracellular matrix degradation by *Chlamydia pneumoniae*-infected monocytes. Implications for plaque rupture. *Thromb Haemost*. 2006 January;95:151–158. [PubMed: 16543974]
 22. Tang Y, Nakada MT, Kesavan P, McCabe F, Millar H, Rafferty P, Bugelski P, Yan L. Extracellular matrix metalloproteinase inducer stimulates tumor angiogenesis by elevating vascular endothelial cell growth factor and matrix metalloproteinases. *Cancer Res*. 2005 April 15;65:3193–3199. [PubMed: 15833850]
 23. Bougateg F, Quemener C, Kellouche S, Naimi B, Podgorniak MP, Millot G, Gabison EE, Calvo F, Dosquet C, Lebbe C, Menashi S, Mourah S. EMMPRIN promotes angiogenesis through hypoxia-inducible factor-2{alpha}-mediated regulation of soluble VEGF isoforms and their receptor VEGFR-2. *Blood*. 2009 December 24;114:5547–5556. [PubMed: 19837976]
 24. Kandalam V, Basu R, Abraham T, Wang X, Soloway PD, Jaworski DM, Oudit GY, Kassiri Z. TIMP2 Deficiency Accelerates Adverse Post-Myocardial Infarction Remodeling Because of Enhanced MT1-MMP Activity Despite Lack of MMP2 Activation. *Circ Res*. 2010 Mar 5;106:796–808. [PubMed: 20056917]
 25. Marks AR. Cellular functions of immunophilins. *Physiol Rev*. 1996 July;76:631–649. [PubMed: 8757784]
 26. Sherry B, Yarlett N, Strupp A, Cerami A. Identification of cyclophilin as a proinflammatory secretory product of lipopolysaccharide-activated macrophages. *Proc Natl Acad Sci U S A*. 1992 April 15;89:3511–3515. [PubMed: 1565646]
 27. Jin ZG, Melaragno MG, Liao DF, Yan C, Haendeler J, Suh YA, Lambeth JD, Berk BC. Cyclophilin A is a secreted growth factor induced by oxidative stress. *Circ Res*. 2000 October 27;87:789–796. [PubMed: 11055983]
 28. Seko Y, Fujimura T, Taka H, Mineki R, Murayama K, Nagai R. Hypoxia followed by reoxygenation induces secretion of cyclophilin A from cultured rat cardiac myocytes. *Biochem Biophys Res Commun*. 2004 April 23;317:162–168. [PubMed: 15047162]
 29. Wang L, Wang CH, Jia JF, Ma XK, Li Y, Zhu HB, Tang H, Chen ZN, Zhu P. Contribution of cyclophilin A to the regulation of inflammatory processes in rheumatoid arthritis. *J Clin Immunol*. 2010 January;30:24–33. [PubMed: 19789967]
 30. Yang Y, Lu N, Zhou J, Chen ZN, Zhu P. Cyclophilin A up-regulates MMP-9 expression and adhesion of monocytes/macrophages via CD147 signalling pathway in rheumatoid arthritis. *Rheumatology (Oxford)*. 2008 September;47:1299–1310. [PubMed: 18567920]
 31. Igakura T, Kadomatsu K, Kaname T, Muramatsu H, Fan QW, Miyauchi T, Toyama Y, Kuno N, Yuasa S, Takahashi M, Senda T, Taguchi O, Yamamura K, Arimura K, Muramatsu T. A null mutation in basigin, an immunoglobulin superfamily member, indicates its important roles in peri-implantation development and spermatogenesis. *Dev Biol*. 1998 February 15;194:152–165. [PubMed: 9501026]
 32. Weig HJ, Bott-Flugel L, Stadele C, Winter K, Schmidt R, Gawaz M, Laugwitz KL, Seyfarth M. Impaired platelet function reduces myocardial infarct size in Galphaq knock-out mice in vivo. *J Mol Cell Cardiol*. 2008 January;44:143–150. [PubMed: 18021799]
 33. Renno T, Wilson A, Dunkel C, Coste I, Maisnier-Patin K, oit de CA, Aubry JP, Lees RK, Bonnefoy JY, MacDonald HR, Gauchat JF. A role for CD147 in thymic development. *J Immunol*. 2002 May 15;168:4946–4950. [PubMed: 11994445]
 34. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, Just S, Rottbauer W, Frantz S, Castoldi M, Soutschek J, Koteliansky V, Rosenwald A, Basson MA, Licht JD, Pena JT, Rouhanifard SH, Muckenthaler MU, Tuschl T, Martin GR, Bauersachs J, Engelhardt S. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. *Nature*. 2008 December 18;456:980–984. [PubMed: 19043405]

35. Seizer P, Borst O, Langer HF, Bultmann A, Munch G, Herouy Y, Stellos K, Kramer B, Bigalke B, Buchele B, Bachem MG, Vestweber D, Simmet T, Gawaz M, May AE. EMMPRIN (CD147) is a novel receptor for platelet GPVI and mediates platelet rolling via GPVI-EMMPRIN interaction. *Thromb Haemost*. 2009 April.101:682–686. [PubMed: 19350111]
36. Colgan J, Asmal M, Yu B, Luban J. Cyclophilin A-deficient mice are resistant to immunosuppression by cyclosporine. *J Immunol*. 2005 May 15.174:6030–6038. [PubMed: 15879096]
37. Gwinn WM, Damsker JM, Falahati R, Okwumabua I, Kelly-Welch A, Keegan AD, Vanpouille C, Lee JJ, Dent LA, Leitenberg D, Bukrinsky MI, Constant SL. Novel approach to inhibit asthma-mediated lung inflammation using anti-CD147 intervention. *J Immunol*. 2006 October 1.177:4870–4879. [PubMed: 16982929]
38. Damsker JM, Okwumabua I, Pushkarsky T, Arora K, Bukrinsky MI, Constant SL. Targeting the chemotactic function of CD147 reduces collagen-induced arthritis. *Immunology*. 2009 January. 126:55–62. [PubMed: 18557953]
39. Agrawal SM, Silva C, Tourtellotte WW, Yong VW. EMMPRIN: a novel regulator of leukocyte transmigration into the CNS in multiple sclerosis and experimental autoimmune encephalomyelitis. *J Neurosci*. 2011 January 12.31:669–677. [PubMed: 21228176]
40. Satoh K, Nigro P, Matoba T, O'Dell MR, Cui Z, Shi X, Mohan A, Yan C, Abe J, Illig KA, Berk BC. Cyclophilin A enhances vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms. *Nat Med*. 2009 June.15:649–656. [PubMed: 19430489]
41. Doerries C, Grote K, Hilfiker-Kleiner D, Luchtefeld M, Schaefer A, Holland SM, Sorrentino S, Manes C, Schieffer B, Drexler H, Landmesser U. Critical role of the NAD(P)H oxidase subunit p47phox for left ventricular remodeling/dysfunction and survival after myocardial infarction. *Circ Res*. 2007 March 30.100:894–903. [PubMed: 17332431]
42. Venkatesan B, Valente AJ, Reddy VS, Siwik DA, Chandrasekar B. Resveratrol blocks interleukin-18-EMMPRIN cross-regulation and smooth muscle cell migration. *Am J Physiol Heart Circ Physiol*. 2009 August.297:H874–H886. [PubMed: 19561311]
43. Tang Y, Nakada MT, Rafferty P, Laraio J, McCabe FL, Millar H, Cunningham M, Snyder LA, Bugelski P, Yan L. Regulation of vascular endothelial growth factor expression by EMMPRIN via the PI3K-Akt signaling pathway. *Mol Cancer Res*. 2006 June.4:371–377. [PubMed: 16778084]
44. Bukrinsky MI. Cyclophilins: unexpected messengers in intercellular communications. *Trends Immunol*. 2002 July.23:323–325. [PubMed: 12103338]
45. Skyschally A, van CP, Boengler K, Gres P, Musiolik J, Schilawa D, Schulz R, Heusch G. Ischemic postconditioning in pigs: no causal role for RISK activation. *Circ Res*. 2009 January 2.104:15–18. [PubMed: 19038864]
46. Nigro P, Satoh K, O'Dell MR, Soe NN, Cui Z, Mohan A, Abe J, Alexis JD, Sparks JD, Berk BC. Cyclophilin A is an inflammatory mediator that promotes atherosclerosis in apolipoprotein E-deficient mice. *J Exp Med*. 2011 January 17.208:53–66. [PubMed: 21173104]
47. Skyschally A, Schulz R, Heusch G. Cyclosporine A at reperfusion reduces infarct size in pigs. *Cardiovasc Drugs Ther*. 2010 February.24:85–87. [PubMed: 20182781]
48. Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung TT, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G, Finet G, ndre-Fouet X, Revel D, Kirkorian G, Monassier JP, Derumeaux G, Ovize M. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *N Engl J Med*. 2008 July 31.359:473–481. [PubMed: 18669426]

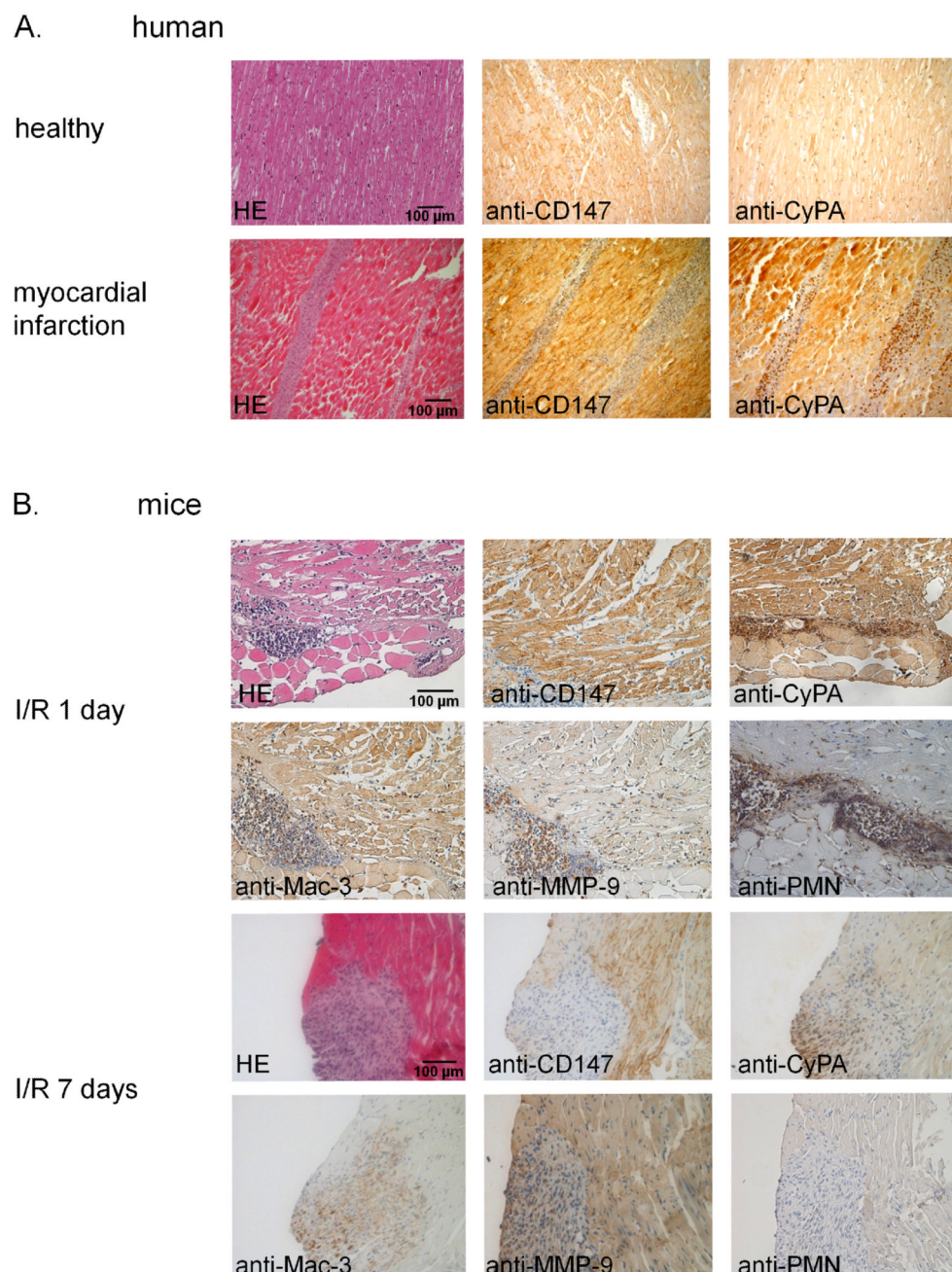


Figure 1. CD147 and CyPA in acute myocardial infarction and I/R injury

(A) Cardiac sections from human autopsies of patients who had died of acute myocardial infarction (MI) ($n = 5$) were stained with hematoxylin and eosin (HE) and immunostained for CyPA and CD147 and compared with specimen from autopsies of three patients, who had died of non-cardiac reasons and who had a “normal” histology. (B) Serial cardiac sections of mice after I/R injury at day 1 and day 7 were stained with HE and immunostained for CD147, CyPA, macrophages (anti-Mac3), neutrophils (anti-PMN) and MMP-9. Representative images of $n \geq 3$ are shown, respectively.

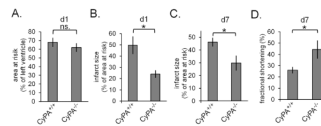


Figure 2. CyPA^{-/-} mice are protected from myocardial I/R injury

I/R injury was initiated in CyPA^{+/+} and CyPA^{-/-} mice as described in methods. After 1 day (d1) or 7 days (d7) mice were sacrificed and analyzed for area at risk and infarct size A) Evans Blue staining showed a comparable area at risk (related to the left ventricle in %) after I/R injury at day 1 (d1). B/C) Quantitative analysis of infarct size (related to area at risk in %) at day 1 (d1, B) and day 7 (d7, C) (n=7). D) Echocardiographic analysis of fractional area shortening as an indicator for left ventricular systolic function after 7 days (d7) (n=5). * indicates p<0.05 between groups.

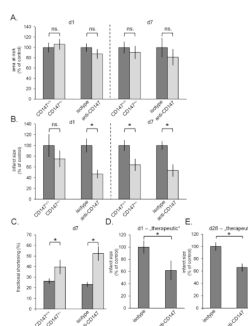


Figure 3. CD147^{+/-} mice and mAb anti-CD147-treated mice are protected from myocardial I/R injury

I/R injury was initiated in CD147^{+/+} and CD147^{+/-} mice (C57BL/6) as well as in C57BL/6 mice with treatment with mAb anti-CD147 or isotype control mAb (1 µg/g i.p., each) as described in methods. After 1 day (d1) or 7 days (d7) mice were sacrificed and analyzed for area at risk and infarct size (n≥5, each). (A) Evans Blue staining showed a comparable area at risk (in % of the respective control) after I/R injury in all groups of mice. (B) Quantitative analysis of infarct size in % of the respective control (C) Echocardiographic quantitative analysis of fractional area shortening at day 7 (n≥5), (D/E) C57BL/6 mice were treated with mAb anti-CD147 or an isotype control mAb (1 µg/g i.p., each) in a more “therapeutic setting”. First application of the antibody was performed directly before reopening of the LAD. Quantitative analysis of infarct size (D) at day 1 (n=7) and (E) day 28 (n=5), * indicates p<0.05.

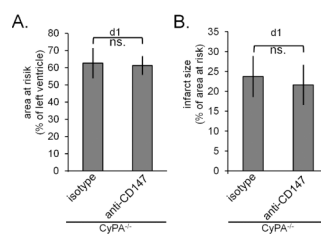


Figure 4. Anti-CD147 treatment does not yield further protection from I/R in $CyPA^{-/-}$ mice
 I/R injury was initiated in $CyPA^{-/-}$ mice which were treated with mAb anti-CD147 or isotype control mAb (1 μ g/g i.p., each) as described in methods. After 1 day mice were sacrificed and analyzed for area at risk and infarct size (n=6, each). (A) Evans Blue staining showed a comparable area at risk (in % of the respective control) after I/R injury. (B) Quantitative analysis of infarct size in % of area at risk; n.s. indicates not significant ($p > 0.05$)

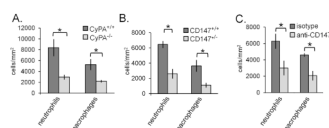


Figure 5. CyPA and CD147 regulate macrophage and neutrophil recruitment in I/R injury
I/R injury was initiated as described in methods. After 1 day (d1) of reperfusion cardiac sections of mice were analyzed for neutrophil (anti-PMN) and monocyte (anti-Mac3) infiltration into the infarct border zones. (A) $CyPA^{+/+}$ vs. $CyPA^{-/-}$ (n=4); (B) C57BL/6 $CD147^{+/+}$ vs. $CD147^{+/-}$ (n=4); (C) anti-CD147 treated mice vs. isotype treated C57BL/6 mice (n=5). * indicates $p < 0.01$.

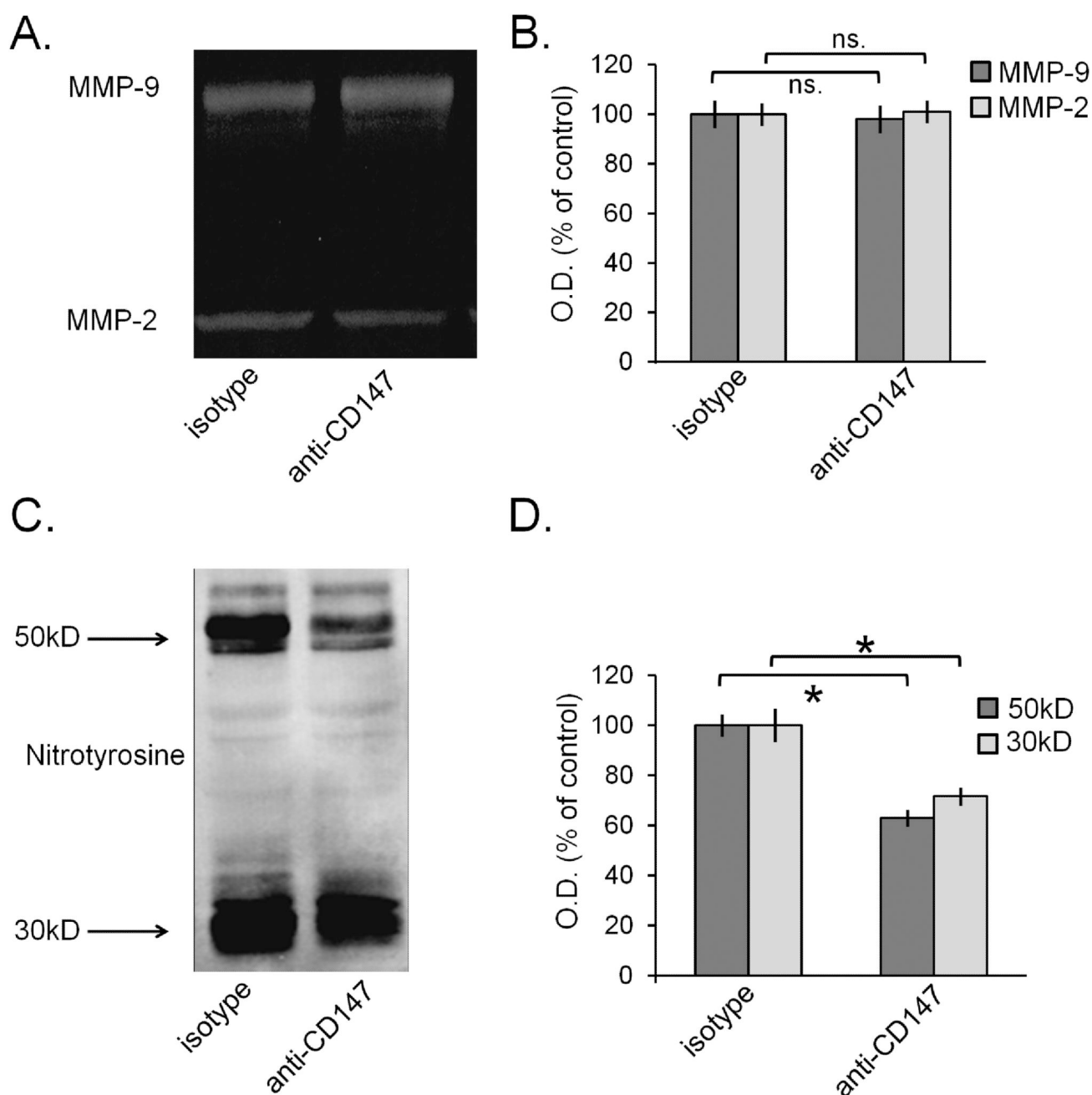


Figure 6. Myocardial MMP activity and oxidative stress in anti-CD147 treated mice after I/R Mice were treated with mAb anti CD147 or isotype IgG and underwent I/R injury as described in methods. After 24 hours mice were sacrificed and left ventricular myocardial tissue (free wall) was analyzed. (A) Representative zymography (n=4) and (B) analysis by optical density (O.D.) shows no difference for MMP-2 and MMP-9. (C/D) Representative Western blot analysis (n=3) for 3-nitrotyrosine residues and quantification by optical density * indicates $p<0.05$

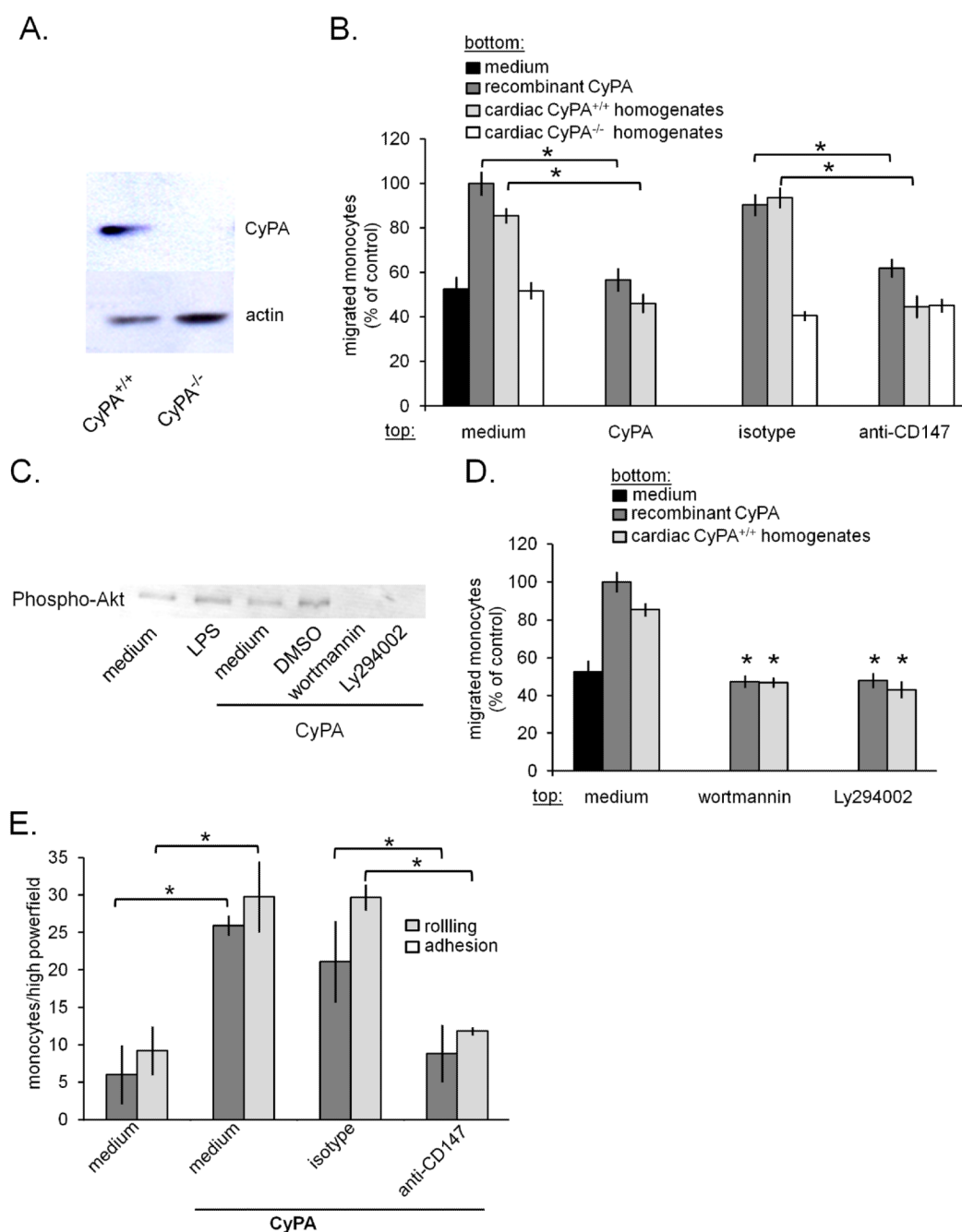


Figure 7. Cardiac-derived CyPA induces chemoattraction and adhesion of monocytes

(A) Expression of CyPA in cardiac homogenates of CyPA^{+/+} vs. CyPA^{-/-} mice. One representative immunoblot (of three) is shown. Actin was used as internal loading control. (B) Chemotaxis of human monocytes from the upper compartment (top) containing medium, recombinant CyPA (100nM), isotype-control mAb or anti-CD147 (20μg/ml each) as indicated towards the lower compartment (bottom) containing medium (negative control, black bar), recombinant CyPA (100nM, dark bars), medium containing homogenates from CyPA^{+/+} (grey bars) or CyPA^{-/-} hearts (white bars) (n=6). (C) Human isolated monocytes were pretreated with medium, DMSO, wortmannin (100nM) or LY294002 (25μM) as indicated. Then cells were treated with medium, lipopolysaccharide (positive control, 100ng/

ml), or CyPA (200nM) as indicated over night. Cells were lysated and Phospho-Akt expression was analyzed by Western blotting. (D) Chemotaxis of human monocytes from the upper compartment (top) containing medium, wortmannin (100nM) or Ly294002 (25μM) as indicated towards the lower compartment (bottom) containing medium (negative control, black bar), recombinant CyPA (100nM, dark bars), or medium containing homogenates from CyPA^{+/+} hearts (grey bars) (n=6). (E) After stimulation with medium or CyPA (100nM, 12h) human monocytes (2×10⁵/ml) were perfused over activated (TNFα and INFγ as described in material and methods) human umbilical vein endothelial cells (HUVEC) under arterial shear conditions. Cells were pretreated with medium, isotype or anti-CD147 mAb (20mg/ml each) as indicated. Monocyte rolling and firm adhesion were counted for 1 minute (n=5). * indicates p<0.05 between groups.