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The role of inflammation in cardiac and vascular remodelling

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Chapter 2

Anti phosphorylcholine antibodies reduce infarct size and left ventricular dilatation by inhibiting the early and late inflammatory response following myocardial infarction

Submitted for publication

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Abstract

Background: PC-mAb is a human IgG1 directed against phosphorylcholine that has anti-inflammatory properties. In this study, we hypothesized PC-mAb treatment would reduce adverse cardiac remodeling and infarct size (IS) following myocardial infarction (MI).

Methods: MI was induced by permanent ligation of the LAD coronary artery in hypercholesterolemic ApoE*3-Leiden mice. Cardiac function and IS were assessed using cardiac magnetic resonance imaging (MRI). Using (immuno)histological analysis we determined left ventricle (LV) fibrous content, LV wall thickness and leukocyte infiltration. ELISA and FACS analyses were used to study the systemic inflammatory response.

Results: We found a 21% reduced LV diastolic volume and 31% reduced IS following PC-mAb treatment after three weeks compared to untreated mice. The decreased IS was confirmed histologically, since LV fibrous content was decreased, and LV wall thickness was preserved. CCL2 concentrations were decreased two days after MI induction, while no difference was observed three weeks post MI. Furthermore, percentage of circulating monocytes were decreased two days post MI. Finally, leukocyte influx was decreased three weeks post MI, but not after two days.

Conclusions: PC-mAb treatment reduces local and systemic inflammatory response resulting in limitation of adverse cardiac remodeling and IS following MI. This indicates that PC-mAb might be a promising therapeutic agent following MI and subsequent cardiac remodeling.

Introduction

Over the last decades much improvements have been made to treat patients suffering MI, using percutaneous coronary interventions[1] or coronary artery bypass grafting[2]. However, revascularization is not possible in a significant portion of the patients suffering from chronic coronary artery disease[3], due to anatomical or clinical complications or simply because the possibilities for providing this kind of clinical care are not available. Therefore, it is still relevant in these times where most of the studies focus on ischemia reperfusion studies in relation to myocardial infarction, to study the effects permanent ischemia on post infarctional cardiac remodeling. MI can lead to adverse left ventricular (LV) remodeling, characterized by LV dilatation and reduced LV wall thickness, which successively leads to heart failure[4], one of the leading causes of death worldwide[5].

Following a MI, an intense inflammatory response is triggered, which helps to clear the injured myocardium from dead cardiomyocytes and matrix debris, and ultimately leads to infarct healing and scar formation[6]. However, when the inflammatory response is extended it may cause viable cardiomyocytes to die[7]. Necrotic cardiomyocytes release damage-associated molecular patterns (DAMPs), like high mobility group box-1 (HMBG1), heat shock protein (HSP), interleukin-1α (IL-1α) and extracellular RNA (eRNA), which trigger the innate immune system[7] via TLR activation[8-10]. The role of apoptotic cells seems to be more complicated. Uptake of apoptotic cells by macrophages might have anti-inflammatory effects[11], on the other hand it has been suggested that apoptotic cells are immunogenic and pro-inflammatory[12]. Wan and colleagues found that effective efferocytosis of apoptotic cardiomyocytes, promotes the resolution of inflammation after a MI[13]. However, the main part of apoptotic cells in the healing infarct are non-cardiomyocytes. For instance apoptotic neutrophils represent a large part of the apoptotic cells in the healing infarct, and their role in inflammation resolution is not yet known[7].

Following a MI, the production of reactive oxygen species (ROS) by endothelial cells, circulating phagocytes and cardiomyocytes is increased as a result of the ischemic event[14]. These ROS are responsible for generating oxidative damage, and thereby producing oxidation-specific epitopes (OSEs) on apoptotic cells, which can act as DAMPs and are recognized by innate immunity[15]. Further research revealed that the polar headgroup, phosphorylcholine (PC), of oxidized phospholipids (oxPLs), is an important OSE, which is present on apoptotic cells, but not on viable cells[12]. Moreover, PC is present on oxidized LDL (oxLDL), a key player in atherogenesis because of its pro-inflammatory properties[16]. It has been shown that a specific clone of IgM autoantibodies against PC, termed EO6 or T15 antibodies[17], can inhibit the uptake of both apoptotic cells[18] and oxLDL[19], and has anti-inflammatory properties[12]. Furthermore, it has been shown that B-1a and B-1b cells produce OSE specific IgM antibodies which protect against atherosclerosis[20-22] and it has been found that splenic B-cells display an OSE

associated atheroprotective effect which is initiated through sterile inflammation[23].
Low levels of natural IgM anti-PC antibodies are associated with increased risk for cardiovascular events[24-28] and resulted in a worsened prognosis regarding patients with an acute coronary syndrome[29]. In addition, both active and passive immunization with antibodies against PC ameliorates atherosclerosis development and is proven to be atheroprotective[30-32]. Taken together, these data show that blocking PC might be an interesting therapeutic approach to treat cardiovascular disease. However, IgM antibodies are not optimal for therapeutic use, particularly because of its difficulty to produce recombinant IgM antibodies with their pentamer structures.
We previously developed a fully human IgG type1 directed against human PC (PC-mAb), which has anti-inflammatory properties, blocks oxLDL uptake by macrophages and inhibits vascular remodeling in a mouse model for accelerated atherosclerosis[33]. In the current study we aimed to investigate the effect of PC-mAb treatment on cardiac function, LV remodeling and the inflammatory response following MI. To study this, we administrated PC-mAb to hypercholesterolemic APOE*3-Leiden mice after induction of a permanent MI.

Methods

Animals and diets

All animal experiments were approved by the Institutional Committee for Animal Welfare of the Leiden University Medical Center (LUMC) and in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Transgenic female APOE*3-Leiden mice[34], aged 8-10 weeks at the start of a dietary run-in period were used for this experiment. Mice were fed a semisynthetic Western-type diet supplemented with 0.4% cholesterol (AB Diets, Woerden, The Netherlands) four weeks prior to surgery which was continued throughout the complete experiment. Mice were housed under standard conditions in conventional cages and received food and water ad libitum.

Plasma lipid analysis

Plasma levels of total cholesterol (TC) and triglycerides (TG) were determined for randomization one week before surgery. After a four hours fasting period, plasma was obtained via tail vein bleeding (approximately 50 μL) and assayed for total cholesterol (TC) and triglycerides (TG) levels using commercially available enzymatic kits according to the manufacturer's protocols (11489232; Roche Diagnostics, Mannheim, Germany, and 11488872; Roche Diagnostics, Mannheim, Germany, respectively)

Induction of myocardial infarction and PC-mAb treatment

Myocardial infarction (MI) was induced by ligation of the left anterior descending (LAD) coronary artery at day 0 in 12-14 weeks old female APOE*3-Leiden mice as described

previously[35,36]. Briefly, mice were pre-anesthetized with 5% isoflurane in a gas mixture of oxygen and placed in a supine position on a heating pad (37°C). After endotracheal intubation and ventilation (rate 160 breaths/min, stroke volume 190µL; Harvard Apparatus, Holliston, MA, USA), mice were kept anesthetized with 1.5-2% isoflurane. Subsequently a left thoracotomy was performed in the 4th intercostal space and the LAD coronary artery was permanently ligated using a 7-0 prolene suture. Subsequently, the thorax was closed in layers with 5-0 prolene suture and mice were allowed to recover. Analgesia was obtained with buprenorfine s.c. (0.1mg/kg) pre-operative and 12h post-operative.

After surgery animals were randomly grouped to receive administration of i.p. injections with 10 mg/kg PC-mAb (Athera, Biotechnologies, Solna, Sweden) every 3rd day or NaCl 0.9% w/v (vehicle) as a control (Figure 1B). Sham operated animals were operated similarly but without ligation of the LAD and received i.p. injections with NaCl 0.9% w/v (sham).

After two days or three weeks were euthanized by bleeding and explantation of the heart under general anesthesia with 1.5-2% isoflurane. Hearts were immersion-fixated in 4% paraformaldehyde for 24 hours and embedded in paraffin. Blood samples were collected and used for serum analysis. The heart and body weights were measured from all animals using a digital scale.

Cardiac magnetic resonance imaging

Left ventricular (LV) dimensions, function and infarct size (IS) were assessed two days and three weeks after surgery (Figure 1A) by using a 7-Tesla magnetic resonance imaging (MRI) (Bruker Biospin, Ettlingen, Germany) to obtain contrast-enhanced and cine MRI images. Mice were pre-anesthetized with 5% isoflurane in a gas mixture of oxygen and kept anesthetized with 1.5-2% isoflurane. Respiratory rate was monitored by a respiration detection cushion, which was place underneath the thorax and connected to a gating module to monitor respiratory rate (SA Instruments, Inc., Stony Brook, NY). Image reconstruction was performed using Bruker ParaVision 5.1 software.

Infarct size

Infarct size was determined with contrast-enhanced MR imaging after injection of 150 µl (0.5 mmol/ml) of gadolinium-DPTA (Gd-DPTA, Dotarem, Guerbet, The Netherlands) via the tail vein. A gradient echo sequence (FLASH) was used to acquire a set of 14 contigious 0.7 mm contrast-enhanced slices in short-axis orientation. Imaging parameters were: echo time of 1.9 ms, repetition time of 84.16 ms, field of view of 33 mm^2, and a matrix size of 192x256.

Left ventricular function

Left ventricular function was assessed with a high resolution 2D FLASH cine sequence

to acquire a set of 9 contigious 1 mm slices in short axis orientation covering the entire heart. Imaging parameters were: echo time of 1.49 ms, repetition time of 5.16 ms, field of view of 26 mm^2, and a matrix size of 144x192.

Image analysis

Image analysis was performed with the MR Analytical Software System (MASS) for mice (MEDIS, Leiden, The Netherlands). LV endo- and epicardial borders were delineated manually and a reference point was positioned by an investigator blinded to treatment. End-diastolic and end-systolic phases and the contrast enhanced areas were identified automatically, and the percentage of infarcted myocardium, LV end-diastolic volume (EDV), LV end-systolic volume (ESV), and LV ejection fraction (EF) were computed.

LV fibrous content and LV wall thickness

Five µm thick transverse sections were made along the entire long-axis of the LV and every 50^{th} section was stained with Sirius Red. Collagen deposition was used as an indicator of the fibrotic area and LV fibrous content was determined by planimetric measurements of all sections and calculated as fibrotic area divided by the total LV wall surface area.

LV wall thickness was analyzed in five different sections centralized in the infarct area. Per section wall thickness was measured at three places in the infarct area, both border zones, and at two places in the intraventricular septum. All measurements were performed using the ImageJ 1.47v software program (NIH, USA).

Local inflammatory response

For analysis of the cardiac inflammatory response a subpopulation was selected, and sections were stained using antibodies against leukocytes (anti-CD45, 550539; BD Pharmingen, San Diego, CA, USA). The number of leukocytes was expressed as a number per 0.25 mm^2 in the septum (2 areas), border zones (2 areas), and infarcted myocardium (3 areas).

FACS analysis

To examine the effect of PC-mAb therapy on the acute inflammatory response, mice were sacrificed and blood samples were collected at day two. To study the systemic effects whole blood was analyzed for monocytosis. Total circulating leukocytes were determined using a semi-automatic hematology analyzer F-820 (Sysmex; Sysmex Corporation, Etten-Leur, The Netherlands).

For FACS analysis, 35µL of whole blood was incubated for 30 min on ice with directly conjugated antibodies directed against Ly6C-FITC (AbD Serotec, Dusseldorf, Germany), Ly6G-PE (BD Pharmingen, San Diego, CA, USA), CD11b-APC (BD Pharmingen, San Diego, CA, USA), and CD115-PerCP (R&D Systems, Minneapolis, MN, USA). Monocytes were

gated based on their expression profile: CD11b-positive, Ly6G-negative, and CD115-positive. Data was analyzed using FlowJo software (Tree Star Inc.)

CCL2 and PC-mAb ELISA

A PC-mAb ELISA kit (Athera Biotechnologies, Solna, Sweden) was used to determine serum PC-mAb concentrations. To study the effects of PC-mAb on systemic inflammation, inflammatory cytokine concentration of chemokine (C-C motif) ligand 2 (CCL2) was determined using an ELISA kit (Cat. No. 555260, BD Biosciences, San Diego, CA, USA).

TLR4 and PC co-localization

The presence of Toll-like receptor 4 (TLR4) and PC co-localization in the infarct area, was investigated by immunohistochemistry. TLR4 was stained using specific antibodies against TLR4 (anti-CD284, AHP1822, Bio-Rad Laboratories Inc.). PC was stained using the same antibody (Athera, Biotechnologies, Solna, Sweden) as was used for treatment.

Statistical analysis

Values were expressed as mean ± SEM. Comparisons of parameters between the sham, PC-mAb, and vehicle groups were made using 1-way analysis of variance (ANOVA) with Tukey's correction or 2-way ANOVA with repeated measures and Tukey's posttest in case of multiple time points. Comparisons between PC-mAb and vehicle were made using unpaired t-tests. A value of $P \leq 0.05$ was considered to represent a significant difference. Statistical procedures were performed using IBM SPSS 23.0.0 (SPSS Inc – IBM, Armonk, NY, USA) and Graphpad Prism 6.02 (Graphpad software Inc, La Jolla, CA, USA).

Results

Animal characteristics

No differences in bodyweight (BW) were observed following PC-mAb (20.4±0.3 g) treatment compared to vehicle (20.5±0.4 g) and sham (19.6±0.3 g). Possible cardiac hypertrophy was assessed by determining heart weight (HW) and heart to body weight (HW-BW) ratio. Following PC-mAb treatment both HW (134±5 mg) and HW-BW ratio (6.6±0.3) were reduced compared to vehicle (HW: 167±9 mg, $P<0.01$; HW-BW ratio: 8.2±0.4, $P<0.01$), to levels similar to those observed in the sham group (HW: 144±8 mg; HW-BW ratio: 7.3±0.3) (Table 1).

PC-mAb concentrations

To validate that the observed data is the result of PC-mAb treatment, we determined serum PC-mAb concentrations using ELISA. PC-mAb levels were only detectable in PC-mAb treated mice (32±8 μg/ml after two days, and 36±6 μg/ml after three weeks) but

not in vehicle or sham-operated animals after both two days and three weeks following MI.

Table 1: Plasma lipids & animal characteristics

	T (wk)	sham	MI PC-mAb	MI vehicle
N		*13*	*14*	*16*
TC (mmol/L)	*0*	17.5±1.7	15.0±1.4	14.8±1.0
TG (mmol/L)	*0*	2.5±0.2	3.0±0.2	2.8±0.1
BW (g)	*0*	20.7±0.5	21.1±0.3	20.9±0.5
	3	19.6±0.3	20.4±0.3	20.5±0.4
HW (mg)	*3*	144±8	134±5$^{\#\#}$	167±9
HW/BW ratio (mg/g)	*3*	7.3±0.3	6.6±0.3$^{\#\#}$	8.2±0.4

Table 1: Plasma lipid levels and animal characteristics: plasma total cholesterol (TC), triglycerides (TG), body weight (BW), heart weight (HW). Values are means ± SEM. $^{\#\#}P<0.01$ vehicle.

TLR4 and PC co-localization

Since our rationale for using PC-mAb to treat adverse cardiac remodeling following MI was to inhibit the pro-inflammatory response, we investigated if TLR4 was localized at the same areas as PC. As can be appreciated from Figure 1 TLR4 staining is indeed localized at comparable areas in the infarct area as is PC staining.

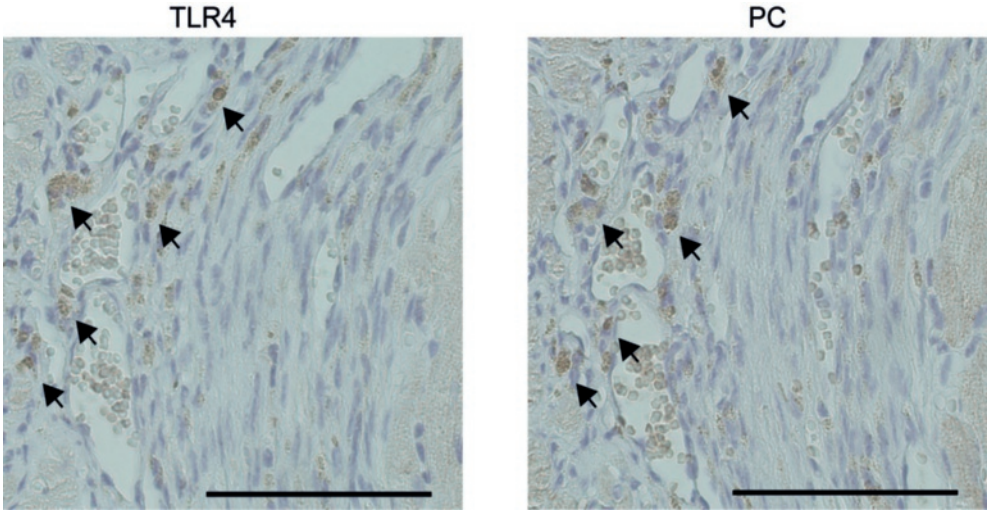

Figure 1: TLR4 and PC co-localization. Representative images of TLR4 (left) and PC (right) staining in the infarct area of an untreated mice. Scale bar = 50 μm.

PC-mAb reduces contrast-enhanced MRI assessed LV infarct size

Baseline infarct size (IS) was assessed using contrast-enhanced MRI two days post MI. No differences in IS could be observed between the PC-mAb treated group and the vehicle group at baseline (30.9±3.2% vs. 36.7±2.7%). However, three weeks post MI, PC-mAb treated mice showed significantly smaller IS compared to vehicle treated mice

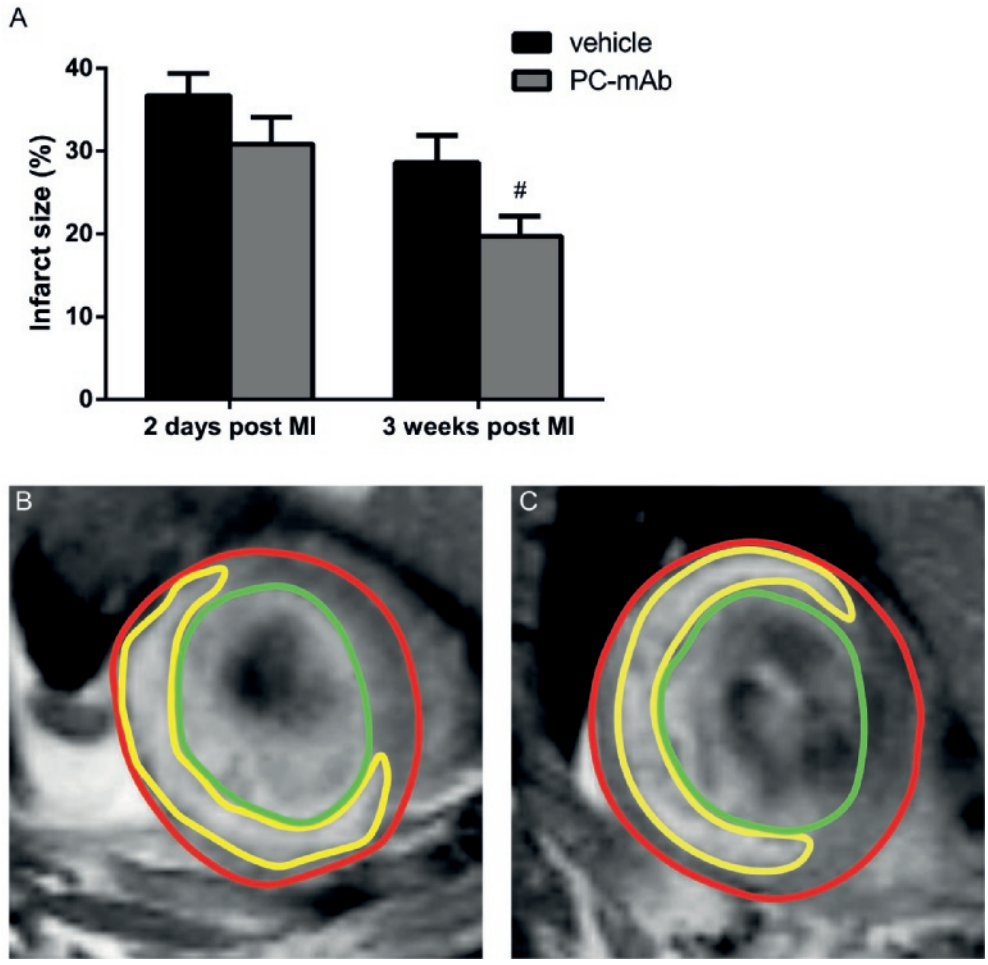

Figure 2: Quantification of infarct size using contrast-enhanced MR imaging. Infarct size was measured at baseline (two days post MI) and at sacrifice (three weeks post MI) and quantified as percentage of the LV mass (A). Representative Gd-DPTA-enhanced MR images 2 days post MI of vehicle (B) and PC-mAb treated (C) mice. Red line indicates epicardial border, green line indicates endocardial border and yellow line indicates infarct area. Data are mean ± SEM. #P≤0.05 vs. vehicle.

(19.7±2.4% vs. 28.6±3.3%, P<0.01; Figure 2A).

Interestingly, IS was significantly smaller three weeks post MI compared to two days post MI in both the vehicle and PC-mAb group, indicating some amount of infarct healing and loss of acute infarct edema took place in both groups.

PC-mAb reduces LV dilatation, but does not affect LV function

To investigate the effect of PC-mAb treatment on LV dilatation serial cardiac cine MRI images were made two days and three weeks following MI. Two days post MI no differences could be observed between vehicle and PC-mAb treatment regarding EDV

(39.7±2.8 µl vs. 36.7±2.2 µl; Figure 3A) and ESV (25.7±3.1 µl vs. 23.5±2.7 µl; Figure 3B). Three weeks post MI ESV was not significantly different in the PC-mAb group compared to the vehicle group (32.9±5.5 µl vs. 44.0±7.0 µl; Figure 3B). Interestingly, PC-mAb treated animals showed a significantly smaller EDV three weeks post MI compared to untreated animals (48.6±4.7 µl vs. 61.3±6.3 µl, P=0.05; Figure 3A), indicating a reduction in LV dilatation in the PC-mAb treated group compared to the vehicle group.
Next, ejection fraction (EF) was measured as indication of LV function. No differences could be observed between PC-mAb treated mice and untreated mice two days post MI (38.3±4.0% vs. 37.8±4.1%) and three weeks post MI (37.4±4.7% vs. 34.4±5.1%), indicating PC-mAb treatment does not affect LV function (Figure 3C).

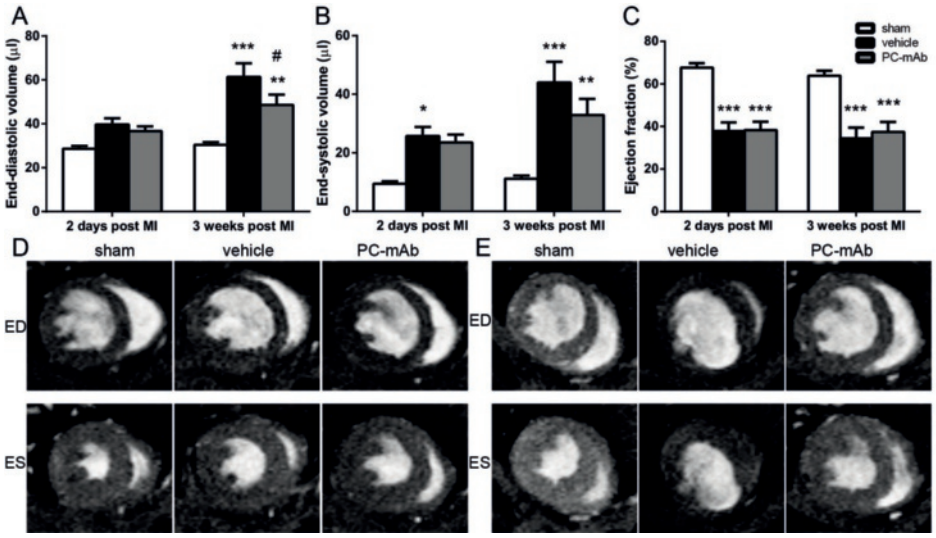

Figure 3: Quantification of LV volumes and function using cardiac MR imaging. LV volumes, EDV (A) and ESV (B), and function, EF (C), were assessed two days and three weeks after MI (n=12-16 per group). Representative transversal short-axis MR images at end-diastole (ED) and end-systole (ES) two days (D) and three weeks (E) post MI in the sham, vehicle and PC-mAb groups. Data are mean ± SEM. #P≤0.05 vs. vehicle, *P≤0.05, **P≤0.01, ***P≤0.001 all vs. sham.

PC-mAb reduces LV fibrous content and ameliorates LV wall thickness

To validate the decreased IS by immunohistochemistry, cross-sections from the hearts were stained with Sirius Red to measure fibrous content of the LV as a measure for IS. PC-mAb treated animals showed a strong trend towards decreased LV fibrous content compared to untreated animals (18.6±1.4% vs 25.5±3.4%, P=0.07; Figure 4A), confirming the decreased IS measured with contrast-enhanced MRI.
Next, we assessed the LV wall thickness in different areas of the heart; the septum, the border zones and the infarct area. LV wall thickness was significantly increased in the septum of both the PC-mAb (1.22±0.04 mm, P<0.001) and the vehicle group (1.10±0.05

mm, P<0.01) compared to sham (0.85±0.04 mm; Figure 4B). This indicates compensatory cardiac hypertrophy in this area, suggesting the viable myocardium to compensate for the infarcted myocardium. Interestingly, LV wall thickness was increased in the border zone and infarct area in the PC-mAb treated animals compared to untreated animals (border zones: 1.17±0.03 mm vs. 0.99±0.06 mm, P<0.05; infarct area: 0.66±0.06 mm vs. 0.45±0.08 mm, P<0.05; Figure 4B). The increased LV wall thickness in the border zone is likely the result of cardiac hypertrophy, since it is also significantly increased compared to the sham group (1.01±0.03 mm). Since the LV wall thickness in the infarct area was significantly decreased in both the PC-mAb and vehicle group compared to sham, the observed reduced decrease in the PC-mAb treated animals compared to untreated animals indicates preserved LV wall thickness following PC-mAb treatment.

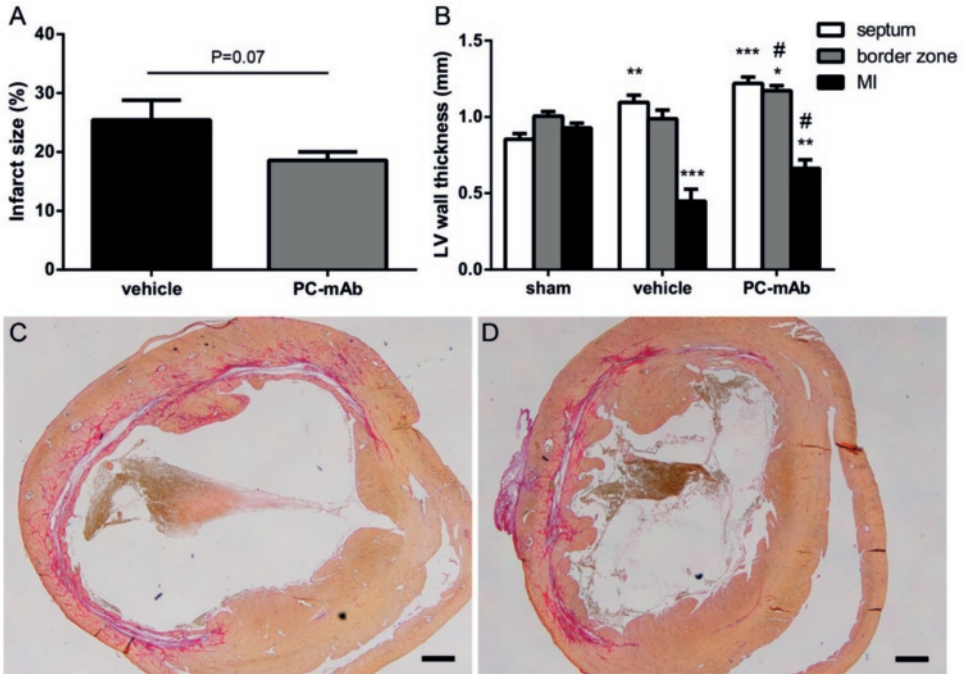

Figure 4: Histological quantification of LV fibrous content and LV wall thickness three weeks post MI. LV fibrous content (A) was measured by Sirius red staining and quantified as the area of the LV occupied by collagen (n=9-10 per group). LV wall thickness (B) was assessed in 3 specific areas: interventricular septum, border zone and infarct area (n=9-10 per group). Representative images of Sirius Red staining of untreated (C) and PC-mAb treated (D) mice. Scale bar = 500 µm. Data are mean ± SEM. #P≤0.05 vs. vehicle, *P≤0.05, **P≤0.01, ***P≤0.001 all vs. sham.

PC-mAb causes reduction of the local inflammatory response

To investigate the local inflammatory response, cross-sections from the hearts were immune-stained for CD45 (leukocyte marker), and the number of CD45 positive cells

were counted in the septum, border zones and infarct area. We observed a striking decrease in number of leukocytes in all areas following PC-mAb treatment compared to the vehicle group three weeks after MI induction (septum: 1.2±0.2 vs. 3.0±0.6, P<0.01; border zones: 1.4±0.2 vs. 3.3±0.7, P<0.05 and infarct area: 1.3±0.2 vs. 3.4±0.7 per 0.25 mm^2, P<0.01; Figure 5B), suggesting PC-mAb treatment reduces the local inflammatory response following MI. Moreover, numbers of leukocytes in the PC-mAb group were comparable with the sham group (septum: 1.2±0.3, border zones: 1.0±0.2, and infarct area: 0.8±0.1 per 0.25 mm^2), suggesting, to some degree, an accelerated and better resolution of the inflammatory response.

Since the acute phase of the inflammatory response is crucial in MI we investigated the number of leukocytes that infiltrated the cardiac tissue two days post MI. In the infarct area an increased number of leukocytes was observed in the vehicle group compared to the sham group (20.5±4.0 vs. 4.9±0.4 per 0.25 mm^2, P<0.01), while no difference was observed between the PC-mAb group and the sham group (11.3±3.3 vs. 4.9±0.4 per 0.25 mm^2, P=0.33; Figure 5A), suggesting that PC-mAb treatment dampened the acute local inflammatory response. In all other areas no differences could be detected between the PC-mAb and vehicle group (Figure 5A).

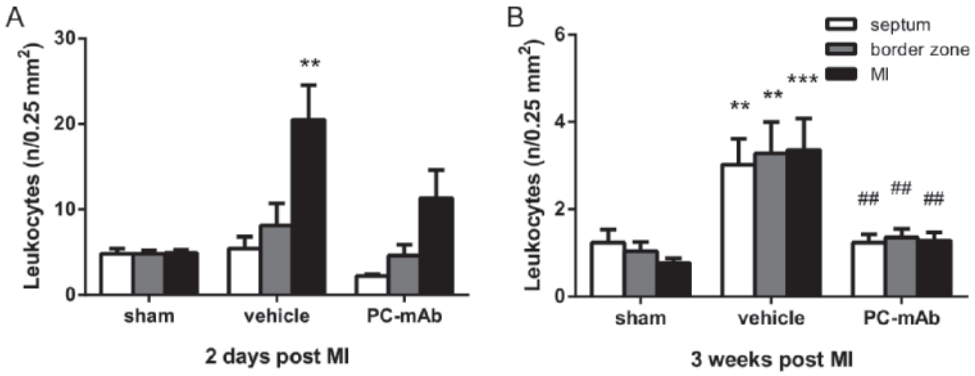

Figure 5: Histological quantification of the local inflammatory response. The number of CD45 positive cells (leukocytes) were counted per specific area: interventricular septum, border zone and infarct area, as measure of local inflammation. Each bar represents the average number of leukocytes per field of view in the specific areas three weeks post MI (A; n-9-10 per group) and two days post MI (B; n=4-5 per group). Data are mean ± SEM. ##P≤0.01 vs. vehicle, **P≤0.01, ***P≤0.001 both vs. sham.

PC-mAb attenuates the systemic inflammatory response

To further unravel the effect of PC-mAb treatment following MI, we investigated the systemic inflammatory response. Serum concentrations of CCL2 (a pro-inflammatory cytokine) were measured two days and three weeks after MI induction. Two days post MI CCL2 concentrations were significantly reduced in the PC-mAb group (18.3±13.7 pg/ml) compared to both the sham (80.5±14.5 pg/ml, P<0.01) and vehicle group (96.8±4.4

pg/ml, P<0.01; Figure 6A), suggesting that PC-mAb treatment reduces the systemic inflammatory response. However, three weeks post MI no significant differences could be observed in serum CCL2 concentrations between all groups suggesting a transient effect on the systemic inflammatory process (Figure 6B).
Furthermore, we investigated the effect of PC-mAb treatment on circulating monocytes two days after induction of MI using FACS analysis. We found a decrease in percentage of circulating monocytes following PC-mAb treatment compared to the vehicle group (1.6±0.2% vs. 4.4±0.7%, P<0.01; Figure 6C). Moreover, percentage of circulating monocytes was comparable to the sham group (2.0±0.5%). Taken together these data demonstrate that PC-mAb treatment attenuates monocytosis.

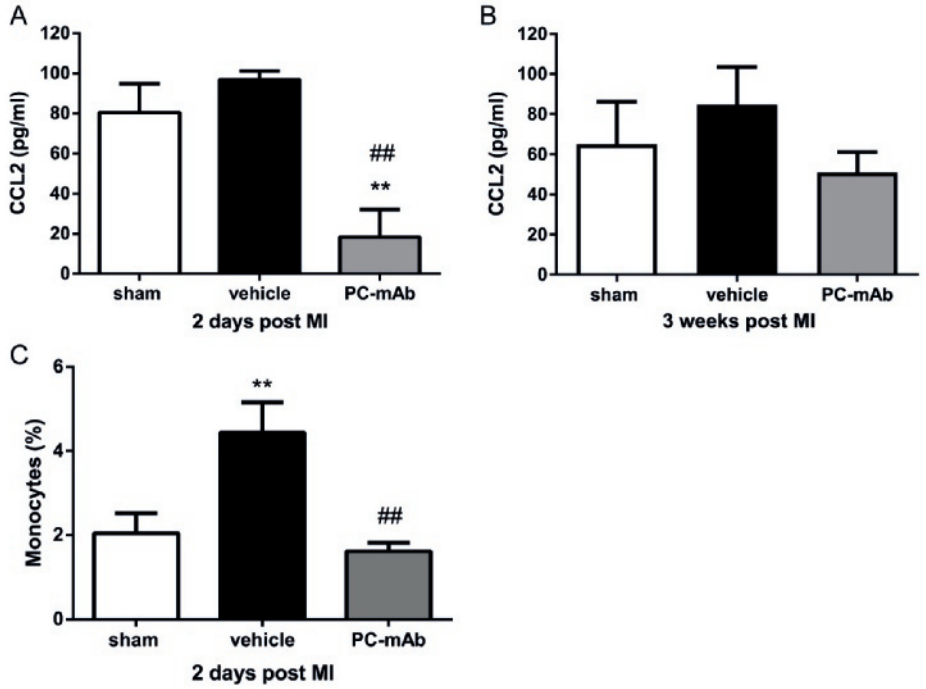

Figure 6: Quantification of the systemic inflammatory response. Serum levels of CCL2 were determined using ELISA as measure of systemic inflammation, two days post MI (A; n=6-8 per group) and three weeks post MI (B; n=9-10 per group). Circulating monocytes were determined two days post MI using FACS analysis and expressed as percentage of total leukocytes (C; n=6-8 per group). Data are mean ± SEM. ##P≤0.01 vs. vehicle, **P≤0.01 vs. sham.

Discussion

PC-mAb is a human IgG directed against PC with anti-inflammatory properties[33]. This study demonstrates a beneficial effect of PC-mAb treatment following permanent MI. We show that PC-mAb treatment decreases HW, infarct size and LV dilatation, while

preserving LV wall thickness. This effect is most likely due to the reduced systemic inflammatory response two days post MI and the reduced local inflammatory response after three weeks.

Adverse left ventricular remodeling is one of the mechanisms responsible for development of heart failure[4], which is one of the leading causes of morbidity and mortality worldwide[5]. We demonstrate a reduction of adverse LV remodeling following administration of PC-mAb, as observed by restricted LV dilatation. Furthermore, we found that HW and HW/BW ratio was reduced following PC-mAb treatment, indicating reduced compensatory cardiac hypertrophy, which is another hallmark of adverse cardiac remodeling and heart failure[37]. Moreover, PC-mAb treatment decreases IS and it has been postulated that IS can be directly linked to heart failure and mortality following MI[38]. These results suggest PC-mAb treatment might be a promising therapeutic agent against heart failure in ischemic heart disease.

Inflammation plays an important role following MI, being responsible for removing necrotic and apoptotic cells, thereby improving infarct healing and scar formation[6]. On the other side, extensive inflammation may cause death of viable cardiomyocytes and enhances LV remodeling[7]. We demonstrate that PC-mAb treatment reduces the local inflammatory response, indicated by reduced leukocyte infiltration, three weeks post MI, but not two days after MI. This suggests that PC-mAb treatment does not inhibit the early beneficial local inflammatory response, but interestingly, it inhibits the deleterious extensive inflammatory response, limiting adverse LV remodeling[7].

Although context dependent, it has been shown that oxPLs are agonists for TLR4 signaling resulting in production of cytokines and chemokines like, CCL2, interleukin- 6 (IL-6) and interleukin (IL-8)[39,40]. Here we show that TLR4 and PC are localized at similar areas in the infarct area following MI, supporting a role of PC as a ligand for TLR4 signaling in the infarcted myocardium. This justifies our choice to use PC-mAb as potential therapy following MI. By expressing anti-inflammatory properties PC-mAb seems to reduce LV remodeling as shown by decreased IS and LV dilatation, while preserving LV wall thickness. Following MI a portion of the cardiomyocytes undergo apoptosis[6]. Apoptotic cells express oxidized lipids on their outer membrane[41], which is immunogenic[12]. It has been demonstrated that natural and monoclonal EO6/T15 antibodies against PC bind to apoptotic cells[18,41], thereby reducing the inflammatory response[12]. Therefore, we suggest that the observed reduced IS is the result of a dampened inflammatory response, thereby sparing viable cardiomyocytes. The preserved LV wall thickness might also be a result of the dampened inflammatory response, but it can also be a direct result of the decreased LV dilatation[42].

CCL2 is a chemoattractant known for its ability to attract inflammatory leukocytes to sites of tissue injury[43], for example after a MI followed by reperfusion[44]. Although it is believed that these attracted leukocytes promote removal of dead tissue and infarct healing, it has been shown that CCL2 null mice show decreased macrophage recruitment

to the infarcted myocardium that coincides with decreased LV remodeling following myocardial ischemia-reperfusion (MI-R)[44]. This is in agreement with our finding of decreased CCL2 serum concentration, and decreased LV remodeling. Interestingly, CCL2 serum concentrations in the PC-mAb treated group were also decreased compared to the sham group indicating PC-mAb reduces both surgery-induced systemic inflammation and infarction-induced local inflammation.

Previously, we showed PC-mAb to reduce CCL2 levels produced by human monocytes stimulated with oxLDL *in vitro* and in our model for accelerated atherosclerosis local expression of CCL2 in the vessel wall was inhibited[33]. Furthermore, it is known that blood CCL2 levels are increased in ApoE*3-Leiden mice when fed a high fat diet[45]. We suggest PC-mAb treatment reduces the systemic inflammatory response by binding to PC on apoptotic cells and/or oxLDL, which might contribute to the reduced LV remodeling, as observed in reduced serum CCL2 concentrations.

Hypercholesterolemia causes a pro-inflammatory state which is characterized by a monocytosis[46]. This increase in monocytes is mainly caused by an increase in the pro-inflammatory Ly6C^{hi} subset. It has been shown that following MI in hypercholesterolemic ApoE$^{-/-}$ mice more Ly6C^{hi} monocytes are recruited into the infarct area which resulted in decreased LV function[47] and impaired infarct healing[48]. In the current study we found a reduction of total monocytes in the PC-mAb treated group, accompanied with a decrease in IS and LV dilatation. However, the reduction of total monocytes could not be assigned to one of the different monocyte subsets (Ly6C^{hi}, Ly6C^{med} and Ly6C^{low}; data not shown).

In conclusion, PC-mAb treatment following permanent MI reduces adverse cardiac remodeling and IS, likely by reducing the inflammatory response. Therefore PC-mAb treatment might be a potential therapy to reduce inflammation and adverse cardiac remodeling, thereby preventing heart failure in ischemic heart disease.

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