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PRECLINICAL RESEARCH

Phosphorylcholine Antibodies Preserve Cardiac Function and Reduce Infarct Size by Attenuating the Post-Ischemic Inflammatory Response

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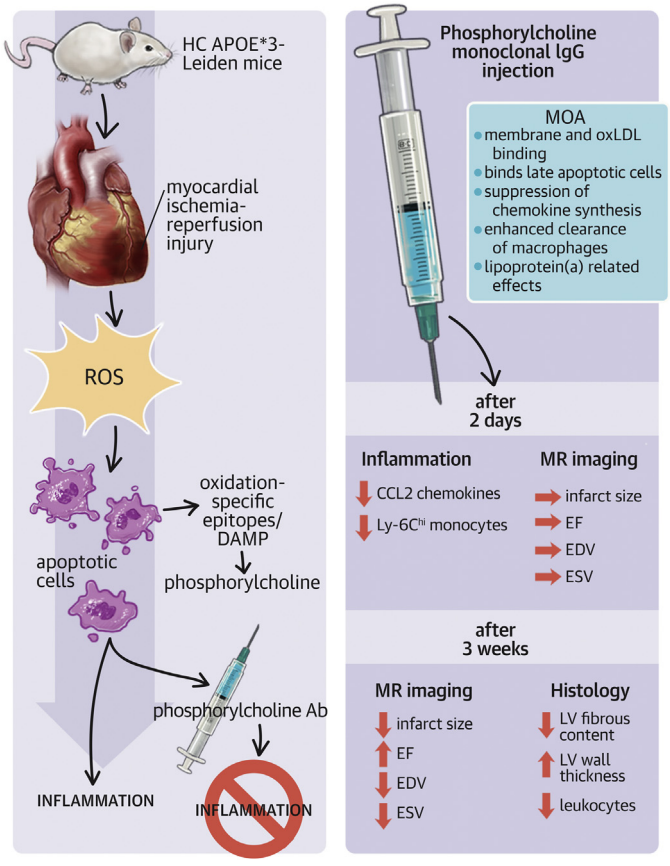
VISUAL ABSTRACT



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HIGHLIGHTS

- **Phosphorylcholine is a proinflammatory epitope exposed on the outer membrane of apoptotic cells.**
- **This study investigated the modulatory effects of a fully human IgG1 monoclonal antibody directed against phosphorylcholine (PC-mAb) on myocardial remodeling and cardiac function following myocardial ischemia-reperfusion injury.**
- **PC-mAb attenuates the immediate post-ischemic inflammatory response by reducing the proinflammatory CCL2 chemokine and circulating Ly-6C^{hi} monocytes. This subsequently enhances the post-ischemic repair process resulting in limited adverse cardiac remodeling and preservation of cardiac function.**
- **PC-mAb therapy may be a valid therapeutic approach against myocardial ischemia-reperfusion injury.**

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SUMMARY

Phosphorylcholine monoclonal immunoglobulin G antibody attenuates the immediate post-ischemic inflammatory response by reducing the proinflammatory chemokine (C-C motif) ligand 2 chemokine and circulating Ly-6C^{hi} monocytes. This subsequently enhances the post-ischemic repair process, resulting in limited adverse cardiac remodeling and preservation of cardiac function. Therefore, phosphorylcholine monoclonal immunoglobulin G antibody therapy may be a valid therapeutic approach against myocardial ischemia-reperfusion injury. (J Am Coll Cardiol Basic Trans Science 2020;5:1228–39) © 2020 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

ABBREVIATIONS AND ACRONYMS

CCL2 = chemokine (C-C motif) ligand 2
CMR = cardiac magnetic resonance
EDV = end-diastolic volume
EF = ejection fraction
ESV = end-systolic volume
Ig = immunoglobulin
IS = infarct size
LV = left ventricular/ventricle
MI = myocardial infarction
MI-R = myocardial ischemia-reperfusion
PC = phosphorylcholine
PC-mAb = phosphorylcholine monoclonal immunoglobulin G antibody

Ischemic heart disease remains one of the leading causes of death worldwide (1). Currently, the preferred therapy to treat acute myocardial infarction (MI) is rapid revascularization therapy to salvage myocardium (2). However, revascularization causes a subsequent problem of myocardial ischemia-reperfusion (MI-R) injury, in which an additional wave of damage is inflicted to the myocardium due to an increased inflammatory response (3) and generation of reactive oxygen species (4), ultimately leading to increased cell death. In a clinical perspective, MI-R injury contributes to adverse cardiac remodeling, which subsequently leads to chronic heart failure, known as an important contributor of morbidity and mortality worldwide (3). Currently, ameliorating this post-ischemic inflammatory process and healing of ischemic myocardium to inhibit left ventricular (LV) remodeling remains a challenge. Though, large randomized controlled trials such as the CANTOS (Canakinumab Antiinflammatory Thrombosis Outcomes Study) (5) and COLCOT (Colchicine Cardiovascular Outcomes) (6) trials showed the therapeutic potential of anti-inflammatory therapies in reducing cardiovascular events after MI as well as a role for autoantibodies in the prior process of atherogenesis (7).

The increased inflammatory response during MI-R injury is partially responsible for the increased generation of reactive oxygen species (4). These reactive oxygen species are responsible for the formation of oxidation-damaged molecules, which can be recognized by the innate immune system. Oxidation-damaged molecules are recognized by the innate immune system via oxidation-specific epitopes (8), which can act as endogenous danger-associated molecular patterns and trigger Toll-like receptors, which play an important role in post-MI remodeling (9,10) or trigger a chronic inflammatory process as atherosclerosis when the innate immune system is dysfunctional or overwhelmed (11). In this way, inflammation and reactive oxygen species generation increase each other's adverse effects following MI-R injury and in chronic inflammation.

Following MI-R injury, ischemic cells express oxidation-specific epitopes on their outer membrane in the form of oxidized membrane phospholipids. Phosphorylcholine (PC) is the polar head group of the membrane phospholipid phosphatidylcholine and an example of such an oxidation-specific epitope, which is an epitope on apoptotic cells but, interestingly, not on viable cells. Furthermore, apoptotic cells expressing PC, or other oxidation-specific epitopes, when present in high quantities are known for their immunogenic and proinflammatory properties (12). Interestingly, PC is also expressed by oxidized low-density lipoprotein, an important lipoprotein in the development of atherosclerosis due to its proinflammatory properties (8). Plasma levels of oxidized phospholipids present on lipoprotein(a) are in turn related to an increased risk of coronary artery disease events (13). These PC-containing proteins are targeted by innate immunity through recognition by scavenger receptors and natural antibodies (14).

Natural antibodies against PC, in mice known as EO6 or T15 antibodies (15), are capable to inhibit oxidized low-density lipoprotein and apoptotic cell uptake by macrophages in vitro (16) and in vivo (17). However,

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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TABLE 1 Plasma Lipid Profiles and Animal Characteristics

	T (weeks)	Sham (n = 13)	MI-R Vehicle (n = 15)	MI-R PC-mAb (n = 14)	p Value (Overall Chi-Square Test)
TC, mmol/l	0	17.5 ± 1.7	16.8 ± 1.3	17.4 ± 1.0	0.932
	3	13.1 ± 1.1	14.0 ± 1.2	12.2 ± 0.5	0.388
TG, mmol/l	0	2.5 ± 0.2	2.6 ± 0.2	3.0 ± 0.2	0.109
	3	2.4 ± 0.2	1.8 ± 0.1*	1.6 ± 0.1†	0.005
BW, g	0	20.7 ± 0.5	21.1 ± 0.4	21.5 ± 0.3	0.361
	3	19.6 ± 0.3	20.2 ± 0.4	20.8 ± 0.3	0.076
HW, mg	3	144 ± 8	140 ± 7	123 ± 2	0.053
HW/BW ratio, mg/g	3	7.3 ± 0.3	6.9 ± 0.3	5.9 ± 0.1‡§	0.002

Values are mean ± SEM. *p = 0.037 vs. sham. †p = 0.004 vs. sham. ‡p = 0.025 vs. vehicle. §p = 0.002 vs. sham.

BW = body weight; HW = heart weight; MI-R = myocardial ischemia-reperfusion; PC-mAb = phosphorylcholine monoclonal immunoglobulin G antibody; T = time; TC = total plasma cholesterol; TG = triglycerides.

with intact effector functions and cascade systems, E06 enhances efferocytosis of apoptotic cells in vivo (18,19). Furthermore, it has been shown that E06 and T15 antibodies block the proinflammatory effects of PC-expressing oxidation-damaged molecules (12,20). In addition, it has been shown that B-1a and B-1b cells produce atheroprotective oxidation-specific epitope-specific antibodies (21–23), and sterile inflammation in the spleen initiates oxidation-specific epitope-specific antibody production by splenic B cells, which reduce the development of atherosclerosis (24). Moreover, low concentrations of immunoglobulin M (IgM) PC antibodies are associated with increased risk for cardiovascular diseases (25–27), and acute coronary syndrome patients with low PC antibody levels experience a worsened prognosis (28). Active and passive immunization with antibodies against PC reduces atherosclerosis development (29,30) and vein graft plaque size (31). Recently, transgenic mice with high levels of a single chain variable fragment of E06 were shown to reduce infarct size (IS) following MI-R (32). Altogether, these data indicate that blocking PC using IgM antibodies may be an interesting approach to treat cardiovascular disease. However, IgM antibodies are not optimal for therapeutic use, because they are, compared with IgG antibodies, rapidly eliminated from plasma, unstable, relatively expensive, and difficult to produce.

Previously we developed PC monoclonal IgG antibody (PC-mAb), a fully human IgG1 against human PC with anti-inflammatory properties, which reduces accelerated atherosclerosis development (33). Moreover, recently we were able to demonstrate that this antibody preserves coronary vascular function and attenuates vascular ^{18}F-fluorodeoxyglucose uptake in atherosclerotic mice (34). In the current study, we used PC-mAb to investigate its long-term effect against MI-R injury with special attention to the development of heart failure. This study was performed in a model trying to resemble the clinical setting of patients experiencing from MI-R injury as a result of urgent revascularization therapy after a temporary thrombotic occlusion of a coronary artery due to an atherosclerotic plaque rupture. Therefore, we used an MI-R model in hypercholesterolemic APOE*3-Leiden mice starting treatment just after reperfusion with a follow-up period of 3 weeks.

METHODS

MI-R injury was induced in 12- to 14-week-old female APOE*3-Leiden mice as described previously (35). Subsequently mice were treated with 10-mg/kg PC-mAb (also known as ATH3G10) (Athera Biotechnologies AB, Stockholm, Sweden) every third day or NaCl 0.9% w/v as a control (vehicle) intraperitoneally. Sham-operated animals were operated similarly but without ligation of the left anterior descending coronary artery, and received injections with NaCl 0.9% w/v. After 2 days and 3 weeks, LV function and IS were assessed by cardiac magnetic resonance (CMR) imaging. Three weeks post-reperfusion, LV fibrous content and LV wall thickness were evaluated histologically. Local inflammatory response was investigated 2 days and 3 weeks after MI-R injury using immunohistochemistry. The systemic inflammatory response was analyzed using enzyme-linked immunosorbent assay and fluorescence-activated cell sorting. All animal experiments were approved by the Institutional Committee for Animal Welfare of the Leiden University Medical Center. For further details see the Supplemental Appendix.

STATISTICAL ANALYSIS. Values were expressed as mean ± SEM. Comparisons of parameters between the sham, PC-mAb, and vehicle groups were made using 1- or 2-way analysis of variance with repeated measures and Tukey's post hoc correction for multiple pairwise comparisons. Comparisons between PC-mAb and vehicle were made using unpaired Student's *t*-tests. A value of $p < 0.05$ was considered to represent a significant difference. All statistical procedures were performed using SPSS 26.0 (IBM Corporation, Armonk, New York) and GraphPad Prism 8.0 (GraphPad Software, San Diego, California).

RESULTS

ANIMAL CHARACTERISTICS AND PC-mAb CONCENTRATIONS AND CELLULAR MECHANISMS. Body weight, heart weight, total plasma cholesterol, and triglyceride

concentrations were not affected following PC-mAb treatment (Table 1). To confirm that the observed effects were the result of PC-mAb treatment, we measured circulating PC-mAb levels 2 days and 3 weeks after MI-R injury. PC-mAb was not detectable in the sham and vehicle group at both time points. In the PC-mAb–treated group, PC-mAb levels were 45 ± 10 μg/ml after 2 days and 40 ± 10 μg/ml after 3 weeks, endorsing the absence of an immune response against PC-mAb. During the development and production process, PC-mAb was shown to bind late apoptotic cells with strong affinity (Supplemental Figure 1). In addition, cultured peripheral blood mononuclear cells isolated from human blood treated with oxidized low-density lipoprotein showed suppression of chemokine (C-C motif) ligand 2 (CCL2) production levels following concomitant treatment with PC-mAb (Supplemental Figure 2).

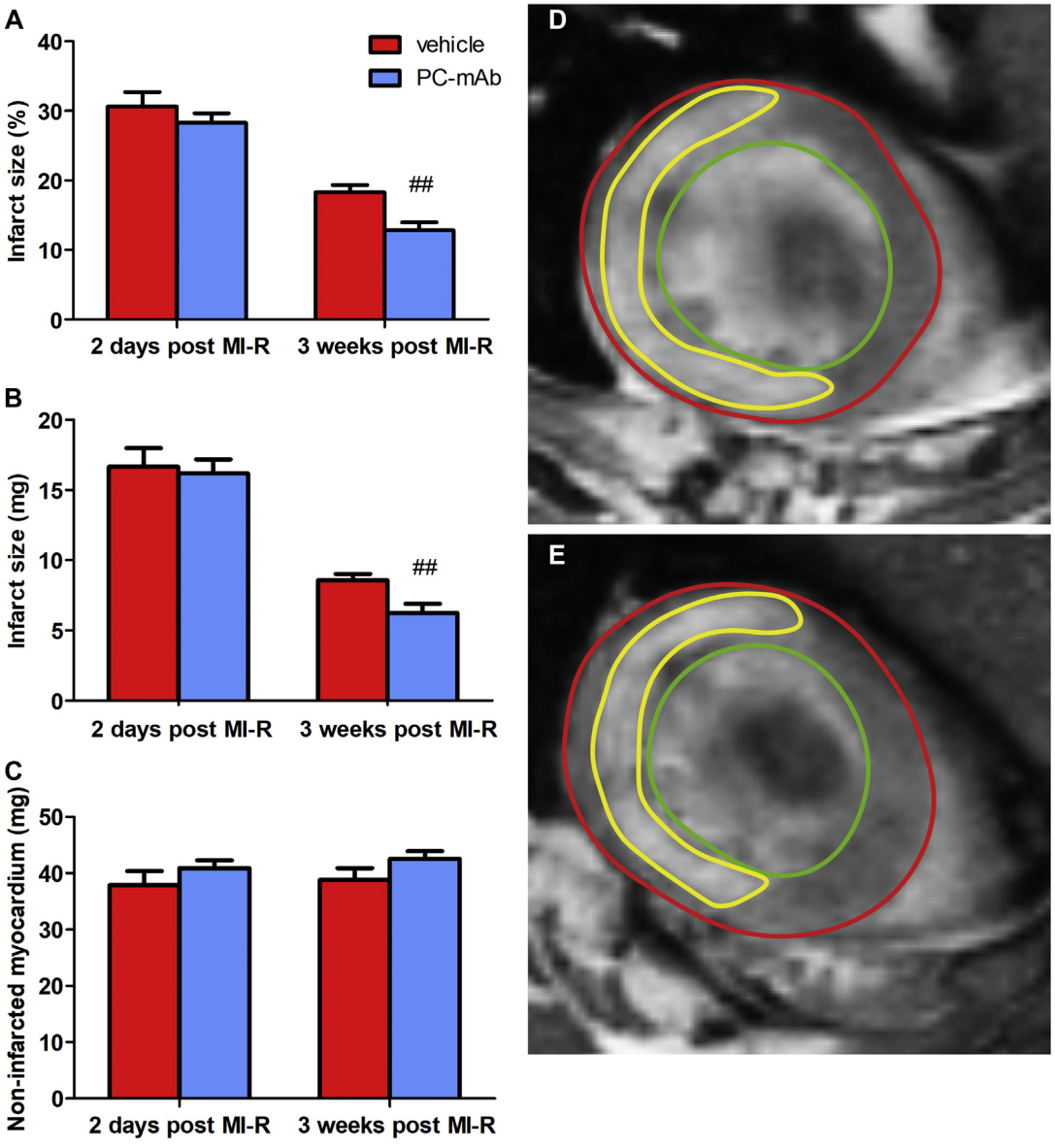
CONTRAST-ENHANCED CMR ASSESSED INFARCT SIZE. First, we assessed baseline IS 2 days post MI-R injury using contrast-enhanced CMR imaging. No difference was observed between PC-mAb (28.3 ± 1.4%) compared with vehicle (30.6 ± 2.1%) treatment (Figure 1A). Three weeks after MI-R injury, IS was significantly reduced in the PC-mAb group (12.8 ± 1.2%) compared with the vehicle group (18.3 ± 1.1%; $p = 0.002$). Likewise, absolute IS in the PC-mAb and vehicle groups was similar 2 days after MI-R (16.2 ± 1.0 mg vs. 16.7 ± 1.3 mg) (Figure 1B) but was significantly reduced 3 weeks after MI-R in the PC-mAb group (6.3 ± 0.6 mg) compared with the vehicle group (8.6 ± 0.5 mg; $p = 0.006$). Noninfarcted myocardium (Figure 1C) was not significantly different between the PC-mAb and vehicle groups after both 2 days (40.8 ± 1.4 mg vs. 37.9 ± 2.5 mg) and 3 weeks (42.5 ± 1.4 mg vs. 38.8 ± 2.1 mg). As expected, some amount of infarct healing was observed in both groups, as IS was significantly smaller 3 weeks after MI-R when compared with 2 days after MI-R ($p < 0.001$) as a result of transitory early infarct edema. Taken together, PC-mAb treatment seems to significantly decrease IS 3 weeks after MI-R injury.

LV DILATATION AND FUNCTION. To investigate the effect of PC-mAb treatment on LV dilatation and function, we made serial cine MR images 2 days and 3 weeks post MI-R injury. Two days after MI-R end-diastolic volume (EDV) (Figure 2A) was not affected following PC-mAb treatment (30.8 ± 0.9 μl) when compared with sham (28.7 ± 1.2 μl) and vehicle (34.4 ± 2.3 μl) treatment. However, 3 weeks after MI-R, PC-mAb treatment resulted in significantly smaller EDV compared with vehicle treatment (33.7 ± 1.4 μl vs. 44.4 ± 2.4 μl; $p < 0.001$), which was statistically not different from the EDV of sham animals (30.4 ± 1.2 μl). End-systolic volume (ESV) (Figure 2B) was significantly increased 2 days after MI-R in both the vehicle (19.4 ± 2.0 μl; $p < 0.001$) and PC-mAb (14.7 ± 0.7 μl; $p = 0.047$) groups compared with the sham group (9.4 ± 0.8 μl), while no significant difference could be observed between the vehicle and PC-mAb groups ($p = 0.066$). Interestingly, 3 weeks post MI-R, ESV was markedly reduced following PC-mAb treatment (15.5 ± 0.9 μl) compared with vehicle treatment (26.6 ± 2.2 μl; $p < 0.001$), while no significant difference was observed when compared with sham treatment (11.2 ± 1.0 μl; $p = 0.123$). Taken together, these results suggest that PC-mAb treatment prevents LV dilatation to a level comparable to animals without MI-R injury.

Ejection fraction (EF) as a measure of LV function (Figure 2C) was significantly decreased 2 days post MI-R injury in both the vehicle (44.7 ± 3.0%; $p < 0.001$) and PC-mAb (52.1 ± 1.8%; $p < 0.001$) groups when compared with the sham group (67.6 ± 2.1%), while no significant difference was observed between vehicle and PC-mAb treatment ($p = 0.072$). Three weeks after MI-R, EF was still decreased in both the vehicle (40.8 ± 2.9%; $p < 0.001$) and PC-mAb (54.1 ± 1.8%; $p = 0.020$) groups when compared with the sham group (63.9 ± 2.3%). However, PC-mAb treatment significantly increased EF compared with vehicle treatment ($p < 0.001$), indicating preservation of LV function by PC-mAb treatment, whereas EF further deteriorated in the vehicle group compared with the day 2 time point.

LV FIBROUS CONTENT AND LV WALL THICKNESS. To confirm the effect of PC-mAb on contrast-enhanced CMR assessed IS, we measured LV fibrous content, as a measure of IS, using Sirius red staining. LV fibrous content was significantly reduced following PC-mAb treatment (12.9 ± 1.0%) compared with vehicle treatment (19.8 ± 1.8%; $p = 0.004$) (Figure 3A), confirming the earlier obtained CMR data. Accordingly, 3 weeks after MI-R, LV wall thickness (Figure 3B) in the PC-mAb group compared with the vehicle group was increased in the infarct area (0.87 ± 0.03 mm vs. 0.75 ± 0.04 mm; $p = 0.045$) and border zones (1.13 ± 0.02 mm vs. 1.03 ± 0.03 mm; $p = 0.041$). LV wall thickness in the interventricular septum was significantly increased in both the PC-mAb (1.18 ± 0.04 mm) and vehicle (1.10 ± 0.04 mm) groups compared with the sham group (0.85 ± 0.04 mm; both $p < 0.001$). These results indicate cardiac hypertrophy, probably caused by compensation of healthy cardiomyocytes to maintain cardiac function.

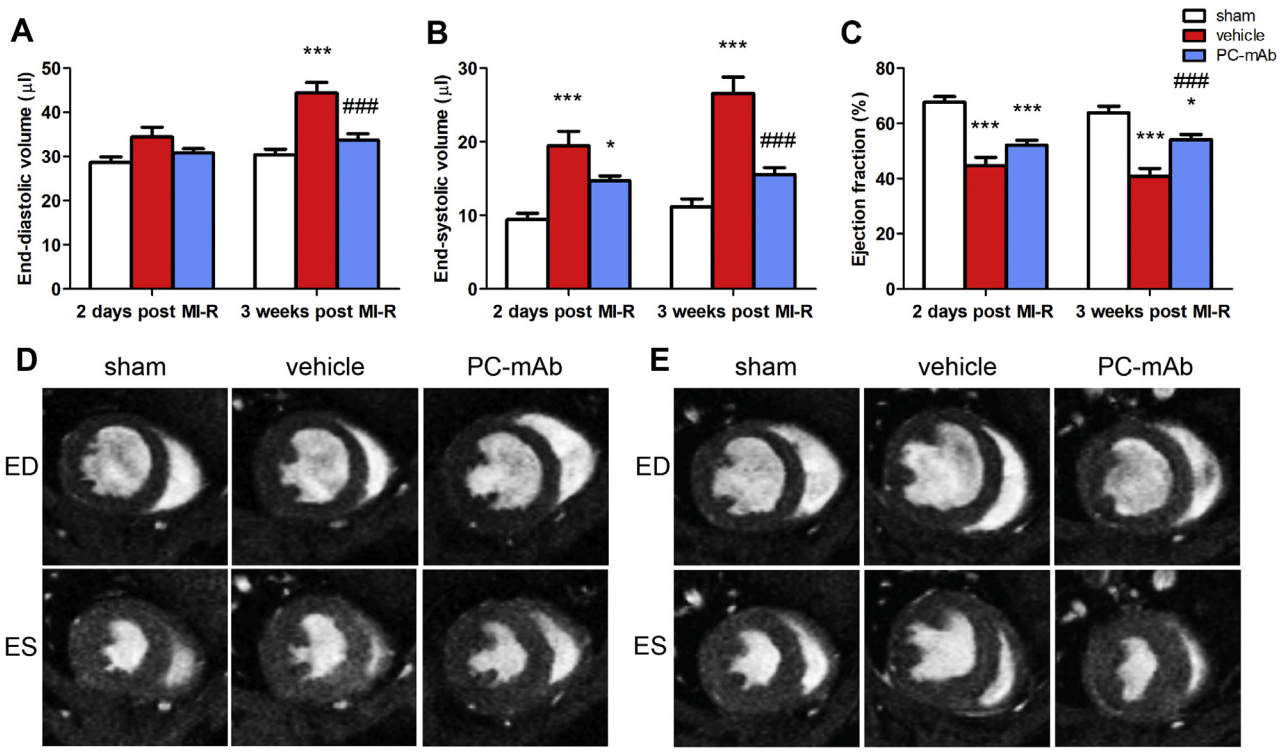
FIGURE 1 Quantification of Infarct Size Using Contrast-Enhanced Cardiac Magnetic Resonance Imaging



Infarct size was measured at baseline (2 days following myocardial ischemia-reperfusion [MI-R] injury) and at sacrifice (3 weeks post MI-R), and infarct size is quantified **(A)** as percentage of the left ventricular mass or **(B)** as absolute mass of the infarcted myocardium ($n = 14$ to 15 per group). **(C)** Absolute mass of the noninfarcted myocardium. Representative Gd-DPTA–enhanced magnetic resonance images 2 days post MI-R injury of **(D)** vehicle-treated mice and **(E)** phosphorylcholine monoclonal immunoglobulin G antibody (PC-mAb) therapy–treated mice. **Red line** indicates epicardial border, **green line** indicates endocardial border, and **yellow line** indicates infarct area. Values are mean ± SEM. $^{\#\#}p < 0.01$ vs. vehicle.

LOCAL INFLAMMATORY RESPONSE. To unravel the mechanism of PC-mAb treatment against MI-R injury, we investigated leukocyte infiltration 2 days and 3 weeks after MI-R using immunohistochemistry. First, we studied the early leukocyte infiltration 2 days post MI-R in different areas of the LV wall: the interventricular septum, border zones, and infarct area (Figure 4A). There were no differences observed in leukocyte infiltration into the interventricular septum between all groups (sham: 4.8 ± 0.7

FIGURE 2 Quantification of LV Volumes and Function Using Cardiac Magnetic Resonance Imaging



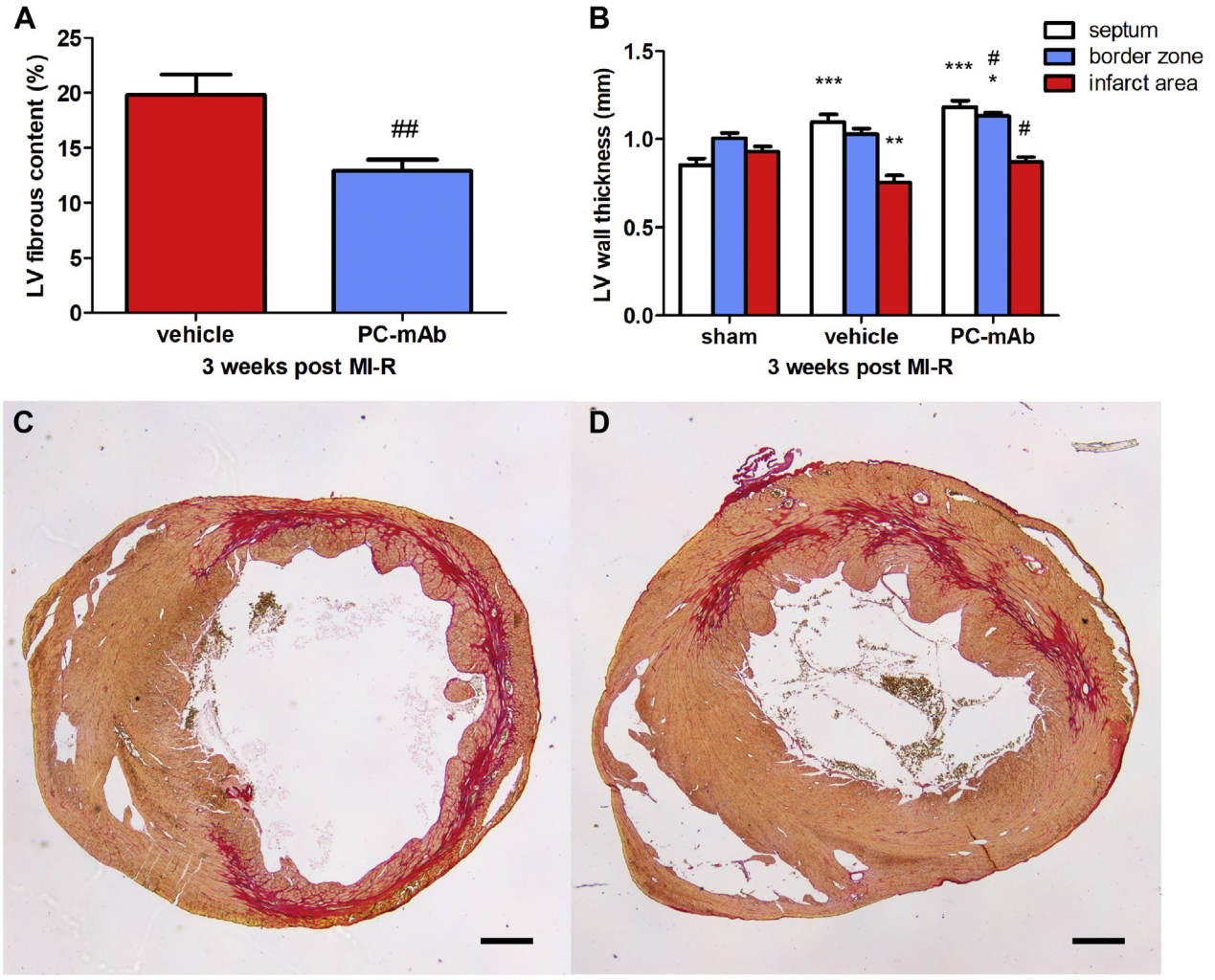
Left ventricular (LV) volumes, **(A)** end-diastolic volume and **(B)** end-systolic volume, and function, **(C)** ejection fraction, were assessed 2 days and 3 weeks after MI-R (n = 12 to 15 per group). Representative transversal short-axis magnetic resonance images at end-diastole (ED) and end-systole (ES) **(D)** 2 days and **(E)** 3 weeks after MI-R in the sham, vehicle, and PC-mAb groups. Values are mean ± SEM. ###p < 0.001 vs. vehicle, *p < 0.05, ***p < 0.001 both vs. sham. Abbreviations as in Figure 1.

leukocytes per 0.25 mm^2, vehicle: 3.3 ± 1.1 leukocytes per 0.25 mm^2, and PC-mAb: 2.7 ± 0.6 leukocytes per 0.25 mm^2). Compared with the sham group there was an increased number of leukocyte infiltration in the infarct area of the vehicle group (15.8 ± 4.7 leukocytes per 0.25 mm^2 vs. 4.9 ± 0.4 leukocytes per 0.25 mm^2; p = 0.014) and a trend toward increased leukocyte infiltration in the border zones (7.7 ± 1.6 leukocytes per 0.25 mm^2 vs. 4.8 ± 0.4 leukocytes per 0.25 mm^2). PC-mAb treatment showed a trend toward attenuated leukocyte infiltration in the infarct area and especially in the border zones (9.5 ± 1.6 leukocytes per 0.25 mm^2 and 4.2 ± 0.6 leukocytes per 0.25 mm^2; p = 0.153 and p = 0.080, consecutively).

Next, we investigated the leukocyte infiltration 3 weeks post MI-R injury in the same areas as mentioned previously (Figure 4B). In line with previous observations (36), leukocyte infiltration was found in the infarcted and noninfarcted myocardium. We observed a significant reduction of leukocyte infiltration in all areas following PC-mAb treatment compared with vehicle (septum: 0.8 ± 0.1 vs. 3.2 ± 0.7 leukocytes per 0.25 mm^2; p = 0.001; border zones: 1.1 ± 0.3 vs. 3.1 ± 0.4 leukocytes per 0.25 mm^2; p < 0.001; infarct area: 0.8 ± 0.2 vs. 3.4 ± 0.7 leukocytes per 0.25 mm^2; p < 0.001), while no differences were observed between the PC-mAb and sham group (septum: 1.2 ± 0.3 leukocytes per 0.25 mm^2; border zones: 1.0 ± 0.2 leukocytes per 0.25 mm^2; infarct area: 0.8 ± 0.1 leukocytes per 0.25 mm^2). Taken together, these results indicate that PC-mAb treatment reduces local leukocyte infiltration.

SYSTEMIC INFLAMMATORY RESPONSE. To investigate the effect of PC-mAb treatment on the systemic inflammatory response after MI-R injury, we analyzed serum CCL2 levels 2 days and 3 weeks post MI-R injury. Two days after MI-R, CCL2 levels (Figure 5A) were significantly reduced following PC-mAb treatment (13.4 ± 10.0 pg/ml) compared with both vehicle (74.3 ± 6.6 pg/ml; p = 0.007) and sham (80.5 ± 14.5 pg/ml; p = 0.002) treatment. Three weeks after MI-R, the effect of PC-mAb treatment on CCL2 levels was less obvious (Figure 5B). Although not significantly, CCL2 levels were decreased following PC-mAb treatment

FIGURE 3 Histological Quantification of LV Fibrous Content and LV Wall Thickness 3 Weeks Following MI-R

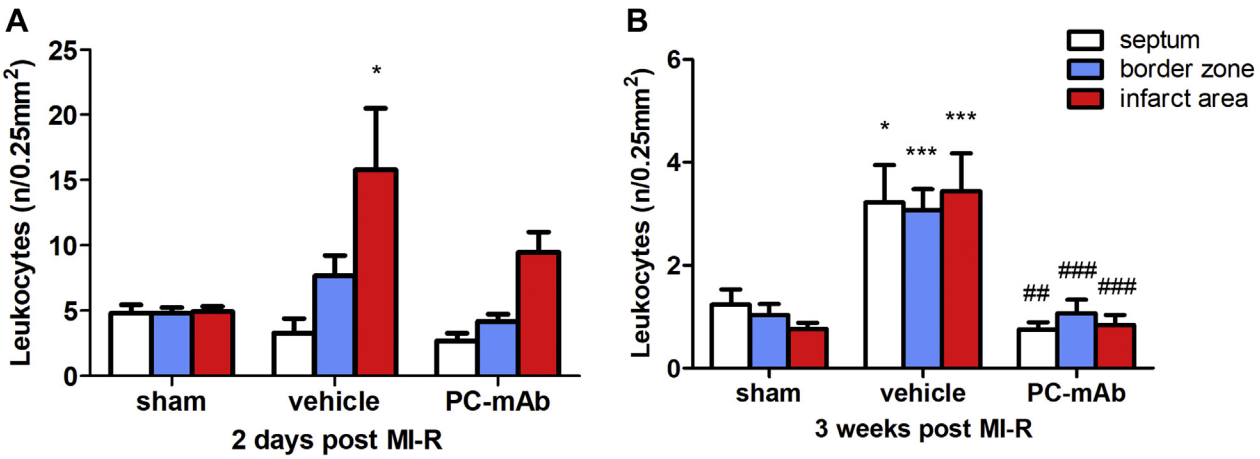


(A) Left ventricular (LV) fibrous content was measured by Sirius red staining and quantified as the area of the LV occupied by collagen. **(B)** LV wall thickness was assessed in 3 specific areas: septum, border zone, and infarct area (n = 9 to 10 per group). Representative images of Sirius red staining of **(C)** vehicle-treated mice and **(D)** PC-mAb–treated mice. Scale bar = 500 μm. Values are mean ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ both vs. vehicle; *p < 0.05, **p < 0.01, ***p < 0.001 all vs. sham. Abbreviations as in Figure 1.

(20.1 ± 7.5 pg/ml) compared with the vehicle (54.3 ± 6.0 pg/ml) and sham (64.9 ± 21.3 pg/ml) groups.

Finally, we investigated the effect of PC-mAb treatment on circulating monocytes 2 days after MI-R. The percentage circulating monocytes (of total leukocytes) was significantly increased following MI-R injury in the vehicle group (4.3 ± 0.8%) compared with the sham group (2.0 ± 0.5%; p = 0.030) (Figure 5C), but total circulating monocytes were not significantly reduced following PC-mAb treatment (2.5 ± 0.4%; p = 0.090) as compared with vehicle treatment. However, the percentage circulating proinflammatory Ly-6C^{hi} monocytes was significantly reduced in the PC-mAb group (1.2 ± 0.2%) compared with the vehicle group (2.5 ± 0.6%; p = 0.017), while no significant difference was observed when compared with the sham group (0.8 ± 0.2%) (Figure 5D). Regarding the percentage circulating reparative Ly-6C^{lo} monocytes, no significant differences were observed between all groups 2 days after MI-R (sham: 0.9 ± 0.2%; vehicle: 1.3 ± 0.5%; PC-mAb: 1.0 ± 0.3%) (Figure 5E). Taken together, these results suggest that PC-mAb treatment especially reduces the early inflammatory response following MI-R injury.

FIGURE 4 Histological Quantification of the Local Inflammatory Response



The number of CD45-positive cells (leukocytes) were counted per specific area (septum, border zone, and infarct area) as a measure of local inflammation. Each bar represents the average number of leukocytes per field of view in the specific areas **(A)** 2 days post MI-R (n = 3 to 5 per group) and **(B)** 3 weeks post MI-R (n = 9 to 10 per group). Values are mean ± SEM. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ both vs. vehicle; $^{*}p < 0.05$, $^{***}p < 0.001$ both vs. sham. Abbreviations as in Figure 1.

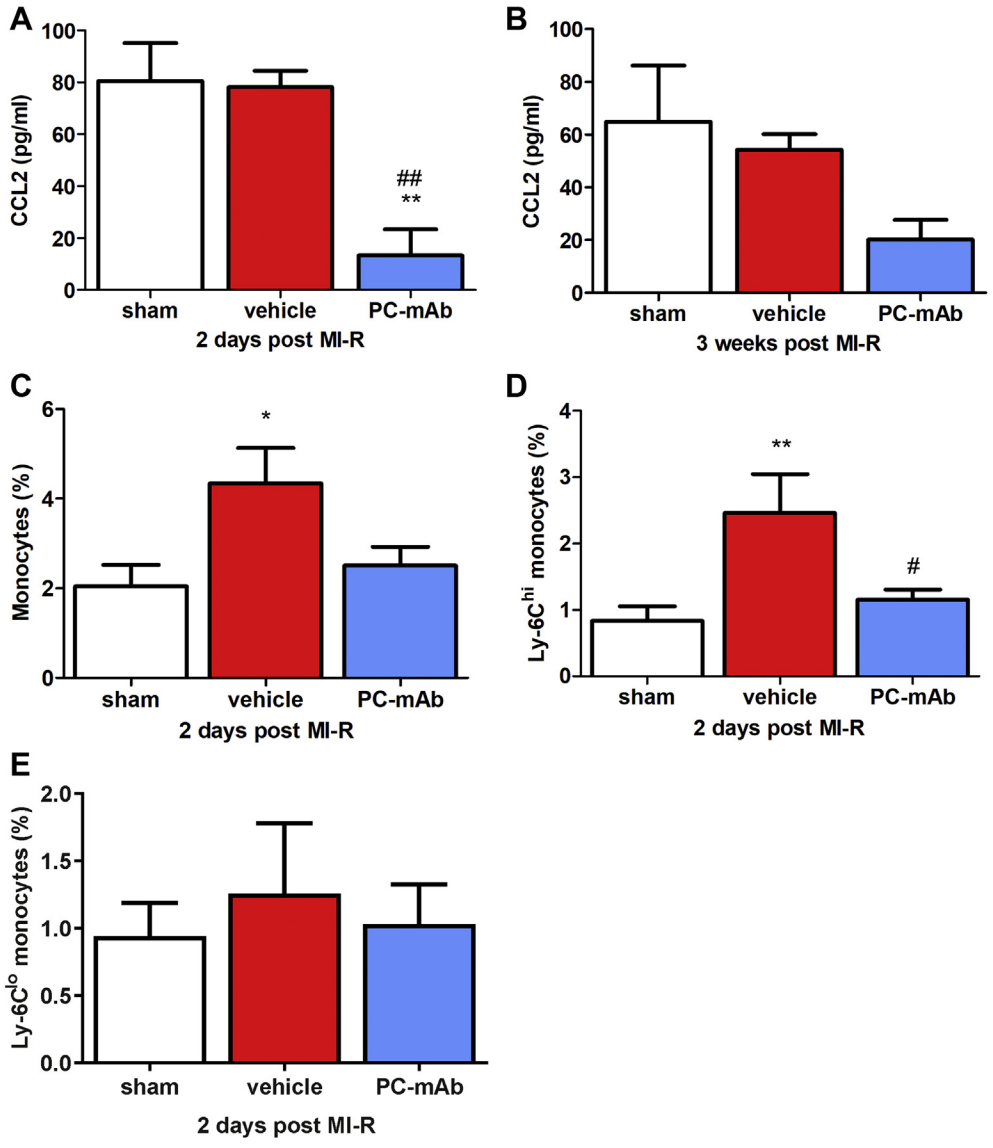
DISCUSSION

This study shows a therapeutic effect of PC-mAb treatment after MI-R injury. Administration of PC-mAb after MI-R injury attenuated the early systemic inflammatory response, by reduction of serum CCL2 levels and circulating Ly-6C^{hi} monocytes after 2 days, as well as the late local inflammatory response by decreased myocardial leukocyte infiltration after 3 weeks. This prevented excessive cardiomyocyte cell death, as expressed by a decreased IS and preservation of LV wall thickness, which eventually caused restricted LV dilatation and preserved LV function. Therefore, this fully human monoclonal IgG1 PC antibody might be a potential future clinical therapeutic agent for the prevention of post-ischemic myocardial remodeling and development of heart failure, and is currently used in a phase 2a randomized, double-blind, placebo-controlled multicenter pilot study in patients with acute MI.

POST-ISCHEMIC LV REMODELING AND FUNCTION. Adverse cardiac remodeling after MI is characterized by an increase of both EDV and ESV, normally followed by a reduced EF (37). In this study, we assessed EDV, ESV, and EF and found PC-mAb treatment to significantly restrict the increase in EDV and ESV following MI-R injury accompanied by a significant increase in EF, suggesting limitation of adverse LV remodeling with preservation of LV systolic function.

We investigated the effect of PC-mAb treatment on IS using contrast-enhanced CMR and showed PC-mAb treatment to significantly decrease IS 3 weeks after MI-R injury. Previous research showed that IS is directly related to LV remodeling and clinical outcome following MI (38,39). This suggests the observed preservation of LV function to be the result of improved infarct healing as demonstrated by the reduced IS. In addition, we histologically supported this observation demonstrating a decreased LV fibrous content as a measure of IS and increased LV wall thickness following PC-mAb treatment. LV wall thickness is affected following MI because of the loss of viable cardiomyocytes, which are replaced by collagen (40). The preservation of LV wall thickness might indicate that PC-mAb treatment restricts loss of viable cardiomyocytes. Because IS was not affected 2 days after administration of PC-mAb, the reduced adverse remodeling is likely to be the effect of attenuating the post-ischemic inflammatory response, rather than direct survival of ischemic cardiomyocytes. This is also supported by our data of PC-mAb binding late apoptotic cells as well as suppressed CCL2 levels after treating cultured activated monocytes with PC-mAb. Furthermore, we observed an increase in LV wall thickness in the interventricular septum in both the PC-mAb and vehicle groups, most likely the result of cardiac hypertrophy caused by compensation of healthy cardiomyocytes to maintain cardiac function (41).

FIGURE 5 Quantification of the Systemic Inflammatory Response



Serum levels of chemokine (C-C motif) ligand 2 (CCL2) were determined using enzyme-linked immunosorbent assay as a measure of systemic inflammation, **(A)** 2 days after MI-R (n = 4 to 8 per group) and **(B)** 3 weeks after MI-R (n = 9 to 10 per group). **(C)** Circulating monocytes and different monocyte subsets, **(D)** Ly-6C^{hi} and **(E)** Ly-6C^{lo}, were determined 2 days after MI-R using fluorescence-activated cell sorting analysis and expressed as percentage of total leukocytes (n = 4 to 8 per group). Values are mean ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ both vs. vehicle; $^{*}p < 0.05$, $^{**}p < 0.01$ both vs. sham. Abbreviations as in Figure 1.

POST-ISCHEMIC INFLAMMATORY RESPONSE. Inflammation plays an important role in the repair process following MI leading to a matured scar formation (42). Reperfusion itself causes additional damage to the myocardium by the formation of reactive oxygen species (4) and accelerates cell membrane damage of cardiomyocytes (43), making ischemic-reperfused myocardium amenable to anti-inflammatory interventions. We assessed the effect of PC-mAb treatment on the post-reperfusion inflammatory response by quantification of local infiltration of leukocytes in the LV wall, which was significantly decreased following PC-mAb treatment after 3 weeks. Infarct healing following MI-R injury

can be divided into 2 phases, the first being the early inflammatory phase, in which leukocytes are predominantly present in the infarct area playing an important role by removing dead cells and matrix debris, with the second being the reparative phase, in which scar tissue is formed (42). Besides the infiltration into the infarct area, innate immune cells are also known for their recruitment into the remote myocardium (36). Our results suggest PC-mAb treatment to reduce the adverse inflammatory response, while the beneficial early inflammatory response is less affected, as demonstrated by a nonsignificant difference in early leukocyte infiltration after 2 days.

CCL2 is an important chemokine responsible for the recruitment of leukocytes to injured tissue (44). Even though leukocytes remove possible immunogenic cell components and promote infarct healing after MI, CCL2 knockout mice experience reduced macrophage recruitment to the infarcted myocardium, which resulted in decreased adverse ventricular remodeling following MI-R injury (45). In agreement with the previously mentioned study, we demonstrated that PC-mAb treatment resulted in significant reduction of CCL2 serum levels 2 days after MI-R injury. Previously, we demonstrated PC-mAb to reduce CCL2 production of monocytes stimulated with oxidized low-density lipoprotein in vitro and CCL2 expression in cuffed femoral arteries in vivo (33). Systemic CCL2 levels are increased in APOE*3-Leiden mice when fed a high-fat diet (46). We assume that PC-mAb is capable of binding to PC-expressing cells, thereby attenuating the initial immediate systemic inflammatory response or proinflammatory reaction, as observed by reduced CCL2 levels, and subsequently enhances the repair process mediated by M2 macrophages, also supported by the reduced CCL2 levels following PC-mAb treatment of cultured activated monocytes. This finally contributed to the reduction of adverse LV remodeling and preservation of systolic function.

In addition, we observed a decrease of the percentage of monocytes in blood following PC-mAb treatment 2 days after reperfusion. As mentioned previously, infarct healing can be divided in 2 different phases, in which both phases a different subset of monocytes plays its own specific role. In the inflammatory phase, proinflammatory Ly-6C^{hi} monocytes, which can differentiate into proinflammatory M1 macrophages, contribute by clearing the infarct site from necrotic cells and matrix debris. In the reparative phase, anti-inflammatory Ly-6C^{lo} monocytes, which can differentiate into repair associated M2 macrophages, play an important role in scar formation and infarct healing (42). In this study we observed a decrease in Ly-6C^{hi} monocytes, but not in Ly-6C^{lo} monocytes. Thus, despite Ly-6C^{hi} monocytes play an important role in clearing the infarct site from cell debris, we found a beneficial effect on infarct healing and LV function following a PC-mAb induced reduction of circulating Ly-6C^{hi} monocytes. In agreement, it has been shown that hypercholesterolemia results in increased numbers of Ly-6C^{hi} monocytes (47), thereby influencing infarct healing and cardiac function following MI (48–50). Furthermore, hypercholesterolemia affects MI-R injury in mice (51–53) and it is an important risk factor of MI in human (54). Vice versa, MI has been shown to accelerate atherosclerosis (55), indicating important interactions between both inflammatory processes. This makes them both amenable to anti-inflammatory and immunomodulatory treatment (56).

Upon a myocardial ischemic event, affected cardiomyocytes can undergo apoptosis (57), thereby expressing oxidation-specific epitopes, like PC, on their outer membrane (58), which are immunogenic (12). Previous research showed that natural and monoclonal EO6/T15 antibodies against PC are capable to bind apoptotic cells and oxidized low-density lipoprotein (16,58,59) thereby dampening the inflammatory response (12,17). Therefore, we postulate that PC-mAb tempers the immediate systemic inflammatory response following MI-R injury by binding PC-expressing cells before they trigger the innate immune system and enhances the repair process mediated by M2 macrophages, finally preventing cardiomyocyte cell death and increasing apoptotic cell clearance which subsequently leads to reduced adverse cardiac remodeling and preservation of cardiac function.

STUDY LIMITATIONS. Although using a translational animal model considering a hypercholesterolemic phenotype and MI-R injury following rapid reperfusion to attempt mimicking the clinical setting, additional human studies are needed to test the clinical relevance of PC-mAb therapy. Further research in atherosclerotic large animal models with human-like plaques or human atherosclerotic patients is needed to investigate the effect on atherosclerotic plaque development. In addition, as a result of the multiple effects of PC-mAb such as direct cellular membrane binding, oxidized low-density lipoprotein binding with inhibition of Toll-like receptor 4 mediated activation,

lipoprotein(a)-related effects, and effects via apoptosis, the exact cellular mechanisms remain partially uncertain, although positive clinical effects seem likely. Until now, phase 1 studies showed good safety and tolerability and currently a phase 2a, placebo-controlled, double-blind, randomized multicenter pilot study in patients with acute MI is running. In this study, we used a fully human IgG monoclonal antibody in a murine MI-R model, however, previous research confirmed the absence of an immune response. So thus far, PC-mAb therapy seemed safe and promising regarding translation toward the clinical situation.

CONCLUSIONS

PC-mAb treatment attenuates the post-ischemic inflammatory response following MI-R injury as demonstrated by a reduction of systemic CCL2 levels and circulating Ly-6C^{hi} monocytes resulting in impaired myocardial leukocyte infiltration and preservation of LV wall thickness. In a hypercholesterolemic mouse model mimicking the clinical setting, this resulted in limited adverse cardiac remodeling, with a decreased infarct size causing reduced LV dilatation, preventing development of heart failure and preserving LV function. Therefore, PC-mAb therapy may be a valid therapeutic approach against MI-R injury.

AUTHOR DISCLOSURES

Dr. Pettersson is listed as an inventor on patent for and is minor shareholder in Athera Biotechnologies. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The therapeutic potential of anti-inflammatory therapies is of interest in reducing cardiovascular events following MI. Antibodies against phosphorylcholine are known to have anti-inflammatory properties by blocking the uptake of oxidized low-density lipoprotein by macrophages.

TRANSLATIONAL OUTLOOK: PC-mAb therapy may be a valid therapeutic approach against MI-R injury.

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KEY WORDS cardiac function, infarct size, inflammation, myocardial infarction, myocardial ischemia-reperfusion

APPENDIX For expanded Methods and References sections as well as supplemental figures, please see the online version of this paper.