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Impaired cardiac function in hypercholesterolemic APOE*3-Leiden mice after myocardial ischemia-reperfusion injury despite reduced infarct size and inflammation

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Abstract

Objective: Hypercholesterolemia is a major risk factor for ischemic heart disease including acute myocardial infarction (MI). However, long-term effects of hypercholesterolemia in a rodent ischemia-reperfusion MI model are unknown. We therefore investigated the effects of diet-induced hypercholesterolemia in APOE*3-Leiden mice on cardiac function up to 8 weeks after myocardial ischemia-reperfusion (MI-R) injury.

Methods: In normocholesterolemic (NC-MI, n=16) and hypercholesterolemic (HC-MI, n=18) mice myocardial ischemia was induced by coronary ligation for 45 minutes followed by permanent reperfusion. Left ventricular (LV) dimensions were serially assessed using parasternal long-axis echocardiography (0, 1, 3, and 8 weeks after MI-R) followed by LV pressure-volume measurements 8 weeks post-MI-R. Subsequently, infarct size and the inflammatory response were analyzed by histology and fluorescence-activated cell sorting (FACS) analysis.

Results: Intrinsic LV function 8 weeks after MI-R was impaired in HC-MI compared to NC-MI mice as assessed by end-systolic pressure (73±3mmHg vs. 89±2mmHg, P=0.001), dP/dt_{MAX} (6.1±0.4mmHg/ms vs. 8.3±0.4mmHg/ms, P=0.001), and -dP/dt_{MIN} (5.3±0.4mmHg/ms vs. 6.8±0.2mmHg/ms, P=0.005). Paradoxically, infarct size was decreased in HC-MI compared to NC-MI mice (12.7±2.0% vs. 22.2±2.9%, P=0.017), which was accompanied by increased wall thickness (0.81±0.05mm vs. 0.57±0.05mm, P=0.007). Hypercholesterolemia caused a pre-ischemic peripheral monocytosis, in particular of Ly-6C^{high} monocytes whereas accumulation of macrophages in the ischemic-reperfused myocardium of HC-MI mice was decreased.

Conclusion: Diet-induced hypercholesterolemia caused an impaired LV function 8 weeks after MI-R injury despite of a reduced post-ischemic infarct size. This was preceded by a pre-ischemic peripheral monocytosis, while there was a suppressed accumulation of inflammatory cells in the ischemic-reperfused myocardium after 8 weeks.
Introduction

Hypercholesterolemia plays an important role in the occurrence of atherosclerosis (1) and is a major risk factor for ischemic heart disease (2). Several clinical studies demonstrated an adverse effect of hypercholesterolemia on coronary heart disease events and left ventricular (LV) systolic function after suffering a myocardial infarction (MI) (3, 4) and reported positive effects of lipid lowering therapies (5, 6).

Experimental MI studies have reported controversial findings regarding the effect of diet-induced hypercholesterolemia on cardiac function following myocardial ischemia-reperfusion (MI-R). Acute cholesterol feeding, up to 3 weeks, is associated with increased MI-R injury (7, 8). Also, prolonged exposure to diet-induced hypercholesterolemia, during up to 20 weeks, followed by MI-R injury with reperfusion periods up to 24 hours showed a reduced hemodynamic performance (9) and a negative inotropic effect (10). In addition, myocardial injury was exacerbated by increased cardiomyocyte apoptosis (11), upregulation of the myeloperoxidase-related inflammatory response (12, 13), reduced myocardial nitric oxide synthesis (14), and increased myocardial necrosis (15, 16). Conversely, other studies reported hypercholesterolemia to cause an improved mechanical recovery (9, 10), a positive inotropic effect (15), and decreased cardiac necrosis (17). However, information on follow-up periods longer than 24 hours is lacking.

To study the effects of hypercholesterolemia on cardiovascular outcome after MI, animal models should mimic the clinical setting. The apolipoprotein E3-Leiden (APOE*3-Leiden) transgenic mouse (18) develops advanced aortic atherosclerotic lesions resembling their human counterparts when exposed to cholesterol feeding (19, 20). On normal chow diet these mice develop no hypercholesterolemia or atherosclerosis. Thus, this hypercholesterolemic mouse model proved to be useful in studying the environmental and genetic factors in the occurrence of hyperlipidemia (20, 21) and the development of atherosclerosis (20, 22). In addition, APOE*3-Leiden mice have been used to study the effects of various lipid lowering therapies (23-25). Although APOE*3-Leiden mice differ from the human situation, because of the absence of coronary atherosclerosis resulting in coronary plaque formation and the lack of rupture followed by thrombus formation, it does provide an excellent model to study the effects of hypercholesterolemia on the pathophysiological processes in an animal model after surgical interventions (26-29) including induction of MI. Furthermore, hypercholesterolemic-induced atherosclerosis itself is considered to be an inflammatory disease which contributes to and affects the post-ischemic inflammatory response (1, 30).

Therefore, the aim of the present study was to investigate the long-term effects of hypercholesterolemia on MI-R induced injury in APOE*3-Leiden mice with a follow-up period of 8 weeks after MI-R, focusing on cardiac function, infarct size, and the post-ischemic inflammatory response.
Materials and Methods

Animals and diets. Transgenic female APOE*3-Leiden mice (18), backcrossed for more than 40 generations on a C57Bl/6J background, aged 8-10 weeks at the start of a dietary run-in period (bred in the animal facility of the Leiden University Medical Center), were used for this experiment. Mice were randomly assigned to either a normal chow (normocholesterolemic, NC) or a semisynthetic Western-type diet supplemented with 0.4% cholesterol (hypercholesterolemic, HC) (AB Diets, Woerden, The Netherlands). The diet was started 4 weeks prior to surgery and was continued until the end of the experiment. Mice were housed under standard conditions in conventional cages and received food and water ad libitum. All animal experiments were approved by the Institutional Committee for Animal Welfare and conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Plasma lipid analysis. Plasma total cholesterol (TC) and triglyceride (TG) levels were determined prior to diet exposure, before induction of MI, and 4 and 8 weeks after surgery. After a 4 hours fasting period, blood was obtained via tail vein bleeding (~50μl) and assayed for plasma total cholesterol (TC) and triglyceride (TG) levels using commercially available enzymatic kits according to the manufacturer’s protocols (11489232 and 11488872; Roche Diagnostics, Mannheim, Germany, respectively)

Surgical MI-R protocol. Surgical ligation of the left anterior descending (LAD) coronary artery followed by permanent reperfusion was performed at day 0 in 12-14 weeks old female APOE*3-Leiden mice as described previously (31). Briefly, mice were pre-anesthetized with 5% isoflurane in a gas mixture of oxygen and room air 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 2% isoflurane. Subsequently, a left thoracotomy was performed in the 4th intercostal space and the LAD coronary artery was ligated using a 7-0 prolene suture. A knot was tied on a 1mm section of a plastic tube placed on top of the LAD to occlude the coronary artery during 45 minutes. Ischemia was confirmed by myocardial blanching and ECG changes. Muscle flaps were folded back, covered with a pre-warmed wet surgical mesh, and body temperature was kept constant between 35-37°C during this period. After 35 minutes of ischemia mice received an intraperitoneal injection of lidocaine (6mg/kg) (32) to prevent cardiac arrhythmias caused by reperfusion. After 45 minutes of ischemia permanent reperfusion was established. 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. The experimental protocol consisted of a normal diet MI-R group (NC-MI, n=16) and cholesterol-enriched diet MI-R group (HC-MI, n=18).
Echocardiography. To evaluate LV function, in vivo transthoracic echocardiography was performed in anesthetized (2% isoflurane) mice using a 15-45MHz RMV707B probe interfaced with a Vevo 770 imaging system (VisualSonics Inc, Toronto, Canada). Two-dimensional echocardiography was achieved in all mice before induction of MI (week 0) to assess baseline cardiac function and serve as an internal control. Subsequently, LV function was measured at 1, 3, and 8 weeks after MI-R. Mice were placed on a heating table in a supine position, with their extremities fixed to four electrocardiography leads. The chest was shaved to minimize ultrasound attenuation and warmed Aquasonic gel (Parker Laboratories Inc, Fairfield, USA) was applied to optimize visibility.

Parasternal long-axis B-Mode, M-Mode, and EKG-gated Kilohertz Visualization (EKV) images were obtained with appropriate angulation and acquisition of maximum LV length, from apex to aortic valve. Datasets were analyzed in a blinded manner, using Visual Sonics software version 3.0.0 (2008). After tracing the end-systolic and end-diastolic endocardial LV area of parasternal long-axis EKV images (33) LV end-diastolic volume (EDV), LV end-systolic volume (ESV), LV ejection fraction (EF), and cardiac output (CO) were calculated.

Hemodynamic measurements. After 8 weeks hemodynamics and LV function indices were determined by invasive LV pressure-volume (PV) relationships. After induction of anesthesia a midline neck incision was performed, and a 1.2F PV catheter (FTS-1212B-4518; Scisense Inc, Ontario, Canada) connected to an ADV signal processor (Scisense Inc) was inserted via the right carotid artery and positioned optimally into the LV to generate high-fidelity PV signals. On-line display and acquisition of the signals (2000 samples·s⁻¹) was obtained with a PowerLab 8/30 system and LabChart Pro software (AD Instruments GmbH, Spechbach, Germany). Parallel conductance was obtained with the hypertonic saline method using intravenous bolus injections of ~5μl 10% saline (34) and calibrated with corresponding echocardiographic values of CO. All data were analyzed off-line in a blinded fashion with custom-made software.

PV signals were obtained in steady state to measure heart rate (HR), CO, ESV, EDV, end-systolic and end-diastolic pressure (ESP and EDP), maximal and minimal rates of LV pressure change (dP/dt MAX and dP/dt MIN), isovolumetric relaxation time constant (Tau), stroke work (SW), effective arterial elastance (Ea), end-systolic peak isovolumic pressure (ESP iso), end-systolic elastance (E Es), end-systolic intercept volume (ESV int), end-diastolic stiffness (E ED), and end-diastolic intercept volume (EDV int).

After measurements, the heart and lungs were quickly excised. Hearts were immersion-fixated in 4% paraformaldehyde for 24 hours and embedded in paraffin. The body weight and wet lung weight were measured from all animals and lungs were then freeze-dried. The difference between wet and dry lung-weight was used as a measure of pulmonary congestion.
Infarct size, LV wall thickness and vascular density. Paraffin-embedded hearts were cut into serial transverse sections of 5μm along the entire long-axis of the LV and subsequently mounted on slides (n=8 for each group). To analyze collagen deposition as an indicator of the fibrotic area, every 50th section of each heart was stained with Sirius Red resulting in approximately 15 stained sections of each heart. Infarct size was determined by planimetric measurement of all sections and calculated as fibrotic area divided by the total LV wall surface area including the interventricular septum. LV wall thickness was measured in five different sections equally distributed through the infarcted area. Per section, wall thickness was analyzed in the mid-infarcted area, both border zones, and interventricular septum. Measurements were performed perpendicular to the ventricular or septal wall.

To determine the vascular profile, serial sections were stained for PECAM-1 (CD31, clone MECA13.3, 550274; BD Pharmingen, San Diego, CA, USA). Subsequent to incubations with an appropriate biotinylated secondary antibody and the signal amplifying ABC system (Vectastain; Vector Laboratories, Burlingame, CA, USA), the reaction product was visualized with 3,3’-diaminobenzidine and counterstained with Mayer’s hematoxillin. Vascular density was determined by quantifying the number of PECAM-1 positive blood vessels per 0.25mm² per section, differentiating between small (<20μm) and large (>20μm) vessels, in the infarcted border zones (4 areas), and infarcted myocardium (5 areas). All measurements were performed by an observer blinded to the groups, using the Image-Pro Plus software package (Media Cybernetics Inc, Bethesda, MD, USA).

Inflammatory response. To study the in vivo effects of the cholesterol enriched diet and MI-R injury, whole blood was analyzed for peripheral monocytosis one week before induction of MI (NC-MI, n=11, and HC-MI, n=15) and 3 weeks after MI-R (NC-MI, n=6, and HC-MI, n=7). Hematological values obtained were white blood cell counts (WBC, x10⁶/ml), red blood cell counts (RBC, x10⁹/ml), and platelets (PLT, x10⁶/ml) 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 minutes on ice with directly conjugated antibodies directed against Ly-6C-FITC (AbD Serotec, Dusseldorf, Germany), Ly-6G-PE (BD Pharmingen, San Diego, CA, USA), CD11b-APC (BD Pharmingen, San Diego, CA, USA), CD115-PerCP (R&D Systems, Minneapolis, MN, USA), and CD45R-APC-Cy7 (eBioscience, San Diego, CA, USA). Monocytes were gated based on their expression profile: Ly-6G-negative, CD11b-positive, and CD115-positive. Pro-inflammatory monocytes were identified based on high Ly-6C expression levels.

For analysis of the local cardiac inflammatory response 8 weeks after MI-R, paraffin sections of the mid-infarct region of the heart were stained using antibodies against leukocytes (anti-CD45, 550539; BD Pharmingen, San Diego, CA, USA) and macrophages (anti-Mac-3, 550292; BD Pharmingen, San Diego, CA, USA). The number of leukocytes and macrophages were expressed as a number per 0.25mm² in the septum (2 areas), border zones (2 areas), and infarcted myocardium (5 areas).
**Statistical analysis.** Values were expressed as means±SEM. Comparisons of parameters between the NC-MI and HC-MI groups were made using independent samples \( t \)-test or 2-way analysis of variance with repeated measures and Bonferroni correction in case of multiple time points. A value of \( P<0.05 \) was considered to represent a significant difference. All statistical procedures were performed using SPSS 20.0.0 (SPSS Inc – IBM, Armonk, NY, USA).

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**Results**

**Plasma lipid profiles and animal characteristics.** Total cholesterol plasma levels in the HC-MI group were increased after exposure to the cholesterol-enriched diet for 4 weeks compared to the normal chow diet group (18.2±1.1mmol/L vs. 2.0±0.3mmol/L, \( P<0.001 \)) and remained stable during the experimental protocol. Triglycerides levels (3.0±0.1mmol/L vs. 2.5±0.3mmol/L) were not significantly different between groups (Figure 1).

![Lipid profiles](image)

**Figure 1.** Lipid profiles. Plasma (A) total cholesterol and (B) triglycerides levels in the NC-MI (□) and HC-MI (■) group. Data are means±SEM. ***\( P<0.001 \) vs. NC-MI group.
No difference in body weight (BW) was observed between groups prior to the induction of MI-R. However, 8 weeks after MI-R weight gain as expressed by BW change was increased in HC-MI as compared to NC-MI mice (12.1±1.1% vs. 7.3±1.3%, \(P=0.009\)). No difference in the amount of lung fluid was observed between both groups (Supplementary material, Table 1). In addition, cumulative survival was comparable between both groups (data not shown).

**Echocardiography.** Serial echocardiography 8 weeks post MI-R revealed an increase in LV dimensions in both groups as compared to baseline cardiac function before MI-R. In the HC-MI group EDV (49.6±2.1μl vs. 41.4±0.5μl, \(P<0.001\)) and ESV (27.4±1.7μl vs. 16.6±0.6μl, \(P<0.001\)) were increased. In the NC-MI group the EDV (52.0±2.7μl vs. 39.4±0.9μl, \(P<0.001\)) and ESV (30.3±2.7μl vs. 14.5±0.5μl, \(P<0.001\)) were increased as well (Figure 2A and 2B). This was accompanied by a progressive impairment of LV function 8

| Table 1. Pressure-volume loops-derived LV function indices. |
|-----------------|-----------------|
|                 | NC-MI            | HC-MI            |
| HR (beats min \(^{-1}\)) | 551 ± 18         | 531 ± 20         |
| CO (ml min \(^{-1}\))     | 13.5 ± 2.5       | 11.4 ± 1.0       |
| ESV (μl)              | 29 ± 5           | 28 ± 5           |
| EDV (μl)              | 53 ± 7           | 49 ± 6           |
| ESP (mmHg)            | 89 ± 2           | 73 ± 3***        |
| EDP (mmHg)            | 4.4 ± 0.6        | 5.9 ± 0.8        |
| \(dP/dt_{max}\) (mmHg ms \(^{-1}\)) | 8.3 ± 0.4        | 6.1 ± 0.4***    |
| \(-dP/dt_{min}\) (mmHg ms \(^{-1}\)) | 6.8 ± 0.2        | 5.3 ± 0.4**     |
| Tau (ms)              | 9.8 ± 0.3        | 11.1 ± 0.8       |
| SW (mmHg.ml)          | 1.9 ± 0.2        | 1.6 ± 0.0        |
| \(E_a\)              | 4.8 ± 1.1        | 3.7 ± 0.3        |
| ESP \(_{in}\)         | 164 ± 5          | 131 ± 5***       |
| ESPVR                 |                 |                 |
| slope: \(E_{es}\) (mmHg μl \(^{-1}\)) | 3.8 ± 0.6        | 2.8 ± 0.3        |
| intercept: ESV \(_{int}\) (μl) | 26.3 ± 4.9       | 29.9 ± 5.1       |
| EDPVR                 |                 |                 |
| slope: \(E_{ed}\) (mmHg μl \(^{-1}\)) | 0.18 ± 0.03      | 0.26 ± 0.05      |
| intercept: EDV \(_{int}\) (μl) | 61.1 ± 10.9      | 46.7 ± 4.0       |

CO, cardiac output; \(dP/dt_{max}\), maximum rate of pressure increase; \(-dP/dt_{min}\), maximum rate of pressure decrease; \(E_a\), effective arterial elastance; EDP, end-diastolic pressure; ESPVR, end-diastolic pressure-volume relationship; EDV, end-diastolic volume; ESV, end-systolic volume; \(E_{es}\), end-systolic elastance; \(E_{ed}\), end-systolic pressure; \(E_{es-p}\), end-systolic peak isovolumic pressure; \(E_{es-p}\), end-systolic pressure-volume relationship; \(E_{es-i}\), end-systolic intercept volume; HR, heart rate; SW, stroke work; Tau, relaxation time constant. Values are means±SEM. **\(P<0.01\), ***\(P<0.001\) vs. NC-MI.
weeks after MI-R as indicated by a decrease in EF (Figure 2C) in the HC-MI (45.0±2.0% vs. 60.1±1.3% before MI-R, \(P<0.001\)) and NC-MI group (43.3±2.5% vs. 63.3±0.7% before MI-R, \(P<0.001\)). There were no differences observed between the HC-MI and NC-MI group during the experiment.

**Hemodynamic measurements.** The functional PV loop-derived data of the groups are presented in Table 1. In accordance with the echocardiographic data, LV volumes did not differ between the HC-MI and NC-MI group. However, when compared to the NC-MI group, a marked impaired intrinsic LV function was found in the HC-MI group as demonstrated by a significantly depressed ESP (73±3mmHg vs. 89±2mmHg, \(P=0.001\)), \(\frac{dP}{dt}_{\text{MAX}}\) (6.1±0.4mmHg/ms vs. 8.3±0.4mmHg/ms, \(P=0.001\)), \(-\frac{dP}{dt}_{\text{MIN}}\) (5.3±0.4mmHg/ms vs. 6.8±0.2mmHg/ms, \(P=0.005\)).

**Infarct size, LV wall thickness, and vascular density.** Histological evaluation 8 weeks after MI-R showed a smaller infarct area in the HC-MI group compared to the NC-MI group (12.7±2.0% vs. 22.2±2.9%, \(P=0.017\), Figure 3A). The subendocardial and epicardial
surviving borders of cardiomyocytes in the HC-MI group (Figure 3D) were larger compared with the NC-MI group (Figure 3C). This resulted in an increased LV wall thickness in the mid-infarct area in the HC-MI group as compared to the NC-MI group (0.81±0.05mm vs. 0.57±0.05mm, \( P=0.007 \), Figure 3B).

**Figure 3.** Infarct size, LV wall thickness, and vascular profile 8 weeks after MI-R. Infarct size was significantly smaller in the HC-MI group compared with the NC-MI group (A). Wall thickness of the infarct area was significantly larger in the HC-MI group (B). Sirius red staining in the HC-MI group showed a more pronounced subendocardial and epicardial border of surviving cardiomyocytes in the HC-MI group (D) compared to the NC-MI group (C). Vascular analysis showed no differences in number of small capillaries (<20μm) or large vessels (>20μm) in the border zone (BZ) or infarct area (MI) between both groups (E). Data are means±SEM. *\( P<0.05 \), **\( P<0.01 \).
Analysis of the vascular profile in HC-MI and NC-MI groups showed no significant differences in the number of small capillaries (<20μm) and large vessels (>20μm) between the groups (Figure 3E).

**Inflammatory response.** After a dietary run-in period the HC-MI group revealed a pre-ischemic peripheral monocytosis as compared to the NC-MI group as expressed by the percentage of monocytes (41.6±2.1% vs. 33.6±1.6% of total leukocytes, \( P=0.009 \), Figure 4A), which was normalized 3 weeks after MI-R. Furthermore, the HC-MI group showed a higher percentage pro-inflammatory Ly-6C\(^{\text{high}}\) monocytes of the total monocyte population in peripheral blood prior to MI-R (40.8±2.7% vs. 30.6±2.2% of total monocytes, \( P=0.01 \), Figure 4B), which was normalized as well 3 weeks after MI-R. These results suggest a loss of total and in particular Ly-6C\(^{\text{high}}\) monocytes from peripheral blood of HC-MI mice due to MI-R. In contrast, 8 weeks after MI-R a non-significant decreased number of infiltrated CD45\(^{+}\) leukocytes was observed in the infarct area of the HC-MI group as

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**Figure 4.** Inflammatory response as a result of the cholesterol enriched diet and MI-R. Quantification of monocytes as a percentage of total leukocytes (A) and activated (Ly-6C\(^{\text{high}}\)) monocytes as a percentage of total monocytes (B) in whole blood 1 week before induction of MI-R and 3 weeks after MI-R. **\( P<0.01 \) vs. time-corresponding NC-MI group. Quantification of leukocytes (C) and macrophages (D) 8 weeks after MI-R in the infarct area, border zone and septum, expressed as the number per area. Data are means±SEM. *\( P<0.05 \).
compared to the NC-MI group (4.7±0.4 vs. 7.0±2.0 cells per 0.25mm², P=0.27, Figure 4C) whereas the number of macrophages was significantly reduced (7.9±1.8 vs. 20.7±5.4 cells per 0.25mm², P=0.049, Figure 4D).

**Discussion**

Key findings of the present study are that diet-induced hypercholesterolemia in APOE*3-Leiden mice caused 1) a pre-ischemic peripheral monocytosis, in particular of the Ly-6C<sup>high</sup> pro-inflammatory monocytes, and 2) an impaired intrinsic LV function 8 weeks after acute MI-R, paradoxically accompanied by 3) a reduced infarct size and 4) a suppressed accumulation of infiltrated inflammatory cells in the ischemic-reperfused myocardium after 8 weeks. This study is the first to extend the follow-up after MI-R to 8 weeks.

**Impaired cardiac function as a result of hypercholesterolemia.** In the present study, hypercholesterolemia resulted in an impaired intrinsic LV function 8 weeks after MI-R as reflected by a reduced left ventricular ESP, dP/dt<sub>MAX</sub> and dP/dt<sub>MIN</sub>. No differences of LV dimensions were observed between the HC-MI and NC-MI groups as assessed by cardiac echocardiography and PV loop measurements. These results are in line with previous studies reporting that unreperfused MI in HC animals results in depressed LV function in rabbits (35) and exacerbated LV diastolic dysfunction in rats (36) at least 8 weeks after ischemia. In contrast, intrinsic cardiac function in a HC pig model was preserved 24 hours after MI-R as compared to NC animals, though follow-up was not extended up to 8 weeks (15). Thus, short-term (24 hours) results differ markedly from long-term (8 weeks) results.

Hypercholesterolemia itself was suggested to cause cardiomyopathy by formation of myocardial cholesterol deposits. Shifting of ATP production from glucose to free fatty acids increased free radicals which resulted in myocardial injury (37). A reduced hemodynamic performance caused by hypercholesterolemia may lead to decreased metabolic demand. This is proposed to confer a cardioprotective state (9, 38) that may lead to improved post-ischemic functional recovery in HC versus NC rabbit hearts. In this way, HC may increase myocardial tolerance against myocardial ischemia (10).

**Reduction of infarct size.** In this study, the reduced infarct size accompanied by preserved LV wall thickness of the infarcted myocardium in the HC animals may have been caused by the aforementioned reduced myocardial metabolic demand and resulting increased tolerance against myocardial ischemia. Differences in myocardial perfusion (reserve) are not likely, since vascular density was similar in both groups. Another explanation could be a different course of the post-ischemic inflammatory response. In accordance with the decreased infarct size, our study showed a reduced influx of leukocytes and macrophages in the post-ischemic myocardium of HC mice 8 weeks following MI-R.
A positive correlation between infiltrated inflammatory cells and infarct size has been reported by previous studies in humans and rats (39, 40).

In contrast to our long-term results 8 weeks after MI-R, previously reported short-term (24 hours or less) experimental studies using HC MI-R models reported an increased infarct size in rabbits (16) and pigs (15) accompanied by increased myeloperoxidase expression in rats (13) and rabbits (12) and increased cardiomyocyte apoptosis (11). In contrast, MI-R in HC mice resulted in smaller infarcts 2 hours after onset of reperfusion and was concluded to provide cardioprotection in mice (17). After permanent ischemia without reperfusion in rabbits (35) or rats (36), hypercholesterolemia caused no difference in infarct size 8 weeks after MI.

Pre-ischemic monocytosis followed by reduced integrated inflammatory cells in the ischemic-reperfused myocardium. In parallel with the decreased infarct size, our study shows a reduced influx of leukocytes and macrophages in the ischemic-reperfused myocardium of HC mice 8 weeks post MI-R. This finding is preceded by a pre-ischemic hypercholesterolemia-associated peripheral monocytosis. Hypercholesterolemia has been reported to cause a peripheral monocytosis with regard to the Ly-6C<sup>high</sup> subset in HC mice (41). In addition, the inflammatory response after MI-R is more complex as compared to MI without reperfusion, since reperfusion itself induces a pathophysiological process of reperfusion injury (42). The pro-inflammatory state resulting from this monocytosis could affect the post-ischemic inflammatory response following MI-R since Ly-6C<sup>high</sup> monocytes are involved in the initial inflammatory response against ischemia. Previous studies demonstrated that within the first hours after MI, monocytes and their lineage descendant macrophages infiltrate the infarcted myocardium resulting in the release of cytokines and growth factors, phagocytosis of debris, clearance of apoptotic cells, and the release of proteases (43, 44). Especially the pro-inflammatory Ly-6C<sup>high</sup> monocyte subset is known to promote digestion of infarcted tissue and clearance of necrotic debris (45). After myocardial ischemic injury, a baseline Ly-6C<sup>high</sup> monocytosis could favorably affect the subsequent Ly-6C<sup>low</sup>-mediated reparative phase, accelerating repair and thereby limiting tissue damage on the long-term. Conversely, atherosclerosis-related leukocytosis was found to disturb the acute post-ischemic healing process (30). Following permanent ischemia, Ly-6C<sup>high</sup> monocytosis has been reported to disturb infarct healing and enhance left ventricular remodelling after 3 weeks (46). This underscores that the post-ischemic inflammatory response after permanent MI differs from the inflammatory response after MI-R (43).

Conclusion

In summary, hypercholesterolemia in an in vivo APOE<sup>*3-Leiden</sup> mouse model causes a pre-ischemic peripheral monocytosis and an impaired systolic and diastolic cardiac function 8 weeks after myocardial ischemia-reperfusion injury. This is accompanied
however with a decreased myocardial infarct size and a reduced accumulation of inflammatory cells in the ischemic-reperfused myocardium.

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**Disclosures.** The authors confirm that there are no conflicts of interest.
Reference List


32. Tarnavski O, McMullen JR, Schinke M, Nie Q, Kong S, Izumo S. Mouse cardiac surgery: comprehensive techniques for the generation of mouse models of human diseases and their


Supplementary material

Results

Table 1. Animal characteristics. Body weight (BW), heart weight (HW). Values are means±SEM. **P<0.01 vs. NC-MI.

<table>
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<td>lung fluid (mg)</td>
<td>208±11</td>
<td>205±11</td>
</tr>
</tbody>
</table>