CHAPTER 4

ABCA1 deficiency protects the heart against injury following myocardial infarction

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ABCA1 deficiency protects the heart against injury following myocardial infarction

Abstract

ATP-binding cassette transporter A1 (ABCA1) exerts anti-atherogenic functions in the pathogenesis of atherosclerosis. We aimed to explore the role of ABCA1 after acute myocardial infarct (MI) induction.

In ABCA1 knockout (KO) mice, wild-type (WT) controls and in WT mice transplanted with ABCA1 KO or WT bone marrow an MI was induced and mice were allowed to recover for two weeks. In addition, isolated hearts from ABCA1 KO and WT mice were subjected to MI induction in a Langendorff perfusion system.

MI size was reduced in both ABCA1 KO mice (-59%, p=0.03) and WT mice transplanted with ABCA1 KO bone marrow (-43%, p=0.12) both compared to their WT controls. MI induction in isolated hearts by Langendorff perfusion showed no effect of ABCA1 deficiency on infarct size. The smaller infarct size *in vivo* in ABCA1 KO mice is thus likely not due to a direct effect of ABCA1 deficiency on myocyte function. Interestingly, after MI, ABCA1 KO mice compared to WT controls, showed higher levels of $CD19^+$ B-lymphocytes (+300%, p=0.02) and $CD3^+$ T-lymphocytes (+420%, p=0.002). Both $CD4^+$ and $CD8^+$ lymphocytes (+460% and +370%; both p=0.002) contributed to the observed increase in $CD3^+$ T-lymphocytes. There were no differences in leukocyte numbers after bone marrow transplantation. However, white blood cell counts were increased 2-7 fold compared to the first experiment.

Although ABCA1 has a protective role in atherosclerosis, it exerts detrimental effects on cardiac function after MI, possibly caused by a reduced activation status of immune cells resulting in less efficient repair after MI.

Introduction

Tangier Disease (TD), resulting in extreme HDL deficiency, is caused by detrimental mutations in the ABCA1 gene.[1-3] Since HDL plays a key protective role in atherosclerosis, by exerting several cardioprotective functions, including anti-oxidative, anti-inflammatory and vasomotor activities[4], the mechanism of action of ABCA1 and its regulation have been investigated extensively.[5-8] Mice deficient for ABCA1 exhibit low plasma HDL levels as well as cholesterol accumulation in peripheral macrophages, a phenotype similar to that of TD patients.[9] Although atherosclerosis-prone mouse models deficient for ABCA1 display impaired cellular cholesterol efflux[10], atherosclerotic lesion development does not increase in these mice[11], probably due to a less atherogenic lipid profile despite the almost complete absence of protective HDL. Deletion of ABCA1 in bone marrow-derived cells, however, did increase atherosclerotic lesion development, coinciding with increased numbers of peritoneal foam cells and impaired cholesterol efflux from macrophages towards apoA-I and HDL, indicating a pronounced anti-atherosclerotic effect of leukocyte ABCA1.[12, 13] In humans, low HDL levels have been correlated to an increased risk of MI; an acute cardiovascular event, often resulting from rupture of advanced atherosclerotic lesions and superimposed thrombus formation.[14]

Inflammatory responses are a critical factor in the balance between adverse ventricular remodeling induced by MI on the one hand[15, 16], and cardiac repair on the other hand.[17] Ischemia induces the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway, an important regulator of cytokine signaling, which plays a vital role in cardioprotection by inducing cytoprotective and survival signals in infarcted hearts.[18] Interestingly, ABCA1 acts as an anti-inflammatory mediator in baby hamster kidney (BHK) cells by inducing signaling through the JAK2/STAT3 pathway in response to binding of lipid-poor apoA-I.[19] Furthermore, ABCA1 exerts important anti-inflammatory properties, due to its key role in modulating the cholesterol content of the plasma membrane and within intracellular compartments.[20, 21] ABCA1 is thus anticipated to be cardioprotective during MI, indirectly by generating HDL as well as directly by its anti-inflammatory effects through JAK2/STAT3 signaling. The actual role of ABCA1 during MI, however, is currently unknown.

In order to investigate the importance of ABCA1 with respect to MI, we performed permanent coronary artery ligation experiments in ABCA1 KO and WT control mice as well as in WT mice transplanted with ABCA1 KO or WT bone marrow. Our results evidently show that ABCA1 has unanticipated unfavorable cardiac effects after MI.

Materials and methods

Animals and bone marrow transplantation

Female WT mice (C57Bl/6J background) and ABCA1 KO mice (kindly provided by Dr. G. Chimini, Centre d'Immunologie de Marseille-Luminy; more than 7 times backcrossed onto a C57Bl/6J background) were used.

To generate mice that specifically lack ABCA1 in bone marrow-derived cells, bone marrow from WT mice and ABCA1 KO mice was transplanted into WT mice as previously described.[22] Briefly, irradiated WT recipients received 5×10^6 bone marrow cells by intravenous injection into the tail vein. After 8 weeks, myocardial infarctions were induced or mice were subjected to a sham operation.

All animals had *ad libitum* access to food and water. At the end of each experiment the mice were sacrificed and hearts and/or blood were isolated for further examination. Animal experiments were approved by the Ethics Committee for Animal Experiments of Leiden University and performed at the Gorlaeus Laboratories of the Leiden Academic Centre for Drug Research in accordance with the National Laws.

Induction of myocardial infarctions

Mice were anesthetized by intraperitoneal injection of a mixture of dormicum (0.7 mg/kg b.w.), dexdomitor (7.2 mg/kg b.w.), and fentanyl (0.07 mg/kg b.w.). Body temperature was maintained at 37°C with an automatic heating pad. Mice were artificially ventilated using a dedicated rodent ventilator (model 845, Harvard Apparatus, Holliston, MA). The left anterior descending (LAD) coronary artery was ligated with a 7-0 Ethilon suture (Johnson and Johnson, New Brunswick, NJ, USA), just distal to the left atrial appendix. All mice that had ischemia, confirmed by bleaching of the left ventricle (LV), and the emergence of arrhythmias, were included in the study. The thorax was closed and the mice received a subcutaneous injection of Anexate (0.5 mg/kg b.w.), Antisedan (2.5 mg/kg b.w.), Naloxon (1.2 mg/kg b.w.), and 50 µL Temgesic/PBS (1.5 µg/50 mL PBS). Thereafter, the mice were allowed to recover on a temperature-controlled heating pad. Mice received another 50 µL Temgesic/PBS (1.5 µg/50 mL PBS) at 24 h after surgery.

Infarct size and immunohistochemistry

Two weeks after MI induction, mice were sacrificed and the arterial tree was perfused *in situ* with PBS (100 mm Hg) for 10 min via a cannula in LV apex. Subsequently, hearts were isolated and cut into four equal 1-2 mm thick slices, perpendicular to the long axis of the heart. The two lower slices, from the middle of the heart to the apex, represent the infarcted area, and were used for infarct quantification. These slices were flat embedded and serial sections (10 µm) were cut using a Leica CM3050S cryostat. To delineate the LV area and infarct area, sections were stained immunohistochemically with Sirius red for collagen. Total LV wall area (including septum) and infarct area were measured with cell^D imaging software (Olympus Soft Imaging Solutions, Tokyo, Japan). Infarct areas were normalized to total LV areas and averaged for individual hearts.

Leukocyte content and flow cytometry

Upon sacrifice, 2 weeks after MI, blood was collected by retro-orbital venous plexus puncture. Leukocyte content was analyzed using an automated Sysmex XT-2000iV Veterinary Hematology analyzer (Sysmex Corporation, Kobe, Japan). For fluorescent activated cell sorting (FACS) analysis, erythrocytes were lysed using erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Blood cells were subsequent stained (0.25 µg Ab/200,000 cells) for T-lymphocytes (CD3, CD4, and CD8), B-lymphocytes (CD19), monocytes/macrophages (F4/80) and dendritic cells (DC; CD11c). Antibodies were purchased from eBioscience (Vienna, Austria). FACS analyses were performed on a FACS Canto II (BD Biosciences, Mountain View, CA, USA). Data were analyzed using FACSDiva software (BD Biosciences).

Ex vivo Langendorff perfusion

To isolate the heart for Langendorff perfusion, the chest was opened excising the sternum and attached costal cartilages to give adequate access to the mediastinum. The heart was rapidly removed and placed in ice cold (4°C) Krebs–Henseleit buffer and the aorta cannulated. Hearts were then perfused with a Krebs–Henseleit buffer (118.0 mM NaCl; 24.0 mM $NaHCO_3$; 4.0 mM KCl; 1.0 mM NaH_2PO_4; 2.5 mM $CaCl_2$; 1.2 mM $MgCl_2$; 0.5 mM EDTA.Na_2; 10 mM glucose, pH 7.4; gassed with 95% O_2/5% CO_2 at 37°C) in a retrograde fashion with a constant pressure of 110 cm H_2O. The coronary flow rate was measured by timed collection of the perfusate. The hearts were stabilized for 20 min and subsequently exposed to 35 min of no-flow global ischemia followed by 45 min of reperfusion. At the end of the reperfusion period, the heart was immediately frozen. Frozen hearts were cut into 6-7 slices, perpendicular to the long axis of the heart, and incubated with triphenyltetrazolium chloride (TTC) to stain viable myocardium. Total myocardium and infarcted areas were measured from computed images using NIH Image software.

Statistical analysis

Statistically significant differences among the means of the different populations were tested using the unpaired Student's t-test (GraphPad InStat and Prism 4 software). The probability level (alpha) for statistical significance was set at 0.05. Results are expressed as mean ± SEM.

Results

ABCA1 KO mice show decreased coronary artery ligation-induced myocardial infarction

To investigate the effects of ABCA1 deficiency on MI-induced damage in vivo, we subjected ABCA1 KO and WT mice to LAD coronary artery ligation. Two weeks after induction of MI, infarct size was quantified. Surprisingly, despite the anticipated cardioprotective functions of ABCA1, ABCA1 KO mice displayed a substantial 59% reduction in MI size as compared to WT mice (p=0.03; figure 1, left panel). No differences in total LV wall area were found (figure 1, right panel), indicating that the observed reduction in MI did not result from alterations in LV size.

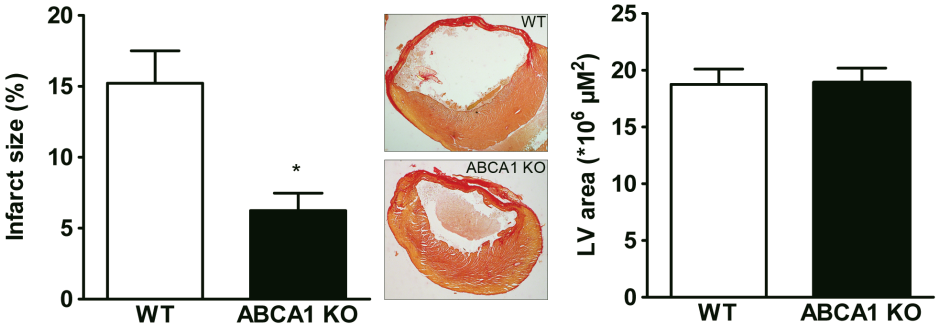

Figure 1 **Reduced myocardial infarct size in ABCA1 KO mice after coronary artery ligation.** Two weeks after induction of myocardial infarction (MI) by ligation of the left anterior descending (LAD) coronary artery, infarct size was determined (left panel). Representative cross sections are shown, stained with Sirius red to visualize the collagen-rich infarcted area (middle panels). Total left ventricle (LV) wall area was determined as a general indicator of heart size (right panel). Values are means ± SEM (n≥4 per group). *p<0.05

ABCA1 KO mice have higher circulating white blood cell and lymphocyte numbers after coronary artery ligation-induced myocardial infarction

Before MI induction, no differences in leukocyte subsets were observed between ABCA1 KO and WT mice (data not shown). Two weeks after MI, however, total leukocyte counts were 2.9-fold higher in ABCA1 KO mice (p<0.05; figure 2), which was primarily the result of augmented numbers of circulating lymphocytes (4.6-fold; p=0.002). To further investigate the increased lymphocyte population in the circulation of ABCA1 KO mice after MI, blood cells were subjected to FACS analysis (figure 3). Before MI, no differences

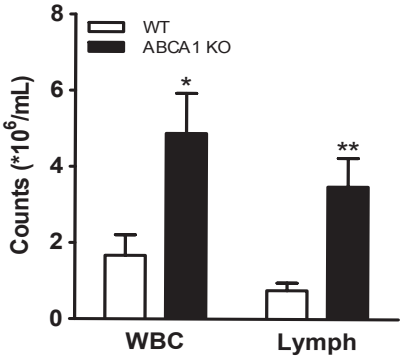

Figure 2 **Increased WBC and lymphocytes in ABCA1 KO mice after MI induction by coronary artery ligation.** Two weeks after induction of a myocardial infarction (MI), total white blood cells (WBC) and lymphocytes in plasma were determined with a hematology analyzer. Values are means ± SEM (n≥4 mice per group). *p<0.05, **p<0.01

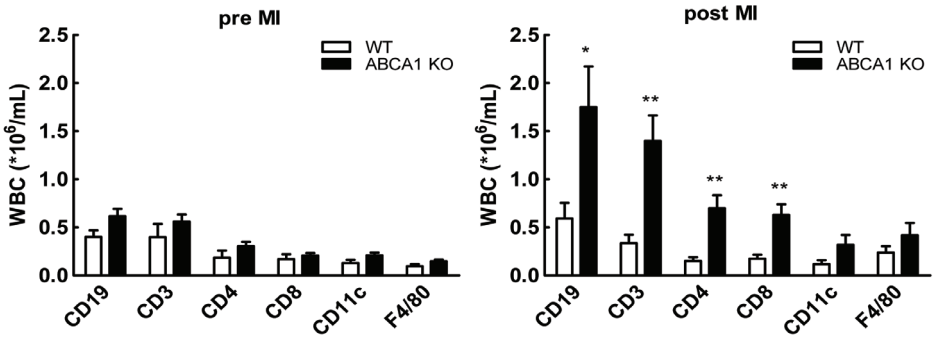

Figure 3 **Increased circulating T- and B-cells in ABCA1 KO mice after MI induction by coronary artery ligation *in vivo*.** Isolated white blood cells (WBC) were stained for T-cells (CD3+, CD4+, and CD8+), B-cells (CD19+), monocytes/macrophages (F4/80+) and dendritic cells (CD11c+) and analyzed by flow cytometry before (left panel) and after myocardial infarction (MI) (right panel). Values are means ± SEM (n≥4 mice per group). *p<0.05, **p<0.01

in T-lymphocytes ($CD3^+$, $CD4^+$, and $CD8^+$), B-lymphocytes ($CD19^+$), dendritic cells ($CD11c^+$) or monocytes/macrophages ($F4/80^+$) were observed between both genotypes (figure 3 left panel). After MI, however, $CD3^+$ T-lymphocytes increased a striking 4.2-fold (p=0.002; figure 3 right panel). $CD4^+$ T-helper lymphocytes (4.6-fold increase; p=0.002) and $CD8^+$ cytotoxic T-lymphocytes (3.7-fold increase; p=0.002) both contributed to this phenomenon. In addition, ABCA1 KO mice displayed a clear 3.0-fold increase (p=0.02) in $CD19^+$ B-lymphocytes after MI. In contrast, monocytes/macrophages ($F4/80^+$) and dendritic cells ($CD11c^+$) did not change between both genotypes upon MI. This indicates that the induction of MI in ABCA1 KO mice primarily induced common lymphoid progenitor (CLP)-derived cells, such as T- and B-lymphocytes, rather than common myeloid progenitor (CMP)-derived cells including monocytes/macrophages and dendritic cells.

ABCA1 KO hearts show unaltered myocardial infarction in a Langendorff perfusion system *ex vivo*

ABCA1 KO mice have been shown to develop cardiomegaly.[23] To determine the direct effects of ABCA1 deficiency on myocyte function during ischemia, MI was induced *ex vivo* in isolated hearts from ABCA1 KO mice and WT controls using the Langendorff perfusion method. After 35 minutes of no-flow global ischemia followed by 45 minutes of reperfusion, only a tendency towards a 15% decrease (p=0.47; figure 4) in infarct size was observed in hearts from ABCA1 KO mice. These data thus indicate that ABCA1-deficiency in cardiomyocytes is not contributing to the smaller infarct size observed in ABCA1 KO mice after coronary artery ligation.

Hematopoietic deficiency for ABCA1 tends to reduce coronary artery ligation-induced myocardial infarction

Next, we investigated if the observed decrease in MI in ABCA1 KO mice results from ABCA1-deficiency in bone marrow-derived cells that may be involved in MI-induced cardiac remodeling. Hereto, we induced MI in WT mice, transplanted with bone marrow from ABCA1 KO vs WT mice. Quantification of infarct size two weeks after MI induction showed a strong trend towards a reduction in MI size (-43%; p=0.12) (figure 5, left panel) without an effect on LV wall area (figure 5, right panel) in mice transplanted with ABCA1 KO as compared to WT bone marrow.

Hematopoietic deficiency for ABCA1 does not affect circulating white blood cell numbers after coronary artery ligation-induced myocardial infarction

Two weeks after MI induction, blood cells were subjected to FACS analysis to investigate the numbers of circulating lymphocytes (figure 6). No difference in T-lymphocytes ($CD3^+$, $CD4^+$, and $CD8^+$), B-lymphocytes ($CD19^+$), dendritic cells ($CD11c^+$) or monocytes/macrophages ($F4/80^+$) were observed between mice transplanted with ABCA1 KO vs WT bone marrow. Strikingly, the number of white blood cell (WBC) counts *per se* was 2-7 fold higher in mice transplanted with WT bone marrow compared to the WT mice in the first *in vivo* experiment. Since a WT control group without bone marrow transplantation in the second experiment also did not show an increase in WBC counts (data not shown) this indicates that the observed increase in WBC counts is induced by the transplantation procedure, combined with MI induction.

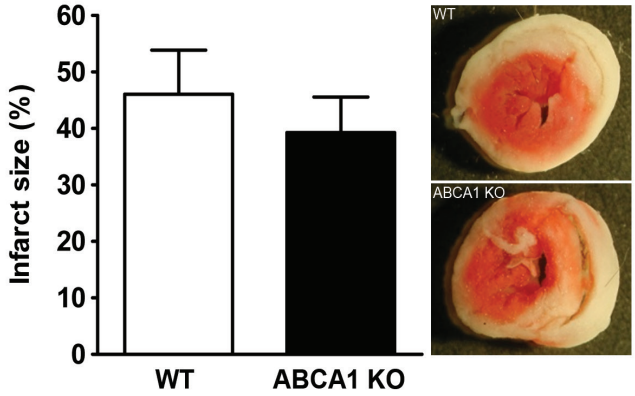

Figure 4 **Unaltered infarcted area of isolated hearts from ABCA1 KO mice subjected to ischemia/reperfusion *ex vivo*.** Isolated hearts were stabilized for 20 minutes in a Langendorff perfusion system, followed by 35 minutes of no-flow global ischemia, and 45 minutes of reperfusion. Infarct size was measured with cell^D imaging software. Infarct areas were normalized to total left ventricular areas and averaged for individual hearts (left panel). Values are means ± SEM (n≥5 mice per group). Representative cross sections are shown, stained with TTC to determine viable myocardium (red staining; right panels).

Discussion

In the current study we show for the first time that mice lacking ABCA1, thereby having reduced HDL levels, are protected against cardiac damage after permanent coronary artery ligation. At first sight, this is remarkable as several studies have revealed protective effects of HDL after MI.[24, 25] HDL protects against MI by inhibiting ischemia-induced cardiomyocyte apoptosis and by reducing the recruitment of inflammatory neutrophils into the infarcted area.[25] In addition, intravenous injection of apoA-I before the onset of reperfusion after MI reduced TNF-α and IL-6 expression in the heart, as well as suppressed ICAM-1 expression in the heart, thereby diminishing neutrophil adherence and subsequent reduced myocyte injury.[24] On the other hand, mice lacking the HDL receptor scavenger receptor BI (SR-BI), have high levels of HDL but spontaneously develop MI.[26, 27] Deficiency of ABCA1 not only results in the lack of HDL, but also highly attenuates total cholesterol levels.[28] Although cholesterol levels are positively correlated with MI risk in humans[29], differential results have been obtained in murine MI models.[30-32]

Interestingly, induction of MI in ABCA1 KO mice resulted in a substantial increase in circulating leukocytes due to higher numbers of circulating B- and T-lymphocytes. No differences in leukocyte numbers or subsets were found prior to coronary artery ligation. Therefore, the increased inflammatory environment, caused by the induction of MI, might have attributed to induce this phenotype in ABCA1 KO mice. Proliferation of LSK stem cells and committed CMPs is regulated by cholesterol efflux mechanisms, whereby HDL suppresses proliferation by facilitating cellular cholesterol efflux via the ABC-transporters ABCA1 and ABCG1.[33] However, mice lacking ABCA1 and ABCG1 did not show an increase in CLP cells that give rise to B-and T-lymphocytes.[33]

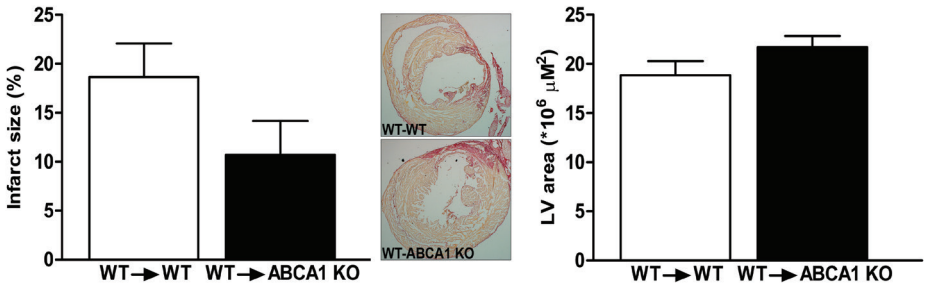

Figure 5 **MI size tends to be lower in WT mice transplanted with ABCA1 KO bone marrow.** WT mice were transplanted with bone marrow from WT or ABCA1 KO mice. After 8 weeks, myocardial infarction (MI) was induced by ligation of the LAD coronary artery. Two weeks later, infarct size was determined (left panel). Representative cross sections are shown, stained with Sirius red to visualize the collagen-rich infarcted area (middle panels). Total left ventricle (LV) wall area was determined as a general indicator of heart size (right panel). WT->WT, WT mice transplanted with WT bone marrow, ABCA1 KO->WT, WT mice transplanted with ABCA1 KO bone marrow. Values are means ± SEM (n≥4 per group).

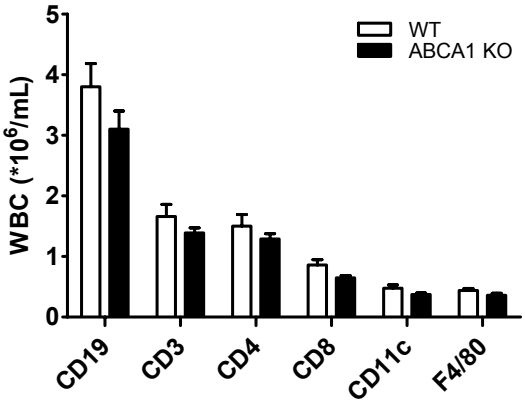

Figure 6 **Comparable circulating T- and B-lymphocytes in WT mice transplanted with ABCA1 KO and WT bone marrow after MI induction by coronary artery ligation *in vivo*.** Isolated white blood cells (WBC) were stained for T-cells (CD3+, CD4+, and CD8+), B-cells (CD19+), monocytes/macrophages (F4/80+) and dendritic cells (CD11c+) and analyzed by flow cytometry after MI. Values are means ± SEM (n≥4 mice per group).

Importantly, we have previously shown that deficiency of leukocyte ABCA1 increases circulating lymphocytes upon induction of atherosclerosis[12], similarly as observed in the current study upon coronary artery ligation. Since the infarct size in ABCA1 KO mice was drastically attenuated, the higher number of circulating lymphocytes might have been mediating tissue repair. This hypothesis is strengthened by the fact that studying the effect of ischemia on isolated hearts from ABCA1 KO and WT mice, thus in absence of blood cells, did not show differences in infarct size using the Langendorff perfusion system.

T-lymphocytes rapidly accumulate in the heart after ischemia/reperfusion injury. Specifically, $CD4^+$ T-lymphocytes were identified as protective mediators of myocardial perfusion injury after MI.[33,34] Recently, Wara *et al.* showed that LSK-derived CMPs, but not CLPs, can differentiate into proangiogenic cells, thereby promoting neovascularization.[34] Instead, $CD4^+$ lymphocytes modulate the influx of among others monocytes, which is a prerequisite for proper myocardial wound healing.[35, 36] Moreover, intramyocardial injection of B-lymphocytes into early post-ischemic myocardium has been shown to preserve cardiac function[37], emphasizing the protective roles of B- and T-lymphocytes upon MI.

The most pronounced lipid phenotype of ABCA1 deficiency is the near absence of HDL cholesterol in the circulation.[23] It is therefore plausible that the absence of HDL, at least in part, promoted the observed secondary effects on lymphocyte numbers upon MI. In agreement, Wilhelm *et al.* observed increased circulating lymphocytes in Western-type diet fed LDLr KO mice lacking apoA-I.[38] ApoA-I KO mice, like ABCA1 KO mice, have virtually no circulating HDL. To provide definitive proof for the distinct importance of ABCA1 and HDL, MI should be induced in apoA-I KO mice, which express ABCA1, but have virtually no HDL.

To elucidate the effect of ABCA1-deficiency in leukocytes on MI, bone marrow was transplanted from ABCA1 KO and WT mice to WT mice, after which MI was induced by coronary artery ligation. A strong trend was observed towards a reduction in MI size (-43%), which just did not reach statistical significance because of large interindividual variation. However, these data strongly suggest that hematopoietic ABCA1 deficiency underlies the difference in MI size observed in the first experiment. No differences were observed in CLP- nor in CMP-derived cells after bone marrow transplantation. However, it should be noted that WBC numbers were dramatically higher in both transplanted groups compared to the non-transplanted animals in the first experiment. Hofmann *et al.* previously showed that the mere presence of CD4+ T-lymphocytes was not sufficient for proper wound healing and that T-cell receptor activation by released cardiac autoantigens after MI is a prerequisite.[35] Furthermore, Wilhelm *et al.* was the first to show that not only monocytes/macrophages become cholesterol enriched but also T- and B-lymphocytes and this cholesterol enrichment seems to be the stimulus that initiates T-lymphocyte activation.[38, 39] One could hypothesize that the inability of immune cells to efflux cholesterol could enhance the activation status of these cells towards more efficient repair of damage induced by MI. However, it remains to be determined how ABCA1 expressing leukocytes exactly exert their detrimental effects during cardiac wound healing.

In conclusion, despite its protective effects regarding the development of atherosclerosis, ABCA1 has adverse effects on cardiac function after MI, which is possibly related to an increased activation status, rather than an increase in the absolute numbers of B- and T-lymphocytes. Importantly, although ABCA1 is considered a potential therapeutic target to treat atherosclerosis, strategies aiming at upregulation of ABCA1 function should be pursued with care in the light of potential adverse effects on cardiac remodeling following MI.

Acknowledgements

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