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BASIC SCIENCE

Neutrophils orchestrate post-myocardial infarction healing by polarizing macrophages towards a reparative phenotype

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Aims Acute myocardial infarction (MI) is the leading cause of mortality worldwide. Anti-inflammatory strategies to reduce neutrophil-driven acute post-MI injury have been shown to limit acute cardiac tissue damage. On the other hand, whether neutrophils are required for resolving post-MI inflammation and repair is unknown.

Methods and results We show that neutrophil-depleted mice subjected to MI had worsened cardiac function, increased fibrosis, and progressively developed heart failure. Flow cytometry of blood, lymphoid organs and digested hearts revealed reduced numbers of Ly6C^{high} monocytes in infarcts of neutrophil-depleted mice, whereas the number of macrophages increased, which was paralleled by reduced splenic Ly6C^{high} monocyte mobilization but enhanced proliferation of cardiac macrophages. Macrophage subtype analysis revealed reduced cardiac expression of M1 markers, whereas M2 markers were increased in neutrophil-depleted mice. Surprisingly, we found reduced expression of phagocytosis receptor myeloid-epithelial-reproductive tyrosine kinase, a marker of reparative M2c macrophages which mediate clearance of apoptotic cells. In agreement with this finding, neutrophil-depleted mice had increased numbers of TUNEL-positive cells within infarcts. We identified neutrophil gelatinase-associated lipocalin (NGAL) in the neutrophil secretome as a key inducer of macrophages with high capacity to engulf apoptotic cells. The cardiac macrophage phenotype in neutrophil-depleted mice was restored by administration of neutrophil secretome or NGAL.

Conclusion Neutrophils are crucially involved in cardiac repair after MI by polarizing macrophages towards a reparative phenotype. Therapeutic strategies to reduce acute neutrophil-driven inflammation after MI should be carefully balanced as they might interfere with the healing response and cardiac remodelling.

Keywords Neutrophils • Myocardial infarction healing • Adverse remodelling • Macrophages • Efferocytosis • Lipocalin

Translational perspective

Neutrophils are generally considered to play a detrimental role after post-myocardial infarction (MI) revascularization, but their role in myocardial ischaemia by itself has not been fully elucidated. Here, we provide novel evidence that neutrophils, despite being short-living innate immune responders, could contribute to improve cardiac healing and outcomes by influencing macrophage polarization towards a 'reparative' phenotype, via neutrophil gelatinase-associated lipocalin release. A better knowledge of the role of neutrophils in MI healing could help predicting the net results of neutrophil-targeted therapies in MI.

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Introduction

Myocardial infarction (MI) induces an inflammatory response which is required for the induction of cardiac repair processes. Various cell types, including neutrophils and macrophages, are involved at different stages of infarct healing, ultimately leading to scar formation and adaptive remodelling to preserve cardiac function.[1] Attracted by cell debris, danger-associated molecular patterns and cytokines of activated neighbouring cells, neutrophils massively infiltrate the infarct area in the first few hours following onset of ischemia.[1] They generate high levels of reactive oxygen species and secrete proteases, which exacerbates local vascular and tissue injury.[2] Subsequently, monocyte-derived macrophages are recruited to the heart to remove debris and apoptotic neutrophils, which leads to activation of reparative pathways necessary for scar formation.[1,3] Macrophages in the ischaemic myocardium exhibit high plasticity and are involved in both inflammatory as well as reparative processes. In the context of post-MI inflammation resolution, pro-inflammatory M1 macrophages are thought to undergo local conversion to M2 resolution–mediating macrophages.[4]

The crucial importance for efficient clearance of cell debris in resolving post-MI inflammation has been highlighted in mice with genetic deficiency of myeloid-epithelial-reproductive tyrosine kinase (MertK). Insufficient clearance of apoptotic cells by MertK-deficient macrophages led to delayed inflammation resolution after MI, adverse remodelling, and decreased cardiac function.[5] Hence, reprogramming macrophages in the heart to a reparative state seems to be an attractive therapeutic strategy to improve infarct repair, e.g. as previously reported by systemic administration of phosphatidylserine-decorated liposomes.[6] In this regard, further studies are warranted to better understand the signalling pathways and local regulators in the cardiac microenvironment promoting resolution of inflammation and macrophage differentiation to a reparative phenotype.

Less is known about the role of neutrophils in post-MI healing. High neutrophil counts are considered as predictor of adverse clinical outcomes and mortality in patients with acute coronary syndromes,[7,8] and their contribution in the acute inflammatory phase after MI is generally considered detrimental. However, in acute inflammation, neutrophils are not only vital for the clearance of pathogens or debris, but also for the resolution of inflammation and return to tissue homeostasis.[9] Macrophages engulfing apoptotic neutrophils activate an anti-inflammatory response by inhibiting pro-inflammatory cytokines and inducing the production of interleukin (IL)-10, transforming growth factor (TGF)-β and pro-resolving lipid mediators.[10] Consequently, anti-inflammatory strategies reducing neutrophil influx in order to limit acute post-ischaemic tissue injury might also inhibit the subsequent healing response. In the present study, we therefore aimed to clarify the contribution of neutrophils in post-MI healing.

Methods

An expanded version is provided in Supplementary material online, *Methods*.

Animal experiments

Female 10- to 12-week-old C57BL/6J mice were used in this study. Myocardial infarction was induced by permanent ligation of the left anterior descending coronary artery (LAD). In additional experiments, mice were subjected to transient LAD occlusion for 45 min followed by reperfusion. Neutrophil depletion was performed by intraperitoneal (i.p.) injection of monoclonal antibody clone 1A8 (50 μg; *Figure 1A*). Control mice received corresponding isotype i.p. injections. In some experiments, mice received intramyocardial injections of neutrophil supernatant in the ischaemic LAD territory.[11] Control animals received intramyocardial injections of vehicle (saline). In other experiments, recombinant murine neutrophil gelatinase-associated lipocalin (NGAL) (100 μg) or vehicle (saline) was injected i.p. after 1, 3, and 4 days LAD ligation. We performed echocardiography, quantification of infarct size, histology, flow cytometry, real-time PCR, western blot, multiplex, and ELISA as described in Supplementary material online. All animal experiments were approved by the local ethical committee.

Macrophage polarization

Murine bone marrow-derived macrophages (BMM) were left untreated or converted to M1 macrophages by stimulation with IFN-γ (10 ng/mL for 72 h). M2a or M2c were obtained by a 72 h treatment with IL-4 (20 ng/mL) or dexamethasone (20 nM), respectively. To assess the effect of neutrophil secretome or recombinant proteins on macrophage polarizaton, BMM were polarized in the presence of 100 μL of neutrophil supernatant or with recombinant mouse NGAL (100 ng/mL), lactoferrin (10 μg/mL), Cramp (1 μg/mL), neutrophil elastase (5 μM), or myeloperoxidase (MPO, 5 μM). Human monocytes were isolated from blood of healthy donors and macrophages were differentiated from monocytes by culturing for 6 days in the presence of recombinant human macrophage colony-stimulating factor (50 ng/mL). Human macrophages were polarized as described above.

Western blot

MertK in heart or cell lysates was detected with anti-MertK antibody (R&D Systems) followed by anti-goat-horseradish peroxidase as secondary antibody.

Neutrophil gelatinase-associated lipocalin immunoprecipitation

Protein G-conjugated magnetic beads were incubated with goat polyclonal anti-NGAL antibody (R&D Systems) or normal goat IgG as negative control. The antibody-conjugated beads were incubated with 500 μL of conditioned medium obtained from activated mouse neutrophils, magnetically separated and supernatants collected for subsequent treatment of macrophages.

Efferocytosis

Macrophages were co-cultured with apoptotic mouse cardiomyocytes (stained with calcein AM) at 37°C for 60 min. Phagocytosis efficiency (%) was quantified by flow cytometry and calculated as calcein positive divided by the total number of macrophages.

Statistical analysis

All data are expressed as mean ± SD, and statistical analysis was performed with Prism Software (version 6; GraphPad, CA, USA). Endpoint comparisons between 2 groups were performed using unpaired 2-tailed Student's *t*-test. For multiple comparisons, false discovery rate according to Benjamini–Hochberg was applied to control type I false positive errors, and FDR was set to 0.05. For parallel repeated-measures studies, 2-way ANOVA was used with Bonferroni *post hoc* evaluation to determine the significance for individual time points. A 2-tailed $P < 0.05$ was considered as significant.

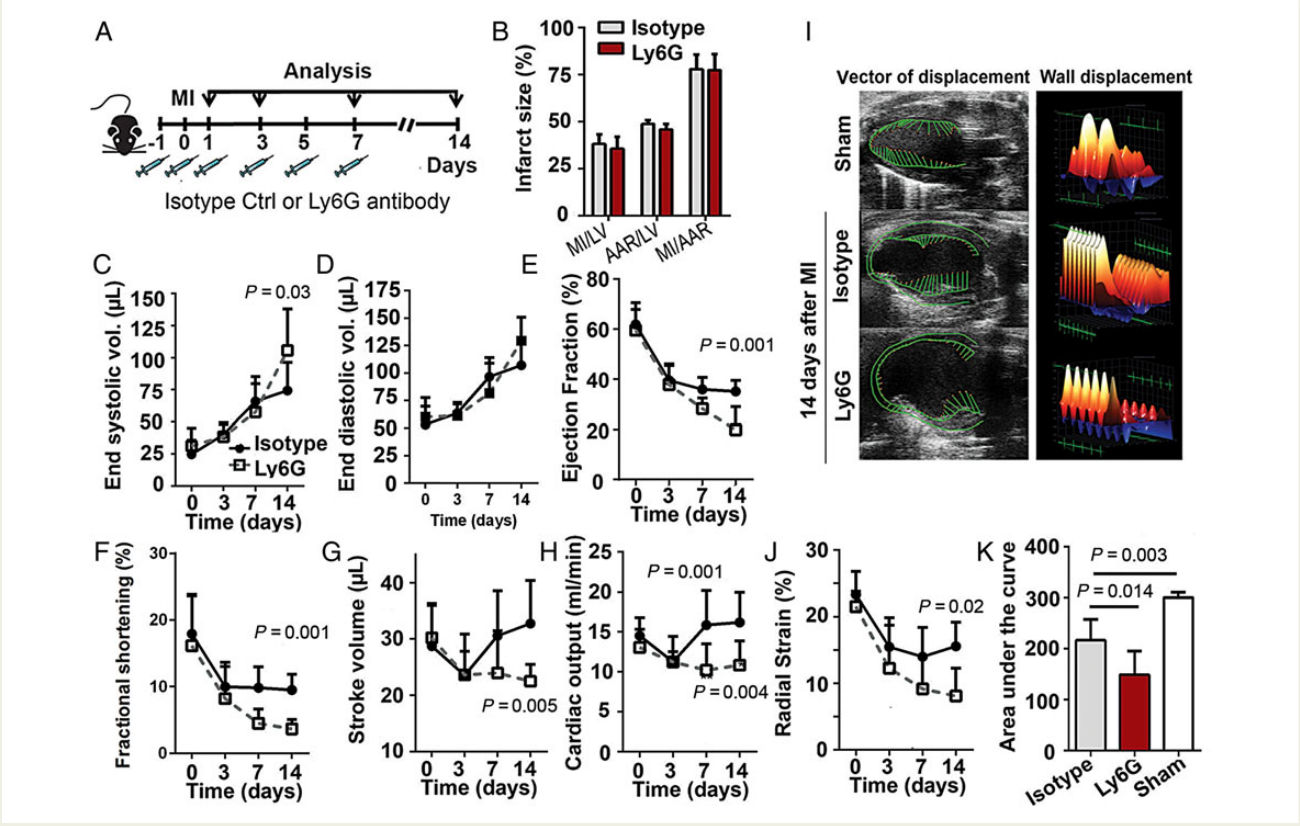

Figure 1 Neutrophil depletion does not affect infarct size but progressively worsens cardiac function. (*A*) Experimental protocol for treatment with neutrophil-depleting antibody (Ly6G) or isotype. (*B*) Quantification of ratios between infarct size (myocardial infarction), area at risk, and left ventricular area, 24 h after myocardial infarction. (*C–H*) Quantification of functional parameters after sham operation (Day 0) or 3, 7, 14 days after myocardial infarction. (*I*) Vector diagrams showing the direction and magnitude of myocardial contraction at midsystole. Three-dimensional regional wall displacement illustrations, showing contraction (positive values, yellow–red) or relaxation (negative values, blue) of consecutive cardiac cycle results. (*J*) Global averages of radial strain and (*K*) measurement of the area under the curve of radial strain. Data are mean ± SD from 8 mice/group.

Results

Neutrophil depletion worsens cardiac function and promotes heart failure

Depletion with anti-Ly6G resulted in significant reduction of circulating neutrophil counts, whereas blood monocytes were unaffected at steady state (Supplementary material online, *Figure S1*). In agreement with previous observations after prolonged ischaemia,[12] we found no difference in infarct size between control and neutrophil-depleted mice 24 h post-infarction (*Figure 1B*). However, 7–14 days after infarction, neutrophil depletion led to worsening of heart function. Characteristic features of neutrophil depletion were larger end-systolic left ventricular dimensions, a significant reduction of left ventricular ejection fraction and cardiac output (*Figure 1C–H* and Supplementary material online, *Table S1*).

In support of reduced global contractility, infarcted hearts of neutrophil-depleted mice had pronounced hypokinesia of left ventricular walls (*Figure 1I*). The radial strain was significantly more decreased in infarcted hearts of depleted animals compared with controls (*Figure 1J* and *K*). Worsening of global contractility might lead to heart failure (HF). In support of this hypothesis, neutrophil-depleted mice had up-regulated cytokine and chemokine levels associated with HF[13–15] 7 days post-MI (*Figure 2A–F*). Moreover, the kidneys had macroscopic lesions and increased expression of galectin-3, a marker of kidney injury (*Figure 2G* and *H*)[16] as well as increased plasma levels of creatinine (*Figure 2I*).

Neutrophil depletion promotes excessive fibrosis

Histological analysis of the myocardium revealed an increased percentage of α-smooth muscle actin-positive myofibroblasts 7 days post-MI in neutrophil-depleted mice (*Figure 3A* and *B*), which was confirmed at the mRNA level (*Figure 3C*). The number of CD31-stained microvessels in healing infarcts of control and neutrophil-depleted mice was comparable (*Figure 3D*). Consistent with a higher amount of cells promoting fibrosis, the collagen content, in particular of thicker type I collagen fibres, was higher in infarcts of depleted mice (*Figure 3E–H*). These findings suggest that depletion of neutrophils leads to excessive fibrosis that might explain the progressive worsening of cardiac function.

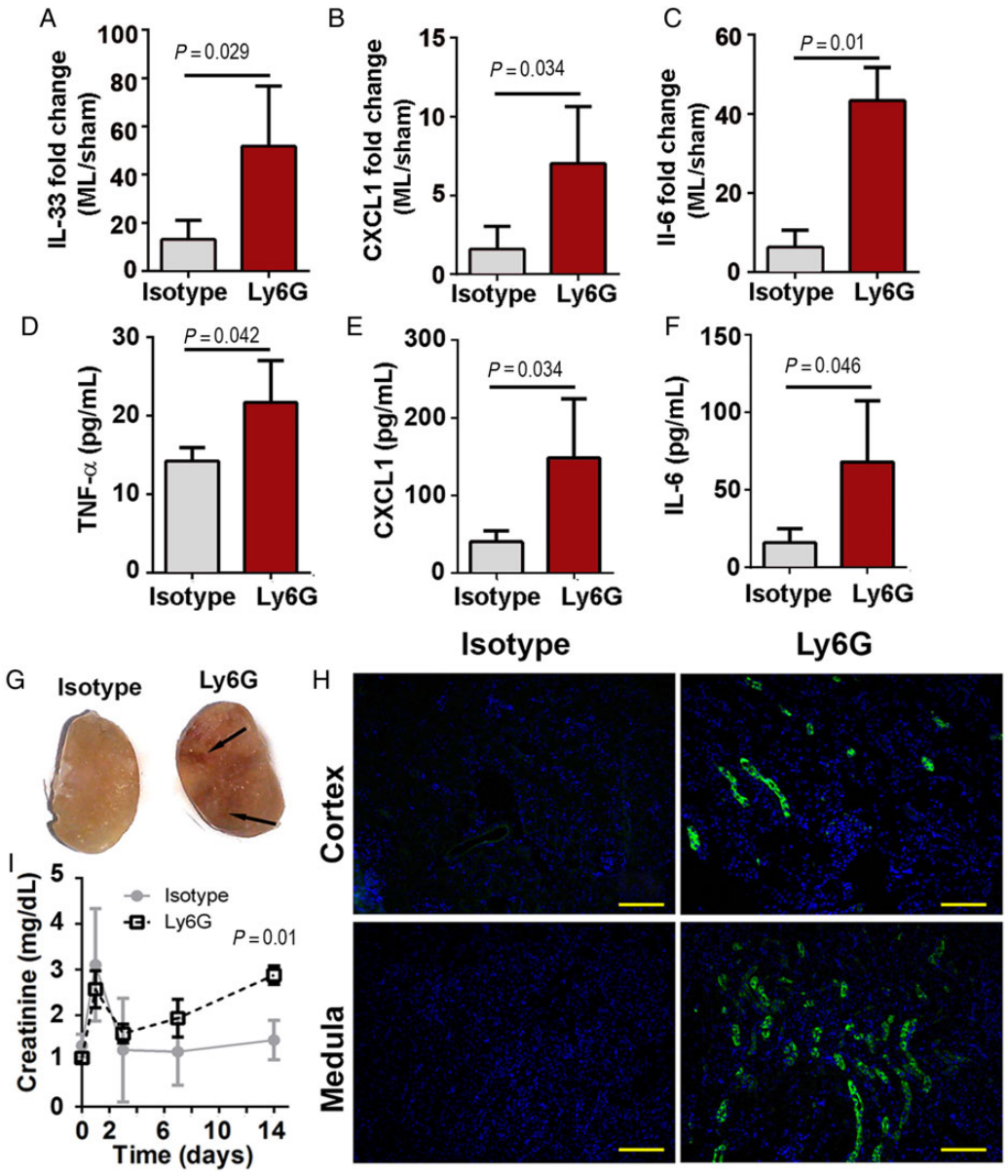

Figure 2 Neutrophil depletion leads to up-regulation of markers for heart failure. (*A*–*C*) Quantification of cardiac mRNA expression in isotype-treated and neutrophil-depleted (Ly6G) mice 7 days after myocardial infarction. (*D*–*F*) Plasma levels 7 days after MI. (*G*) Macroscopic view of the kidney 5 days after myocardial infarction. Arrows indicate macroscopic lesions observed in neutrophil-depleted mice. (*H*) Representative galectin-3 immunostaining 5 days after myocardial infarction. (*I*) Plasma creatinine levels before and 1, 3, 7, 14 days post-myocardial infarction. Data are mean ± SD from 3–4 mice/group.

Neutrophil depletion modulates cardiac monocyte und macrophage profiles

Unfavourable repair in the absence of neutrophils might be a consequence of impaired resolution of inflammation due to insufficient monocyte recruitment or activation of reparative macrophages. We therefore analysed leucocyte profiles in hearts and lymphoid organs. As expected, treatment with depleting antibody Ly6G potently reduced cardiac neutrophil counts (*Figure 4A* and *B*). We detected an overall reduction of blood leukocytes in neutrophil-depleted mice up to 7 days after MI (Supplementary material online, *Figure S1*), probably due to reduced neutrophil-mediated inflammation. Flow cytometric analysis of digested hearts revealed less Ly6C^{hi} monocytes 3 days post-infarction, whereas the number of macrophages was increased at day 3 to 7 post-infarction (*Figure 4C* and *D*).

In response to MI, monocytes are mobilized from the bone marrow and spleen into the blood stream and subsequently recruited into the ischaemic myocardium.[17] In agreement with reduced recruitment of monocytes into infarcts of neutrophil-depleted mice,

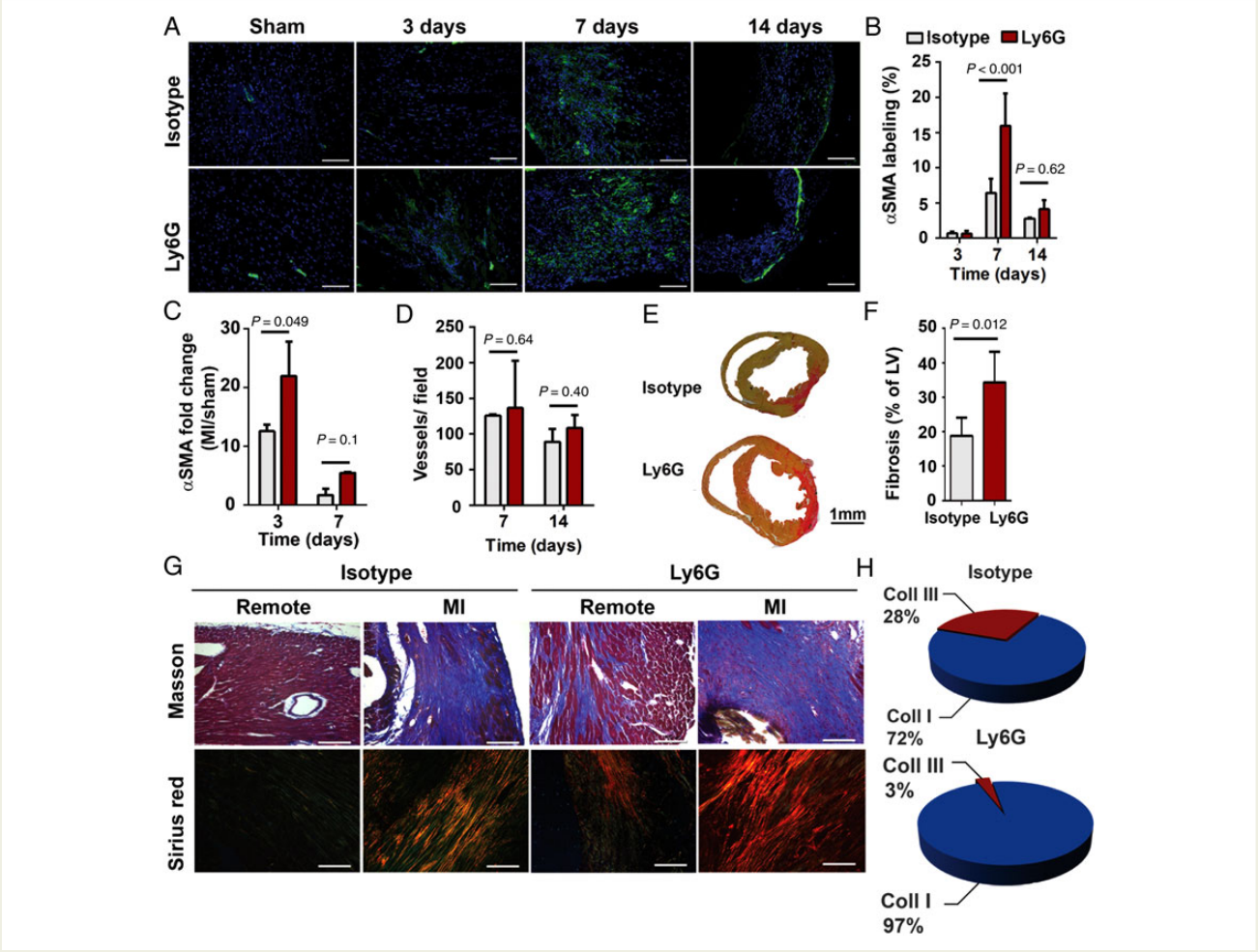

Figure 3 Neutrophil depletion increases cardiac fibrosis. (*A*) Myofibroblasts within infarct areas identified by α-smooth muscle actin labelling (green) and 4′,6-diamidino-2-phenylindole staining of nuclei (blue) in hearts from neutrophil-depleted (Ly6G) or isotype-treated mice. Scale bar, 50 μm; ×20 magnification. (*B*) Quantification of α-smooth muscle actin staining as ratio between stained and total area of myocardium within randomly selected fields. (*C*) Quantification of α-smooth muscle actin mRNA levels, represented as fold change compared with sham-operated hearts. (*D*) Quantification of CD31-stained microvessels within infarct areas of randomly selected fields. (*E* and *G*) Representative collagen staining with Sirius red and Masson trichome (collagen in blue) at day 7 after infarction. Images were taken at ×2.5 magnification (whole hearts, Sirius red) and ×20 magnification of infarct area and remote zone (scale bar, 100 μm). (*F*) Quantification of fibrosis as ratio between collagen-stained area and total area of the left ventricle (LV). (*H*) Relative content of type I and III collagen fibres within infarcts. The graphs show mean ± SD from 5 mice/group.

the spleens contained higher numbers of the Ly6C^{hi} subset (*Figure 4E*). The bone marrow mobilization of monocytes was not different between depleted and control group (data not shown). In support of reduced splenic monocyte mobilization, we found decreased plasma levels of chemokines involved in monocyte recruitment[1,18] one day post-MI (*Figure 4F*).

Neutrophil depletion promotes local macrophage proliferation

To explain the increased number of macrophages in the heart, we may speculate that the microenvironment in the absence of neutrophil-driven inflammation supports an expansion by local proliferation. In support of this hypothesis, we found decreased plasma levels of pro-inflammatory cytokine levels IL-1β, IL-12, TNF-α, and IFN-γ in neutrophil-depleted mice (*Figure 4G*). Conversely, plasma levels of IL-4, a cytokine that induces macrophage proliferation and M2 polarization,[19,20] were increased (*Figure 4H*). Moreover, the flow cytometric analysis revealed a higher number of proliferating macrophages, evidenced by higher percentage of KI-67-positive macrophages in G1 and G2/S/M phase, whereas the percentage in G0 decreased in infarcted hearts of neutrophil-depleted mice (*Figure 4I* and *J*). Conversely, we determined a lower percentage of monocytes in G1 with concomitant increase of monocytes in the G0 phase (*Figure 4K*). Therefore, our data suggest that neutrophils are crucial regulators of the microenvironment driving polarization and proliferation of macrophages involved in cardiac repair.

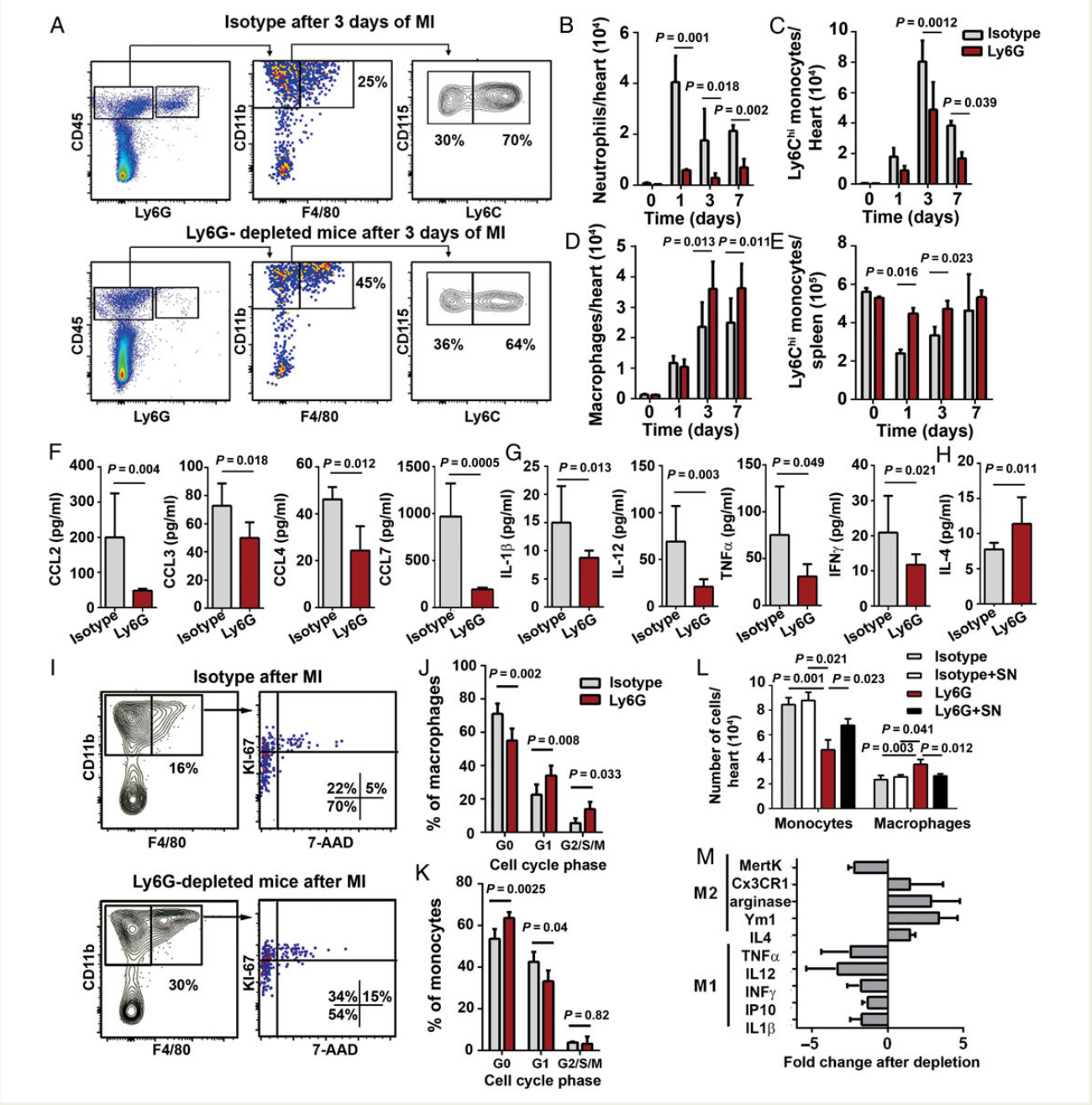

Figure 4 Neutrophil depletion modulates cardiac monocyte/macrophage profiles, splenic mobilization, and local proliferation. (*A–D*) Representative FACS plots and quantification of neutrophils, Ly6C^{high} monocytes and macrophages in digested hearts of neutrophil-depleted (Ly6G) or isotype-treated mice at baseline (0) or 1–7 days after myocardial infarction. (*E*) Quantification of Ly6C^{high} monocytes in the spleen. (*F*) Plasma levels of chemokines involved in monocyte recruitment 24 h after myocardial infarction. Plasma levels of (*G*) pro- and (*H*) anti-inflammatory cytokines 24 h after MI. (*I–K*) Quantification of proliferating (KI67 positive) Ly6C^{high} monocytes and macrophages in the heart 3 days after myocardial infarction, based on flow cytometric cell cycle analysis using 7-AAD for nuclear DNA staining. (*I*) Representative dot plots with quadrants to identify cells in G0 (lower left), G1 (upper left), and G2/S/M (upper right) phase. (*L*) Quantification of Ly6C^{high} monocytes and macrophages in the heart 3 days after myocardial infarction with or without intramyocardial injection of neutrophil supernatant. (*M*) Cardiac mRNA expression levels of M1 and M2 markers, normalized to hypoxanthine phosphoribosyltransferase (fold change compared with isotype group, $P < 0.05$ for all represented factors). (*B–H*) Data are mean ± SD from 8 (*J* and *K*) 5 or (*L* and *M*) 3 mice/group.

The neutrophil secretome changes cardiac monocyte and macrophage profiles

To strengthen this finding, we asked whether neutrophil supernatant was sufficient to restore the cardiac macrophage profile. Indeed, local administration of neutrophil supernatant into infarcted hearts of neutrophil-depleted mice partially reversed the phenotype by increasing the number of Ly6C^{hi} monocytes, while macrophage numbers decreased (*Figure 4L*).

Neutrophil depletion affects macrophage polarization

Polarized macrophages are generally referred to as M1 and M2 macrophages.[21] M1 macrophages are found in the early stages after MI injury and play a key role in acute inflammation, followed by reparative M2 macrophages that mediate resolution of inflammation.[4] We characterized the transcriptional profile in Ly6G depleted vs. control hearts 7 days post-MI and found a significant down-regulation of M1 markers (IL-12, TNFα, IFNγ, IP-10, IL1β) and up-regulation of M2 signature markers (CX3CR1, arginase, YM1, IL-4) compared with controls (*Figure 4M*). Surprisingly, expression of macrophage marker MertK, a phagocytosis receptor mainly expressed by M2c macrophages,[22,23] was decreased (*Figure 4M*).

The neutrophil secretome promotes macrophage polarization towards M2c

We hypothesized that factors released by neutrophils might promote an M2c phenotype with enhanced ability to phagocyte dead cardiomyocytes. To verify this possibility, we tested the influence of neutrophil supernatant on murine macrophage polarization. We confirmed that MertK was mainly expressed by *in vitro* polarized M2c macrophages, whereas M1 and M2a macrophages expressed only low levels of MertK (*Figure 5A* and *B*). Addition of neutrophil supernatant induced MertK expression in M2a macrophages, indicating a polarization from M2a toward M2c (*Figure 5A* and *B*). To test a potential relevance for humans, we incubated human primary macrophages with human neutrophil supernatant and found a comparable induction of MertK expression in M2a polarized macrophages (*Figure 5C* and *D*).

Neutrophil-derived neutrophil gelatinase-associated lipocalin promotes macrophage polarization towards M2c

We further screened the effect of various recombinant neutrophil-related proteins on macrophage polarization. Strikingly, the effect of neutrophil supernatant on MertK expression was reproduced by incubating *in vitro* polarized murine macrophages with recombinant NGAL (*Figure 5A* and *B*). To validate this finding, we performed immunodepletion of NGAL in neutrophil supernatant, which blunted the M2c-polarizing effect (*Figure 5A* and *B*). No change in macrophage polarization from M2a to M2c was observed when cells were treated with other neutrophil-released proteins, i.e. recombinant Cramp, lactoferrin, neutrophil elastase, or MPO (Supplementary material online, *Figure S2*).

In agreement with our *in vitro* data, we found reduced cardiac protein levels of the M2c marker MertK in hearts of neutrophil-depleted mice (*Figure 5E* and *F*). Neutrophil gelatinase-associated lipocalin plasma levels were up-regulated 1–3 days after MI, but were significantly lower in neutrophil-depleted mice (*Figure 5G*), indicating neutrophils to be an important source for NGAL expression after MI. Systemic administration of NGAL in neutrophil-depleted mice not only restored normal plasma levels (Supplementary material online, *Figure S4*) but also cardiac protein levels of MertK (*Figure 5E* and *F*). This effect was confirmed by flow cytometric analysis of digested hearts, revealing a lower percentage of MertK^{hi} expressing macrophages in neutrophil-depleted hearts, while their percentage was restored when injecting NGAL (*Figure 5H* and *I*).

M2c polarization is required for apoptotic cell clearance

An important role of M2c macrophages is the clearance of apoptotic cells (efferocytosis),[22] which is of crucial importance for wound healing after MI.[5] To assess the role of neutrophil-derived NGAL on efferocytosis capacity, we treated *in vitro* polarized macrophages with neutrophil supernatant or NGAL. After incubation with apoptotic cardiomyocytes, we observed a higher efficiency of M2c to phagocyte apoptotic cells compared with M1 and M2a macrophages (*Figure 5J* and *K*), as previously reported.[22] The efferocytosis capacity of M2a was increased after incubation with neutrophil supernatant or NGAL, which is in agreement with the up-regulation of phagocytosis receptor MertK expression (*Figure 5A* and *B*). To test the hypothesis that neutrophil depletion leads to a defect of dying cardiomyocyte clearance in neutrophil-depleted infarcts, we performed histological analysis and found an accumulation of apoptotic cells in infarcts of mice treated with depleting antibody (*Figure 5L* and *M*).

Neutrophil depletion affects macrophage MertK expression after ischaemia reperfusion

Finally, to verify whether the effect of neutrophils in regulating MertK expression is also detectable in reperfused hearts, we subjected mice to transient ischaemia and reperfusion. The flow cytometric analysis confirmed a strong reduction of cardiac neutrophil counts in Ly6G-treated mice. Similar to the effect after permanent ligation, reperfused hearts of neutrophil-depleted mice had less Ly6C^{hi} monocytes 3 days post-infarction, but the number of macrophages was not significantly changed (*Figure 6A* and *B*). The latter effect might be explained by a reduction of infarct size (28 ± 5%) that we observed after neutrophil depletion in the reperfusion model (data not shown). Strikingly, the number of MertK^{hi} expressing macrophages was significantly lower in neutrophil-depleted reperfused hearts (*Figure 6C*), which was confirmed by western blot analysis in heart lysates (*Figure 6D* and *E*).

Discussion

Acute MI leads to death of a large number of cardiomyocytes, which induces an inflammatory process in order to remove the damaged

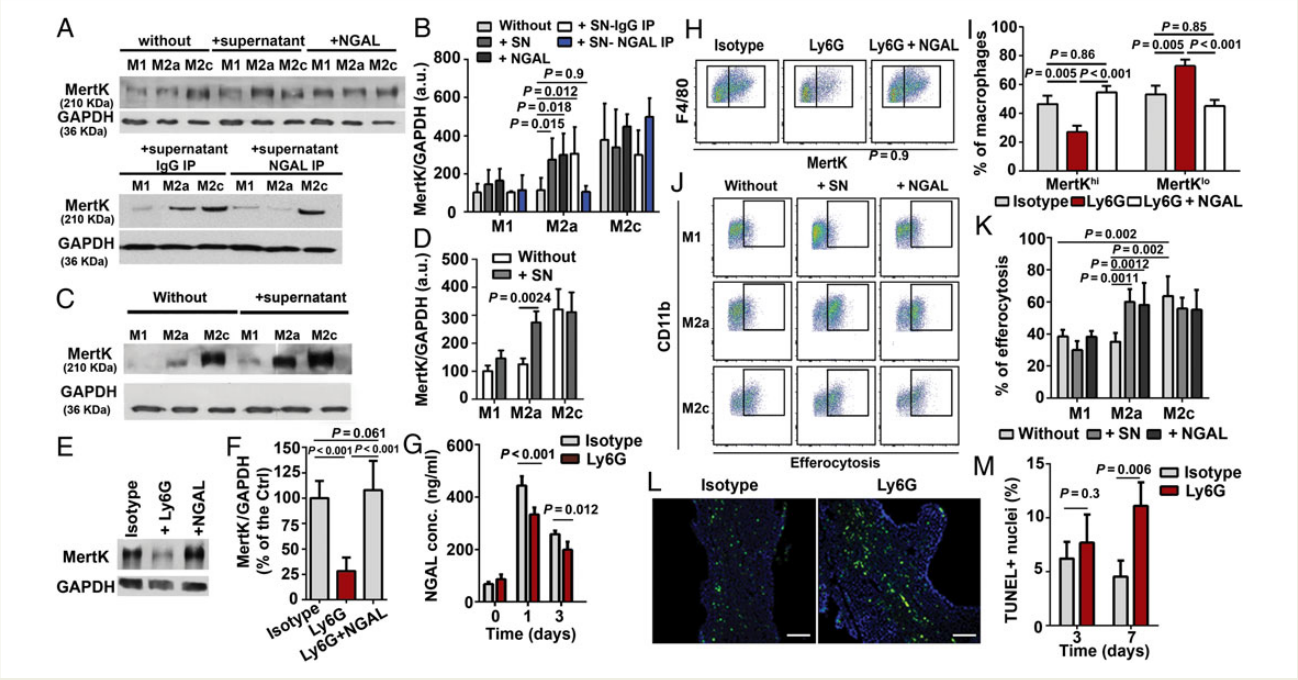

Figure 5 Neutrophils regulate macrophage polarization and efferocytosis. (*A* and *B*) Detection of MertK and GAPDH by western blot in murine bone marrow-derived macrophages. Upper blot: bone marrow-derived macrophages were polarized with IFN-γ (M1), IL-4 (M2a), or dexamethasone (M2c) alone or in the presence of neutrophil supernatant or recombinant murine neutrophil gelatinase-associated lipocalin (100 ng/mL). Lower blot: bone marrow-derived macrophages were polarized in the presence of neutrophil supernatant immunoprecipitated with anti-IgG or anti-neutrophil gelatinase-associated lipocalin. (*B*) Quantification of MertK band density, normalized to GAPDH, shown as arbitrary units. (*C* and *D*) Detection of MertK and GAPDH in polarized human macrophages incubated with human neutrophil supernatant ($n = 2$ donors). (*E* and *F*) Detection of MertK and GAPDH by western blot in hearts of isotype-treated, neutrophil-depleted (Ly6G), or Ly6G-treated mice co-injected with neutrophil gelatinase-associated lipocalin, 7 days after myocardial infarction. (*F*) Quantification of MertK band density, normalized to GAPDH and shown as relative expression compared with isotype. (*G*) Plasma levels of neutrophil gelatinase-associated lipocalin at baseline or after myocardial infarction. (*H* and *I*) Flow cytometric quantification of M2c (MertK^{hi}) and M2a macrophages (MertK^{lo}) in hearts of isotype-, Ly6G-, or Ly6G-treated mice co-injected with neutrophil gelatinase-associated lipocalin 5 days after myocardial infarction. (*J* and *K*) Efferocytosis of fluorescence-labelled apoptotic cardiomyocytes was measured by flow cytometry, after co-incubation of apoptotic cardiomyocytes with polarized bone marrow-derived macrophages. Polarization was performed in the presence or absence of neutrophil supernatant or recombinant mouse neutrophil gelatinase-associated lipocalin (100 ng/mL). (*L*) Representative TUNEL staining in infarcts of isotype or Ly6G-treated mice, 7 days after myocardial infarction. Scale bar, 200 μm; ×5 magnification. (*M*) Quantification of TUNEL-positive apoptotic cells per field of view. Data are mean ± SD from (*B, F, I*, and *K*) three experiments and donor mice, (*G*) 8, or (*M*) 5 mice/group.

tissue. In acute inflammation, neutrophils are not only vital for clearing the wound from pathogens or debris but also for the resolution of inflammation and return to tissue homeostasis.[9] In the context of MI, however, neutrophils are generally considered detrimental. They are recruited few hours after onset of MI and contribute to acute tissue injury, whereas their role in infarct healing has been largely neglected so far. Here, we provide evidence that neutrophils are required for resolving post-MI inflammation and cardiac healing. We show that neutrophil-depleted mice subjected to MI had worsened cardiac function, increased fibrosis, and a progressive increase in biomarkers associated with HF. This was accompanied by reduced cardiac expression of phagocytosis receptor MertK by macrophages and a worsened capacity to clear apoptotic cardiomyocytes.

The clearance of dead cardiomyocytes and inflammatory neutrophils is orchestrated by macrophages which are thought to derive from recruited Ly6C^{hi} monocytes.[3,4] After an acute inflammatory phase mainly driven by pro-inflammatory macrophages, these cells are replaced by reparative macrophages which facilitate wound healing and regeneration by promoting myofibroblast accumulation, collagen deposition, and angiogenesis. Wan and co-workers convincingly demonstrated that accurate clearance of dead cells is a prerequisite for favourable MI healing, whereas failed resolution promotes unfavourable cardiac remodelling which may ultimately result in HF.[5,24] In agreement with this study, our data suggest that inefficient removal of dead cells due to impaired macrophage phenotypic shift in the absence of neutrophil secretome leads to a dysregulated healing response, excessive fibrosis, and progressive loss of ventricular function.

While sufficient myofibroblast density is important for replacing dead cardiac muscle by a robust scar, excessive myofibroblast numbers or inappropriate composition of collagen fibres in the infarcted ventricle might lead to myocardial stiffness,[25] contractile dysfunction, and progression of HF.[26–28] In support of the hypothesis that neutrophil depletion may lead to HF, we found an up-regulation

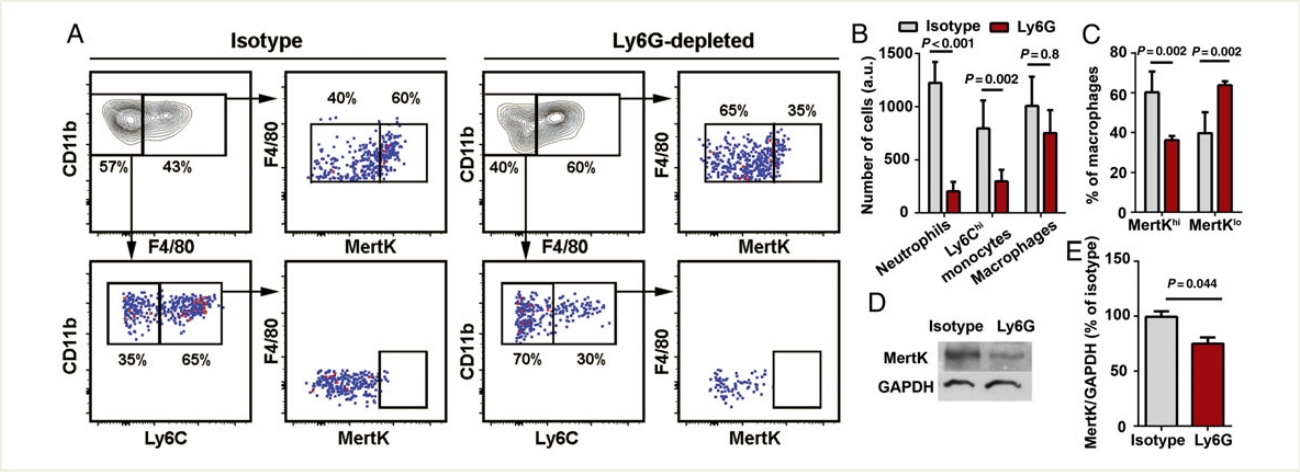

Figure 6 Neutrophil depletion modulates cardiac monocyte/macrophage profiles and MertK expression in reperfused hearts. (*A*–*C*) Representative flow cytometry results and quantification of neutrophils, Ly6C^{hi} monocytes and macrophages, as well as MertK^{hi} and MertK^{lo} macrophages in digested hearts of neutrophil-depleted (Ly6G) or isotype-treated mice 3 days after reperfusion. (*D* and *E*) Detection of cardiac MertK and GAPDH by western blot, 3 days after reperfusion. Data are mean ± SD from 5 mice/group.

of cardiac and systemic markers associated with HF.[14,29] Cardio-renal syndrome may occur as a result of cardiac dysfunction and hypoperfusion of the kidney leading to medullary ischaemia.[30] Indeed, we detected signs of acute kidney injury, which by itself is a prognostic factor associated with adverse outcome.[16]

We identified NGAL in the neutrophil secretome as a key inducer of macrophages with high efferocytosis capacity. Neutrophil gelatinase-associated lipocalin is released by activated neutrophils from specific granules, but also from other cells including macrophages, ischaemic cardiomyocytes, or injured kidney epithelial cells in acute renal injury.[31–33] In the setting of *Streptococcus pneumonia* infection, it was recently shown that NGAL skewed activated alveolar macrophages to a resolving phenotype expressing high levels of IL-10.[34] In skeletal muscle injury, the macrophages involved in muscle regeneration have been characterized as M2c macrophages that exhibit anti-inflammatory properties by releasing TGF-β and by neutralizing pro-inflammatory M1 macrophages.[35] In post-MI repair, M2c marker MertK-expressing macrophages play a crucial role in the clearance of cell debris.[5]

Our findings that neutrophils could contribute to improve cardiac healing and the outcome by influencing macrophage polarization are somewhat surprising, given that they are generally considered to play a detrimental role after post-MI revascularization. In fact, elevated circulating neutrophil counts or plasma levels of their released factors, including NGAL, are associated with poor prognosis and mortality in MI patients.[7,36] We may speculate that a certain number of neutrophils and secretion products are required in the local cardiac microenvironment to promote reparative macrophage polarization. However, there might be a threshold level where the acute tissue damaging effects of neutrophils outweigh their resolving properties during MI healing. This is supported by clinical data reporting that associations between blood neutrophil counts and clinical outcomes are mainly evident in patients with neutrophilia (neutrophil counts > 65%)[37] or in the highest tertile,[38,39] respectively.

Our study has several limitations: First, the mouse model of permanent LAD occlusion, although widely used to study post-MI repair and remodelling, may have limited predictive value for human pathophysiology. Nevertheless, it is a valuable tool to dissect the function of, e.g. specific-cell populations and molecular pathways involved in post-MI healing, which provides the basis for more defined therapeutic approaches. In view of potential relevance for humans, it is promising that we could confirm the effect of neutrophil depletion on macrophage MertK expression in the ischaemia-reperfusion model, and we were also able to show the polarizing effect of human neutrophil secretome in human primary macrophages. In the future, additional studies with clinically more relevant large animal models are warranted before extrapolating a potential significance of our findings for humans. Second, we cannot exclude that additional factors other than NGAL might contribute to the polarizing effects of the neutrophil secretome on macrophages. Nevertheless, we identified NGAL as a key regulator of macrophage reprogramming both *in vitro* and *in vivo*. Finally, it is conceivable that neutrophil-derived factors might promote autocrine release of factors by the macrophages themselves that might facilitate their own phenotypic switch.[34] In the future, a better knowledge of the molecular regulators involved in macrophage reprogramming will help to answer these unresolved questions.

In conclusion, our data suggest that neutrophils participate in MI repair in a mouse model through the secretion of NGAL, thereby skewing macrophages towards a resolving phenotype which mediates efficient clearance of cell debris. This is a prerequisite for regulated fibrosis, scar formation and favourable cardiac remodelling.[5] Our findings therefore have important clinical implications. Beyond their established pro-inflammatory role in acute post-MI injury, we identify neutrophils as pivotal modulators of the healing response after MI and consequently cardiac repair and function. This novel role for neutrophils should be taken in account when designing and applying 'aggressive' anti-neutrophil treatments in the setting of MI.

Authors' contributions

M.H. and D.S. performed statistical analysis. S.S., O.S., and C.W. handled funding and supervision. M.H., L.R., D.S., and M.S. acquired the data. S.S., O.S., and M.H. conceived and designed the research. S.S. drafted the manuscript. M.H., J.D., O.S., M.D., and D.S. made critical revision of the manuscript for key intellectual content.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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