Macrophage Mineralocorticoid Receptor Is a Pleiotropic Modulator of Myocardial Infarct Healing

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Abstract—Myocardial infarction (MI) is a major cause of death worldwide. Here, we identify the macrophage MR (mineralocorticoid receptor) as a crucial pathogenic player in cardiac wound repair after MI. Seven days after left coronary artery ligation, mice with myeloid cell–restricted MR deficiency compared with WT (wild type) controls displayed improved cardiac function and remodeling associated with enhanced infarct neovascularization and scar maturation. Gene expression profiling of heart-resident and infarct macrophages revealed that MR deletion drives macrophage differentiation in the ischemic microenvironment toward a phenotype outside the M1/M2 paradigm, with regulation of multiple interrelated factors controlling wound healing and tissue repair. Mechanistic and functional data suggest that inactivation of the macrophage MR promotes myocardial infarct healing through enhanced efferocytosis of neutrophils, the suppression of free radical formation, and the modulation of fibroblast activation state. Crucially, targeted delivery of MR antagonists to macrophages, with a single administration of RU28318 or eplerenone-containing liposomes at the onset of MI, improved the healing response and protected against cardiac remodeling and functional deterioration, offering an effective and unique therapeutic strategy for cardiac repair. **(*Hypertension*. 2019;73:102-111. DOI: 10.1161/HYPERTENSIONAHA.118.12162.)** ● **Online Data Supplement**

Key Words: liposomes ■ macrophages ■ myocardial infarction ■ receptors, mineralocorticoid ■ wound healing

Myocardial infarction (MI) and ensuing heart failure are leading causes of death worldwide. Ischemic cell death triggers a cascade of cellular and molecular events that promote wound healing and structural cardiac remodeling. Immune mechanisms/mediators regulating ischemic injury and tissue repair are considered important therapeutic targets to prevent cardiac functional decline and are the focus of intensive research efforts.[1–4]

MR (mineralocorticoid receptor) blockade reduces morbidity and mortality in the setting of myocardial infarction. Emerging evidence suggests that administration of MR antagonists early in the course of acute MI offers the greatest benefit in clinical outcome.[5,6] Modulation of the inflammatory response, improvement of cardiac structural and electrical remodeling, and prevention of life-threatening arrhythmia appear to play an important role.[7–10]

Monocytes/macrophages act as key players in the wound repair process through the clearance of apoptotic cells and the release of cytokines/chemokines, proteases, growth factors, and oxygen-derived free radicals.[2,11,12] Experimental studies using cell-specific MR knockout highlighted the cell-specific effects of MR activation in the cardiovascular system and identified the myeloid MR as a critical regulator of cardiovascular inflammation, fibrosis, and hypertrophy.[7,13,14]

However, it remains unknown whether the benefits of early MR blockade post-MI can be attributed to inhibition of the MR signaling in macrophages. Using mice with myeloid cell–restricted MR deficiency, we investigated the relevance of the macrophage MR for cardiac repair and remodeling, dissecting the pathogenic mechanisms associated with MR activation after acute MI. Moreover, we explored whether the targeted delivery of MR antagonists to macrophages at the onset of MI improved the healing response and protected against cardiac remodeling and functional deterioration.

Materials and Methods

The authors declare that all supporting data are available within the article and its online-only Data Supplement.

Detailed Materials and Methods are available in the online-only Data Supplement.

Study Protocol

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85–23, revised 1985). All procedures were approved by the Regierung von Unterfranken (Würzburg, Germany; permit No. 54–2531.01-15/07) and by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Oldenburg, Germany; permit No.

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33.12-42502-04-11/0644 and 33.9-42502-04-13/1124). Adult Nr3c2^{tm2Gsc} (MR^{flox}, WT controls), Nr3c2^{tm2Gsc}Lyz2tm1(cre)Ifo/J (MR^{LysMCre}), and C57Bl/6 mice of both sexes were used in this study. Liposome-encapsulated RU28318 (2 µmol/kg) or liposome-encapsulated eplerenone (20 µmol/kg) were administered intraperitoneally (100 µmol lipid per kg) in C57BL/6 mice at the onset of myocardial infarction. Control C57BL/6 mice were injected with equal volumes of plain (MR antagonists lacking) liposome. In additional experimental groups, starting immediately after coronary ligation, C57BL/6 mice were randomly selected for eplerenone (100 mg/kg of body weight) or placebo treatment (5% arabic gum) administered by gavage once daily. Mice subjected to coronary artery ligation were sacrificed at days 1, 3, or 7 after surgery. Mice were excluded from the analyses for 2 reasons: perioperative death (within the first 12 hours after surgery) and MI size <40.

Statistical Analysis

Results are reported as mean±SEM. One-way ANOVA with Tukey post hoc test or with the unpaired *t* test was performed using GraphPad Prism 6.01 (GraphPad Software, Inc). Values of $P < 0.05$ were considered statistically significant.

Results

The schematic representation of the MR deletion strategy is shown in Figure S1A through S1C in the online-only Data Supplement. Quantitative reverse-transcriptase polymerase chain reaction revealed downregulation of MR expression (Figure S1D) in cardiac macrophages from mice with myeloid cell–restricted MR deficiency (hereafter referred to as MR^{LysMCre}) compared with WT (wild type) controls (hereafter referred to as MR^{flox}).

Mice With Myeloid Cell–Restricted MR Deficiency Displayed Improved Cardiac Function and Remodeling After MI

Infarct size was similar among MR^{flox} and MR^{LysMCre} mice (Figure 1A). We did not detect differences between MR^{flox} and MR^{LysMCre} sham-operated mice regarding left ventricular (LV) systolic or diastolic pressure, cardiac volume, and function (Figure 1B; Figure S1E). Myeloid cell–restricted MR deficiency prevented the rightward shift of the pressure-volume curve 7 days after left coronary artery ligation (Figure 1B). LV end-diastolic pressure, LV end-diastolic volume, and LV end-systolic volume were significantly decreased compared with MR^{flox} (Figure 1C). Amelioration of LV remodeling in infarcted MR^{LysMCre} mice was associated with a significant improvement in LV ejection fraction (Figure 1C). Correspondingly, MR^{LysMCre} mice exhibited enhanced LV dP/dt_{min}, LV dP/dt_{max}, and LV dP/dt_{max} divided by instantaneous pressure—a load-independent measure of contractile function. Moreover, the time constant of LV pressure isovolumic decay (τ)—a relatively load-independent index of LV relaxation—was significantly shortened in MR^{LysMCre} mice compared with control animals (Figure 1C). These results indicate that myeloid cell–restricted MR deficiency prevents early post-MI cardiac dilation, functional deterioration, and failure.

Enhanced Infarct Neovascularization and Scar Maturation in MR^{LysMCre} Mice After Ischemic Injury

Neovascularization of the ischemic myocardium plays an essential role in fibrous tissue formation and infarct healing. Seven days post-infarction, MR^{LysMCre} mice displayed an enhanced angiogenic response to ischemic injury (Figure 2A through 2C). Immunofluorescence analysis showed an increased number of capillaries, identified as small lumen vessels positively staining for CD (cluster of differentiation) 31 (Figure 2C). As the scar matures, many vessels acquire a pericyte coat that stabilizes the infarct vasculature. Many more coated vessels, identified as thin-walled α-SMA (α-smooth muscle actin)–positive vascular structures, were observed in the infarcted myocardium of MR^{LysMCre} mice compared with MR^{flox} animals (Figure 2A and 2B).

Immunofluorescence staining for α-SMA also demonstrated the presence of myofibroblasts, identified as extramural spindle-shaped α-SMA–positive cells, within the infarct region 7 days post-MI (Figures 2A and 3A; enlarged insets). Recent data indicated that in the infarcted myocardium, fibroblasts become activated, differentiate to a α-SMA–positive myofibroblast phenotype, and after the first 7 days, when the infarct scar is mature, begin to lose their α-SMA expression.[15] It is noteworthy that in MR^{LysMCre} infarcts, the expression of α-SMA was reduced in fibroblasts (Figures 2B and 3B; enlarged insets) and α-SMA–positive cells were mostly restricted to vessels.

Scar collagen content was similar among MR^{flox} and MR^{LysMCre} mice, yet collagen maturation was improved in MR^{LysMCre} infarcts (Figure 3C through 3G). Sirius red polarization microscopy revealed a mostly well-aligned collagen matrix with thick, tightly assembled mature collagen fibers in MR^{LysMCre} mice (Figure 3D and 3F) in contrast to the matrix with a predominance of thin, loosely packed immature fibers observed in control-MI mice (Figure 3C and 3E). The ratio of thick (orange-red)-to-thin (yellow-green) birefringent collagen fibers and fiber straightness and alignment were significantly increased in MR^{LysMCre} compared with MR^{flox} infarcts (Figure 3G). Taken together, these findings show MR in myeloid cells to be a pathogenic player and important therapeutic target in cardiac wound repair.

MR Inactivation Drives Macrophage Differentiation in the Ischemic Microenvironment Toward a Phenotype Outside the M1/M2 Activation Paradigm

To delineate the macrophage-specific mechanisms underlying the improved healing response to ischemic injury by myeloid cell–restricted MR deficiency, we investigated phenotypic differences between macrophages isolated from MR^{flox} and MR^{LysMCre} hearts. Importantly, using a modified Langendorff perfusion system, the hearts were perfused for 6 minutes to remove blood cells and subsequently digested for 8 minutes to preserve cell surface antigens along with gene expression profile.[2] Infarct macrophages were identified by flow cytometry as CD45$^+$/CD11b$^+$/Ly6G$^-$/F4/80$^+$ cells and then further stratified by Ly6C expression (Figure 4A). As shown in Figure S2A, infarct macrophages were Mertk$^+$ and CD64$^+$, whereas infiltrating monocytes (CD45$^+$/CD11b$^+$/Ly6G$^-$/F4/80$^-$) were Mertk$^-$. At day 3 post-MI, corresponding to transition from the inflammatory to the reparative phase of cardiac wound repair, we showed that macrophages in the infarcted myocardium of MR^{LysMCre} mice almost exclusively consisted of Ly6C^{low} cells (Figure 4A; Figure S2B). Interestingly, the number of infarct macrophages was similar in MR^{LysMCre} versus MR^{flox} mice, but the amount of CD45$^+$/

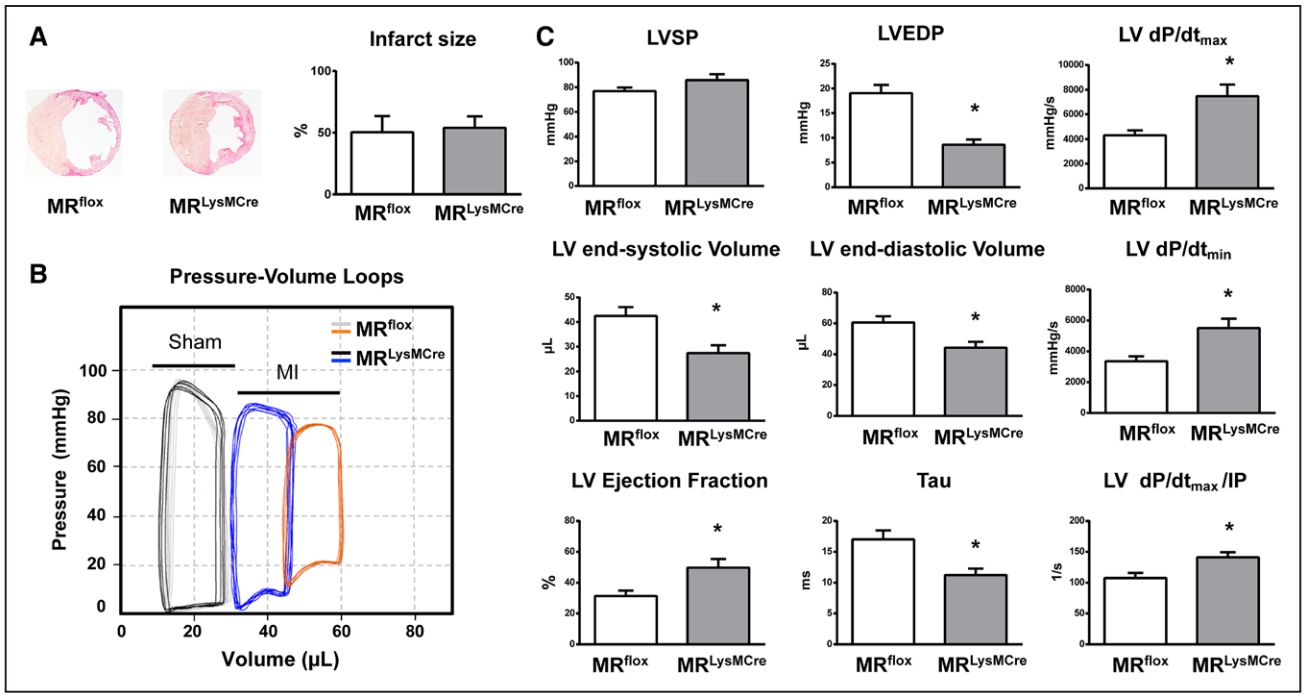

Figure 1. Mice with myeloid cell–restricted MR (mineralocorticoid receptor) deficiency display improved cardiac function and remodeling after myocardial infarction (MI). **A**, Representative sections from MR^{flox} and $MR^{LysMCre}$ infarcted hearts and infarct size. **B**, Representative left ventricular (LV) pressure-volume loops measured in vivo with conductance catheter in sham-operated MR^{flox} (gray) and $MR^{LysMCre}$ (black) mice and in MR^{flox} (orange) and $MR^{LysMCre}$ (blue) mice with MI. **C**, LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), LV end-systolic and end-diastolic volumes; LV ejection fraction, LV maximal rate of pressure rise (LV dP/dt_{max}), maximal rate of pressure decline (LV dP/dt_{min}), and LV dP/dt_{max} divided by instantaneous pressure (IP) and the time constant of LV pressure isovolumic decay (Tau). Mean±SEM (n=14–16). *P<0.01 vs MR^{flox}.

$CD11b^+/F4/80^-/Ly6G^+$ cells was significantly lower, indicative of reduced neutrophil accumulation in the ischemic myocardium of $MR^{LysMCre}$ mice (Figure 4A).

Next, we performed RNA sequencing of fluorescence-activated cell sorting (FACS)–isolated resident macrophages at steady state and of infarct macrophages sorted from the ischemic region at day 3. As shown in the Figure S3A principal component analysis comparing transcriptional profile determined by RNA-seq of heart-resident macrophages at steady state, infarct macrophages and blood monocytes revealed strong dissimilarity between circulating monocytes and cardiac macrophages in steady state and after infarction. Moreover, MA plot of gene expression from infarct macrophages versus resident macrophages at steady state (Figure S3B) showed significant regulation of genes detected by single-cell RNA-seq in macrophages present in the infarct region (from published single-cell RNA-seq data GSE106473[16]).

Comprehensive transcriptome analysis highlighted that MR inactivation induces macrophage differentiation toward a phenotype outside the M1/M2 classification (Figure 4B; Figures S4A through S4D, S5, and S6).[17,18]

Bioinformatic analysis revealed that several genes regulated by MR deletion in infarct macrophages contain at least 1 consensus mineralocorticoid response element motif[19] (Figure S5). Notable, among the most downregulated genes containing a mineralocorticoid response element motif located close to the transcription-starting site, we found 2 key molecules (lipocain-2 [*Lcn2*], amphiregulin [*Areg*]) involved in fibroblast activation and fibrosis post-MI (Figure S5B). Gene set enrichment analysis, able to individuate though small but cumulatively significant changes on sets of functionally related genes, revealed Tnfα signaling via NF-κB (nuclear factor-kB; Figure S6A through S6C), one of the most significantly enriched pathways (Normalized enrichment score, −2.81; P_{adj}=0.009). Among genes of leading edge mainly contributing to the enrichment score, *Ptx3*, *Cxcl5*, *Il1b*, *Sphk1*, *Areg*, and *Tnc* were significantly downregulated in infarct macrophages from $MR^{LysMCre}$ versus MR^{flox} infarct macrophages (Figure S6A through S6C).

Overall, gene expression profiling of infarct macrophages revealed that multiple factors known to mediate tissue repair and wound healing[20] were differently regulated in MR-deficient versus WT macrophages (Figure 4C). Noteworthy, we found that several of these genes were similarly downregulated/upregulated in the infarcted myocardium by eplerenone treatment (Figure S7A through S7C), thereby establishing a relationship between MR signaling in macrophages and the protective effects of MR antagonism after MI.

Transcriptome profiling of infarct and heart-resident macrophages (Figure 4D and 4E) also showed the upregulation of receptors and molecules involved in the phagocytosis of apoptotic cells.[21] The enrichment of efferocytosis-related transcripts included receptors that are able to recognize the chemotactic find-me signals (*Stab1*, *Cx3cr1*, and *S1pr1-2*), receptors able to recognize eat-me signals (*Mertk* and *Scarf1*), and their associated opsonins (*Gas6* and C1q). Also regulated were the scavenger receptor that mediates opsonin-independent phagocytosis (*Marco*), the ATP-binding cassette transporter (*Abca1*), which plays a critical function in the postengulfment response, and the RXR (retinoid X receptor), an obligatory heterodimeric partner for the activity of the peroxisome proliferator activated

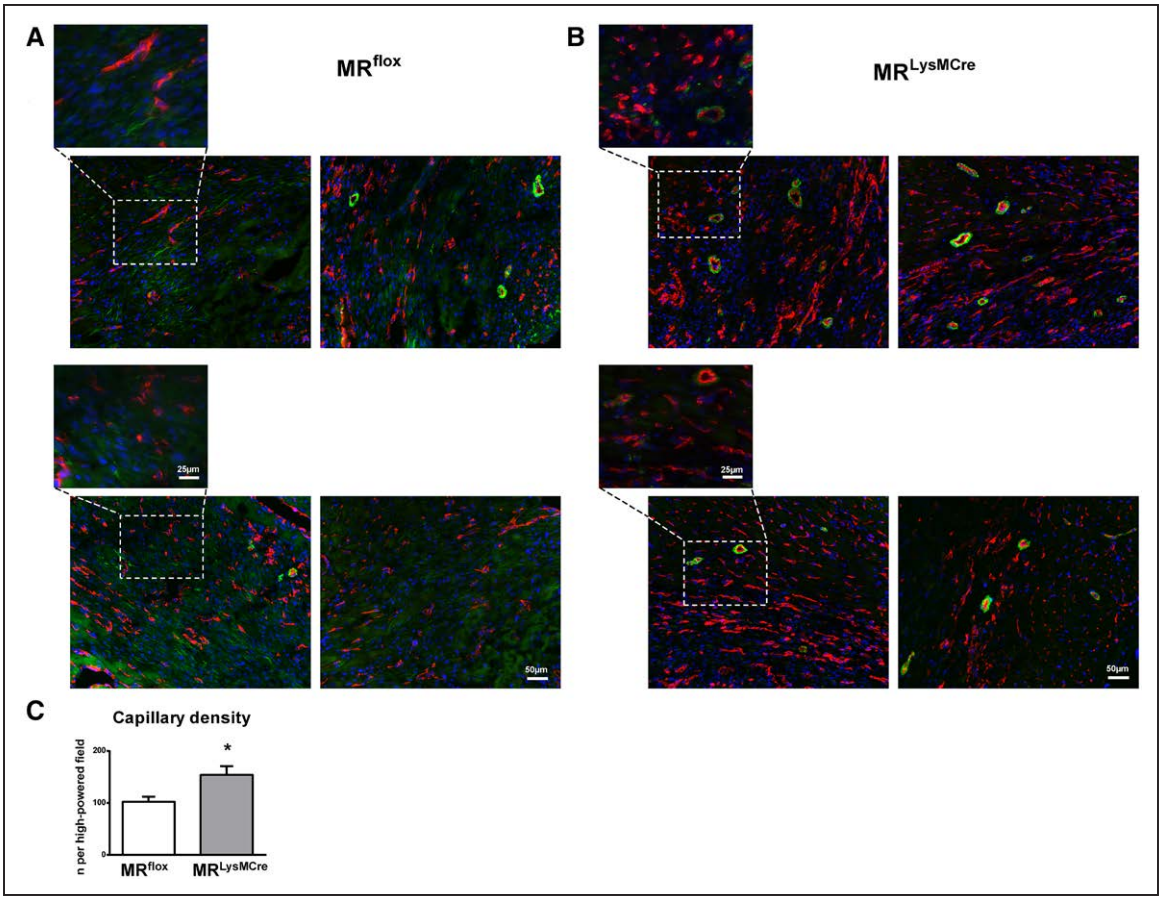

Figure 2. Enhanced cardiac neovascularization after ischemic injury in $MR^{LysMCre}$ mice. **A** and **B**, Immunofluorescence double staining (CD [cluster of differentiation] 31, red; α-SMA [α-smooth muscle actin], green) showing capillaries, coated vessels, and (myo)fibroblasts in the healing myocardium of MR^{flox} and $MR^{LysMCre}$ mice, 7 d after myocardial infarction. In $MR^{LysMCre}$ infarcts, the expression of α-SMA was mostly restricted to pericytes and arterioles. **C**, Capillary density quantification in MR^{flox} and $MR^{LysMCre}$ mice. Mean±SEM (n=6). *P<0.01 vs MR^{flox}.

receptor. Other important prophagocytic genes (*Axl*, *Mefge8*, and *Trem2*) showed a trend toward upregulation contributing to the positive enrichment of efferocytic pathway (Figure 4D and 4E). Of note, Ingenuity pathway analysis identified role of pattern recognition receptors, that includes several genes involved in the clearance of apoptotic cells, among the significantly regulated pathways (Figure S6D).

The regulation of key molecules involved in efferocytosis (*Mertk*, *C1qa*, and *C1qb*), inflammation resolution (*Gpnmb* [glycoprotein nmb] and *Apoe* [apolipoprotein E]), oxidative stress (peroxiredoxin 4 and catalase), angiogenesis/wounding responses (*Vegfb* and *Igf1*), and fibroblast homeostasis (lipocalin-2) was confirmed by quantitative reverse-transcriptase polymerase chain reaction (Figure 5A).

Macrophage MR Deficiency Is Associated With Enhanced Neutrophil Efferocytosis

To define the functional role of the MR in macrophage phagocytic activity in the ischemic microenvironment, we isolated $CD45^+/CD11b^+/Ly6G^-/F4/80^+$ macrophages from MR^{flox} and $MR^{LysMCre}$ infarcts. Because the FACS data showed a significantly lower amount of neutrophils in the ischemic myocardium of $MR^{LysMCre}$ mice, we examined the potential involvement of the MR in modulating neutrophil efferocytosis. As shown in Figure 5B, the phagocytosis of apoptotic neutrophils (isolated from C57BL/6 mice) was significantly increased in macrophages sorted from $MR^{LysMCre}$ infarcts. Furthermore, MR deficiency significantly enhanced the phagocytic capacity of infarct macrophages for zymosan particles (Figure 5C). Collectively, these findings highlight a novel role for the macrophage MR in orchestrating phagocytosis after MI.

Reduced Superoxide Production in Infarct Macrophages Lacking the MR

Oxidative stress triggers a cascade of molecular alterations, leading to impaired wound repair. We assessed superoxide production by $CD45^+/CD11b^+/Ly6G^-/F4/80^+$ macrophages using a highly sensitive isocratic ion-pair high-performance liquid chromatography-electrochemical method (Figure 5D). FACS-isolated macrophages from $MR^{LysMCre}$ infarcts showed significantly decreased $O_2^{\cdot-}$ production (Figure 5D). It is noteworthy that transcriptional profiling revealed upregulation of the antioxidant enzymes, catalase and peroxiredoxin 4, in macrophages lacking the MR (Figures 4C and 5A).

Macrophage MR Affects Interactions Between Fibroblasts and Macrophages

Prompted by the histological and immunofluorescence results that showed enhanced scar collagen maturation and loss of α-SMA expression in infarct fibroblasts from

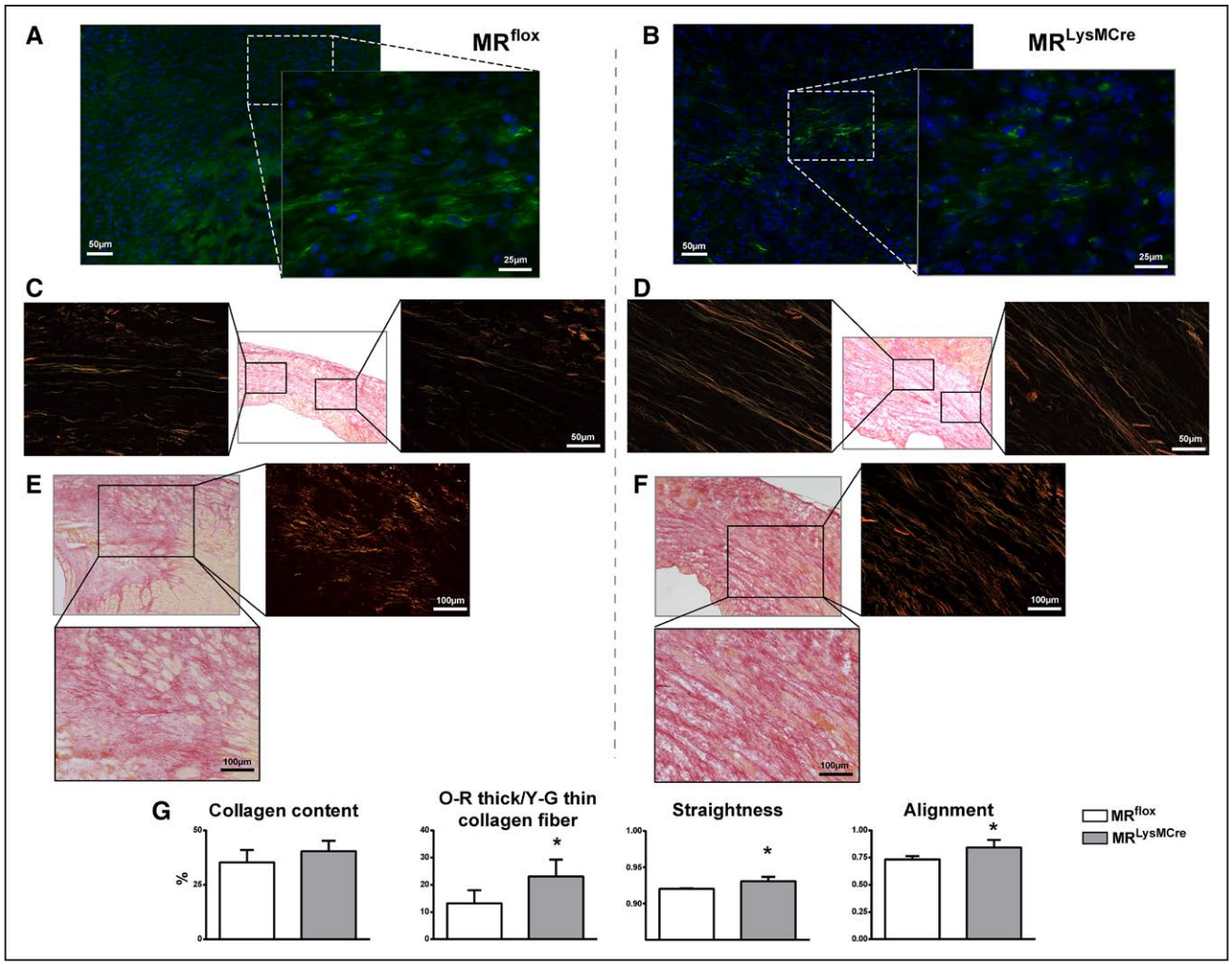

Figure 3. Enhanced collagen scar formation in $MR^{LysMCre}$ mice. Immunofluorescence staining for α-SMA (α-smooth muscle actin; green) showing the presence of myofibroblasts (spindle-shaped α-SMA–positive cells) within the infarct region of (**A**) MR^{flox} and (**B**) $MR^{LysMCre}$ mice. $MR^{LysMCre}$ infarcts exhibited a loss of α-SMA expression in (myo)fibroblasts. **C**–**F**, Sirius red polarization microscopy of scar sections revealed a matrix with a predominance of thin and loosely assembled collagen fibers in MR^{flox} mice (**C** and **E**) and well-aligned and tightly packed collagen fibers in $MR^{LysMCre}$ infarcts (**D** and **F**), 7 d after myocardial infarction. **G**, Infarct collagen content, ratio of orange-red (O-R) thick to yellow-green (Y-G) thin collagen fibers, and fiber straightness and alignment. Mean±SEM (n=6). *P<0.05 vs MR^{flox}.

$MR^{LysMCre}$ mice, we investigated whether the macrophage MR affects the interactions between fibroblasts and macrophages in the early phase of healing.

Recent studies have highlighted that infarct fibroblasts are derived from proliferation and migration of resident cardiac fibroblasts.[15,22] In the ischemic wound environment, fibroblasts differentiate into myofibroblasts mostly driven by paracrine mediators produced by cardiac M(IL [interleukin]-4)-like macrophages.[23] To consider extrinsic factors (released by infarct macrophages) and intrinsic changes in the fibroblast itself, macrophages and fibroblasts were isolated from the infarct myocardium of MR^{flox} and $MR^{LysMCre}$ mice 3 days post-MI. We observed that fibroblasts in coculture with macrophages differentiate into α-SMA–positive myofibroblasts. Interestingly, the coculture of macrophages and fibroblasts led to an increase in MMP (matrix metalloproteinase) secretion into the media, as assessed by zymography (Figure 5E). In contrast, an increase in MMP secretion was not induced by the coculture of macrophages and fibroblasts isolated from $MR^{LysMCre}$ ischemic myocardium. (Figure 5E). These data suggest that dynamic interactions between macrophages and fibroblasts seem to be critically regulated by the macrophage MR

Targeted Delivery of MR Antagonists to Macrophages Improved Postischemic Wound Healing and Protected Against Cardiac Dysfunction and Remodeling

Next, we examined whether the inhibition of MR signaling in macrophages by targeted delivery of MR antagonists (RU28318/eplerenone encapsulated within liposomes) promotes cardiac wound repair. A single dose of liposomal RU28318 or liposomal eplerenone or empty (MR antagonist lacking) liposomes was injected into mice at the onset of MI.

The targeted delivery to macrophages in the infarcted myocardium was investigated with liposomes containing fluorescein isothiocyanate (FITC)-BSA.[11] The liposomes were injected intraperitoneally at the onset of coronary ligation, and the in vivo uptake of liposomes encapsulating FITC-BSA was analyzed by FACS analysis and immunofluorescence (Figure S8). We observed that the overwhelming majority of infarct

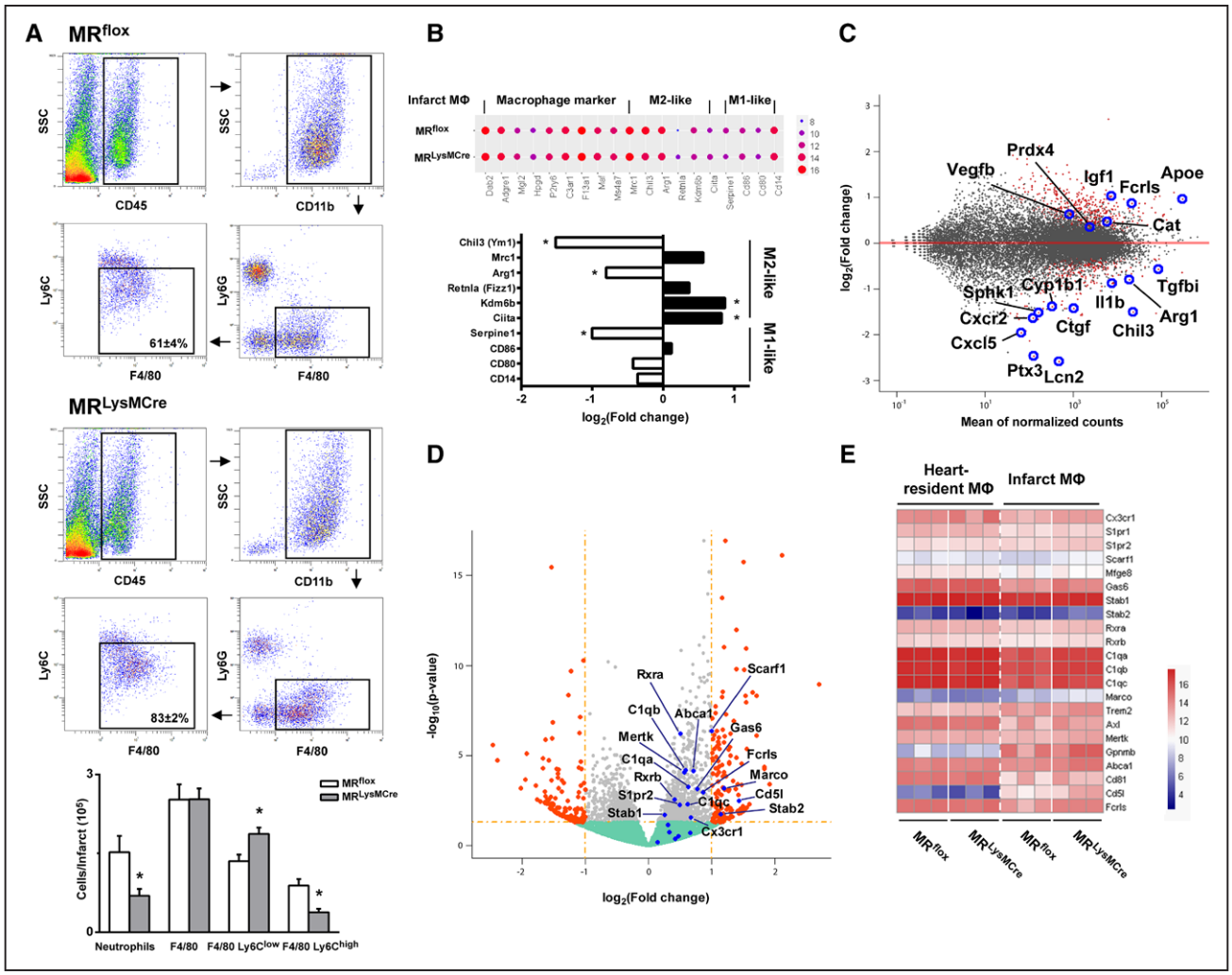

Figure 4. MR (mineralocorticoid receptor) inactivation drives macrophage differentiation in the ischemic microenvironment toward a phenotype outside the M1/M2 activation paradigm. **A**, Flow cytometry and gating strategy identifying macrophages (CD [cluster of differentiation] 45$^+$/CD11b$^+$/Ly6G$^-$/F4/80$^+$ cells –Ly6C^{low} and Ly6C^{high}) and neutrophils (CD45$^+$/CD11b$^+$/F4/80$^-$/Ly6G$^+$ cells) in MR^{flox} and MR^{LysMCre} infarcts, 3 d after coronary artery ligation. SSC indicates side scatter. Mean±SEM (n=5). *P<0.05 vs MR^{flox}. **B**–**E**, Comparison of gene expression profiles of infarct macrophages from MR^{LysMCre} and MR^{flox} mice (n=3). **B**, Dot plot showing gene expression of macrophage markers; fold changes of M1/M2-like markers are reported (*P_{adj}<0.1). **C**, MA plot showing significantly regulated genes with P_{adj} <0.1 (red). **D**, Volcano plot; significantly regulated genes with P<0.05 (gray) and with fold change >2 (orange) are shown; upregulated efferocytic genes are highlighted (blue). **E**, Heatmap showing efferocytic gene expression.

macrophages (Figure S8A) had uptaken FITC liposomes. In contrast, FACS analysis showed that almost all neutrophils, endothelial cells, fibroblasts, and vascular cells were FITC negative (Figure S8B). Moreover, immunofluorescence revealed that FITC-labeled cells in the infarcted heart were CD68-positive macrophages (Figure S8C), demonstrating the selectivity of the approach.

At 7 days post-MI, mice receiving empty liposomes developed elevated LV filling pressure, LV end-systolic and end-diastolic volumes, and marked cardiac dysfunction, as assessed by LV ejection performance, dP/dt_{max}, dP/dt_{min}, and LV dP/dt_{max} divided by instantaneous pressure (Figure 6B and 6C). Intriguingly, the administration of RU28318 or eplerenone-containing liposomes significantly decreased LV end-diastolic pressure, LV end-systolic volume, and end-diastolic volume, associated with a downward and leftward shift of the pressure-volume curve (Figure 6B and 6C). LV contractile function and relaxation were also significantly improved after targeted delivery of MR antagonists (Figure 6C). In addition, the time constant of LV pressure isovolumic decay was significantly shortened by administration of RU28318/eplerenone-loaded liposomes compared with mice receiving empty liposomes (Figure 6C). Of note, nearly identical hemodynamic data were obtained in infarcted MR^{LysMCre} mice (Figure 1B and 1C).

In addition, the administration of RU28318-loaded liposomes enhanced infarct neovascularization (Figure S9A). Similar to the effects observed in MR^{LysMCre} infarcts, mice receiving RU28318-loaded liposomes exhibited more α-SMA–positive cells restricted to pericytes/vascular smooth muscle cells and a loss of α-SMA expression in fibroblasts within the infarcted myocardium (Figure S9A). Further, we performed flow cytometry analysis of fibroblasts isolated from the infarct region of mice receiving empty liposomes and liposomal RU28318 (Figure S9B). Quantitative analysis showed that liposomal RU28318 did not affect the number of fibroblasts present in the infarct scar (Figure S9B).

Taken together, these results suggest that inhibition of MR activation in macrophages by a targeted delivery of MR

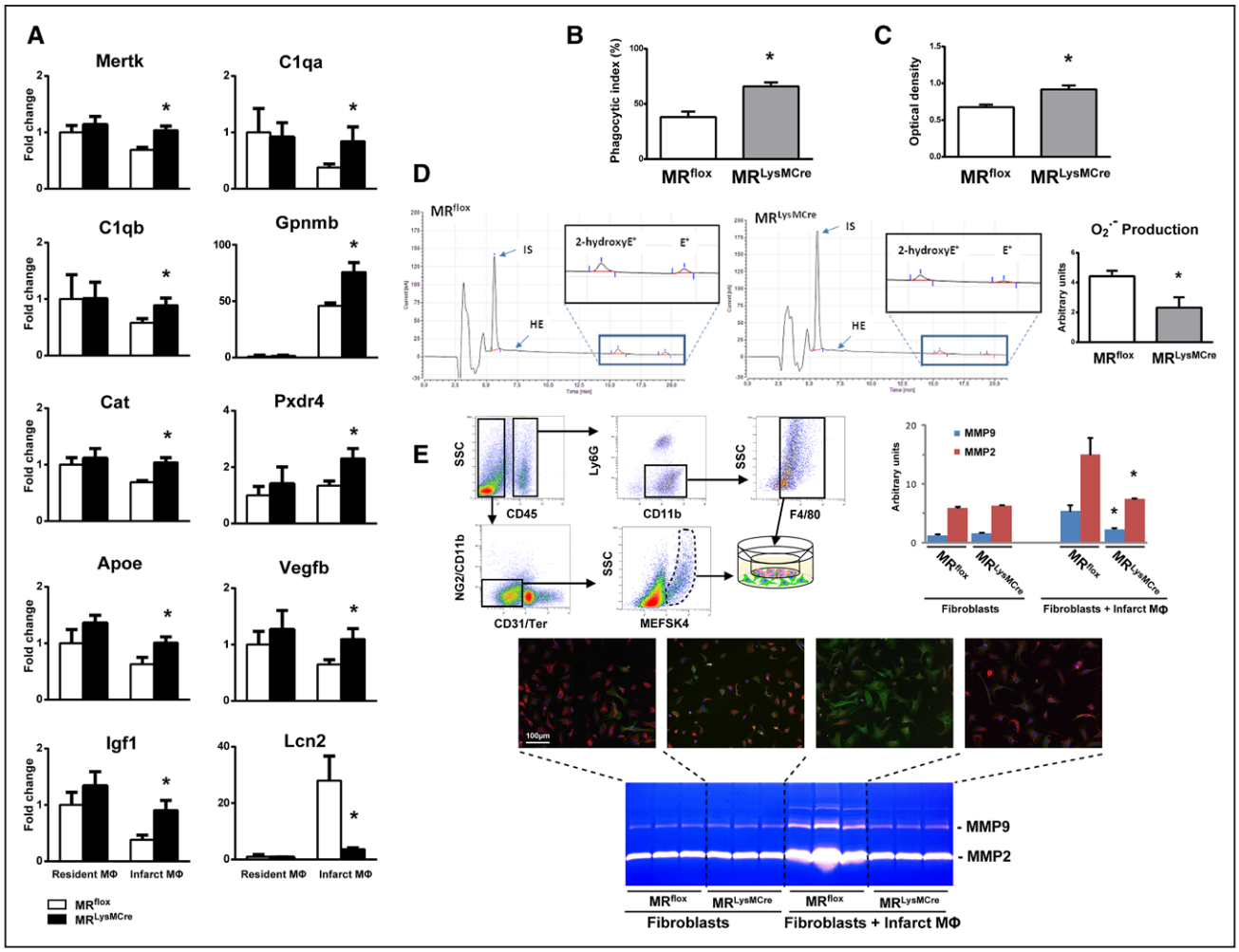

Figure 5. Macrophage MR (mineralocorticoid receptor) deficiency is associated with enhanced neutrophil efferocytosis, suppression of free radical formation and the modulation of fibroblast activation. **A**, Quantitative reverse-transcriptase polymerase chain reaction was used to detect the relative gene expression of *Mertk*, *C1qa*, *C1qb*, *Gpnmb*, *Cat*, *Pxdr4*, *ApoE*, *Vegfb*, *IGF1*, and *Lcn2*. **B**, Phagocytic index for apoptotic neutrophils and (**C**) phagocytic capacity for zymosan particles of macrophages (CD [cluster of differentiation] 45$^+$/CD11b$^+$/Ly6G$^-$/F4/80$^+$ cells) isolated by cell sorting from MR^{flox} and MR^{LysMCre} infarcts, 3 d after coronary artery ligation. **D**, Superoxide production by CD45$^+$/CD11b$^+$/Ly6G$^-$/F4/80$^+$ macrophages fluorescence-activated cell sorting–isolated from MR^{flox} and MR^{LysMCre} infarcts, assessed using a highly sensitive isocratic ion-pair high performance liquid chromatography–electrochemical method. **E**, Immunocytochemical staining showing fibroblasts and differentiated α-SMA (α-smooth muscle actin)–positive myofibroblasts (vimentin, red; α-SMA, green) and zymography of conditioned media. Macrophages (CD45$^+$/CD11b$^+$/Ly6G$^-$/F4/80$^+$ cells) and fibroblasts (CD45$^-$/CD11b$^-$/CD31$^-$/TER-119$^-$/NG2$^-$/MEFSK4$^+$ cells) were isolated by cell sorting from the infarct myocardium of MR^{flox} and MR^{LysMCre} mice and cocultured using the Boyden chamber system. Mean±SEM (n=4–5). MMP indicates matrix metalloproteinase. *P<0.05 vs MR^{flox}. HE indicates hydroethidine; IS, internal standard; NG2, chondroitin sulfate proteoglycan; MEFSK4, anti-feeder antibody, clone mEF-SK4; and SSC, side scatter.

antagonists could represent a unique therapeutic strategy to promote healing and infarct scar maturation and to prevent cardiac dysfunction and remodeling after MI.

Discussion

This study highlights the pathogenic role of the macrophage MR in tissue repair mechanisms after acute MI. Our data strongly suggest that MR deficiency in macrophages improves healing and cardiac remodeling after ischemic injury through multiple mechanisms, including the promotion of neutrophil efferocytosis, the suppression of free radical formation, and the modulation of fibroblast activation state.

Previous studies in isolated peritoneal thioglycollate-elicited macrophages showed that loss of MR activity promotes an alternatively activated M2 macrophage phenotype and identified the myeloid MR as a regulator of macrophage polarization.[14] Macrophages are sentinel cells that can respond to microenvironmental cues and polarize to different phenotypes. A combination of transcription factors and signaling pathway modulators dictate distinct functional responses.[24] Classically activated M1 and alternatively activated M2 macrophages represent the extreme states of macrophage polarization, and this classification unmistakably belies the spectrum of macrophage activation.[24–26] Our transcriptome analysis revealed that MR inactivation drives macrophage differentiation in the ischemic microenvironment toward a phenotype unrelated to the M1/M2 paradigm with regulation of factors promoting wound healing and tissue repair.

Monocytes/macrophages are essential for myocardial infarct healing.[2,11,12,20] Depletion of monocytes/macrophages or defective macrophage recruitment impairs repair and promotes adverse remodeling post-MI.[1,20,27] However, emerging

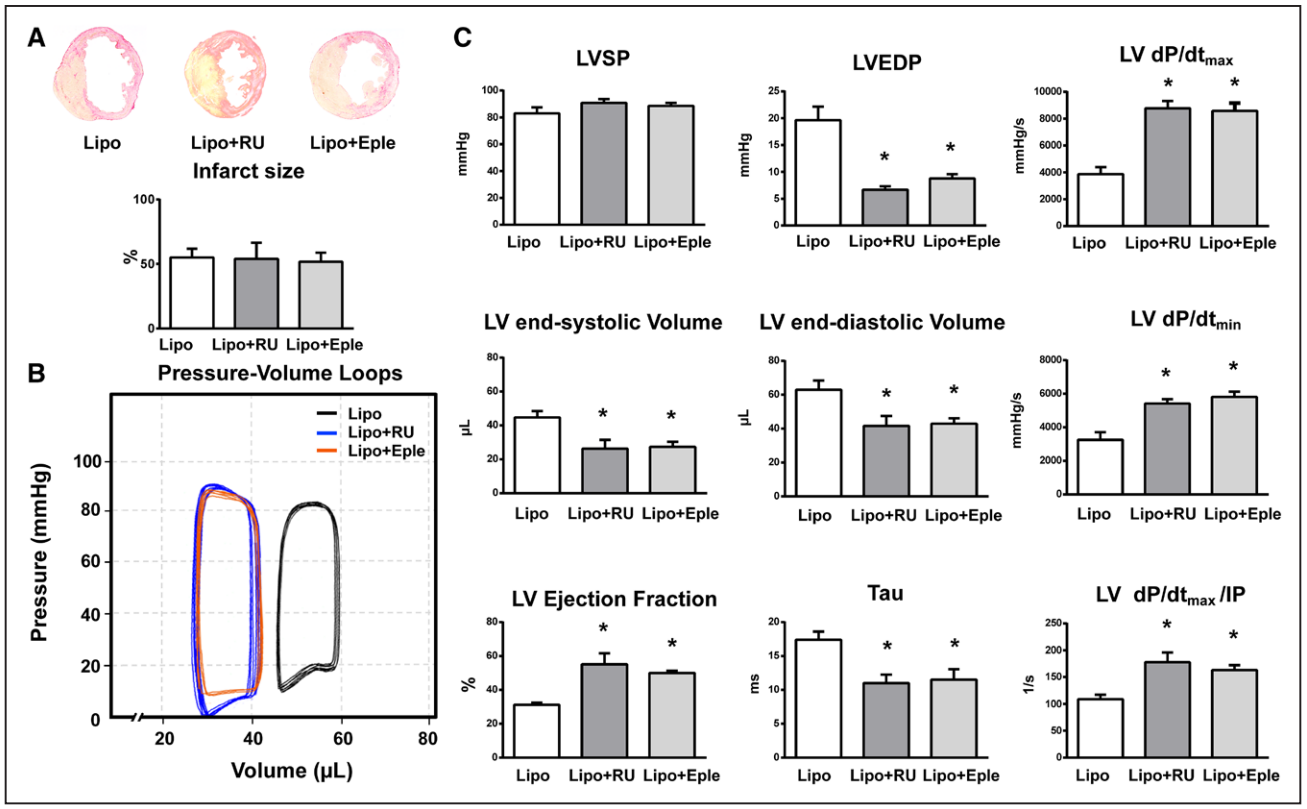

Figure 6. Targeted delivery of MR (mineralocorticoid receptor) antagonists to macrophages protects against cardiac dysfunction and remodeling after myocardial infarction. A single dose of liposomal RU28318 (Lipo+RU) or liposomal eplerenone (Lipo+Eple) or empty (MR antagonists lacking) liposomes (Lipo) were injected intraperitoneally into mice at the onset of myocardial infarction. **A**, Sections of infarcted hearts and infarct size. **B**, Representative left ventricular (LV) pressure-volume loops measured in vivo with conductance catheter, 7 d after myocardial infarction. **C**, LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), LV maximal rate of pressure rise (LV dP/dt_{max}), maximal rate of pressure decline (LV dP/dt_{min}), LV end-systolic and end-diastolic volumes, LV ejection fraction, LV dP/dt_{max} divided by instantaneous pressure (IP), and the time constant of LV pressure isovolumic decay (Tau). Mean±SEM (n=6). *P<0.05 vs Lipo.

evidence suggests that the number of infarct macrophages is not as important as the dynamic changes in macrophage differentiation state for wound healing and functional outcomes after ischemic injury.[2,12,20] Modulation of macrophage activation/function and consequently of their trophic factor secretion could be an important therapeutic target for prevention of progressive functional deterioration and heart failure.

In addition to inducing an optimal pattern of pro-wound healing mediators, macrophage MR deficiency also regulates the expression of receptors and molecules involved in the clearance of apoptotic cells. We report that critical components of the phagocytosis machinery were upregulated in infarct macrophages from $MR^{LysMCre}$ hearts and that MR deficiency in macrophages was associated with enhanced neutrophil efferocytosis in the infarct area. In line with this, MR loss in macrophages was shown to decrease the number of apoptotic cells in atherosclerotic lesions by affecting efferocytosis.[28,29] Moreover, Montes-Cobos et al[30] recently showed that phagocytic activity of bone marrow-derived macrophages and peritoneal macrophages is increased in the absence of the MR. It is important to note that efficient phagocytic clearance of apoptotic/necrotic cells is crucial for the resolution of inflammation and for favorable cardiac repair.[11,21] Increased expression of cell surface receptors (*Cx3cr1* and *S1pr1-2*) probably rendered macrophages more sensitive to soluble find-me signals, such as fractalkine and sphingosine-1-phosphate released by apoptotic cells. In addition, the augmented expression of specific receptors (*Mertk*, *Scarf1*, and stabilin) and bridging molecules (*Gas6* and C1q) likely promoted apoptotic cell clearance by an improved recognition of apoptotic cell–associated molecular pattern, like externalized phosphatidylserine. Also noteworthy is the upregulation of type I surface receptor stabilin-2, which triggers efferocytosis through a direct interaction with phosphatidylserine. Moreover, enhanced expression of the innate immune protein C1q may have promoted M2-like macrophage activation and clearance of apoptotic cells[31] triggering macrophages to produce factors that positively regulate inflammation resolution.

Oxidative stress caused by the excessive formation of reactive oxygen species by inflammatory cells impairs wound healing.[4] Aldosterone/MR activation has been shown to stimulate macrophage oxygen radical generation in mouse peritoneal macrophages from ApoE knockout mice.[32] We found reduced superoxide production in infarct macrophages lacking the MR, most likely because of an increased expression of catalase and peroxiredoxin 4, antioxidant enzymes involved in maintaining cellular redox balance.

Our in vitro studies suggest that the macrophage MR could also play an active role in scar remodeling by controlling the secretion of matrix-degrading MMPs and fibroblast activation state. After infarction injury, cardiac fibroblasts become activated upon sensing microenvironmental factors and critically regulate the wound healing response.[33] However, unrestrained myofibroblast differentiation can lead to fibrotic remodeling and impaired heart function. MR signaling in macrophages

regulates phenotypic changes of fibroblasts in the infarcted heart most probably through mechanisms involving oxidative stress and lipocalin-2. Indeed, MR knockout prevented the extensive upregulation of lipocalin-2 in infarct macrophages. This is of particular relevance considering that lipocalin-2—a downstream MR activation target—is a key mediator of aldosterone/MR profibrotic effects in cardiac fibroblasts and that lipocalin-2 inactivation improves cardiac function and remodeling after MI.[34]

In an exciting recent study, Fu et al[15] investigating the dynamics of fibroblast activation post-MI disclosed that myofibroblasts do not disappear but rather persist within the scar and lose α-SMA expression as the extracellular matrix and scar matures (7–10 days after MI). Therefore, the loss of the α-SMA–positive myofibroblast phenotype within the infarcted myocardium of $MR^{LysMCre}$ mice may be also indicative of a mature scar and an accelerated wound healing process. Interestingly, interventions impairing scar formation are associated with the persistence of α-SMA–expressing myofibroblasts within the infarct region and adverse cardiac remodeling.[15,35]

Despite strong clinical evidence that MR blockade improves outcome in patients with acute MI, MR antagonists are still underused.[5,6] Individual participant data analysis of the REMINDER and ALBATROSS (Aldosterone Lethal Effects Blocked in Acute MI Treated With or Without Reperfusion to Improve Outcome and Survival at Six Months Follow-Up) trials suggests a significant reduction of death or resuscitated sudden death by MR antagonists given early (within 72 hours) after low-risk ST-segment–elevation myocardial infarction.[5] Moreover, a recent meta-analysis of data from 10 randomized placebo-controlled trials (including patients with ST-segment–elevation myocardial infarction without evidence of heart failure or severe LV dysfunction) showed that the use of MR antagonists was associated with a 38% reduction in mortality and a significant increase in LV ejection fraction during follow-up.[36,37]

Decreased blood volume secondary to diuresis may have contributed to beneficial effects of MR antagonists in patients with acute MI. However, Rossignol et al[38] showed that the benefit of MR antagonism on cardiovascular outcomes was independent from the early diuretic and K-sparing effects. Extrarenal effects on myocardial structural and electrical remodeling, on sympathoadrenergic stimulation, platelet activation, and endothelial dysfunction, appear to be important mechanisms underlying the benefits of MR antagonists in the post-MI setting.[5–10,39] The present study identifies the macrophage MR as a pleiotropic modulator of myocardial infarct healing.

Our data also show that targeted delivery of MR antagonists to macrophages promotes wound healing, thus identifying a promising translational strategy for cardiac repair. Remarkably, a single administration of RU28318 or eplerenone-containing liposomes at the onset of MI was able to improve the post-ischemic angiogenic response and to protect against cardiac dysfunction and remodeling. Recent data indicate that myeloid MR activation contributes to progressive kidney disease and that macrophage deficiency of MR provides protection against declining renal function without affecting tubular regulation of salt balance.[40,41] Thus, targeting of MR antagonists to macrophages after acute MI could be a strategic approach to avoid the side effects on regulation of salt homeostasis, particularly in patients with compromised renal function.

Perspectives

We provide important new insights into the mechanisms underlying the clinical benefits of early MR antagonist administration in patients with ST-segment–elevation myocardial infarction and demonstrate that MR signaling in macrophages may be a novel therapeutic target to prevent cardiac dysfunction and failure after ischemic injury.

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Disclosures

None.

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Novelty and Significance

What Is New?

- MR (mineralocorticoid receptor) inactivation drives macrophage differentiation in the ischemic microenvironment toward a phenotype outside the M1/M2 paradigm, with regulation of multiple interrelated factors controlling inflammation resolution and tissue repair.
- Macrophage MR deficiency promotes wound healing through enhanced efferocytosis of neutrophils, the suppression of free radical formation, and the modulation of fibroblast activation state.
- Targeted delivery of MR antagonists to macrophages protects against cardiac dysfunction and remodeling after myocardial infarction.

What Is Relevant?

- The MR in macrophages is a pathogenic player and important therapeutic target in cardiac repair after myocardial infarction.

Summary

This study identifies the MR in macrophages as a pleiotropic modulator of myocardial infarct healing. Macrophage MR deficiency or targeted liposomal delivery of MR antagonists to macrophages improves cardiac repair and remodeling after ischemic injury.