In vivo silencing of the transcription factor IRF5 reprograms the macrophage phenotype and improves infarct healing

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Abstract

Objectives—The aim of the study was to test whether silencing of the transcription factor Interferon Regulatory Factor 5 (IRF5) in cardiac macrophages improves infarct healing and attenuates post-MI remodeling.

Background—In healing wounds, M1→M2 macrophage phenotype transition supports resolution of inflammation and tissue repair. Persistence of inflammatory M1 macrophages may derail healing and compromise organ functions. The transcription factor IRF5 promotes genes associated with M1 macrophages.
Methods—Here we used nanoparticle-delivered siRNA to silence the transcription factor IRF5 in macrophages residing in myocardial infarcts (MI) and in surgically induced skin wounds in mice.

Results—Infarct macrophages expressed high levels of IRF5 during the early inflammatory wound healing stages (day 4 after coronary ligation) whereas expression of the transcription factor decreased during the resolution of inflammation (day 8). Following in vitro screening, we identified an siRNA sequence that, when delivered by nanoparticles to wound macrophages, efficiently suppressed expression of IRF5 in vivo. Reduction of IRF5 expression, a factor that regulates macrophage polarization, reduced inflammatory M1 macrophage markers, supported resolution of inflammation, accelerated cutaneous and infarct healing and attenuated development of post-MI heart failure after coronary ligation as measured by protease targeted FMT-CT imaging and cardiac MRI (p<0.05 respectively).

Conclusion—This work identifies a new therapeutic avenue to augment resolution of inflammation in healing infarcts by macrophage phenotype manipulation. This therapeutic concept may be used to attenuate post-MI remodeling and heart failure.

Introduction

Wound healing follows a general program that comprises distinct stages (1). In the first few days after injury, inflammatory activity dominates the injured tissue. Inflammatory monocytes and classical M1 type macrophages rapidly invade the wound to defend against pathogens, phagocytose and lyse debris, and thus pave the way for tissue regeneration. Mononuclear phagocytes, the most abundant leukocytes in the wound, provide a rich source for proteases, other inflammatory enzymes, and cytokines. During subsequent healing, classical macrophages retreat and give way to M2 type macrophages which exhibit a less inflammatory panel of functions that supports tissue regeneration (2, 3). While inflammation resolves, M2 macrophages elaborate signals that direct endothelial cells, fibroblasts, parenchymal and local progenitor cells which rebuild damaged tissue. This archetypical program unfolds after many different types of injury, most visibly in skin wounds. A frequent, and too often deadly wound in contemporary humans results from ischemic injury to the heart (4). As in other wounds, a transition from M1 towards M2 macrophages predominance follows the initial phase of injury (5–7). The chronic inflammation associated with atherosclerosis (8, 9), the usual cause of myocardial infarction, may delay the resolution of inflammation in the ischemic myocardium. Continued dominance of M1 macrophages may impede tissue regeneration and can have devastating consequences such as infarct rupture, ventricular septal defect, aneurysm formation, acute mitral regurgitation, and heart failure. A delayed M1→M2 macrophage transition, for instance caused by prolonged recruitment of inflammatory monocytes into the cardiac wound (10), may interfere with the healing of the infarct predisposing to adverse ventricular remodeling and to the development of heart failure (4). Other comorbidities such as diabetes, obesity or rheumatoid arthritis may interfere with wound healing via similar mechanisms. These recent insights into monocyte and macrophage heterogeneity (2, 11) should now be translated into therapeutic approaches, as there is currently no clinical therapy to usher in resolution of inflammation and support wound healing in the heart or other tissues, for instance after trauma or surgery.
We chose to investigate Interferon Regulatory Factor 5 (IRF5) during wound healing because this transcription factor serves as a master regulator of macrophage polarization (12, 13). IRF5 translates danger signals, including toll like receptor ligands, into inflammatory gene expression, giving rise to M1 macrophages (12, 14). In humans, polymorphisms in the IRF5 gene have been associated with auto-immune disorders (15–17). IRF5 deficient mice are protected against lupus and display a significantly weakened type I interferon signature (18, 19). Using these data, we formulated and tested the hypothesis that in vivo RNAi silencing of IRF5 in macrophages reprograms macrophage polarization towards the M2 phenotype and thus changes the course of healing in two types of wounds (heart and skin). Small interfering RNA (siRNA) targeting IRF5 was delivered to wound macrophages after incorporation into lipidoid nanoparticles (LNP) (20, 21), which were injected intravenously. Silencing of IRF5 modulated macrophage functions and promoted resolution of inflammation. In mice treated with LNP-encapsulated siRNA, wound inflammation subsided more rapidly and skin wounds closed faster. Silencing IRF5 accelerated resolution of inflammation in the heart after coronary ligation in mice, improved infarct healing, and thus attenuated post-MI heart failure.

METHODS

A method section is provided in the online only appendix.

RESULTS

Expression pattern of IRF5 in infarct macrophages

IRF5 augments inflammatory gene expression in macrophages, and this transcription factor regulates macrophage polarization (13). Its expression in myocardial macrophages in infarcts, however, was previously unknown. Therefore, we first examined IRF5 expression in the heart following coronary ligation in mice. Macrophages isolated on day 4 after MI, i.e. at the peak of infarct inflammation, expressed IRF5 at high levels when compared to steady state resident macrophages retrieved from the heart of naive control animals (Fig. 1). The expression of IRF5 was significantly lower in neutrophils (macrophages 0.019 ± 0.002 versus neutrophils 0.010 ± 0.0007; P = 0.002). On day 8 post coronary ligation, the expression of IRF5 in macrophages fell drastically (Fig. 1b), in parallel with the transition from M1 to M2 macrophages previously observed in healing infarcts (6, 22). Macrophages in the remote myocardium also showed increased IRF5 on day 4 after MI, although to a lesser extent (Fig. 1b). These data, together with the notion that prolonged inflammatory monocyte and macrophage activity after ischemic injury impairs infarct healing and promotes heart failure (10), led to exploration of RNAi silencing of IRF5 in macrophages. We hypothesized that silencing IRF5 would support the M1→M2 macrophage phenotype switch, promote resolution of inflammation, improve healing and attenuate post-MI heart failure.

Towards this goal, we first explored if lipidoid nanoparticle carriers (LNP) (20) deliver fluorescently labeled siRNA to infarct macrophages. Alexa Fluor-647-labeled siRNA was loaded into 70nm nanoparticles and injected into mice 4 days after coronary ligation. Two hours after intravenous injection, ex-vivo fluorescence reflectance imaging (FRI) of short
axis slices produced from hearts revealed a strong fluorescent signal in the infarct area identified on TTC stained sections (Fig. 2a). Immunofluorescence staining for the myeloid cell marker CD11b colocalized with siRNA-reporting fluorescence in these infarcts (Fig. 2b). Flow cytometric analyses of cells dissociated from infarcts confirmed the highest siRNA uptake by F4/80\textsuperscript{high} macrophages and F4/80\textsuperscript{int} Ly-6C\textsuperscript{high} monocytes, whereas the uptake into lymphocytes and neutrophils was minimal (Fig. 2c).

**In vivo knock-down of IRF5 expression in monocytes and macrophages**

To silence IRF5, we first designed specific siRNA sequences against the murine IRF5 transcript. These contained 2′-methoxy (2′-OMe) modifications to mitigate non-specific immunostimulation and improve siRNA stability (23). In vitro screening of twenty-four siRNA candidate sequences designed in silico identified the most promising siRNA duplex (Fig. 3a). This siRNA (5′-cuGcAGAGAAuAAccuGAdTsdT-3′, 5′-UcAGGGuAUUCUCUGcAGdTsdT-3′), dubbed siIRF5, had the highest in vitro silencing efficiency and was thus selected for scale-up. Intravenous administration of a single dose of LNP-encapsulated siIRF5 (0.5mg/kg) resulted in efficient IRF5 gene silencing in splenic Ly-6C\textsuperscript{high} monocytes in steady state (Fig. 3b). Thus encouraged, we investigated IRF5 gene silencing efficiency in the infarcted heart. Treatment with siIRF5 decreased IRF5 expression in macrophages isolated from the infarcts by more than 70% at both mRNA and protein levels (Fig. 3c,d).

**Modulation of infarct inflammation with IRF5 gene silencing**

Next we investigated the impact of IRF5 silencing on infarct healing. To mimic infarct healing in patients with atherosclerosis, we ligated coronary arteries in ApoE\textsuperscript{-/-} mice on high fat diet. These atherosclerotic mice have impaired resolution of inflammation post coronary ligation, due to increased and prolonged recruitment of inflammatory monocytes to the heart (10). The delayed M1$\rightarrow$M2 macrophage phenotype transition leads to impaired infarct healing and increased incidence of post-MI heart failure (22).

Treatment with siIRF5 in atherosclerotic mice with MI resulted in efficient knock-down of IRF5 expression in macrophages (Fig. 4a,b), whereas no silencing occurred in neutrophils and lymphocytes (Supplemental Figure 1). Mice treated with siIRF5 showed a decreased inflammatory leukocyte content in their infarcts (Fig. 4c). This motivated us to study chemokines involved in recruitment of leukocytes after MI; however, we did not detect that RNAi changed their mRNA levels (Supplemental Figure 2). Likewise, apoptotic rates of macrophages were not different in siCON and siIRF5 treated cohorts (Supplemental Figure 3). As suggested by Yang et al. (19), the observed differences in leukocyte numbers may be caused by cell-intrinsic mechanisms.

To determine whether silencing IRF5 can change macrophage polarization, we evaluated the expression levels of M1 and M2-related genes by qRT-PCR analyses of cells isolated from infarct tissue. IRF5 silencing decreased the expression of proinflammatory M1 markers, including TNF\textsubscript{\alpha} and IL-1\textsubscript{\beta}, without reducing M2 gene expression (Fig. 4d).

Histological biomarkers of infarct healing were assessed on day 7 after MI. Analysis of tissue biomarkers of infarct healing on day 7 after MI revealed a reduction of neutrophils

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and macrophages in the infarct while neovascularization, the number of fibroblasts, and collagen deposition did not decline in mice treated with siIRF5 (Fig. 5). The net extracellular matrix production in healing infarcts is a product of collagen synthesis and degradation (24). We thus determined the impact of IRF5 silencing on matrix metalloproteinase (MMP) and their tissue inhibitors (TIMP) on the mRNA level. While expression of MMP2, MMP3, TIMP1, and TIMP2 was unchanged by IRF5 silencing, MMP9 was reduced significantly (Supplemental Figure 4). This resulted in a significantly reduced MMP9/TIMP1 ratio (25), reflecting lower matrix degradation.

**Functional impact of IRF5 silencing on the evolution of post-MI heart failure**

Investigation of the long-term impact of IRF5 gene silencing in macrophages on the evolution of heart failure used multimodal serial imaging in ApoE^{-/-} mice that received siIRF5 for five days after coronary ligation. Serial cardiac magnetic resonance imaging (MRI) on day 1 and day 21 after MI monitored left ventricular dilation. One day after coronary artery ligation, siIRF5 and siCON treated mice had similar infarct size measured by delayed enhancement MRI (Fig. 6a). Non-invasive assessment of infarct inflammation used fluorescence molecular tomography co-registered with X-ray computed tomography (FMT/CT). Activation of a protease sensor fell on day 4 after MI in mice treated with siIRF5 (Fig. 6b). The cohorts underwent follow-up imaging by cardiac MRI three weeks later. Mice treated with siIRF5 had diminished post-MI increase of the left ventricular volume between the first MRI on day 1 and day 21 follow-up imaging, reflecting reduced post-MI dilatation (Fig. 6a). Because initial infarct size was similar, and because mice were only injected with siRNA for day 1–5 after coronary ligation, this difference in left ventricular remodeling likely resulted from improved infarct healing in siIRF5 treated mice.

**Silencing of IRF5 accelerates skin wound healing**

We also evaluated the effect of RNAi-mediated IRF5 inhibition in excisional skin wounds. One day after creation of full-thickness skin wounds, C57BL/6 mice received AF647-labeled siRNA LNPs intravenously. Imaging of the wounds 2 hours later showed strong fluorescent signal, indicating enrichment of siRNA (Fig. 7a). Flow cytometric analysis confirmed cellular uptake of siRNA into monocytes and macrophages in the skin wound (Fig. 7b). Protease activity imaging identified day 4 after injury as the peak of enzyme activity (Fig. 7c), hence we chose this time point to assess therapeutic effects of IRF5 silencing. We then injected mice with full-thickness skin wounds (6 mm in diameter) for 4 consecutive days after wounding either with control- or IRF5 targeted siRNA encapsulated in LNP. Injection of siIRF5 reduced IRF5 gene expression in macrophages isolated from skin wounds (Fig. 8a). Wounds were imaged and the open wound area measured digitally every day. Wound closure was accelerated in mice treated with siIRF5 (Fig. 8b). Flow cytometric analyses of wounds showed that IRF5 gene silencing decreased monocyte and neutrophil content (Fig. 8c). In vivo fluorescence imaging showed significantly lower protease activity in wounds of siIRF5 treated mice (Fig. 8d). Consistent with the efficient IRF5 silencing, qRT-PCR analyses of wound macrophages revealed a significant decrease of pro-inflammatory gene expression, including lower mRNA levels of TNFα, IL-1β and IL-6 in mice treated with siIRF5 (Fig. 8e).
DISCUSSION

With the recent insight into the molecular mechanisms governing macrophage heterogeneity, polarization and function (26), it has become feasible to modulate macrophages’ actions in interventions that might optimize healing of injured tissues. One attractive option is to harness the endocytic machinery of macrophages to deliver drugs or siRNA. Progress in siRNA delivery, which relies increasingly on smart materials such as nanoparticles (27), spawned clinical trials using intravenous injection of nanoparticle-encapsulated siRNA (28, 29). The ease of delivering nanomaterials to phagocytic immune cells (30), and the central position of monocytes and macrophages in many key disease areas (4) including atherosclerosis and myocardial infarction, render inflammatory myeloid cells a prime target for in vivo RNAi (21, 31–33). Advantages of applying RNAi to target immune reactions include the selectivity for specific gene products (thereby avoiding unwanted side effects of broad immunosuppression) and the ability to reach intracellular decision nodes such as transcription factors.

In the present study, silencing the transcription factor IRF5 regulated a range of inflammatory genes, an approach that may have broader efficiency than silencing a single effector gene. IRF5 mediates inflammatory and immune responses by controlling expression of pro-inflammatory cytokines downstream of MyD88-dependent TLR signaling (12). Among many other inflammatory genes, IRF5 directly regulates the expression of TNFα and IL1β (13). As a result of siIRF5 treatment, we observed significantly lower expression of these proteins in the infarct. IL1β and TNFα may increase matrix metalloproteinase activity (34, 35), inline with the decreased MMP9 expression and lower protease imaging signal we detected in wounds of siIRF5 treated mice. IRF5 silencing thus changed the extracellular matrix turnover in the healing infarct: while the production of new matrix was not affected, the MMP9/TIMP1 ratio was reduced. Thus, the net effect of IRF5 silencing may favor matrix accumulation. TNFα also has pro-apoptotic (36) and negative inotropic (37) effects on cardiomyocytes. Genes promoted by IRF5 may therefore hinder healing in the infarct and favor post-MI heart failure.

Knock down of the transcription factor IRF5 changes macrophage polarization in human macrophages (13). Considerable contemporary data implicate the participation of macrophage subsets in the promotion of diseases and inflammation (M1) on the one hand, and in healing and resolution of inflammation (M2) on the other (3, 11). While this appealing concept has propelled the field, it also seems unlikely that modulating in vivo macrophage polarization works like ‘flipping a switch’. However, even if macrophage phenotypes represent a sliding scale between classical M1 and non-classical M2 functions, and macrophages from humans and mice may have different programs, it is clear that variations in macrophage character influence outcome in many disease settings (2).

We studied here how modulating the macrophage functional program impacts acute inflammation during the healing of injured tissues. Indeed, we found that in vivo silencing of IRF5, a transcription factor serving as a master regulator of macrophage polarization, reduced the typical genes expressed by M1 macrophages without affecting levels of those ascribed to M2 macrophages. The attenuation of M1 macrophage polarization, which
typically dominates in wounds shortly after injury, supported resolution of inflammation and accelerated tissue regeneration in skin wounds. The observed acceleration of skin wound closure in C57BL/6 mice suggests that silencing IRF5 may have broader implications in optimizing the repair of injured tissues. In addition, the skin wound healing assay, unlike coronary ligation, allows one to directly study the wound healing tempo, which was accelerated in siIRF5 treated mice.

Enforcing the natural transition of M1→M2 macrophages in wounds may thus usher in resolution of inflammation and speed healing, especially if acute wound inflammation exists in the setting of an underlying chronic inflammatory disease. A prolongation of the inflammatory phase of wound healing inhibits regenerative processes and may compromise tissue integrity. ApoE−/− mice with atherosclerosis and blood monocytosis (10), as well as infarct patients with leukocytosis (38), may have a higher risk of developing heart failure post-MI, possibly due to compromised infarct healing (4). Indiscriminate or blunt immunosuppression may be detrimental for wound healing, as many leukocyte actions are essential for an efficient repair program and rapid reconstitution of tissue integrity (22, 39, 40). Likely, any therapeutic intervention may have to be tailored in timing and in dose, or only target specific cellular functions that are detrimental while sparing others that are beneficial. An early and brief intervention after acute MI aimed to reprogram macrophage function and to improve infarct healing might be such an avenue to reduce post-MI remodeling and benefit long term prognosis.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**ABBREVIATIONS**

- FMT-CT: fluorescence molecular tomography - computed tomography
- FRI: fluorescence reflectance imaging
- IRF5: Interferon Regulatory Factor 5
- LNP: lipidoid nanoparticles
- M1: classical inflammatory macrophages
- M2: non-classical, alternatively activated macrophages
- MI: myocardial infarction
References


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Figure 1. Upregulation of IRF5 in heart macrophages after MI

(A) Representative flow cytometric dot plots show samples from day 4 after MI, stained with lineage cocktail and CD11b antibodies before and after MACS separation. (B) Unlabeled fractions were processed for western blot analysis of IRF5 in isolated cells from naive hearts, infarct tissue and remote myocardium. Liver mouse tissue was used as a positive control.
Figure 2. Infarct uptake of LNP-siRNA nanoparticles

(A) TTC staining and ex-vivo fluorescence reflectance imaging (FRI) of 4 day-old infarcts, 2 hours following i.v. injection of LNP-encapsulated AF647-labelled siRNA. (B) Immunofluorescence microscopy of infarcts showing colocalization of CD11b expressing cells (green) and fluorescently labeled siRNA (red). Scale bar indicates 50 μm. (C) FACS of siRNA uptake by lymphocytes (i), neutrophils (ii), macrophages (iv) and Ly-6C<sup>high</sup> monocytes (v) isolated from infarcts.
Figure 3. LNP-IRF5 siRNA treatment results in efficient silencing in macrophages

(A) IRF5 siRNA candidates were ordered by their silencing efficiency at 10 nM concentration. The red frame highlights the siRNA selected for subsequent in vivo experiments. (B) Mice were injected i.v. with either the LNP-IRF5 siRNA (siIRF) or control siRNA (siCON). Twenty-four hours later, splenic Ly-6C<sup>high</sup> monocytes were isolated by FACS to quantify IRF5 mRNA (n=7 per group). (C) IRF5 mRNA and (D) IRF5 protein levels in infarct macrophages on day 4 after MI. *P<0.05, **P<0.01, ***P<0.001.
Figure 4. IRF5 silencing modulates macrophage polarization in apoE−/− mice with MI

(A) FACS plots of infarct tissue and analysis of IRF5 protein in macrophages and Ly-6C^{high} monocytes. Histogram plots display control siRNA (red), IRF5 siRNA (blue) and isotype control (grey). Bar graph shows mean fluorescence intensity (MFI) for IRF5 (n=8 per group). (B) IRF5 gene expression in infarct macrophages (n = 6–8 per group). (C) Neutrophils (ii), macrophages (iv) and Ly-6C^{high} monocytes (v) in infarcts on day 4 after MI. (D) M1 and M2 genes in macrophages isolated from 4 day old infarcts (n = 8–11 per group). *P<0.05, **P<0.01.
Figure 5. Histological assessment of infarct healing on day 7 after MI in ApoE\(^{-/-}\) mice treated with siCON or siIRF5

Immunohistochemistry staining for myeloid cells (NIMP-R14; CD11b), macrophages (MAC-3), neo-vascularization (CD31), collagen deposition (collagen I) and myofibroblasts (\(\alpha\)-SMA). Bar graphs display region of interest (ROI, n=5–7 per group). *P<0.05, **P<0.01, ***P<0.001.
Figure 6. siIRF5 improves infarct healing in ApoE−/− mice

(A) Serial cardiac MRI (day 1 and day 21) in ApoE−/− mice treated with siIRF5 or siCON. The dotted line highlights the infarct. Insets show the infarct-induced thinning of the left ventricular wall in apical short axis slices. (B) FMT-CT on day 4 after MI. Circles indicate infarct signal (n=5–9 per group). *P<0.05.
Figure 7. LNP-siRNA delivery to monocytes/macrophages in skin wounds
(A) On day 4 after wound induction, mice were injected i.v. with AF647 labeled siRNA encapsulated in lipid-nanoparticles. Fluorescence reflectance imaging (FRI) revealed a strong siRNA signal in the wound one hour after injection. (B) FACS analysis of siRNA uptake by both F4/80\textsuperscript{high} macrophages and Ly-6C\textsuperscript{high} monocytes in the wound. (C) Quantitation of protease activity in skin wounds by FRI revealed the highest signal peaking on day 4 post-injury (n=6 per time point). *P <0.01.
Figure 8. Treatment with siIRF5 accelerates skin wound healing
(A) IRF5 silencing in monocytes/macrophages isolated from the wound (n=9 per group). (B) Wound area (n = 7–8 per group). (C) FACS of wound leukocyte content on day 4 (n=5 per group). (D) Protease activity by FMT and FRI. TBR, target to background ratio (n=8 per group). (e) M1 and M2 genes in wound macrophages on day 4 (n=10–12 per group).

*P<0.05, **P<0.01, ***P<0.001.