Critical Role for Leukocyte Hypoxia Inducible Factor-1α Expression in Post–Myocardial Infarction Left Ventricular Remodeling

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Rationale: Hypoxia inducible factor (HIF)-1α is a transcription factor stabilized by hypoxia. It regulates cytokines involved in the inflammatory response after ischemia and affects white blood cell (WBCs) function. The effect of HIF-1α on WBC function and inflammation following myocardial infarction (MI) is unknown.

Objective: We assessed peritoneal and myocardial inflammation in the setting of low WBC HIF-1α expression through bone marrow transplantation of hematopoietic stem cells transfected with scramble or HIF-1α small interfering (si)RNA.

Methods and Results: Rosa hematopoietic stem cells (lin−, cKit+) were transfected with a green fluorescent protein (GFP) reporter lentivirus encoding a siRNA to HIF-1α or scramble. Irradiated 6- to 8-week-old C57/BL6J mice received 50 000 GFP+ HIF-1α or scramble siRNA–transfected hematopoietic stem cells. Peritonitis or myocardial infarction via left anterior descending coronary artery ligation was induced 6 weeks after bone marrow transplantation. In the peritonitis model, HIF-1α siRNA group exhibited a significant decrease in neutrophil and monocyte entry to the peritoneum compared to scramble mice. Similarly neutrophil infiltration into the infarct zone was decreased in the HIF-1α siRNA group. No difference of myocardial infarct size was observed between groups. Interestingly, the ejection fraction were similar in both groups at baseline and 3 days post-MI but increased significantly in the HIF-1α siRNA group compared to control beginning 7 days after MI. Gene array studies demonstrated that downregulation of WBC HIF-1α was associated with decreased WBC CCR1, -2, and -4 expression. Chemotaxis assay results confirmed that decreased monocyte migration induced by downregulation of HIF-1α was partially reversed by overexpression of CCR2.

Conclusions: Downregulation of leukocyte HIF-1α expression resulted in decreased recruitment of WBC to the sites of inflammation and improvement in cardiac function following MI. Downregulation of HIF-1α suppressed WBC cytokine receptors CCR1, -2, and -4, which are necessary for WBC mobilization and recruitment to inflammatory cytokines following MI. The effects of downregulation of leukocyte HIF-1α on WBC migration are attributable, at least in part, to the decreased CCR2 expression. These results demonstrate that WBC infiltration into the newly injured myocardium plays a significant role in left ventricular remodeling, but not infarct size. (Circ Res. 2010;106:601-610.)

Key Words: leukocyte ■ hypoxia inducible factor-1α ■ myocardial infarction ■ left ventricular remodeling ■ inflammation

Left ventricular (LV) remodeling post myocardial infarction (MI) determines myocardial performance and residual LV function. The remodeling process can cause myocardial rupture, lead to LV dilation and ultimately cause decreased functional reserve and congestive heart failure.1 We have previously suggested that inflammation plays a critical role in post-MI LV remodeling2 but not infarct size. Increased WBC count is linked to increased LV size in patients with MI3,4 and inflammation most likely associates with unfavorable outcomes by increasing the infarct size or altering ventricular remodeling.5 Direct inhibition of WBC infiltration through the administration of an antibody to CD11b failed to reduce infarct size; however, inhibition of WBC infiltration remains a potential target to optimizing cardiac remodeling post-MI.

Chemokines play an important role in the myocardial healing after MI.6,7 Earlier data demonstrated that blocking CC chemokines is helpful in the prevention of post ischemic
organ dysfunction.8 However, clinical trials of WBC adhesion blockade did not succeed in MI.9,10 Understanding the genes involved in the regulation of inflammation and remodeling processes after MI is key to effective therapeutic targets that slow or stop the progression of patients to heart failure after MI.

Sites of inflammation are characterized by high levels of lactate and reductive metabolites and low levels of oxygen and glucose. For this reason, the cells involved in the inflammation should be able to sense and respond to changes in oxygen tension.11 Hypoxia inducible factor (HIF)-1 plays an important role in response to changes in available oxygen, specifically, to hypoxia in the cellular environment. The HIF-1 heterodimer consists of 2 helix–loop–helix proteins, HIF-1α and HIF-1β. HIF-1α is the oxygen-responsive component and can only be detected under hypoxia. Under normoxic conditions, HIF-1α is rapidly degraded by the ubiquitin–proteasome pathway.12,13 Recent studies have demonstrated that HIF-1α is involved in the induction of several cytokines involved in inflammation and important for the function of the WBCs. It was reported that loss of HIF-1α in myeloid cells can decrease the macrophage migration by 50% and 75% at normoxic and hypoxic conditions, respectively.12 HIF-1α is also involved in enhancing production of enzymes responsible for glycolysis.11,12,14,15 HIF-1α is important not only for hypoxic adaptation but also for physiological function in many cell types.15

Several studies have demonstrated the beneficial effects of upregulation of HIF-1α in myocardial tissue for ischemic preconditioning16,17 and in determining the vascular response and infract size following MI.18 These effects may be attributable to the ability of HIF-1α to turn on vascular endothelial growth factor and then promote angiogenesis post-MI. Because HIF-1α can also activate stromal cell–derived factor-1 and CXC chemokine receptor 4 pathway, increased recruitment of endothelial progenitor cells to the infarcted heart may also play an important role.19

Although emerging evidence shows that HIF-1α is important for the function of the WBC, the effects of modulating WBC HIF-1α on the inflammatory response following MI are unknown. We hypothesized that downregulation of HIF-1α in WBC may improve cardiac function post-MI by inhibition of inflammation. HIF-1α whole-animal knockouts are embryonic lethal because of abnormal vascular development.11,15 Therefore, to investigate the effects of HIF-1α, HIF-1α knockdown in a highly specific organ or tissue of interest becomes necessary. We developed a novel strategy for downregulating WBC protein expression through the transplantation of hematopoietic stem cells (HSCs) engineered with small interfering (si)RNA to targets of interest. The major reason for using HSCs is that they have the ability of self-renewing and no need for repeated administrations of the gene transfer. HSCs are stem cells that give rise to all the blood cell types including WBCs. Knocking out HIF-1α in HSCs downregulates HIF-1α in WBCs. In addition, HSCs can be easily manipulated in the laboratory and then returned to recipients by bone marrow or intravenous injection. These studies examine the effects of decreasing HIF-1α in HSCs on inflammation and cardiac function following MI, as well as begin to determine the relevant mechanisms responsible.

### Methods

#### Experimental Animals

All animal protocols described in this study were approved by the Animal Research Committee. C57BL/6j and Rosa male mice were obtained from the Jackson Laboratory (Bar Harbor, Me) at 4 to 6 weeks of age and were housed with free access to food and water in the American Association for Accreditation of Laboratory Animal Care–approved animal facility of the Cleveland Clinic Foundation. One hundred C57BL/6j mice were randomly divided into 2 experimental groups: group 1: HIF-1α siRNA group (mice in this group received 50 000 HIF-1α siRNA transfected HSCs); group 2: HIF-1α SCR group (mice in this group received 50 000 scrambled (SCR) HIF-1α siRNA transfected HSCs). Mice from each group underwent bone marrow transplantation to insert the respective siRNA into its genome. After the animals reconstitute their bone marrow, as evidenced by complete blood count, they underwent either peritonitis induction or left anterior descending coronary artery (LAD) ligation to induce MI. Mice in HIF-1α siRNA SCR group were used as control for all the experiments.

#### Bone Marrow Harvesting and HSC Isolation

Four- to 6-week-old Rosa mice were euthanized and the hind limbs were removed. The femurs and tibias were cut from both ends and were flushed into a 50-mL falcon with flush medium (Alpha Medium with 2 g/L NaHCO3, 10% horse serum, 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin). After collection of bone marrow from femur and tibia, red blood cells were lysed by red blood cell lysis buffer (155 mmol/L NH4Cl, 12 mmol/L NaHCO3, 0.1 mmol/L EDTA). The bone marrow cells underwent magnetic cell sorting to isolate lineage negative and cKit positive HSCs using mouse hematopoietic progenitor cell enrichment kit and mouse CD117-positive selection kit (Stem Cell technologies Inc).

#### Vector Constructs

The green fluorescent protein (GFP) reporting lentivirus encoding the siRNA to HIF-1α and SCR were custom-constructed into the
GTGGCTCATAA-3. Gapdh SYBR Green primers were purchased from Invitrogen, Carlsbad, Calif.

The deletion efficiency was determined by measuring the HIF-1 α levels using Western blot analysis in bone marrow transplantation studies. HSCs (Lin−, cKit+) were transfected with a GFP reporting lentivirus encoding a siRNA to HIF-1α. Cells expressing GFP were sorted by fluorescence-activated cell sorting (FACS). Six- to 8-week-old C57/BL6J mice were pretreated with acidified water (pH 2 to pH 3) and neomycin 1.1 g/L of water and Polymixin 1 million unit/L of water for 7 to 10 days. After pretreatment, the mice underwent irradiation at a total dose of 850 rads divided into 2 equal doses of 425 rads separated by 4 hours. Then the irradiated C57/BL6J mice received 50 000 HIF-1 α or SCR siRNA transfected HSCs by intrafemoral injection. After 4 to 6 weeks, the transplanted mice were assessed for hematologic reconstitution by complete blood count.

Echocardiography

Six weeks after bone marrow transplantation, anterior wall MI was induced in those mice as described.1,2 Briefly, animals were endotracheal intubated and ventilated with room air at 100 breaths per minute using a rodent ventilator (Harvard Apparatus). Animals were endotracheal intubated and ventilated with room air at physiological pressures 3 days after LAD ligation. Fixed hearts were perfusion fixed with HistoChoice at physiological pressures. Fixed hearts were embedded in paraffin and serially cut at 4 μm from the apex to the level just below the coronary artery ligation site. Alternating sections were stained with Masson trichrome. The infarcted area was measured by planimetry using computer-assisted image analysis software Image-Pro Plus (Media Cybernetics). Parameters were calculated using the equations: % infarct size=epicardial infarct length/epicardial LV circumferences×100.

Myocardial Infarct Size Measurement

Neutrophil Infiltration in the Heart Following MI

WBCs from each group were collected and cultured in 1% oxygen for 6 hour. Cells were sonicated in a lysis buffer containing 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitor mixture. Equal amounts of protein lysates were separated on 10% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad) and transferred to nitrocellulose membranes. The membranes were blocked in 5% nonfat milk in Tris-buffered saline-T buffer and then incubated with anti-HIF-1α (1:500, Bethyl Laboratories Inc, Montgomery, Tex) at 4°C overnight. After incubation with primary antibody, blots were incubated with anti-rabbit IgG horseradish peroxidase–linked antibody at a dilution of 1:5000 for 100 minutes at room temperature. Immunoreactive bands were detected using the Super Signal West Dura Extended Duration Substrate (Pierce). The intensity of bands was measured with a scanning densitometer (Model GS-800; Bio-Rad) coupled with Bio-Rad PC analysis software. α-Tubulin was used as an internal loading control.

LAD Ligation

Six weeks after bone marrow transplantation, anterior wall MI was induced in those mice as described (n=20 in each group).1,2 Briefly, animals were endotracheal intubated and ventilated with room air at 100 breaths per minute using a rodent ventilator (Harvard Apparatus). Sternotomy was performed and the proximal LAD was identified using a surgical microscope (Leica M500) after retraction of the left atrium and ligated with 7-0 Prolene. Blanching and dysfunction of the anterior wall verified LAD ligation. LAD ligation was performed by a surgeon blinded to the identity of the mice. After LAD ligation, the animals were evaluated with echocardiography or euthanized at different time points (3 days, 7 days, and 4 weeks) for organ harvest and staining.

Echocardiography

Two-dimensional echocardiography was performed using a 15-MHz linear array transducer interfaced with a Sequoia C256 (Acuson) as previously described.2 Briefly, 3 days before LAD ligation as well as 3, 7, and 28 days after LAD ligation, LV dimensions were quantified by digitally recorded 2D clips and M-mode images in a short-axis view from the mid-LV just below the papillary muscles to allow for consistent measurements from the same anatomic location in different mice. Measurements were made by 2 independent blinded researchers off-line using ProSolv echocardiography software. Each measurement in each animal was made from 3 randomly chosen M-mode clips out of 5 recorded by observers blinded to the strain of the mice. Dimensions were measured between the anterior wall and posterior wall from the short-axis view just below the level of the papillary muscle. The LV mass (LVM) was calculated with the following formula: LV mass index (LVMi) (mg/g) = 1.055 × [(LVSD + LVPWd + LVIDD) − (LVEDD)2] / (BW (body weight, g), where 1.055 is the density of mouse myocardium; LVPWd, diastolic thickness of the LV posterior wall; LVIDD, diastolic LV internal dimension.

Neutrophil infiltration was assessed in the heart 3 days after LAD ligation. Mice were anesthetized and perfusion fixed with 10% phosphate-buffered formalin at physiological pressures. Fixed hearts were embedded in paraffin and serially cut at 4 μm from the apex. Infiltrating neutrophils were observed and counted in the area surrounding the infarct zone. WBC chemokine receptor expression between groups was assessed using computer-assisted image analysis software Image-Pro Plus (Media Cybernetics).

Peritonitis Induction and WBC Mobilization and Migration Measurement

The animals were given an intraperitoneal injection of either 1 mL of 4% thioglycollate broth. Six hours or 3 days after the injection, the animals were euthanized and the peritoneum was washed by 10 mL of 4% thioglycollate broth. Six hours or 3 days after the injection, the animals were euthanized and the peritoneum was washed by 10 mL of sterile PBS. The wash fluid was aspirated and centrifuged at 600g for 5 minutes and then resuspended in 1 mL of PBS. The numbers of WBCs in the 1 mL of PBS were assessed by the HemaVet Instrument and macrophage counts were made by viewing 100 cells on a Wright’s-stained smear. To determine whether HIF-1α is critical for egress of monocytes and neutrophils or their precursors from the bone marrow, we analyzed leukocyte populations in peripheral blood with the HemaVet Instrument in the peritonitis model.

Real-Time PCR Analysis of CC Chemokine Receptor Expression on WBCs

Gene arrays and real-time PCR were performed to assess differences in WBC chemokine receptor expression between groups. After peritonitis induction, total RNA was extracted from isolated WBCs using a RNA isolation kit (SuperArray, Frederick, Md) according to the instructions of the manufacturer, and any contaminating DNA was degraded by a 15-minute incubation with RNase-free DNase. Cytokines gene array was performed to assess the modulation of cytokines genes in both groups using a Mouse Inflammatory Cytokines & Receptors PCR Array Kit (SuperArray). CCR1, CCR2,
CCR4, and Gapdh SYBR Green primers were purchased from SuperArray Bioscience for real-time PCR.

Real-Time PCR Analysis of Monocyte Chemotactrant Protein-1 and -3 Expression in the Infarcted Heart

Real-time PCRs were performed to assess differences in monocyte chemotactrant protein (MCP)-1 and MCP-3 expression in the infarcted mice hearts from HIF-1α siRNA group and HIF-1α SCR group. Total RNA was extracted from heart tissue using a RNA isolation kit (SuperArray), and any contaminating DNA was degraded by 15-minute incubation with RNase-free DNase. MCP-1, MCP-3, and Gapdh SYBR Green primers used for real-time PCR were purchased from SuperArray Bioscience.

Isolation of Peritoneal Neutrophils

Peritoneal neutrophils were isolated as previously described. Briefly, peritoneal fluid was collected 6 hours after thioglycollate injection then mixed 1:1 with PBS and layered over Histopaque (Sigma) in 15-mL centrifuge tubes. The tubes were centrifuged at 400g for 30 minutes at room temperature, and the bottom pellet containing the neutrophils and red blood cells were dissolved in lysis buffer to lyse any contaminating erythrocytes. Isolated neutrophils were used for chemotaxis assays.

Isolation of Monocytes and Overexpression of CCR2 in Monocytes

Mice were given an intraperitoneal injection of 1 mL of 4% thioglycolate broth. Three days after the injection, blood were drawn and mixed 1:1 with PBS and layered over Histopaque (Sigma) in 15-mL centrifuge tubes. The tubes were centrifuged at 600g for 30 minutes at room temperature, and the layer containing the monocytes were washed twice with PBS. Then the cell pellet was resuspended in RPMI 1640 (10% FCS, 1% penicillin-streptomycin, and 1% glutamine) and were incubated at 37°C and allowed to adhere for 1 hour. Nonadherent cells were washed twice with PBS, and new culture medium were added. More than 95% of these cells were monocytes, as evidenced by a Wright’s-stained smear. Isolated monocytes from the HIF-1α siRNA group were transfected with the RFP reporting lentivirus encoding CCR2. Cells expressing RFP were sorted by FACS.

Assessment of Expression of CCR2 on Monocytes by Flow Cytometry and Western Blot Analysis

Isolated monocytes with or without transfection were prepared for flow cytometry and Western blot analysis. For flow cytometry, cells washed with PBS and fixed with 4% PFA. Then cells were incubated with antibodies against the mouse CCR2 (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) for 45 minutes. Cells were washed and stained in the dark for 60 minutes with FITC-labeled donkey anti-rabbit IgG (Santa Cruz Biotechnology Inc). Flow cytometry were performed on a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software. The expression of CCR2 on monocytes was compared among the groups. Isolated monocytes for Western blot analysis were sonicated in a lysis buffer and equal amounts of protein lysates were separated on 10% SDS-polyacrylamide gels. The intensity of bands was measured with the same method described earlier. α-Tubulin was used as an internal loading control.

Chemotaxis Assay

Chemotaxis assay of monocytes and neutrophils was performed using a QCM cell migration assay kit (Chemicon International) following the recommended protocol of the manufacturer. Briefly, cells (5×10^4 in 100 µL) were loaded onto migration chamber plate (5- or 3-µm pore size; Millipore) along with serum-free medium, whereas 150 µL serum-free medium containing 20 ng/mL MCP-1 were placed in the lower wells. The cells were allowed to migrate at 37°C in a CO2 incubator (5% CO2) for 4 hours. Then the nonmigrating cells remaining on the upper surface of the insert were removed. Cells migrated to the lower chamber and attached to the lower surface of the insert were collected and incubated with Lysis Buffer/Dye Solution (CyQuant GR Dye) for 15 minutes at room temperature. Then the plates were read with a fluorescence plate reader using 480/-520-nm filter set, and the migratory cell number was determined using a fluorescent cell dose standard curve.

Statistical Analysis

Data were presented as means±SEM. Data were analyzed by repeated measures 2-way ANOVA or Student t test (2-tailed) where appropriate. A Dunnett’s test was used for post hoc analysis. All differences were considered statistically significant at P<0.05 level.

Effect of Downregulation of Leukocyte HIF-1α on WBC Migration and Mobilization in the Peritonitis Model

To determine whether HIF-1α is critical for recruitment of monocytes and neutrophils from the bone marrow to sites of inflammation, we analyzed leukocyte populations in peripheral blood and peritoneal fluid following the induction of peritonitis 6 hours and 3 days after the thioglycollate broth injection. WBC number in both peritoneal fluid and blood was assessed by the HemaVet Instrument and a Wright’s-stained smear. HIF-1α siRNA mice (mice in this group received 50 000 HIF-1α siRNA transfected HSCs) showed a significant decrease in mobilization (neutrophils and monocytes) compared to mice in HIF-1α siRNA SCR group (n=10 to 13 each group). Figure 2A and 2B). We also found that HIF-1α siRNA mice had a significant decrease in WBC migration (neutrophils and macrophages) to the peritoneum compared to HIF-1α SCR mice (Figure 2C and 2D). Our data indicate that downregulation of leukocyte HIF-1α expression resulted in decreased recruitment of WBCs to the sites of inflammation.

Effect of Downregulation of Leukocyte HIF-1α on Neutrophil Infiltration in the Heart Following MI

In the MI model, infiltrating neutrophils were stained with Naphthol AS-D Chloroacetate esterase (Sigma) 3 days post-MI. We found a 53% decrease of HIF-1α siRNA mice compared to the MI control group (n=4 to 6 each group) (Figure 3).
systolic and diastolic LV internal dimensions (LVISD and LVIDD), interventricular septal thickness in systole and diastole, LVMI, and ejection fraction were similar in both groups at baseline and 3 days post-MI. However, at 7 and 28 days, ejection fraction became significantly higher and LVIDD and LVISD became significantly lower in the HIF-1α/H9251 siRNA group compared with HIF-1α/H9251 SCR group. Interestingly, at 7 days post-MI, interventricular septal thickness in systole, interventricular septal thickness in diastole, and LVMI became significantly larger in the HIF-1α siRNA group. However, at 28 days post-MI, LVMI became significantly smaller in the HIF-1α siRNA group compared with HIF-1α SCR group (n=9 each group, Figure 4). No difference in the diastolic and systolic thicknesses of the LV posterior wall were found between HIF-1α SCR group and HIF-1α siRNA group (data not show). These data indicate that downregulation

Figure 1. Deletion efficiency of HIF-1α in WBCs. A, Representative gel blots of HIF-1α and α-tubulin. B and C, The mRNA and protein expression of HIF-1α in WBCs isolated from HIF-1α siRNA mice showed a 74% and 67% decrease. The appropriate bands were quantitated and normalized to α-tubulin. Data are means±SEM (n=4 to 5 each group). *P<0.05 vs HIF-1α SCR group.

Figure 2. Effect of downregulation of leukocyte HIF-1α on WBC migration and mobilization in the peritonitis model. A, Differential cell count of peripheral blood 6 hours after the thioglycollate injection. B, Differential cell count of peripheral blood 72 hours after the thioglycollate injection. C, Differential cell count of peritoneal fluid 6 hours after the thioglycollate injection. D, Differential cell count of peritoneal fluid 72 hours after the thioglycollate injection. Data are means±SEM (n=10 to 13 each group). *P<0.05 vs corresponding HIF-1α SCR group.
of leukocyte HIF-1α expression resulted in improvement in LV remodeling and cardiac function following MI.

Effect of Downregulation of Leukocyte HIF-1α on Myocardial Infarct Size
To determine whether downregulation of HIF-1α has an effect on infarct size, some animals were euthanized 3 and 28 days after LAD ligation. Paraffin-embedded heart sections were stained with Masson’s trichrome. No difference of myocardial infarct size (as percentage of left ventricle) was found between HIF-1α SCR group and HIF-1α siRNA group. (27.8±8.1 versus 26.4±8.1 at 3 days and 35.5±12.1 versus 32.9±9.6 at 28 days, respectively; n=4 to 5 each group; Figure 5).

Effect of Downregulation of Leukocyte HIF-1α on WBC Cytokine Receptors CCR1, -2, and -4 Expression
In the peritonitis model, gene arrays and real-time PCR were performed to assess differences in WBC receptor expression between groups. Gene array studies comparing circulating leukocytes following intraperitoneal thioglycolate injection demonstrated decreased WBC cytokine receptors CCR1, -2, and -4 expression in HIF-1α siRNA group. The mRNA expression of CCR1, -2, and -4 decreased 97%, 95%, and 91%, respectively. Our data indicate that downregulation of HIF-1α results in suppression of the cytokine receptors CCR1, -2, and -4 expression in WBCs (n=4, Figure 6A).

Effect of Downregulation of Leukocyte HIF-1α on MCP-1 and MCP-3 Expression in the Infarcted Heart
Real-time PCR was performed to assess differences in MCP-1 and MCP-3 expression in hearts of mice from HIF-1α siRNA group and HIF-1α SCR group. No difference of MCP-1 and MCP-3 expression was found between the 2 groups (n=4, Figure 6B).

Overexpression of CCR2 in Monocytes
Flow cytometry and Western blot was used to characterize the expression of CCR2 on monocytes with or without lenti-CCR2 transfection. Monocytes from HIF-1α siRNA group showed significant decrease (43%) in log fluorescence intensity and 64% decrease in protein expression compared with monocytes from HIF-1α SCR group. After being transfected with lentivirus encoding CCR2, monocytes from the HIF-1α siRNA mice showed a 2-fold increase in log fluorescence intensity and 1.6 fold increase in protein expression (Figure 7A through 7D).

Chemotaxis Assay
To determine the relative importance of CCR2 downregulation on HIF-1α siRNA inhibition of WBC migration, we overexpressed of CCR2 in HIF-1α siRNA leukocytes. In response to MCP-1, the migration of HIF-1α–null monocytes and neutrophils decreased significantly (53% and 40%, respectively) compared with monocytes isolated from HIF-1α SCR mice. Suggesting a limited role for the downregulation of CCR2 by HIF-1α siRNA, the migration of HIF-1α–null monocytes showed a small (20%) but statistically significant
Discussion

The major findings of our study are that downregulation of leukocyte HIF-1α expression results in decreased recruitment of WBCs to sites of inflammation and improvement in LV remodeling and cardiac function following MI. The improvement in cardiac parameters occurred despite no difference in myocardial infarct size. Results from our study provide new evidence for the beneficial effects of inhibition of WBC infiltration through modulation of HIF-1α on post-MI LV remodeling. Importantly, we observed decreased WBC infiltration and improved cardiac function without any evidence for increased rate of myocardial rupture.

Our data demonstrate that downregulation of leukocyte HIF-1α decreases WBC recruitment to the sites of inflammation. In the peritonitis model, we observed that mice with WBC engineered with HIF-1α siRNA had a significant decrease in WBC (monocytes and neutrophils) mobilization and WBC (macrophage and neutrophils) migration to the peritoneum compared to HIF-1α SCR mice. In the chemotaxis assay, downregulation of leukocyte HIF-1α decreased macrophage and neutrophil migration by 50% and 40% at normoxic conditions, respectively. In the MI model, we observed decreased neutrophil infiltration in the area surrounding the infarct zone in hearts from HIF-1α siRNA group. Although loss of HIF-1α in myeloid cells was found to decrease macrophage migration at both normoxic and hypoxic conditions.12 Peyssonnaux et al21 reported that HIF-1α–null neutrophils can migrate to sites of microbial infection and generate reactive oxygen species in vivo normally. Our results provided new evidence that downregulation of leukocyte HIF-1α can decrease both macrophage and neutrophil recruitment to the sites of inflammation. The difference between our results and the previously published results could be attributable to different methods to generate HIF-1α–null animal models and attributable to different stimulus to neutrophil migration. We tested neutrophil migration with a MI model and a thioglycollate-induced peritonitis model, whereas Peyssonnaux et al measured the neutrophil migration induced by bacterial infection. Bacterial infection would lead to endotoxin-induced neutrophil activation, which may be more robust or a HIF-1α–independent pathway compared to ischemia-induced activation. Consistent with this hypothesis, Peyssonnaux et al observed decreased neutrophil infiltration in of HIF-1α deficient neutrophils with a chemical irritation model of skin injury.
Our laboratory has previously demonstrated that inflammation is more important in leukocyte trafficking and is upregulated during myocardial infarction. We chose to focus on CCR2 because CCR2 is the most abundant cytokine receptor on the surface of macrophages/monocytes and has an important role in mediating monocyte/macrophage migration. Therefore, we isolated monocytes from animals transplanted with HIF-1α siRNA HSCs and transfected those cells with lentivirus encoding CCR2. Both our flow and Western results confirm the overexpression of CCR2 on these HIF-1α–null monocytes. The results from our in vitro chemotaxis assay demonstrate that the decreased WBC migration induced by downregulation of HIF-1α can be partially recovered (~15% to 20%) by the overexpression of CCR2. The migration of HIF-1α–null monocytes showed small but consistent increase following the overexpression of CCR2. This is the first study that demonstrates decreased recruitment of WBCs induced by downregulation of leukocyte HIF-1α is attributable, at least in part, to decreased CCR2 expression.

MCP-1 is a member of the CC family of chemokines that mediate leukocyte chemotaxis and CCR2 is the only functional receptor for MCP-1. MCP-1/CCR2 are believed to play an important role in leukocyte trafficking and is upregulated during myocardial infarction. Consistent with our findings is the recent study showing that activation of MCP-1 is involved in the LV remodeling after MI. Inhibition of the MCP-1/CCR2 pathway has been shown to attenuate myocardial ischemia/reperfusion injury. Several studies with CCR2–/– mice and MCP-1–/– mice have shown that decreased leukocytes infiltration can attenuate LV remodeling and have beneficial effects on myocardial ischemia/reperfusion injury. Although inhibition of the MCP-1/CCR2 pathway using MCP-1–/– mice did not show modulation of infarct size following 60 minutes ischemia/reperfusion model, CCR2 deficiency decreased infarct size and reduced the degree of cardiac fibrosis following 45 minutes ischemia then reperfusion. We have previously suggested that inflammation modulates LV remodeling and not infarct size; thus, we anticipate in the setting of HIF-1α–null WBC, similar findings would be seen in an ischemia/reperfusion model as we have seen in the ischemia model implemented here. Our data demonstrate that the effects of downregulation of leukocyte HIF-1α on WBC migration may be attributable, at least in some limited part, to the decreased CCR2 expression.

To examine the mechanism involved in the antiinflammatory effect induced by downregulation of HIF-1α, gene arrays and real-time PCR were performed to assess differences in WBC receptor expression between groups. Downregulation of WBC HIF-1α results in suppression of the expression of WBC cytokine receptors CCR1, -2, and -4, which are critical for WBC mobilization and recruitment to inflammatory cytokines following MI. The results from our in vitro chemotaxis assay demonstrate that decreased WBC migration induced by downregulation of HIF-1α can be partially recovered (~15% to 20%) by the overexpression of CCR2. The migration of HIF-1α–null monocytes showed small but consistent increase following the overexpression of CCR2. This is the first study that demonstrates decreased recruitment of WBCs induced by downregulation of leukocyte HIF-1α is attributable, at least in part, to decreased CCR2 expression.

Our data also demonstrate that downregulation of leukocyte HIF-1α improves cardiac function and LV remodeling but not infarct size following MI. In the MI model induced by LAD ligation, besides decreased neutrophil infiltration, our data showed enhanced ventricular function in the HIF-1α siRNA group compared with HIF-1α siRNA group at 7 and 28 days post-MI. Our data indicate beneficial effects of inhibition of WBC infiltration on the cardiac function post-MI may be attributable to modulation of LV remodeling as evidenced by greater preservation of septal thickness and significantly decreased LVIDD and LVISD in the HIF-1α siRNA mice. Twenty eight days post-MI, LVMI is significantly less in the HIF-1α siRNA group compared with HIF-1α SCR group, suggesting the pathological hypertrophy in response to MI is decrease in the HIF-1α siRNA group. These findings are consistent with our previous work that suggests inflammation is more important in LV remodeling than infarct size.1

Our laboratory has previously demonstrated that inflammation regulates LV remodeling, not infarct size,1,2 following MI, findings consistent with results of this study. Taken together, these data indicate that downregulation of leukocyte HIF-1α expression resulted in improvement in cardiac function following MI by regulation of LV remodeling, not infarct size. Leukocytes can cause oxidative stress and myocardial ischemia/reperfusion injury is associated with inflammation. Several studies with CCR2–/– mice and MCP-1–/– mice have shown that decreased leukocytes infiltration can attenuate LV remodeling and have beneficial effects on myocardial ischemia/reperfusion injury. Although inhibition of the MCP-1/CCR2 pathway using MCP-1–/– mice did not show modulation of infarct size following 60 minutes ischemia/reperfusion model, CCR2 deficiency decreased infarct size and reduced the degree of cardiac fibrosis following 45 minutes ischemia then reperfusion. We have previously suggested that inflammation modulates LV remodeling and not infarct size; thus, we anticipate in the setting of HIF-1α–null WBC, similar findings would be seen in an ischemia/reperfusion model as we have seen in the ischemia model implemented here. Our data demonstrate that the effects of downregulation of leukocyte HIF-1α on WBC migration may be attributable, at least in some limited part, to the decreased CCR2 expression.

To examine the mechanism involved in the antiinflammatory effect induced by downregulation of HIF-1α, gene arrays and real-time PCR were performed to assess differences in WBC receptor expression between groups. Downregulation of WBC HIF-1α results in suppression of the expression of WBC cytokine receptors CCR1, -2, and -4, which are critical for WBC mobilization and recruitment to inflammatory cytokines following MI. We chose to focus on CCR2 because both MCP-1 (signals via CCR2) and MCP-3 (signals via CCR1/2) are both expressed following myocardial infarction. Moreover, the CCR2- and MCP-1–null mice have been shown to have significantly altered LV remodeling.22,23 whereas the overexpression of MCP-3 alone has not been shown to alter cardiac remodeling.24

Figure 6. Gene array and real-time PCR quantification of percentage mRNA expression compare with HIF-1α SCR group. A, mRNA expression of WBC cytokine receptors CCR1, -2, and -4 in HIF-1α siRNA group. B, mRNA expression of MCP-1 and MCP-3 in the mice heart from HIF-1α siRNA mice. *P<0.05 vs corresponding HIF-1α SCR group (n=4).
injury and LV remodeling after MI. Both MCP-1 and CCR2 mice demonstrate decreased leukocyte infiltration and exhibited attenuated ventricular dilation. Decreased leukocyte infiltration in the infarcted myocardium, suppression of matrix metalloproteinase activity, and reduced collagen accumulation in injured myocardium may be important mechanisms responsible for attenuated LV remodeling. Although we found that changed CCR2 expression is among the mechanisms of decreased WBC migration induced by downregulation of HIF-1, it is obviously not the only mechanism involved. Several studies showed that other mechanisms, including decreased energy production in WBCs, have important roles in decreased WBC migration in the setting of downregulation of HIF-1. HIF-1 can affect the transcription of key glycolytic enzymes and is essential for the regulation of glycolytic capacity in WBCs. Loss of HIF-1α in myeloid cells leads to decreased cellular ATP in both macrophages and neutrophils. Interestingly, the reduction of cellular ATP is also found under normoxic conditions, indicating that HIF-1α play an important role in the energy metabolism of WBCs even in oxygenated environments. HIF-1α also plays a role in the mitochondrial respiration. HIF-1α may reduce the mitochondrial oxygen-consumption and decrease the reactive oxygen species generation. Because of the vital role of HIF-1α in cellular ATP production of WBCs, decreased energy production in WBCs induced by downregulation of HIF-1α may be an important reason for decreased WBC migration. However, further investigation is needed to confirm the causal relationship between them.

In summary, our study further demonstrates that migration of neutrophils and monocytes is regulated by leukocyte HIF-1α expression resulting in decreased recruitment of WBCs to the sites of inflammation and improvement in cardiac remodeling and function following MI in the setting of decreased WBC HIF-1α. Downregulation of HIF-1α reduces the expression of WBC cytokine receptors CCR1, -2, and -4. The effects of downregulation of leukocyte HIF-1α on WBC migration are attributable, at least in part, to the decreased CCR2 expression; although, other important mechanisms are clearly involved.

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Disclosures
None.

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Novelty and Significance

What Is Known?

- HIF-1alpha is important for WBC response to injury.
- Inflammation plays a critical role in ventricular remodeling.

What New Information Does This Article Contribute?

- Inhibition of WBC HIF-1alpha decreases myocardial inflammation and improved cardiac function.
- Inhibition of WBC HIF-1alpha results in decreased WBC CCR1, CCR2, and CCR4 expression.
- Restoration of WBC CCR2 expression modestly improves WBC migration in the setting of decreased WBC HIF-1alpha.

Inflammation plays a critical role in cardiac remodeling following acute ischemic injury. Indices of increased inflammation in the perinfarct period are associated with myocardial rupture, heart failure, and mortality. HIF-1alpha is known to regulate WBC migration and response to tissue inflammation. In this study, we observed that downregulation of WBC HIF-1alpha expression resulted in decreased recruitment of neutrophils and monocytes to sites of inflammation in models of peritonitis and acute myocardial infarction. The downregulation of WBC HIF-1alpha did not alter myocardial infarct size but significantly modulated left ventricular remodeling, leading to improved cardiac function 21 days after myocardial infarction. Our study further demonstrates that downregulation of WBC HIF-1alpha leads to downregulation of WBC chemokine (CC motif) receptor (CCR)1, CCR2, and CCR4 in response to inflammatory stimuli, which, in part, is responsible for the impaired WBC migration in response to HIF-1alpha downregulation. This study indicates that transient inhibition of WBC migration after acute myocardial infarction can lead to improved left ventricular remodeling and suggests that WBC HIF-1alpha as a therapeutic target for the inhibition of WBC migration following acute myocardial infarction.
Critical Role for Leukocyte Hypoxia Inducible Factor-1α Expression in Post-Myocardial Infarction Left Ventricular Remodeling

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