Macrophage Migration Inhibitory Factor Deficiency Ameliorates High-Fat Diet Induced Insulin Resistance in Mice with Reduced Adipose Inflammation and Hepatic Steatosis

Orla M. Finucane1,2, Clare M. Reynolds2,3, Fiona C. McGillicuddy2, Karen A. Harford2, Martine Morrison2, John Baugh4, Helen M. Roche2*

1 Institute of Molecular Medicine, School of Medicine, Trinity Centre for Health Sciences, St. James Hospital, Dublin 8, Ireland, 2 Nutrigenomics Research Group, School of Public Health & Population Science, UCD Conway Institute, University College Dublin, Dublin 4, Ireland, 3 Liggins Institute, University of Auckland, Auckland, New Zealand, 4 School of Medicine and Medical Science, UCD Conway Institute, University College Dublin, Dublin 4, Ireland

Abstract

Macrophage infiltration is a critical determinant of high-fat diet induced adipose tissue inflammation and insulin resistance. The precise mechanisms underpinning the initiation of macrophage recruitment and activation are unclear. Macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine, displays chemokine-like properties. Circulating MIF levels are elevated during obesity however its role in high-fat diet induced adipose inflammation and insulin resistance remains elusive. Wildtype and MIF−/− C57Bl/6J mice were fed chow or high-fat diet. Body weight and food intake was assessed. Glucose homeostasis was monitored by glucose and insulin tolerance tests. Adipose tissue macrophage recruitment and adipose tissue insulin sensitivity was evaluated. Cytokine secretion from stromal vascular fraction, adipose explants and bone marrow macrophages was measured. Inflammatory signature and insulin sensitivity of 3T3-L1 adipocytes co-cultured with wildtype and MIF−/− macrophage was quantified. Hepatic triacylglyceride levels were assessed. MIF−/− mice exhibited reduced weight gain. Age and weight-matched obese MIF−/− mice exhibited improved glucose homeostasis coincident with reduced adipose tissue M1 macrophage infiltration. Obese MIF−/− stromal vascular fraction secreted less TNF-α and greater IL-10 compared to wildtype. Activation of JNK was impaired in obese MIF−/− adipose, concomitant with pAKT expression. 3T3-L1 adipocytes cultured with MIF−/− macrophages had reduced pro-inflammatory cytokine secretion and improved insulin sensitivity, effects which were also attained with MIF inhibitor ISO-1. MIF−/− liver exhibited reduced hepatic triacylglyceride accumulation, enhanced pAKT expression and reduced NFκB activation. MIF deficiency partially protects from high-fat diet induced insulin resistance by attenuating macrophage infiltration, ameliorating adipose inflammation, which improved adipocyte insulin resistance ex vivo. MIF represents a potential therapeutic target for treatment of high-fat diet induced insulin resistance.

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Introduction

Adipose tissue inflammation is central to the pathogenesis of obesity associated insulin resistance (IR), type 2 diabetes and hepatic steatosis [1–3]. High-fat diet (HFD)-induced adipose tissue expansion is accompanied by a progressive infiltration of macrophages [4,5]. Resident macrophages present immense heterogeneity and are broadly classified as pro-inflammatory M1 and anti-inflammatory M2 [6,7]. At the onset of obesity M2 macrophages acquire an M1 phenotype [8,9]. Pro-inflammatory cytokines including TNFα [10] and IL-6 produced by adipose tissue macrophages (ATM) exacerbate local inflammation promoting IR via down-regulation of IRS-1 and GLUT-4 [2]. Furthermore abrogation of Jun NH2-terminal kinase (JNK), a critical inflammatory regulator, in hematopoietic-derived cells protects mice from obesity-induced inflammation and IR [11]. Indispensably ATM infiltration and subsequent local inflammation is paramount for induction of IR, however the signals responsible for triggering macrophage recruitment remain ambiguous. Emerging data has highlighted the significance of adipose tissue-derived chemokines in driving macrophage recruitment during obesity. Deletion of CC-chemokine ligand CCL2/monocyte chemoattractant protein-1 (MCP-1) or its receptor CCR2 attenuates ATM recruitment concurrent with improved adipose tissue inflammation and systemic insulin sensitivity in vivo [12–15]. Several reports have challenged these findings indicating...
macrophage recruitment may occur independently of MCP-1 [15–
17]. Furthermore, the importance of C-X-C chemokine ligand
c(XCL)–5 [18], IL-6 [19] and CX3CL1 [20] has become
apparent in both human and rodent models. Macrophage
migration inhibitory factor (MIF) represents another potential
candidate.

MIF is a pleiotropic cytokine, ubiquitously expressed and
paramount in regulating inflammatory responses [21,22]. Clinical
studies suggest a link between MIF and adiposity. Circulating
plasma MIF and mononuclear cell MIF mRNA expression are
associated with increasing BMI and fatty acid concentration,
impaired glucose tolerance and T2D [23–25], whereas reducing
body weight or metformin treatment decreases serum MIF levels
[26,27]. Human and murine adipocytes express and secrete MIF
[24,28,29]. Moreover glucose and insulin regulate MIF expression
in adipocytes [30]. Recently MIF was identified as a non-cognate
ligand of CXC chemokine receptors CXCR2 and CXCR4 in
macrophages and T-cells respectively [31]. More recently in an
atherosclerotic mouse model (LDLR
−/−MIF
−/−) maintained on
a standard chow diet, absence of MIF reduced monocyte
adhesion, macrophage lesion content, and atherosclerotic lesion
size; coincident with improved glucose homeostasis [32].

Our study was undertaken to further define the impact of the
obesogenic environment induced by high-saturated fat feeding on
insulin sensitivity in a MIF-deficient setting. We hypothesized that
lack of MIF protein would protect mice from the adverse effects of
high-fat feeding by reducing ATM recruitment and improving
adipose tissue immunophenotype. Additionally we examined the
functional consequences of MIF
−/−
macrophages on adipocyte
biology and explored whether inhibition of exogenous MIF could
block macrophage-induced adipocyte IR in vitro. This study
highlights MIF as a critical mediator of ATM recruitment and
regulator of adipose tissue inflammation during HFD-induced
obesity.

Methods

Ethics statement

Ethical approval was obtained from UCD Ethics Committee
and mice were maintained according to European Union and Irish
Department of Health regulations. Body weight was monitored
prior to and after all metabolic procedures to ensure full recovery
of the animals. Any animals found to be exhibiting symptoms of
pain or distress during or after procedure were euthanized
immediately.

Materials

Deoxy-D-glucose 2-[1,2-3H(N)]-was purchased from Perkin-
Elmer Analytical Sciences (Dublin, Ireland). Cell culture material
was purchased from Lonza (Slough, UK). All other reagents unless
otherwise stated were from Sigma Aldrich Ltd.

Animals

C57BL/6j wildtype (WT) mice were purchased from Charles
River, Ireland. C57BL/6j MIF
−/− mice were generous gift from
Dr. Baugh and bred at University College Dublin (UCD) under
pathogen free conditions. MIF
−/− mice were backcrossed for 10
generations onto C57BL/6j background. Male mice aged 8–9
weeks were fed HFD (45% kcal from palm oil, 20% kcal from
protein, 35.1% kcal from carbohydrates) [Research Diets Inc.,
USA] or chow diet (17% kcal from fat, 25% kcal from protein,
58% kcal from carbohydrates) [Harlan Teklad UK] ad libitum
for 16 weeks. Body weight and food intake were recorded weekly.
Mice were deemed to be obese when they weighed greater than
35g. Lean and obese mice were overnight fasted and injected with
NaCl (pH 5.0) or insulin (0.75 U insulin/kilogram [kg], body-
weight [bw]); Actrapid, Novo Nordisk, Denmark). After 15
minutes, mice were killed and plasma and tissue harvested.

Body mass composition

Body mass composition was measured using Bruker’s minispec
LF30 body composition analyzer (Bruker Optik GmbH, Germany).
Lean tissue, fat and fluid was calculated based on body
weight of mouse (University College Cork/Alimentary Pharma-
biotic Centre).

Glucose and insulin tolerance test (GTT/ITT)

GTT and ITTs were performed on 4–6 h fasted mice. Mice
were intraperitoneally injected (i.p.) with 25% (w/v) glucose (1.5 g
glucose/kg bw); Braun Medical Ltd, Dublin, Ireland) or insulin
(0.75 U/kg bw) respectively. Blood glucose levels were measured
prior to administration and 15, 30, 60, 90, 120 minutes post
insulin/glucose challenge using Accu-Chek glucometer (Roche
Ltd, Dublin, Ireland).

Insulin secretory response

Overnight fasted mice were subjected to i.p. glucose challenge
(1.5 g glucose/kg bw). Blood samples were collected by tail vein
bleed prior to and 30, 60 minutes post glucose challenge. Plasma
insulin secretory response was determined using an ultra-sensitive
insulin ELISA kit (Crystal Chem Inc., USA).

Isolation of stromal vascular fraction (SVF) and flow
cytometry

To separate the SVF from adipocytes, epididymal adipose tissue
(EAT) was minced then collagenase (2 mg/ml) digested for
70 minutes. Adipocytes were removed and digested EAT suspension
was filtered and centrifuged for 5 minutes at 1,700 rpm. The
SVF was re-suspended and blocked in 2% BSA/PBS. Cells were
stained with fluoresently labelled antibodies; F4/80-FITC, CD11B-AF647/PE,
CD11C-RPE, CD3-APC, CD4-FITC,
CD8-PE, (AbD Serotec, Kidlington, UK). Unstained, single
stained and fluorescence minus one controls were used for setting
compensation and gates. Flow cytometry was performed on Dako
CyAn ADP platform and analyzed using Summit v4.3 software
(Beckman-Coulter Ltd., UK). Adipocytes and remaining SVF
were seeded at 1×106 cells/ml and cultured in serum rich media
(DMEM, 10% FBS and 1% penicillin/streptomycin) for 24 h.
Media was harvested for cytokine profiling. Cells were re-
suspended in TRI Reagent® for gene expression analysis.

Ex vivo adipose tissue culture

EAT explants harvested from lean and obese mice (50 mg/well)
were cultured in serum rich media for 24 h. Tissue was
homogenized in 1 ml radioimmunoprecipitation assay (RIPA)
buffer using a tissue lyser (Qiagen, West Sussex, UK).

Insulin-stimulated glucose uptake into adipose explants

Adipose explants (30 mg) were placed in PBS+0.2% BSA prior
to stimulation ± insulin (100 nM) for 15 min. 3H-glucose (0.1 mM
2-deoxyglucose+0.5 μCi/ml 3H-deoxyglucose) was added for
45 min. Tissue was washed, lyzed in RIPA buffer and homoge-
nized using a tissue lyser (Qiagen, West Sussex, UK). Glucose
uptake was measured by liquid scintillation counting. Fold increase
in glucose uptake over basal was calculated for each individual
mouse.
Cell culture

Bone marrow derived cells were isolated from femurs and tibias of 8–11 week old lean WT and MIF−/− mice. Cells were cultured in serum rich media supplemented with 30% L929 conditioned medium for 7 days at 37°C to differentiate to bone marrow macrophage (BMM). On day 7 of differentiation macrophages were treated with lipopolysaccharide (LPS) (10 ng/ml) for 30 minutes; cells were harvested for protein or incubated with fresh media for 6–24 h to measure cytokine secretion. 3T3L1-fibroblasts and 4) J774.2 macrophages pretreated with ISO-1 containing 10% FBS, 0.5 mM isobutylmetylxanthine, 1 mM dexamethasone and 10 μg/ml insulin to induce differentiation.

Co-culture assay

WT and MIF−/− BMM were seeded on 0.4 μm Corning transwell filters, LPS-stimulated for 30 minutes and washed with PBS. Fresh media was added; transwells were transferred onto 3T3-L1 adipocytes (day 7 differentiation) and incubated for 72 h. Media was collected for cytokine analysis and 3T3-L1 adipocytes were harvested for gene expression analysis. On an identically treated 3T3-L1 adipocytes culture plate insulin-stimulated 3H-glucose transport assay was conducted.

Conditioned media experiments

J774.2 macrophages were grown in serum rich media in T75 culture flask at a density of 2×10⁶ cells/ml. Media was collected after 24 h from 1) unstimulated J774.2 macrophages, 2) J774.2 macrophages treated with recombinant (r) MIF (100 ng/ml), 3) J774.2 macrophages treated with the commercially available MIF inhibitor (S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid, methyl ester (ISO-1) (50 μM) (Merck, Ireland) and 4) J774.2 macrophages pretreated with ISO-1 (50 μM) for 1 h followed by rMIF (100 ng/ml) treatment for 24 h. Mature 3T3-L1 adipocytes were subsequently exposed to the collected conditioned media (CM) for 72 h. Cells were lysed in RIPA buffer and insulin-stimulated 3H-glucose transport assay into adipocytes was conducted.

Glucose transport assay

Glucose transport assay was performed on 3T3-L1 adipocytes co-cultured with WT or MIF−/− BMM or incubated with CM for 72 h. Cells were incubated in serum free media (DMEM +1% penicillin/streptomycin) for 2–5 h followed by glucose free incubation (0.2%BSA/PBS). Cells were stimulated with insulin (100 nM) then [3H] Deoxyglucose (5 μCi/ml) cold glucose (1 mM) was added. Cells were washed, lysed in RIPA buffer at 4°C. [3H] Deoxyglucose uptake was measured by liquid scintillation counting.

Statistical analysis

Data are reported as mean ± SEM. For GTT/ITT studies with multiple time-points an ANOVA was used to test for differences in means between WT and MIF−/− groups. When ANOVA was significant post-hoc Bonferroni corrected t-tests were applied. AUC analysis was performed on GTT and ITT curves using Graphpad Prism 5 software (GraphPad Software Inc., San Diego, CA) calculated using the trapezoidal method. For comparison of data between two groups at a single time-point unpaired t-tests were performed. Statistical significance is presented as *p<0.05, **p<0.01 and ***p<0.001 in all figures.

General laboratory techniques

Detailed description of plasma, gene expression, immunohistochemistry and immunoblot analysis are supplied in File S1.

Results

MIF deficiency partially protects mice from HFD-induced obesity and insulin resistance

The severity of HFD-induced IR was less in obese MIF−/− mice, with significantly lower GTT and ITT compared to obese WT mice (Figure 1A–D). Fasting plasma insulin levels increased following the HFD irrespective of genotype, however obese MIF−/− mice secreted significantly less insulin in response to glucose compared to obese WT mice (Figure 1E). Baseline GTT and ITT were not different between WT and MIF−/− mice. Age-matched chow-fed WT and MIF−/− mice GTTs and ITTs were equivalent and significantly lower than obese WT and MIF−/− mice (Figure S1A–D). Despite equivalent body weight at baseline and comparable food intake during the intervention, MIF−/− mice gained significantly less weight than WT mice in response to HFD (Figure 1F–G). Body mass composition confirmed the lower body weight was due to reduced fat mass (Figure 1H), however we could not determine the body regions of fat distribution. Nevertheless, we measured the weight of various organs and observed both liver and epididymal weights were significantly greater in obese WT compared to MIF−/− mice (Table 1). Histological analysis confirmed obese MIF−/− mice display a hyperplastic morphology (Figure S2A). Since weight is a key determinant of insulin sensitivity, we sought to distinguish between direct effects of MIF deficiency on insulin sensitivity from secondary effects of reduced weight. Weight-matched (45–47 g) obese MIF−/− mice had significantly lower GTT and ITT, compared to equivalently obese WT mice, indicating that improved glucose homeostasis in obese MIF−/− mice is independent of body weight (Figure S2B–E). Fasting plasma triglycerides (TAG), NEFA, IL-6 IL-10, IL-12p70 and MCP-1 levels increased equivalently in both genotypes following HFD compared to lean counterparts. Plasma leptin and keratinocyte chemoattractant (KC) levels were lower in HFD MIF−/− mice compared WT mice following feeding of a HFD (Table 1).

Adipose tissue MIF expression is elevated in obese WT mice while lack of MIF reduced ATM recruitment following HFD

To confirm the positive relationship between obesity and heightened levels of adipose tissue MIF, we profiled the expression of MIF and its corresponding receptors. Obese EAT expressed significantly higher protein MIF levels compared to lean EAT (Figure 2A–B). Intriguingly, obese EAT and visceral adipose tissue have exhibited reduced Mif mRNA expression compared to lean adipose tissue (Figure 2C). In terms of defining the primary cellular source (adipocyte or SVF) of the enhanced adipose tissue MIF, we demonstrated that Mif mRNA expression was markedly increased in the adipose SVF but not adipocyte fraction of the obese mice compared to lean mice (Figure 2C). In contrast, Mif expression was not altered in liver in response to HFD (Figure 2C). Further gene expression analysis revealed expression of known MIF receptors Cxcr2, Cxcr4 and Cd74 was significantly upregulated in the EAT in response to HFD (Figure 2D). Protein levels of CXCR2, CXCR4 and CD74 could not be detected by immunoblot analysis. This may be attributed to reduced levels of stromal vascular cells in whole adipose tissue sections.
Figure 1. MIF deficiency partially protects from HFD-induced obesity and improves glucose homeostasis. (a) GTT (1.5 g glucose/kilogram (kg) body weight (BW)) in fasted lean and obese WT and MIF−/− mice (white circles = WT lean; black circles = MIF−/− lean; white squares = WT obese; black squares = MIF−/− obese). Lean n = 9, obese n = 9. (b) ITT (0.75 U insulin/kg BW) in fasted lean and obese WT and MIF−/− mice (white circles = WT lean; black circles = MIF−/− lean; white squares = WT obese; black squares = MIF−/− obese). Lean n = 18, obese n = 18–33. (c&d) AUC for WT (white bars) and MIF−/− (black bars) mice over the course of GTT and ITT expressed as arbitrary units (AU). Lean n = 9, obese n = 18–33. (e) Plasma insulin levels over time in response to glucose challenge (white circles = WT lean; black circles = MIF−/− lean; white squares = WT obese; black squares = MIF−/− obese).
Next, we speculated that a reduction in adipose tissue immune cell infiltration would explain the attenuated IR phenotype observed in MIF−/− mice. Both SVF and whole adipose tissue F4/80 mRNA expression were decreased in MIF−/− mice (Figure 3A-B). Concomitantly, the recruitment of M1 ATM in response to HFD was markedly reduced in obese MIF−/− mice compared to obese WT (Figure 2E). M2 ATM number decreased in response to the HFD, irrespective of genotype (Figure 2E). Correspondingly, M2 ATM marker Gld206 was equivalently expressed in adipose of WT and MIF−/− mice (Figure 3B). No difference in either CD8α or CD4+ T-cell number was evident between genotypes (Figure 2F). The inflammatory signature of SVF displayed a reduction in TNFα (Figure 2G) and IL-1β (Figure 3C) secretion compared to obese WT. Conversely IL-10 (Figure 2H) and MCP-1 secretion (Figure 3D) was increased in obese MIF−/− SVF; while IL-6 secretion was equivalent between genotypes (Figure 3E). No difference in cytokine secretion was evident between genotypes from lean or obese adipocytes.

MIF deficiency attenuates adipose tissue inflammation and improves insulin sensitivity *ex vivo*

Adipose tissue inflammation was assessed in explants from lean and obese WT and MIF−/− mice Tnfα and IL-1β secretion was significantly lower from obese MIF−/− explants compared to obese WT explants (Figure 3A-B), with reduced Tnfα and Il-1β mRNA expression in obese MIF−/− adipose tissue (Figure 3C-D). Furthermore, phosphorylated JNK, but not Nfkbp65, p38 and ERK, was significantly reduced in obese MIF−/− versus WT adipose tissue (Figure 3E; Figure S4A-C). Subsequently, we investigated if reduced adipose tissue inflammation translated to improved adipose tissue insulin sensitivity in MIF−/− mice. Insulin-stimulated 3H-glucose uptake into adipose explants was reduced in WT obese adipose compared to MIF−/− obese adipose (Figure 3F). Coincident with HFD, Glut-4 mRNA expression decreased in both genotypes; however Glut-4 mRNA expression remained higher in obese MIF−/− mice compared to obese WT (Figure 3G). A comparable reduction in adipose *Irs-1* mRNA expression was observed in obese WT and MIF−/− mice following HFD (Figure 3H). To investigate the effect of MIF on insulin signaling lean and obese WT and MIF−/− animals were injected with or without insulin. Obese MIF−/− adipose tissue displayed markedly increased levels of phosphorylated AKT compared to corresponding obese WT adipose tissue (Figure 3I).

**MIF−/−** BMM have improved adipocyte-macrophage crosstalk, while ISO-1 ameliorates the adverse effects of MIF on adipocyte insulin sensitivity

Given the improved immunophenotype observed in obese MIF−/− SVF we hypothesized that MIF−/− macrophages would have an attenuated pro-inflammatory phenotype and improve cross-talk with adipocytes *in vitro*. We determined the effect of MIF−/− BMM on adipocyte biology as a surrogate for ATMs; as the number of ATM were limited for mechanistic studies. MIF−/− BMM secreted significantly less IL-6, IL-1β and MCP-1 and exhibited reduced Il-6 and Il6s mRNA expression compared to WT (Figure 4A-D; Figure S3E). While anti-inflammatory IL-10 demonstrates a marked increase in MIF−/− BMM compared to WT (Figure S3F). Furthermore, activation of MAPKs, p38, JNK, ERK, and Nfkb pathways was significantly impaired in MIF−/− BMM compared to WT (Figure 4E). Exogenous rMIF directly impaired insulin-stimulated glucose transport in 3T3-L1 adipocytes (Figure 3A) which corroborates previous work [33]. Furthermore we demonstrated that 3T3-L1 adipocytes, co-cultured with WT BMM, but not with MIF−/− BMM, had reduced adipocyte insulin-stimulated glucose uptake, with decreased Glut-4 and *Irs-1* mRNA expression (Figure 3B-D). Co-culture of adipocytes with WT BMM significantly reduced insulin-stimulated phosphorylation of AKT, an effect which was not observed when co-cultured with MIF−/− BMM (Figure 5E). Also, adipocytes co-cultured with MIF−/− BMM secreted less TNFα and IL-6 compared to adipocytes co-cultured with WT BMM (Figure 5F-G). Lastly we examined if ISO-1, a MIF inhibitor which targets MIFs D-dopachrome tautomerase enzymatic activity would inhibit MIF inflammatory effects in macrophages and improve macrophage-adipocyte crosstalk. Pre-treatment with ISO-1 blocked MIF-induced TNFα cytokine secretion from J774A1.

![Table 1. Plasma metabolic profile and adipose tissue depot weights.](https://www.plosone.org/). Plasma was isolated from overnight fasted WT and MIF−/− mice by cardiac puncture and metabolic markers were analyzed enzymatically. (*p<0.05, **p<0.01 w.r.t. WT obese; p<0.05, ##p<0.01 and ###p<0.001 w.r.t. respective lean). doi:10.1371/journal.pone.0113369.t001
Figure 2. MIF mRNA is elevated in adipose tissue of obese WT mice, while adipose tissue lack of MIF reduced ATM recruitment into adipose tissue following HFD. (a) Immunoblot analysis of MIF and control β-actin in adipose of lean and obese WT mice (n = 4). (b) Densitometry analysis quantifying phosphorylated MIF protein expression relative to β-actin expressed in arbitrary units (AU) in lean (white bars) and obese (black bars) mice. (c) Gene expression analysis determined by RT-PCR of Mif in the epididymal adipose tissue (EAT), visceral adipose tissue (VAT), stromal vascular fraction (SVF), adipocyte fraction and liver of lean (white bars) and obese (black bars) WT mice. Lean n = 3–6, obese n = 4–7. (c) Gene expression analysis of known MIF receptors Cxcr4, Cxcr2, Cd74 in EAT of lean (white bars) and obese (black bars) mice, lean = 4–8, obese n = 3–5. (d) Recruitment of ATM into adipose tissue: cells triple positive (F4/80+/CD11B+/CD11C+) were classified as M1 macrophages. Cells double positive (F4/80+/CD11B+/CD11C-) were classified as M2 macrophages. (e) Recruitment of T-cells into adipose tissue: Cells double positive (CD3+/CD4+ /CD8+) were classified as cytotoxic T-cells. Cells double positive (CD3+/CD4+ /CD8-) were classified as helper T-Cells. Recruitment of cells is presented as percentage of total SVF cells. Lean WT (white bars) = 9, lean MIF−/− (grey bars) = 9, obese WT (dark grey bars) n = 15–17, obese MIF−/− (black bars) n = 15–17. (f) TNFα and (g) IL-10 cytokine secretion from SVF and adipocyte fraction from lean and obese WT (white bars) and MIF−/− (black bars). Lean = 12, obese n = 12. Data are mean ± SEM, *p<0.05, **p<0.01 and ***p<0.001 w.r.t obese WT.

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Figure 3. Obese MIF \(^{-/-}\) mice exhibit reduced adipose tissue inflammation and improved adipose tissue insulin sensitivity compared to obese WT mice. Levels of pro-inflammatory (a) TNF\(\alpha\) and (b) IL-1\(\beta\) secretion into media from adipose tissue explants was measured by ELISA. Lean = 17, obese n = 17. Gene expression analysis of (c) Tnf\(\alpha\), (d) Il-1\(\beta\) in lean and obese adipose tissue from WT and MIF \(^{-/-}\) mice. Lean = 5, obese n = 5. (e) Immunoblot analysis of phosphorylated JNK and control \(\beta\)-actin and corresponding densitometry analysis expressed in arbitrary units (AU) Lean = 3, obese n = 3. (f) Ex vivo insulin (100 nM)-stimulated \(^3\)H-glucose transport into whole adipose tissue (50 mg) harvested from obese WT and MIF \(^{-/-}\) mice was evaluated. Fold increase in \(^3\)H-glucose transport into adipose in response to insulin over basal (non-insulin-stimulated) is presented (white bars = + insulin, black bars = + insulin) n = 6. Gene expression analysis of (g) Glut-4 in adipose tissue from lean and obese WT and MIF \(^{-/-}\) mice. Lean = 5, obese n = 5. (h) Immunoblot analysis of phosphorylated AKT levels and control \(\beta\)-actin. Lean = 3, obese n = 3. WT mice represented by white bars, MIF \(^{-/-}\) mice represented by black bars in all graphs. Data are mean \(\pm\) SEM, *p < 0.05, **p < 0.01 and ***p < 0.001 w.r.t obese WT.

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macrophages (Figure S5A). Subsequently, 3T3-L1 adipocytes were incubated with MIF treated CM in the presence or absence of ISO-1. CM from unstimulated macrophages had no effect on insulin-stimulated glucose uptake into adipocytes while pretreatment with ISO-1 reversed the insulin desensitizing effects of MIF in 3T3-L1 adipocytes (Figure 5H).

Short-term ISO-1 treatment does not impede HFD-induced IR or alter immune cell recruitment

We next assessed if short-term treatment with ISO-1 would mirror our results observed in MIF<sup>−/−</sup> mice. As obesity is a low grade chronic inflammatory state we first speculated a low dose of 10 mg/kg over 14 consecutive days would have therapeutic benefits [34,35]. ISO-1 treated animals exhibited similar glucose tolerance to vehicle control treated animals (Figure S5B). As such a higher dose was proposed. A subset of WT mice were treated with

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**Figure 4. MIF<sup>−/−</sup> bone marrow macrophages have reduced inflammatory signature compared to WT mice.** Levels of pro-inflammatory (a) IL-6, (b) IL-1β (c) MCP-1 secretion into media from lean WT and MIF<sup>−/−</sup> BMM stimulated ±LPS, (d) Gene expression analysis of Il-6 in WT and MIF<sup>−/−</sup> BMM ±LPS, n = 5/group. (e) Immunoblot analysis of phosphorylated and whole cell ERK, JNK, p38, NFkB and β-actin stimulated ±LPS, n = 3/group. WT mice represented by white bars, MIF<sup>−/−</sup> mice represented by black bars in all graphs. Data are mean ± SEM, *p<0.05, **p<0.01 and ***p<0.001 w.r.t WT+LPS.

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Figure 5. MIF<sup>-/-</sup> macrophages have altered adipocyte-macrophage crosstalk compared to WT macrophages, while ISO-1 blocks MIFs insulin desensitizing capacity in adipocytes. (a) Chronic treatment of 3T3-L1 adipocytes with rMIF (100 ng) and its effect on insulin (100 nM)-stimulated 3H-glucose uptake was evaluated. Data are mean ± SEM, *p<0.05, **p<0.01 and ***p<0.001 w.r.t untreated +Insulin. (b) The effect of WT and MIF<sup>-/-</sup> BMM on insulin (100 nM)-stimulated 3H-glucose transport into 3T3-L1-adipocytes was evaluated. Fold increase in 3H-glucose transport into adipocytes in response to insulin over basal (non-insulin-stimulated) is presented (white bars = -insulin, black bars = + insulin). Data are mean ± SEM, *p<0.05, **p<0.01 and ***p<0.001 w.r.t adipocytes co-cultured with WT BMM+insulin. The effect of BMM co-culture on adipocyte on (c) Glut-4 and (d) Irs-1 mRNA expression was determined by real-time PCR, n = 4/group. (e) Immunoblot analysis of phosphorylated AKT, whole-cell AKT and β-actin in co-cultured adipocytes stimulated with insulin (100 nM). Levels of (f) TNFα (g) IL-6, were measured in media from co-cultured cells.
MIF deficiency alleviates hepatic steatosis and improves hepatic insulin sensitivity in response to HFD

Adipose tissue expansion exacerbates lipolysis and elevates NEFA influx into liver. MIF−/− mice had reduced liver weight compared to WT mice following HFD indicating partial protection from HFD-induced hepatomegaly (Figure 6A). Furthermore, fasting plasma alanine aminotransaminase (ALT) levels were considerably lower in obese MIF−/− mice compared to obese WT, indicative of reduced liver tissue injury (Figure 6B). Hepatic TAG content was significantly lower in obese MIF−/− mice compared to obese WT mice (Figure 6C). Hematoxylin and eosin (H&E) studies confirmed that obese MIF−/− mice have reduced lipid accumulation compared to obese WT after HFD (Figure 6D). Also lipogenic genes Cds16, Dgat-1, Fasn, Srebp-1c, Pgc-1α, Lpl and Pparγ mRNA expression was significantly lower in obese MIF−/− mice compared to obese WT (Figure 6E). Lastly, we investigated the effect of MIF on hepatic insulin signaling and inflammation. Insulin-stimulated hepatic phosphorylated AKT expression was significantly greater in both lean and obese MIF−/− mice compared to WT controls (Figure 6F). Furthermore phosphorylated Nfkb p65 levels were markedly lower in obese MIF−/− mice compared to obese WT mice (Figure 6G).

Discussion

Expanding adipose tissue mass in response to HFD is accompanied by a progressive infiltration of macrophages and T-cells which contribute significantly to inflammation and IR [4]. The mediators which initiate ATM recruitment remain to be fully deciphered. Here we have shown that HFD augments local adipose tissue MIF expression, which is primarily attributed to the adipose SVF. We demonstrate that MIF is pivotal for HFD-induced ATM recruitment and adipose tissue inflammation. Impeding MIF with ISO-1 attenuates MIFs pro-inflammatory, insulin desensitizing effects in a macrophage-adipocyte conditioned media system. However the beneficial effects of ISO-1 were not evident in vivo. Furthermore MIF deficiency partially protects from HFD-induced obesity, IR and hepatic steatosis.

MIF affects glucose metabolism at several levels, including pancreatic insulin secretion [36], TNFα mediated adipocyte IR and glucose uptake and glycolysis in skeletal muscle [37]. Recent reports present conflicting data regarding its role in glucose homeostasis in vivo. Deletion of MIF in LDLR−/− mice impeded the development of atherosclerosis, glucose intolerance and IR without altering adiposity [32]. We extend this knowledge by demonstrating that obese MIF−/− mice are partially protected from HFD-induced IR, are less hyperinsulenic in response to a glucose load and exhibit improved glucose homeostasis. Increased levels of MIF can enhance insulin secretion in a glucose dependent manner [36] and this may in turn explain the reduced hyperinsulimemia observed in obese MIF−/− mice. Consistent with Verschuren et al., findings MIF−/− mice display adipocyte hyperplasia. However unlike Verschuren et al we illustrate MIF−/− mice exhibit reduced weight gain attributable to lower fat mass, without altered feeding behavior in response to HFD. Plasma insulin levels in chow fed animals are equivalent, suggesting that absence of MIF does not impair insulin production/secretion but reduction may be reflective of reduced weight gain and improved insulin sensitivity in HFD-fed MIF−/− mice. Improved glucose tolerance was still evident in obese MIF−/− mice compared to weight-matched obese WT, thus we speculate that MIF has additional beneficial mechanisms beyond reduced weight-gain. Conversely, Serre-Beiner et al. showed that absence of MIF led to impaired glucose tolerance, hyperinsulimemia and increased body fat mass in mature mice compared to WT mice in response to chow diet [38], however interactions with HFD were not examined. These contradictory findings underscore the complexity of MIF inflammatory signals within glucose and energy metabolism depending on diet and age. Further, increases in plasma leptin levels observed in the obese state are usually directly proportional to increases in plasma insulin levels and expanding adipose tissue mass [39]. It is therefore likely that the reduced adipose tissue mass in MIF−/− mice accounts for differences in plasma leptin levels.

ATM infiltration and inflammation are crucial to the pathogenesis of HFD-induced IR. Ablation of pro-inflammatory CD11c+ [M1] macrophages ameliorates IR coincident with diminished systemic inflammation in obese mice [40]. Furthermore mice lacking PPARγ in myeloid cells exhibit reduced numbers of M2 macrophages and are susceptible to HFD-induced IR [41]. We postulated that given the potent chemotactic properties of MIF [31], reduced ATM accumulation would explain the partial protection from HFD-induced IR observed in MIF−/− mice. In support of our hypothesis, M1 ATM number was lower in obese MIF−/− adipose tissue compared to WT coincident with reduced adipose F4/80, while M2 ATM number was equivalent. Our study demonstrated reduced circulating levels of keratinocyte-derived chemokine (KC), a mouse ortholog for human IL-8, in MIF−/− mice after HFD compared to WT mice. KC signals via a known MIF receptor CXCR2 which is involved in chemotaxis. It is plausible that the combination of reduced KC and lack of MIF contributed to reduced inflammatory cell recruitment into MIF−/− adipose tissue and preservation of insulin sensitivity. Furthermore, a previous study by Lin et al., demonstrated that administration of exogenous MIF increased circulating KC in mice indicative of a direct capacity of MIF to regulate KC levels [42].

Obese MIF−/− SVF secreted less TNFα and IL-1β compared to WT SVF. IL-10 secretion was increased from MIF−/− SVF compared to WT SVF. In contrast lack of MIF did not alter adipose T-cell CD8+ or CD4+ populations as illustrated in Figure 2F. Recent studies have implicated the immunogenic phenotype of ATM is of critical importance in dictating the severity of adipose tissue inflammation and IR [43]. We speculated that the improved inflammatory profile was macrophage derived, however given that we are limited to interpreting the specific cellular source of cytokines while working within the mixed cell pool of the SVF, we investigated the response of a more purified.
macrophage population from WT and MIF$^{-/-}$ mice to an LPS stimulus by utilizing BMM. Furthermore, MIF$^{-/-}$ macrophages have previously displayed an attenuated inflammatory phenotype in response to the TLR4 ligand LPS in vitro [44,45]. Correspondingly MIF$^{-/-}$ BMM demonstrated improved immunogenic phenotype with reduced expression of M1 marker iNOS and heightened IL-10 secretion. Furthermore co-culture of MIF$^{-/-}$ BMM with adipocytes resulted in reduced inflammation.

**Figure 6. Lack of MIF improves HFD-induced hepatic steatosis and hepatic insulin sensitivity.** Liver tissue was harvested from lean and obese WT and MIF$^{-/-}$ mice. (a) Weight of liver tissue expressed as a percentage of total body weight. Lean = 4, obese = 4. (b) Fasting plasma ALT levels in lean and obese mice. (c) Hepatic triacylglyceride levels, lean = 4, obese = 4. (d) Hematoxylin & eosin staining to visualize hepatic lipid accumulation (representative of n = 3 images per group). (e) Gene expression analysis of markers of lipogenesis and lipid storage as determined by RT-PCR. Lean = 4, obese = 4. (f) Unstimulated tissue phosphorylated-NFkB were determined by immunoblot analysis. Lean = 3, obese = 3. WT mice represented by white bars, MIF$^{-/-}$ mice represented by black bars in all graphs. Data are mean ± SEM, *p < 0.05, **p < 0.01 and ***p < 0.001 w.r.t obese WT. doi:10.1371/journal.pone.0113369.g006
and preservation of adipocyte insulin sensitivity compared to co-culture with WT BMM. This data highlights the significance of MIF in the pathophysiology of HFD-induced IR not only as a controller of M1 ATM macrophage recruitment but also as a modulator of macrophage activation status and subsequent insulin de-sensitizing capacity. Recently, Saksida and co-workers showed inhibition of MIF \textit{in vitro} utilizing MIF inhibitor ISO-1 reduced palmitic acid-induced pancreatic beta cell dysfunction [46]. We also have highlighted the potential therapeutic potential of MIF inhibitors by demonstrating that ISO-1 successfully blocked MIF-induced macrophage inflammation and reversed the detrimental effects of MIF-stimulated macrophages on adipocyte function \textit{in vitro}. On the contrary, short term treatment with ISO-1 failed to attenuate HFD-induced IR and or impede immune cell infiltration \textit{in vivo}. It is probable that the timing and dosing of the ISO-1 inhibitor accounted for lack of efficacy in the obese phenotype. An alternative MIF antibody with greater specificity may provide greater efficacy.

Several models of inflammation have indicated MIF as a central controller of systemic inflammation [21,47,48]. This study extends our current understanding of MIF as an inducer of local inflammation within the context of HFD-induced adipocyte dysfunction. We hypothesized that reduction of M1 ATM number and diminished immunogenic phenotype of adipose tissue SVF in obese MIF−/− mice may attenuate whole adipose tissue inflammation and further dissipate adipocyte dysfunction. Obese MIF−/− whole adipose tissue cultured \textit{ex vivo} exhibited a marked reduction in TNFα and IL-1β secretion accompanied by reduced Tnfα and Il-1β mRNA expression compared with obese WT. Moreover JNK activation was impaired in obese MIF−/− adipose tissue, while no difference in pNFkBp65 or pp38 activity was observed. Attenuated TNFα and IL-1β secretion may account for the observed increase in JNK activity. Interestingly, deletion of JNK-1 in obese mice reduced adiposity and alleviated IR [49], we could therefore speculate that diminished JNK activation in obese MIF−/− adipose tissue may contribute to reduced adiposity. Concomitant with attenuated local adipose tissue inflammation and corroborating our hypothesis we observed improved insulin sensitivity in obese MIF−/− adipose tissue, whereby in insulin-stimulated H-glucose uptake into adipose explants was increased in obese MIF−/− compared to WT mice.

The contribution of MIF to HFD-induced hepatic steatosis has been relatively unexplored. We demonstrate that obese MIF−/− mice have reduced hepatomegaly, lower systemic ALT levels and are partially protected from HFD-induced hepatic steatosis; coincident with reduced Pparg and Srebp-1c mRNA expression compared to WT. Pparg regulates expression of the fatty acid translocase protein Cd36, expression of which is also reduced in obese MIF−/− mice compared to WT. Previous studies have reported that lean mice lacking MIF have improved hepatic insulin sensitivity [38]. In addition, we demonstrated that obese MIF−/− liver tissue has heightened insulin sensitivity compared to obese WT. Overflow of NEFA from obese, insulin resistant adipose tissue to liver contributes to hepatic steatosis and resultant IR, an effect which is markedly improved upon disruption of AT1 recruitment [30,51] [12]. Whether MIF directly induces hepatic lipid accumulation or indirectly via AT1 recruitment is yet to be established.

In conclusion, this study illustrates a direct role for MIF signaling in HFD-induced ATM recruitment, adipose dysfunction and glucose homeostasis. However, there were limitations to our study. These studies were performed in whole-body MIF deficient mice, whether lack of MIF within the immune or non-immune system is primarily governing these protective effects remains to be addressed. Furthermore the significant reduction in body weight warrants further investigation to decipher whether this is an intrinsic characteristic of this mouse model or if MIF regulates energy homeostasis and/or gut assimilation. Nevertheless, given the body of evidence presented, we speculate that small molecule MIF inhibitors may have therapeutic potential to ameliorate obesity-induced IR, halting the progression to T2D and non-alcoholic fatty liver disease.

**Supporting Information**

Figure S1 Glucose tolerance (GTT) and insulin tolerance tests (ITT) in WT and MIF−/− mice after high-fat and after chow diet. (A) GTT (1.5 g/kg glucose) in 4–6 hour fasted age-matched chow-fed lean and high-fat fed obese Wild-type (WT) and MIF−/− animals (black circles = WT lean; open circles = MIF−/− lean; black squares = WT obese; open squares = MIF−/− obese; \( \ast p < 0.05, \ast \ast p < 0.01 \) w.r.t. MIF−/− obese; \( n = 9 \)). (B) ITT (0.75 U/kg insulin) in 6 h fasted lean and obese WT and MIF−/− animals (black circles = WT lean; open circles = MIF−/− lean; black squares = WT obese; open squares = MIF−/− obese; \( \ast \ast \ast p < 0.001 \) w.r.t. MIF−/− obese; \( n = 7–9 \)). (B&D) Area under the curve (AUC) for lean and obese animals over course of GTT and ITT was calculated and expressed as arbitrary units (AU), \( (\ast p < 0.5, \ast \ast \ast p < 0.001 \) w.r.t. WT, \( \# p < 0.01, \# \# \# p < 0.001 \) w.r.t. corresponding lean; \( n = 7–9 \)). (TIF)

Figure S2 MIF deficiency improves glucose homeostasis in response to HFD in weight-matched animals. (A) Histological analysis of paraffin embedded adipose tissue (image representation of \( n = 6 \)/group). (B) GTT (1.5 g/kg glucose) in 6 hour fasted lean and obese WT and MIF−/− animals (\( n = 9 \)/group; white circles = WT lean; black circles = MIF−/− lean; white squares = WT obese; black squares = MIF−/− obese; \( \ast \ast \ast p < 0.001 \) w.r.t. WT, \( \ast \ast \ast p < 0.001 \) w.r.t. MIF−/− obese; \( n = 9 \)). (B&D) Area under the curve (AUC) over course of GTT was calculated and expressed as arbitrary units (AU). (A) ITT (0.75 U/kg insulin) in 6 h fasted lean and obese WT and MIF−/− animals (\( n = 9 \)/lean group, \( n = 18–33 \)/obese group; \( \ast \ast \ast \) w.r.t. WT, \( \ast \ast \ast \ast \) w.r.t. MIF−/−). (B) Area under the curve (AUC) over course of ITT was calculated and expressed as arbitrary units (AU), \( (\ast p < 0.5, \ast \ast \ast p < 0.001 \) w.r.t. WT, \( \# \# \# p < 0.001 \) w.r.t. corresponding lean). (TIF)

Figure S3 Stromal vascular fraction (SVF) inflammatory and adipocyte inflammatory signature is altered in MIF−/− mice compared to WT mice in response to HFD. (A) F4/80 mRNA expression in SVC from obese mice only (\( n = 4 \)/group; \( \ast \ast \ast \) p < 0.001 w.r.t. WT obese). (B) Adipose tissue F4/80 and Cd206 mRNA expression (\( n = 6 \)/group; \( \ast \ast \ast \) p < 0.001 w.r.t. WT obese). (C) IL-1β, (D) MCP-1 and (E) IL-6 and cytokine secretion from SVF cells and adipocytes from lean and obese mice cultured in serum rich media for 24 hours (seeded 1million cells/1 ml) (\( n = 12 \)/group; \( \ast \ast \ast \) p < 0.001 w.r.t. WT obese). (TIF)

Figure S4 Adipose tissue inflammation. (A) Immunoblot analysis of phosphorylated NFkB (p65), (B) p38, (C) ERK and control β-actin in adipose tissue of WT and MIF−/− mice. Densitometry analysis illustrates expression relative to β-actin expressed in arbitrary units (AU) (\( n = 3 \)/group), (C) Iris-1 mRNA in adipose tissue of lean and obese animals (\( n = 5 \)/group). (D) BMM ± LPS (10 ng/ml) iNos gene expression (\( n = 6 \)/group,\( p <

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**MIF and High-Fat Diet Induced Insulin Resistance**
were pre-treated with ISO-1 (50 μM) for 4–6 hours fasted mice treated with or without ISO-1 (n = 10/group).

(A) TNF-α (100 ng/ml) stimulation for 3 hours. Media was harvested for ELISA (n = 4/group).

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