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MIF Deficiency Does Not Alter Glucose Homeostasis or Adipose Tissue Inflammatory Cell Infiltrates during Diet-Induced Obesity

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Abstract

Objective—Circulating Macrophage Migration Inhibitory Factor (MIF) levels have been shown to positively correlate with body mass index (BMI) in humans. Our objective in this study was to determine the effects of MIF deficiency in a model of high fat diet-induced obesity.

Design and Methods—MIF wild type (MIF WT) and MIF deficient (MIF^{-/-}) C57Bl/6J mice were fed a high fat diet (HFD) for up to 15 weeks. Weight and metabolic responses were measured over the course of the disease. Immune cell infiltrates in visceral and subcutaneous adipose tissue were examined by flow cytometry.

Results—There was no difference in weight gain or adipose tissue mass in MIF^{-/-} mice compared to MIF WT mice. Both groups fed HFD developed glucose intolerance at the same rate and had similar elevations in fasted blood insulin. MDSC abundance was evaluated and showed no MIF-dependent differences. Macrophages were elevated in the visceral adipose tissue of obese mice, but there was no difference between the two groups.

Conclusions—While high fat diet feeding induced obesity with the expected perturbations in glucose homeostasis and adipose tissue inflammation, the presence or absence of MIF had no effect on any parameter examined.

Keywords

MIF; obesity; MDSC; macrophage; metabolism

Introduction

Obesity is a multifactorial disease affecting numerous tissues and physiological processes(1). Metabolic dysregulation during obesity leads to problems such as glucose intolerance, insulin resistance, and Type 2 diabetes (2, 3). Chronic inflammation also arises as a result of excessive weight gain (4, 5). Adipocytes that are damaged or stressed by high lipid intake produce signals that reprogram the inflammatory response from a maintenance

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Disclosure

The authors declared no conflict of interest.

Th2/M2 response to a proinflammatory Th1/M1 response (6, 7, 8). While not completely understood, these immunological changes are thought to contribute to the damage of adipocytes that aids in metabolic disruption resulting in problems such as insulin resistance(9).

Researchers and clinicians alike have become very interested in the potential roles of Macrophage Migration Inhibitory Factor (MIF) in the development of obesity. Human studies have demonstrated a strong positive correlation between body mass index (BMI) and circulating MIF levels (10, 11). It has also been shown that MIF mRNA levels in the visceral white adipose tissue (vWAT) positively correlate with percentage of body fat (12). Mice that are deficient in MIF are protected from the onset of glucose intolerance in the context of a pro-atherogenic model (LDLR^{-/-}) (13). These data have led to speculation that MIF contributes to some of the complications of obesity and that inhibition of MIF may provide a therapeutic approach for some of the co-morbidities of the disease (14, 15).

MIF is a widely expressed protein that participates in pathways significant for both metabolism and regulation of the immune system. MIF promotes insulin secretion in the pancreas (16). The absence of MIF protects beta cells from fatty acid-induced apoptosis and helps preserve beta cell function (16, 17). MIF deficient mice have a more rapid onset of age-induced glucose intolerance, although they exhibit no differences in glucose metabolism compared to WT mice throughout most of their lives (18). MIF deficient mice are also less susceptible to streptozotocin (STZ)-induced diabetes, in that multiple low doses of STZ failed to elevate blood glucose in MIF^{-/-} mice to the same degree as MIF WT (19, 20).

MIF has been shown to regulate several distinct inflammatory processes (21). The protein was originally identified and named for its ability to inhibit the random migration of macrophages in vitro (22) and has been shown to recruit macrophages in a CCL2-dependent manner in vivo (23). Using histologic staining for the MAC-3 surface marker, the study in LDLR^{-/-} mice demonstrated that mice that also lacked MIF (LDLR^{-/-}MIF^{-/-}) exhibited fewer macrophages infiltrating into epididymal adipose tissue (13). In the 4T1 breast cancer model, our group has shown that tumor-derived MIF positively regulates the abundance of monocytic MDSCs into the tumor (24). MIF has also been shown to promote alternative activation of tumor associated macrophages (TAMs) and suppressive function of both TAMs and MDSCs (25).

MDSCs are known to suppress T cells, and to some extent NK cells, and are best characterized in the context of cancer (26, 27). MDSCs are understudied in obesity; however, Xia *et al.* have demonstrated that MDSCs are increased during obesity and that they contribute to protection from both inflammation and development of insulin resistance that result from obesity (28). The monocytic subtype (mMDSC) is known to be more suppressive of T cells than the granulocytic population (gMDSC), and can also be induced to differentiate into macrophages under certain conditions (26, 29, 30). To our knowledge, the potential roles of the two recognized MDSC subsets, granulocytic MDSCs and monocytic MDSCs, have not been explored in any model of obesity.

The clinical data from obesity studies and the potential importance of MIF in both metabolism and immune cell infiltration led us to explore the effects of MIF deletion in the context of diet-induced obesity. Based on our observations in the 4T1 tumor model, we hypothesized that MIF would promote the prevalence of mMDSCs in obese vWAT and that this would contribute to the proinflammatory environment that promotes diet-induced glucose intolerance. Thorough studies of high fat diet-fed MIF WT and MIF^{-/-} mice demonstrate that the absence of MIF does not alter obesity, inflammatory cell accumulation in vWAT, or the development of high fat diet-induced glucose intolerance.

Methods

Animals

Heterozygous MIF^{+/-} Balb/c mice (31) were obtained from cryopreserved stocks maintained at Jackson Labs (Bar Harbor, ME). Mice were backcrossed onto C57Bl/6 using speed congenic microsatellite marker screening to allow completion of the backcross in six generations. Male 6- to 8-week old homozygous MIF wild type (MIF WT) and MIF deficient (MIF^{-/-}) C57Bl/6 mice were fed either normal chow (Harlan 7012) or 60% fat/kcal diet (High Fat Diet (HFD), Research Diets, New Brunswick, NJ) for up to 15 weeks. Mass of the animals was monitored over the course of HFD feeding. All experiments were conducted in accordance with guidelines of the University of Virginia Animal Care and Use Committee.

Isolation of Stromal-vascular Fraction (SVF)

At time of harvest, mice were perfused immediately post-mortem with PBS and visceral and subcutaneous adipose depots collected. Weights of these adipose depots were recorded, and lymph nodes were excised from the sWAT prior to further processing. Adipose tissue was finely minced in a solution of Krebs-Ringer-HEPES (KRH) Buffer plus 2.5% BSA and then digested with 2 mg of collagenase I (Worthington Biochemical, Lakewood, NJ) per gram of fat at 37°C for 45-60 minutes.

Tissue fragments were removed from the digests by straining through a 400µm pore mesh (Sefar, Buffalo, NY). The stromal-vascular cell fraction (SVF) was pelleted by centrifugation at 400xg for 10 minutes at 4°C. Floating cells (adipocytes) were removed by aspirating the supernatant and the SVF pellet was washed again before filtration through a 70µm pore filter to obtain a final single cell suspension.

Flow Cytometry

Flow cytometry was used to immunophenotype the isolated SVF with the following stains/antibodies: LIVE/DEAD Fixable Red Dead Stain (Invitrogen), CD45 PerCP (clone 30-F11, BD Biosciences), CD11b Pacific Blue (M1/70.15, Invitrogen), F4/80 APC-eFluor780 (BM8, eBioscience), Ly6C APC (HK1.4, Biolegend) and Ly6G FITC (1A8, BioLegend). Single cells were gated by forward and side scatter followed by gating for live, CD45+, CD11b+ to identify the myeloid cells. Further gating of Ly6C and Ly6G was utilized to discern mMDSC and gMDSC subtypes, while F480+ cells were defined as macrophages. A representative gating strategy is provided in Supplemental Figure 3. CountBright counting

beads (Invitrogen) were included in all samples to allow for determination of absolute cell numbers. Flow cytometry was performed using the CyAN ADP LX 9 Color Flow Cytometer (Beckman Coulter). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR) and gates were set based on fluorescence minus one (FMO) controls.

Liver Histology

Livers from mice that had been fed a HFD for 15 weeks were harvested following perfusion. The tissue was fixed for 24 hours in formalin, embedded in paraffin, sectioned, and stained with H&E.

Measurement of Blood Glucose and Blood Insulin Levels

To obtain random-fed blood samples, mice that had been on HFD for 12 weeks were separated into individual cages the night before blood collection and provided food and water *ad libitum*. The following morning (15-16 hours later) 2-3 mm of the tail of each mouse was amputated, and blood was collected to measure blood glucose using a glucose meter (Accu-Chek Advantage, Roche), following which 25-40 μ l of blood was drawn into a heparinized capillary tube. Plasma was isolated by centrifugation at 1600xg for 10 minutes at 4°C. Samples were stored at -20°C for batched assay.

For blood collection from fasted mice, animals fed a HFD for 11 weeks were separated into individual Sani-Chip (Harlan) bedding cages with water, but no food and fasted for 15-16 hours (overnight). Blood samples and glucose measurements were taken as described above, except that 50-75 μ l of blood was required from fasted mice. Plasma was isolated as described above and stored at -20°C.

Blood insulin levels in both fasted and random-fed mice were measured using an Ultrasensitive Insulin ELISA kit (ALPCO, Salem, NH) per manufacturer's instructions, except that detection was enhanced by substituting Amplex Red in phosphate buffer in place of the substrate provided (32).

Intraperitoneal Glucose Tolerance Tests

Mice were weighed and separated into individual Sani-Chip cages to fast for 15-16 hours with water, but no food. Baseline blood glucose measurements were taken using a glucose meter and blood from the tail of each mouse. Immediately after the baseline reading, each mouse was given an intraperitoneal (IP) injection of 1.4 g/kg of D-glucose. Subsequent blood glucose readings were taken at 15, 30, 45, 60, 90, and 120 minutes post-injection. Data were averaged and graphed as blood glucose as a function of time.

Statistics

Data are displayed as mean \pm SEM and were analyzed using one-way ANOVA, two-way ANOVA, or unpaired two-tailed Student's t-test as indicated in the figure legends. Significance was defined as $P < 0.05$ and is indicated by a single asterisk in the figures.

Results

MIF Deficiency Does Not Impact Weight Gain During Diet-Induced Obesity

The observed correlations between MIF levels and obesity in both humans and other animal models prompted us to determine if absence of MIF in a mouse model of diet-induced obesity would result in changes in weight gain over the course of the disease (10, 11, 17, 33). MIF WT and MIF^{-/-} C57Bl/6 mice were placed on HFD for 15 weeks. Total weight was tracked throughout the course of HFD feeding. We found that HFD-fed mice gained significantly more weight than their chow-fed controls; however, there was no difference in weight gain between the MIF WT and MIF^{-/-} groups (Fig 1 A).

It is possible that while there was no difference in the overall weight gain between MIF WT and MIF^{-/-} mice, these two groups might vary in the distribution of increased fat mass between the primary adipose tissue depots. Mice were sacrificed after 15 weeks on either HFD or normal chow diet for quantification of visceral adipose tissue (vWAT) and subcutaneous adipose tissue (sWAT) masses. While both MIF WT and MIF^{-/-} mice fed HFD had significantly larger fat depots compared to their chow-fed counterparts, both obese WT and obese MIF^{-/-} animals gained sWAT and vWAT masses equivalently (Fig 1 B and C).

To determine if MIF could be important in the early stages of obesity, we placed 4 week old mice on HFD for either 1 or 4 weeks. We found no difference between the weights of MIF WT and MIF^{-/-} mice at these stages, nor was there any difference in vWAT mass (Supplemental Figure 1 A and B). Taken together, these data suggest that MIF does not play a direct role in weight gain or distribution of expanding adipose tissue mass during the development of obesity in this model.

MIF Does Not Influence MDSC or Macrophage Infiltration into vWAT During Weight Gain

The inflammation that results from increased weight gain has pleiotropic effects on the organism, but is still poorly understood. MIF has been shown to be important in the recruitment of immune cells such as macrophages, neutrophils, and MDSCs in models of cancer and arthritis (23, 24, 34). Knowing that MIF is involved in the regulation of many inflammatory processes in various disease settings, we set out to determine if MIF influences MDSC or macrophage prevalence in obese adipose tissue.

Visceral and subcutaneous adipose tissues were harvested from MIF WT and MIF^{-/-} mice that had been fed a HFD for 15 weeks. The stromal-vascular fraction (SVF) from these depots was isolated and stained for monocytic MDSCs (Ly6C^{hi}, Ly6G^{lo}), granulocytic MDSCs (Ly6G^{hi}, Ly6C^{lo}), and macrophages (F4/80+). Neither diet nor genotype induced differences in mMDSC or gMDSC prevalence in the vWAT (Fig 2 A, Supplemental Fig 2 A), although the obese vWAT showed an increase in total MDSCs compared to chow controls (Supplemental Fig 2 E). As expected, we observed an increase in F4/80+ macrophages in the vWAT of HFD-fed mice; however, the absence of MIF did not lead to any change in the macrophage population as compared to the WT HFD group (Fig 2B, Supplemental Fig 2C). No differences in any of the three immune cell populations were found between MIF WT and MIF^{-/-} mice on HFD in the sWAT (Fig 2 B and D,

Supplemental Fig 2 B and D). Interestingly, MIF^{-/-} mice on HFD demonstrated a statistically significant increase in the percentage of infiltrating F480+ cells compared to the chow controls. This difference was not seen between the MIF WT HFD and MIF WT chow mice (Fig 2 D). Immunophenotyping of the visceral fat depots of mice that had been fed HFD for only 1 or 4 weeks also demonstrated no significant differences in MDSC infiltration at earlier time points (Supplemental Figure 1 C and D). These data demonstrate that the absence of MIF does not lead to differences in the abundance of MDSCs or macrophages in obese adipose tissue.

Knowing that excess lipids that are not successfully stored in the adipose depots are often deposited in the liver (35), we sought to determine if MIF deficiency might alter lipid deposition in the liver. We harvested livers from mice that had been on HFD for 15 weeks and stained sections with H&E. There was a marked increase in lipid accumulation in the livers of all obese animals; however, we saw no difference due to the presence or absence of MIF (Fig 3).

MIF Deficiency Does Not Influence the Development of Glucose Intolerance

Given the apparent absence of differences in weight gain and immune cell populations in the adipose tissue, we wanted to determine if there was a difference in the metabolic responses of MIF WT and MIF^{-/-} mice after an extended period on HFD. As a functional readout of glucose metabolism, intraperitoneal glucose tolerance tests (IPGTTs) were performed on cohorts of mice that had been on a HFD for 5, 8, or 12 weeks. As expected, a delay in the response to glucose challenge was observed in obese mice at all three time points. However, there was no difference in glucose response between the MIF WT and MIF^{-/-} mice, suggesting that the absence of MIF neither inhibits nor promotes the development of glucose intolerance (Fig 4 A-C).

Differences in insulin secretion could account for similar IPGTT curves that mask an underlying metabolic perturbation. Therefore, we measured both random-fed and fasted blood insulin levels in these mice. HFD groups showed slight elevations in their blood insulin levels in both random-fed and fasted conditions. However, there was no significant difference in insulin levels between the obese MIF WT and MIF^{-/-} groups (Fig 5 B and D). Random-fed and fasted blood glucose concentrations were monitored concurrently with insulin levels and while there was a slight elevation of fasted HFD glucose levels in obese mice compared to chow fed controls, again, there was no indication that the absence of MIF had any effect (Fig 5 A and C). Taken together, these data suggest that the presence or absence of MIF alone is not sufficient to change the development of insulin resistance during diet-induced obesity.

Discussion

We have shown that in a mouse model of diet-induced obesity, the absence of MIF does not impact weight gain or adipose tissue mass. The human data showing correlations between BMI and MIF (10, 11) might lead one to expect that lack of MIF would result in decreased weight gain compared to WT. Given our data, we believe that the increase in MIF seen during obesity may be an effect, rather than a cause of weight gain and increased adiposity.

For example, it is known that preadipocytes increase MIF secretion during the differentiation process in which mature adipocytes are formed(36). Obesity results in an increased rate of preadipocyte differentiation to accommodate increased nutrient intake, which might be predicted to lead to more MIF secretion from the adipose tissue (37).

In this study, we have demonstrated that MIF does not impact the abundance of mMDSC, gMDSC, or F4/80+ macrophage populations in the vWAT. These data do not support our initial hypothesis that MIF expression would positively regulate the prevalence of mMDSCs in adipose tissue during obesity, as we have shown in a breast cancer model (24). We did see the expected increase in macrophages and total MDSCs due to the onset of obesity (Fig. 2, Supplemental Fig 2); however, we did not observe any MIF-dependent changes in myeloid cell infiltration into vWAT during obesity. While our study carefully examined the number of cells infiltrating into the fat, we did not address the function of these cells. Therefore, it remains possible that MIF could exert some effect on the role that MDSCs or macrophages play once they enter into the vWAT. In particular, it is possible that MIF may influence the relative abundance of M1 vs M2 macrophages within the adipose tissue. The M1 vs M2 phenotype has been shown to play a role in the pathogenesis of obesity (38) however, even if there is a MIF dependent alteration in MDSC and/or macrophage function, that difference does not manifest in any changes in weight gain or in the metabolic effects of glucose intolerance as obesity develops.

Others have shown that MIF is a positive regulator of insulin secretion from pancreatic islets (16). It has also been demonstrated that MIF deficiency protects pancreatic islets from fatty acid induced apoptosis *in vitro* (17). These studies are compatible with our hypothesis that MIF^{-/-} mice fed a HFD would develop glucose intolerance at a slower rate than obese WT mice. Instead, we observed that both groups develop similar glucose intolerance and increased insulin levels on HFD regardless of MIF expression. This suggests that MIF is not a key regulator of the damage that leads to obesity-related disruption of glucose homeostasis and therefore is not a promising therapeutic target for modulation of conditions such as Type 2 diabetes. It is possible that we did not see the predicted metabolic changes in MIF^{-/-} mice because of compensatory pathways that could make up for MIF deficiency or MIF overexpression.

The role of MIF in the development of glucose intolerance has been studied in the context of an atherogenic mouse strain (LDLR^{-/-}). In this model, MIF deficient LDLR^{-/-} mice fed a chow diet developed glucose intolerance at a slower rate than LDLR^{-/-} mice that express MIF. The two groups of mice became obese at the same rate and had no differences in fat mass (13). This study differs from ours in that the effect of MIF deficiency was examined on an atherosclerosis prone background (LDLR^{-/-}) and the composition of the diets was not the same. Either of these factors could result in alterations in both the metabolic and immune responses of the mice. Based on our results, we would contend that while MIF deficiency is protective in the context of genetic predisposition for atherosclerosis, it does not exert an effect in a high fat diet model of obesity.

Serre-Beinier *et al.* demonstrated that MIF^{-/-} mice develop age-induced glucose intolerance. Their data showed that MIF WT and MIF^{-/-} mice had no significant differences in glucose

tolerance at 2 or 4 months of age. The latest timepoint tested, 12 months, was the only one to show that MIF^{-/-} mice were more glucose intolerant than MIF WT mice (18). It is possible that our mice would have shown similar changes over time had our study extended as long. In a different model, Harper *et al.* demonstrated that MIF^{-/-} mice on a C57BL/6J x 129/SvJae background exhibit increased longevity and are significantly smaller when compared to MIF wild type controls (39). This may be a strain-specific difference, as MIF^{-/-} mice on a pure C57BL/6J background are no different in size than their MIF wild type litter mate controls (40), though this study did not address longevity. It is important to note that while there are several studies that have looked at the role of MIF in control of various metabolic parameters, none have compared obese MIF WT and MIF^{-/-} animals to assess diet-induced glucose intolerance, and our data clearly point toward MIF expression not being a key factor in the dysregulated glucose homeostasis resulting from diet-induced obesity.

Our study was designed to determine if MIF would be a good therapeutic target for treating obesity-related metabolic and immunologic co-morbidities. There has been much speculation in the literature that controlled regulation of MIF during obesity may benefit patients; however, up until this point no one had investigated the impact of MIF deletion in an animal model of diet-induced obesity. We provide the first report that the absence of MIF alone is not sufficient to alter either glucose homeostasis or the inflammatory changes that accompany HFD-induced obesity. Taken together, our results suggest that MIF inhibition may not be a useful approach for correction of these aspects of obesity-related disease development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What is already known about this subject area:

- Circulating levels of macrophage migration inhibitory factor (MIF) positively correlate with BMI in patients.
- MIF functions within the metabolic system to regulate processes such as insulin secretion and has been shown to be able to both protect against and promote the development of glucose intolerance in various disease settings.
- MIF has been implicated in regulating the abundance of many immune cell types in models such as breast cancer and rheumatoid arthritis.

What this study adds to the field:

- Ours is the first study to examine the effect of MIF deficiency alone in a high fat diet (HFD) model of obesity.
- We demonstrate that the absence of MIF neither promotes nor inhibits the development of glucose intolerance in a HFD model of obesity.
- We show that the absence of MIF does not influence the abundance of myeloid derived suppressor cells or macrophages in the visceral white adipose tissue during HFD-induced obesity.

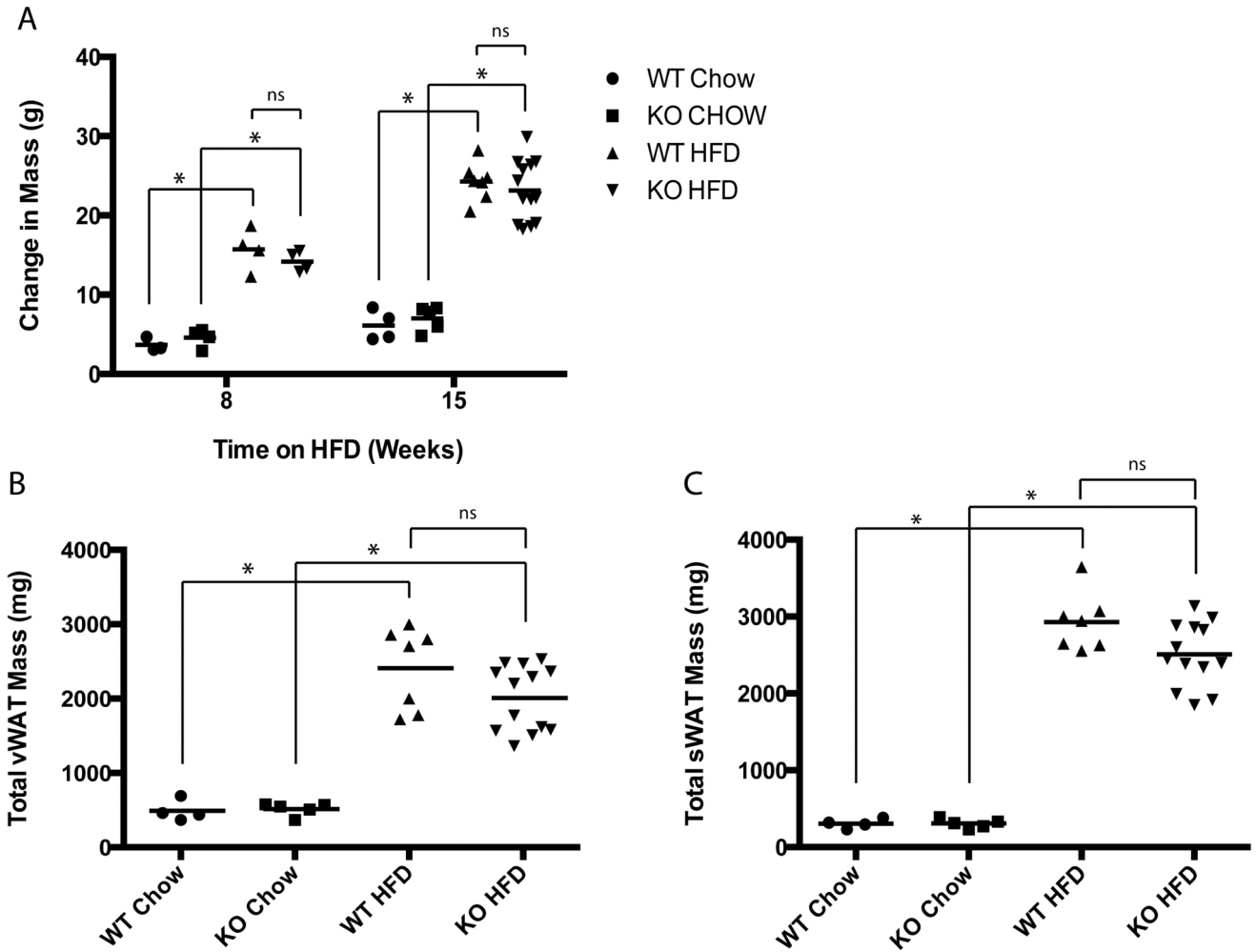


Figure 1. MIF deficiency does not impact weight gain or WAT mass during high fat diet-induced obesity

A) 6-8 week old WT and MIF^{-/-} C57Bl/6 mice were fed a normal chow or HFD for 15 weeks. Change in total mass of chow-fed or HFD-fed WT and MIF^{-/-} C57Bl/6 mice is shown after 8 and 15 weeks of diet. HFD-fed mice are significantly heavier than chow controls ($p < 0.05$), however, there are no differences between WT and MIF^{-/-} mice. B) Total vWAT and C) sWAT mass are not different between WT and MIF^{-/-} mice after 15 weeks of HFD. N= 4-13 per group. Statistical significance was tested using one-way ANOVA..

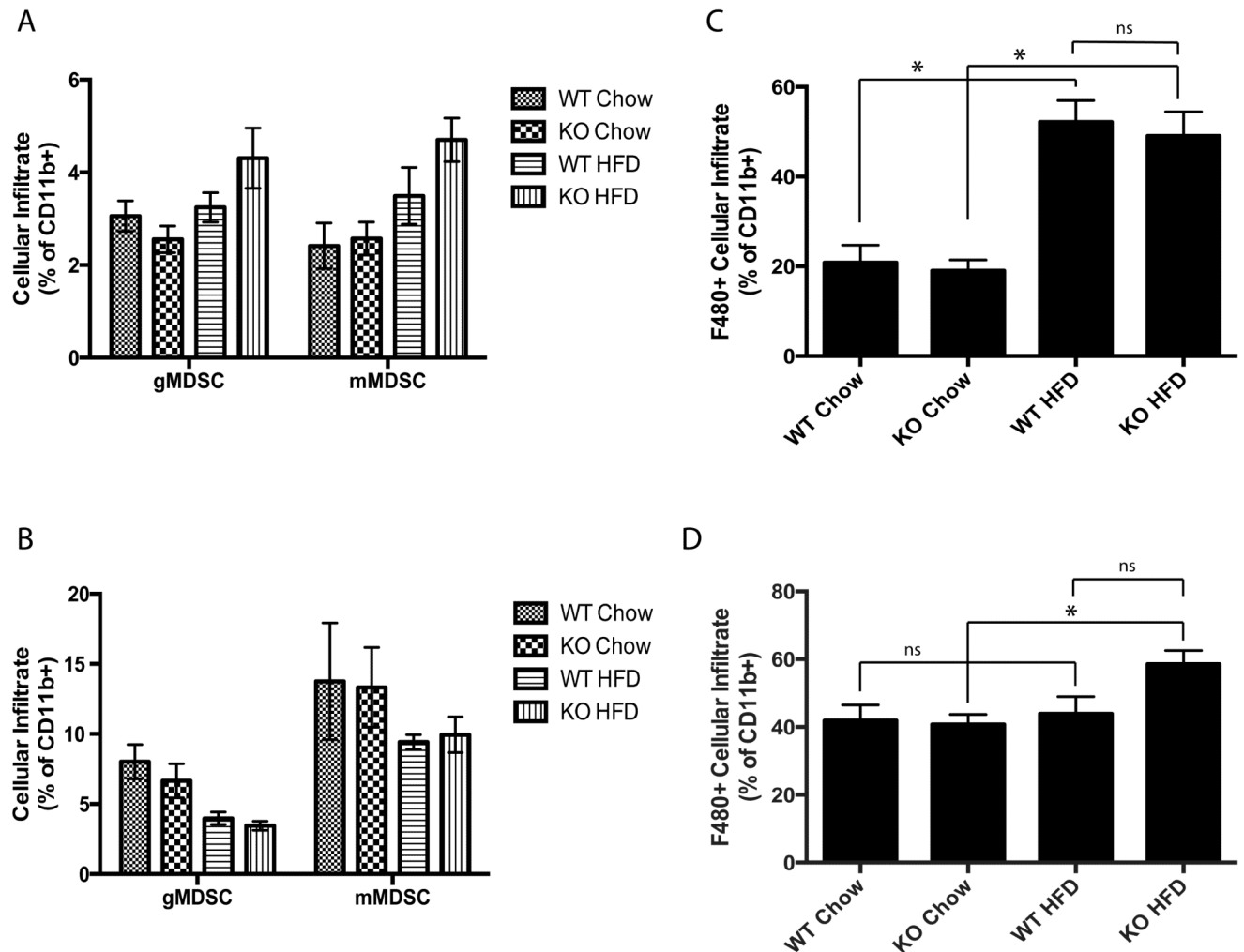


Figure 2. MIF deficiency does not affect MDSC or macrophage abundance during diet-induced obesity

A-D) The stromal-vascular fraction (SVF) was purified from the vWAT and sWAT of WT and MIF^{-/-} mice that had been fed either chow or HFD for 15 weeks. Cells were stained with a live/dead marker along with fluorescent antibodies for the cell surface markers CD45, CD11b, Ly6C, Ly6G, and F480. Among the live, CD45⁺, CD11b⁺ cell populations, no significant differences were seen in the gMDSC (Ly6G^{hi}, Ly6C^{lo}) or mMDSC (Ly6C^{hi}, Ly6G^{lo}) populations in the vWAT (A) or sWAT (B) of MIF WT versus MIF^{-/-} mice. Macrophages (F480⁺) were elevated in the vWAT (C) of obese mice ($p < 0.05$), however, no significant differences were seen in the vWAT (C) or sWAT (D) between WT and MIF^{-/-} mice on HFD. $N = 4-13$ per group. Statistical significance was tested using one- or two-way ANOVA. Asterisks indicate differences from chow controls.

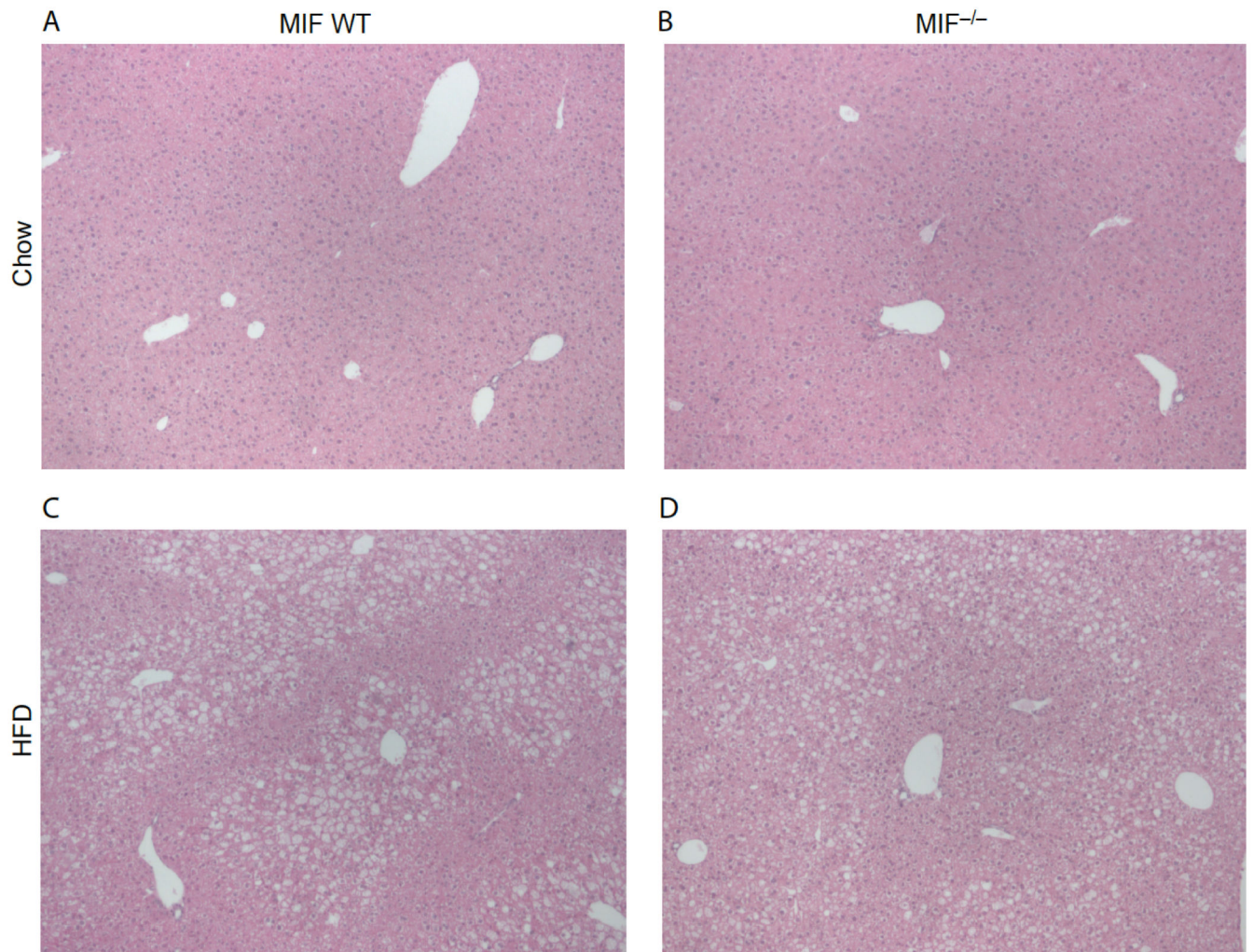


Figure 3. Fat deposition is the same in the livers of obese WT and MIF^{-/-} mice
Livers from 15 week chow-fed and HFD-fed WT and MIF^{-/-} mice were processed for histologic sections and stained with H&E. Chow-fed livers appear normal (A and B) compared to HFD-fed livers (C and D), which have marked lipid deposition. No MIF-dependent differences were observed in any condition tested. 8× magnification.

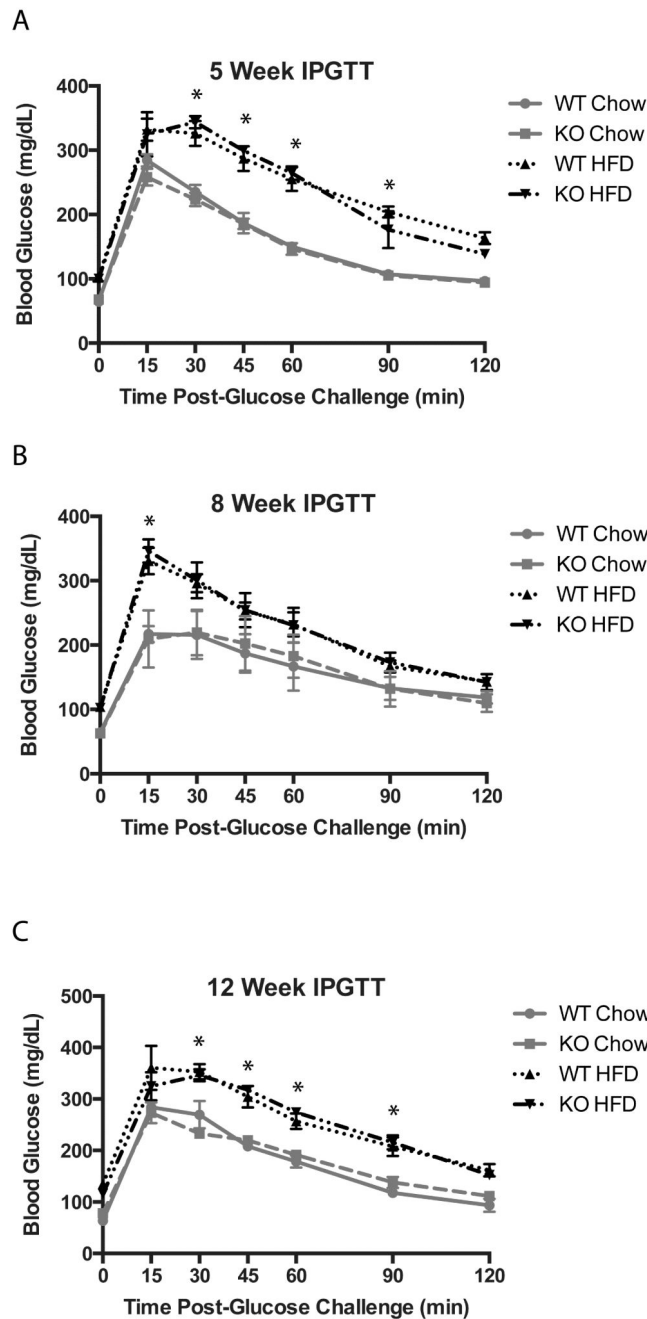


Figure 4. MIF deficiency does not impact the development of glucose intolerance during diet-induced obesity

Intraperitoneal glucose tolerance tests (IPGTTs) were performed on WT and MIF^{-/-} mice after 5 (A), 8 (B), and 12 (C) weeks of HFD. At all time points, chow-fed controls respond normally to glucose challenge, whereas HFD-fed mice have a delayed response in normalizing blood glucose levels, indicating the development of glucose intolerance. No significant differences were measured between the WT and MIF^{-/-} groups on either diet. Asterisks indicate time points at which both HFD groups were significantly different from

both chow groups ($p < 0.05$). $N = 3-4$ per group. Statistical significance was tested using one-way ANOVA.

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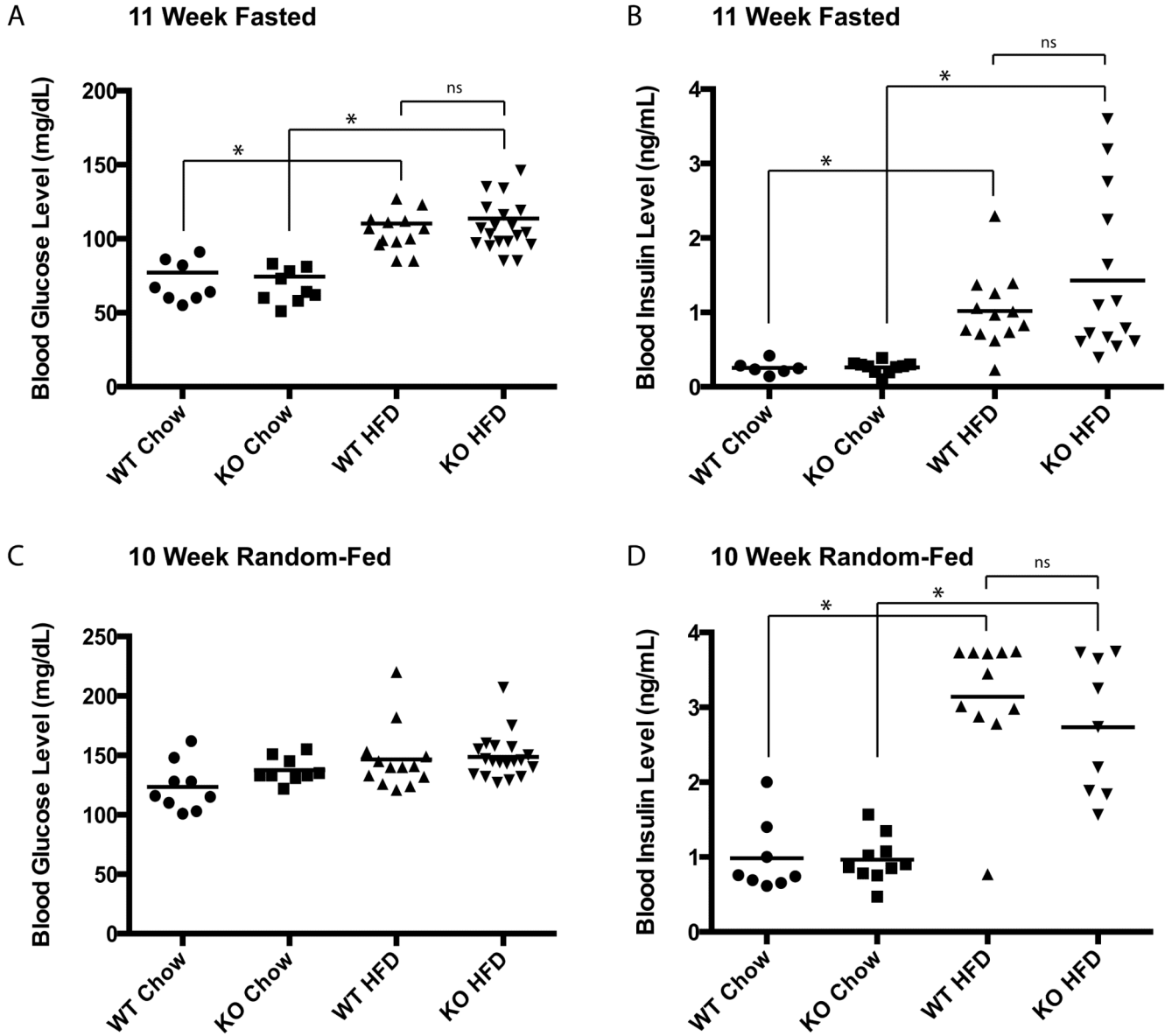


Figure 5. WT and MIF^{-/-} mice have comparable fasted and random-fed blood glucose and blood insulin levels in response to HFD

A and B) After 11 weeks of HFD, WT and MIF^{-/-} mice were fasted overnight (approximately 16 hours). Blood glucose levels (A) and blood insulin (B) were slightly elevated in obese compared to normal mice, however, no significant MIF-dependent differences were observed. C and D) During week 10 of HFD feeding, random-fed blood glucose (C) and blood insulin levels (D) were quantified. Random-fed blood insulin was increased in the chow compared to HFD groups (p<0.05); however, there were no significant differences between the WT and MIF^{-/-} groups. N= 8-19 per group. Statistical significance was tested using one-way ANOVA.