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## Sex differences in macrophage responses to obesity-mediated changes determine migratory and inflammatory traits

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### Abstract

The mechanisms whereby obesity differentially affects males and females are unclear. Since macrophages are functionally the most important cells in obesity-induced inflammation, we sought to determine reasons for male-specific propensity in macrophage migration. Whereas we previously determined that male mice fed high fat diet (HFD) exhibit macrophage infiltration into the hypothalamus, while females were protected irrespective of ovarian estrogen, here we show that males accumulate more macrophages in adipose tissues that are also more inflammatory. Using bone marrow cells or macrophages, differentiated *in vitro*, from male and female mice fed control and HFD, we demonstrated that macrophages derived from male mice are intrinsically more migratory. We determined that males have higher levels of leptin in serum and adipose tissue. Serum CCL2 levels, however, are the same in males and females, although increased in obese mice compared to lean mice of both sexes. Leptin receptor and free fatty acid receptor, GPR120, are upregulated only in macrophages derived from male mice, when cultured in the presence of free fatty acids (FFA) to mimic hyperlipidemia of obesity. Unless previously stimulated with LPS, CCL2 did not cause migration of macrophages. Leptin, however, elicited migration of macrophages from both sexes. Macrophages from male mice maintained migratory capacity when cultured with FFA, while female macrophages failed to migrate. Therefore, both hyperlipidemia and hyperleptinemia contribute to male macrophage specific migration, since increased FFA induce leptin receptors, while higher leptin causes migration. Our results may explain sex differences in obesity-mediated disorders caused by macrophages infiltration.

### Keywords

macrophage; obesity; leptin; high fat diet

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#### Author Contributions

KHE Chen performed most of the experiment reported herein and data analyses; N. Lainez performed cytokine analyses. D. Coss guided the study and wrote the manuscript.

#### Statement of Ethics

All experiments were performed with approval from the University of California (Riverside, CA) Animal Care and Use Committee and in accordance with the National Institutes of Health Animal care and Use Guidelines.

#### Disclosure Statement

The authors have no conflicts of interest to declare

## Introduction

The prevalence of obesity has increased steadily over the last 30 years (1). Currently, over half of the U.S. population is classified as overweight and a third is classified as obese (2). This obesity epidemic caused an increase in associated diseases, such as type 2 diabetes, cardiovascular disease, stroke, and dementia. Obesity is characterized by chronic inflammation and altered metabolic markers, such as hyperinsulinemia, hyperglycemia and hyperlipidemia (3). Increased adiposity causes an increase in inflammatory cytokines in the circulation, primarily due to infiltration and activation of macrophages in adipose tissue (4–9). The vast majority of studies that analyzed macrophages in adipose tissues during diet induced obesity (DIO) were conducted using only male mice. However, there are profound sex differences in adiposity and obesity-associated diseases (10). To address the gap, we analyzed sex differences in this study.

In men, obesity is associated with heart disease and myocardial infarction, while obese women are more prone to ischemic stroke. Although normal weight females have higher proportion of fat than normal weight males, when fed high fat diet (HFD), males accrue more adipose tissue (11). Differential accumulation of adipose tissue may contribute to sex differences in obesity-associated disease, but mechanisms that contribute to higher risk for diseases, such as heart disease and metabolic syndrome, in men are not clear (12). The presence of sex steroid hormones, specifically estrogen, was postulated to contribute to these sex differences (11, 13, 14). Alternatively, sex chromosome complement, in particular X-inactive specific transcript (XIST) expression in females, was shown to be critical for some sex differences (15). In support for the role of estrogen are observations that post-menopausal weight gain in women coincides with a loss of estrogen. Similarly, an increase in adiposity following ovariectomy and removal of ovarian estrogen was observed in rodents (16, 17) and in monkeys (18). Our previous results concur that ovarian estrogen is protective from obesity, but do not support the assumption that ovarian estrogen is necessary for protection from inflammation (19). We demonstrated that females are protected from immunological changes, in cytokine levels, and neuroendocrine changes, in hormone levels, regardless of the gonadal status. For example, males on HFD have increased levels of tumor necrosis factor (TNF)- $\alpha$ , leukemia inhibitory factor (LIF) and interleukin (IL)-6, while in females, unmodified or ovariectomized, TNF $\alpha$  and LIF are unchanged (19, 20). This may indicate that chromosomal differences contribute to intrinsic sex differences in the immune system.

Intrinsic sex differences in immune system and macrophages may contribute to sex differences in obesity-associated diseases, which were reported in human population and in response to DIO in rodent animal models (13, 21). Macrophages, particularly, demonstrate profound sex differences (22). Intrinsic differences in myeloid cells, irrespective of sex-steroid hormones, include enhanced myelopoiesis leading to proinflammatory responses in obese males (23). Macrophage infiltration into insulin-target tissues causes insulin resistance in obesity (8, 9), and insulin resistance maintains a greater incidence in men than women, after adjusting for age and adiposity (10). Male mice fed HFD have a higher propensity for insulin resistance than females, regardless of the strain (24). Myeloid-/macrophage-specific knock-outs of inflammatory pathway signals ameliorate hyperglycemia

and hyperinsulinemia, without preventing obesity when mice are fed HFD (9, 25, 26). Additionally, macrophage-derived molecules that are increased in obesity, such as resistin, contribute to insulin resistance (27). Correspondingly, neutralization of the elevated levels of proinflammatory cytokine TNF $\alpha$  improves insulin resistance (28). Therefore, we postulate that intrinsic sex differences in macrophages contribute to sex differences in macrophage infiltration to various tissues in obesity.

Macrophages infiltrate insulin-target tissues and other tissues in obesity (29). Our recent study demonstrated that peripheral macrophages infiltrate the hypothalamus of obese male mice, contributing to neuroinflammation that is associated with obesity (19). Infiltration of peripheral, circulatory macrophages occurs in addition to activation of resident immune cells, microglia (30, 31). Macrophages infiltrate muscle in mice and humans, where they contribute to insulin resistance and impede muscle regeneration and myogenesis (32, 33). Although the liver contains a resident macrophage population known as Kupffer cells, peripheral monocytes infiltrate the liver and give rise to proinflammatory macrophages, contributing to non-alcoholic fatty liver disease (NAFLD) (34). Several cytokines and chemokines are postulated to serve as chemoattractants for macrophages in obesity. Increase in free fatty acids or leptin in the circulation in obesity, may lead to recruitment of circulatory monocytes to tissues and lead to their activation (8, 35). Monocyte chemoattractant protein-1 (MCP-1, or CCL2 chemokine) is expressed by a variety of cell types upon stimulation with inflammatory signals and stimulates extravasation across the endothelium (36). In obesity, following increase in their size, adipose tissues produce CCL2, and recruit monocytes (37, 38). Another study demonstrated that CCL5, but not CCL2, contributes to macrophage accumulation and survival in adipose tissue (39). CCL5 is also produced by hepatocytes in obesity to stimulate immune cell infiltration and NAFLD (40). Therefore, it is still not clear which adipokine, cytokine or chemokine causes macrophage accumulation in adipose tissue. Furthermore, whether there are sex differences in macrophage response to these stimuli is not known. Herein, we provide answers to both of these questions.

To analyze sex differences and diet-induced changes in monocytes and macrophages, we compared chemoattractive molecules and macrophages in adipose tissues *in vivo*; and macrophages from male and female, control and diet-induced obese (DIO) mice that were differentiated in culture. We used C57BL/6J mice, the most commonly used strain to study DIO and metabolic syndrome because mice become obese, insulin resistant, and hypertensive, when fed HFD (41, 42). We used bone marrow cells and bone marrow derived macrophages (BMDM) from control-fed and HFD-fed males and females, and analyzed them separately to determine sex differences and diet-induced differences. BMDM from males and females were differentiated using standard protocol in the same medium, without the presence of sex steroids to analyze intrinsic sex differences in macrophages. Since obesity is a state of low grade chronic inflammation, we determined sex differences in obesity, without prior infection or activation by LPS (43–45), which may mask obesity-mediated changes. Herein, we demonstrate profound sex differences in macrophage migration and in their response to increased free fatty acids (FFA). Male macrophages express more inflammatory cytokines and are more migratory in obesity, due to upregulation of both leptin and free fatty acid receptors. These intrinsic differences may contribute to

higher infiltration of macrophages in peripheral tissues in obesity that leads to insulin resistance and pathophysiologies of obesity. A better understanding of sex differences with respect to macrophage responses may contribute to better prevention and treatment for obesity and related diseases.

## Materials and Methods

### Animals

C57BL/6J mice were maintained under a 12-h light, 12-h dark cycle and received food and water *ad libitum*. All experiments were performed with approval from the University of California (Riverside, CA) Animal Care and Use Committee and in accordance with the National Institutes of Health Animal care and Use Guidelines. C57BL/6J mice were placed on either high fat diet (HFD, D12492, 60% kcal from fat; 5.21 kcal/g; protein 20% kcal; carbohydrate 20% kcal; fat 60% kcal (lard 0.32 g/g diet, soybean oil 0.03 g/g) Research Diet, New Brunswick, NJ) or control diet (CTR, D12450J, 10% kcal from fat; matching sucrose levels to HFD; 3.82 kcal/g; protein 20% kcal; carbohydrate 70% kcal; fat 10% kcal (lard 0.02 g/g diet, soybean oil 0.025 g/g) Research Diet, New Brunswick, NJ) from weaning age for 15 weeks. We reported before that females on HFD have irregular estrous cycles (19). Females were collected in diestrus after 15 weeks on control or HFD. Each animal was processed independently.

### Cytokine assay

For serum collection, mice were sacrificed by isoflurane inhalation and blood was obtained from the inferior *vena cava*. The blood was left to coagulate for 15 min at room temperature, and then centrifuged at 2000 RCF for 15 min for serum separation. Adipose tissue was collected from gonadal fat pads and processed for protein extraction. Fat tissue was homogenized using a bead beater in lysis buffer (PBS with 0.1% NP-40 and protease inhibitors, Sigma Cocktail P8340), centrifuged at 12,000g for 20 minutes 4°C. Protein lysate was separated from fat layer using a 27 gauge needle (repeated twice). Protein concentration was determined using Bradford assay, and 50 ug of protein was used in the Luminex assay. Levels of leptin and CCL2 in serum and gonadal fat tissue protein lysates were measured using Luminex MagPix instrument and mouse Adipokine or Chemokine panel (Millipore, Burlington MA).

### Flow cytometry

Tissues from each mouse were processed separately as part of a 5-mouse cohort per group, with each experiment repeated 3 times. In brief, mice were perfused with ice cold PBS, adipose tissue was collected from gonadal fat pads, rinsed in cold PBS, weighted, minced with razor blade and digested enzymatically with 3 mg/mL collagenase at 37 C for 2 hour. Suspension was passed through 40um cell strainer, centrifuged to pellet stromal vascular fraction, cells collected and 2 million cells labeled for flow cytometry analyses. Cells were Fc-blocked with anti-CD16/CD32 (1:100, 553141, BD Biosciences, San Jose, CA) followed by surface marker staining with antibodies to F4/80 (Brilliant Violet 650; 1:400, 123149, Biolegend, San Diego, CA) and CD11b (Brilliant Violet 605; 1:400, Biolegend, San Diego, CA). Flow analysis was performed with BD LSR II Flow

Cytometer using the following gating strategy: cells, singlets, live cells, F4/80+CD11b+. For intracellular staining, cells were pretreated with 0.0026% Golgistop (BD Biosciences, CA) for 4 hours to block cytokine release, fixed, permeabilized with Cytofix/Cytoperm Fixation/Permeabilization solution (BDB555028, BD Biosciences, San Jose, CA) and stained with fluorophore-conjugated antibodies specific for IL-6 (anti-IL6 APC, 1:400, 504507, Biolegend, San Diego, CA), IL-10 (anti-IL10 PerCP/Cy5.5, 1:400, 505027, Biolegend, San Diego, CA) and TNF $\alpha$  (anti-TNF $\alpha$  FITC, TN3–19.12, 11–7423-82 eBioscience, San Diego, CA). Results were analyzed using FlowJo software (Tree Star, Inc.).

### **Collection of bone marrow cells and adipocytes, and ex vivo derivation of bone marrow derived macrophages (BMDM)**

For cell collection, animals were perfused with ice cold phosphate buffer saline (PBS). Bone marrow cells were collected from hind limb long bones as previously described (46). Briefly, the femurs were dissected using sterile surgical scissors. Muscles connected to the bone were removed, and the femurs then were placed in sterile PBS with 1% Penicillin-Streptomycin on ice. In a tissue culture hood, epiphyses were removed using sterile scissors and forceps and the femurs were then placed in sterile 200ul pipet tips. A brief centrifugation was performed using table top centrifuge to collect the bone marrow cells from the femurs. For gene expression analyses, cells were lysed using Trizol. Bone marrow derived macrophages (BMDM) are differentiated from femoral bone marrow cells for 7 days in RPMI 1640 with 10% FBS, in the presence of 30% conditioned medium from the L929 cell cultures that produce macrophage colony-stimulating factor (M-CSF). When indicated, cells were treated with 5% free fatty acid supplement (Sigma, MO) for 7 days, or co-cultured with adipocytes. Matured adipocytes were collected from visceral fat pad as described by (47). Briefly, after fat depot digestion as described above, the supernatants that contain adipocytes were cultured in an inverted completely filled flask. Adipocytes and bone marrow cells (and later their derivatives to BMDM) for co-cultures were collected from the same mouse to ensure isogenic conditions. To analyze a role of estrogen in sex differences, BMDM with and without free fatty acids were also cultured in the presence of 1 $\mu$ M ICI 182,780 (48, 49) for the duration of free fatty acid treatment.

### **qPCR Analysis**

Total RNA was extracted using Trizol (Invitrogen, CA), quantified using Nanodrop and the same amount per sample reverse transcribed using Superscript IV (Invitrogen, CA). qPCR was performed using an iQ SYBR Green supermix and an IQ5 real-time PCR machine (Bio-Rad Laboratories, Hercules, CA), with primers listed in Table 1, under the following conditions: 95 °C for 15 min, followed by 40 cycles at 95 °C for 20 s, 56 °C for 30 s, and 72 °C for 30 s. The amount of the gene of interest was calculated by comparing the threshold cycle obtained for each sample with the standard curve generated in the same run and normalized to the TATA-binding protein (TBP) or GAPDH housekeeping genes, as indicated, in the same sample using Ct method. Preliminary experiments analyzed several housekeeping genes and determined that these housekeeping genes do not change with treatments. Replicates were averaged. After each run, a melting curve analysis was performed to confirm that a single amplicon was generated in each reaction.

## Transwell™ Migration Assay

Two million BMDM, collected using accutase (Innovative Cell Technologies, CA), in 300  $\mu$ L were placed in the upper chamber of Boyden chamber / Transwell™ dish (5  $\mu$ m pore size, Corning 3421, NY) and 1mL medium containing chemoattractant (either 50  $\mu$ g/mL MCP-1 (479-JE, R&D Systems, Tustin, CA) or 100 ng/mL Leptin (ab9750, Abcam, Cambridge, MA)) or medium with vehicle control was added to the lower chamber. Cells that migrated into the lower chamber during 2 hour were collected and counted.

## Statistical analyses.

We focused on differences between control and HFD within males and females, and sex differences between males and females. Statistical differences ( $p < 0.05$ ) were determined by ANOVA followed by Tukey's test for multiple comparisons using Prism software (GraphPad, CA). Interaction between sex and diet was analyzed by two-way ANOVA using Prism software (GraphPad, CA).

## Results

### Males accumulate more macrophages in adipose tissue

Chronic inflammation, elicited by high fat diet (HFD), is a hallmark of obesity. Macrophages that are activated in obesity infiltrate not only the adipose tissue but other tissues as well and contribute to insulin resistance and potentially other negative effects. We demonstrated profound sex differences in the diet-induced inflammation in obese mice, that were independent of ovarian estrogen (19). To investigate if these sex differences stem from intrinsic differences in the immune system, we initiated our studies with an analysis of macrophage numbers in the adipose tissue of C57BL/6 male and female mice, fed control and HFD for 15 weeks. Male mice weighed 47.94 grams after 15 weeks on HFD, compared to 27.53 grams for mice on control diet. HFD female mice weighed 29.28 grams, while control-fed female mice weighed 21.2 grams. Macrophages accumulate in adipose tissue in male mice in agreement with previously published reports. Earlier studies that determined macrophage numbers used a single marker to characterize adipose tissue macrophages in male mice (7, 50); here we analyzed double positive F4/80+CD11b+ population in both males and females using flow cytometry. The proportion of macrophages in the stromal vascular fraction of gonadal fat tissue reached 14.2% in HFD male mice (M hfd, black squares), compared to 5% in control male mice (M ctrl, white squares, Fig. 1A, each square represents one animal; a bar represents group mean; star indicates statistical significance between groups). Despite the weight increase, females did not exhibit significant increase in the proportion of macrophages in the adipose tissue (circles, Fig. 1A). We then determined the absolute number of macrophages (Fig. 1B). Male mice fed HFD had 5-fold more macrophages than lean male mice fed control diet, while in females the number doubled. These cohorts of mice exhibited the same increase in fat pads' weight as we reported before (19). In both male and female mice, fat pad weight increased by ~2.5 fold with HFD. When macrophage numbers were normalized to milligrams of fat tissue, HFD males had 10 times more macrophages than HFD females, while control males had 2 times more macrophages than control females. Therefore, male mice accumulate more macrophages in adipose tissues when exposed to HFD.



## Macrophages from male mice remain more inflammatory and more migratory after *in vitro* differentiation

Previously, we reported that females synthesize higher levels of anti-inflammatory IL-10 than males, in the hypothalamus of control diet fed mice, a difference that is exacerbated in HFD mice. That led us to postulate IL-10 as a reason for female protection from neuroinflammation (19). Here, we analyzed sex differences in the expression of prototypical inflammatory cytokine, TNF $\alpha$ , and anti-inflammatory cytokine, IL-10, in the adipose tissues of control and HFD males and females (Fig. 2A, B). We determined that HFD males increased the expression of TNF $\alpha$  in fat tissue, while the increase in TNF $\alpha$  in females was not significant. Thus, males on HFD expressed a higher level of TNF $\alpha$  than females on HFD (Fig. 2A). IL-10 expression in adipose tissue, on the other hand, increased in female mice on HFD, coinciding with our previous observation in the hypothalamus (Fig. 2B, (19)). We then analyzed expression of these cytokines in the bone marrow cells from males and females following control and HFD (Fig. 2C, D). Similar to adipose tissue, bone marrow cells from male mice fed HFD expressed higher levels of TNF $\alpha$  than control diet males, while in female cells, diet did not cause a change in TNF $\alpha$  expression (Fig. 2C). Expression in control males was higher than in control females, indicating that males expressed higher level of TNF $\alpha$  pro-inflammatory cytokines in basal conditions. Bone marrow cells from HFD males expressed more TNF $\alpha$  than HFD females. IL-10 expression, on the other hand, was higher in control female bone marrow cells than in control male bone marrow cells (Fig. 2D). Males on HFD increased IL-10 expression in bone marrow cells compared to males on control diet, while in females this trend was not significant. We utilized flow cytometry to analyze intracellular TNF $\alpha$  and IL-10 to assess a percentage of adipose tissue macrophages that synthesized these cytokines (Fig 2E, F, G). Adipose tissue macrophages were identified as double positive F4/80+CD11b+ cells and from that population, percentages of adipose tissue macrophages that contained intracellular TNF $\alpha$  and IL-10 were determined. As described in Fig 1, males have an increased percentage of F4/80+CD11b+ positive cells in the stromal vascular fraction (Fig. 2G top panels). To analyze percentages of these cells that synthesize cytokines, we prevented the secretion of cytokines using Golgi block in order to successfully stain for intracellular cytokines. A larger percentage of male macrophages contained TNF $\alpha$  than female macrophages (Fig. 2E and middle panels in Fig. 2G), while higher proportion of female macrophages contained IL-10 (Fig. 2F and bottom panels in Fig. 2G). However, there were no differences in the proportions of positive cells between diets (Fig. 2E, F). There was also no difference in the intensity of staining between sexes or between diets (MFI, data not shown), indicating lack of differences in the amount of either cytokine in each macrophage. Our results demonstrate that the increase in TNF $\alpha$  mRNA in adipose tissue in HFD males (Fig. 2A) stems from the influx of macrophages (Fig 1A and Fig. 2G, top panel), since there is no increase with HFD in the proportion of macrophages in the adipose tissue that contain the cytokine (Fig. 2E) or in the cytokine level per cell (not shown). Significantly, our data also show that male macrophages are more inflammatory than female macrophages.

Since macrophages in obesity infiltrate not only adipose tissue, but other tissues such as liver, muscle and hypothalamus, we then determined the migratory capacity of bone marrow derived macrophages (BMDM) from female and male mice fed control and HFD. Bone



marrow cells were differentiated into macrophages according to the published protocol (46) and transferred into Transwell Boyden chamber dishes and their migration monitored after 2 hours. Naïve macrophages derived from male mice were 4 times more migratory than those derived from female mice (Fig. 3). There was no difference between macrophages derived from lean or obese mice in either sex. Taken together, our results indicate that male mice have more macrophages in their adipose tissues than females, likely due to males having macrophages that are more inflammatory and more migratory.

### **Males have higher levels of leptin that may attract macrophages**

We previously reported that males on HFD have higher levels of inflammatory cytokines than control-fed lean male mice. Female mice, on the other hand, although gained weight when fed HFD, did not show an increase in serum levels of TNF $\alpha$  or LIF (19). Females were protected irrespective of ovarian estrogen, and neither unmodified nor ovariectomized females on HFD had elevated cytokines. This prompted us to investigate sex differences in adipokines and/or cytokines that were postulated to contribute to macrophage recruitment to adipose tissue. We measured leptin, CCL2 / MCP-1 and CCL5 / RANTES in the serum and adipose tissue of control and HFD male and female mice (Fig. 4). There was a significant increase in serum leptin levels in HFD males (6.2 ng/ml) compared to control males (1.3 ng/ml; Fig. 4A). Importantly, HFD males had higher serum leptin (6.2 ng/ml) than HFD females (3.1 ng/ml). In fat tissues, leptin also exhibited sex differences. At the mRNA levels, on control diet, males express higher levels of leptin mRNA compared to females on control diet (Fig. 4B). Both males and females up-regulate leptin expression on HFD. At the protein level, only in males did the increase in leptin reach significance following HFD (35.3  $\mu$ g/g in HFD compared to 10.6  $\mu$ g/g in controls), while in females there was no statistical difference in leptin levels between controls and HFD (3.2  $\mu$ g/g and 6.2  $\mu$ g/g, respectively) (Fig. 4C). Our results demonstrate that male adipose tissue in control conditions synthesizes higher levels of leptin than female control adipose tissue. Furthermore, HFD males have higher levels of leptin than HFD females in fat tissue.

CCL2 / MCP-1, as expected, increases in serum in HFD males compared to control males from 20.6 pg/ml to 32.9 pg/ml, and in HFD female mice compared to control female mice from 23.1 pg/ml to 42.4 pg/ml, but there was no difference between control diet females and control diet males, or HFD females and HFD males (Fig. 4D). Thus, there is no sex difference in serum levels of CCL2. In the fat tissue, at mRNA (Fig 4E) and protein levels (Fig. 4F), males increased CCL2 levels 3-fold and 6-fold on HFD, respectively, compared to control diet, while females increased CCL2 levels in fat 1.3-fold on HFD which was not significant (Fig. 4E, F). Thus, males on HFD had significantly higher levels of CCL2 in adipose tissues than females on HFD, while there was no sex difference in the control mice.

Serum CCL5 / RANTES on the other hand, is the same in both males and females on HFD compared to control diet and was the same between sexes (Fig. 4G). In the adipose tissue, both males and females increased expression of CCL5 mRNA on HFD, but there were no sex differences (Fig. 4H). CCL5 protein in adipose tissues of control males was higher than in control females and HFD males had higher levels than HFD females (Fig. 4I). Although

there was no difference between control and HFD in either males or females, males had 2-fold higher levels than females.

Next, we assessed receptor levels for the ligands analyzed above, in bone marrow cells and bone marrow derived macrophages (BMDM) from males and females fed control or HFD. There was no difference in the expression level of leptin receptor (LEP-R) in bone marrow cells between diets, however female mice had higher expression than males (Fig. 5A). This sex difference was lost during differentiation into BMDM (Fig. 5B). Thus, although leptin concentration is elevated in the serum and adipose tissue in males fed HFD, the levels of leptin receptor in bone marrow cells or macrophages do not change with diet.

CCR2 receptor is expressed on macrophages and binds MCP-1 / CCL2 ligand. CCR2 expression was higher in bone marrow cells from HFD males than control males and in cells from HFD females than control females, but there were no sex differences (Fig. 5C). Therefore, both the ligand, CCL2, and its receptor CCR2, increase with HFD in both sexes. The difference with diet was lost during differentiation *in vitro* and BMDM expressed similar levels of CCR2 regardless of sex or diet (Fig. 5D). Expression of CCR5 that binds CCL5 / RANTES was higher in bone marrow cells from female mice than in bone marrow cells from male mice, however there was no difference between diets (Fig. 5E). In BMDM this sex difference was lost, and there was no difference between sexes or between diets in CCR5 expression (Fig. 5F). These results indicate that, as expected, leptin is increased in obesity, and that this increase is much higher in males than in females.

### Hyperlipidemia of obesity increases leptin receptor levels in male macrophages

Increase in leptin concentration in obese males is not accompanied with increased levels of receptors (Fig. 5A). To determine how macrophages respond to increased leptin concentration, we cultured BMDM in the presence or absence of free fatty acids (FFA) to mimic hyperlipidemia present in obesity. Macrophages derived from male mice exhibited 6-fold increase in the expression of leptin receptor in response to FFA, compared to controls (LEP-R, Fig. 6A). Macrophages from female mice increased LEP-R 1.7-fold in response to FFA, but changes from control culture conditions did not reach significance (Fig. 6A). Moreover, macrophages derived from male mice fed HFD and in the presence of FFA had higher levels of LEP-R than macrophages derived from male mice fed control diet in the presence FFA. When cultured with FFA, macrophages from male mice on HFD exhibited increased expression of CCR2, compared to macrophages cultured in the vehicle control or those derived from male mice on control diet (Fig. 6B). Female-derived BMDM did not increase levels of CCR2 expression compared to controls.

We also analyzed expression levels of GPR120 (FFAR4), which is a receptor for saturated and unsaturated long chain free fatty acids (51). Expression of GPR120 increased 3.5-fold in the male BMDM cultured in the presence of FFA, but not in female-derived BMDM (Fig. 6C). There were no differences in the GPR120 expression in BMDM from control or HFD fed mice regardless of sex.

Surprisingly, when cultured in the presence of FFA, regardless of sex or diet, BMDM lost expression of CCR5 (Fig. 6D). We further analyzed insulin receptor and IGF-1 receptor.

Expression of insulin or IGF-1 receptors in BMDM was the same regardless of sex, diet or culture conditions (data not shown). To determine if increases in LEP-R and GPR120 in male BMDM occur due to hyperlipidemia or adipocyte hypertrophy, which are hallmarks of obesity, we co-cultured BMDM with adipocytes. Adipocytes were purified from the same mouse as BMDM to assure isogenic conditions. There were no changes in the expression in LEP-R or GPR120 receptors when BMDM were co-cultured with adipocytes (data not shown). Therefore, BMDM from male mice in particular those fed HFD, increase expression of leptin receptor, free fatty acid receptor GPR120 and chemoattractant receptor CCR2 when cultured with FFA, while female derived BMDM do not. These results also demonstrate that hyperleptinemia of obesity is not sufficient to increase leptin receptor levels in macrophages, but increased free fatty acids lead to elevated leptin receptor levels particularly in male macrophages.

To determine if estrogen plays a role in observed sex differences, we cultured BMDM with ICI 182,780 (ICI), an estrogen receptor (ER)  $\alpha$  antagonist (48, 49). We focused on ER $\alpha$ , since ER $\alpha$  has a larger role in myeloid cells responses to obesity (52) and is expressed at a higher level in mouse and human macrophages than ER $\beta$  (53). When cultured with FFA, both male and female BMDM increase expression of TNF $\alpha$ , regardless of the presence of ICI (Fig. 7A). However, cells derived from different animals had varied degree of response, eliciting a lack of significant changes in expression. IL-10 was expressed at a higher level in female BMDM than male BMDM, consistent with results using bone marrow cells presented in Fig. 2D (Fig. 7B). After exposure to FFA, IL-10 expression in male BMDM increased 5.5-fold, while IL-10 expression in female BMDM increased 23-fold. Higher increase in response to FFA in female-derived BMDM than in male-derived BMDM, corresponds to higher IL-10 induction in female adipose tissue than in male adipose tissue in obesity *in vivo*, presented in Fig. 2B. In the presence of ICI, inhibiting estrogen signaling, increase in IL-10 expression in response to FFA was 40% lower in both males and females (Fig. 7B). Thus, estrogen contributes to maximal induction of IL-10 by FFA, but sex differences in IL-10 expression remain. As presented in Figs 6A and 6B, expression of LEP-R and GPR120 increased in male BMDM in response to FFA, and ICI treatment didn't cause significant differences (Figs. 7C, 7D). Therefore, estrogen does not play a role in male macrophage induction of leptin and GPR120 in response to FFA, but it may partially contribute to IL-10 expression in females, which may provide protection.

### Macrophages from male mice are more migratory in response to leptin

Given that we determined that naïve macrophages from male mice are intrinsically more migratory than macrophages from female mice, we wanted to compare migration in the presence of FFA. Since both leptin and MCP-1 / CCL2 are increased in obese males, and macrophages from male mice cultured in the presence of FFA increase expression levels of leptin receptor, GPR120 and CCR2, we analyzed migration in response to leptin and CCL2 with and without FFA. In agreement with previous studies, CCL2 causes macrophage migration after their activation: we observed BMDM migration in response to CCL2 when cells were pretreated with LPS (Fig. 8A). Given that LPS is not normally present in the adipose tissue in the absence of infection, while increase in FFA is a hallmark of obesity,

we assessed if macrophages are migratory in response to CCL2 in the presence of FFA (Fig. 8B). CCL2 failed to cause migration in the absence of LPS, with or without FFA.

We then analyzed leptin-stimulated migration and observed an increased migration in response to leptin (Fig. 8C). Presence of FFA prevented migration of macrophages in response to leptin from female mice, but not from males (Fig. 8C). Therefore, male macrophages maintain migratory capacity in culture conditions mimicking hyperleptinemia and hyperlipidemia of obesity, while female macrophages do not. Taken together, our results demonstrate that 1) male mice have higher leptin in adipose tissue than female mice and further increase leptin synthesis in obesity, to a higher level than females; 2) macrophages derived from male mice increase the expression of leptin receptor when cultured in the presence of FFA and 3) male macrophages are intrinsically more migratory, migrate in response to leptin and maintain migration in the presence of FFA. Our results may explain higher infiltration of macrophages in obese adipose tissue in males.

## Discussion

We sought out to uncover mechanisms of sex differences in obesity-mediated inflammation that contribute to male propensity for insulin resistance. While it is known that males on HFD gain more adipose tissue than females, that recruits and activates macrophages, the reasons for these differences are only beginning to emerge. Our previous study determined that major obesity-mediated sex differences in inflammatory and endocrine changes occur irrespective of ovarian estrogen (19). Herein, we identify several differences between male and female macrophages and in the expression of cytokines in male and female adipose tissues. We determined that male adipose tissues accumulate more macrophages even when the numbers are normalized to the adipose tissue weight. We also demonstrate that male macrophages are more inflammatory and more migratory, while females may be protected due to higher expression of anti-inflammatory cytokines. Significantly, obese males have much higher levels of leptin than obese females, and macrophages from male mice increase migration in response to leptin. However, this elevated leptin is not sufficient to induce leptin receptor in male macrophages. When cultured in the presence of free fatty acids, which mimics hyperlipidemia of obesity, male macrophages increase expression of leptin receptor, which may allow macrophage migration to various tissues in response to higher leptin in males. A previous study that analyzed immune cell autonomous response to obesity determined intrinsic sex differences in myeloid cell, and posited that males increase myelopoiesis leading to inflammation (23). We postulate that males exhibit higher leptin levels, due to differential deposition to fat depots as we demonstrated before (19). Higher leptin, combined with intrinsically higher migratory capacity and higher leptin receptor induction in response to free fatty acids in male macrophages, leads to more macrophage accumulation in male adipose tissues and higher inflammation.

Leptin is produced by adipocytes and its expression increases in response to adiposity and adipocyte hypertrophy. In humans, elevated leptin leads to monocyte activation and proliferation (54). Males especially increase levels of leptin in the serum following exposure to HFD, corresponding to the increase in weight. In the females, 2-fold increase in leptin didn't reach significant levels. It is possible that the increase in leptin, in the circulation and

in adipose tissue, is higher in males than in the females, because males on HFD gain more weight (175% compared to males on control diet) than females on HFD (140% compared to females on control diet). Bone marrow cells of female mice express higher levels of leptin receptor, and in neither sex the expression changes in response to diet. This difference was lost during differentiation into BMDM and there was no difference in leptin receptor expression in BMDM. This also shows that higher leptin in obese males is insufficient to increase leptin receptor levels in male bone marrow cells or BMDM. However, when BMDM were cultured with free fatty acids (FFA) to mimic hyperlipidemia of obesity, macrophages derived from male mice increased expression of leptin receptor. Induction of leptin receptor by FFA was larger than the induction of CCR2 expression. This may indicate that male macrophages may be more responsive to the increase in leptin during obesity. Indeed, male macrophages are more migratory in response to leptin when cultured with FFA. This may be a reason that males accumulate more macrophages in the adipose tissues in obesity. Leptin was postulated to contribute to macrophage accumulation by promoting adhesion of macrophages to endothelial cells and their transport to adipose tissue (8). Although males and females increase adipose tissue weight approximately 2.5-fold, males accumulate 10 times more macrophages, and increase responsiveness of male macrophages to leptin may be one of the reasons. A role for leptin receptor in macrophages was analyzed in the presence of active infection. In response to Salmonella infection, leptin receptor was necessary for maximal cytokine expression, while macrophage-specific leptin signaling inhibited their bactericidal functions (45). On the other hand, lack of leptin receptor, though it had no effect on cytokine production, impaired phagocytosis of Streptococcus (44). We analyzed a role for leptin in sex differences in the naïve physiology of obesity-induced low grade chronic inflammation, without infections; and in macrophage migration. We determined that male macrophages, in the state of hyperlipidemia, augment expression of leptin receptor, which leads to increased migration in response to leptin. This implies that in obesity, both increased leptin and increased fatty acids contribute to certain male-specific pathologies. Since infiltration of macrophages to the liver contributes to insulin resistance (29), our findings may explain higher incidence of insulin resistance in males than females (10, 24).

CCL2, identified as monocyte chemotactic protein (MCP) 1 (55), increases in obesity (7) and contributes to insulin resistance, hepatic steatosis, and macrophage accumulation in adipose tissue (50, 56). In agreement with these studies, concentration of CCL2 increases in serum in both males and females with HFD in our study. On the other hand, we determined that only males had significant increase in CCL2 in adipose tissue. This may cause higher accumulation of macrophages in male adipose tissues as discussed above. It is unlikely, given that CCL2 is not necessary for macrophage recruitment into the adipose tissue of obese mice, since CCL2 null mice had the same proportion of adipose tissue macrophages as controls, following HFD (57). We further show that increased concentration of the ligand in the serum corresponds to increased expression of the receptor, CCR2 in bone marrow cells with HFD in both males and females. After differentiation to BMDM, there was no difference in CCR2 expression, however, HFD male-derived BMDM increased CCR2 expression when they were cultured in the presence of FFA. Significantly, there was a lack of migration in response to CCL2 with and without FFA, if BMDM were not activated

previously with LPS, consistent with previous studies (58). Cells that normally express high levels of CCR2, such as THP-1 human immortalized monocytic cell line (59), migrate in response to CCL2 (35). However, these cells shows substantial differences from circulating monocytes or macrophages (60). Bone marrow cells, in obese or genetically modified mice, migrate in response to CCL2 (56), likely because they upregulate CCR2 in response to HFD as we show here. We used BMDM, which as we show lose induction of CCR2 during differentiation, and thus need to be stimulated prior to migration in response to CCL2 (61). LPS activates macrophage migration in response to CCL2, but LPS is not present in normal, physiological conditions in the absence of infection. Given that FFA levels are increased in obesity, we wanted to assess if FFA can activate macrophage migration in response to CCL2. However, neither macrophages from female nor male mice migrated in response to CCL2, with and without FFA, which may indicate that FFA does not crosstalk with CCL2-CCR2 pathway.

Previously a study demonstrated that CCL5 and its receptor, CCR5, expression increased in adipose tissue following HFD (62). Further studies demonstrated that CCL5, rather than CCL2, contributes to macrophage accumulation in adipose tissue in obesity (39). Our results do not support a role for CCL5. CCL5 levels in serum do not change with diet, nor do they exhibit sex differences. In our study, in agreement with previous studies (62, 63), expression of CCL5 mRNA in adipose tissues is increased following HFD. However, this increase is not reflected at the protein level. While in the adipose tissues, males have higher levels of CCL5 than females, which may correspond to higher accumulation of macrophages in males, there is no difference with diet or sex in the expression of its receptor, CCR5, in BMDM. Moreover, the CCR5 expression is completely lost in the presence of FFA that mimic conditions of obesity. It is possible that CCL5 may play a role in the liver and NAFLD (40), but our results indicate that it doesn't play a role in sex differences in macrophage accumulation to adipose tissue.

FFA increase in the circulation is a hallmark of obesity. Long chain FFA bind GPR40 and GPR120 (64). Since neither macrophages nor adipocytes express GPR40, GPR120 is the relevant receptor that conveys increase in FFA in obesity to these tissues (65). GPR120 binds both unsaturated and saturated long chain FFA (51). Since GPR120 binds omega-3 polyunsaturated fatty acids that have an anti-inflammatory role, GPR120 signaling was also postulated to be anti-inflammatory. However, some studies dispute a role for GPR120 in an anti-inflammatory function of unsaturated fatty acids (66). In human adipose tissues, GPR120 is involved in the induction of both pro-inflammatory and anti-inflammatory cytokines by fatty acids (67). GPR120 levels increase following HFD in adipose tissue stromal vascular fraction, which contains macrophages, and GPR120 activation decreases expression of inflammatory cytokines in response to LPS (65). Similarly to CCL2, macrophages had to be stimulated with LPS to observe the effect of GPR120 on inflammatory cytokine expression (65). We analyzed the levels of GPR120 expression in cultured BMDM in the presence of FFA, and determined that only male-derived macrophages increase the expression of GPR120. Thus, we postulate that male-specific increase in GPR120 may contribute to higher migration of male macrophages in the presence of hyperlipidemia of obesity. Increased leptin receptor and increased free fatty



receptor may work together to facilitate larger macrophages infiltration into male adipose tissue.

Some of identified sex differences may depend on sex steroid hormones (11, 13, 14), while others may depend on sex chromosomes (15) or other intrinsic differences (23). Since estrogen is protective from DIO, but is not necessary for protection from neuroinflammatory changes (19), to investigate the contribution of estrogen in sex differences observed here, we treated cells with estrogen receptor antagonist. Consistent with previous studies in which estrogen may either induce or repress TNF $\alpha$ , depending on the duration or concentration of treatment, model or design, or inter-animal variability (30, 49, 68–75), our results with estrogen inhibition of TNF $\alpha$  induction in response to FFA were inconclusive. Since cells from different animals, males or females or control or HFD, were processed and differentiated into BMDM separately, this may have contributed to variability. On the other hand, induction of IL-10 in response to FFA was higher in female-derived BMDM, consistent with IL-10 increase in adipose tissues of obese females *in vivo*. Disruption of estrogen signaling, prevented maximal induction of IL-10 in both male and female BMDM, but the sex differences remained. Role of estrogen in IL-10 production was shown before (71, 76); as was protection by IL-10 in obesity-mediated inflammation (77). Here we show that female macrophages synthesize more IL-10 than male macrophages, which may contribute to female protection in obesity and relatively fewer pathophysiological outcomes. Even in the absence of estrogen signaling, in the presence of ICI estrogen antagonist, females synthesized more IL-10 than males, which may account for female protection. Since the induction of leptin receptor or GPR120 free fatty acid receptor by FFA was specific for male BMDM, estrogen does not play a role in leptin receptor or GPR120 increases in hyperlipidemic conditions.

Leptin has been shown to promote macrophage migration (35, 78), and our results agree with these reports. Leptin knockout mice and leptin receptor null mice exhibit reduced macrophage infiltration and lower inflammatory gene induction in adipose tissues, despite higher obesity (79). Studies using bone marrow transplants from leptin receptor null mice obtained conflicting results, and thus, the role of leptin receptor specifically in bone marrow cell recruitment into adipose tissues remains unclear. One study that transplanted leptin receptor-deficient bone marrow cells into irradiated wild-type recipient mice observed no differences in immune cell infiltration into adipose tissue following HFD (80). Another study performed the same transplant but observed significantly fewer CCL+ macrophages in adipose tissues and reduced macrophage-forming crown like structures around adipocytes (81). Studies that showed macrophage migration in response to leptin in the transwell dishes used THP-1 cell line that, as discussed above, constitutively expresses high CCR2 levels and was derived from a male (82), while herein, we compared male and female macrophage migration in response to leptin. Furthermore, a role of leptin receptor in macrophages was analyzed before in the context of infection (44, 45). We compared side by side unstimulated BMDM derived from males and females, which were differentiated using the same conditions without the presence of sex steroid hormones, and determined that male BMDM migrate more in response to leptin, especially in the hyperlipidemic conditions. BMDM may differ from resident or recruited tissue macrophages *in situ* (83–85), since during differentiation, BMDM lose sex differences observed in bone marrow cells, as we



show here. However, although differentiated *in vitro*, BMDM are primary cells, as opposed to transformed cell lines mentioned above. Macrophages are, at any rate, a heterogeneous cell population, which is further confounded with their high plasticity in response to the changing environment in tissues or varied stimuli under different conditions (86). Utilizing BMDM allowed us to control culture conditions and focus on the roles of free fatty acids and leptin in macrophage migration, in addition to differences we determined in bone marrow cells and adipose tissue macrophages *in vivo*.

Intrinsic sex differences in macrophage migration or sex differences in response to leptin were not analyzed before. Additionally, a role of FFA in macrophage migration or expression of receptors was not analyzed in detail before, although FFA are increased in obesity. We determined that male macrophages increase expression of GPR120 FFA receptor 4, and that the presence of FFA contributes to sex differences in macrophage migration. Our results demonstrated that 1) males have higher leptin levels, 2) male macrophages increase leptin receptor expression in response to free fatty acids, and 3) male macrophages migrate in response to leptin. These results may explain male propensity for insulin resistance that is a hallmark of metabolic syndrome and is elicited by macrophage infiltration. Therefore, our results indicate that hyperleptinemia combined with hyperlipidemia may be the critical condition in obesity that leads to accumulation of macrophages in adipose tissue. Since males increase leptin to much higher levels than females, this causes disproportionately more macrophages in male adipose tissue, which in turn, leads to elevated levels of inflammatory cytokines, such as TNF $\alpha$ . Female macrophages, however, express higher levels of anti-inflammatory IL-10, which may provide protection to females. Together, these differences that lead to chronic inflammation in males, may contribute to disproportionately increased risk of cardiovascular diseases and insulin resistance in obese males than females.

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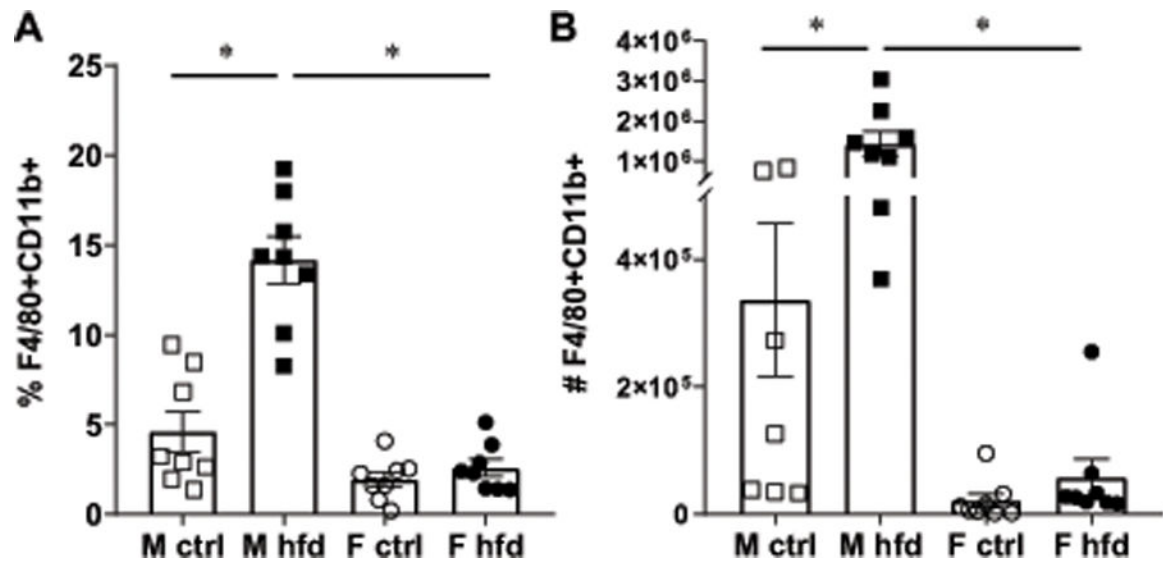
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**Key points**

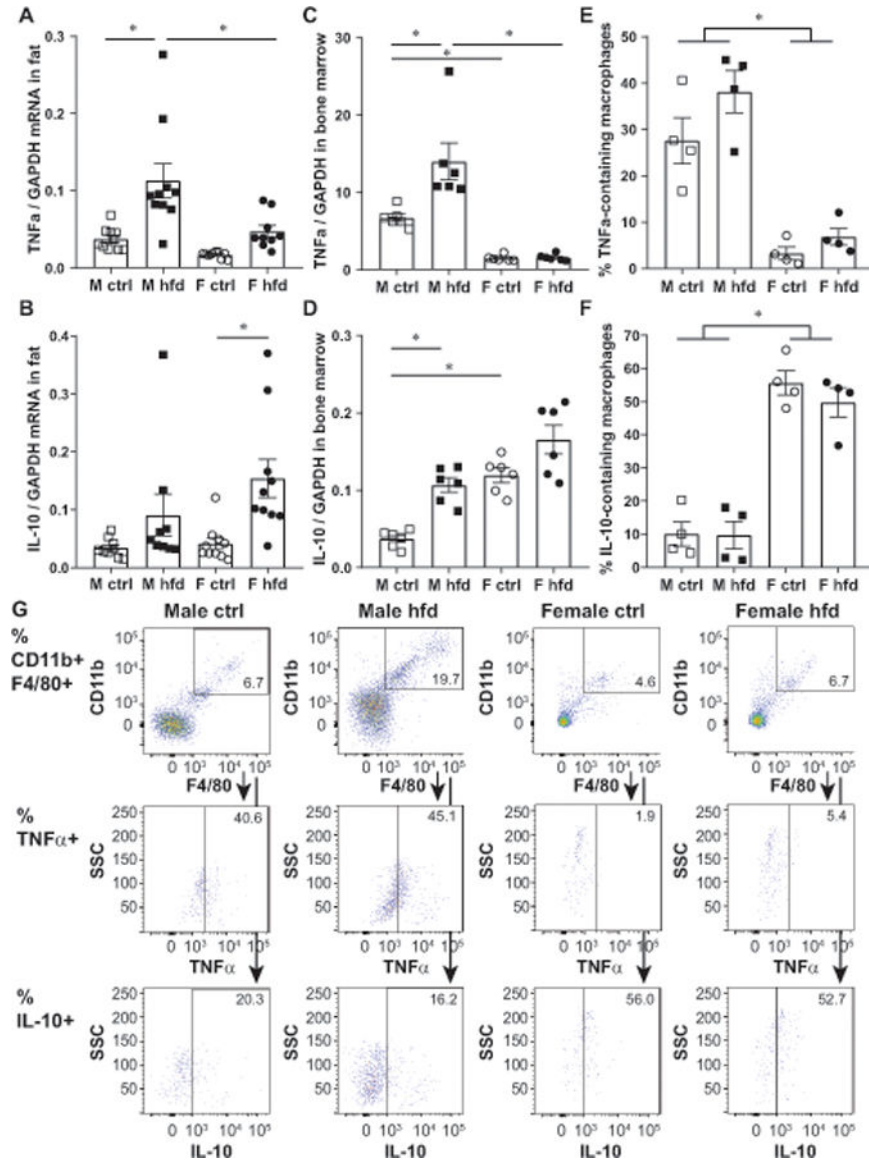
- Male macrophages are intrinsically more migratory in the same culture conditions
- Male bone marrow cells and male adipose tissue macrophages are more inflammatory
- Male macrophages induce leptin receptor and GPR120 in response to free fatty acids





**Figure 1.**

Males accumulate more macrophages in adipose depots. Macrophages identified as F4/80+ and CD11b+ positive cells using flow cytometry in the stromal vascular fraction from gonadal fat tissues from male mice fed control diet (white squares), male mice fed HFD (black squares), female mice fed control diet (white circles) and female mice fed HFD (black circles) for 15 weeks. Each point represents one animal, processed independently, while bars represent group means with standard error; n=8. Statistical significance (\*,  $p < 0.05$ ) was determined with ANOVA followed by Tukey's post hoc test. A, percentage of F4/80+ CD11b+ positive cells of all cell in stromal vascular fraction. B, absolute cell numbers of F4/80+CD11b+ positive in one gonadal fat depot.



**Figure 2.**

Fat-infiltrated macrophages from male mice are more inflammatory. A, TNFα mRNA expression in gonadal fat tissues from male mice fed control diet (white squares), male mice fed HFD (black squares), female mice fed control diet (white circles) and female mice fed HFD (black circles) for 15 weeks. 2 ug of total mRNA was reverse transcribed and qPCR performed with primers listed in Table 1. B, IL-10 mRNA expression in the same tissues as A. C, TNFα expression in bone marrow cells from the same mice as above. D, IL-10 expression in bone marrow cells. E-G, stromal vascular fraction cells from adipose tissue were stained as described in materials and methods, and analyzed by flow cytometry. Macrophages were identified as F4/80+CD11b+ cells as illustrated in G, top panels, and proportions of these double positive cells that additionally stained with TNFα (C, and G, middle panels) and IL-10 (D and G, bottom panels) antibodies determined. A-F, Each point represents one animal, processed and analyzed separately (A-D, n=6-10; E-F,

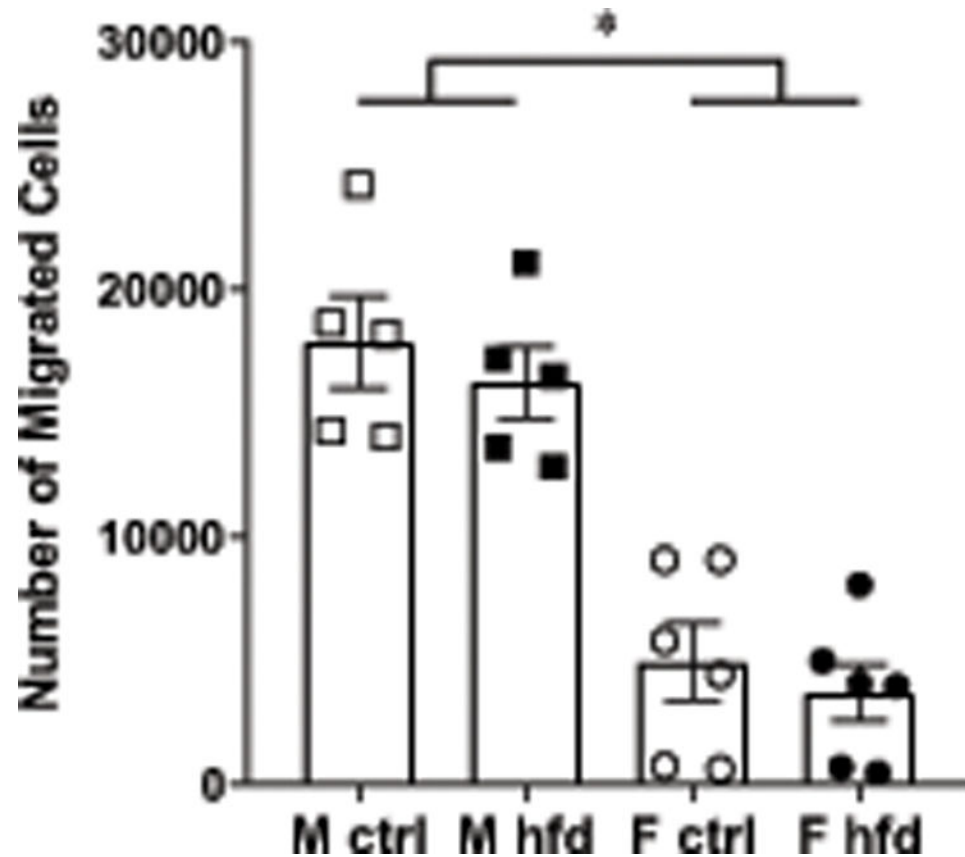
n=4). G, representative example of one animal per group, which was included in E and F. Statistical significance, indicated with a \* ( $p < 0.05$ ) was determined with ANOVA followed by Tukey's post hoc test.

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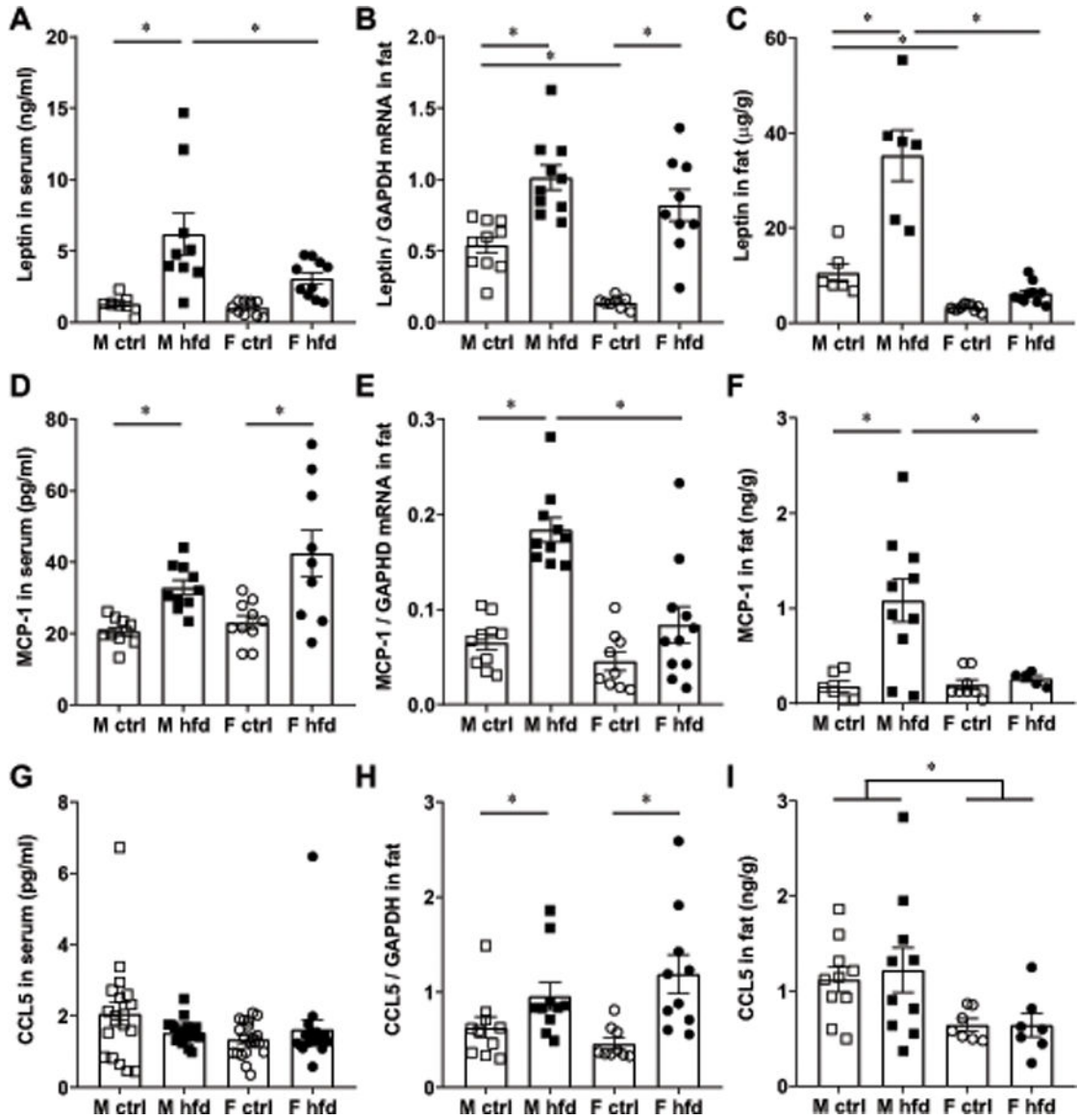
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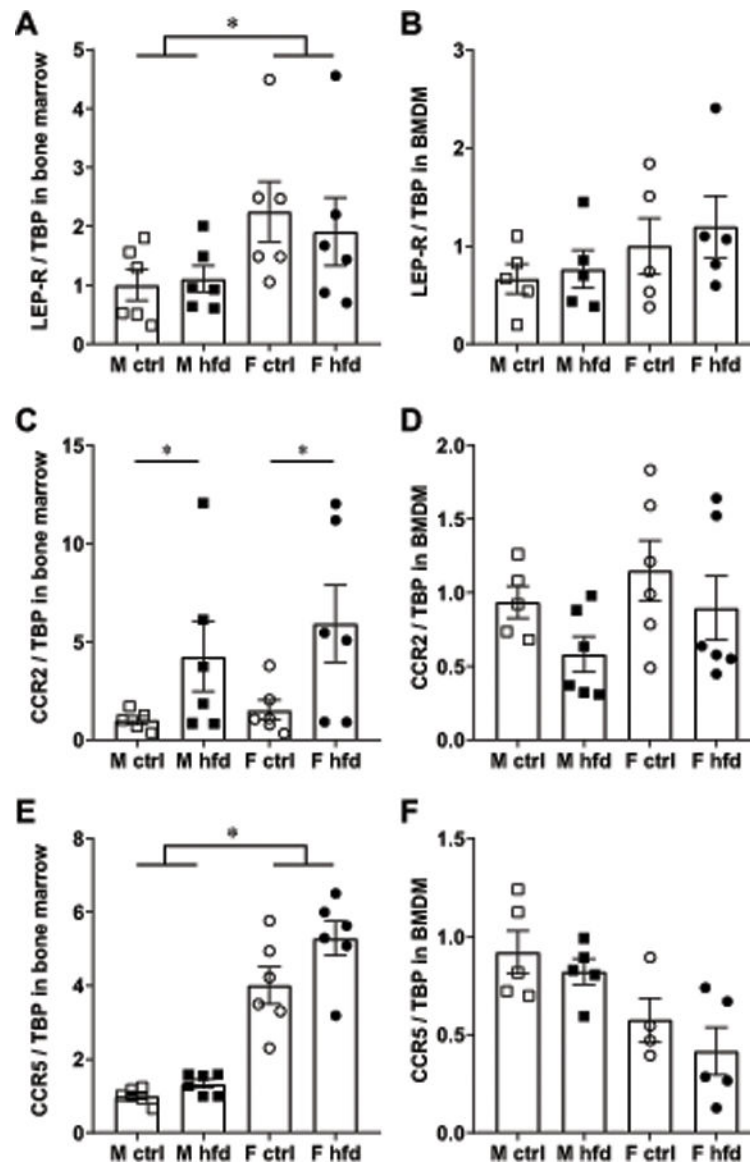
**Figure 3.**

Bone marrow derived macrophages (BMDM) from male mice are more migratory. BMDM differentiated from bone marrow cells for 7 days, as described in materials and methods, from male mice fed control diet (white squares), male mice fed HFD (black squares), female mice fed control diet (white circles) and female mice fed HFD (black circles) for 15 weeks. Each point represents cells isolated from one animal (n=5–6), and differentiated into BMDM *in vitro*, in the same medium using standard conditions. After differentiation, cells were placed in Transwell Boyden chambers in the top well, and migration to the bottom counted after 2 hours. Statistical significance, indicated with a \* ( $p < 0.05$ ) was determined with ANOVA followed by Tukey's post hoc test.

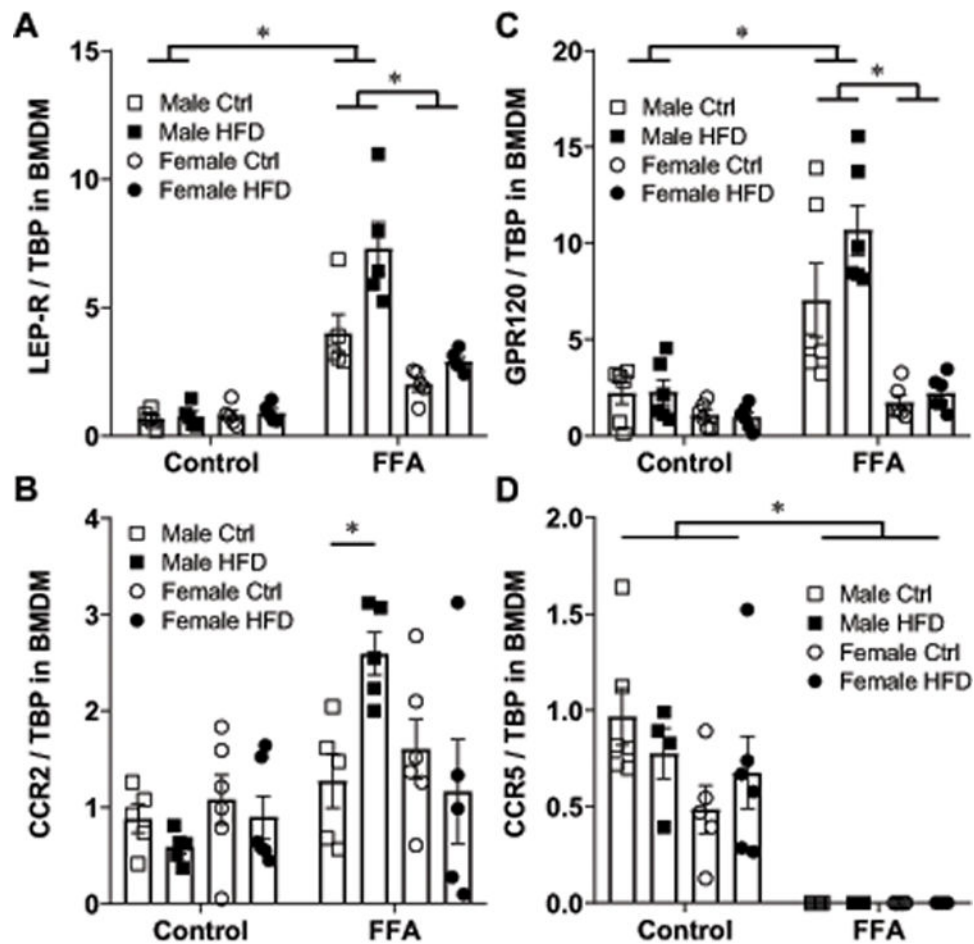


**Figure 4.**

Male adipose tissues have higher levels of leptin and MCP-1 / CCL2. A, D, G, levels in serum of male mice fed control diet (white squares), male mice fed HFD (black squares), female mice fed control diet (white circles) and female mice fed HFD (black circles) determined by Luminex assay; n=10–20. B, E, H, mRNA levels normalized to GAPDH in the gonadal depot determined by RT-qPCR, n=9–12. C, F, I, protein levels normalized to grams of total protein in gonadal adipose tissue determined by Luminex assay, n=6–10. Each point represents one animal, while bars represent group means. Statistical significance ( $p < 0.05$ ), determined with ANOVA followed by Tukey's post hoc test, is indicated with a \*.

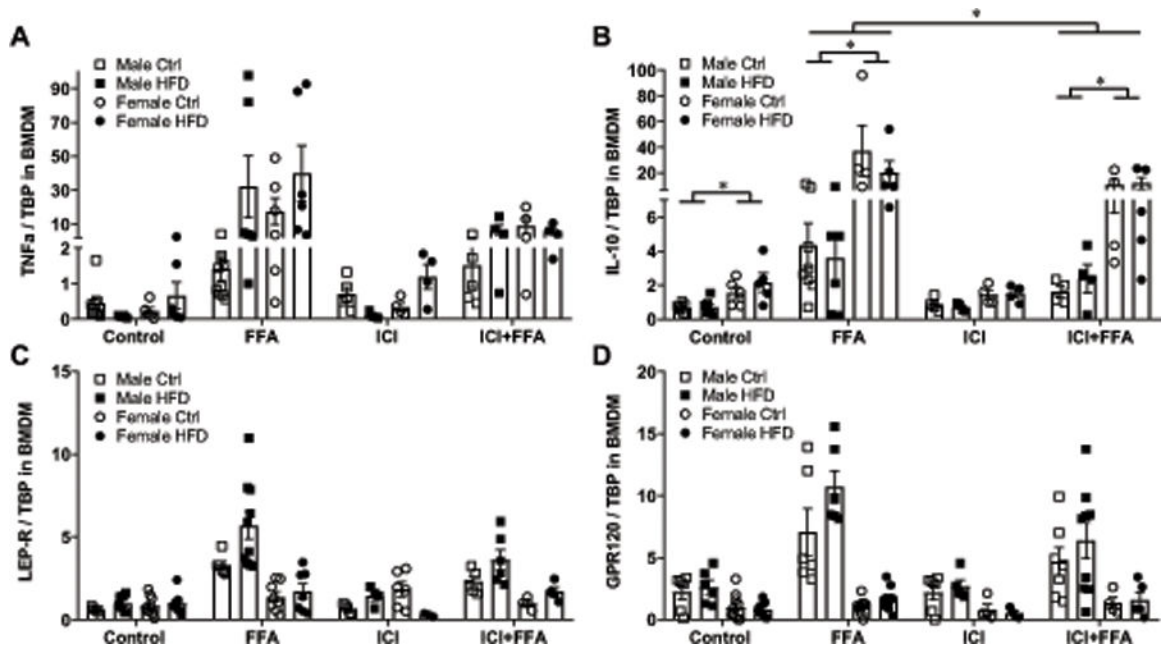


**Figure 5.** Expression of leptin receptor mRNA (A, B), CCR2 mRNA (C, D) and CCR5 mRNA (E, F) in bone marrow cells (A, C, E) and BMDM (B, D, F) from male mice fed control diet (white squares), male mice fed HFD (black squares), female mice fed control diet (white circles) and female mice fed HFD (black circles), for 15 weeks, determined by RT-qPCR. Each point represents cells isolated from one animal, n=5–6, and bars represent group means. Statistical significance ( $p < 0.05$ ), determined with ANOVA followed by Tukey's post hoc test, is indicated with a \*.

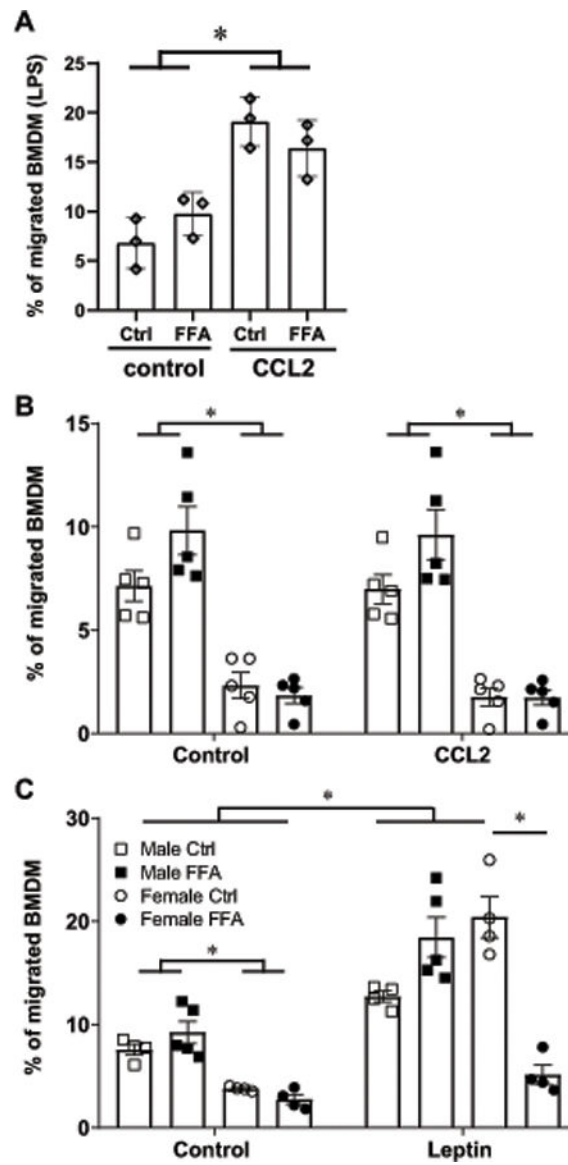


**Figure 6.** BMDM from male mice cultured in the presence of free fatty acids (FFA) up-regulate expression of leptin receptor, GPR120 and CCR2. A, leptin receptor mRNA; B, CCR2 mRNA; C, GPR120 mRNA, and D, CCR5 mRNA in BMDM from male mice fed control diet (white squares), male mice fed HFD (black squares), female mice fed control diet (white circles) and female mice fed HFD (black circles) determined by RT-qPCR. Cells derived from one animal, n=5–6, were split, and one half cultured under control conditions in differentiation medium, and the other half in the same differentiation medium, with addition of FFA. Each point represents cells from one animal that was processed independently. Statistical significance ( $p < 0.05$ ), determined with ANOVA followed by Tukey's post hoc test, is indicated with a \*.





**Figure 7.** Estrogen is necessary for the maximal induction of IL-10 by FFA, but not for sex differences. A, TNF $\alpha$  mRNA, B, IL-10 mRNA C, leptin receptor (LEP-R mRNA; D, GPR120 mRNA, produced by BMDM from male mice fed control diet (white squares), male mice fed HFD (black squares), female mice fed control diet (white circles) and female mice fed HFD (black circles) determined by RT-qPCR and normalized to TATA-binding protein (TBP). Cells derived from one animal, n=5–6, were split, and one quarter cultured under control conditions in differentiation medium, and second quarter in the same differentiation medium, with addition of FFA for 7 days, third quarter in the presence of 1 $\mu$ M ICI 182,780 (Fulvestrant) estrogen receptor antagonist, and the final quarter with ICI and FFA. Each point represents cells from one animal that was processed independently. Statistical significance ( $p < 0.05$ ), determined with ANOVA followed by Tukey’s post hoc test, is indicated with a \*.



**Figure 8.**

Male macrophages are more migratory in response to leptin. A, percent of BMDM, cultured in control conditions or in the presence of FFA, activated by LPS overnight, that migrate in response to vehicle or CCL2. B, percent of BMDM from male mice cultured in control conditions (white squares), male mice cultured with FFA (black squares), female mice cultured in control conditions (white circles) and female mice cultured with FFA (black circles) that migrated in response to vehicle control or CCL2 in 2 hours. C, percent of BMDM that migrated in response to vehicle control or leptin in 2 hours. Cells derived from one animal were split, and one half cultured under control conditions in differentiation medium, and the other half in the same differentiation medium with FFA. After differentiation, cells were again divided, counted, and  $2 \times 10^6$  cells placed in the Boyden chamber with vehicle control, and  $2 \times 10^6$  in the chamber containing CCL2 or leptin to monitor migration after 2 hours. Each point represents cells from one animal,  $n=5$ , that

was processed and analyzed independently. Statistical significance,  $p < 0.05$ , indicated with a \*, was determined with ANOVA followed by Tukey's post hoc test.

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**Table 1.**

## Primers

Primers	Forward	Reverse
CCL2 / MCP-1	CACTCACCTGCTGCTACTCA	CAGCACAGACCTCTCTTGA
CCL5 / RANTES	GCAAGTGCTCCAATCTTGCA	CTTCTCTGGGTTGGCACACA
Leptin	TTCCTGTGGCTTTGGTCCTAT	TGCAGCACATTTTGGGAAGG
CCR2	TCAACTTGGCCATCTCTGACC	AGACCCACTCATTTGCAGCAT
CCR5	AGACATCCGTTCCCCCTACA	GCAGGGTGCTGACATACCAT
LEPR	GGTCCTCTTCTTCTGGAGCCT	AGAACTGCTTTCAGGGTCTGG
IL10	GCTGGACAACATACTGCTAACC	ATTTCCGATAAGGCTTGCAA
TNF $\alpha$	ATGTCTCAGCCTTCTCTCAITCC	GCTTGTCACCTCGAATTTGAGAA
TBP	CAAACCTGACCACTGCACCGTTG	GAAGCTGGTGTGGCAGGAGT
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTT

Table 1. List of primers used to analyze expression by quantitative RT-PCR. Primers were used at 400 nM final concentration in the PCR reaction, as described in Materials and Methods section.