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Effects of Obesity-Associated Chronic Inflammation on Peripheral Blood Immunophenotype Are Not Mediated by TNF in Female C57BL/6J Mice

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ABSTRACT

Chronic low-grade systemic inflammation in obesity contributes to the development and progression of aspects of metabolic syndrome. In obese male mice, expanded adipose tissue releases proinflammatory cytokines, including TNF, which promotes an increase in immature, proinflammatory, peripheral blood Ly-6C\textsuperscript{high} monocytes. The aim of this study was to characterize how TNF alters circulating cellular immunity in female mice with diet-induced obesity. We initially quantified peripheral blood immune cells by flow cytometry in female wild-type C57BL/6J mice after 3–30 wk of allocation to a high-fat (HF) or standard chow diet. We assessed effects of diet and time on neutrophil, monocyte, B cell, NK cell, CD4\textsuperscript{+} T cell, and CD8\textsuperscript{+} T cell populations. There was a significant interaction of the effects of diet type and time on the numbers and prevalence of circulating total monocytes and Ly-6C\textsuperscript{high}, Ly-6C\textsuperscript{low}, and Ly-6C\textsuperscript{−} subsets. Circulating monocytes, in particular Ly-6C\textsuperscript{high} monocytes, were increased in HF-fed mice compared with chow-fed mice. Ly-6C\textsuperscript{high} monocytes from HF-fed mice also had a more immature phenotype yet were highly responsive to the chemotactic ligand CCL2 and had greater intracellular production of TNF. Comparisons of the effects of HF diet feeding in littermate wild-type (TNF\textsuperscript{+/+}) and TNF\textsuperscript{−/−} female mice showed that genetic ablation of TNF did not protect from higher adiposity or an increase in circulating, immature, proinflammatory Ly-6C\textsuperscript{high} monocytes during HF diet-induced obesity. These data emphasize the importance of considering biological sex when determining the mechanisms of TNF action in obesity-induced cellular inflammation and in other chronic inflammatory conditions. ImmunoHorizons, 2021, 5: 370–383.

INTRODUCTION

Obesity is a complex chronic disease that is driven by many factors that influence energy intake and expenditure. Obesity is associated with an increased risk of morbidity and early mortality (1) and is often associated with other chronic inflammatory disorders, including cardiovascular disease (1), nonalcoholic fatty liver disease (2), and certain cancers and their metastasis (3), as well as type 2 diabetes and insulin resistance (1). Improvements in physical and mental health and well-being...
are important in obesity management (4, 5). However, a better understanding of the causes of obesity will inform novel interventions.

A characteristic of the pathophysiology of obesity-induced insulin resistance and low-grade chronic inflammation is the accumulation of macrophages within metabolic tissues, including skeletal muscle, liver, and adipose tissue (6, 7). Experiments in obese male mice have shown that most of these macrophages are derived from Ly-6C<sub>high</sub> monocytes in the blood, which increase in circulation within 6 wk of diet allocation (8) and remain elevated after 24 wk (9–11). These Ly-6C<sub>high</sub> monocytes primarily enter the adipose tissue via CCR2/CCL2-mediated chemotaxis (6, 12–14). Accordingly, reduction of monocyte recruitment into adipose tissue, via genetic global or hematopoietic ablation of CCR2 or CCL2 (12, 15, 16), treatment with a CCR2 antagonist (17), or genetic ablation of macrophage migration inhibitory factor (MIF) (7) decreases male mouse adiposity, tissue inflammation, and metabolic dysfunction. Together, these data implicate monocyte recruitment as a contributing factor to the adipose tissue inflammation that accompanies obesity.

Adipose tissue macrophages contribute to local and systemic inflammation, as well as metabolic dysfunction, by producing large quantities of the proinflammatory cytokine TNF (18–21). TNF has pleiotropic roles in regulating myeloid cell survival and function and their responses to inflammation (22, 23), as well as in modulating systemic inflammation and metabolism in chronic inflammatory disorders like obesity (24). We previously reported in male mice that lowering obesity-associated inflammation via genetic ablation of TNF reduces circulating monocytes and that the prevalence of peripheral blood immature, proinflammatory, migration-primed Ly-6C<sub>high</sub> monocytes correlates with insulin resistance in those male mice (10). Multiple studies in male rodent models of obesity have shown that genetic ablation of TNF (25–27) and Ab-mediated (28) or small interfering RNA–mediated (18) blockade of TNF can reduce adiposity and improve glucose homeostasis and insulin resistance, whereas administration of TNF has the opposite effect (29–31). It has been suggested that in TNF<sup>−/−</sup> mice, the absence of TNF helps maintain insulin sensitivity in the first weeks of high-fat (HF) diet feeding, which may also attenuate later development of adiposity and inflammation, protecting against insulin resistance (27). These investigations, however, have been restricted to the study of male mice, and few researchers have examined whether these observations are also applicable in female mice.

Epidemiological data show that the global incidence of obesity is similar in both sexes (1), yet most preclinical studies, including those described above, use male rodent models to evaluate the adverse effects of HF diet consumption (32). Basic features of obesity, like adipocyte hypertrophy and hyperplasia, leading to excess visceral adiposity and ectopic fat accumulation, are apparent in both sexes (33). Despite this, women of reproductive age are often described to be protected from obesity-associated comorbidities, including cardiovascular disease and metabolic syndrome (34, 35). Male mice, compared with female mice, generally show more extreme phenotypes of adiposity, metabolic dysfunction, and inflammation in response to diet-induced obesity (32, 36), supporting the notion that biological sex impacts fat deposition, metabolism, and energy balance (34, 35, 37). Sexual dimorphism also influences regulation of hematopoietic stem cells and innate immune signaling pathways (8, 38, 39), which may contribute to findings that male mice with diet-induced obesity have enhanced bone marrow myelopoiesis, as well as enhanced macrophage accumulation within adipose tissue, compared with their female counterparts (6, 8). Males generally have higher levels of circulating monocytes under conditions of homeostasis (40, 41) and PBMC production of TNF in response to acute inflammation is generally lower in females compared with males (40, 42). Together, these data emphasize the sex-specific differences that exist in immunometabolic regulation and highlight the fact that researchers often overlook sex differences when considering mechanisms that contribute to the development and progression of obesity.

In this study, we aimed to examine if female mice, with their differences in TNF-mediated cellular inflammation and partial protection from obesity-associated phenotypes, have TNF-dependent changes to peripheral monocytes in response to diet-induced obesity, as reported in male mice. We assessed changes to peripheral immune cell population dynamics and the effects of immunomodulation by TNF. We show that obese female mice, like male mice, have time-dependent changes to peripheral blood immunophenotype composition, including an increase in immature, proinflammatory, migration-primed Ly-6C<sub>high</sub> monocytes. However, the essential role for TNF in mediating adiposity and cellular inflammation in obesity in male mice is not apparent in females.

**MATERIALS AND METHODS**

**Ethical approval**

All animal experiments were performed in accordance with Institutional Animal Utilization Protocols approved by McMaster University’s Animal Research Ethics Board following the recommendations of the Canadian Council on Animal Care.

**Animals**

Wild-type (WT) C57BL/6J mice (catalog no. 000664) and TNF<sup>−/−</sup> mice (catalog no. 003008) were originally purchased from The Jackson Laboratory and bred at the McMaster University Central Animal Facility. Littermate TNF<sup>+/+</sup> and TNF<sup>−/−</sup> mice were generated from F2 TNF<sup>+/−</sup> heterozygotes (43), and genotype was determined by PCR on tail snips at weaning as per standard protocols. All mice used in this study were females. Mice were caged two to five per cage under specific pathogen–free conditions in vent/rack cages with constant ambient temperature (22°C) on a 12-h light–dark cycle.
Mice were provided with a plastic tube and cotton and paper bedding material for enrichment. WT mice were fed a standard chow diet upon weaning (catalog no. 8640 Teklad 22/5 Rodent Diet; Envigo). Littermate WT and TNF^{−/−} mice were fed Teklad irradiated global 14% protein diet (catalog no. 2914; Envigo) upon weaning. To assess effects of diet-induced obesity in WT mice, mice were fed the standard chow diet (17% kcal fat, 29% kcal protein, and 54% kcal carbohydrates; catalog no. 8640; Envigo) or a low-fiber, HF diet (60% kcal fat, 20% kcal protein, and 20% kcal carbohydrates; catalog no. D12492, Research Diets) and provided water ad libitum beginning at 5 wk of age. To assess effects of TNF in diet-induced obesity, littermate TNF^{−/−} and TNF^{+/+} mice were fed the low-fiber, HF diet and provided water ad libitum beginning at 8 wk of age. Following retro-orbital blood collection, mice were administered eye gel and an s.c. injection of normal saline solution (0.9% NaCl) and were monitored to ensure recovery. At the end of the experimental period, all mice were sacrificed by exsanguination under isoflurane anesthesia. All sample sizes represent individual mice. Experimental groups are described in figure and table legends.

**Body composition assessments**

Mouse body weight and food intake were monitored weekly. Caloric intake was calculated based on the amount of food consumed and individual mouse weight per experiment cage ([(food eaten/number of mice in cage) × (grams per kilocalorie food energy density)]/mouse weight). Adiposity was measured as a percentage of body fat calculated from whole-body EchoMRI imaging (Bruker Minispec LF90-II).

**Adipose tissue histology and immunohistochemistry**

Gonadal adipose tissue was fixed in 10% neutral-buffered formalin at room temperature for 24 h and processed and embedded into paraffin. Sections of 5 μm were cut at 50 μm intervals. For quantification of adipocyte cross-sectional area, slides were stained with H&E at the Core Histology Facility, McMaster Immunology Research Centre. For each mouse, three adipose tissue sections were used for analysis, and three fields of view were captured at 20× magnification per section such that at least 30 adipocytes could be measured within each image. Measurement of cross-sectional area was performed with ImageJ (44). Adipose tissue macrophages were identified by immunohistochemistry using an anti-F4/80 mAb (1:100; rabbit anti-rat; catalog no. ab6640; Abcam), as previously described (45). For each mouse, two sections of adipose tissue were used for analysis, and four fields of view were captured per section at 20× magnification. The fraction of F4/80^{+} cells was calculated as the sum of the number of nuclei of F4/80^{+} cells divided by the total number of nuclei for each field of view, and ratios were averaged across each section and for each mouse and expressed as a percentage of F4/80^{+} cells per total cells (6). For both adipocyte quantification and macrophage immunohistochemistry, slides were visualized with a Nikon Eclipse NI microscope (960122, Nikon Eclipse NI-S-E). Images were captured using a Nikon DS-Qi2 Color Microscope Camera and Nikon NIS Elements Imaging Software (v4.30.02).

**Metabolic assessments**

Blood glucose was measured via tail vein using the Accu-Chek Inform II system glucometer and test strips (Roche Diagnostics). For the glucose tolerance test, mice were fasted for 6 h (9 AM to 3 PM). Blood glucose concentrations were measured via tail nick samples before i.p. injection of 1.5 g/kg glucose (catalog no. G-7528; Sigma-Aldrich) in sterile 0.9% saline and after 20, 30, 40, 60, 90, and 120 min, as previously described (46, 47). For the glucose-stimulated insulin secretion test, mice were fasted for 12 h (9 PM to 9 AM). Blood was collected from a tail nick using a heparinized capillary tube before and at 10, 60, and 120 min after i.p. injection of 3 g/kg glucose in sterile 0.9% saline. Serum was collected and frozen at −80°C after centrifugation at 8000 × g for 10 min. Insulin concentrations were measured using a commercial ELISA kit according to the manufacturer’s instructions (catalog no. 33270; AIS Toronto Biosciences). Assay results were recorded using the BioPlex 200 system and BioPlex Manager software v6.1 (Bio-Rad Laboratories). For the insulin tolerance test, mice were fasted for 6 h (9 AM to 3 PM). Blood glucose concentrations were measured via tail nick samples before i.p. injection of 1 U/ml/kg insulin (catalog no. G-7528; Sigma-Aldrich) in sterile 0.9% saline and postinjection after 20, 30, 40, 60, 90, and 120 min, as previously described (46, 47).

**Cytokine ELISA**

Whole blood was retro-orbitally collected into heparin, centrifuged at 10,000 × g for 10 min, and plasma was frozen until analysis. Plasma levels of TNF and IL-6 were assessed as per the manufacturer’s instructions with a high sensitivity Milliplex MAP Kit (catalog no. MCYTOMAG-70K; EMD Millipore). Assay results were recorded using the BioPlex 200 system and BioPlex Manager software v6.1 (Bio-Rad Laboratories).

**CCL2-elicited monocyte response assay**

Sterile saline or 200 nM of murine rCCL2 (endotoxin-free, catalog no. 250-10; PeproTech) diluted in sterile saline were administered i.p. as previously described (48). Blood and femur bone marrow were collected 4 h later for assessment by flow cytometry, as described below.

**Flow cytometry of bone marrow and peripheral blood leukocytes**

Blood was collected retro-orbitally in heparinized capillary tubes under isoflurane anesthesia. Peripheral blood leukocytes (monocytes, neutrophils, NK cells, CD4^{+} T cells, CD8^{+} T cells, and B cells) were analyzed by flow cytometry as previously described (41). Bone marrow leukocytes were flushed from femurs, disrupted with an 18-gauge needle and analyzed by flow cytometry as previously described (49). Stains were prepared in FACS buffer (PBS, 0.5% w/v BSA, and 1% EDTA [pH

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HF diet increases adiposity, blood glucose, and insulin, but not peripheral soluble inflammation, in WT C57BL/6J female mice

We allocated young female WT C57BL/6J mice to either a commonly used chow diet or an obesogenic HF diet for up to 30 wk. On a weekly basis, we monitored body weight (Fig. 1A) and calculated energy consumption from food intake (Fig. 1B). We observed that there was a significant interaction between the effects of diet type (i.e., chow or HF diet) and time of diet allocation on body weight as well as energy consumption. HF-fed mice reduced their caloric intake despite continued increases in body weight and adiposity (Fig. 1C). Within 6 wk, we observed that HF-fed mice had ~20% greater adiposity than chow-fed mice (mean ± SD; chow: 15.0 ± 3.2%; HF: 35.9 ± 8.6%), as well as increased gonadal fat weights (Fig. 1D) and adipocyte sizes (Fig. 1E). Increased adipose tissue accumulation of F4/80+ macrophages was also apparent after 24 wk in HF-fed mice compared with chow-fed mice (Fig. 1F).

We measured peripheral blood TNF and IL-6 cytokine levels at multiple time points in a cohort of chow-fed and HF-fed mice as an indication of soluble inflammation but were only able to detect TNF in 10/78 assessed samples (data not shown). Although we were able to measure detectable levels of IL-6 in 75/78 samples (Fig. 1G), there were no statistically significant differences between the diet groups after 6, 12, 18, or 24 wk of diet allocation. We also assessed metabolic parameters and observed that by 6 wk, HF-fed mice compared with chow-fed mice had significantly increased fasting blood glucose levels, which persisted to 30 wk of HF diet feeding (Fig. 1H). Between 14 and 18 wk of diet allocation, we performed glucose tolerance tests (Fig. 1I, 1J) and an insulin tolerance test (Fig. 1K). Female mice with HF diet-induced obesity had a slower rate of glucose clearance compared with chow-fed mice despite having significantly greater insulin secretion, suggesting that HF-fed mice were glucose intolerant and hyperinsulinemic. Insulin tolerance was similar between diet groups. These data were consistent with a study using a similar model in female mice after 12–15 wk of HF diet (50). In summary, feeding female mice an HF diet, compared with a chow diet, induced changes to body composition that were characteristic of obesity, including increases in adiposity, adipocyte hypertrophy, hyperinsulinemia, and hyperglycemia, but did not result in insulin resistance or elevate peripheral blood soluble inflammation.

Statistical analysis

Data normality and variance were considered in choosing statistical tests, and data were analyzed and plotted with GraphPad Prism version 9 (GraphPad Software). Comparisons of leukocyte populations at discrete time points between mice fed chow or HF diet or between WT and TNF−/− littermate mice fed HF diet were analyzed by unpaired two-tailed Student t test (parametric) with Welch correction (for unequal variances) or Mann–Whitney U test (nonparametric) according to normality. Main effects of diet type and time of diet allocation for mice fed chow or HF diet or time of diet allocation and genotype for littermate mice were assessed by two-way ANOVA with Sidak post hoc correction for multiple comparisons. Statistical significance was defined as a p value <0.05.
FIGURE 1. Measures of body weight, adiposity, glucose, insulin, and soluble inflammation in C57BL/6J female mice fed a chow or HF diet. Mice were placed on a standard chow diet (Chow) or HF diet. Physiological, inflammation-associated, and metabolic measures were assessed between 0 and 30 wk of diet allocation. Body weight (A) and energy consumption from food (B) were assessed weekly. (C) Adiposity was assessed after 6, 12, 18, 24, and 30 wk of diet allocation by EchoMRI. (D) Weight of gonadal fat after 6, 12, or 24 wk of diet allocation. (E) Cross-sectional area of adipocytes in gonadal fat after 6 and 24 wk of diet allocation. (F) Macrophages (F4/80+ cells) in gonadal adipose tissue after 24 wk of diet allocation. (G) IL-6 assessed by ELISA from serum collected prediet through to 24 wk of diet allocation. Metabolic phenotype: (H) fasting blood glucose; (I) glucose tolerance test. (J) Glucose-stimulated insulin secretion test. (K) Insulin tolerance test. Data in (A) and (B) and (I)–(K) are shown as a dot at the mean with error bars at ± SD. Data in (C)–(G) and area under the curve in (I)–(K) are shown with box height at the mean with error bars at ± SD, with each data point (Continued)
Flow cytometry analysis of peripheral blood leukocyte numbers in WT mice fed a standard chow (Chow) or HF diet from 3 wk (wk3) through 30 wk (wk30) of diet allocation. Absolute cell counts (numbers) of the following: (A) neutrophils, (B) total monocytes, (C) Ly-6C<sup>high</sup> monocytes, (D) Ly-6C<sup>low</sup> monocytes, (E) Ly-6C<sup>−</sup> monocytes, (F) B cells, (G) NK cells, and (H) T cells. (I) Ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells. (J) Ratio of lymphoid to myeloid cells. (K) Total leukocytes. Data are presented with a dot at the mean with error bars of ± SD. Data are pooled from one to four independent experiments with a total of n = 4–8 mice per diet group. Main effects of diet and time of diet allocation were assessed by two-way ANOVA with Sidak post hoc correction for multiple comparisons. Boldface text indicates significant main effects. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

FIGURE 2. Peripheral blood immune cell numbers in C57BL/6J female mice fed a chow or HF diet. Indicating an individual mouse. Data in (A)–(D) and (H) were pooled from one to four independent experiments of n = 4–8 mice per diet group. Data in (E) and (F) are from a subset of mice from one independent cohort. Data in (G) and (I)–(K) are from one independent cohort with Chow n = 7 and HF n = 8. (A–C) Main effects of diet and time of diet allocation were assessed by two-way ANOVA with Sidak post hoc correction for multiple comparisons, and statistical significance was assessed in (D)–(H) and area under the curve in (I)–(K) by two-tailed Student t test with Welch correction or Mann–Whitney U test between diet groups at each time point. For (A)–(C), boldface text indicates significant main effects. For (A), p < 0.05 from week 3 through week 30, and for (B), p < 0.05 for weeks 1–7. (C–K) *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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diet allocation, likely due to diet type as well as biological age, on all immune cell populations, except for numbers of B cells and total leukocytes. We observed an interaction between the effects of diet type and time of diet allocation on numbers of total monocytes (Fig. 2B) and Ly-6C<sup>high</sup> (Fig. 2C), Ly-6C<sup>low</sup> (Fig. 2D), and Ly-6C (Fig. 2E) monocyte subsets. HF-fed female mice had an increase in circulating monocytes between 3 and 30 wk of diet allocation, whereas this was not observed in chow-fed mice. In particular, numbers of Ly-6C<sup>high</sup> monocytes were lower in HF-fed mice compared with chow-fed mice after 3 wk of diet allocation, similar after 6 wk, and were consistently higher after 12 wk through 30 wk (Fig. 2C). Assessment of leukocyte population relative prevalence (as a proportion of total leukocytes) also revealed a significant interaction of the main effects of diet type and time of diet allocation on circulating monocyte populations, with an increase in monocyte prevalence in HF-fed mice compared with chow-fed mice (Supplemental Fig. 3). Therefore, there was a time-dependent increase in peripheral blood monocytes in female mice with HF diet-induced obesity.

**HF diet alters blood monocyte phenotype, function, and inflammatory response in WT C57BL/6J female mice**

Under conditions of homeostasis, Ly-6C<sup>high</sup> monocytes transition into Ly-6C<sup>−</sup> monocytes, which patrol the vasculature and remove damaged cells and debris, as well as promote angiogenesis and endothelial cell proliferation (51, 52). When homeostasis is perturbed, Ly-6C<sup>high</sup> monocytes are preferentially recruited to the site of inflammation, adopt proinflammatory effector functions, and may differentiate into tissue macrophages, as is observed within obese adipose tissue (53, 54). Chronic inflammation is also associated with changes to monocyte phenotype and function. We assessed expression of surface proteins associated with monocyte maturity (Ly-6C and F4/80) and migration (CCR2, CD11b, and CX<sub>3</sub>CR<sub>1</sub>) after 3, 6, 12, 18, 24, and 30 wk of diet allocation (Table I). In HF-fed mice compared with chow-fed mice, Ly-6C<sup>high</sup> monocytes generally had higher expression of Ly-6C (<i>downregulated</i> in the transition of monocytes from Ly-6C<sup>high</sup> to Ly-6C<sup>−</sup>) (51, 54) and decreased expression of CX<sub>3</sub>CR<sub>1</sub> (<i>upregulated</i> in the transition of monocytes from Ly-6C<sup>high</sup> to Ly-6C<sup>−</sup>) (51) and F4/80 (<i>upregulated</i> upon differentiation into macrophages) (53, 54), which suggests they are more immature. Expression of CD11b, involved in monocyte vascular movement and endothelial transmigration (53, 55), was higher on Ly-6C<sup>high</sup> monocytes after 3 and 6 wk of diet allocation in HF-fed mice. Ly-6C<sup>low</sup> monocytes had increased expression of Ly-6C<sup>−</sup> after 3 and 6 wk, although this decreased by 18 wk. Ly-6C<sup>−</sup> monocytes had decreased expression of CCR2, F4/80, and CX<sub>3</sub>CR<sub>1</sub> after 3 wk, but this was not consistently observed thereafter. In summary, in addition to increased numbers of circulating monocytes, diet-induced obesity in female mice altered monocyte expression of surface markers associated with the Ly-6C<sup>high</sup> to Ly-6C<sup>−</sup> transition, migration, and macrophage differentiation.

In male mice, CCR2/CCL2-mediated chemotaxis of Ly-6C<sup>high</sup> monocytes into obese adipose tissue, and their differentiation into proinflammatory TNF-producing macrophages, is a key contributor to the development of insulin resistance and systemic inflammation (7, 12–16). CCR2/CCL2 signaling mediates the movement of Ly-6C<sup>high</sup> monocytes from bone marrow into the periphery (53, 56). However, we found that there were no differences in Ly-6C<sup>high</sup> monocyte CCR2 surface expression between HF-fed and chow-fed female mice. We next determined if Ly-6C<sup>high</sup> monocytes from both diet groups were similarly responsive to the chemotactic ligand CCL2. Mice fed chow or HF diet were injected i.p. with CCL2 or saline, and we measured changes in bone marrow and peripheral blood monocyte populations 4 h later (Fig. 3A, 3B) (48). We found

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**Table I. Blood monocyte surface phenotype in WT mice fed an HF or chow diet**

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>Population</th>
<th>3</th>
<th>6</th>
<th>12</th>
<th>18</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly-6C</td>
<td>Ly-6C&lt;sup&gt;high&lt;/sup&gt;</td>
<td><strong>p &lt; 0.0001</strong></td>
<td><strong>p = 0.0001</strong></td>
<td><strong>p = 0.0041</strong></td>
<td><strong>p &lt; 0.0001</strong></td>
<td><strong>p = 0.0001</strong></td>
</tr>
<tr>
<td></td>
<td>Ly-6C&lt;sup&gt;low&lt;/sup&gt;</td>
<td><strong>p = 0.0006</strong></td>
<td><strong>p = 0.0021</strong></td>
<td>NS</td>
<td><strong>p = 0.0424</strong></td>
<td><strong>p = 0.0218</strong></td>
</tr>
<tr>
<td>CCR2</td>
<td>Ly-6C&lt;sup&gt;high&lt;/sup&gt;</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Ly-6C&lt;sup&gt;low&lt;/sup&gt;</td>
<td>p = 0.0452</td>
<td>NS</td>
<td>NS</td>
<td><strong>p = 0.0104</strong></td>
<td>NS</td>
</tr>
<tr>
<td>F4/80</td>
<td>Ly-6C&lt;sup&gt;high&lt;/sup&gt;</td>
<td><strong>p = 0.0064</strong></td>
<td><strong>p &lt; 0.0001</strong></td>
<td><strong>p = 0.0156</strong></td>
<td><strong>p = 0.0063</strong></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Ly-6C&lt;sup&gt;low&lt;/sup&gt;</td>
<td><strong>p = 0.0093</strong></td>
<td><strong>p &lt; 0.0001</strong></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CX&lt;sub&gt;3&lt;/sub&gt;CR&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Ly-6C&lt;sup&gt;high&lt;/sup&gt;</td>
<td><strong>p = 0.0248</strong></td>
<td><strong>p = 0.0303</strong></td>
<td><strong>p &lt; 0.0003</strong></td>
<td><strong>p &lt; 0.0001</strong></td>
<td><strong>p = 0.0018</strong></td>
</tr>
<tr>
<td></td>
<td>Ly-6C&lt;sup&gt;low&lt;/sup&gt;</td>
<td><strong>p = 0.0020</strong></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD11b</td>
<td>Ly-6C&lt;sup&gt;high&lt;/sup&gt;</td>
<td><strong>p = 0.0160</strong></td>
<td>NS</td>
<td>NS</td>
<td><strong>p = 0.0439</strong></td>
<td><strong>p = 0.0026</strong></td>
</tr>
<tr>
<td></td>
<td>Ly-6C&lt;sup&gt;low&lt;/sup&gt;</td>
<td><strong>p = 0.0123</strong></td>
<td><strong>p = 0.0035</strong></td>
<td>NS</td>
<td><strong>p = 0.0087</strong></td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are shown comparing WT HF-fed mouse monocyte surface expression to Chow-fed mouse monocyte surface expression, with arrows denoting the direction of significant differences (e.g., Ly-6C<sup>high</sup> monocytes from HF-fed mice, compared with chow-fed mice, consistently have higher Ly-6C expression). Geometric mean expression of each marker was compared from two to four independent experiments of n = 4–8 mice per group by normalizing the data from each mouse to the mean of the chow diet mouse group in each independent experiment. Statistical significance was assessed by two-tailed Student t test or Mann-Whitney U test between diet groups at each time point.

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that the prevalence of bone marrow Ly-6C^high monocytes in chow-fed and HF-fed female mice treated with CCL2 tended to decrease, compared with mice treated with saline (Fig. 3A), suggesting that there was CCL2-elicited egress of Ly-6C^high monocytes from bone marrow into circulation. There was a significantly higher fold difference in circulating Ly-6C^high monocytes between CCL2- and saline-treated, HF-fed mice (2.58 ± 0.29), compared with the fold difference in CCL2- and salinetreated, chow-fed mice (1.83 ± 0.37) (Fig. 3B). The fold differences of Ly-6C^- monocytes were similar between diet groups, as expected, given that they do not use CCR2/CCL2 for bone marrow egress or migration to tissues (51, 53), and the fold differences of Ly-6C^low monocytes were also similar between diet groups. Therefore, despite similar surface expression of CCR2 on Ly-6C^high monocytes from chow-fed mice, Ly-6C^high monocytes from HF-fed mice were more responsive to CCL2 in the periphery. We also assessed Ly-6C^high monocyte intracellular production of IL-6 and TNF. There were no significant differences in IL-6 production between diet groups after 6, 12, 18, and 24 wk of diet allocation (data not shown). TNF production was similar in LPS-stimulated Ly-6C^high monocytes from HF-fed mice compared with chow-fed mice after 6 and 12 wk of diet allocation (Fig. 3C, 3D) but increased in HF-fed mouse Ly-6C^high monocytes after 18 and 24 wk (Fig. 3E, 3F). Therefore, HF-fed female mice, compared with chow-fed mice, had a higher prevalence of circulating, immature, migration-primed Ly-6C^high monocytes, with increased production of TNF.

**Genetic ablation of TNF does not protect female mice from the effects of HF diet-induced obesity on peripheral blood immunophenotype**

Littermate female TNF^-/- and WT (TNF^+/+) mice were placed on an HF diet to assess if obesity-associated changes to peripheral immunophenotype, and Ly-6C^high monocytes in particular, are mediated by TNF. We found that there was a main effect of the time of diet allocation on body weight (Fig. 4A) and changes in whole-body adiposity (Fig. 4B) in the TNF^-/- and WT mice. There was a main effect of genotype on body weight, but post hoc analyses showed no differences between

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**FIGURE 3. Peripheral monocyte response to chemotactic ligand CCL2 and Ly-6C^high monocyte TNF production in C57BL/6J female mice fed a chow or HF diet.**

Mice were placed on a standard chow diet (Chow) or HF diet for 6 wk. Mice were injected i.p. with CCL2 or saline, and 4 h later bone marrow (A) and/or blood (B) were collected for assessment by flow cytometry. Data in (B) are shown as fold differences in immune cells between mice injected with CCL2 and the mean of mice injected with saline for both of the diet groups. (C–F) Flow cytometry analysis of Ly-6C^high monocyte intracellular production of TNF in unstimulated (Unstim) and LPS-stimulated (Stim) whole blood after (C) 6, (D) 12, (E) 18, and (F) 24 wk of diet allocation. Data are shown with box height at the mean, with error bars indicating ± SD. Each data point in (C)–(F) indicates an individual mouse. Data are from one independent experiment for (A) and (B), with CCL2 n = 6 and saline n = 4 for both diet groups in blood. Bone marrow was collected from n = 3–4 mice per treatment for both diet groups. Data in (C)–(F) are from a single experiment but are representative of one to four independent experiments of n = 4–8 mice per group and are reported as geometric mean fluorescence intensity. Statistical significance was assessed by two-tailed Student t test between diet groups at each time point. *p < 0.05, **p < 0.01.
FIGURE 4. Body weight, adiposity, and fasting blood glucose in WT and TNF−/− mice fed an HF diet. WT and TNF−/− (KO) mice were placed on an HF diet for up to 30 wk. (A) Body weight prior to diet allocation and every 6 wk thereafter. (B) Increase in adiposity (fat as a percentage of total body weight) prior to diet allocation and every 6 wk thereafter. (C) Six hours of fasting blood glucose after 12 or 28 wk of diet allocation. Data in (A) and (B) are presented with box height at the mean, with error bars indicating ± SD. Data in (C) are presented in box and whisker plots, minimum to maximum, in which the center line indicates the median. Data are pooled from one to three independent experiments of n = 4–8 mice per group. Main effects of time of diet allocation and genotype were assessed by two-way ANOVA with Sidák post hoc correction for multiple comparisons in (A) and (B), and statistical significance in (C) was assessed by two-tailed Student t test between diet groups at each time point. Boldface type indicates significant main effects.

We assessed peripheral blood immune cell numbers (Fig. 5) and relative prevalence (Supplemental Fig. 4) by flow cytometry in the HF-fed WT and TNF−/− mice prior to diet allocation, and every 6 wk thereafter through to 30 wk of diet allocation. We observed that there was no significant interaction between the main effects of genotype and time of HF diet allocation on numbers of neutrophils (Fig. 5A), total monocytes (Fig. 5B), and monocyte subsets (Fig. 5C–E), as well as NK cells (Fig. 5F), B cells (Fig. 5G), T cells (Fig. 5H), the ratio of CD4+ T cells to CD8+ T cells (Fig. 5I), the ratio of lymphocytes to myeloid cells (Fig. 5J), and total numbers of leukocytes (Fig. 5K). In addition, there was no interaction between the main effects of genotype and time of diet allocation on the prevalence of monocytes or other immune cell populations (Supplemental Fig. 4). Although a cursory inspection suggested that HF-fed TNF−/− mice had a lower prevalence of monocytes (as a proportion of total leukocytes) in comparison with HF-fed WT mice with increasing time of diet consumption (Supplemental Fig. 4B), the numbers of monocytes were similar between the WT and TNF−/− mice (Fig. 5B–E). Numbers of Ly-6Chigh monocytes increased from week 0 to week 30 of HF diet allocation over 2.2-fold in WT mice (mean ± SD; week 0: 68 ± 30 cells/μl; week 30: 155 ± 30 cells/μl) and over 2.5-fold in TNF−/− mice (week 0: 83 ± 37 cells/μl; week 30: 212 ± 57 cells/μl). However, we observed that there was a main effect of time on numbers and prevalence of all assessed immune cell populations because of the length of HF diet allocation and likely increasing biological age. We also observed a main effect of genotype on numbers of neutrophils (Fig. 5A), Ly-6Chigh monocytes (Fig. 5C), Ly-6Clow monocytes (Fig. 5D), NK cells (Fig. 5F), B cells (Fig. 5G), T cells (Fig. 5H), the ratio of lymphocytes to myeloid cells (Fig. 5J), and total numbers of leukocytes (Fig. 5K). Differences in peripheral blood immune cells by genotype were not unexpected, as TNF influences bone marrow hematopoietic progenitor proliferation and differentiation (23), the survival, activation, and functions of immune cells (22, 57), as well as cellular and structural organization of B cell follicles and germinal centers in secondary lymphoid tissues (58). In addition, we assessed the surface phenotype of Ly-6Chigh, Ly-6Clow, and Ly-6C− monocytes in the HF-fed WT and TNF−/− mice (Table II). Once mice were placed on the HF diet, there was some variability in monocyte surface marker expression over time, although monocyte surface phenotype generally became more similar between WT and TNF−/− mice with increasing time of HF diet allocation, with the exception of CX3CR1. In summary, there was a similar directional change to peripheral blood monocyte numbers and surface phenotypes over time in HF-fed WT and TNF−/− female mice.

DISCUSSION

To date, few studies have investigated whether obesity produces the same or similar outcomes in female animal models as in male models. To gain insight into the effects of obesity on

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peripheral cellular changes in obese female mice, we used WT and TNF\(^{-/-}\) mouse models of HF diet-induced obesity and performed assessments by flow cytometry at multiple time points. We found that diet-induced obesity in female mice led to changes in peripheral blood immunophenotype, with an increase in circulating immature, proinflammatory, migration-primed Ly-6\(\text{C}\)\(^{\text{high}}\) monocytes. We previously showed, consistent with other studies (25–27), that whole-body genetic ablation of TNF in male mice reduced adiposity, macrophage accumulation in adipose tissue, and insulin resistance (10). We also found that TNF\(^{-/-}\) male mice fed an HF diet were protected from an obesity-associated increase in circulating Ly-6\(\text{C}\)\(^{\text{high}}\) monocytes (10). We observed in female mice in this study that even after 30 wk of HF diet feeding, genetic ablation of TNF did not protect against diet-induced adiposity or changes to peripheral blood Ly-6\(\text{C}\)\(^{\text{high}}\) monocytes. To our knowledge, this is the first observation of sexual dimorphism in TNF-induced effects on immunological changes in diet-induced obesity. Our data from

\[ \text{FIGURE 5. Peripheral blood immune cell numbers in WT and TNF}^{-/-} \text{ mice fed an HF diet.} \]

Flow cytometry analysis of peripheral blood leukocyte numbers in WT and TNF\(^{-/-}\) (KO) mice on an HF diet prior to diet allocation (wk0) and every 6 wk through to 30 wk of diet allocation (wk30). Absolute cell counts (numbers) of the following: (A) neutrophils, (B) total monocytes, (C) Ly-6\(\text{C}\)\(^{\text{high}}\) monocytes, (D) Ly-6\(\text{C}\)\(^{\text{low}}\) monocytes, (E) Ly-6\(\text{C}\)\(^{-}\) monocytes, (F) NK cells, (G) B cells, and (H) T cells. (I) Ratio of CD4\(^{+}\) to CD8\(^{+}\) T cells. (J) Ratio of lymphocytes to myeloid cells. (K) Absolute cell count of leukocytes. Data are presented with a dot at the mean with error bars of ± SD. Data are pooled from one to three independent experiments with \(n = 4–8\) mice per genotype. Main effects of time of diet allocation and genotype were assessed by two-way ANOVA with Sidák post hoc correction for multiple comparisons. Boldface type indicates significant main effects. *\(p < 0.05\), **\(p < 0.01\), ****\(p < 0.0001\).
this study therefore emphasize that research from male mouse models is not always applicable in female models or vice versa (Fig. 6).

We found that WT female mice have time-dependent changes in numbers and surface phenotype of Ly-6C\textsuperscript{high} monocytes in response to 3–30 wk of allocation to an HF or chow diet. It was recently shown in male mice that within 1 wk of diet feeding, HF-fed mice, compared with chow-fed mice, have decreased circulating monocytes but increased \textit{ccl2} mRNA expression and accumulation of macrophages in adipose tissue (59). Our data showing an acute reduction in circulating monocytes after 3 wk of diet may indicate Ly-6C\textsuperscript{high} monocytes also preferentially travel to adipose (or other metabolic tissues) as an early effect of HF diet feeding in female mice. We also observed that total monocytes increased in prevalence within the blood of HF-fed female mice compared with chow-fed mice within 12 wk of diet allocation. These observations were in contrast to a study in female mice that found no differences in circulating monocyte prevalence after 12 wk diet feeding (8), possibly due to differences in age and chow diet composition. However, these data were consistent with previous data, including our own, from male mice (8–11). Monocytosis is also recognized to occur in obese humans, irrespective of sex (60–62).

Increased surface expression of chemokines and enhanced cytokine responses to bacterial LPS or viral ssRNA stimulation have been reported in classical monocytes from obese female humans compared with lean controls (60, 61). We found that Ly-6C\textsuperscript{high} monocytes (similar to human classical monocytes) from HF-fed female mice were highly primed for CCL2-mediated chemotaxis compared with Ly-6C\textsuperscript{high} monocytes from chow-fed mice, despite similar surface expression of CCR2 on Ly-6C\textsuperscript{high} monocytes in both diet groups. We previously found that CCR2 expression was elevated on Ly-6C\textsuperscript{high} monocytes from male mice fed an HF diet compared with a chow diet (10). It has been reported that \textit{ccl2} mRNA expression is lower in obese adipose tissue of females compared with males (8). Other chemokine receptors, such as CCR5, contribute to monocyte recruitment into adipose tissue in male mice (63), so our data could indicate that monocytes in female mice rely more on CCR2-independent signaling for recruitment to adipose tissue, although it is unknown whether there are sex differences in the regulation of monocyte chemotaxis in obesity. In addition, we detected similar serum levels of IL-6 between chow and HF-fed female mice from 6 through 24 wk of diet allocation and were not able to detect TNF, consistent with previous observations (64). We further showed that Ly-6C\textsuperscript{high} monocytes

![Summary of monocyte-related sex differences in obesity.](https://www.immunohorizons.org/)
from HF-fed female mice have the capacity to produce more TNF in response to LPS stimulation after an extended period of HF diet allocation (i.e., 18 or 24 wk). Collectively, these data suggest that although monocytes have the capacity to produce TNF, and TNF influences their function, monocytes are not major contributors to TNF within circulation, and most TNF produced within tissues is used locally and/or remains bound to receptors in female mice with diet-induced obesity. Furthermore, proinflammatory cytokines can modulate expression of chemokine receptors and ligands. We confirmed in our experiments that TNF-mediated tonic stimulation of monocytes is important for their expression of the chemokine receptor CX3CR1 (65, 66), as we observed that Ly-6C<sup>low</sup> and Ly-6C<sup>+</sup> monocytes retained lower expression of CX3CR1 in HF-fed TNF<sup>−/−</sup> mice compared with HF-fed WT mice through to 24 or 30 wk of diet allocation. TNF has also been reported to reduce monocyte expression of CCR2 (67, 68). Although expression of CCR2 expression was higher on peripheral Ly-6C<sup>high</sup> monocytes from TNF<sup>−/−</sup> mice compared with WT mice in our experiments, CCR2 expression was similar between genotypes with extended HF diet allocation, suggesting that TNF is not a major mediator of CCR2 expression in female mice with diet-induced obesity.

The factors that contribute to sex differences in TNF action during obesity on local and peripheral cellular inflammation, as well as insulin resistance, are unclear. Within obese adipose tissue, TNF promotes transcription of proinflammatory cytokines (including itself) via NF-κB, and TNF also, via TNFRI, blocks downstream signaling of the insulin receptor, leading to insulin resistance (24). The magnitude of TNF expression in adipose tissue in HF-fed male mice has been reported to be significantly higher than in HF-fed female mice, possibly due to an autoamplification effect of TNF expression within adipose tissue of obese male mice, but not obese female mice (69). In male, but not female, rodents, a paracrine loop of TNF production from macrophages and free fatty acid production from adipocytes has also been described that exacerbates inflammation within adipose tissue (70–72). As suggested by our data, genetic ablation of TNF may, as a consequence, have a greater impact on local and peripheral cellular inflammation, as well as insulin sensitivity, in male mice than in female mice in the context of diet-induced obesity.

Although few clinical studies have addressed whether biological sex contributes to variable outcomes of immunotherapies, there is evidence that anti-TNF treatments for rheumatoid arthritis show sex-specific differences in efficacy (73). Male patients have higher remission rates and less-adverse outcomes compared with female patients (74, 75). The underlying biological basis for why anti-TNF treatments are less efficacious in females is unknown. However, the success of anti-TNF therapy in rheumatoid arthritis has been linked to changes in peripheral blood monocyte numbers and activation phenotype (76) and a reduction in macrophage accumulation in the synovium (77). Future studies into the mechanisms underlying sex differences in response to TNF in obesity-associated chronic inflammation and changes to monocyte and macrophage populations may therefore provide insights into other chronic inflammatory disorders.

**DISCLOSURES**

J.D.S., D.M.S., and D.M.E.B. hold Canada Research Chairs. The other authors have no financial conflicts of interest.

**REFERENCES**


Supplementary Figure 1. Surface expression of monocyte/macrophage maturity and migration markers assessed by flow cytometry.
Supplementary Figure 2. Gating strategy for Ly6C^{high} monocyte intracellular TNF production in response to LPS stimulation.
Supplementary Figure 3. Peripheral blood immune cell prevalence in mice fed a chow or HF diet.

Flow cytometry analysis of peripheral blood leukocytes in wildtype mice on standard chow (Chow) or a high fat (HF) diet from 3 weeks (wk3) through 30 weeks (wk30) diet allocation. Prevalence (as a proportion of CD45^+ leukocytes) of: (A) neutrophils, (B) total monocytes, (C) Ly6C^high monocytes, (D) Ly6C^low monocytes, (E) Ly6C^-monocytes, (F) NK cells, (G) B cells, (H) T cells, (I) CD4^+ T cells, (J) CD8^+ T cells. (K) CD4^+ T cells as a proportion of total CD3^+ T cells. Data are presented with a dot at the mean with error bars of ± standard deviation. Data are pooled from 1-3 independent experiments with a total of n=4-8 mice per diet. Main effects of diet and time of diet allocation were assessed by two-way ANOVA with Šidák’s post-hoc correction for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Supplementary Figure 4. Peripheral blood immune cell prevalence in WT and TNF^{-/-} mice fed a HF diet.

Flow cytometry analysis of peripheral blood leukocytes in wildtype (WT) and TNF^{-/-} (KO) mice on a high fat diet prior to diet allocation (wk0) and every 6 weeks through to 30 weeks diet allocation (wk30). Prevalence (as a proportion of CD45^{+} leukocytes) of: (A) neutrophils, (B) total monocytes, (C) Ly6C^{high} monocytes, (D) Ly6C^{low} monocytes, (E) Ly6C^{-} monocytes, (F) NK cells, (G) B cells, (H) T cells, (I) CD4^{+} T cells, (J) CD8^{+} T cells. (K) CD4^{+} T cells as a proportion of total CD3^{+} T cells. Data are presented with a dot at the mean with error bars of ± standard deviation. Data are pooled from 1-3 independent experiments with n=4-8 mice per genotype. Main effects of time of diet allocation and genotype were assessed by two-way ANOVA with Šidák’s post-hoc correction for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001.