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Original Research

Comparison of Blood Monocytes and Adipose Tissue Macrophages in a Mouse Model Diet-Induced Weight Gain

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Diet-induced weight gain causes changes in adipose tissue that alter blood monocytes and adipose tissue macrophages, increasing disease risk. The purpose of this study was to compare the effects of 24 wk of diet-induced weight gain on the percentages of blood monocytes and adipose tissue macrophages as well as the cell-surface expression of toll-like receptors 2 and 4 and leptin receptor, which are associated with inflammation and homing to adipose tissue. Crl:CD1(ICR) male mice were assigned to either a diet-induced weight gain (60% of calories from fat; *n* = 12) or control (10% of calories from fat; *n* = 13) group. After 24 wk of dietary treatment, whole blood and bilateral perigonadal fat pads were collected. Whole blood or SVF were separately labeled for monocytes (CD11b$^+$CD14-) or macrophages (CD11b$^+$F4/80$^+$) and receptor expression by using 3-color flow cytometry. Data were analyzed by using univariate ANOVA. Compared with control mice, those in the weight-gain group had greater body weight, fat mass, and percentages of monocytes and macrophages compared with CN. Regardless of cell type, monocytes and macrophages from mice in the weight-gain group expressed significantly less toll-like receptor 2 and leptin receptor than did control mice. The present study demonstrates that monocytes and macrophages are similarly affected by diet-induced weight gain. More research is needed to confirm how monocytes might be used as a proxy measure of macrophages.

Abbreviations: gMFI, geometric mean fluorescence intensity; SVF, stromal vascular fraction; TLR, toll-like receptor.

Diet-induced weight gain causes changes in adipose tissue that contributes to increased disease risk via an elevation in systemic inflammation.[1,13] Obese subjects have elevated concentrations of chemoattractants, which recruit naïve blood monocytes from the blood into adipose, where they mature into adipose tissue macrophages and 'inflame' adipose tissue.[2,7,8,26] The principal recruitment signal for monocytes is elevated leptin and monocyte chemoattractant protein 1 and reduced adiponectin.[4,28] Inflammation, as measured by an increase in proinflammatory cytokine (IL1β, IL6, and TNFα) production, is involved in the pathophysiology of cardiovascular disease and type 2 diabetes mellitus.[9,11] Proinflammatory cytokine production in monocytes and macrophages is mediated by toll-like receptors (TLR). Of the 9 known TLR, TLR2 and TLR4 are involved in adipose tissue inflammation because they are responsive to leptin and other biomarkers associated with weight gain.[3,5,14,23,24] Despite the known association of both monocytes and macrophages with inflamed adipose tissue, monocytes and macrophages in lean and obese mice have not been compared.

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Such a comparison would be advantageous because the collection of adipose tissue macrophages requires a terminal procedure, whereas blood monocytes can be harvested by using a nonlethal technique. We previously suggested that the use of nonlethal collection is desirable because it allows a researcher to collect longitudinal data over time and to reduce the number of animals that are required for a given experiment.[7,14] Because blood monocytes may be a naïve version of adipose tissue macrophages, their measurement may provide a suitable proxy for the assessment of adipose tissue macrophages. With this knowledge, future studies might improve outcome measures related to diet-induced obesity, with fewer animals required for a given experiment. The purpose of the current study was to evaluate blood monocytes and their cell-surface receptors (leptin receptor, TLR2, and TLR4) as a possible proxy measure of adipose tissue macrophages in lean and obese mice.

Materials and Methods

Animals. All protocols used in the present study were approved by the University of Houston IACUC. We certify that all applicable institutional and governmental regulations concerning the use of animals were followed during this research. Male Crl:CD1(ICR) mice (age, 6 to 8 wk) were purchased from Charles Rivers Labs (Wilmington, MA). It has been previously suggested that outbred mice are a better model for longitudinal studies because they typically live longer and experience fewer age-related

complications than do inbred mice.[25] All mice were free of mouse parvovirus (general [NS1 antigen] and types 1 and 2), minute virus of mice, mouse hepatitis virus,Theiler murine encephalomyelitis virus, epidemic diarrhea of infant mice virus, pneumonia virus of mice, reovirus, and *Mycoplasma pulmonis*. Mice were acclimated to a reversed light:dark cycle (lights off at 0900) in a temperature-controlled (22 ± 1 °C) room of the central animal care facility for 14 d prior to additional treatment. After acclimation, mice were tattooed with a study identification number on the tail. Mice were housed 4 or 5 per microisolation cage (65 cm^3; Techniplast, Maggio, Italy) containing aspen straw bedding at the AAALAC-accredited animal care facility. Cages were changed twice weekly. Enrichment was provided in the form of a chew pad and a house in each cage. Body weight and food intake were determined weekly by using a digital scale. Mice had ad libitum access to water throughout the study.

Conditions and treatment. The 24-wk treatment was selected in light of findings of our previously published weight-gain study.[5] After acclimation, mice were randomized to either a diet-induced weight-gain ($n = 12$) or a control ($n = 13$) group. Mice in the weight-gain group were provided ad libitum access to a 60% fat diet (catalog no. 12492, Research Diets, Brunswick, NJ), whereas control mice had ad libitum access to a 10% fat diet (catalog no. 12450B, Research Diets). Both commercially available diets were based on the AIN76A formulation. We opted to use the designation 'diet-induced weight gain' rather than 'diet-induced obesity' because there is no universally accepted standard for defining obesity in mice.

Chow was presented as a weekly bolus dose in the food rack in the wire-bar lid of the homecage. Water bottles were held in the wire-bar lid also. Food was replaced each week, and intake was monitored by using a digital scale. Body weight was also measured on a weekly basis using a digital scale. Mice were treated with the 2 diets for 24 wk.

Blood and tissue collection. Mice were fasted (except for water) for at least 6 h prior to blood and tissue collection. Venous blood (approximately 50 µL) was collected from a peripheral leg vein as described previously and treated with lithium heparin to prevent clotting.[14] Whole blood was used as a source of monocytes for subsequent experiments. After venous blood collection, fasted (more than 8 h) mice were injected with a near-lethal dose of xylazine–ketamine solution (5 mg/kg IP), which incapacitated them within 3 min of injection. After loss of the plantar reflex and before cessation of respiration, the thoracic cavity was opened, and the inferior vena cava was severed as a secondary euthanasia method. The abdominal cavity then was opened, and the bilateral perigonadal fat pads were removed and placed in a tube containing Kreb Ringer Lactate Buffer (Sigma-Aldrich, St Louis, MO) until digestion to isolate macrophages. Care was taken during fad pad removal to dissect away connective tissue and the epididymis. Blood samples and adipose tissue were stored on wet ice and processed within 2 h of collection.

Adipose tissue digestion. Both bilateral perigonadal fat pads were minced with sterile scissors in a 50-mL centrifuge tube filled with 10 mL of a 1-mg/mL collagenase type I solution (Worthington Biomedical, Lakewood, NJ) and incubated for 30 min in a 37 °C shaker to separate the mature adipocytes from the stromal vascular fraction (SVF). The floating mature adipocyte fraction was removed and discarded, and the SVF was washed twice and resuspended in 500 µL PBS with 10% BSA (Sigma-Aldrich) and used a source of adipose tissue macrophages in subsequent flow cytometric experiments.

Labeling for flow cytometry. All labeling for flow cytometry was completed as described previously.[6,14] The concentration of blood leukocytes and SVF cells were determined using an automated procedure (Millipore–Guava Viacount, Hayward, CA). All antibodies and reagents were purchased from eBioscience (San Diego, CA). Lithium heparin-treated whole blood was aliquoted into 5 tubes (40 µL per tube). The first 3 tubes contained both CD14–FITC (clone Sa2-8) and CD11b–PECy5.5 (clone M1/70) to identify monocytes and one of each of the following cell-surface markers: TLR2–PE (clone 6C2), TLR4–PE (clone UT41), or PE-conjugated leptin receptor (clone 55305). Similar to blood labeling, SVF suspensions were aliquoted into 5 tubes (40 µL per tube). The first 3 tubes contained F4/80–FITC (clone BM8) and CD11b–PECy5.5 (clone M1/70) to identify macrophages and one of each of the following cell-surface markers: TLR2–PE (clone 6C2), TLR4-PE (clone UT41), or PE-conjugated leptin receptor (clone 55305). Tubes 4 and 5 for both blood and SVF contained isotype-matched control antibodies and cells only, respectively. Signals in lysed and fixed cell suspensions were acquired on a flow cytometer (EasyCyte Mini, Millipore–Guava Viacount). Daily variation was tracked by using calibrated bead standards (Guava Check Beads) and was less 5% for all fluorescent analyses. Prior to acquisition, protocols were established to adjust photomultiplier voltages such that the negative population (from isotype control and cells-only tubes) was placed in the first log-decade.

Samples were analyzed uncompensated, and FACS data files were exported for digital compensation and analysis offline by using FCS Express (version 3; De Novo Software, Los Angeles, CA). Compensation was completed by using single-color tubes for the described antibody panels as done previously.[6] Care was taken to carefully compensate the spectral overlap between FITC and PE. Monocytes ($CD14^+CD11b^+$) and macrophages ($F4/80^+CD11b^+$) were identified by using primary 2-parameter dot plots, and secondary histograms were used to determine geometric mean fluorescence intensity (gMFI) for TLR2, TLR4, and leptin receptor.

Statistical analysis. All statistical analysis was completed by using PASW software (version 18; Somers, NY). Prior to statistical analysis, data were assessed for normality and constant error variance. Data that were in violation were log-transformed before additional testing. All dependent variables were analyzed using separate 2 (treatment: weight-gain or control) × 2 (cell type: monocyte or macrophage) ANOVA with repeated measures on the second factor. Significance was set at a P value of less than 0.05, and location of significant effects was determined by using a Tukey post hoc test. Data are presented as group mean ± 1 SD. Linear regression was used to further compare the relationship between blood monocytes and adipose tissue macrophages. Body weight was included as a variable in the model.

Results

Body weight and dietary intake. Consistent with our previous findings, mice fed the weight-gain diet had significantly greater body weight ($P < 0.001$) and perigonadal fat weight ($P = 0.036$) than did control mice. Weight-gain mice weighed 70.7 ± 1.7 g compared with control mice, which weighed 52.7 ± 1.8 g, representing a weight increase of 34% with high-fat feeding. Perigonadal fat weight of weight-gain mice was 5.09 ± 0.56 g compared with 2.69 ± 0.58 g for control mice, representing an 89% increase with high-fat feeding. These results are consistent with the pattern the response to diet-induced weight gain in Crl:CD1(ICR) male mice that we previously reported.[5]

Change in monocyte/macrophage percentage. The percentage of monocytes was significantly ($P = 0.031$) increased in weight-gain (3.32% ± 0.48%) compared with control (1.89% ± 0.41%) mice. A similar increase was observed for the percentage of SVF cells that were macrophages ($P = 0.004$), with that in weight-gain mice (3.26% ± 0.40%) greater than that for control mice (1.25% ± 0.46%). The percentage increase in monocytes and macrophages in weight-gain mice was similar.

Cell-surface receptor expression. TLR2 cell-surface expression was significantly reduced on the cell-surface of both monocytes (17.2 ± 3.2 gMFI; $P = 0.002$) and macrophages (95.2 ± 24.9 gMFI; $P = 0.002$) from weight-gain mice compared with control mice (monocytes, 43.2 ± 3.0 gMFI; macrophages, 120.1 ± 21.9 gMFI). In addition, regardless of dietary treatment, TLR2 cell-surface expression was significantly ($P = 0.002$) greater in macrophages than monocytes. Of the cell-surface receptors measured, the largest effect size was associated with TLR2 expression.

TLR4 cell-surface expression demonstrated a similar trend that for cell-surface TLR2 expression, but differences were not significant at the present sample size ($P = 0.226$). The pattern of TLR4 expression differed from that of TLR2 in that, regardless of group, monocytes and macrophages appeared to express similar amounts of TLR4. Of the cell-surface receptors measured, the smallest effect size was associated with cell-surface TLR4 expression.

Leptin receptor expression demonstrated a similar pattern to that of TLR2 expression. Leptin receptor expression was decreased on both monocytes (39.5 ± 9.4 gMFI; $P = 0.045$) and macrophages (60.2 ± 6.0 gMFI; $P = 0.045$) in weight-gain compared with control mice (monocytes, 69.0 ± 9.0 gMFI; macrophages, 85.9 ± 5.0 gMFI). In addition, macrophages expressed more leptin receptor than did monocytes. The effect size associated with leptin receptor expression was intermediate to that of TLR2 and TLR4.

Discussion

The present study used our validated model of diet-induced weight gain to evaluate similarities between blood monocytes and adipose tissue macrophages in lean and obese mice.[5] To assess this relationship, we evaluated the abundance of monocytes and macrophages and the expression of receptors that may reflect functional capacity. We found that obese mice had twice as many monocytes and macrophages as did lean mice. In addition to difference in cell abundance, monocyte and macrophage expression of TLR2 and leptin receptor were lower in the diet-induced weight-gain group. Although TLR4 expression did not change significantly, it showed a trend toward a similar pattern of change as that of TLR2 and leptin receptor. The findings of the present study provide new information supporting the principal of the '3Rs.' Specifically, using blood collection in lieu of terminal procedures would substantially reduce the number of animals required for a particular experiment. In addition, collection of nonlethal blood samples replaces traditional blood collection methods, which tend to be more invasive.

Our findings with respect to the relationship between monocyte–macrophage proportion and weight gain are consistent with previous reports from our laboratory and others.[5,14,16] We previously demonstrated that as little as 6 wk of diet-induced weight gain elevates blood monocyte concentration, and this increase is maintained for at 12 wk.[14,30] Consistent with our hypothesis, monocytes and macrophages demonstrated nearly identical responses and were in greater abundance in obese compared with lean mice. The greater numbers of monocytes and macrophages in obese mice are interrelated in that monocytes increase the adipose tissue macrophage pool, which creates a stimulus for further release of monocytes into circulation. The similar changes in the percentages of monocytes and macrophages suggest that measuring changes in blood monocyte percentage may reflect changes in macrophage accumulation.

In addition to comparing differences in monocyte–macrophages abundance, we included a measure of cell-surface receptors associated with inflammatory potential or homing to adipose tissue. Our laboratory and others have demonstrated that diet-induced weight gain is commonly associated in alterations in TLR2 and TLR4 expression.[14,30] In the present study, we found that excessive weight gain in mice reduced TLR2 and tended to reduce TLR4 expression on both monocytes and macrophages, suggesting that their cytokine-producing capacity had been reduced. We previously reported that 12, but not 6, wk of diet-induced weight gain was associated with a decrease in monocyte TLR4 expression.[14,30] The present study represents a continuation of this work, because it demonstrates that 12-wk reductions in TLR4 expression can be maintained until at least 24 wk. Several previous studies have reported that TLR4 knockout mice are resistant to diet-induced obesity.[10,20,31] In addition, obese children who carry a mutant allele of TLR4 have lower TNFα and less insulin resistance than do their lean counterparts.[19] Therefore, the reduction in cell-surface receptors we observed in the present study may indicate reduced functionality in the monocytes and macrophages of obese mice.

Collectively, TLR appear to play several different roles in metabolism, weight gain, and disease.[23-27] Further interpretation of the data from the previous[25-30] and current studies suggests that TLR may play a role in the underlying physiology of diet-induced weight gain. Applying these findings to the present study suggests that the reduction in monocyte–macrophage TLR expression with diet-induced weight gain may represent an attempt by the body to curb additional weight gain. In addition, TLR play a key role in mediating inflammatory capacity,[15,24] development of metabolic syndrome,[17,22] and insulin resistance.[21] Given these known effects, a reduction in TLR expression may be another mechanism to reduce the onset of obesity-associated disease, although others have reported that such an approach is ineffective.[32]

Although considerable previous research has explored the link between TLR and obesity, much less is known about the implications of changes in monocyte–macrophage leptin receptor expression. Leptin is a metabolic hormone that is released in high concentrations from hypertrophied adipose tissue.[12,18,29] In addition, leptin has been demonstrated to increase proinflammatory cytokine production in other tissues from obese mice.[27] In light of these previous findings, it is plausible that leptin receptor plays a similar inflammation-mediating role in monocytes and macrophages during obesity. The differences in leptin receptor expression that we found were similar to those observed for TLR in obese mice. To our knowledge, the present study is the first to measure monocyte–macrophage leptin receptor expression in obese and lean mice. Given this information, future research is needed to further expand our understanding of the roles of leptin receptor expression in a diet-induced weight gain model.

An obvious limitation of the present study is that we did not measure disease outcomes in our model. Now that we have established the similarity of the relationship between monocytes and macrophages in obese mice, we can explore longitudinally how changes in monocytes contribute to disease risk in a diet-induced weight

gain model. Because the present study was completed in outbred mice, we believe that the findings would translate well to other inbred mouse strains that are likely to have less variability. Obviously, more research is needed to confirm whether this hypothesis is true. Due to the variability in cell-surface marker expression levels described herein, future studies may still require some direct testing of expression in blood and tissue to confirm inferences made with blood monocyte phenotyping in healthy and disease states.

In summary, the key findings of the present study were that the percentages of monocytes and macrophages were similar but greater in diet-induced weight-gain mice compared with control mice. Diet-induced weight-gain mice had approximately twice as many monocytes and macrophages as did control mice. In addition, these cells in obese mice display similar reductions in receptors that are associated with inflammatory capacity and migration into adipose tissue. These changes could contribute to adipose tissue inflammation, elevated systemic inflammation, and eventually increased disease risk. The present study effectively evaluated monocytes as a proxy measure of macrophages, but more research is needed to refine these measures.

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