

Longitudinal, Diet-induced Weight Gain is Associated with Increased Blood Monocytes and Reduced TLR4 Expression

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ABSTRACT

Int J Exerc Sci 3(3): 134-142, 2010. Excessive weight gain increases systemic inflammation resulting in increased disease risk. Toll-like receptor 4 (TLR4) reportedly mediates increases in inflammation; however, its role in obesity-induced inflammation has not been fully evaluated. The purpose of this study was to determine the longitudinal effect of diet-induced weight gain on blood monocyte concentration and cell-surface TLR4 expression. Male CD-1 mice were randomly assigned to high-fat (HF, $n = 12$) or low-fat (LF, $n = 13$) groups. Non-lethal, saphenous vein blood samples were collected at 0, 4, 8 and 12 weeks of treatment. Three-color flow cytometry was used to measure monocyte (CD11b⁺/CD14⁺) concentration and TLR4 cell-surface expression. Data were analyzed with a repeated measures ANOVA; significance was set at $P < 0.05$. Body weight at week 12 was 21% greater in HF than LF ($P < 0.05$). At week 12 HF had 155% more monocytes ($P < 0.05$) with 24% less TLR4 than LF; Monocyte concentration and body weight at week 12 was negatively correlated with TLR4 gMFI ($P < 0.05$). The observed effects of high-fat feeding on blood monocytes are consistent with a phenotype, which may be associated with premature morbidity. The observed monocyte responses may be associated with immune dysfunction and diminished response to infection.

KEY WORDS: 60% fat diet, CD-1 mice, obesity, non-lethal, flow cytometry

INTRODUCTION

Weight gain following consumption of a high-calorie, high-fat diet is known to cause premature morbidity and mortality in a variety of mammals (30). Alfune Neto et al. reported that in humans an elevated monocyte concentration was associated with an increased risk of cardiovascular disease (1). Monocytes are also a source of pro-inflammatory cytokines that have been implicated in insulin resistance and initiating the hepatic acute phase response

(20, 23). The functional capacity of macrophages has been associated with expression of cell-surface receptors (3, 19), suggesting that functional changes of monocytes may be estimated by assessing such expression. Toll-like receptor 4 (TLR4) is responsible for mediating monocyte response to a variety of endogenous and exogenous substances (6). Weight gain is associated with an increase in endogenous lipopolysaccharide (5) and glucose, which can alter monocyte TLR4 expression (8) and thus monocyte functional capacity. TLR4

also appears to play a role in body fat accumulation, because mice with a functional knockout of TLR4 develop the Adonis phenotype, which is characterized by low body fat and high bone mineral density regardless of dietary fat content (13, 14). In our lab, we have reported that sedentary non-obese adults have higher monocyte TLR4 expression than matched physically active individuals (11, 15, 16). More research is needed to compare and contrast the effect of physical inactivity and weight gain on monocytes in humans and mice.

The key aim of the present study was to examine the effect of diet-induced weight gain on blood monocyte concentration and cell-surface TLR4 expression in mice. A unique aspect of the present study is that we used a longitudinal survival model, which allowed us to evaluate changes over time within in each animal. We hypothesized that diet-induced weight gain would be associated with an increase in monocyte concentration and an increase in cell-surface TLR4 expression. The purpose of this study was to determine the effects of 12-weeks of diet-induced weight gain on monocyte concentration and cell-surface TLR4 expression in male CD-1 mice. A secondary purpose was to evaluate the effectiveness of a longitudinal survival model to evaluate changes in monocytes over time.

METHODS

Reduction and Refinement of Existing Models

The present study was designed to reduce the number of animals needed and refine existing experimental methods. The sample size for the present study was selected

using an *a priori* calculation that was completed using preliminary weight gain data from our lab (17). Despite the modest sample size of the present study (N=25), we had sufficient statistical power due to the robust, repeated measures design. The dependent variable with the smallest effect size was monocyte concentration (0.45, moderate effect), which had an associated *post hoc* statistical power of 85%. The statistical power for the other dependent variables exceeded 90%. If the present study had been completed using an end-point design, we would have needed 100 mice compared to the 25 that we used. In order to utilize the survival design, we had to modify existing mouse flow cytometry techniques to use small blood volumes (<50 μ L), which was possible using micro capillary flow cytometry and the Millipore-Guava EasyCyte Mini. The present study demonstrates that it is possible to track changes in monocytes over time, thus reducing the number of animals needed and refining existing measurement techniques. While we used the present design in the context of diet-induced weight gain, we anticipate that our approach would be very useful in other experimental contexts.

Animal Subjects

All methods were reviewed and approved by the UH Institutional Committee for the Care and Use of Animals and principles of laboratory animal care were followed, as well as specific national laws as governed by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Outbred, specific-pathogen free CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA) and were 22-24 weeks of

age at the start of the study. Routine sentinel animal testing did not reveal the presence of any pathogens during the 12-weeks of the study. We selected outbred mice because they are believed to be better suited for longitudinal experiments than inbred mice (18). After arrival at the facility (24-h), an ID number was tattooed on the tail (AIMS, Hornell, NY). Mice were housed 4-5 per cage in a temperature-controlled room (22°C) at the UH Animal Care facility. Mice were kept on a 12:12-h light/dark cycle and were provided *ad libitum* access to water and food for the duration of the study. Body weight and food intake were recorded on a weekly basis using a digital scale. Caloric intake was calculated using information provided by the food manufacture (Research Diets, Inc).

Group Assignment and Diet Treatment

Following a 2-week acclimation, mice were randomly assigned by cage to either high-fat (HF, N=12) or low-fat (LF, N=13) groups. High-fat (60% kcal from fat, 20% from carbohydrate and 20% from protein) and low-fat (10% kcal from fat, 70% from carbohydrate and 20% from protein) diets were purchased from Research Diets, Inc. (New Brunswick, NJ). Each cage was provided an 80-100g bolus per week for *ad libitum* access to the food.

Saphenous Vein Blood Collection

Non-lethal venous blood samples were collected at 0, 4, 8 and 12 weeks^(10, 12). Mice were placed in a modified 50 mL centrifuge tube, exposing one of the hind limbs. Hair was removed using an electric clipper and a thin layer of petroleum jelly was applied to allow blood to bead on the skin. A sterile 5 mm lancet (Medipoint, Inc.; Mineola, NY) was used to puncture the saphenous vein.

A 40 µL aliquot of blood was drawn into a capillary tube treated with lithium heparin for flow cytometry analysis.

Flow Cytometry for Monocytes

Prior to labeling, total leukocyte concentration was determined using an automated assay (ViaCount; Millipore-Guava; Hayward, CA). All antibodies and reagents were purchased from e-Bioscience (San Diego, CA) unless otherwise noted. Heparin-treated whole blood (10 µL) was transferred to 1.2 mL library tubes (VWR Scientific; West Chester, PA) and treated with 10 µL FC (CD16/32) blocking cocktail for 10-min. FC blocked cells were washed with PBS (Sigma-Aldrich; St. Louis, MO) and re-suspended in staining buffer with CD14-FITC, TLR4-PE, and CD11b-PECy5.5 anti-mouse monoclonal antibodies at the titration recommended by the manufacturer. Following a 30-min incubation, red blood cells were lysed by incubating cells with a commercial lysing buffer for 10-min followed by centrifugation (2000 x g, 10-min). Cells were washed with PBS and re-suspended in 100 µL of staining buffer and 100 µL of 1% formalin (Electron Microscopy Sciences; Hatfield, PA) to fix cells. Additional tubes were included for isotype antibodies (Rat IgG1), which served as a negative control. Uncompensated flow cytometry data were acquired using a Millipore-Guava EasyCyte Mini flow cytometer (Heyward, CA) equipped with a solid-state 488 nm laser. Instrument variability was <2% as tracked using standard sized polystyrene beads. Electronic compensation and analysis of flow data were completed using FCS Express (DeNovo Software; Los Angeles, CA). Primary plots (CD14 vs. SSC and CD14 vs. CD11b) were used to identify

monocytes and secondary histograms were used to determine monocyte cell surface TLR4 expression as geometric mean fluorescent intensity (gMFI), which is the accepted way to express cell-surface receptor changes.

Statistical Analysis

All statistical testing was completed using SPSS (v. 17.0; Chicago, IL). Prior to formal statistical testing, data were analyzed for normality and constant error variance between groups and sphericity among the repeated measures. Non-normal data were log-transformed prior to analysis of variance (ANOVA) testing. Body weight and energy intake were analyzed using a 2 (Group: HF and LF) \times 12 (Time: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 weeks) ANOVA with repeated measures on the second factor. Monocyte concentration and cell-surface TLR4 expression were analyzed using a 2 (Group) \times 4 (Time: 0, 4, 8, 12 weeks) ANOVA with repeated measures on the second factor. Significance was set at a $P \leq 0.05$. Location of significant effects was determined in a *post hoc* manner using individual *t*-tests with a Bonferroni correction for multiple comparisons. Pearson's correlation coefficient was used to evaluate the associations between all dependent variables.

Results

Weight Gain and Energy Intake

At baseline, there was no significant difference in body weight between groups. A significant group \times time interaction was found for body weight (Figure 1; $F=14.996$, $P=0.001$). Starting at week 3, HF weighed more than LF. Over the course of the 12-week treatment period, both LF (41%) and

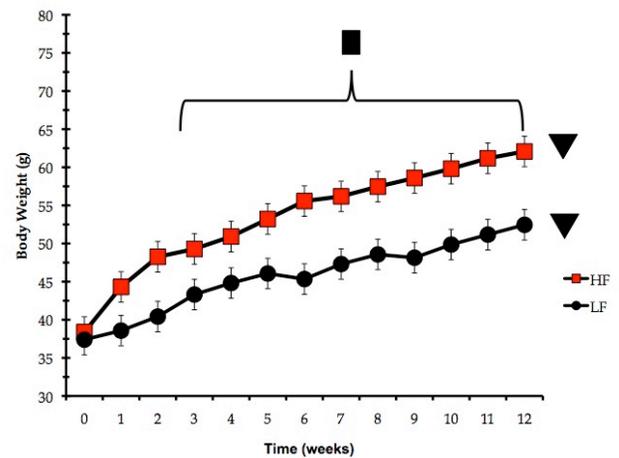


Figure 1. Body Weight. The present study examined peripheral blood monocyte TLR4 expression in male CD-1 mice gaining weight by consuming either a high-fat (HF, 60% kcal) or a low-fat (LF, 10% kcal) diet for 12 weeks. The results presented are for body weight in grams. Values are expressed as mean \pm SE. ■ indicates a significant difference between HF and LF ($P < 0.05$). ▼ indicates a significant change from baseline in the same group ($P < 0.05$).

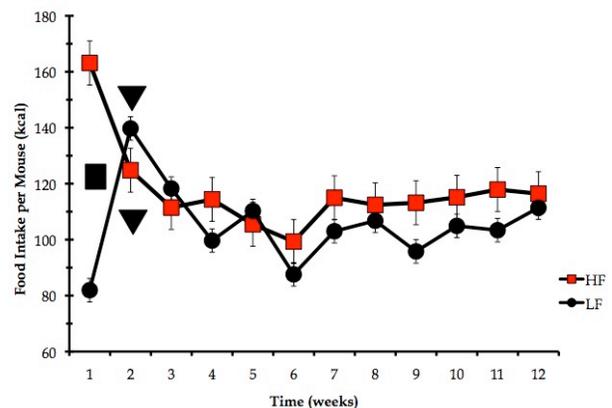


Figure 2. Food Intake. The present study examined peripheral blood monocyte TLR4 expression in male CD-1 mice gaining weight by consuming either a high-fat (HF, 60% kcal) or a low-fat (LF, 10% kcal) diet for 12 weeks. The results presented are for food intake per mouse in kilocalories. Values are expressed as mean \pm SE. ■ indicates a significant difference between HF and LF ($P < 0.05$). ▼ indicates a significant change from baseline in the same group ($P < 0.05$).

HF (62%) significantly increased their body weight compared to baseline. At week 12, HF was 21% heavier than LF. A significant group x time interaction was found for energy intake (Figure 2; $F=67.103$, $P=0.001$). HF consumed more calories than LF during week 1-2; however, from week 2-12 energy intake did not differ between groups.

Monocyte Concentration and TLR4 Expression
 A significant group x time interaction was found for monocyte concentration ($F=7.219$; $P=0.003$; Figure 3). HF and LF did not differ at baseline, however; HF was greater than LF at weeks 4 (118%), 8 (90%), and 12 (155%). Also, at week 12, HF was 35% greater than baseline and LF was 61% less than baseline. A significant main effect for group was found (Figure 4; $F=16.073$, $P=0.001$) such that HF had 24% lower monocyte cell-surface TLR4 expression than LF.

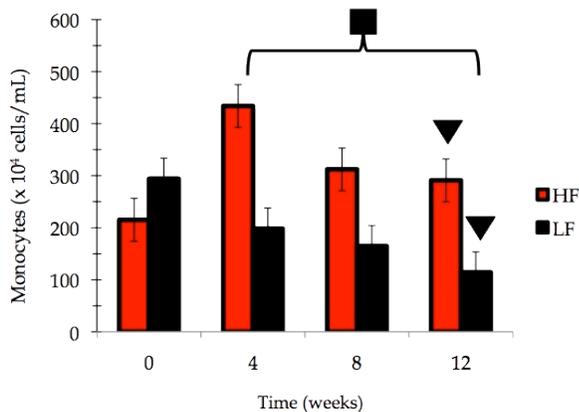


Figure 3: Monocyte Concentration. The present study examined peripheral blood monocyte TLR4 expression in male CD-1 mice gaining weight by consuming either a high-fat (HF, 60% kcal) or a low-fat (LF, 10% kcal) diet for 12 weeks. The results presented are for the concentration of CD11b⁺/CD14⁺ monocytes (x10⁴ cells/mL). Values are expressed as mean ± SE. ■ indicates a significant difference between HF and LF ($P<0.05$). ▼ indicates a significant change from baseline in the same group ($P < 0.05$).

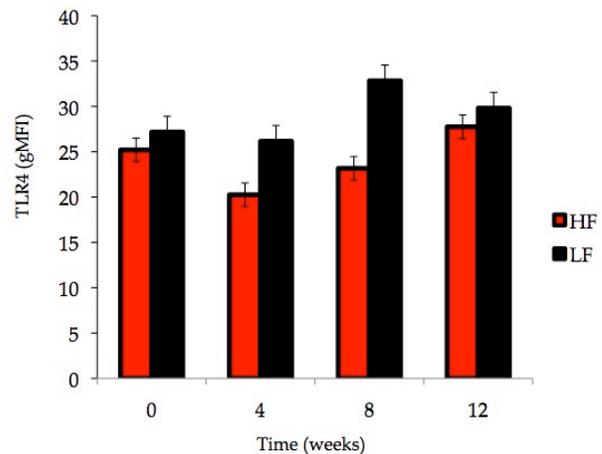


Figure 4: Monocyte Cell-Surface TLR4 Expression. The present study examined peripheral blood monocyte TLR4 expression in male CD-1 mice gaining weight by consuming either a high-fat (HF, 60% kcal) or a low-fat (LF, 10% kcal) diet for 12 weeks. The results presented are for cell-surface TLR4 gMFI on CD11b⁺/CD14⁺ monocytes in panel B. Values are expressed as mean ± SE. ■ indicates a significant difference between HF and LF ($P<0.05$). ▼ indicates a significant change from baseline in the same

Correlations

At week 12, body weight and monocyte concentration were positively correlated ($r=0.478$, $P=0.016$). Conversely, body weight was negatively correlated to monocyte TLR4 gMFI at week 12 ($r=-0.438$, $P=0.032$). Monocyte concentration was negatively correlated to TLR4 gMFI at week 12 ($r=-0.559$, $P=0.005$).

DISCUSSION

The key finding of the present study was that high-fat fed mice had significantly more blood monocytes and less cell-surface TLR4 expression than low-fat fed mice, which were both correlated with body weight. Our findings with respect to monocyte concentration are consistent with previous studies (26-28). To our knowledge the present study is the first published

report to document the effect of diet-induced weight gain on monocyte TLR4 expression.

As expected, high-fat feeding caused significantly greater weight gain than low-fat feeding; however, we did not find any differences for energy intake between groups. Thus, it is likely that HF mice gained more weight due to increased feeding efficiency and the high fat content. It is also plausible that HF gained more weight because they were less active. Sullivan et. al. reported that weight gain during high-fat feeding can be attributed to sedentary behavior (25). Although we did not directly measure energy expenditure, we did observe HF were less active in their cages and more compliant during weighing/blood draw procedures whereas LF mice exhibited more activity such as climbing on in-cage shelters, burrowing and movement during blood draws. Our anecdotal observation is consistent with Storlien *et al* who reported that rats had decreased energy expenditure during high fat feeding (24).

Our finding of increased monocyte concentration in the diet-induced weight gain group was consistent with previously published cross-sectional studies in humans and mice (21, 27, 28)). It is important to note the novel nature of the present study because to our knowledge we are the first to report longitudinal changes in monocyte concentration that were derived from a non-lethal blood collection technique. Takahashi et al. used a cross-sectional model and found that 6-months of *ad libitum* access to a 33% fat diet (33% kcal from fat) was associated with a 50% increase monocyte concentration compared

to a low-fat control group (21, 28). In humans, it has been reported that an increase in blood monocyte concentration is associated with an increased incidence of cardiovascular disease (1). It is likely that an expanded monocyte pool may reflect the need for increasing monocyte-macrophage transmigration into inflamed tissue compartments (7). In the case of the HF mice, it is reasonable to speculate that increasing amounts of blood monocytes are recruited into adipose tissue (29) and plaque deposits in coronary arteries (22). Such transmigration of monocytes is likely to contribute to elevated disease risk. While the increase in HF monocytes was expected, we had not expected to see a significant decrease in LF monocytes. It is possible that an absence of systemic inflammation paired with a disease-free environment resulted in a reduction of circulating monocytes. Based on this finding, future studies should anticipate this reduction and assess its contribution to immune function in control mice.

In addition to finding an increased monocyte concentration, we found that both body weight and monocyte concentration was negatively correlated with monocyte cell-surface TLR4 expression. These findings contradict what has been observed in pro-inflammatory states such as physical inactivity and Type 1 Diabetes Mellitus in humans (9, 15). However, alterations in monocyte TLR4 *during* weight gain have not previously been assessed in mice or humans; it is possible that a reduction in monocyte TLR4 may occur prior to increases as observed the aforementioned reports. Since, TLR4 is known to play a role in body fat accumulation and distribution (13), it is

reasonable to speculate that reduced TLR4 cell-surface expression may reflect an attempt by the body to prevent additional weight gain and excess inflammation. Further weight gain despite these signals may eventually lead to the increases in TLR4 expression observed in established inflammatory states. Exposure to low-levels of LPS may have lead to reduced TLR4 during high-fat feeding.

Others have reported that high-fat feeding increases endogenous LPS concentration (5) and prolonged *in vitro* stimulation of monocytes with LPS causes decreased TLR4 expression (19). Thus, it is plausible that changes in endogenous LPS may explain our observed differences in monocyte TLR4 expression since high-fat feeding has been associated with metabolic endotoxemia in mice (5). In the present study, HF and LF had the greatest difference in TLR4 at week 8 and consequently during weeks 8-12 HF mice had a lower percent weight gain (20%) than during week 1-8 (42%). Thus, it is plausible in our model that decreased monocyte TLR4 expression was associated with a decrease in the rate of weight gain. More research is needed to validate the direct effects of reduced TLR4 expression on weight gain in mice using repeated measures models. Despite the possible beneficial effects of decreased TLR4 expression on weight gain, decreased TLR4 cell-surface expression compromises the ability of the immune system to respond to gram-negative pathogens (2). Such functional declines in immunity via disruption of TLR4 lead to premature mortality (4).

To our knowledge, the present study is the first to use a longitudinal, repeated

measures design to examine the effects of diet-induced weight gain in outbred mice. There are a few minor limitations worth noting because they will be addressed soon in future projects from our laboratory. It has been recently reported in humans that it is important to consider changes in monocytes subsets when examining their response to weight gain and disease (21). Few studies have taken this approach in mice and thus, we aimed to first establish if diet-induced weight gain affected the whole monocyte population, prior to completing more complicated subset analysis. Future research is also needed to determine if changes in endogenous LPS are responsible for decreased monocyte TLR4 expression with diet-induced weight gain.

CONCLUSION

In summary, we found that weight gain was associated with increased monocyte concentration and lower TLR4 expression. Future studies should consider the influence of monocyte subsets and endogenous LPS on the observed responses. Further research is necessary to understand how such findings translate to immune dysfunction in human obesity.

ACKNOWLEDGMENTS

This research was funded by the American College of Sports Medicine, Texas Chapter (TACSM) Student Development Research Award and the University of Houston Health and Human Performance Graduate Student Research Fund. No author on this manuscript reported any conflict of interest. K.S. was responsible for study design, data collection, mouse care, statistical analysis

and manuscript preparation. R.J.S aided in data analysis and manuscript preparation. K.C.C and W.B participated in mouse care, data collection, analysis, and manuscript preparation. B.K.M oversaw all aspects of this research as mentor and corresponding author for the project.

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