T Cell Changes Induced by Dietary/Weight Change

Aljowhara Aljeraiwi

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T CELL CHANGES INDUCED BY DIETARY/WEIGHT CHANGE

by

Aljowhara Ibrahim Aljeraiwi

A Thesis/Dissertation
Submitted in Partial Fulfillment of
the Requirements for the Degree of

Master of Science.

Major: Nutrition.

The University of Memphis

May 2022
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ABSTRACT

Obesity is a condition that affects many people and is associated with numerous health issues and chronic diseases, including viral infections. For example, during the SARS-CoV2 epidemic, obesity emerged as a major risk for severe COVID 19. Obesity has been shown to increase T cell exhaustion resulting in loss of CD8⁺ T cell functions and reducing the immune system's capacity to fight infectious diseases. Exhausted T cells can be identified by the increased expression of the membrane protein Programmed Cell Death 1 (PD-1). While many studies demonstrate that a high-fat diet and an increase in weight can affect the immune system, there is limited knowledge of whether improved metabolic function through dietary change/weight loss can restore immune function. Therefore, the aim of the current studies was to assess if dietary change/weight loss in obese mice can improve immunity through reduced PD1 expression and restore activation-induced IFNγ. C57Bl/6 mice were fed a high-fat diet (HFD) (60% kcal) for 5 months to induce obesity. Half of the mice were then switched to a low-fat diet (LFD) (10 % kcal) for the next 9 weeks to induce weight loss. Switching mice to the LFD significantly reduced body weight, decreased adiposity, and improved fasting glucose levels and glucose clearance. However, there were no improvements in PD1 status with weight loss for CD4⁺ and CD8⁺ T cells in the spleen. As leptin is a major obesity-induced hormone that has been shown to affect immune cells, we also determine if activation of T cells in the presence of leptin would alter PD1 status. In a pilot study, human peripheral blood mononuclear cells (PBMCs) were stimulated in the presence of increasing concentrations of leptin, and the levels of intracellular IFNγ and PD1 were measured using flow cytometry. Activation in the presence of leptin (100 ng) showed limited increase in IFNγ production and expression of PD1 in both CD4⁺ and CD8⁺ cells. In conclusion, we demonstrated that weight loss did not appear to improve PD1 status, although PD1 a
# Table of Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>v</td>
</tr>
<tr>
<td><strong>1. Introduction.</strong></td>
<td></td>
</tr>
<tr>
<td>Obesity.</td>
<td>1</td>
</tr>
<tr>
<td>Leptin.</td>
<td>2</td>
</tr>
<tr>
<td>Immune system and PD1.</td>
<td>3</td>
</tr>
<tr>
<td>Fat diet and the immune system.</td>
<td>5</td>
</tr>
<tr>
<td><strong>2. Methods.</strong></td>
<td></td>
</tr>
<tr>
<td>Experimental animals.</td>
<td>7</td>
</tr>
<tr>
<td>Experimental diets.</td>
<td>8</td>
</tr>
<tr>
<td>Food consumption and body mass.</td>
<td>9</td>
</tr>
<tr>
<td>Glucose tolerance test.</td>
<td>9</td>
</tr>
<tr>
<td>Blood and Tissue collection.</td>
<td>10</td>
</tr>
<tr>
<td>Leptin mRNA expression and qPCR Analysis.</td>
<td>11</td>
</tr>
<tr>
<td>Human cell isolation (PBMCs).</td>
<td>12</td>
</tr>
<tr>
<td>Statistical Methods.</td>
<td>14</td>
</tr>
<tr>
<td><strong>3. Results.</strong></td>
<td></td>
</tr>
<tr>
<td>Switching to a low-fat diet (LFD) induced weight loss.</td>
<td>14</td>
</tr>
<tr>
<td>Food and calorie Intake.</td>
<td>16</td>
</tr>
<tr>
<td>Effect of dietary change and weight loss on metabolic profile.</td>
<td>17</td>
</tr>
<tr>
<td>Low fat diet reduced the adipose size.</td>
<td>18</td>
</tr>
<tr>
<td>Dietary change altered lymphocytes and granulocytes percentage.</td>
<td>19</td>
</tr>
<tr>
<td>Weight loss, dietary change hematology and PD-1 in T cells.</td>
<td>20</td>
</tr>
<tr>
<td>Leptin mRNA expression.</td>
<td>22</td>
</tr>
<tr>
<td>Leptin pretreated of peripheral blood T cells might enhances the production of proinflammatory cytokine IFNγ.</td>
<td>23</td>
</tr>
<tr>
<td><strong>4. Discussion.</strong></td>
<td>25</td>
</tr>
<tr>
<td><strong>5. Conclusion.</strong></td>
<td>27</td>
</tr>
<tr>
<td><strong>6. References.</strong></td>
<td>28</td>
</tr>
</tbody>
</table>
## List of figures.

<table>
<thead>
<tr>
<th>List of figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Animal experiment</td>
<td>9</td>
</tr>
<tr>
<td>2. Human experiment</td>
<td>15</td>
</tr>
<tr>
<td>3. Weight and weight change</td>
<td>16</td>
</tr>
<tr>
<td>4. Food and calorie intake</td>
<td>17</td>
</tr>
<tr>
<td>5. Pre and post blood glucose tolerance and change in fasting glucose</td>
<td>18</td>
</tr>
<tr>
<td>6. Mice organs size</td>
<td>19</td>
</tr>
<tr>
<td>7. WBC count</td>
<td>20</td>
</tr>
<tr>
<td>8. PD1 expression in mice T cell and IFNγ release</td>
<td>21</td>
</tr>
<tr>
<td>9. Leptin mRNA expression</td>
<td>22</td>
</tr>
<tr>
<td>10. PD1 expression in human PBMC</td>
<td>23</td>
</tr>
</tbody>
</table>
1. Introduction

Many people around the world suffer from obesity. Obesity has been linked to serious health problems, including type 2 diabetes, cardiovascular disease, and cancers.\(^{(1)}\) In addition, various studies have shown altered immune cell functions in obese humans compared to those with a healthy weight.\(^{(2,3,4)}\) For example, Aguilar et al. found significant discrepancies between lean and obese individuals in circulating leukocyte numbers and leukocyte subset counts, as well as phagocytic activity of monocytes.\(^{(3)}\) Impaired immune cells are also observed in people with type II diabetes, one of the common complications associated with obesity and excess weight.\(^{(4)}\) Moreover, ex vivo studies using human peripheral blood mononuclear cells (PBMCs), demonstrate that obesity results in a low-grade pro-inflammatory state compared with healthy-weight people.\(^{(2)}\)

Specific to the adaptive immune system, it has been demonstrated that obesity can promote thymic aging and reduce T-cell repertoire diversity, thus impacting immune surveillance.\(^{(5)}\) Besides fast-tracking T-cell aging, obesity has been shown to diminish T-cell functionality in numerous experimental models. Aguilar and Murphy posit that chronic T-cell stimulation results in exhausted T-cell phenotypes that can be distinguished by decreased synthesis and proliferation of effector biomolecules and increased expression of inhibitory receptors.\(^{(3)}\) While a chronic inflammatory milieu largely accounts for the exhausted T-cell phenotype, this dysfunction is largely induced by the dysregulation of leptin, glucose, adiponectin, TNFα, IL-6, and other immune-active molecules that are typically associated with obese individuals.\(^{(6,7)}\)

In individuals with a genetic mutation that prevents leptin from being appropriately produced, morbid obesity occurs, along with weakened immune defenses.\(^{(8)}\) Using obese mice, it was also found that obesity may impair host defense since immune cells are activated, which may increase risk of infection.\(^{(1)}\) And indeed, studies have linked obesity with an increased risk of
infection.\(^{(9,10)}\) In addition, obesity has also been identified as a significant risk factor for the surgical and post-operative sites, \(^{(11)}\) nosocomial, \(^{(12)}\) periodontal, \(^{(13)}\) and viral respiratory infections in various studies.\(^{(14,15,16)}\) According to Anaya et al., obese individuals are at a greater risk of morbidity and mortality from infection with the pandemic novel influenza H1N1 strain.\(^{(17)}\) Recently, a strong association between the severity of COVID-19 disease and obesity was also demonstrated.\(^{(18)}\) Infected patients who are either nutritionally deficient or obese may have an inadequate inflammatory reaction causing more severe adverse clinical outcomes. A recent study demonstrated that obese patients tended to have more severe cough and fever symptoms than patients with an average weight.\(^{(19)}\)

**Leptin**

Obesity and overweight result in chronic metabolic dysfunction that alter insulin-glucose levels, disrupt adiponectin-leptin equilibrium, and influence other adipokines and hormones.\(^{(20)}\) As a hormone responsible for regulating satiety with immune stimulating capabilities, leptin increases T cell activation through metabolic reconfiguring. Leptin levels are increased during obesity \(^{(21)}\) and these elevated levels among obese individuals are associated with the mechanism underlying immune dysregulation in high-adiposity conditions.\(^{(3,22,23)}\)

Leptin is secreted and synthesized primarily from white adipose tissue. Leptin acts in the brain but also in several peripheral tissues. It signals by interacting with leptin receptors (LepRs) in both peripheral tissues and the brain leading to activation of numerous signaling pathways \(^{(24)}\) The LepRs isoform that primarily mediates leptin transduction in the nervous system is called LepRb, which is expressed mainly in the hypothalamus and other parts of the brain. LepRb regulates neuroendocrine function, hedonic control of feeding, energy homeostasis, learning, and memory. In addition, peripheral leptin receptors regulate cellular lipid balance also modulate glucose metabolism and insulin action.\(^{(25)}\) In addition, the leptin
receptors LepRs have also been identified on immune cells including T cells, B cells, and natural killer (NK) cells, which are directly affected by leptin in normal and pathological conditions, resulting in various adaptive immune responses. Moreover, the interaction between leptin and its receptors stimulates Mitogen-Activated Protein Kinase (MAPK), Signal Transducer and Activator of Transcription 3 (STAT3), and Janus Kinase 2 (JAK2), which all have a significant role in increasing IFNγ, TNF-α and IL6 in memory T cells. Thus, this interaction promotes the proliferation of naive T cells and the switch toward Th-1 cell immune responses. It has been shown that leptin promotes the proliferation of activated T cells. The increase in leptin levels might increase inflammation since the leptin receptor is highly expressed during inflammatory conditions and is involved in numerous immune pathways and cell types. Obesity and overweight are proven determinants of meta-inflammatory reactions that induce the production of specific pro-inflammatory cytokines, namely Interleukin (IL-6) and Tumor Necrosis Factor α (TNFα). The inflammatory condition is associated with elevated levels of leptin, fatty acids, and glucose.

**Immune system and PD1**

The immune system is one of the most complex and intricate body systems. Human bodies are exposed to many potentially harmful microbes, including viruses, bacteria, parasites, which are recognized as foreign invaders. This system works very efficiently with immediate action to protect the human body against harmful microbes. The immune system cells may be divided into innate and adaptive immune cells. The innate immune system is the first, rapid response to an invading pathogen and includes phagocytes, neutrophils, mast cells, eosinophils, etc. The adaptive immune system includes B, T, and natural killer cells and have a lag period before a response. In general, T cells are divided into CD4+ helper T cells, and CD8+ cytotoxic T-cells. The CD4+ helper T cells are important in coordinating immune cell responses, while T-cells with CD8+ receptors can direct the killing of infected, damaged or
transformed cells.\(^{(30)}\) CD8\(^+\) T cell cytotoxicity requires activation in order to produce high amounts of IFN\(\gamma\) and kill other cells in an antigen-specific manner.\(^{(31)}\) Zou et al. demonstrated that weight gain suppressed the effective functioning of CD4\(^+\) T cells, which protect the body against chronic ailments including cancer and diabetes.\(^{(22)}\) Also, they found that chronic systemic inflammation was linked to obesity.

With obesity, activated CD8\(^+\) T cells accumulate in visceral adipose tissue and contribute to the inflammatory milieu.\(^{(29)}\) Obesity negatively affects T-cell functionality through the dysregulation of PD1 expression. PD-1 is shown to augment energy metabolism and mitochondrial biogenesis of exhausted T cells.\(^{(30)}\) However, the loss of mitochondrial mass and function correlated with the upregulation of co-inhibitory receptors, including PD1, therefore, blocking the PD-1 pathway will restore T-cell function and improve pathogen control.\(^{(32,33)}\) Evidence demonstrates that consistent alteration in T-cell stimulation and metabolic state among obese individuals triggers the exhausted T-cell phenotype.\(^{(34)}\) PD1 is a cell-surface biomolecule and has a role in controlling adaptive immune reactions by interacting with its ligands PD-L1 and PD-L2 receptors to limit T-cell proliferation, inhibit cytokine synthesis, and limit cytolytic functions.\(^{(35)}\) PD1 is also expressed by macrophages, dendritic cells, B cells, and activated T cells.\(^{(36,37)}\) The expression of PD1 is tightly and dynamically regulated. It has been observed that PD1 is expressed at relatively low levels in resting T cells and certain populations of developing thymocytes.\(^{(30,31)}\) PD1 is seen as an exhaustion marker as low-level expression is correlated with improved immunity.\(^{(30)}\) To regulate effector functions in T cells, \(^{(38)}\) PD1 primarily suppress CD28 signaling when bound to its ligand PD-L1.\(^{(39)}\) PD-L1 is ubiquitously expressed, whereas PD-L2 is found on antigen-presenting cells (APCs). Inflammation will increase levels of both ligands PD-L1 and PD-L2.\(^{(36)}\) An increase in the PD1/PD-L1 signaling pathway has been shown to inhibit autoimmune responses.\(^{(40,41)}\)
The PD1/PD-L1 receptor pathway is dysregulated by diet-induced obesity (DIO), with subsequent increased frequency of PD1 T cells in peripheral blood, liver, and spleen of rodents as well as non-human primate models.\(^{(2)}\) Moreover, in vivo studies show in DIO mice increased CD4\(^+\) T cell senescence phenotypes, reduced IFN-production, and upregulation of PD1 in visceral fat.\(^{(42)}\) As a consequence, obesity results in increased responsiveness to anti-PD1 immunotherapy, in part due to leptin-dependent mechanisms, leading to greater impairment of T cell function.\(^{(2)}\) Interestingly, high blood level of leptin among obese individuals influences T-cell functions and increases PD1 expression.\(^{(2)}\) Hence, there is a positive correlation between higher leptin serum levels and increased PD1 expression on CD8\(^+\) T-cells. Besides generating similar findings on DIO mice, the same study demonstrated that reversing obesity through calorie-restricted diet reduced visceral adiposity, limited T-cell exhaustion, and lowered leptin levels in mice.\(^{(2)}\) Although some investigation suggests that PD-mediated T-cell dysfunction is confoundedly influenced by gender, genetics, dietary differences, age, and metabolic deregulation, obesity-stimulated leptin production results in upstream regulation of PD1 genes that consequently triggers T-cell dysfunction.\(^{(43)}\) Despite these overlying leptin regulation factors, evidence indicates that obesity induces PD1 expression through an under studied leptin-mediated pathway. It has been demonstrated the level of serum leptin in obese donors correlated with the expression of PD-1 on CD8\(^+\) T cells. DIO mice also showed elevated levels of leptin that correlated with CD8\(^+\) T-cell PD-1 levels.\(^{(2)}\) Since findings reveal that previously obese animals may successfully regain their immunity through lowered leptin levels and reversed T-cell dysfunction, the consumption of a low-fat diet and the use of other anti-obesity strategies may potentially resuscitate human immunity by reducing leptin synthesis, stopping meta-inflammation, and subsequently limiting PD1 expression.
Fat diet and the immune system

Nutrition and diet are vital for proper immune function, reducing inflammation and oxidative stress caused by different factors. The immune system is activated during infection and the energy demand increases accordingly. The best way to strengthen the immune system and maintain the proper function of the immune cells is by consuming a well-balanced, sufficient diet, which is geared to achieving rapid and effective resolution of infections preventing any further chronic inflammation. The immune system can be influenced by a variety of micronutrients and dietary components throughout life. For example, the amino acid arginine is essential for the generation of nitric oxide by macrophages. Additionally, dietary fats are necessary for absorbing liposoluble vitamins A, D, E, and K, which are involved in the immune system and permeability and stability of immune cell membranes. Fatty acids are known to perform several roles in immune cells. Short chain fatty acids (SCFAs) exert immune-modulating properties in vitro studies. SCFAs can regulate the activation of the immune cells, such as macrophages and T lymphocytes. In a study conducted by Liu et al., SCFAs decreased the production of pro-inflammatory factors, including TNF-, IL-1, IL-6, and increased production of anti-inflammatory cytokines, including IL-10. Additionally, PUFA such as omega-3 supplements reduced the level of inflammatory mediators, i.e., IL-6 in healthy adults. Researchers have shown that omega-3 fatty acids, like DHA and EPA, can reduce inflammatory cytokines by activating the peroxisome proliferator-activated receptors (PPARs). However, not all PUFA acids act as an anti-inflammatory agents, omega-6 fatty acids can increase pro-inflammatory molecules, which have essential roles in regulating and increasing inflammation. A previous study demonstrated that consuming a high-fat diet reduced the response to pandemic H1N1 influenza A vaccination by increasing levels of pro-inflammatory cytokines and neutrophils. According to Milner and colleagues, a high-fat diet (HFD) causing obesity can exacerbate inflammation or infection even in
Obese mice had increased inflammatory cytokine levels, and more memory CD8+ cells in the lung airways and, serum leptin levels were significantly elevated. In mice on a high-fat diet, Trottier et al. observed induced inflammation. This change was accompanied by a modest increase in the percentage of lymphocytes.

Obesity is a worldwide epidemic that continues to increase at an alarming rate. An increase in body weight may often be caused by poor eating habits, particularly from consuming highly processed, nutrient deficient food containing high amounts of saturated fat and excess calories. In vitro and in vivo studies showed that obesity and a high-fat diet affect the immune response and influence infection susceptibility. In addition, obesity negatively affects T-cell functionality through the dysregulation of PD1 expression. Since controlling PD1 expression is feasible by reducing adipose tissue, consuming a low-fat diet, and using healthy weight-loss measures may restore human immunity by reversing T-cell exhaustion, and reinstating the homeostasis of both T-cell and PD1 expression. Therefore, the first aim of the study will allow for a better understanding of whether a low-fat diet that promotes weight loss could improve T-cell function. We expect to observe that the dietary changes in obese mice will result in altered body mass index, an indication of weight and adiposity, which will impact immunity. Based on these reports, we will investigate the relationship between obesity, weight loss with dietary changes, and T cell function.

2. Materials and Methods

1st Hypothesis:

We hypothesized that weight loss in obese mice would reduce PD1 expression on T-Cells, which would positively improve T-cells function (as measured through increase IFN-γ expression).
a. **Experimental set up.**

20 C57Bl/6 mice were transferred from a protocol where they were fed either a custom-made high fiber diet (DF, Research Diets, custom-D13092801, 8 animals; 4 male and 4 female) or a 60% high fat diet (HFD, Research Diets-D12492, 12 animals; 6 male and 6 female). At the start of the current study, the mice were 7 months of age and were fed their respective diets for 5 months. All mice consuming the DF remained on their diet for the duration of the study. Mice that consumed the high fat diet were divided into two groups (1:1 male to female randomized for each group) where half of the mice (n=6) continued consuming the 60% HFD, while the second group (n=6) were switched to a low-fat diet containing 10% fat (LFD, Research Diets-D12450J), but were similar to the high fat diet in nutrient composition. All mice continued with their respective diets for 9 weeks. All animals were housed in a USDA-approved animal facility on the University of Memphis Campus with a 12h light-dark schedule and ad libitum access to food and water. All experiments were approved by the Institutional Animal Care and Use committee.

**Experimental diets:**

Experimental diets were purchased from Research Diets, Inc (New Brunswick, NJ) and comprised of the following:

1. **Daniel Fast** -25% kcal fat (flax and safflower oil), 15% kcal protein, 59% kcal carbohydrate (high amounts of Corn Starch- High Maize 260 and inulin) 3.9 kcal/gm. (Research Diets, custom – D13092801).

2. **High Fat Diet** - 60% kcal fat (lard, soybean oil), 20% kcal protein, 20% kcal carbohydrate. (High amounts of sucrose, corn, and starch) 5.21 Kcal/g.

   (Research Diets, D12492).

3. **Low Fat Diet** - 10% kcal fat (lard, soybean oil), 20% kcal protein, 70% kcal carbohydrate (High amounts of sucrose, corn, and starch) 3 kcal/gm. (Research Diets, D12450J).
Food consumption and body mass

Food consumption and body mass were monitored three times a week in the morning (on Monday, Wednesday, and Friday) for 9 weeks.

Glucose Tolerance Test

A fasting glucose tolerance test was performed prior to switching to the LFD and again after all animals were on their respective diets for 9 weeks to determine differences in glucose clearance. The second glucose tolerance test was performed 2 days prior sacrifice. Briefly, mice were fasted for 6 hours during their inactive (lights on) phase. (This resembles an overnight fast in humans). At time 0, 10 ul of blood was collected from the tail vein and glucose level measured using a (One Touch Ultra 2 glucometer). Mice were then intraperitoneally injected with a sterile solution containing water and glucose at a
concentration of 1g glucose/kg body weight. Blood glucose concentrations were measured via the tail vein and every 30 minutes up to 120 minutes after injection.

**Blood and Tissue collection**

After 9 weeks on their respective diets, animals were euthanized, and blood and tissue collected. After an overnight fast, mice were euthanized using CO2 inhalation and cervical dislocations. Immediately prior to sacrifice, blood was collected from the facial vein and into EDTA containing tubes for white blood cells analysis (lymphocytes, monocytes, granulocytes) using a hematology analyzer (VetScan HM2, Zoetis, US). Samples of liver, adipose tissue and intestines were collected and either immediately frozen in liquid nitrogen prior to storage or stored in formalin for histological analysis. Fecal samples from the cecum and large intestine were also collected. All samples were stored at -80°C until analysis.

The spleen was immediately processed after harvest for immune cell isolation, analysis, and functional assay. Spleens were minced and passed through a 40µ sterile strainer to generate single cell suspensions. Red blood cells were removed using ACK lysis buffer (Gibco, Thermo Scientific, Waltham, MA) and white blood cells counted using a hemacytometer. Cells from the spleen and blood were stained with the following fluorochrome-conjugated antibodies: eFluor 450-anti-CD4 (Biolegend, clone RM4-5), APC/Cy7-anti-CD8a (Biolegend, clone 53-6.7), FITC-anti-PD-1 (Biolegend, clone 29F.1A12); from R&D: anti-IFN-gamma (R7d, clone IC485F) and PE-anti-CD19 (Invitrogen, clone Bio1D3). For intracellular cytokines staining, samples were resuspended in fixation and permeabilization solution for 20 minutes at 4 degrees. Samples were resuspended in PBS containing 1% FBS and incubated with fluorescently labelled anti-cytokine antibodies IFNg for 30 min in 4 degrees in the dark. After incubation, cells were washed 2 times with 1x BD Prem/Wash
buffer (250ul) then resuspended in PBS containing 1% FBS. Cells were analyzed using an Invitrogen Attune Nxt Flow Cytometer (ThermoFisher Scientific, Waltham, MA).

**Leptin mRNA expression and qPCR Analysis**

Epididymal white adipose tissue were harvested, frozen in liquid nitrogen and stored −80 °C. Total RNA was isolated from the adipose tissue using TRIzol® Reagent (Invitrogen) followed by further purification using RNeasy mini kits (Qiagen). Reverse transcription was performed using a high-capacity RNA to cDNA kit (Applied Biosystems). Briefly, 1 μg of total RNA was reverse-transcribed into cDNA. Quantitative RT-PCR was performed in a final reaction volume of 20 µL using SYBR Green PCR master mix (Thermo Fisher Scientific). Each real-time PCR solution contained 10 µL of SYBR Green qPCR, 0.6 µL each primer, and 8.8 µL an RNA template. Real-time PCR was carried out in duplicate for each sample, the samples were then normalized using the housekeeping gene. Two genes were tested to identify the appropriate transcript for normalization. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was then used as an internal control to determine mRNA levels of leptin. Primer sequences for leptin, and GAPDH are listed in (Table.1) and mRNA levels for genes of interest were expressed as a ratio of leptin to GAPDH mRNA to normalize for initial RNA input.

**Table.1**

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<td></td>
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11
Table 1 (continued)

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<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>GCCTTCCGTGTTCCTACC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCTCAGTGTAGCCCAAGTG</td>
</tr>
</tbody>
</table>

**Pilot project:** Changes in PD1 levels when T cell are stimulated in the presence of a high amount of leptin.

**2nd Hypothesis:**

Leptin Exposure of T cells during ex vivo stimulation will increase PD1 expression.

**Isolation of human PBMCs**

Protocol was approved by the IRB at The University of Memphis (PRO-FY2022-2). Human blood was collected via venipuncture into EDTA containing tubes. PBMCs were isolated from blood using Ficoll-Paque (1.077) density centrifugation. The cell number was determined using Trypan Blue and microscopy. The following fluorochrome-conjugated monoclonal antibodies were purchased from BioLegend: Pacific Blue-anti-CD8 (SK1), PE-anti-CD4 (A161A1), FITC-anti-CD3 (OKT3), and from Invitrogen FITC-anti-IFN gamma (4S.B3). PreCP-eFluore710-anti-CD4 (SK-3), PreCP-eFluore710-anti-PD-1 (M1H4). Human recombinant leptin was from R&D Systems (Minneapolis, MN, USA). Invitrogen eBioscience cell stimulation cocktail – cat number 50-930-
Phorbol 12-myristate 13 acetate (PMA), ionomycin (I), and brefeldin-A were from Sigma Chemical Company (St Louis, MO, USA).

**T cell stimulation**

PBMCs (1X10^6 cells/well) were cultured in U-bottom 96-well plates. Cells were stimulated with PMA and ionomycin cocktail (Invitrogen, eBioscience) for 7 hours at 37°C in absence or presence of 100 ng/ml recombinant human leptin (R&D Systems, Minneapolis, MN, USA). After stimulation, GolgiStop (BD Biosciences) was immediately added to the cells to prevent IFNγ release and intracellular IFNγ measured. After stimulation, media was removed, and surface staining completed with the following antibodies: Pacific Blue-anti-CD8 (clone SK1), PE-anti-CD4 (clone A161A1) and FITC-anti-CD3 (clone OKT3) and PreCP-eFluore710-anti-PD-1 (clone M1H4).

**Intracellular cytokines staining**

After cells were stained, cells were resuspended in Fixation and Permeabilization solution for 20 minutes at 4°C. Then, samples were washed 2 times with 1x BD Prem/Wash buffer (250ul). Samples were resuspended in PBS containing 1% FBS prior to intracellular staining then samples washed again with 1x BD Prem/Wash buffer (250ul). Samples were resuspended in PBS containing 1% FBS and incubated with fluorescently labelled anti-cytokine antibodies IFNγ for 30 min in 4°C in the dark. After incubation, cells were washed 2 times with 1x BD Prem/Wash buffer (250ul) then resuspended in PBS containing 1% FBS prior to analysis using a Invitrogen “Attune” NxT Flow Cytometer (ThermoFisher Scientific, Waltham, MA).
Statistical Methods

t-tests and ANOVA with multiple comparisons were used for all statistical analyses.
Statistical significance was defined as a p-value less than \(0.05\). Graph Pad Prism Software was used for all analysis (Version 7, San Diego, CA).

3. Results

High-fat diet (HFD) induced severe obesity in mice; switching to a low-fat diet (LFD) induced weight loss.

Male and female mice were fed a high-fat diet for 5 months. After being on an HFD for 5 months, we replaced HFD with LFD for half of the mice to induce weight loss for another 9 weeks. There were no significant differences in body weight among the HFD and LFD
groups at baseline week 0 to week 3 (Fig. 3A). However, there was a significant difference between LFD and DF in the first two weeks (p<0.0012, Fig. 3A). As early as the fourth week there was a significant difference in the weight of LFD and HFD mice, the weight of LFD was lower than the weight of HFD mice (p=0.0020, Fig. 3A). Also, there was a significant difference in the weight between HFD and DF throughout the study week 0 to week 9 (p<0.0001, Fig 3A). Up to sacrificing day, HFD fed mice remained heavier than the other groups, although with no significant differences (Fig. 3B). Also, HFD mice continued to gain more weight to the last day of the intervention with an average percentage weight gain 11.59%. HFD mice weighed 60% and 80% more than LFD and lean mice (DF), respectively, after 9 weeks of diet. However, LFD continued to lose weight and they lost more weight at week 4, which significantly differed from week 1. Mice on LFD rapidly lost their weight, and the average weight lost was 1.85 gm per week. After 4 weeks the LFD mice stabilized their weight. The body weight change in grams of the mice throughout the 9 weeks of the intervention is shown in (Fig. 3C). As Fig.3C shows the highly decrease in body weight of LFD mice, whereas increase in the HFD mice. Weight change was significantly difference between LFD and HFD (p=0.018, Fig. 3C). No other significant difference observed between the groups.
Food and calorie Intake.

After the dietary switch from a high fat to a low-fat diet, mice consumed significantly less total energy. The first couple of weeks there was difference in food consumption (Fig. 4A). LFD mice markedly decreased their caloric intake immediately after the nutritional intervention (Fig. 4B) and normalized this parameter 4 week later. The calorie intake of LFD was significantly different of HFD in the first four weeks also, significant difference between DF and HFD in week 1 to week 3 ($p<0.05$, Fig. 4B). Moreover, there was a significant difference in the total calories consumed over the 9 weeks between LFD and HFD ($p=0.017$, Fig. 4C). Even though HFD mice were heavier than DF, there were no significant differences between DF and HFD (Fig. 4C). in total energy consumption over the intervention time.

Fig 3. Body mass was monitored over the 9 week intervention period. Body weight in LFD, HFD and DF mice from the first week to last week of intervention (A). Final weight of mice from each group at last week (B). Change in body weight during intervention over 9 weeks (C). Data are presented as mean ± SD. The significance of difference among the three groups was analyzed by one way ANOVA analysis of variance and 2 way ANOVA.
Effect of dietary change and weight loss on metabolic profile.

Metabolic parameters including fasting blood glucose and glucose clearance were assessed on the first and last days of the nutritional intervention (Fig. 1). At the start of the intervention, all mice consuming a high fat diet had increased fasting glucose and reduced clearance compared to DF mice (Fig. 5A). However, after weight loss both fasting glucose and glucose clearance improved (Fig. 5B) (p=0.0002, Fig. 5B). As (Fig. 5D) shows the increase in fasting blood glucose of HFD, however, LFD mice was less than the other mice groups by week 9. In summary, HFD mice were obese and had an impaired glucose metabolism. Switching to a LFD improved both glucose and glucose clearance.

**Fig 4.** Weekly food intake in grams (A), Weekly caloric intake (B), and total calorie intake over 9 weeks (C). The data are expressed as mean ± SD and were analyzed using 2way ANOVA multiple comparison procedures.
Effects of dietary change and weight loss on adipose and organs size

Total liver size was lower in mice fed DF (Fig. 6A) but did not reach significance when compared to the other groups. Cecum content weight varied with diet (Fig. 6B). LFD fed mice had the lowest cecum weight (1.09 mg), while DF displayed the highest weight (1.8 mg) (Fig. 6B). The size of adipose tissue was clearly larger in HFD mice than in LFD and DF mice. The weight of adipose tissue in the HFD group was significantly greater than in the LFD mice (p=0.0098, Fig. 6C), however, there were no significant differences between HFD and DF mice or LFD and DF. Nevertheless, small, and large intestine were not found to be affected by diet or weight, no significant difference was found among groups (Fig. 6D, E).

Fig 5. Glucose tolerance test pre nutritional intervention (A), after 9 weeks of intervention (B), fasting blood glucose for 6 hours pre and post food intervention (C). Change in fasting blood glucose in mice maintained on LFD, HFD or DF (D). Data were analyzed using 2way ANOVA multiple comparison procedures and were presented as mean ± SD. p<0.05.
Blood Analyses and WBC Count.

Blood collected form mice pre and post intervention were analyzed for total WBC and the following populations: monocytes, lymphocytes and granulocytes. The dietary intervention did not alter the WBC counts (P>0.05, Fig 7 A). For monocyte %, there was no significant difference between all groups (Fig. 7 B). However, the percentage of monocyte showed a slight % increase in the HFD group post food intervention. There was a significant reduction in lymphocyte % after the LFD intervention (p=0.0432, Fig. 7 C) also significant difference between LFD and HFD at the end of the study (p=0.0302, Fig. 7 C). The granulocytes percentage significantly increased with the low-fat diet (p=0.0481, Fig. 7 D) and there was also significant difference between LFD and HFD.
post intervention ($p=0.0268$, Fig. 7 D). In general, the WBC count and monocyte % for all the mice groups were similar in the post-intervention phase, except that the lymphocyte % was reduced in the LFD mice. In contrast, the granulocyte count was very high in the same mice group compared to HFD and DF mice group.

![Graphs showing changes in WBC monocyte, lymphocyte, and granulocyte counts](Image)

**Fig 7.** WBC counts in LFD and HFD mice pre and post intervention. (A) Mice monocyte pre and post intervention. (B) Total lymphocyte pre and post intervention. (C) Lymphocyte % in LFD mice pre- and post-intervention was significantly different. HFD pre granulocyte % was higher than the LFD. However, Post granulocyte % was very high in the LFD compared to HFD and LFD mice group. Also, granulocyte % in LFD mice pre- and post-intervention was significantly different. Data are expressed as mean ± SD. One-way ANOVA with Kruskal-Wallis multiple comparison, and unpaired Welch’s t-test $p<0.05$.

**Weight loss and dietary change might not affect PD-1 expression on the T cell.**

As PD-1 is a T cell exhaustion marker and it has previously been shown to be increased with obesity, we determined if dietary change and weight loss affected PD-1 status of CD8$^+$ and CD4$^+$ T cells collected from the blood or spleen. There was a significant
difference in the percentage splenic PD1+ CD8+ cell between the HFD and DF groups (p=0.04, Fig. 8A), consistent with previous studies showing that PD-1 is increased with obesity. Although not significant, this trend was also seen in T cells collected from blood. No significant differences in PD1 status were detected between LFD and HFD (P>0.05, Fig. 8B). To determine if there was a difference in T cell function, we measured intracellular IFNγ production in response to stimulation (Fig. 8C). Unfortunately, the IFNγ response was not robust. However, HFD mice had decreased in IFNγ production level in the CD8+ cells, this decreased is consistent with PD-1 % in the splenic CD8+ cell since they have been significantly higher in the HFD.
Leptin mRNA expression in the adipose tissue

To determine if there is a correlation between weight and the adipokine leptin, adipose tissue was harvested, and RNA isolated to measure leptin transcript levels. To determine the optimal maintenance gene to use as endogenous control for adipose tissue, both GAPDH (Fig. 9A) and B-actin (Fig 9B) were tested as reference genes. Figure 9A demonstrated that GAPDH can be used as a valid reference. Leptin transcript within adipose tissue (normalized to GAPDH) were not different between the groups, although a difference in leptin mRNA expression existed between groups (Fig 9C).

**Fig 8.** PD1% in the spleen CD8, CD4 cells(A). PD1 % in the CD8, CD4 blood cells(B). Ex vivo stimulated T cells shows IFNγ production in CD8, CD4 cells(C). Data were presented as mean ± SD, each dot represents one mouse: One-way ANOVA with Kruskal-Wallis test p<0.05.
Pilot study:

Increased Leptin exposure during T cell stimulation.

Blood was collected from a healthy human donor and PBMC isolated and stimulated with cocktail of PMA+ ionomycin and GolgiStop for 6 hrs., in the absence or presence of leptin (100 ng/mL). As shown in (Fig.10A), leptin alone had no effect on T lymphocyte activation in PBMC. To determine if leptin was immune stimulatory, we measured the IFNγ response to PMA and Ionomycin stimulation. As shown in (Fig.10B), leptin exposure during stimulation resulted in a slight increase in the IFNγ percentage producing CD8+ and CD4+ T cells. However, no significant changes were detected. Similar to IFNγ, leptin exposure also resulted in a slight, but non-significant increase in PD1 in both CD8+ and CD4+ cells (Fig10.C).
Fig 10. PBMC lymphocytes that were depleted of monocytes in the absence or presence of leptin (100 ng) for 7 h. (A) The effect of leptin on lymphocyte function by measuring the production of cytokines IFNγ. (B) Leptin enhanced the expression of activation markers PD1 in both CD4+ and CD8+ cells after 7-hrs of stimulation. (C) Data are expressed as mean ± SD p<0.05.
4. Discussion

Obesity and its associated metabolic dysfunction are known to alter in the physiologic milieu and immune function. Various hormones and immune modulatory molecules are altered by the state of obesity. What is less well studied is the role of weight loss on immune function. For the current study, we showed that switching obese mice to LFD for 9 weeks significantly reduced body weight and improved glucose clearance. Weight reduction significantly decreased the size of adipose tissue in LFD mice. Moreover, we found that a high-fat diet increased body weight gain, adipose size, liver size, and induced glucose intolerance. This generally supports the findings of a previous study by Lavekar et al. that consuming too many calories from fat may lead to obesity, adipocyte dysfunction, and adverse metabolic effects. In addition, an earlier study found that rats that consumed high-fat diets developed dyslipidemia, oxidative stress, and glucose intolerance. In general, obesity results in an increase in visceral fat, but fatty deposits can be also found in organs such as the liver. In our study, we hypothesized that weight loss might improve immune response on obese mice since obesity resulted in an altered immune cell. In a study by Drew et al. to assess white blood cells in response to acute and gradual weight loss among young males, weight loss can alter the level of white blood cells, which are determined by the immune system and contribute to infectious diseases. Data from our study indicate that despite large differences in body weight between the obese and nonobese groups, WBC counts and monocytes, were similar between all groups. Our finding reveals that even with weight reduction LFD mice experienced significant decreases in total lymphocyte cell counts, but a massive increase in the total number of granulocytes following weight loss. It is believed that neutrophil counts are increasing in response to stress and infectious diseases. It has been suggested that weight loss may cause immune system changes due to a decrease in nutrient intake. As known, obesity results in increased expression of PD-1 on T cells. Our HFD mice showed
increased in PD-1 in the splenic and blood CD8+ cells. We hypothesized that weight loss would restore the function of T cells and reduce the PD-1 expression on T cells in obese mice. Several studies found that weight loss does not completely reverse the effects of weight gain in the function of certain aspects of the immune system. In this study, we found that weight loss could reverse the systemic hyperglycemia seen in obesity. However, CD8+ and CD4+ T cell function did not restore after weight loss. Our finding is consistent with the study by Nieman et al., found that weight loss was associated with significant decreases in T cell and monocyte percentages. Another study demonstrated that obesity was associated with T-cell dysfunction by inducing a unique T-cell metabolic program. They reported that weight loss did not improve the metabolic dysfunction associated with obesity in the CD4+ and CD8+ T cells. Also, weight loss did not decrease infiltration of T cells into adipose tissue. Moreover, our results showed that obese mice had decreased IFNγ production in splenic CD8+ cells, which is consistent with PD-1 % in the splenic CD8+ cell since they have been significantly higher in the HFD. Based on data from DIO mice, CD4+ T cells reduced production of IFNγ and increased PD-1 expression in visceral fat. Indeed, visceral fat is a highly active tissue and excess adipose tissues are responsible for many metabolic complications including the increased production of adipokines and cytokines. Because large adipocytes tend to rupture more frequently, they become a source of inflammation, as demonstrated by a positive correlation between adipocyte size and inflammatory cytokines. As a result of weight loss, adipocytes are believed to produce fewer inflammatory cytokines. It has previously been shown that HFD mice do not stimulate leptin mRNA expression from adipose tissue. A study by Schoof et al. suggests that obesity is associated with decreased leptin expression in the adipocytes when fat mass increased in mice. In addition, leptin is expressed differently depending on where the tissue is located. A study in
human adipose tissue found that leptin mRNA expression was significantly higher in front of stomach and intestine but not in omental fat, thus, leptin mRNA levels were significantly higher in subcutaneous and mesenterial sites in both children and adults. According to four studies published in the literature, recombinant leptin modulates T cell immune responses in vitro by increasing T cell proliferation and inducing the production of proinflammatory cytokines such as IFNγ. In one experiment, leptin presence marginally increased IFNγ levels of the CD8+ and CD4+ cells. T cells are known to be impacted by leptin, yet the literature also suggests that leptin may increase T cell activation. Our results showed that leptin alone had no effect on T lymphocyte activation in PBMC. However, when T lymphocytes are stimulated with PMA-I in the presence of leptin accelerates activation of CD8+ and CD4+ cells. Since both the short and long isoforms of the leptin receptor have been found in both CD4+ and CD8+ T lymphocytes, we agree with the statement suggested that leptin affects T cells through leptin receptor LepRs. These data are complemented by the finding that LepRs is expressed in peripheral T cells.

Limitation of the study

Due to the limited time and funds, we could not have the opportunity to do all the analysis of the PD1 on both human and animal models, also the number of the animals was less than the prospective. Further studies are needed to determine the clinical implications of weight loss / dietary change in immunity and whether various interventions such as exercise or nutrient supplementation can help enhance them. In this regard, it is also necessary to measure how weight loss interacts with changes in psychological stress.

5. Conclusion

In summary, obesity contributes to several health problems. Therefore, dietary changes such as LFD and weight loss will reduce the size of adipose tissue, which will improve metabolic status such as glucose clearance. However, our data are consistent with the viewpoint that
weight loss leads to a reduction of certain immune functions. The results of this study suggest that weight loss and diet intervention might not affect PD-1 expression on the T cell.

**Ethical license and Experimental infections**

The animal experiment for this study was approved by the University of Memphis IACUC (protocol # 0858). In addition, all methods will be performed in accordance with the relevant guidelines and regulations of IACUC. Human blood collection study was approved by the IRB at The University of Memphis IRB ID:(PRO-FY2022-2).

6. References


