Obesity Contributes to an Accumulation of Ly6c^{high} Pro-inflammatory Monocytes in the Spleen and White Adipose Tissue

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KEYWORDS. Immunology, Monocytes, Obesity, Immune System

BRIEF. The purpose of this experiment was to see whether or not obesity correlated with an increase in pro-inflammatory monocytes

ABSTRACT. Obesity is one of the most prevalent health concerns in the United States. Individuals who are obese have an increased risk of developing other health problems such as heart disease, high blood pressure, and type 2 diabetes. It is not completely understood why obese people have this increased risk but some relationship with the immune system has been recently established via a mechanism involving low-grade inflammation. In this project, a high fat diet was used to induce obesity in mice to model obesity in humans, while a low fat diet was used as a control for lean mice. Monocytes expressing high levels of Ly-6C were specifically examined because of their pro-inflammatory effects. Using fluorescence-activated cell sorting and conventional microscopy, the prevalence of Ly-6Chigh monocytes was quantified in the spleen and white adipose tissue of lean and obese mice. The results show that diet induced obesity in mice did increase the number of Ly-6Chigh monocytes in both tissues. These observations suggest that pro-inflammatory monocytes may play a role in generating the chronic inflammation associated with obesity.

INTRODUCTION.

Obesity has become one of the most prevalent global health problems in humans. There are myriad of health concerns associated with the condition because it increases one's risk of developing other diseases such as cardiovascular diseases, metabolic syndrome, and type 2 diabetes [1]. Obesity develops when excessive fat deposits in an organism's white adipose tissue (WAT). It is often characterized by a low-grade chronic inflammation which is a major contributing factor to the development of obesity-related diseases [1].

A long-held hypothesis in the immunological field was that monocytes were one of the few immune cells in constant circulation throughout the whole body. It was thought that only upon tissue entry could these circulatory monocytes differentiate into macrophages or dendritic cells [2]. Contrary to this notion, the spleen was discovered to serve as a reservoir to monocytes, therefore showing monocytes are not strictly circulatory [2]. Furthermore, these splenic monocytes can be deployed from the spleen to various inflammatory sites around the body. There are two subsets of monocytes which can be found in splenic tissues and these are Ly6c^{high} monocytes and Ly6c^{low} monocytes [2]. The pro-inflammatory Ly6c^{high} monocytes are responsible for digesting damaged tissue while the Ly6c^{low} monocytes are anti-inflammatory and primarily are involved in the resolution of inflammation and promotion of wound healing [2].

Another subset of myeloid mononuclear cells, adipose tissue macrophages (ATMs), have become more commonly studied regarding their relationship with obesity. Notably, one found that ATMs accumulate in WAT and play a significant role in obesity-related inflammation [1]. These ATMs can then proliferate and promote myelopoiesis, the production of myeloid immune cells, and monocytosis, the production of monocytes, specifically when an organism is obese [1].

ATMs are more commonly found in visceral adipose tissue rather than subcutaneous fat which is a layer of fat lying directly beneath the skin [3]. Visceral adipose tissue is fat tissue that can be found within the abdominal cavity and can accumulate around organs. This is the type of fat that can accumulate in obese individuals. Although obesity can promote macrophage proliferation in adipose tissue it does not increase their proliferation in other tissues [3]. This evidence leads researchers to hypothesize that within adipose tissue there is a unique environment that is necessary in the promotion of the elevated monocyte and macrophage proliferation and accumulation found in an obese setting [3].

Because of the known contribution of the spleen when deploying pro-inflammatory monocytes under inflammatory conditions, and the fact that monocytes can give rise to macrophages in WAT, this research project sought to assess whether or not pro-inflammatory monocytes are increased in adipose tissue when in an obese setting. The spleen was additionally analyzed to examine whether or not this organ deployed Ly6c^{high} monocytes to adipose tissue like an inflammatory site. It is hypothesized that obesity is both correlated with the localization of pro-inflammatory monocytes to adipose tissue, and also the recruitment of splenic monocytes. This outcome would result in less monocytes to be found in the spleen when an organism is obese. A more complete characterization of immune cell localization will be helpful in guiding researchers to better understand the inflammation associated with obesity and therefore treat one aspect of obesity-related inflammation.

METHODS.

Inducing Obesity.

The study used diet-induced obesity (DIO) in mice to model obesity in humans. Male wild type C57BL/6J (B6) mice were used due to their susceptibility to DIO. Experimental mice had been fed a high fat diet (HFD) for 12 weeks to induce obesity. The control mice received a low fat diet (LFD) to provide a basis for comparison. After 12 weeks on the test diets splenic and adipose tissue was procured for analysis.

Isolation of Stromal Vascular Fraction from White Adipose Tissue.

Peri-gonadal visceral white adipose tissue was minced and digested at 37^0 C for 1 hour using 10 ml of Collegenase I solution (1 mg/ml in RPMI 1640 medium). The digestion process was stopped by adding 40 ml of wash medium (RPMI 1640 medium containing 5% fetal bovine serum (FBS) to each sample. The samples were then centrifuged at 1300 rpm for 10 minutes at 4 ^{o}C. The supernatant was removed and the red blood cells were lysed using 0.5 ml of ACK lysing buffer for 5 minutes. After stopping this reaction by adding 10 ml of wash medium, the cells were then centrifuged once more under the same conditions. The supernatant was removed of and the pellet was resuspended in RPMI containing 10% FBS. The isolated cells (stromal vascular fraction, SVF) consisted of immune cells, vascular cells, and other stromal cells.

Isolation of Splenocytes.

The spleen was pressed through a 70-μm mesh in 10 ml of wash buffer to disassociate the tissue. This solution containing splenocytes was then centrifuged under the same conditions as described above in order to collect the cells from the splenic tissue. In order to lyse the red blood cells in this larger sample, 5 ml of ACK lysing buffer was used and the reaction was stopped after 5 min by adding 10 ml of wash medium. Immune cells were harvested and suspended in RPMI buffer containing 10% FBS as described above.

Cellular Staining.

Fluorescently labeled antibodies and Fluorescence-Activated Cell Sorting (FACS) were used to identify and isolate the Ly6c^{high} pro-inflammatory monocytes. Each staining mixture contained a cocktail of antibodies. The lineage markers CD90, B220, NK1-1, Pan NK, Ly6G, and Siglec F, allowed mature

T and B lymphocytes, NK cells, neutrophils, and eosinophils to be excluded from the cell solutions via various gating parameters. The antibody, CD11b, was used to stain macrophages, dendritic cells, and monocytes among lineage-negative cells and facilitated in their isolation from the other cells. A combination of Ly6C, CD11c, F4/80, MHC class II (I-Ab) aided in the discernment of pro-inflammatory monocytes, the pro-inflammatory phenotype being positive for all of these surface antibodies. A total of 1 x 10^6 SVF cells or splenocytes were calculated through cell counting in a hemacytometer and added to FACS tubes. This cell suspension in the FACS tubes was then centrifuged at 1400 rpm for 10 minutes at 4 °C to remove supernatant, and 30 µl of the above antibody mixture was added and gently vortexed. Then the samples were allowed to incubate at 4 °C for 30 minutes. After incubation 2 ml of FACS buffer (PBS containing 1% FBS and sodium azide) was used to wash off excess antibodies from the samples and all of the samples were once again centrifuged under the same condition as described above. Finally, the stained cells were resuspended in 200µl of FACS buffer before flow cytometry.

Flow Cytometry.

Samples were analyzed by a 4-color flow cytometer. Specific gates were then setup on the flow cytometer was set to collect 500,000 events. The collected data was visualized and analyzed using FlowJo software.

RESULTS.

Antibody Mixture Effectively Allows Flow Cytometry to Identify Cells of Myeloid Lineage.

Cell solutions were supplied to a preparative flow cytometer to isolate separated cell subpopulations for microscopy. Myeloid cells can be seen successfully separated from lymphoid cells in Supplemental Figure 1. The cells pictures were of similar size and only varied in the shape of the nucleus. The presence of smaller cells would have suggested the flow cytometer had not effectively isolated myeloid cells from lymphoid cells.

Flow Cytometry Gating for Ly6c^{high} Pro-inflammatory Monocytes.

For the splenic tissues there was a minor decrease in the number of monocytes, macrophages, dendritic cells when solely separating the myeloid and lymphoid cells as seen in Figure 1. The percentage of the myeloid cells that fell into the first gate on the flow cytometer for the lean mouse was 0.862% and the percentage for the obese mouse was 0.722%. The percent received from the flow cytometer is the total number of cells counted within the gated area over the total number of cells on this specific plot.

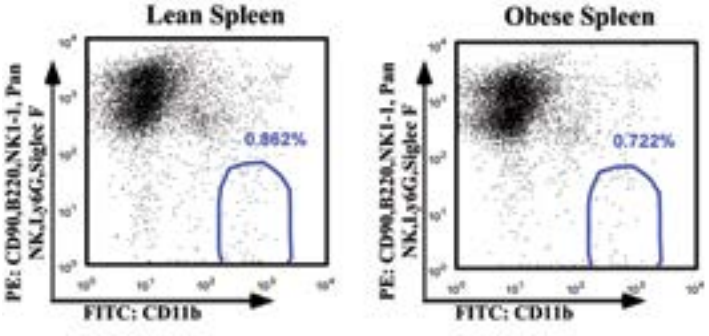

Figure 1. The frequency of splenic macrophages, monocytes, and dendritic cells is comparable in lean and obese mice. Mice were fed a HFD or LFD, and after 12 weeks, the spleens were harvested and analyzed for macrophages, monocytes, and dendritic cells by flow cytometry. Frequencies of macrophages, monocytes, and dendritic cells (CD11b+ CD90- B220- NK1.1-, Ly6G-, Siglec F-) are indicated in blue.

However, when the macrophages and dendritic cells are separated from the pro-inflammatory monocytes, there was found to be an increase of 15.1% in the percentage of pro-inflammatory monocytes when in an obese environment shown in Figure 2.

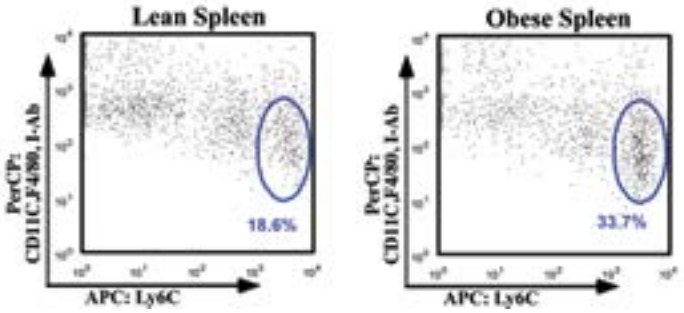

Figure 2. An obese environment in the spleen increased the prevalence of pro-inflammatory monocytes. Although the event count defined that there were nearly 2,000 more cells on the plot for the lean mouse, the obese mouse has a higher percentage of its cells to be found within the Ly6c^{high} monocyte gate.

In white adipose tissue there is an obvious increase (16.62%) in the percentage of monocytes, macrophages, and dendritic cells. (Figure 3). The obese plot shows that there is still a greater percentage of macrophages, monocyte, and dendritic cells than the lean mouse cells, despite lower total cell numbers.

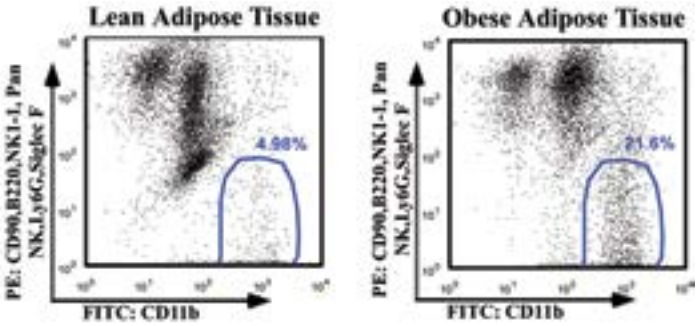

Figure 3. Adipose tissue also displayed an increased prevalence of macrophages, monocytes, and dendritic cells. There was increase that is clearly visible in the obese plot when compared to the lean. Even though more cells were counted in the lean plot there was still a greater percentage in the myeloid immune cell gate for the obese mouse.

This increase tells nothing about the total number of macrophages, monocytes, and dendritic cells that could be found in adipose tissue. Rather it states that in obese mice the immune cells in the fat tissue are more likely to be comprised of macrophages, monocytes, and dendritic cells.

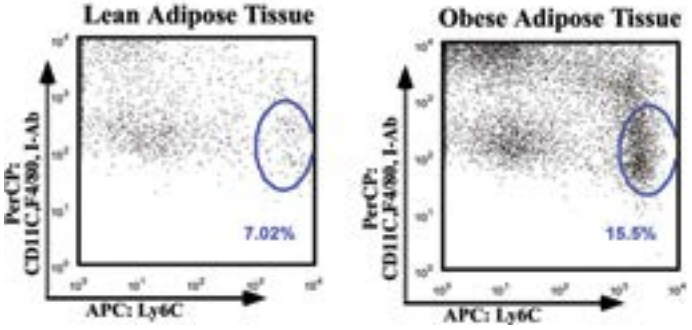

Figure 4. The frequency of pro-inflammatory monocytes within adipose tissue also shows a visible increase. An increase in the percentage of pro-inflammatory monocytes is present but is not as great as in Figure 4. These plots have a different anomaly where the number of cells counted in the obese plot is nearly 3,000 more than the number counted in the lean plot.

There was also found to be an increase in the percentage of pro-inflammatory monocytes in adipose tissue in obese mice compared to the lean controls (Figure 4). The increase was less prominent than the increase of the macrophages,

monocytes, and dendritic cells found in Figure 3, and was smaller than the increase in percentage of pro-inflammatory monocyte in the splenic tissues in Figure 2.

DISCUSSION.

The antibody mixture used in this study was successful in identifying the various types of immune cells that could be found in either splenic tissues or adipose tissue. This was determined by viewing the cells that underwent Fluorescence Activated Cell Sorting (FACS). Morphology of the identified cell subpopulations could be confirmed using a high-powered microscope (Supplemental Figure 1). By systematically viewing each of the samples spun after each gate during flow cytometry, it was determined phenotypically that the antibody solution was sufficient in separating myeloid cells from lymphoid immune cells and specifically monocytes from macrophages and dendritic cells. Also, monocytes were also successfully separated by the intensity in which they expressed Ly-6C.

The flow cytometry results from the spleen were unexpected since it was hypothesized that obesity would increase the amount of myeloid immune cells. The above results are supported by the literature in that the spleen is a reservoir for monocytes and other immune cells and deploys them throughout the body during inflammation [2]. This is a possible explanation for the slight decrease in the percentage of myeloid immune cells in Figure 1. Even though a previous study specifically discussed a drastic decrease in the number of monocytes in the spleen after myocardial infarction (heart attack), this is applicable to this research even though obesity is more commonly associated with chronic inflammation and not the acute inflammation that occurs during myocardial infarction [2]. Though there are differences between the characteristics of acute inflammation and chronic inflammation, the observation that the percentage of myeloid cells in total decreased when in an obese environment is indicative of some state of inflammation happening within the organism.

From the flow cytometry results of splenic tissue, it cannot be determined whether the inflammation that incurred the deployment of immune cells from the spleen was caused by obesity. In Figure 2, the increase in the percentage of myeloid cells and specifically Ly-6C^{high} pro-inflammatory monocytes is indicative of inflammation actually happening within the adipose tissue which gives possible reasoning as to why the spleen in the obese mice had a fewer percentage of myeloid immune cells, if they were sent to adipose tissue to promote inflammation. In both Figure 3 and Figure 4 the percentage of myeloid cells and the percentage of pro-inflammatory monocytes increased giving even further reasoning to a there being some relationship between obesity, inflammation, and myelopoiesis.

Further experimentation needs to be done in order to assess whether or not the inflammatory monocytes found in adipose tissue actually originate from the spleen. By doing this it could rule out the possibility of an infection or another disease causing the deployment of immune cells from the spleen. Because this was not done in this study, an assumption was that the mice were overall healthy, apart from being obese, by their physical characteristics. Also another aspect of this project that could be investigated is determining whether obesity changes the behavior of immune cells from their point of origin within bone marrow. This would allow researchers to see whether or not obesity causes more myeloid progenitors to form out of bone marrow. As a result more myeloid cells would be produced in the spleen and consequently more myeloid cells to be deployed to infiltrate fat tissues throughout the body. A final aspect of the study to be improved upon is to increase the quantity of tissues from lean and obese mice for analysis. Only four lean tissues and four obese tissues were used for this project, and increasing this number would give enough results to perform statistical analysis to determine whether the positive trends found in this study are statistically significant.

Although it was determined that obesity did cause the accumulation of pro-inflammatory monocytes in adipose tissue, more work is required to determine the mechanism behind this. It would also be beneficial to learn whether or not this chronic inflammation caused by obesity ultimately impairs immune cells function and efficiency similar to an autoimmune disease. Even though more research will need to be done to further explain the physiological and biological effects of obesity on the immune system, the information learned from this project provides others with a path to continue stepping in the right direction until obesity's effect on the immune system is completely understood.

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