Video Article

Isolation of Adipose Tissue Immune Cells

Jeb S. Orr[1], Arion J. Kennedy[1], Alyssa H. Hasty[1]

[1]Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine

Correspondence to: Alyssa H. Hasty at alyssa.hasty@vanderbilt.edu

URL: https://www.jove.com/video/50707
DOI: doi:10.3791/50707

Keywords: Immunology, Issue 75, Cellular Biology, Molecular Biology, Biophysics, Physiology, Anatomy, Biomedical Engineering, Surgery, Metabolic Diseases, Diabetes Mellitus, diabetes, Endocrine System Diseases, adipose tissue, AT, stromal vascular fraction, macrophage, lymphocyte, T cells, adipocyte, inflammation, obesity, cell, isolation, FACS, flow cytometry, mice, animal model

Date Published: 5/22/2013

Citation: Orr, J.S., Kennedy, A.J., Hasty, A.H. Isolation of Adipose Tissue Immune Cells. *J. Vis. Exp.* (75), e50707, doi:10.3791/50707 (2013).

Abstract

The discovery of increased macrophage infiltration in the adipose tissue (AT) of obese rodents and humans has led to an intensification of interest in immune cell contribution to local and systemic insulin resistance. Isolation and quantification of different immune cell populations in lean and obese AT is now a commonly utilized technique in immunometabolism laboratories; yet extreme care must be taken both in stromal vascular cell isolation and in the flow cytometry analysis so that the data obtained is reliable and interpretable. In this video we demonstrate how to mince, digest, and isolate the immune cell-enriched stromal vascular fraction. Subsequently, we show how to antibody label macrophages and T lymphocytes and how to properly gate on them in flow cytometry experiments. Representative flow cytometry plots from low fat-fed lean and high fat-fed obese mice are provided. A critical element of this analysis is the use of antibodies that do not fluoresce in channels where AT macrophages are naturally autofluorescent, as well as the use of proper compensation controls.

Video Link

The video component of this article can be found at https://www.jove.com/video/50707/

Introduction

Historically, the adipose tissue (AT) has been viewed as an inert organ of lipid storage, which expands and contracts in response to energy balance. We now understand that AT represents a dynamic endocrine organ that actively secretes a number of hormones, which directly influence feeding behavior and systemic glucose homeostasis. In addition, over the past decade there has been an increasing appreciation for the numerous populations of immune cells residing in the AT stromal vascular fraction (SVF), as well as their contribution to AT homeostasis.

The ability to separate the AT adipocyte and SVF using a collagenase digest followed by differential centrifugation was first described by Rodbell in 1964 [1]. Collagenase II is most often used for adipocyte and SVF separation due to maintenance of adipocyte insulin receptors[1]. Early on, enzymatic fractionation of AT was primarily employed to study adipocyte metabolism and to isolate preadipocytes. More recently, this technique, combined with the widespread availability of flow cytometers and the ever-increasing number of commercially available fluorophore-conjugated antibodies, has facilitated the characterization of AT immune cells.

Although the presence of immune cells in inflamed AT had been described previously [2], the seminal papers by Weisberg *et al*. and Xu *et al*. published in 2003 were the first to document the accumulation of AT macrophages (ATMs) in obesity, which secrete inflammatory cytokines and correlate with AT-specific and systemic insulin resistance [3,4]. These observations served as the basis of a new field of investigation recently coined, "immunometabolism," [5] and have been followed up by studies implicating various immune cell populations, including dendritic cells [6], mast cells [7], T cells [8-10], B cells [11], NKT cells [12], eosinophils [13], and neutrophils [14,15] in the development of obesity associated insulin resistance.

The goal of this article is to provide a detailed description of the collagenase digest technique used to isolate cells of the AT SVF and to characterize ATMs and AT T cells via flow cytometry. This protocol has been optimized for mouse AT; however, viewers may benefit from reading an excellent article providing extensive detail on optimization of this technique for human AT [16]. The target audience of this article includes investigators with limited experience working with mouse AT and performing flow cytometry. Several practical considerations for balancing cellular yield and viability with time and resources are presented as well as optimal flow cytometry controls for characterizing AT immune cell populations. In addition to our protocol, readers are referred to a recent *JoVE* article by Basu *et al.* for an excellent discussion of some of the technical aspects of flow cytometry to include proper controls and compensations [17].

Protocol

1. Reagents and Supplies

Prior to initiating this experimental protocol, prepare the following reagents:

1. 70% ethanol
2. 1X PBS
3. 1X DPBS (without Ca and Mg) supplemented with 0.5% BSA
4. FACS buffer: 1X DPBS (without Ca and Mg), 2 mM EDTA, and 1% FCS
5. ACK buffer: 150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA in water

2. Harvesting and Preparation of Adipose Tissue

1. Euthanize mice according to IACUC-approved procedures specific to each institution.
2. Thoroughly wet the fur with 70% ethanol.
3. Make an incision at the level of the xiphoid process (lower part of the sternum) and open the thoracic cavity to expose the heart, taking care not to sever any major blood vessels.

Note: At this point, it is also helpful to leave the diaphragm intact as much as possible and to cut a notch out of the right side of the rib cage to allow blood and perfusate to flow out of the thoracic cavity.

4. Clip the right atrium to allow blood and perfusate to escape the circulatory system.

Note: When dealing with obese mice, excess pericardial AT may need to be removed to permit access to the heart.

5. Grasp the heart with forceps and gently insert a needle into the left ventricle through the apex. Slowly perfuse the mouse with 15 ml sterile PBS.

Note: Reduce the rate of perfusion if the lungs begin to fill and expand.

6. Open the peritoneal cavity and remove the perigonadal fat pads using care to avoid any gonadal tissues.
7. Place fat pads in a weigh boat on ice containing 2 ml 1X DPBS (without Mg or Ca) supplemented with 0.5% BSA, and mince the AT into fine pieces.

Note: Limit the amount of AT per weigh boat to 1.2 g. If the amount of AT exceeds 1.2 g, divide it evenly between two weigh boats.

8. Keep AT samples on ice and prepare 3 ml collagenase digest solution per AT sample consisting of 1X DPBS supplemented with 0.5% BSA, 10 mM $CaCl_2$, and 4 mg/ml collagenase, type II.

3. Collagenase Digestion

1. Transfer AT to 50 ml conical tubes by pouring the homogenate and rinsing the weigh boat with 1 ml DPBS (0.5% BSA) and 3 ml collagenase II digest solution.
2. Incubate AT homogenate in a rotational shaker (200 rpm) at 37 °C for 20 min.
3. Add 10 ml DPBS (0.5% BSA) to conical tubes and place on ice.
4. Triturate homogenate numerous times using a 10 ml serological pipette, and pass cell suspensions through 100 µm filter in to a new 50 ml conical tube.
5. Centrifuge cell suspension at 500 x g for 10 min at 4 °C.
6. Decant supernatant and resuspend SVF cell pellet in 3 ml ACK buffer to lyse contaminating erythrocytes.
7. Add 12 ml FACS buffer and centrifuge cell suspension at 500 x g for 10 min at 4 °C.
8. Decant supernatant and resuspend SVF cell pellet in FACS buffer.

Note: Use the size of the cell pellet as a guide for how much FACS buffer to resuspend in. If the cell pellet covers the bottom of the 50 ml conical tube, use 0.5-1 ml FACS buffer; otherwise, resuspend in 0.25-0.5 ml.

9. Place samples on ice and prepare 1:10 dilution aliquots of each sample for cell counting by mixing 40 µl FACS buffer, 50 µl trypan blue solution (0.2%), and 10 µl cell suspension.
10. Count viable cells based on trypan blue exclusion and dilute cell suspensions to a final concentration of 5-10 x 10^6 cells/ml.

4. Staining of Cell Surface Antigens

1. Add anti-mouse CD16/CD32 antibody (Fc block) to a final concentration of 0.5-1 µg/10^6 cells, and incubate on ice for 10 min.
2. Transfer samples (≥10^6 cells) to 12 x 75 mm polystyrene round bottom tubes. Prepare separate tubes if analyzing ATMs and T cells, and combine extra cells to prepare an adequate number of tubes to accommodate the required compensation and modified fluorescence minus one (FMO) controls.

Note: As an example, when quantifying the proportion of ATMs based on F4/80 and CD11b, the following compensation and FMO controls will need to be prepared:

- Unstained (cells)

- DAPI or propidium iodide (PI) single stain (cells; do not add viability dye until step **5.1**)

- F4/80 APC single stain (cells or compensation beads)

- CD11b FITC single stain (cells or compensation beads)

- FMO 1 (cells): Rat IgG2a κ isotype control APC + CD11b FITC + viability dye (added at step **5.1**)

- FMO 2 (cells): F4/80 APC + Rat IgG2b κ isotype control FITC + viability dye (added at step **5.1**)

3. Add fluorophore-conjugated primary antibodies and/or isotype controls at the appropriate concentration (see **Table of reagents and materials**).
4. Protect samples from light and incubate at 4 °C for 30 min.
5. Add 2 ml FACS buffer and centrifuge cell suspension at 500 x g for 5 min at 4 °C.
6. Decant supernatant and resuspend SVF cell pellet in 2 ml FACS buffer.
7. Centrifuge cell suspension 500 x g for 5 min at 4 °C, and resuspend SVF cell pellet in ≥400 µl FACS buffer.
8. Transfer samples to 12 x 75 mm polystyrene round bottom tubes equipped with a 35 µm cell strainer tube tops.
9. Protect from light, and store samples at 4 °C until FACS analysis.

Note: For optimum results cells should be analyzed immeadiately; however, FACS analysis with this protocol has been successfully conducted on labeled cells stored in FACS buffer for 1-2 hr at 4 °C. If cells need to be fixed to increase storage time, labeled cells can be fixed with 2% paraformaldehyde at 4 °C for 24 hr prior to FACS analysis. Antibody companies suggest that labeled cells can be stored for up to one week; however, this has not been tested within the context of this procedure.

5. FACS Analysis

1. Prior to FACS analysis, add viability dye to samples and appropriate controls to allow for live/dead cell discrimination.

Note: Numerous viability dyes are commercially available, but DAPI and PI are recommended depending on the excitation/emission profiles of the fluorophore-conjugated antibodies being used. DAPI and Propidium iodide are added to each sample at a final concentration of 0.2 mg/ml.

2. Use an unstained negative control sample, preferably cells isolated from the same tissue as the experimental samples, to adjust side scatter (SSC) and forward scatter (FSC) so that the cell population(s) of interest are on scale.

Note: For the analysis of AT SVF cells, it is recommended that SSC be displayed in a log scale versus FSC in a linear scale. The use of a log scale for SSC is especially important when analyzing ATMs, which are often very large and granular.

3. Draw an initial light scatter gate based on the type of cell(s) being analyzed, and adjust the photomultiplier tube (PMT) gain so that the unstained cells are on the far left of a single-parameter histogram (approximately centered on 10^2) for the appropriate channels.

Note: It is recommended that lymphocytes and macrophages (or other myeloid cells) be analyzed separately due to differences in autofluorescence.

4. Use single stained controls or antibody capture compensation beads to perform multi-color compensation.

Note: The use of compensation beads is recommended; however, compatibility of each antibody must be ensured. For example, compensation beads may not cross-react with rabbit antibodies, in which case isolated cells are required to obtain an appropriate single stain control.

5. Set experimental gates based on modified FMO controls.
6. Set the flow cytometer to collect the appropriate number of events based on the prevalence of the population of interest and record experimental data.
7. Export FCS data files for offline analysis. There are numerous programs available for flow cytometry data analysis. We recommend Cytobank, a web-based platform that allows investigatores to store and analyze data and generate figures from any computer with internet access [18]. Additionally, Cytobank offers the ability to make data public accessible or restrict access to collaborators.

Representative Results

Collagenase digestion of AT followed by differential centrifugation was used to isolate the SVF from epididymal fat pads of male C57BL/6J mice fed a low fat (10% kcal from fat) or high fat (60% kcal from fat) diet (LFD and HFD, respectively) for 16 weeks. Cells of the SVF were then labeled with fluorophore-conjugated primary antibodies to quantify the proportion of viable ATMs (**Figure 1**) and AT T cells (**Figure 2**) via FACS analysis. Initial gating, including light scatter, doublet discrimination, and viability gates are depicted in **Figures 1A** and **2A**. It should be emphasized that the initial light scatter gate in **Figure 2A** is restricted to the lymphocyte population in order to avoid including autofluorescent myeloid cells. An example of using modified FMO controls to set experimental gates is depicted in **Figure 1B**. In this example, the F4/80 (**left column**) and CD11b (**right column**) antibodies are replaced with appropriate isotype controls to account for autofluorescence and non-specific binding. Quadrant gates are then drawn to identify viable ATMs ($F4/80^+CD11b^+$). Gating for $CD4^+$ and $CD8^+$ AT T cells is shown in **Figure 2B**. Viable AT lymphocytes are first gated for TCRβ (**left column**). Subsequently, $TCR\beta^+$ AT T cells are gated for CD4 and CD8 (**right column**).

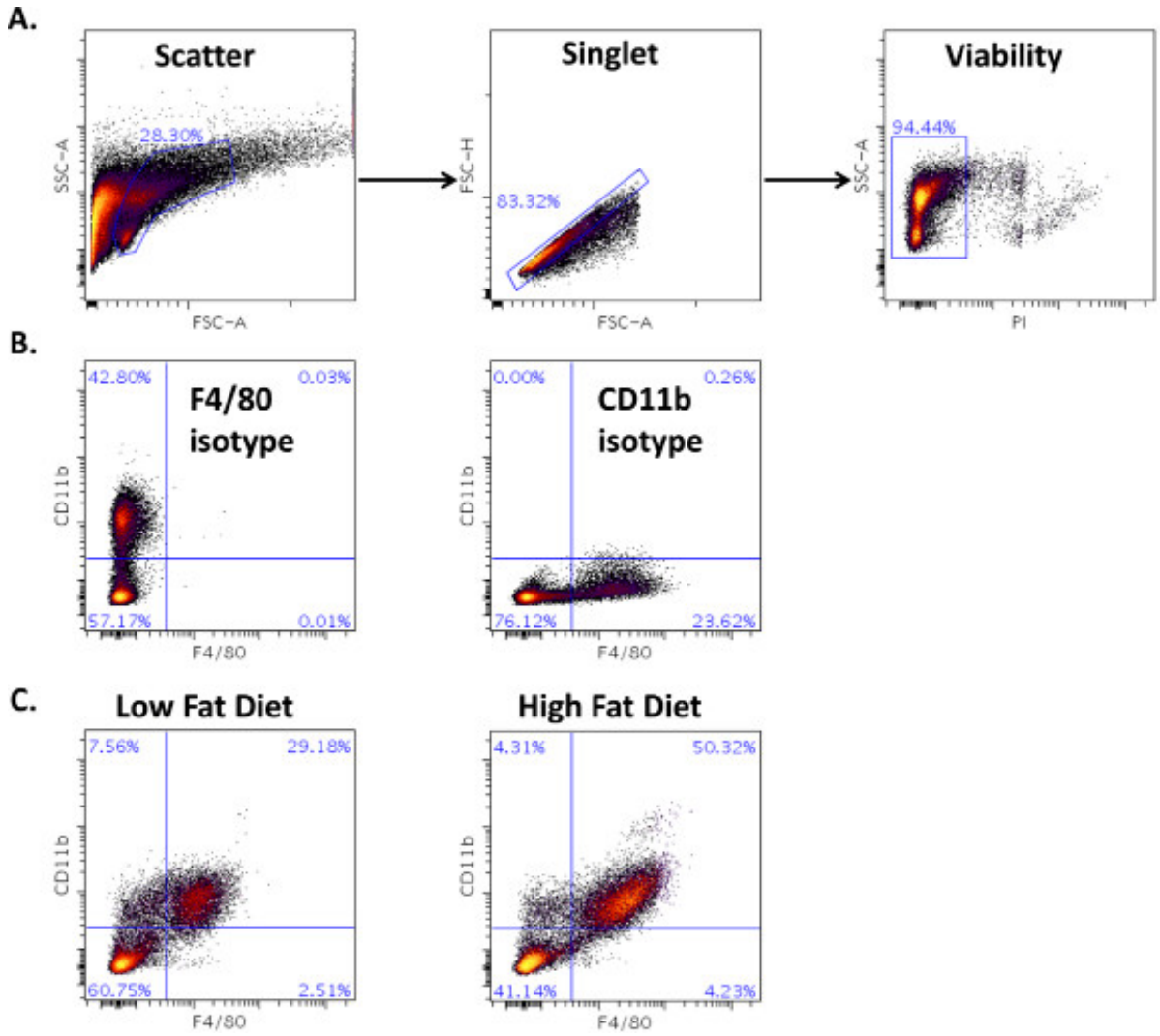

Figure 1. Adipose tissue macrophages. The SVF was isolated from the epididymal AT of male C57BL/6J mice fed a low fat or high fat diet for 16 weeks using the collagenase digest protocol. Subsequently, the SVF cells were stained for F4/80 and CD11b and FACS analysis was performed using a flow cytometer to identify ATMs. (**A**) Initial light scatter and viability gates. (**B**) Modified FMO controls incorporating isotype antibodies for F4/80 and CD11b were used to align quadrant gates. (**C**) Comparison of the proportion of $F4/80^{+}CD11b^{+}$ ATMs from the AT of low fat and high fat fed mice. Click here to view larger figure.

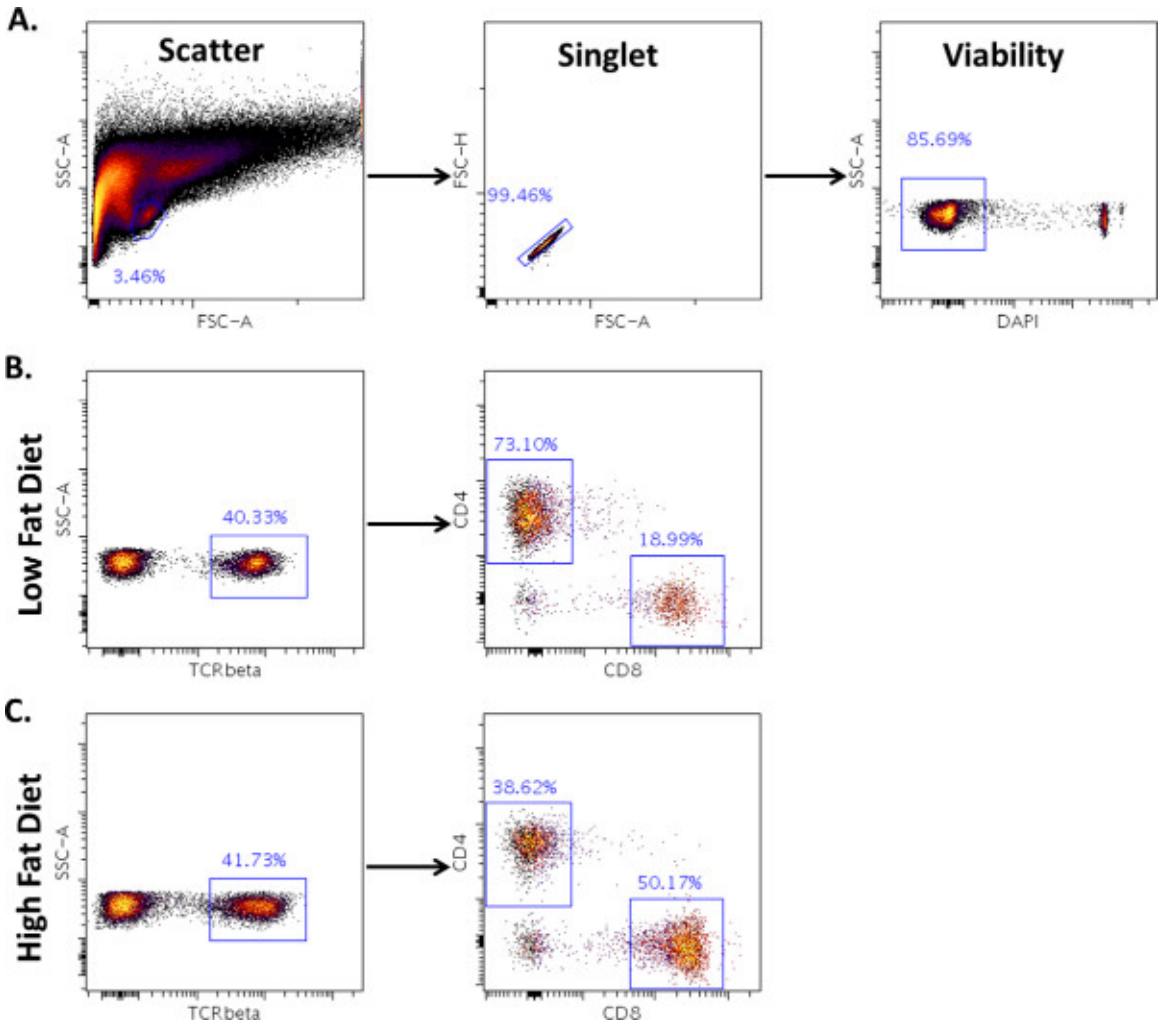

Figure 2. Adipose tissue T cells. The SVF was isolated from the epididymal adipose tissue of male C57BL/6J mice fed a low fat or high fat diet for 16 weeks using the collagenase digest protocol. Subsequently, the SVF cells were stained for TCRβ, CD4, and CD8a and FACS analysis was performed using a flow cytometer to characterize AT T cells. (**A**) Initial light scatter and viability gates. (**B-C**) Comparison of the proportion of $TCR\beta^+$ T cells (left column) and of $CD4^+$ and $CD8^+$ T cells (right column) from the AT of (B) low fat and (C) high fat fed mice. Click here to view larger figure.

Discussion

Increasing interest in the role of the immune system in the metabolic consequences of obesity has led to the widespread use of flow cytometry to characterize immune cells of the AT. Although the exact protocol will vary between laboratories based on their own experience and available equipment, the critical steps include collagenase digestion, differential centrifugation, and cell surface antigen labeling. The goal of the present article is to provide a detailed protocol and practical guide for the isolation of the AT SVF to investigators with limited experience working with AT and/or performing flow cytometry.

As with any experimental technique, there are numerous variations that may or may not significantly impact the desired outcome. Based on our experience, the protocol detailed herein represents the most efficient tradeoff between time, resources, cellular yield, and cell viability. For example, we have found that increasing the duration of collagenase II digestion to as much as 60 min at similar concentrations of collagenase has a negligible impact on cellular yield; thus, we utilize a 20 min digestion in order to reduce the length of collagenase exposure. Collagenase II is the most commonly used collagenase for adipose tissue digestions. A description of all of the different collagenases and their primary uses can be found on Sigma's website. Typical cellular yields from the above protocol are 2-3 x 10^6 and 4-5 x 10^6 cells/g AT from mice fed a low fat (10% kcal from fat) and high fat (60% kcal from fat) diet for 16 weeks, respectively. In addition to varying collagenase digest times, there are a number of potential modifications to the current protocol depending on the experimental model being used, including dietary treatment. First, when harvesting AT from mice fed a diet enriched for cholesterol or saturated fatty acids, avoid keeping the samples on ice prior to mincing because the AT will solidify and make mincing difficult. Second, our protocol includes an erythrocyte lysis step (**3.6**); however, a successful initial perfusion often makes this step unnecessary. Third, care must be taken to not add more than 10 mM $CaCl_2$ to the collagenase digest solution (5 mM final concentration); greater calcium concentrations may result in the formation of a calcium phosphate precipitate. If this precipitate forms following the initial centrifugation step (**3.5**), add two washing steps with 20 ml FACS buffer (containing EDTA) prior to the erythrocyte lysis step to remove the precipitate. Fourth, we recommend that investigators use at least one entire perigonadal fat pad when isolating the SVF. We make this recommendation not because of limitations in cellular yield, but because various cell populations display different regional distributions [19].

Thus, results may be biased when only a portion of the perigonadal fat pad is used to isolate immune cells residing in the AT. If the entire fat pad is larger than 1.2 grams, divide the adipose tissue longitudinally from the rostral to caudal ends. If divided in half this way, each half should provide a representative population of cells and should generally be less than 1.2 grams. However, in the case that this is still more than 1.2 grams, we recommend performing collagenase digestions of 1.2 gram segments in separate tubes, and then combining all of the SVF cells for the flow cytometry. In addition, for lean mice, it may be necessary to combine fat pads from 2 or 3 mice to obtain enough SVF cells for the flow cytometric analysis. The need for additional mice should be accounted for when designing experiments. Finally, another way to display the data is number of cells per gram of tissue. If cell density is desired, the AT should be weighed before mincing and digestion.

In regards to characterizing AT SVF cells via flow cytometry, two points should be emphasized. First, all fluorophore-conjugated primary antibodies should be validated for use with AT SVF cells and properly titrated. Although we demonstrated compensation with beads, we had previously titrated these antibodies with AT SVF. When characterizing the macrophages, we recommend against the use of tandem dyes (*e.g.* PE-Cy7), as we have had mixed results with respect to non-specific binding of ATMs when using antibodies conjugated to these dyes. In addition, fluorophores such as PE and FITC should only be used after careful titration as described above. A list of specific antibodies we commonly used to characterize AT SVF cells along with recommended concentrations can be found in the provided in **Table 1**. Second, because ATMs exhibit strong autofluorescence and a number of antibodies display a low level of non-specific binding when used at a high concentration, we recommend the use of modified FMO controls to set experimental gates. FMO controls are generated by staining a set of control samples with all but one of the antibodies used to label experimental samples [20]. We have modified the FMO controls in our protocol to replace one of the antibodies with an appropriate fluorophore-conjugated isotype control rather than simply removing it. In practice, it may be acceptable to forgo FMO controls for antibodies that provide exceptional separation between positive and negative populations. In addition to these controls, you will note that prior to gating on the antibodies, we preselect for lymphocyte and macrophage populations based on forward and side scatter. This will eliminate the detection of autofluorescent macrophages when analyzing lymphocyte populations with fluorophores such as FITC and tandem dyes.

Although detailed protocols are beyond the scope of this article, the isolation technique described serves as an important first step for numerous techniques aimed at characterizing immune cells residing in the AT. For example, the above isolation and staining protocol can be used to facilitate sorting of specific populations from which mRNA can be isolated to analyze gene expression. In addition, simple modifications to the protocol can be made to permit *ex vivo* treatment of ATMs. Such modifications would include utilizing sterile techniques to obtain the SVF, which would then be washed twice and resuspended in an appropriate cell culture media. Rather than adding fluorophore-conjugated antibodies to stain cell surface antigens, the SVF can be added to non-treated tissue culture plates to enrich for ATMs via plastic adherence (although this cannot be claimed to be a pure population as fibroblasts and preadipocytes can also adhere). This is accomplished by culturing the SVF for 2-4 hr at 37 °C followed by two washing steps to remove non-adherent cells. ATMs can then be treated accordingly and harvested using an EDTA-based cell dissociation solution for flow cytometry or lysed in Trizol or RIPA buffer for the isolation of RNA or protein, respectively. Regardless of the downstream application, the collagenase digest technique for isolating adipocytes and SVF cells first described by Rodbell in 1964 [1] continues to be an invaluable tool for the characterization of AT immune cells and their contribution to the metabolic consequences of obesity.

Disclosures

We have nothing to disclose.

Acknowledgements

JSO is supported by an NIH Ruth L. Kirschstein NRSA (F32 DK091040), AK is supported by a Postdoctoral Fellowship from the American Diabetes Association (7-10-MI-05), and AHH is supported by an American Heart Association Established Investigator Award (12EIA8270000). Flow cytometry experiments were performed in the VMC Flow Cytometry Shared Resource. The VMC Flow Cytometry Shared Resource is supported by the Vanderbilt Digestive Disease Research Center (DK058404).

References

1. Rodbell, M. Metabolism of Isolated Fat Cells. I. Effects of Hormones on Glucose Metabolism and Lipolysis. *The Journal of Biological Chemistry.* **239**, 375-380 (1964).
2. Danse, L.H. & Verschuren, P.M. Fish oil-induced yellow fat disease in rats. III. Lipolysis in affected adipose tissue. *Veterinary Pathology.* **15**, 544-548 (1978).
3. Weisberg, S.P., *et al.* Obesity is associated with macrophage accumulation in adipose tissue. *The Journal of Clinical Investigation.* **112**, 1796-1808, doi:10.1172/JCI19246 (2003).
4. Xu, H., *et al.* Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *The Journal of Clinical Investigation.* **112**, 1821-1830, doi:10.1172/JCI19451 (2003).
5. Mathis, D. & Shoelson, S.E. Immunometabolism: an emerging frontier. *Nature Reviews. Immunology.* **11**, 81, doi:10.1038/nri2922 (2011).
6. Stefanovic-Racic, M., *et al.* Dendritic cells promote macrophage infiltration and comprise a substantial proportion of obesity-associated increases in CD11c+ cells in adipose tissue and liver. *Diabetes.* **61**, 2330-2339, doi:10.2337/db11-1523 (2012).
7. Liu, J., *et al.* Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. *Nature Medicine.* **15**, 940-945, doi:10.1038/nm.1994 (2009).
8. Feuerer, M., *et al.* Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nature Medicine.* **15**, 930-939, doi:10.1038/nm.2002 (2009).
9. Nishimura, S., *et al.* CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nature Medicine.* **15**, 914-920, doi:10.1038/nm.1964 (2009).
10. Winer, S., *et al.* Normalization of obesity-associated insulin resistance through immunotherapy. *Nature Medicine.* **15**, 921-929, doi:10.1038/nm.2001 (2009).

11. Winer, D.A., *et al.* B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies. *Nature Medicine.* **17**, 610-617, doi:10.1038/nm.2353 (2011).
12. Wu, L., *et al.* Activation of invariant natural killer T cells by lipid excess promotes tissue inflammation, insulin resistance, and hepatic steatosis in obese mice. *Proceedings of the National Academy of Sciences of the United States of America.* **109**, E1143-1152, doi:10.1073/pnas.1200498109 (2012).
13. Wu, D., *et al.* Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. *Science.* **332**, 243-247, doi:10.1126/science.1201475 (2011).
14. Elgazar-Carmon, V., Rudich, A., Hadad, N., & Levy, R. Neutrophils transiently infiltrate intra-abdominal fat early in the course of high-fat feeding. *Journal of Lipid Research.* **49**, 1894-1903, doi:10.1194/jlr.M800132-JLR200 (2008).
15. Talukdar, S., *et al.* Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. *Nature Medicine.* **18**, 1407-1412, doi:10.1038/nm.2885 (2012).
16. Hagman, D.K., *et al.* Characterizing and quantifying leukocyte populations in human adipose tissue: impact of enzymatic tissue processing. *Journal of Immunological Methods.* **386**, 50-59, doi:10.1016/j.jim.2012.08.018 (2012).
17. Basu, S., Campbell, H.M., Dittel, B.N., & Ray, A. Purification of specific cell population by fluorescence activated cell sorting (FACS). *J. Vis. Exp.* (41), e1546, doi:10.3791/1546 (2010).
18. Kotecha, N., Krutzik, P.O., & Irish, J.M. Web-based analysis and publication of flow cytometry experiments. *Current Protocols in Cytometry.* **Chapter 10**, Unit 10, 17, doi:10.1002/0471142956.cy1017s53 (2010).
19. Cho, C.H., *et al.* Angiogenic role of LYVE-1-positive macrophages in adipose tissue. *Circulation Research.* **100**, e47-57, doi:10.1161/01.RES.0000259564.92792.93 (2007).
20. Roederer, M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry.* **45**, 194-205 (2001).

