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TITLE:

Characterization of immune cells in human adipose tissue by using flow cytometry

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KEYWORDS:

Flow cytometry, human, obesity, immune cells, macrophages, adipose tissue

SHORT ABSTRACT:

This article describes a method to analyze immune cell content of adipose tissue by isolation of immune cells from adipose tissue and subsequent analysis using flow cytometry.

LONG ABSTRACT:

Infiltration of immune cells in the subcutaneous and visceral adipose tissue deposits leads to a low-grade inflammation contributing to the development of obesity-associated complications such as type 2 diabetes. To quantitatively and qualitatively investigate the immune cell subsets in human adipose tissue deposits, we have developed a flow cytometry approach. The stromal vascular fraction, containing the immune cells, is isolated from subcutaneous and visceral adipose tissue biopsies by collagenase digestion. Adipocytes are removed after centrifugation. The stromal vascular fraction cells are stained for multiple membrane-bound markers selected to differentiate between immune cell subsets and analysed using flow cytometry. As a result of this approach, pro- and anti-inflammatory macrophage subsets, dendritic cells, B-cells, $CD4^+$

and $CD8^+$ T-cells, and NK cells can be detected and quantified. This method gives detailed information about immune cells in adipose tissue and the amount of each specific subset. Since there are numerous fluorescent antibodies available, our flow cytometry approach can be adjusted to measure various other cellular and intracellular markers of interest.

INTRODUCTION:

Obesity is characterized with low-grade adipose tissue (AT) inflammation [1] and infiltration of pro-inflammatory immune cells in both visceral and subcutaneous AT (vAT, sAT). Accumulation of pro-inflammatory immune cells in the vAT leads to insulin resistance which is a primary risk factor for developing type 2 diabetes [2]. Immune cells of both the innate and adaptive immune system are found in the obese adipose tissue, such as macrophages, mast cells, neutrophils, $CD4^+$ and $CD8^+$ T-cells, and B-cells [3-7]. These immune cells, together with endothelial cells, stromal cells, adipocyte progenitors, fibroblasts and pericytes, constitute the stromal vascular fraction (SVF) [8] and are the main source of pro-inflammatory substances in the adipose tissue [9].

The inflammatory status of AT is commonly investigated by techniques including Western blot [10], qPCR [11], and immunohistochemistry [11]. However, when using these techniques, the entire AT, adipocytes and SVF, is used. This makes it difficult to determine the amount and subsets of immune cells present in the AT. Immune cells have various cell markers to define and categorize them, such as macrophages. Macrophages show significant heterogeneity in both function and cell surface marker expression [12]. Therefore, they are often categorized into two macrophage populations: M1 and M2. M2 macrophages are usually called alternatively activated macrophages [12,13] and reside in the adipose tissue of lean, metabolically normal humans [14]. However, during obesity, a phenotypic switch occurs from M2 macrophages to M1 macrophages. These classically activated M1 macrophages express CD11C [12] and accumulate around dead adipocytes to form crown-like structures [13]. It has been shown that $CD11C^+$ macrophages in the adipose tissue impair insulin action and are associated with insulin resistance in obese humans [15]. To identify M1 and M2 macrophages in the AT, one could opt for immunohistochemistry. This technique gives information about the location of the macrophages in the tissue. However, it will limit the amount of markers that can be used in one staining. Moreover, it is also difficult to quantify. Therefore, to investigate the different immune cell subsets in the vAT and sAT deposits, we have developed a flow cytometry approach. This approach gives us the opportunity to use multiple markers per cell with one flow cytometry analysis to define cell subsets and count the numbers of each subset present in the AT deposits.

PROTOCOL:

Visceral and subcutaneous adipose tissue samples were taken from subjects enrolled in the study approved by the Medical Ethical committee Jessa Hospital, Hasselt, and Hasselt University, Belgium, in accordance with the Declaration of Helsinki.

1. Preparation of reagents

1.1 Collagenase solution

1.1.1 Dissolve 1 g of Collagenase I in 10 mL of phosphate buffered saline (PBS, without calcium of magnesium) to make a 100 mg/mL stock solution. Prepare 200 µL aliquots and store at -20 °C.

1.1.2 Dissolve 1 g of Collagenase XI in 10 mL of PBS to make a 100 mg/mL stock solution. Prepare 200 µL aliquots and store at -20 °C.

1.1.3 Dissolve 10 mg of DNase I in 10 mL of PBS to make a 10 mg/mL stock solution. Prepare 180 µL aliquots and store at -20 °C.

1.1.4 Add 100 µL Collagenase I (100 mg/mL), 100 µL Collagenase XI (100 mg/mL), and 90 µL DNase I (10 mg/mL) to 10 mL of DMEM Ham's F12. Make collagenase solution fresh for each isolation.

1.2 Erythrocyte lysis buffer

1.2.1 Dissolve 0.84 g NH_4Cl in 100 mL of ultrapure water.

1.2.2 Set the pH at 7.4 before use. Store in glass flask at 4 °C.

1.2.3 Place erythrocyte lysis buffer on ice before use.

1.3 FACS buffer

1.3.1 Dissolve 0.5 g bovine serum albumin (BSA) in 100 mL of PBS to obtain 0.5 % BSA PBS.

1.3.2 Dissolve 65 mg of NaN_3 in 100 mL 0.5 % BSA PBS to obtain 10 mM NaN_3 0.5 % BSA PBS. Store solution in glass flask at 4 °C.

1.3.3 Place FACS buffer on ice before use.

CAUTION: NaN_3 is highly toxic. Work in a fume hood and wear safety glasses and gloves to protect yourself while handling NaN_3.

1.4 Human IgG block

1.4.1 Dissolve 10 mg of human IgG in 10 mL PBS to obtain 1 mg/mL. Prepare 100 µL aliquots and store at -20 °C.

1.4.2 Place human IgG block on ice before use.

2. Isolation of stromal vascular fraction from adipose tissue

2.1 Cut 1 g of adipose tissue biopsy into small pieces (±2 mm^2) with a scalpel and transfer to

a 50-mL centrifuge tube (*e.g.*, Falcon tube). Add 10 mL of collagenase solution to each adipose tissue sample.

NOTE: Close the lid of the tube completely and turn the lid ¼ turn back.

2.2 Incubate for 60 min at 37 °C in a water-bath under gentle shaking (60 cycles/min).

2.3 Filter the resulting suspension with a 200 µM filter and collect the sample in a new 50-mL centrifuge tube. Add 7 mL PBS on top of the filter to rinse the filter and obtain all the cells.

2.4 Centrifuge the sample at 280 x g for 5 min at 4 °C.

2.5 Remove the floating adipocyte fraction by pipetting. The cell pellet is the stromal vascular fraction.

NOTE: Remove the adipocyte fraction to obtain stromal vascular fraction. Avoid submerging the entire tip in the sample because this will only remove the PBS and not the floating adipocytes.

2.6 Resuspend the stromal vascular fraction in 5 mL of PBS to remove collagenase, filter the suspension with a 70 µM filter, rinse the filter with 5 mL PBS and centrifuge the sample at 280 x g for 5 min at 4 °C.

2.7 Remove the supernatant and resuspend the pellet in 3 mL of erythrocyte lysis buffer.

2.8 Incubate for 5 min on ice. Add 7 mL of PBS after incubation.

2.9 Centrifuge the sample at 280 x g for 5 min at 4 °C.

3. Staining of stromal vascular fraction for flow cytometry analysis

3.1 Dissolve the cell pellet in 90 µL 4 °C FACS buffer and add 10 µL of 1 mg/mL human IgG block. Divide the cell suspension in 2 wells of a 96 v-shape well plate. Place the plate on ice and let the human IgG block incubate for 15 min.

3.2 Add 100 µL FACS buffer to each sample to wash and centrifuge the plate for 5 min with 280 x g at 4 °C. Remove the supernatant by tipping the plate upside down in one smooth movement without tapping the plate.

NOTE: Make sure that you remove any remaining liquid from the top of the plate with a tissue while keeping the plate upside down.

3.3 Prepare antibody cocktails for macrophage and dendritic cell subsets (FACS panel 1) and for T- and B-cell subsets (FACS panel 2) as described in table 1 and 2. The volumes described in table 1 and 2 are selected after optimizing antibody concentration and are sufficient for one

vAT or sAT sample.

NOTE: In FACS panel 1, the markers CD303 and CD141 were used to confirm that $CD11C^{+}$ $CD11B^{low}$ cells were dendritic cells. However, these markers can be excluded from the panel to include a live/dead staining, which is recommended. Both FACS panel 1 and 2 can be combined with the LIVE/DEAD Fixable Red Dead Cell Stain Kit viability staining when excluding CD303 in panel 1 as the PE channel will be unused. Perform viability staining according to manufacturer's instructions.

3.4 Resuspend the pellet in 29.5 µl antibody cocktail for FACS panel 1 and 23 µl antibody cocktail for FACS panel 2 and incubate for 30 min in the dark on ice.

3.5 Add 150 µL FACS buffer to each well and resuspend the cell pellet to perform a second wash step. Centrifuge the plate for 5 min at 280 x g at 4 °C and remove the supernatant by tipping the plate upside down.

3.6 Add 150 µL 1% formaldehyde solution to each well to fix the cells. Transfer the cell suspension from each well to the corresponding FACS tube by pipetting with a P200 pipet. Store FACS tubes at 4 °C in the dark up to 7 days.

NOTE: Direct measurement is also possible. Add 150 µL FACS buffer to each well instead of 1% formaldehyde, transfer the cells by pipetting with a P200 pipet to the corresponding FACS tubes and the cells can be analysed.

CAUTION: Formaldehyde is very toxic. Prepare formaldehyde solutions while working in a fume hood to avoid inhalation and wear gloves and safety glasses to protect yourself.

4. Flow cytometry analysis

4.1 Before the first measurement, use an unstained negative control to set the forward scatter (FSC) and side scatter (SSC). Adjust the voltages of your flow cytometer according to manufacturer's instructions so that all populations of interest are visible in the FSC and SSC graph and a distinction between debris and live cells can be made.

4.2 Perform multi-colour compensation analysis with antibody capture beads following manufacturer's protocol.

4.3 Prepare fluorescence minus one (FMO) controls by making the antibody mix but exclude one antibody from the mix. Do this for every antibody, creating 8 antibody mixes for FACS panel 1 and 6 antibody mixes for FACS panel 2. These FMO antibody mixes are used to stain SVF as described previously in this protocol.

4.4 Measure all FMO controls and set the gating strategy based on FMO controls. Use the FMO controls to detect possible auto-fluorescence of the cells. By removing one antibody from

the mix, any fluorescence level detected in this channel is a background/autofluorescent signal. Hence, by comparing the different FMO control FACS results, gates can be drawn on specific populations ensuring that the gatings are based on positive cells and not based on auto-fluorescence.

4.5 Vortex the FACS tubes at 800 rpm before placing them in the flow cytometer and starting the measurement.

NOTE: A minimum of 50,000 events in the live gate is recommended to ensure enough cells are measured from each subpopulation.

REPRESENTATIVE RESULTS:

The SVF isolated from vAT and sAT was measured using flow cytometry. Flow cytometry measurements generate plots showing different cell populations based on cellular markers (**Figure 1A, B**). First, by plotting the forward scatter width (FSC-W) and forward scatter area (FSC-A), cell aggregates can be eliminated from further analysis by gating the single cells as low FSC-W. Next, live cells are selected, and cellular debris is excluded by gating the cells of the correct size and complexity using FSC-A and the side scatter area (SSC-A), respectively. Dead cells are small and therefore visible as a distinct population with a small FSC-A. Next, immune cells were selected by use of the pan-leukocyte marker CD45 (Panel 1 and 2, **Figure 1A**). To analyze macrophages, other immune cells such as T-cells (CD3), B-cells (CD19), neutrophils ($CD66b^+$ $CD11B^+$) and NK-cells (CD56) were excluded from further analysis by using distinct antibodies targeting these cells, but with the same fluorochrome. Further subdivision of the remaining cells was based on CD11B and CD11C expression. This resulted in the following populations: $CD11B^+$ $CD11C^+$ macrophages, $CD11B^+$ $CD11C^-$ macrophages and $CD11B^{low/-}$ $CD11C^+$ dendritic cells (DCs) (FACS panel 1, **Figure 1B**). Measurement of mean fluorescence intensity allowed us to quantify the expression of CD303 (plasmacytoid DC marker) and CD141 (DC marker), on $CD11B^+$ $CD11C^+$ macrophages and $CD11B^{low/-}$ $CD11C^+$ DCs. Expression of both these markers were higher in $CD11B^{low/-}$ $CD11C^+$ cells confirming that $CD11B^{low/-}$ $CD11C^+$ cells were DCs (**Figure 1C**).

The $CD45^+$ cells (**Figure 1A**) were divided into T-cells and B-cells using CD3 and CD19, respectively. T-cells were subdivided into T-helper cells ($CD4^+$) and cytotoxic T-cells ($CD8^+$). Lastly, $CD3^-CD19^-$ cells were plotted to quantify NK-cells using the marker CD56 (FACS panel 2, **Figure 1D**). The number of cells in each gate is quantified and can be used to calculate the percentage of this cell type of all living cells (**Table 3**).

The percentage of living cells can be calculated for each subject allowing the calculation of an average of all subjects in a group of for example lean or obese men displaying the abundance of a specific immune cell, *i.e.* the pro-inflammatory $CD11B^+$ $CD11C^+$ macrophage in visceral AT (**Figure 2**).

FIGURE AND TABLE LEGENDS:

Figure 1. FACS gating strategy of visceral adipose tissue. (**A**) FACS plot of all events (black) with forward scatter width intensity (FSC-W) and forward scatter area intensity (FSC-A) containing a gate to select only single cells (red) followed by a FACS plot based on FSC-A and side scatter area intensity (SSC-A) containing a gate selecting live cells (lightgreen). Next plot with SSC-A and CD45 fluorescence intensity contains a gate selecting all $CD45^+$ (immune) cells (blue). (**B**) FACS plot of CD19, CD3, CD66b and CD56 fluorescence intensity versus CD11B fluorescence intensity and a gate selecting all cells that are CD19, CD3, CD66b and CD56 negative (brown) for further division into populations. Further subdivision in the next plot based on CD11B and CD11C fluorescence intensity. Gates are displayed containing $CD11B^+$ $CD11C^+$ macrophages (dark green), $CD11B^+$ $CD11C^-$ macrophages (purple) and CD11Blow/$^-$ $CD11C^+$ dendritic cells (blue). (**C**) Amount of $CD11B^+$ $CD11C^+$ or $CD11B^{low/-}$ $CD11C^+$ cells (Y-axis) displaying their levels of fluorescence intensity (X-axis) for CD303 and CD141 and the corresponding quantification of the mean fluorescence intensity (MFI). (**D**) FACS plot displaying the previous $CD45^+$ population (blue) based on CD3 and CD19 fluorescence intensity containing gates selecting T-cells (magenta), B-cells (dark green) and non-autofluorescent cells (green) negative for both CD3 and CD19. The following plot is based on CD4 and CD8 fluorescence with gates selecting $CD4^+$ (lightgreen) and $CD8^+$ (magenta) T-cells. An identical gating strategy is used for subcutaneous adipose tissue. An identical gating strategy is used for subcutaneous adipose tissue. This figure has been modified from Wouters et al.[16].

Figure 2. Obese vAT contains more pro-inflammatory macrophages. The amount of $CD11B^+$ $CD11C^+$ macrophages presented as percentage of all living cells in vAT of lean and obese men. All data are means ± SEM; n=20 for lean and n=31 for obese. **P ≤ 0.01 vs lean.

Table 1. Antibody cocktail for FACS panel 1 to identify macrophage subsets and dendritic cells populations. Amount of antibody described is for the analysis of one sample.

Table 2. Antibody cocktail for FACS panel 2 to identify T and B cell populations. Amount of antibody described is for the analysis of one sample.

Table. 3. Immune cell abundance of different cell types in vAT. Amount of cells in each gate and the percentage of the different cell types based on the total amount of living cells.

DISCUSSION:

These methods describe how to isolate the stromal vascular fraction (SVF) from vAT and sAT and quantify the relative amounts of immune cells within these tissues. Furthermore, the methods state how to determine the expression of markers on specific cell types.

Flow cytometry of tissue immune cells is a powerful technique to phenotype the immunological state of tissues. The quantification of tissue immune cells can have many applications. As described in the results, it is possible to compare the presence of specific immune cells between groups of patients (*e.g.* lean vs obese). In addition, by also performing flow cytometry on blood of the same patients, associations between circulating cells and tissue cells can be investigated. This application allowed our group to determine that a specific subset of

circulating monocytes is associated with pro-inflammatory $CD11C^+$ adipose tissue macrophages[16].

Adjustments to the described protocol will expand applications as the numerous available fluorescent antibodies make flow cytometry very versatile. With different antibodies nearly all cell types can be distinguished and the expression of many markers can be detected. Furthermore, it is possible to stain markers intracellularly by permeabilizing the cell membrane allowing intracellular binding of the fluorescent antibodies. These characteristics allow distinction of the very diverse macrophage populations beyond the overly simplicated M1 and M2 macrophage subtypes. Besides measurement of surface marker expression as we described, proteins (*i.e.* cytokines) can be stained intracellularly providing information on macrophage functionality. In addition, proliferation markers such as Ki67 are used to quantify proliferation rates. As described, distinction between macrophages and DCs was done based on MFI levels of DC markers. A general macrophage marker, such as CD68 can be incorporated into the macrophage panel (FACS panel 1). However, CD68 needs to be stained intracellularly requiring permeabilization of the cell membrane which is not preferable and would extend the protocol. Other macrophage markers are subset markers such as CD163 and CD206 or CD11C, the latter being integrated in our macrophage panel.

In our FACS panels, a marker to distinguish live and dead cells was not included, which would be preferable because it allows a more accurate exclusion of dead cells than the use of FSC and SSC. Frequently used are the DNA staining viability dyes propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI) as well as free amine reacting dyes such as the LIVE/DEAD Fixable Dead Cell Stain Kit, which is available in different dye colours. However, PI and DAPI cannot be used when fixing the cells. As described in the protocol, the LIVE/DEAD Fixable Red Dead Cell viability staining can be integrated into both panels without affecting the overall FACS gating strategy.

In addition, our data is expressed as a percentage of live cells meaning all data is relative. Only by entering an exact and known amount of cells into the flow cytometer, it would be possible to determine the exact numbers of each cell type. An approximate number of cells could be calculated after counting the cells in the SVF fraction by using a counting chamber. However, this number would have to be adjusted for the amount of biopsy tissue used to isolate the SVF but this has limitations when comparing lean to obese AT. A similar mass of obese AT consists of less adipocytes as they are filled with lipids and have expanded greatly. This could lead to an underestimation of immune cell number if presented as number of immune cells per gram of AT or per adipocyte.

In human studies, inclusion of patients is usually done over a longer period of time making standardization of experimental procedures of great importance. For comparison of flow cytometry data between patients, there are several options. As described in this protocol, cells can be fixed before measurement allowing analysis of several samples on the same day. This can also be achieved by freezing the SVF before staining them, which allows even the staining procedure to be equal between all samples, but viability of cells might be affected. Lastly, also employed in our study, are fluorescent beads to install compensation levels and cytometer

tracking beads were used bi-weekly to standardize daily measurements of the cytometer. This last option is the most efficient when measuring samples from a study spanning a long period of time.

A limiting factor for flow cytometry in general is the use of fluorescence. The amount of fluorescent labels that can be detected simultaneously is limited due to overlap in emission spectra. However, with smart FACS panel development and the use of several antibody cocktails per vAT or sAT sample this issue can be overcome as described in this protocol. An important aspect of FACS panel development is fluorescence minus one (FMO) controls. By using all antibodies of the panel except for a specific one, potential autofluorescence levels can be appreciated when comparing the FMO with the full panel. This allows accurate gating of populations and these procedures should be performed when setting up a new FACS panel.
In addition, new generations of FACS devices can detect up to 50 parameters allowing simultaneous detection of many characteristics per cell. Another issue related to the fluorescence aspect is the autofluorescence of cells, particularly macrophages. After excitation of the cells with the FACS laser (mainly with 488 nm wavelength excitation), these cells emit a fluorescent signal (mainly <640 nm) that can overlap with the emission spectra of the antibody labels[17,18]. To account for this, unstained cells should be measured to determine the autofluorescence in each channel. With this knowledge, fluorochromes should be selected that display a signal strength that exceeds the autofluorescent signal. This autofluorescent background signal should be kept into account when determining the gating strategy of the populations. Therefore, by application of this protocol and intelligent FACS panel design it is possible to in depth phenotype macrophage subtypes. New distinct adipose tissue macrophages and their function could be characterized.

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DISCLOSURES:
The authors declare no conflicts of interest.

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