Chapter 10

Isolation and Flow Cytometry Analysis of Macrophages from White Adipose Tissue

Dalila Juliana Silva Ribeiro, Seniz Yüksel, Andreas Dolf, and Dagmar Wachten

Abstract

Macrophages are one of the prominent leukocyte populations in white adipose tissue (WAT) and play an important role during WAT homeostasis and remodeling. Macrophage function in WAT is determined by ontogeny and the local tissue environment. Here, we present a protocol to analyze different macrophage populations from murine WAT using flow cytometry.

Key words White adipose tissue, Macrophages, Flow cytometry

1 Introduction

The white adipose tissue (WAT) is a heterogeneous tissue that consists not only of adipocytes that store energy in form of lipids, but it also contains a variety of other cell types like endothelial cells, fibroblasts, and different immune cells. WAT dynamically adapts to change in whole body homeostasis to maintain tissue function, e.g., during higher energy intake and obesity development [1]. WAT remodeling is functionally coordinated by the different cell types in the tissue [2]. Macrophages are the most prominent leukocyte populations in WAT and play an important role during WAT homeostasis and remodeling [3]. Under lean conditions, 5–10% of the stromal cells are macrophages [4]. Macrophage function in WAT is determined by ontogeny and the local tissue environment. WAT contains yolk sac-derived, tissue-resident macrophages and bone marrow-derived monocytes/macrophages [3, 5], which control WAT development and expansion or diet-associated inflammation, respectively [6]. Different surface marker expression allows to isolate the different macrophage populations from adult WAT in mice (Fig. 1) [6].
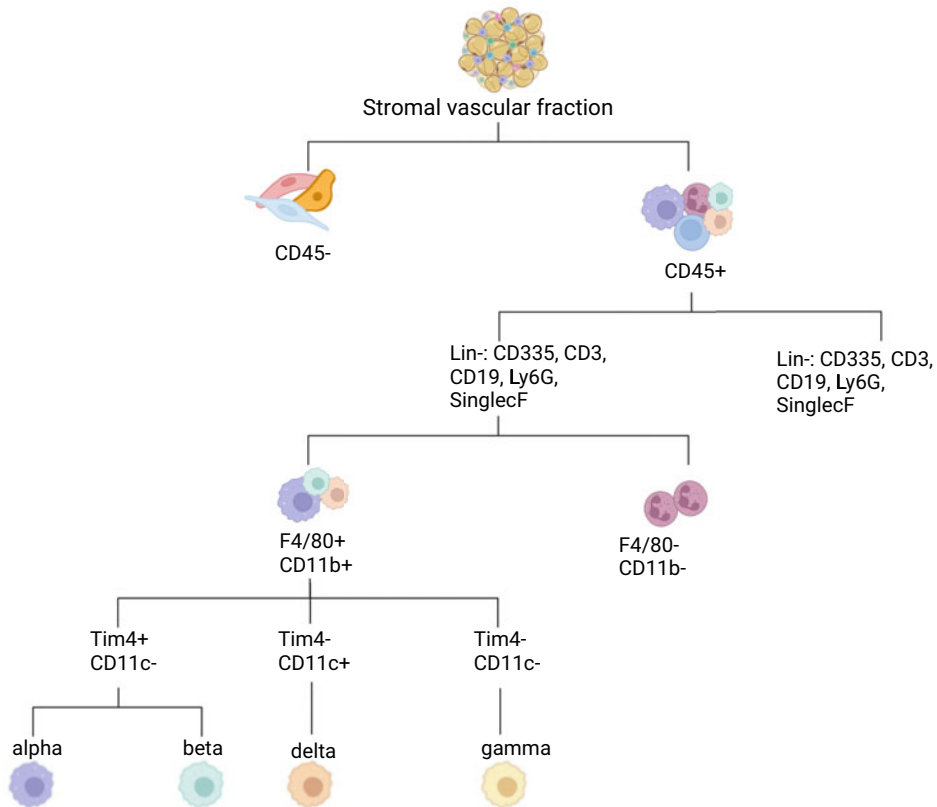
Fig. 1 Schematic overview of adipose tissue-resident macrophage subpopulations. Cell surface markers that allow to delineate between the different cell populations are indicated. The macrophage populations can be divided four subpopulations (α, β, δ, γ) [6]. Figure created with Biorender.com

Macrophages subpopulations in WAT have been distinguished by the expression of TIM4 and CD11c [6] and can be further delineated by the expression of MHCII, resulting in two yolk sac-derived tissue-resident macrophages subpopulations (a, b) and two bone marrow-derived monocyte/macrophage subpopulations (g, d). Upon diet-induced obesity, resulting in adipose tissue inflammation, bone marrow-derived monocytes are recruited to the adipose tissue, increasing the number of the g/d populations in WAT [6].

In this chapter, we describe how to isolate and analyze macrophages from WAT by flow cytometry.

2 Materials

2.1 Consumables	1. 10.0 cm curved scissors.

2.1 Consumables

1. 10.0 cm curved scissors.
2. Sterile 50 mL falcon tubes.
3. Sterile 15 mL falcon tubes.
4. Sterile 1.5 mL microcentrifuge tubes.
5. 2 mL microcentrifuge/reaction tubes.
6. 10 mL Serological pipettes.
7. 96-well microplate round bottom (Corning).
8. Cell strainers: 70 μm and 100 μm.
9. Tubes for flow cytometer acquisition (5 mL round tubes).

2.2 Buffers and solutions

1. 1x Dulbecco's phosphate buffered saline (DPBS), without calcium and magnesium.
2. FACS Buffer: 0.5% (w/v) BSA, 2 mM EDTA in 1x DPBS.
3. Fc Block solution: anti-mouse CD16/32 (Clone 93) 1:100 with 2% rat serum in FACS buffer.
4. Isolation buffer: 0.5% (w/v) BSA in 1x DPBS.
5. Digestion Buffer: 1 mg/mL Collagenase II, 2.5 mM $CaCl_2$ in isolation buffer.
6. Erythrocyte lysis-buffer (e.g., from Biolegend).
7. 70% Ethanol in distilled H_2O.
8. Trypan blue solution.

2.3 Antibodies/Dyes

1. Antibody capture compensation beads.
2. Fluorochrome-labeled anti-mouse antibodies against indicated cell surface markers (*see* Table 1).
3. 7-AAD (7-amino-actinomycin D) Viability Staining Solution.

2.4 Equipment

1. Refrigerated centrifuge.
2. Platform shaker with heating module.
3. Neubauer counting chamber.
4. Micropipettes.
5. Pipette boy.
6. Flow cytometer.

3 Methods

Before starting, it is recommended to set the instruments to their required working temperatures. For optimal tissue digestion, the shaker should be set at 37 °C, and the centrifuge should be cooled down to 4 °C for higher cell survival during the experiment.

Table 1
Antibodies

Antigen	Fluorophore	Concentration	Clone
NKp46 (CD335)	PE	1 μg/mL	29A1.4
CD3	PE	1 μg/mL	17A2
CD19	PE	0.5 μg/mL	1D3/CD19
Ly6G	PE	0.5 μg/mL	1A8
SiglecF	PE	2 μg/mL	S17007L
CD45	APC-Cy7	0.5 μg/mL	30-F11
CD11b	PE-Cy7	0.25 μg/mL	M1/70
F4/80	BV421	0.5 μg/mL	BM8
Tim4	AF647	1.25 μg/mL	F31-5G3
CD11c	BV605	2.5 μg/mL	N418
MHC-II	FITC	1.25 μg/mL	M5/114.15.2

3.1 Harvesting of WAT

1. Euthanize mice according to a locally approved procedure and disinfect the skin with 70% ethanol.

2. Using clean instruments, make a transverse cut in the abdominal area and open the abdominal cavity. Cut out the gonadal WAT (gWAT) from both sides around the testes (Fig. 2) (*see* **Note 1**).

3. Weigh the tissue of interest for each mouse (*see* **Note 2**).

3.2 Tissue Digestion and Preparation of Single-Cell Suspension from WAT

1. Transfer the tissues to a 1.5 mL tube and add 1 mL cold WAT isolation buffer. Cut the tissue into small pieces with the 10.0 cm curved scissor (*see* **Note 3**).

2. Transfer the whole content from the 1.5 mL tube to a sterile 50 mL falcon tube and add 3 mL of digestion buffer to the sample. We advise performing the tissue digestion from individual mice in separate tubes. We recommend a maximum of 0.6 g of gWAT per 4 mL of digestion buffer. Incubate for 30 min at 190 rpm in a shaker at 37 °C (*see* **Note 4**).

3. Stop the enzymatic reaction by adding 12 mL of cold isolation buffer, pipette up and down several times with a pipette boy, place a 100-μm filter to a new empty sterile 50 mL falcon tube, and apply cells to the column (*see* **Note 5**).

4. Collect the cell pellet by spinning the sample for 10 min at $500 \times g$, 4 °C, discard supernatant, resuspend the pellet in 1 mL erythrocyte lysis buffer for 2 min, and stop the reaction by adding 9 mL of isolation buffer.
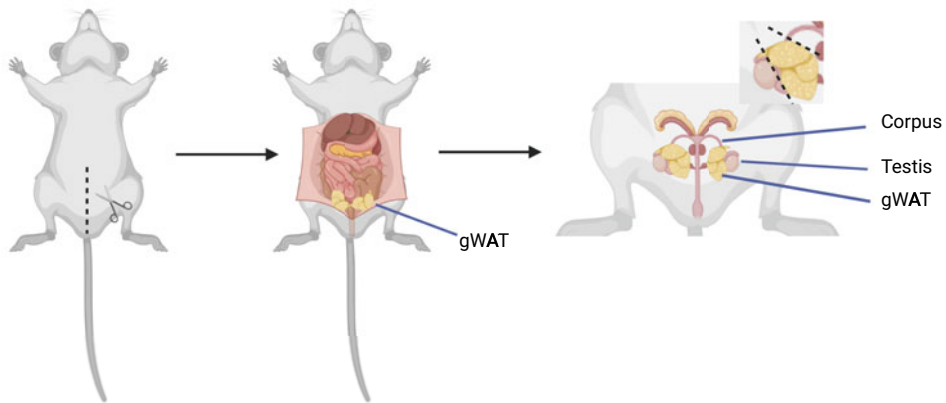
Fig. 2 Representative figure of mice dissection. A transverse cut in the abdominal region is followed by laterally opening the skin, which allows to investigate the adipose tissue. The gonadal WAT (gWAT) is located around the testes. Figure created with Biorender.com

5. Again, collect the cell pellet by centrifuging the sample for 10 min with $500 \times g$, 4 °C, discard supernatant, and resuspend the pellet in 1 mL FACS buffer.

6. Determine the cell count by diluting 10 µL of the cell suspension 1:2 with Trypan Blue Solution and count the cells using a Neubauer counting chamber.

3.3 Antibody Staining of Cell Surface Markers

Before starting the staining procedure, all antibody solutions, i.e., the full staining mix containing all antibodies, single-staining solutions (*see* **Note 6**), and fluorescence minus one (FMO) control (*see* **Note 7**) should be prepared in FACS buffer and kept at 4 °C.

1. Resuspend cells in 100 µL FACS buffer in a 1.5 mL reaction tube to a final concentration of $2–3 \times 10^6$ cells. Centrifuge the cells for 10 min at $500 \times g$ at 4 °C.

2. Resuspend the cell pellet in 100 µL of Fc Block and incubate for 20 min at 4 °C in a 1.5 mL reaction tube.

3. Plate 80 µL of the cell suspension into a round-bottom 96-well microplate and use the remaining 20 µL from each sample for FMOs and unstained controls. Seed them on the same plate.

4. Centrifuge the plate for 10 min at $500 \times g$, 4 °C.

5. Discard the supernatant by flipping the plate on a paper towel (*see* **Notes 8** and **9**).

6. Resuspend the cell pellet in 20 µL of antibody cocktails, as listed in Table 1 for the different stainings and FMO controls, or in FACS buffer alone for the unstained control.

7. Single staining controls should be done with capture beads that are used for compensation and are stained for the same time as regular samples; to this end, add the appropriate quantity of single-staining solutions to match the concentrations indicated in Table 1 into one drop of the capture beads (vortex bead stock first).

8. Incubate samples on ice, protected from light, for 30 min.

9. Washing procedure: add 100 µL of FACS buffer and repeat **steps 5** and **6**.

10. Resuspend cells in 100 µL of FACS buffer.

11. Filter samples through a 70 µm cell strainer (essential to avoid clogging) and place them in the indicated tubes for flow cytometry acquisition. If starting the staining with 2×10^6 cells, dilute the samples to achieve less than 2000 events per second. Finally, add the cell viability 7-AAD dye at a final dilution of 1:50 in the full stained samples and FMO controls. Do not add the dye to the unstained control.

12. Place the samples on ice, protected from light, and proceed to the acquisition in the flow cytometer. Determine forward and side scatters with the unstained control samples (Fig. 3). Use unstained controls to determine the background signal for all the detectors. Acquire all single-stained compensation controls and make the appropriate adjustments. Calculate compensation values and apply them for the real samples as well as for FMO controls.

13. For setting up the gating strategy, scatter parameters must be used to exclude cellular debris and doublets (Fig. 3). Selection of the population without a signal for 7-AAD will exclude dead cells (Fig. 3). FMO controls should be used to determine the placement of the subsequential gates. Figure 4 illustrates exemplary how FMOs were used to determine the BV605 signal, i.e., for the selection of CD11c-positive cells (Fig. 4a), and the FITC signal (Fig. 4b), and for the selection of MHCII positive cells (Fig. 4c, d).

4   Notes

1. Inspect the liver and spleen for possible signs of disease, abdominal carcinomas, or organomegaly. Signs of illness should be infrequent events in young wild-type mice.

2. Flow cytometry data can be analyzed in two different ways: frequency of superordinate group, e.g., frequency of CD45+ cells or cells per tissue depot. Hence, it is fundamental to weigh the tissue and calculate the number of cells per mL. The weight
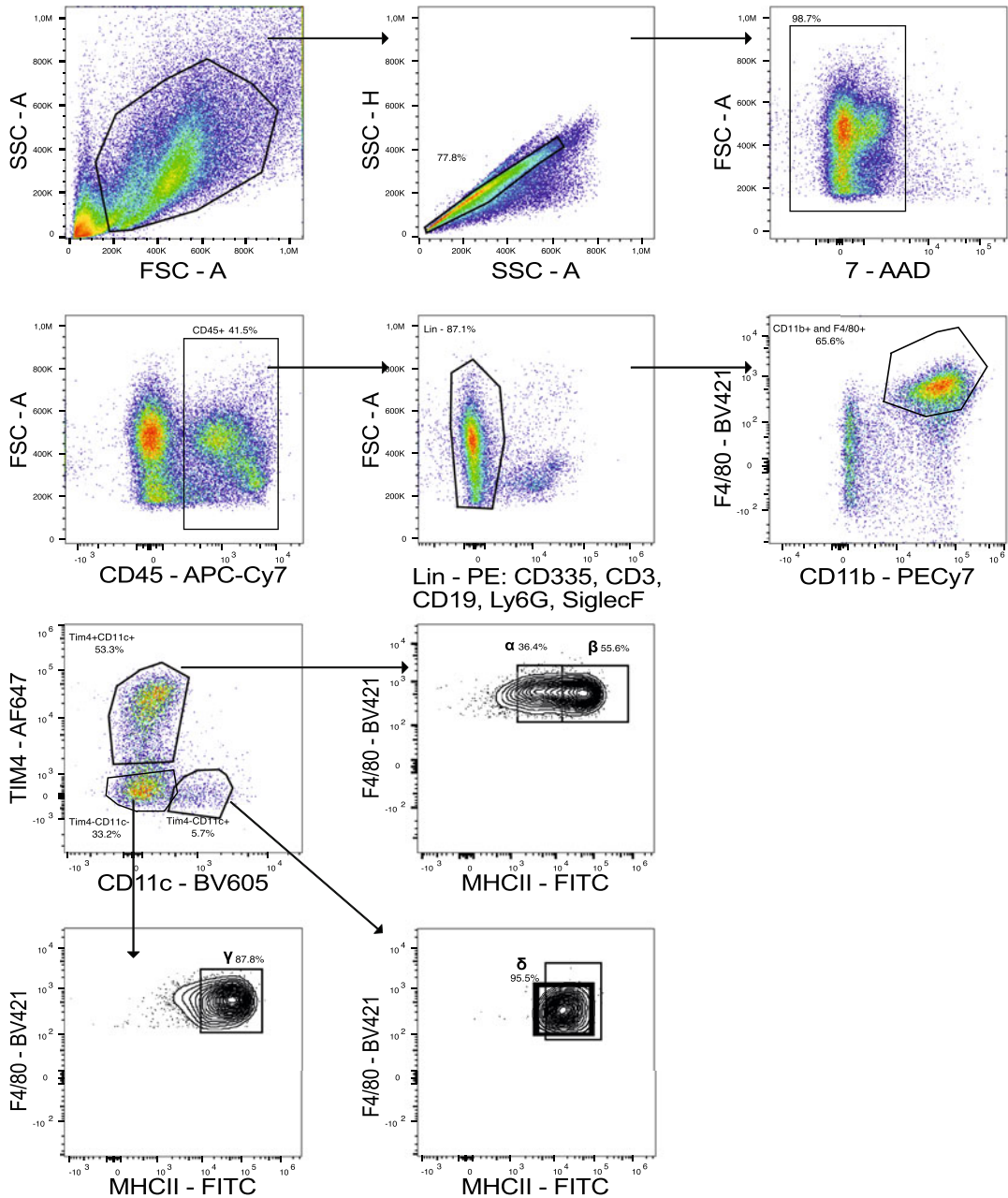
Fig. 3 Representative pseudo-color plots of the gating strategy to discriminate the four subpopulations of ATM. The cells of interest were gated from cells of the stromal vascular fraction (SV), followed by doublets and live cell discrimination. CD45 positive signal was used to select cells from hematopoietic origin. Additional immune cell populations were excluded from the analysis using a "dump channel", in which antibodies from different lineage markers were conjugated with the same fluorochrome. A double-positive signal for F4/80 and CD11b allows the selection of all macrophages, while TIM4, CD11c, and MHCII signals distinguish between the different subpopulations as indicated. For fluorescent channels, parameters were shown using the biexponential scale; this transformation improves the display of negative values, preventing the accumulation of events in the chart edges. The samples for this protocol were acquired in the flow cytometer Attune Nxt (ThermoFisher), following the manufacturers' instructions on flow rate and sample volume
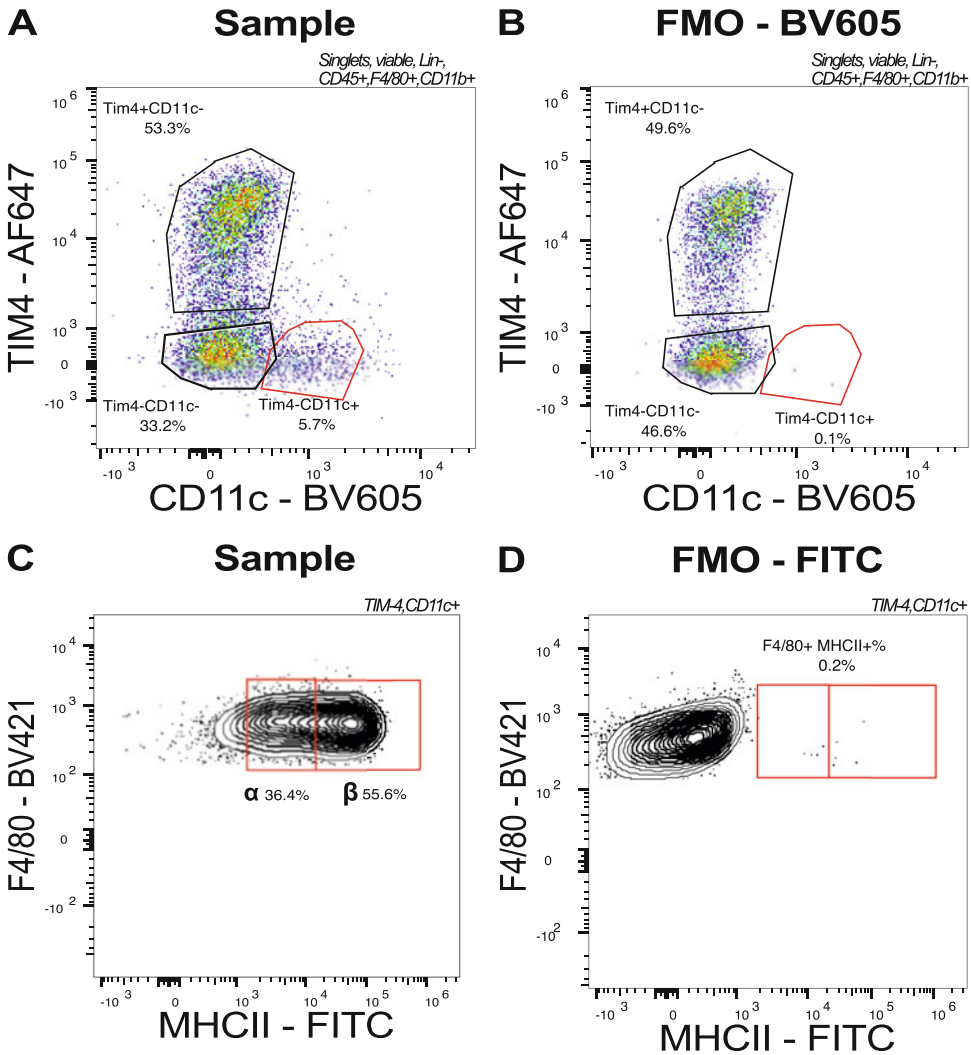
Fig. 4 Example of how FMO controls can be used to determine the gates. (**a** and **c**) were stained with all antibodies and followed the same gating strategy as presented in Fig. 2. (**b** and **d**) were treated as **a** and **c**, except they lack one antibody each, i.e., anti-CD11c conjugated to BV605 (**b**) and anti-MHC-II conjugated to FITC (**d**)

of the fat depot depends on the age and diet of the mice. The final cell frequency may depend on the WAT depot, nevertheless all described markers can be applied for all WAT types.

3. Cold isolation buffer will prevent denaturation of the tissue, and smaller pieces of the tissue will result in better digestion efficiency.

4. Attention should be paid to avoid prolonged incubation times with the digestion buffer, since extensive collagenase digestion can degrade the epitopes on the cell surface.

5. Adjust the pipet boy to low and filter the solution into a new tube. This step will provide a clean pellet and will avoid contamination of reminiscent lipid particles coming from the walls of the falcon tube.

6. Compensation can be performed using the values generated by individual antibody labeling for each parameter using the cells of interest or capture beads. The latter is recommended when dealing with cell surface markers that show a low expression level and/or when the protein expression is limited to sparse cell populations. As the viability dye belongs to the group of nuclei acid binding-dyes, the single staining control can only be performed with cells for compensation. Unstained cells are used to determine morphological parameters and auto-fluorescence.

7. FMO controls consist of cells stained with all fluorophores, except the one being measured, and are essential when running a multicolor flow-cytometry experiment. FMOs help to account for the fluorescence spread generated after compensation to identify possible false-positive signals. When setting up a multicolor flow-cytometry panel for the first time, FMOs should be included for all fluorophores. Afterwards, they can be used to separate only rare populations.

8. Staining in plates can make the procedure faster in terms of pipetting time, especially during washing steps. However, this is also more prone to contamination between adjacent samples, which can be detrimental for single-staining controls. We recommend leaving spaces between the wells. It is also possible to perform the staining procedure directly in flow cytometry tubes.

9. As an alternative to prevent non-specific polymer interaction, Brilliant Stain Buffer (InvitrogenTM) can be added prior to the staining. It is used as a complement for multicolor flow-cytometry experiments and is only necessary when more than one polymer dye-conjugated is included.

Acknowledgments

References

1. Rosen ED, Spiegelman BM (2014) What we talk about when we talk about fat. Cell 156:20–44
2. Choe SS, Huh JY, Hwang IJ, Kim JI, Kim JB (2016) Adipose tissue remodeling: its role in energy metabolism and metabolic disorders. Front Endocrinol (Lausanne) 7:30
3. Chakarov S, Bleriot C, Ginhoux F (2022) Role of adipose tissue macrophages in obesity-related disorders. J Exp Med 219:e20211948
4. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr (2003) Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 112:1796–1808
5. Cox N, Geissmann F (2020) Macrophage ontogeny in the control of adipose tissue biology. Curr Opin Immunol 62:1–8
6. Cox N, Crozet L, Holtman IR, Loyher PL, Lazarov T, White JB, Mass E, Stanley ER, Elemento O, Glass CK et al (2021) Diet-regulated production of PDGFcc by macrophages controls energy storage. Science 373: eabe9383