



Published in final edited form as:

Curr Protoc Immunol. 2019 September ; 126(1): e86. doi:10.1002/cpim.86.

Characterization of Immune Cells from Adipose Tissue:

Adipose Tissue Immune Cell Profiling

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Abstract

Adipose tissue (AT) serves a crucial role in maintaining organismal metabolic homeostasis. Studies have demonstrated that AT is populated with a diverse array of immune cells that coordinate and regulate AT function. This adipo-immune system is highly dynamic, reflecting the physiologic state of the organism (e.g. obese, lean, aged, and young) as well as the constant physiologic remodeling of AT associated with the daily rhythms of fasting and feeding. Many of the adaptive and maladaptive functional changes of AT are regulated by changes in the quantity and quality of distinct sets of AT-resident immune cells. Here we developed a protocol to assess the dynamic state of the immune system within AT by constructing censuses of adipose-resident immune cells (macrophages, dendritic cells, neutrophils, eosinophils, NK cells, innate lymphocytes, T cells, and B cells, etc.) based on flow cytometry, which we term adipo-immune profiles (AIPs). Constructing AIPs can be an integral part of assessment for AT health and function. This unit describes the protocols to generate such AIPs.

Keywords

adipose tissue; immune cell profiling; macrophage; regulatory T cell; flow cytometry

INTRODUCTION

Adipose tissue (AT) function is intimately related with immune cells residing within AT (Hotamisligil, 2017; Y. S. Lee, Wollam, & Olefsky, 2018). Since the initial findings that macrophage number increases in obese AT in mice described in the 1960s (Hausberger, 1966) and again demonstrated in 2003 (Ferrante, 2013; Xu et al., 2003), critical roles for several specific types of immune cells have been implicated in modulating distinct functions of AT, including insulin sensitivity (Lynch et al., 2012; Molofsky et al., 2013; Talukdar et al., 2012; Wu et al., 2011), adaptive thermogenesis (Brestoff et al., 2014; M.-W. Lee et al., 2014; Rao et al., 2014), and immune homeostasis (Han et al., 2017). While these studies have largely focused on the effect of one immune cell type at a time, the immune system usually carries out its function via coordination of an array of immune cell subsets, in AT as well as

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other tissue types. To gain more holistic insight about the composition of the immune system within AT, we found it helpful to assemble an immunological census within a defined AT depot, which we termed an adipo-immune profile (AIP). This method is reliant on conventional flow cytometry and allows for quantitation of numerous distinct immune cell types (macrophages, dendritic cells, neutrophils, eosinophils, NK cells, innate lymphocytes, T cells, and B cells, etc.), and thus allows investigators to understand how immune systems globally change within AT upon a given perturbation. We developed this method while characterizing immune changes in ATs from lean, young mice, obese mice, and lean, old mice and discovered a striking, previously undescribed increase between young and old mice in a unique type of regulatory T cell, we termed the fat-resident regulatory T cell (fTreg). This lynchpin finding was what facilitated our eventual discovery that fTregs drive age-associated insulin resistance (Bapat et al., 2015; Maillard & Saltiel, 2015). We have continued to refine this method and have found construction of AIPs useful in understanding the immune systems of AT from other important physiological contexts. The protocol described below describes in detail how to construct AIPs from murine AT depots.

BASIC PROTOCOL 1 Isolation of immune cell populations from adipose tissue

AT depots are located in multiple defined anatomic sites in the murine and human body. Distinct AT depots function and behave differently under different physiologic stresses, carry out distinct metabolic functions, and possess distinct adipo-immune cell compositions. In general, AT depots can be divided into visceral AT (VAT) and subcutaneous AT (SAT). In this protocol, we use epididymal AT, a visceral AT depot, as an example to construct an AIP. AIPs of other AT depots can be carried out using the same method.

Materials

Mice for Adipo-Immune Profiling (Wild-type C57BL/6 male mice at one year of age were used here, but this protocol can apply to mice of other genetic backgrounds and at different ages.)

Adipose tissue dissociation buffer (see recipe)

RPMI medium (Thermo-Fisher, Cat. No. 11875093)

1× PBS (Fisher Scientific, Cat. No. MT21040CM)

70 µm cell strainer (Fisher Scientific, Cat. No. 22363548)

Scissors and tweezers for mouse dissection

Petri Dish

Conical tubes

Centrifuge (Eppendorf 5810R)

Orbital Shaker

1. Euthanize mouse in a CO₂ chamber. Open the thoracic cavity and cut a small incision in the right atrium of the heart as an outlet for blood. Perfuse the mouse by slowly injecting 10 mL of ice cold 1×PBS into the left ventricle to remove blood from tissues. See UNIT 15.1 (Miller, Karpus, & Davidson, 2010) for detailed instructions on mouse perfusion.
For optimal result, perfuse shortly after euthanization.
2. Use scissors and forceps to open the peritoneal cavity and remove the epididymal AT (Figure 1).
Be careful to dissect the testes away from the epididymal ATs.
3. Measure the weight of the AT (The epididymal AT from a one-year-old mouse is typically 0.4 g to 0.8 g.).
4. In a 10 cm dish, use scissors to mince individual AT depots into fine pieces (2–5 mm³).
5. Transfer the minced ATs to a 15 mL conical tube. Add 10 mL adipose tissue dissociation buffer, and incubate at 37°C with intermittent shaking for 1.5 hrs.
6. Pass the suspension through an 80 µm mesh to remove undigested clumps and debris.
7. Let the flow-through stand for 10 min to separate the floating adipocyte fraction and the infranatant containing the stromal vascular fraction.
8. Carefully transfer the infranatant to a new 15 ml conical tube without disturbing the floating adipocyte fraction.
9. Centrifuge at 400g for 10 min.
10. Wash the pellet once with 10 ml RPMI.
11. The resultant isolated cells can be immediately used for FACS staining and analysis (see basic Protocol 2 for details), or stored on ice for up to 4 hours before FACS staining.

BASIC PROTOCOL 2 Staining and flow cytometric analysis of AT immune cells

Here we describe how to perform staining of the immune cells isolated from AT for FACS analysis. Additionally, we show how FACS data is analyzed to identify each immune cell population.

Materials

Adipose tissue immune cell samples isolated from the Basic Protocol 1

Cell staining buffer (see recipe)

Cell fixation/permeabilization buffer (see recipe)

1× cell permeabilization buffer (see recipe)

Fc block: anti-mouse CD16/CD32 clone 2.4G2 (BioXcell, Cat. No. BE0307) in cell staining buffer

Fluorescently labeled antibodies (Table 3)

Cell fixation buffer (see recipe)

LSR II flow cytometer (Becton Dickinson) or equivalent

FlowJo analysis software

Surface and intracellular stain of the cells

1. Resuspend $0.5\text{--}1\times 10^5$ AT immune cells obtained from Basic Protocol 1 in 200 μL Fc block solution. Incubate on ice for 10 min.

Cell numbers should be counted at this time using a hemocytometer after trypan blue staining. It is important to be able to quantitate the actual number of specific immune cell populations identified by FACS, which generally provides frequencies. Typically, we can harvest $2\text{--}5\times 10^5$ cells per gram of fat at this stage.

2. Prepare antibody cocktails in cell staining buffer at 100 μL volume per sample. The four commonly used antibody cocktails for AT immune cells are listed in Table 1.

Cocktail #1 is used to identify myeloid cell subsets. Cocktails #2 and #3 are used to identify T, B, and NK subsets. Cocktail #4 is used to identify innate lymphocyte subsets. All antibody concentrations should be titrated for optimal results. As a common practice, we use 1:400 dilution of each antibody (~ 1 ng/ml) as a starting point. Design of proper compensation matrices is essential for multiparameter FACS analysis. Fluorescence-minus-one (FMO) controls should be conducted when setting up this analysis for the first time to allow for proper gating. A more detailed discussion on multiparameter FACS analysis is described in UNIT 5.8 (Roederer, 2002).

3. Aliquot immune cells in Fc block solution into three tubes at 50 μL each.
4. Add 50 μL premixed antibody cocktail 1, 2, 3, or 4 (Table 1) into each tube with immune cell samples in Fc block solution to reach final volume at 100 μL .
5. Incubate cells in antibody cocktail for 30 min on ice in the dark.
6. Centrifuge 5 min at 500 g at 4°C.
7. Remove supernatant and wash pellet with 200 μL cell staining buffer.
8. Centrifuge 5 min at 500 g at 4°C.

9. For samples that don't need intracellular staining (Stain 1 and 3), resuspend cell pellet in 100 μ l cell fixation buffer. Store at 4°C in the dark until ready for FACS analysis (Basic Protocol 2 Step 22. For samples that need intracellular staining (Stain 2 and 4), proceed to the next step (Basic Protocol 2, Step 10–21).
10. Resuspend cells in 200 μ l cell fixation/permeabilization buffer.
11. Incubate on ice for 1 hr in the dark.
12. Centrifuge 5 min at 725 g at 4°C.
13. Remove supernatant and wash pellet with 200 μ l 1 \times cell permeabilization buffer.
14. Centrifuge 5 min at 725 g at 4°C.
15. Prepare antibody cocktail for intracellular staining in 1 \times cell permeabilization buffer (Table 1).
16. Remove supernatant and resuspend cell pellet in 100 μ l antibody cocktail.
17. Incubate cells in antibody cocktail for 30 min on ice in the dark.
18. Centrifuge 5 min at 725 g at 4°C.
19. Remove supernatant and wash pellet with 200 μ l 1 \times cell permeabilization buffer.
20. Centrifuge 5 min at 725 g at 4°C.
21. Remove supernatant and resuspend cell pellet in 100 μ l cell fixation buffer. Store at 4°C in the dark until ready for FACS analysis.
22. Stained cells should be analyzed by FACS within 24 hours to achieve optimal result.

FACS data acquisition and analysis

23. Stained cell samples can be acquired on a BD LSRII or equivalent FACS machine with more than 8 color capacity.

During data acquisition, record all events instead of events in selected gates so that there is more flexibility in gating choices during the data analysis step.
24. Typically, 20,000 events are acquired. For analysis of more rare immune cell populations, it is advisable to increase acquisition events to 200,000 or more.
25. For FACS data analysis, use FlowJo software to process FCS sample file. Detailed gating strategy for each immune cell subset is listed in Table 2. A representative FACS analysis of AT immune cells from a one-year old male mouse's epididymal fat is shown in Figure 2.
26. Use total cell number from the hemocytometer cell count and percentages generated from FACS analysis to calculate the number for each immune cell subset. Normalize cell numbers using the weight of the fat pad to generate quantitation in the form of cell numbers per gram of fat.

REAGENTS AND SOLUTIONS

Adipose tissue dissociation buffer

100mM HEPES buffer pH7.4, 120mM NaCl, 50mM KCl, 5mM glucose, 1mM CaCl₂, 1.5% BSA, and 1mg/ml collagenase.

Make 1M stock solutions of NaCl, KCl, glucose, and CaCl₂•2H₂O. To make 50 ml stock solutions, the following amount of materials is needed.

2.922g NaCl

3.727g KCl

9.0g glucose

7.35g CaCl₂•2H₂O (Be certain to use the dihydrate form of CaCl₂ as opposed to the anhydrous form. The anhydrous form has less purity, and the stock solution will form a foggy precipitate in a few days.)

Once the stock solutions are made, make the buffer fresh each time. For 25 ml of adipose tissue dissociation buffer, combine

3 ml of 1M NaCl

1.25 ml of 1M KCl

125 µl of 1M glucose

25 µl of 1M CaCl₂

2.5 ml of 1M HEPES

0.375 g BSA

Adjust pH of the solution to 7.4. Add collagenase at 1mg/ml (Worthington Biochemical, Collagenase Type 1, Cat no LS004196). Dissolve in 37°C water bath. Sterilize the solution through a 0.2µm filtration unit, and put it on ice until use.

FACS staining buffer

0.5% BSA in 1× PBS.

Cell fixation/permeabilization buffer

1 to 4 dilution from Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent. (Thermo Fisher, Cat. No. 00–5521-00)

1× Cell permeabilization buffer

1 to 10 dilution in distilled water from 10x Cell Permeabilization Buffer. (Thermo Fisher, Cat no 00–8333-56)

Cell fixation buffer

1% paraformaldehyde in 1× PBS.

COMMENTARY

Background Information

Since the reports in 2003 of an increase in macrophage content associated with obesity-associated insulin resistance, there has been a steady increase in interest of various immune cell types found in AT, at first focusing on innate immune cells but eventually focusing on adaptive immune cells as well. These studies have largely studied the given immune cell of interest in isolation from other AT-resident immune cells. Yet it is clear that the immune system functions in an interdependent manner with extensive crosstalk between different immune cell subsets to collectively preserve or dysregulate immune-metabolic homeostasis in AT. As such, we developed a method to globally assess immune state within AT at single-cell resolution.

The protocol described herein to assemble an immunological census within AT, which we call an adipo-immune profile or AIP, utilizes conventional flow cytometry. In our own practice, we have found this approach to be affordable, adaptable, and requiring a relatively short amount of time from initial recovery of AT-resident immune cells to data acquisition and analysis (one full working day). Recently, additional methodologies beyond conventional flow cytometry to globally assess immune cells at a single cell resolution have emerged including mass cytometry and single cell RNA-sequencing (scRNA-Seq). In principle, these approaches also will be able to quantitatively define distinct immune cell types within AT. However, caution should be taken to validate that these emerging methodologies accurately preserve relative cell abundance across different immune cell types, perhaps via cross-comparison utilizing conventional flow cytometry. Regardless of methodology, the overarching principle of assembling a holistic immunological census within AT is crucial when assessing AT-immune relationships. In our assessment, for most initial investigations, flow cytometry-based AIPs provide a reliable, reproducible, and affordable snapshot of immune state within a given AT.

Critical Parameters

The immune cell composition in ATs is highly dynamic, and varies depending on the condition of the mouse. Major factors include age, sex, diet, and anatomic site of the AT depot being interrogated. For example, fTreg cell frequency increases as the C57BL/6J mouse gets older, from about 10–15% of total CD4⁺ T cells at 6 weeks of age to 60–70% in one-year old mice. Therefore, it is important to always have an appropriately matched control group for equitable comparison.

One of the critical factors in constructing AIPs involves the process of AT digestion. AT needs to be minced very well into small pieces for digestion to work well. If the AT is under-digested, not enough immune cells will be liberated into the infranatant for downstream analysis. Oppositely, if AT is over-digested, a large portion of the cells will die and detrimentally affect FACS staining and analysis. It is helpful to evaluate the status of the

cells by cell counting after the digestion step before FACS staining. Most of the cells at this stage should be alive as assessed by Trypan Blue stain; live cells will not take up the stain. Additionally, we noticed that certain cell surface markers, such as CD4, are downregulated during digestion, perhaps due to regulated endocytosis and other mechanisms. One way to mitigate this issue is to incubate immune cells in RPMI medium at 37°C for one hour right after they are isolated from AT, and then proceed to FACS staining. Cell surface CD4 expression increases significantly after this recovery step.

Troubleshooting

It can be challenging to identify multiple AT immune cell subsets from FACS data initially. We always stain the splenic cells from the same mouse to help set up gates for our AT-resident immune cell populations. AIP construction might require a new compensation matrix setup on the flow cytometer due to the unique properties of these AT-resident immune cells as well as the different isolation protocols required to analyze the AT-resident immune cells, in contrast to isolation of cells from lymphoid tissues. When it is not clear if a particular antibody is staining correctly, we perform a FMO, “X-1” control stain, a stain with all other antibodies in the cocktail except the one antibody in question. We can validate the antibody by comparing the FACS signal of the experimental stain with the “X-1” stain.

Understanding Results

In general, immune cells are more abundant in visceral ATs than subcutaneous ATs. Obese mice, induced by high-fat diet or genetic mutations, usually have AT depots that are several times larger than lean mice. For meaningful comparisons, we calculate the density of AT infiltrating immune cells based on cell number per gram of fat. The most abundant immune cell population in AT is macrophages. They can be further divided into M1 and M2 subsets, which are shown to be pro-inflammatory or anti-inflammatory, respectively. Although the frequencies of other immune cells in ATs are not as abundant as macrophages, numerous studies have shown the importance of nearly every immune cell subset present in AT, including CD4⁺ T cells, CD8⁺ T cells, Tregs, B cells, dendritic cells, NK cells, Eosinophils, and ILCs, etc. Therefore, if we are wanting to investigate non-macrophage populations more carefully, we find it helpful to gate out the macrophage populations early in our gating schema to better understand how the non-macrophage populations behave within AT.

Time Considerations

Harvesting the AT depots from mouse and processing them into single cell suspension takes about 3 to 4 hours. FACS staining of the immune cells takes about 3 hours. Acquisition of FACS data takes about 3 to 5 minutes per sample. For a typical experiment analyzing AT immune cells from 5–6 mice, the total experimental time is about 8 hours.

ACKNOWLEDGEMENT

This work is partly supported by NOMIS Foundation, Rita Allen Foundation, and National Institutes of Health (R01-A1107027, P30-CA014195, S10-OD023689).

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Significance Statement

Adipose tissue serves an essential function in maintaining organismal metabolic homeostasis. Dysfunction of adipose tissue can lead to metabolic diseases such as type-2 diabetes and atherosclerosis. Studies have demonstrated that adipose tissue is populated with a diverse array of immune cells that coordinate and regulate adipose tissue function. This adipo-immune system is highly dynamic, reflecting the physiologic state of the organism as well as the constant physiologic remodeling of adipose tissue. Here we describe a protocol to assess the dynamic state of the immune system within adipose tissue by constructing censuses of adipose-resident immune cells based on flow cytometry, which we term adipo-immune profiles (AIPs). Constructing AIPs can be an integral part of assessment for adipose tissue health and function.

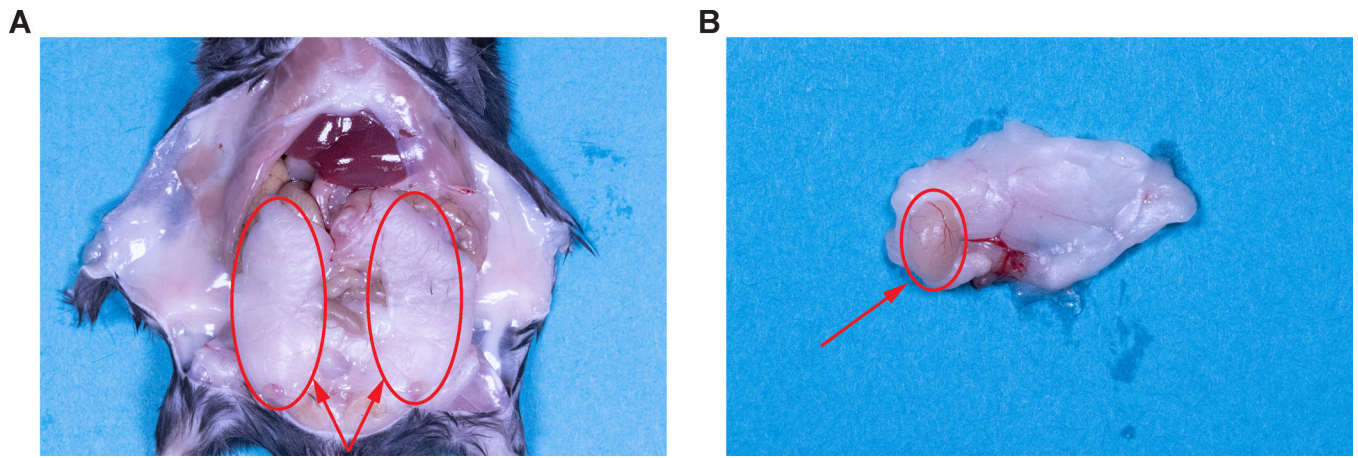


Figure 1. Isolation of the epididymal fat pad.

(A) Location of the two epididymal fat pads marked by the red circles. (B) Location of the testis imbedded in the epididymal fat pad marked by the red circle.

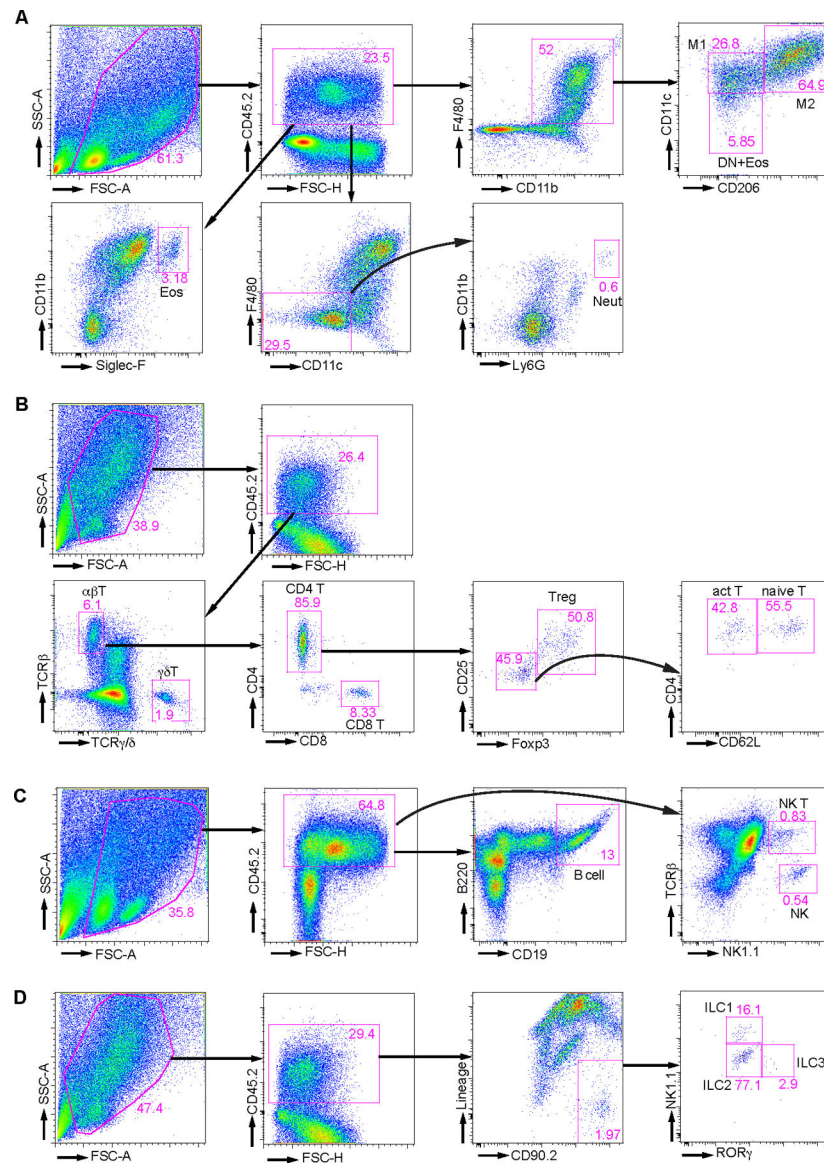


Figure 2. Gating strategy for FACS analysis of AT immune cell subsets.

Typical FACS plots generated from (A) Antibody cocktail #1, myeloid cells; (B) Antibody cocktail #2, T cell subsets; (C) Antibody cocktail #3, B cell, NK cell, and NK T cell; and (D) Antibody cocktail #4, innate lymphocytes. M1: M1 adipose tissue associated macrophage. M2: M2 adipose tissue associated macrophage. DN: double negative adipose tissue associated macrophage. Eos: eosinophil. Neut: neutrophil. Treg: regulatory T cell. Naïve T: naïve CD4+ T cell. Act T: activated CD4+ T cell. NK: natural killer cell. NK T: natural killer T cell. ILC1: group 1 innate lymphocyte. ILC2: group 2 innate lymphocyte. ILC3: group 3 innate lymphocyte.

Table 1.

Antibody cocktails for AT immune profiling.

Antibody Cocktail #1		Antibody Cocktail #2		Antibody Cocktail #3		Antibody Cocktail #4	
Antibody	Color	Antibody	Color	Antibody	Color	Antibody	Color
CD11b	APC-Cy7	CD4	APC	CD19	Alexa 700	CD45.2	Alexa 700
CD11c	PerCP-Cy5.5	CD8	Brilliant Violet 510	CD45.2	PerCP-Cy5.5	CD90.2	FITC
CD45.2	Alexa 700	CD25	FITC	B220	eFluor 450	GATA3 (IC) *	APC
CD206	PE-Cy7	CD45.2	PerCP-Cy5.5	NK1.1	PE	NK1.1	Brilliant Violet 605
F4/80	APC	CD62L	Brilliant Violet 605	TCR β	FITC	ROR γ (IC) *	eFluor 450
Ly6G	FITC	Foxp3 (IC) *	eFluor 450			CD3 ϵ **	PE
Siglec-F	PE	TCR β	PE-Cy7			CD5 **	PE
		TCR γ/δ	PE			CD8 **	PE
						CD11b ***	PE
						CD19 **	PE
						TCR β **	PE
						TCR γ/δ **	PE
						Fc ϵ R1 **	PE
						F4/80 **	PE

* IC: intracellular staining. These antibodies need to be used after permeabilization of the cells.

** These PE-conjugated antibodies are used to gate out lineage positive cells for innate lymphocyte stain.

Table 2.

Cell surface and intracellular markers used to identify immune cell subsets in AT.

Immune cell subsets	Markers for identification of immune cell subsets
M1 macrophage	CD45.2 ⁺ F4/80 ⁺ CD11b ⁺ CD11c ⁺ CD206 ⁻
M2 macrophage	CD45.2 ⁺ F4/80 ⁺ CD11b ⁺ CD11c ⁺ CD206 ⁺
Double negative (DN) macrophage	CD45.2 ⁺ F4/80 ⁺ CD11b ⁺ CD11c ⁻ CD206 ⁻
Eosinophil	CD45.2 ⁺ CD11b ⁺ Siglec-F ⁺
Neutrophil	CD45.2 ⁺ CD11c ⁻ F4/80 ⁺ CD11b ⁺ Ly6G ^{high}
NK cell	CD45.2 ⁺ TCRβ ⁻ NK1.1 ⁺
NK T cell	CD45.2 ⁺ TCRβ ⁺ NK1.1 ⁺
B cell	CD45.2 ⁺ B220 ⁺ CD19 ⁺
γ/δ T cell	CD45.2 ⁺ TCRβ ⁻ TCRγδ ⁺
CD4 ⁺ T cell	CD45.2 ⁺ TCRβ ⁺ CD4 ⁺ CD8 ⁻
CD8 ⁺ T cell	CD45.2 ⁺ TCRβ ⁺ CD4 ⁻ CD8 ⁺
Regulatory T cell	CD45.2 ⁺ TCRβ ⁺ CD4 ⁺ CD8 ⁻ Foxp3 ⁺
Naïve CD4 ⁺ T cell	CD45.2 ⁺ TCRβ ⁺ CD4 ⁺ CD8 ⁻ Foxp3 ⁻ CD62L ^{high}
Activated CD4 ⁺ T cell	CD45.2 ⁺ TCRβ ⁺ CD4 ⁺ CD8 ⁻ Foxp3 ⁻ CD62L ^{low}
Innate lymphocyte group 1 (ILC1)	CD45.2 ⁺ Lineage ⁻ CD90.2 ⁺ NK1.1 ⁺ RORγ ⁻
Innate lymphocyte group 2 (ILC2)	CD45.2 ⁺ Lineage ⁻ CD90.2 ⁺ NK1.1 ⁻ RORγ ⁻
Innate lymphocyte group 3 (ILC3)	CD45.2 ⁺ Lineage ⁻ CD90.2 ⁺ NK1.1 ⁻ RORγ ⁺

Table 3.

Information on antibodies commonly used in AT immune cell profiling by flow cytometry.

Antibody	Clone	Vendor
CD3e	145-2C11	Tonbo
CD4	RM4-5	Biolegend
CD5	53-7.3	Biolegend
CD8a	53-6.7	Biolegend
CD11b	M1/70	Biolegend
CD11c	N418	Tonbo
CD19	1D3	Tonbo
CD25	PC61.5	Biolegend
CD45.2	104	Biolegend
CD62L	MEL-14	Biolegend
CD90.2	53-2.1	Thermo-Fisher
CD206	C068C2	Biolegend
B220	RA3-6B2	Biolegend
F4/80	BM8.1	Biolegend
FcεR1	MAR-1	Tonbo
Foxp3	FJK-16s	Thermo-Fisher
GATA3	TWAJ	Thermo-Fisher
LY6G(Gr-1)	RB6-8CS	Tonbo
NK1.1	PK136	Biolegend
RORγ	Q31-378	BD Biosciences
Siglec-F	E50-2440	BD Biosciences
TCRβ	H57-597	Biolegend
TCRγ/δ	GL3	Biolegend