Unravel the ontogeny and function of adipose tissue macrophage subpopulations in health and obesity

Chen, Qi

2019

Chen, Q. (2019). Unravel the ontogeny and function of adipose tissue macrophage subpopulations in health and obesity. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/136900>

<https://doi.org/10.32657/10356/136900>

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0).

Downloaded on 26 Apr 2023 20:24:48 SGT

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**Unravel the ontogeny and function of adipose tissue
macrophage subpopulations in health and obesity**

Qi Chen

SCHOOL OF BIOLOGICAL SCIENCES

2019

**Unravel the ontogeny and function of adipose tissue
macrophage subpopulations in health and obesity**

Qi Chen

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for
the degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

...2019-08-18..

....
CHEN QI

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it of sufficient grammatical clarity to be examined. To the best of my knowledge, the thesis is free of plagiarism and the research and writing are those of the candidate's except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

20.08.2019
Date

A handwritten signature in dark ink, appearing to read 'Christiane Ruedl', written in a cursive style.

Prof. Christiane Ruedl

Authorship Attribution Statement

Please select one of the following; *delete as appropriate:

*(A) This thesis **does not** contain any materials from papers published in peer-reviewed journals or from papers accepted at conferences in which I am listed as an author.

... 2019-08-18...
Date

... ...
CHEN QI

ACKNOWLEDGEMENTS

I would like thank my supervisor Prof. Christiane Ruedl for giving me the opportunity to study as a PhD student in her lab. During the four years' PhD study, she gave me a lot of instruction, motivation and support for my project, and she is always a good example for me to learn as a scientist. It is really a nice experience to work with her for the past four years.

Thanks to Prof. Klaus Karjalainen for his knowledge, precious discussion and contribution to the project.

Thanks to Dr Lai Si Min, for continuing doing the adipose tissue project when I am away for writing thesis and for being always available to discuss about the experiments and give advises.

Thanks to Dr Sheng Jianpeng, for his help and guidance during the first year of my PhD study, for his valuable discussion and suggestions.

Thanks to Mr Sajith, for his responsibility of daily tasks and maintaining all my mice when I am away for writing thesis.

Thanks to CR/KK lab members: Ms Monika Tetlak for the mice genotyping; Dr Leong Meiling, for discussions and technical help; Dr Irene Soncin, for her help for the colon cancer project during the first two years of my PhD study; Dr Ng See Liang, Dr Teo Yi Juan, Dr Piotr Tetlak, Ms Yolanda Gan, for their technical help and everyday conversations.

I would also like to thank our collaborator Dr Liu De Hua from SIGN, for his help in the UMAP analysis of adipose tissue myeloid cell populations.

Lastly but not least, I would like to thank my family and my friends for always being supportive during my PhD study, especially my husband Dr Xu Shaohai, thanks to him for his great help of analysing the RNAseq data and his support for both my work and life.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	i
TABLE OF CONTENTS	iii
LIST OF FIGURES	ix
LIST OF TABLES	xi
ABBREVIATIONS	xiii
Abstract.....	xv
1 Introduction.....	1
1.1 Adipose tissue	1
1.1.1 Adipose tissue types and distribution	1
1.1.2 Function of the adipose tissue.....	2
1.1.2.1 Energy homeostasis	2
1.1.2.2 Endocrine and metabolic function	4
1.2 Obesity	5
1.2.1 The progression of obesity.....	5
1.2.2 Microenvironment.....	6
1.2.3 Adipose tissue inflammation and macrophages recruitment during obesity	7
1.3 Tissue resident macrophages	8
1.3.1 Tissue-resident macrophages	8
1.3.2 Function of tissue-resident macrophages in disease conditions	11
1.3.3 Ontology of tissue-resident macrophages	11
1.4 Adipose tissue macrophages	13
1.4.1 ATM subsets and their function.....	13
1.4.2 Localization of ATM subsets.....	14
1.5 Clinical meaning of studying ATM subsets during obesity.....	15
1.6 Experimental mouse models to investigate ontogeny, maintenance, and function of ATMs in health and obesity	15
1.6.1 CCR2 ^{-/-} mice	15
1.6.2 Diphtheria toxin receptor mice and CD169-DTR mice.....	16
1.6.3 Inducible Kit ^{MerCreMe/R26} fate mapping mice	16

1.7 Project objectives	17
2 Materials and methods	19
2.1 Materials	19
2.1.1 Mouse strains	19
2.1.2 Antibodies for FACS	19
2.1.3 Antibodies for immunofluorescence staining	19
2.1.4 Materials and reagents	19
2.1.5 Primers	19
2.1.6 Equipment	19
2.1.7 Computer software	19
2.2 Methods	20
2.2.1 High fat diet induced obesity mouse model	20
2.2.2 Tamoxifen-inducible fate mapping mouse model	20
2.2.3 Turnover kinetics of ATM subsets in both lean and obese mice	20
2.2.4 Glucose Tolerance Test (GTT) measurement	20
2.2.5 Diphtheria toxin (DT) injection	21
2.2.6 Anti-CSF1R antibody injection	21
2.2.7 Isolation of leukocytes from adipose tissue	21
2.2.8 Adipose tissue and serum samples preparation for lipid metabolism	22
2.2.9 Lipid metabolites processing and analysis	22
2.2.10 ELISA for serum adipokines	23
2.2.11 FACS staining	23
2.2.12 Adipose tissue preparation for immunofluorescence staining	24
2.2.13 Immunofluorescence cell staining	24
2.2.14 BODIPY staining of ATM subsets	25
2.2.15 RNA isolation from adipose tissue and reverse transcription to cDNA	25
2.2.16 RNA isolation from FACS-sorted cells and reverse transcription to cDNA	25
2.2.17 Quantitative polymerase chain reaction (qPCR)	25
2.2.18 Uniform Manifold Approximation and Projection (UMAP)	26
2.2.19 Statistical analysis	26
3 Results:	27
3.1 Characterization of high fat diet (HFD) treated obese mice	27
3.1.1 Diet-induced obesity mouse model	27

3.1.2 Macrophage infiltration during obesity	28
3.1.3 Gating strategy and definition of distinct myeloid cell populations in eAT	29
3.1.4 Myeloid cell heterogeneity in obese eAT	30
3.1.5 Positive correlation between the number of ATM subsets and the eAT tissue	31
mass during the progression of obesity	31
3.1.6 Different morphology of ATM subsets.....	32
3.1.7 Fluorescent detection of lipid droplets in ATM subpopulations.	33
3.1.8 Phenotypic Kinetics of ATM subsets during the progression of obesity.	34
3.1.9. Different CCR2 dependency of ATM subpopulations	38
3.1.10 Distinct adipose tissue myeloid subset clusters are distinguished by high dimensional analysis	39
3.2 Ontogeny and turnover rate of ATM subpopulations in the fate mapping mouse	42
3.2.1 Embryonic and adult labelling	42
3.2.2 Ontogeny of ATM subsets in embryonic labelled mice	44
3.2.3 Slow turnover rate of ATM subsets in steady state mice.....	45
3.2.4 Obesity accelerates the turnover rate of ATM subsets	47
3.2.5 CCR2-deficient ATMs are BM-independent.....	49
3.2.6. Depleted ATMs are replaced by BM-derived macrophages within 3 days.	50
3.3 Transcriptomics analysis of distinct ATM subsets in lean and obese mice....	53
3.3.1 Distinct transcriptomics of ATM subsets	53
3.3.2 Specific transcriptomics of MHCII ^{low} ATMs	56
3.3.3 MHCII ⁺ CD11c ⁺ ATMs are specialized in lipid metabolism	58
3.4 Functional characterization of ATM subsets	59
3.4.1 Mouse models to study the in vivo function of different ATM subpopulations	59
3.4.2 Investigation of the in vivo function of CD169 ⁺ ATMs in health and obesity.....	60
3.4.2.1 Phenotype of adipose tissue lacking ATMs (CD169-DTR mouse)..	60
3.4.2.2 Myeloid cell profiling in adipose tissue lacking ATMs.....	62
3.4.2.3 Depletion of ATMs leads to the hypertrophy of adipocytes.....	63

3.4.2.4 Ablation of ATMs cause the dysfunction of eAT	64
3.4.2.5 Removing ATMs affects the lipid metabolism of the eAT.....	66
3.4.3 Investigation of the in vivo function of ATM subsets in anti-CSF1R antibody treated mice	67
3.4.3.1 Metabolic phenotypes of the anti-CSF1R antibody treated mice	67
3.4.2.2 Myeloid cell profiling in eAT lacking MHCII ^{low} and MHCII ⁺ CD11c ⁻ ATMs	68
3.4.2.3 Anti-CSF1R injection leads to smaller adipocytes	69
3.4.2.4 Microenvironment change of the anti-CSF1R antibody treated eAT	70
3.4.2.5 Lipid metabolism in eAT lacking MHCII ^{low} and MHCII ⁺ CD11c ⁻ ATMs	72
4 Discussion	74
4.1 HFD induced obesity in mouse.....	74
4.2 Distinct subpopulations of ATMs in lean and obese mice	75
4.3 Causes for augmented macrophage activation and recruitment during obesity	78
4.4 ATM ontogeny and turnover rate and growth factor/chemokine requirements	79
4.5 ATM subsets show distinct transcriptome and cell identity	82
4.6 Regulation of adipose tissue metabolism by distinct ATM subpopulations...	84
5 Conclusion	88
6 Appendix	89
6.1 Permission to reuse Table 1.1	89
6.2 Permission to reuse reuse Figure 1.3	90
6.3 Permission to reuse Figure 1.5.....	91
6.4 Permission to reuse Figure 1.6.....	92
6.5 Antibodies used for FACS	93
6.6 Antibody for immunofluorescence staining.....	94
6.7 Materials and reagents	95
6.8 Primer sequences for qPCR	98
6.9 Equipment.....	99
6.10 Sorting efficiency.....	100
6.11 CD169 expression on myeloid cell populations in adipose tissue.....	101

6.12 Pictures of eAT from obese WT and CD169-DTR mice.....	102
6.13 CD64 and CD206 expression on monocyte-derived cells in eAT	103
6.14 Trem2 expression in sorted eAT myeloid cells	104
7. Author's publications	105
8 Posters and invited oral presentations	106
9 Awards	107
10 Reference:	109

LIST OF FIGURES

Figure 1.1: Distribution of adipose tissue in a mouse.....	2
Figure 1.2: Lipid metabolism and mobilization in the adipose tissue	3
Figure 1.3: Microenvironment changes from lean to obese adipose tissue	6
Figure 1.4: Niche specific factors and transcription factors involved in the maintenance of tissue-resident macrophages.....	9
Figure 1.5: Most tissue-resident macrophages except microglia are derived from fetal hematopoietic stem cells	12
Figure 1.6: Diphtheria toxin/ Diphtheria toxin receptor (DT/DTR) system	16
Figure 3.1: Effects of HFD on weight gain, GTT and fat accumulation	27
Figure 3.2: Effects of obesity on the macrophage infiltration in eAT	28
Figure 3.3: Myeloid cell populations in lean eAT	30
Figure 3.4: Myeloid cell profiling in obese adipose tissue	31
Figure 3.5: The relationship between the absolute cell number of different ATM subsets and eAT mass	32
Figure 3.6: Different morphology of ATM subsets	32
Figure 3.7: Lipid content of ATM subsets	33
Figure 3.8: Phenotypic comparison of different ATM subsets between lean and obese eAT	35
Figure 3.9: CD163 expression on ATM subsets	37
Figure 3.10: Different CCR2 dependency of ATM subsets.....	39
Figure 3.11: High dimensional analysis shows distinct myeloid clusters in eAT	40
Figure 3.12: Kinetics of myeloid cell populations in eAT during the progression of Obesity	41
Figure 3.13: Embryonic and adult fate mapping.....	43
Figure 3.14: Fate mapping of ATM subsets in embryonic labelled mice.....	44
Figure 3.15: Slow turnover rate of ATM subsets in steady state mice	46
Figure 3.16: The expression levels of Tim-4 negatively correlate with the BM Labelling	47
Figure 3.17: Fast turnover rate of ATM subsets during the progression of obesity	48
Figure 3.18: CCR2 deficient ATMs are BM-independent.....	50
Figure 3.19: Replenishment kinetics of ATMs in the empty niche	51
Figure 3.20: ATM emptied niches are refilled efficiently by BM-derived cells	53

Figure 3.21: Distinct transcriptome of ATM subsets in lean and obese mice	54
Figure 3.22: Transcriptional landscape of myeloid cell subsets in lean and obese mice.....	55
Figure 3.23: MHCII ^{low} ATMs express genes involved in Archidonic acid metabolism pathway	57
Figure 3.24: MHCII ⁺ CD11c ⁺ ATMs are involved in the lipid metabolism.....	58
Figure 3.25: Selective depletion of distinct ATM subsets in various mouse models ..	60
Figure 3.26: Metabolic parameters of DT injected CD169-DTR mice	61
Figure 3.27: Skewed representation of myeloid cell subsets in CD169-DTR mice	62
Figure 3.28: Depletion of CD169 ⁺ ATMs leads to the hypertrophy of adipocytes.....	63
Figure 3.29: Quantification of key factors in the eAT microenvironment in the absence of ATMs	65
Figure 3.30: Perturbed lipid metabolisms of eAT in the absence of ATMs	66
Figure 3.31: Metabolic parameters of the Anti-CSF1R injected mice	68
Figure 3.32: CSF1R mediated depletion of ATMs	69
Figure 3.33: Anti-CSF1R antibody treatment leads to smaller adipocytes.....	70
Figure 3.34: Depletion of ATM subsets in anti-CSF1R antibody treated mice did not cause dramatic disorders of the tissue microenvironment	71
Figure 3.25: Lipid metabolism in eAT in absence of MHCII ^{low} and MHCII ⁺ CD11c ⁻ ATMs	73
Figure 6.1: Sorting efficiency of ATM subsets and P3 monocyte-derived cells in adipose tissue	100
Figure 6.2: CD169 is specifically expressed on adipose tissue-resident macrophage Subsets	101
Figure 6.3: Pictures of eAT from CD169-DTR mice	102
Figure 6.4: Gene expression of Trem-2 in different myeloid cell populations from lean and obese mice.....	103
Figure 6.5: CD64 and CD206 expression on monocyte-derived cells (P3).....	104

LIST OF TABLES

Table 1.1: The multiple function of adipokines	4
Table 3.1: Surface markers of ATM subsets in lean and obese mice eAT	38
Table 3.2: Parameters for UMAP analysis.....	40
Table 4.1: Some commonly used animal models of obesity.....	74

ABBREVIATIONS

-/-	Knock-out
AGM	Aortal-gonads-mesonephros
APC	Antigen presenting cell
ANGPYL 2	Angiopoietin-like protein 2
AQP 7	Aquaporin-7
ATGL	Adipose triglyceride lipase
ATM	Adipose tissue macrophage
BAT	Brown adipose tissue
BM	Bone marrow
CCR	C-C chemokine receptor
CCL	Chemokine ligand
CD	Cluster of differentiation
cDNA	Complementary DNA
CLS	Crown like structure
CSF-1	Colony stimulating factor 1
CX3CR1	CX3C chemokine receptor 1
DAMP	Danger-associated molecular patterns
DEGs	Differentially express genes
DGA	Diacylglycerol
DGAT	Diacylglycerol acytransferase
DNA	Deoxyribonucleic acid
Dox	Doxycycline
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
ESCs	Embryonic stem cells
ELISA	Enzyme-linked immuno-absorbent assay
FACS	Fluorescence activated cell sorting
FABP	Fatty acid-binding protein
FATP	Fatty acid transport protein
FL	Fetal liver
FSC	Forward scatter
FFA	Free fatty acid
GLUT 4	Glucose transporter type 4
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	Haematoxylin and eosin
HSL	Hormone-sensitive lipase
IL	Interleukin
ILC	Innate lymphoid cell
IACUC	Institutional Animal Care and Use Committee
IFN	Interferon
i.p.	Intraperitoneal
i.v.	Intravenous
LCs	Langerhans cells
LPL	Lipoprotein lipase
LPS	Lipopolysaccharides
Mφ	Macrophages
MAG	Monoacylglycerol
MARCO	Macrophage receptor with collagenous structure

MCP-1	Monocyte chemoattractant protein-1
M-CSF	Monocyte colony-stimulating factor
MDSC	Myeloid-derived suppressor cells
MERTK	Tyrosine kinase
MHC	Major histocompatibility complex
mL	Milliliter
MGL	Monoglyceride
MSC	Mesenchymal stem cell
MZ	Marginal zone
NAMPT	Nicotinamide phosphoribosyl transferase
NE	Norepinephrine
NLR	Nod-like receptor
OPG	Osteoprotegerin
PRRs	Pattern recognition receptors
PBS	Phosphate buffer saline
qPCR	Quantitative polymerase chain reaction
RBP4	Retinol-binding protein 4
RLR	RIG-like receptor
RNA	Ribonucleic acid
RT	Room temperature
SCFAs	Short chain fatty acids
SFRP	Secreted frizzled-related protein
SSC	Side scatter
sWAT	Subcutaneous white adipose tissue
TAM	Tamoxifen
TetR	Tet repressor DNA binding protein
TF	Transcriptional factor
TG	Triglyceride
TGF	Transforming growth factor
T _H	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRE	Tetracycline-responsive promoter element
T _{reg}	Regulatory T cell
tTA	Tetracycline-controlled trans-activator
UCP-1	Uncoupling protein 1
UMAP	Uniform Manifold Approximation and Projection
VAT	Visceral adipose tissue
VLDL	Very-low-density lipoprotein
vWAT	Visceral white adipose tissue
WAT	White adipose tissue
WT	Wild type
YFP	Yellow fluorescence protein
YS	Yolk sac
γ	Gamma
α	Alpha
β	Beta
μ	Micro

Abstract

Immune cell infiltration in visceral adipose tissue (VAT) during obesity is associated with local chronic inflammation and the development of metabolic syndromes, such as insulin resistance and type 2 diabetes. Among the infiltrating immune cells, adipose tissue macrophages (ATMs) are the most abundant leukocyte fractions, they are not only the key cells for maintenance of VAT homeostasis, but also the major players in controlling metabolic dysregulation during aging and obesity. It is well established that functional distinct ATM subsets exist in VAT, but their specific functional contribution during health and obesity still need to be clarified. In my project, three phenotypically distinct subpopulations of F4/80^{hi} ATMs (MHCII^{low}, MHCII⁺ CD11c⁻ and MHCII⁺CD11c⁺) showing different frequencies, morphology, transcriptomic profiles and lipid content during the progression of obesity are present in VAT. Each of the ATM subset displays characteristic half-lives and turnover rates to ensure their presence in the VAT during obesity-mediated tissue remodeling. The long-lived MHCII^{low} ATMs maintain their embryonic origin even during obesity, while the other two ATM subsets are rapidly replenished by bone marrow-derived infiltrating monocytes upon high-fat feeding. Furthermore, our functional studies show that MHCII⁺CD11c⁺ ATM subset, which is almost absent in normal lean tissue but is massively expanded in the VAT during obesity, not only contains adipocyte hypertrophy and dysfunction, but is also important for lipid buffering, suggesting that it is crucial in regulating adipose tissue function in obesity. Therefore, we provide additional evidence for beneficial functions of macrophages in obesity-induced adipose tissue remodeling.

1 Introduction

1.1 Adipose tissue

1.1.1 Adipose tissue types and distribution

Adipose tissue, also known as fat, is a loose connective tissue distributed all over the body. It contains adipocytes, preadipocytes, blood vessels, nerves, fibroblasts and different leukocytes, such as macrophages, dendritic cells, eosinophils, T cells and B cells. Two types of adipose tissue have been classified, white adipose tissue (WAT) and brown adipose tissue (BAT), according to their anatomical locations, functions, gene expression patterns and morphological structures [1]. White adipocyte contains mainly a single lipid droplet and 90 % of the adipocyte volume consists of triglycerides, which causes its ivory colour. Because of their good contractility, the size of white adipocytes depends mainly on the lipid stored in it, which displays a hypertrophic phenotype during obesity. On the contrary, brown adipocytes store only small amount of triglycerides, but contain a high density of mitochondria, which leads to the brown colour of BAT tissue [2, 3]. Recent studies showed that BAT develops before birth and WAT forms shortly after birth, but BAT and WAT fat cells derived from the same stem cells (Mesenchymal stem cells, MSCs) with different lineages [4, 5]. The MSCs have the potential to differentiate into both adipogenic or myogenic lineages, with adipogenic cells further differentiating into preadipocytes and then mature white adipocytes, while myogenic cells polarizing into mature brown adipocytes [6]. Even though, white adipocytes and brown adipocytes originate from different lineages, they have the same subsequent differentiation steps and share common transcriptional factors such as PPAR γ and C/EBPs, which enable them to transdifferentiate into each other under certain environmental conditions [6, 7].

The distribution of WAT is usually much more than the BAT, as the WAT constitutes roughly 20 % of the body weight [1]. There are two types WAT, subcutaneous WAT (sWAT) and visceral WAT (vWAT), sWAT includes intramuscular and inguinal WAT and vWAT involves mesenteric, retroperitoneal, omental and perigonadal WAT (also known as epididymal adipose tissue). Usually the sWAT is located under the skin to maintain the temperature of the body and the vWAT is distributed around organs to provide protective padding. Among the different types of vWAT, the paired perigonadal WAT is attached to the epididymis and testes in males and uterus and ovaries in females;

the mesenteric WAT is distributed around the intestine; the reproperitoneal WAT is attached to the dorsal wall of the abdomen; and the omental WAT is found around the stomach (Figure 1.1 upper part). There are also two types BAT in mice, interscapular and perirenal BAT. The typical interscapular BAT is located in the interscapular, perirenal, paravertebral and axillary sites. (Figure 1.1 lower part).

Figure 1.1: Distribution of adipose tissue in a mouse. Adipose tissue can be classified into WAT and BAT according to their anatomical locations, functions and morphologies. WAT: white adipose tissue BAT: brown adipose tissue. (Anna Park et al., 2014, *WJSC* and Kenji Ikeda et al., 2018, *Trends Endocrinol Metab*). No permission is required to reuse these figures.

1.1.2 Function of the adipose tissue

1.1.2.1 Energy homeostasis

The traditional function of adipose tissue is to store and release energy by lipogenesis and lipolysis, respectively. Both WAT and BAT are involved in the energy homeostasis, with WAT mainly specialized in energy storage and mobilization and BAT mainly involved in dissipating energy as heat when needed [8]. High circulating glucose level caused by excess of nutrition, stimulates the pancreas to release insulin, which can promote glucose uptake in adipocytes and then trigger the initiation of the glycolytic

and lipogenic pathways in adipocytes. Lipogenesis includes both fatty acid and triglyceride synthesis. The circulating carbohydrate (glucose) can provide metabolite substrates, such as acetyl-CoA, for the synthesis of fatty acids, which can be further esterified into glycerol and triglycerides to be stored in adipose tissue [9, 10].

During fasting, decreased insulin levels deactivate lipogenesis and at the same time activate lipolysis, leading to free fatty acids (FFAs) released through triglyceride breakdown in the adipose tissue. The released FFAs can be transferred to other organs to maintain the energy homeostasis and this lipolysis only take place in adipose tissue [11]. During the lipolysis process, triglyceride is sequentially hydrolyzed to form diacylglycerol (DGA) and monoacylglycerol (MAG), one molecule of fatty acid is released in each step, in the final step MAG is hydrolyzed into free FFA and glycerol [12]. Currently, there are three important lipolytic enzymes involved in the lipolysis pathway: the adipose triglyceride lipase (ATGL), which serves as the major lipase during lipolysis pathway; the hormone-sensitive lipase (HSL), working primarily as a diacylglycerol lipase and the nonoglyceride lipase (MGL). All these enzymes work stepwise and sequentially in regulating the degradation of triglycerides in the adipose tissue [13, 14], as shown in Figure 1.2.

Figure 1.2: Lipid metabolism and mobilization in the adipose tissue. During lipogenesis, carbohydrate is esterified with glycerol-3 phosphate and converted into free FFA and triglyceride which are stored as energy in adipocytes. During fasting stage, triglyceride is hydrolyzed into FFAs and glycerol, which can be released into the circulation and transferred to other organs to serve as energy. TG: triglyceride; HSL: hormone-sensitive lipase; GLUT4:

glucose transporter type 4; FABP: fatty acid-binding protein; AQP7: aquaporin-7; VLDL: very-low-density lipoprotein; FAT/CD36: fatty acid translocase; FATP: fatty acid transport protein. (Lina Badimon et al., 2015, *Rev Esp Cardiol*). Permission is not required to reuse this figure.

1.1.2.2 Endocrine and metabolic function

The adipose tissue is also known as a major metabolic and endocrine organ, plays an important role in physiologic and pathologic processes, including inflammation and immunity. Recent studies showed that white adipose tissue can secrete a variety of factors, such as adiponectin and leptin with important functions in lipid metabolism, as well as some inflammatory cytokines, including IL-6, TNF α , CCL2 and other factors which may play important roles in maintaining the tissue homeostasis or promoting the local inflammation [15, 16]. By releasing these factors, the adipose tissue communicates with other organs controlling appetite, regulating insulin sensitivity and modulating energy expenditure.

Adipokine	Primary source(s)	Binding partner or receptor	Function
Leptin	Adipocytes	Leptin receptor	Appetite control through the central nervous system
Resistin	Peripheral blood mononuclear cells (human), adipocytes (rodent)	Unknown	Promotes insulin resistance and inflammation through IL-6 and TNF secretion from macrophages
RBP4	Liver, adipocytes, macrophages	Retinol (vitamin A), transthyretin	Implicated in systemic insulin resistance
Lipocalin 2	Adipocytes, macrophages	Unknown	Promotes insulin resistance and inflammation through TNF secretion from adipocytes
ANGPTL2	Adipocytes, other cells	Unknown	Local and vascular inflammation
TNF	Stromal vascular fraction cells, adipocytes	TNF receptor	Inflammation, antagonism of insulin signalling
IL-6	Adipocytes, stromal vascular fraction cells, liver, muscle	IL-6 receptor	Changes with source and target tissue
IL-18	Stromal vascular fraction cells	IL-18 receptor, IL-18 binding protein	Broad-spectrum inflammation
CCL2	Adipocytes, stromal vascular fraction cells	CCR2	Monocyte recruitment
CXCL5	Stromal vascular fraction cells (macrophages)	CXCR2	Antagonism of insulin signalling through the JAK-STAT pathway
NAMPT	Adipocytes, macrophages, other cells	Unknown	Monocyte chemotactic activity
Adiponectin	Adipocytes	Adiponectin receptors 1 and 2, T-cadherin, calreticulin-CD91	Insulin sensitizer, anti-inflammatory
SFRP5	Adipocytes	WNT5a	Suppression of pro-inflammatory WNT signalling

Table 1.1: The multiple function of adipokines. RBP4: retinol-binding protein 4; ANGPTL2: angiopoietin-like protein 2; TNF: Tumor necrosis factor; IL: Interleukin; CCL: CC-chemokine ligand; CXCR: CXC-chemokine ligand; NAMPT: nicotinamide phosphoribosyltransferase; SFRP: secreted frizzled-related protein. (Noriyuki Ouchi et al., 2011, *Nature Reviews*). Permission to reuse this table is showing in Appendix 6.1.

Adipokines

The biological mediators secreted by adipocytes are called adipokines. Leptin as one of the first discovered adipokine, is a critical hormone involved in energy homeostasis and is mainly produced by adipocytes [17]. The discovery of leptin changed the general perspectives on WAT, giving an endocrine function to the adipose tissue. Its main function is to control appetite and its circulating levels correlates closely with the adipose tissue mass. The loss of leptin function or of its receptor can lead to massive obesity [18, 19]. Recent studies showed that leptin can acts as an adipose tissue derived messenger signalling to other tissues, including the brain. Through binding its receptors in liver and muscle, leptin promotes energy expenditure by stimulating the oxidation of FFAs [20-22].

Adiponectin, another important adipokine, is an anti-inflammatory molecule, which protects against the obesity-related metabolic syndrome and improves insulin activity by inhibiting the production of IL-6 and promoting the production of IL-10 [23]. Several important cytokines are also secreted by adipocytes, such as IL-6 and TNF α , both of their levels increase during the progression of obesity and are involved in the regulation of insulin resistance [24]. Previous investigations demonstrated that WAT contributes 30 % of the circulating IL-6, and within the adipose tissue, both adipocytes as well as macrophages are able to secrete IL-6. Increased IL-6 levels in obese subjects down regulate the enzyme lipoprotein lipase and the production of adiponectin as well as promote the release of more FFAs from the WAT influencing the local inflammation and insulin resistance [25]. Also TNF α can down regulate the production of adiponectin and induce the serine phosphorylation of the insulin receptor to inhibiting the insulin signaling pathway and, as a consequence, leading to insulin resistance [24-26]. A summary of the source, function and binding receptors of other adipokines secreted by adipocytes is shown in Table 1.1.

1.2 Obesity

1.2.1 The progression of obesity

Obesity is a medical condition when a person is overweight and has an high body mass index (>30). It is not just a simple cosmetic issue, since obesity can cause a series of metabolic syndromes, such as diabetes, cardiovascular diseases and liver disease. Nowadays, more and more people are becoming obese, making obesity a worldwide

concern and a crisis of human health. Energy imbalance is the main reason of obesity, by taking excessive nutrition but at the same time insufficient physical activities. The progression of obesity does not rely on the body weight, but depends on the accumulation of fat, which involves both the hypertrophy of pre-existing adipocytes and the hyperplasia of the newly differentiated adipocytes from the pre-adipocytes [27]. However, various adipose tissues are functionally different during the progression of obesity, with BAT and sWAT are performing the beneficial metabolism, while the vWAT, especially the visceral adipose tissue, is closely related with obesity-induced complications [28].

1.2.2 Microenvironment

The microenvironment of a lean healthy adipose tissue is rich in classical anti-inflammatory leukocytes (anti-inflammatory macrophages, type 2 immune cells (ILCs) and type 2 helper $CD4^+$ T cells) as well as a variety of anti-inflammatory cytokines, such as IL-10, along with IL-4, IL-13 and IL-33, together these leukocytes and cytokines maintain a healthy adipose tissue microenvironment and control the insulin sensitivity [29].

Figure 1.3: Microenvironment changes from lean to obese adipose tissue. During the progression of obesity, more immune cells infiltrate the adipose tissue, especially the pro-inflammatory macrophages, which can form crown-like structure around the necrotic adipocytes. The chemokines MCP-1, pro-inflammatory cytokines such as IL-6, TNF α and IL-1 β and circulating FFAs all increased concomitantly, which can negatively regulate the insulin sensitivity. There is also a disorder in adipokines secretion, with the levels of leptin increased and adiponectin decreased in the progression of obesity, which can further promote the insulin resistance. DAMP: damage-associated molecular patterns; CLS: crown like structure; CAM Φ : crown like structure associated macrophages; MDSC: myeloid-derived suppressor cells; ILC1:

innate lymphoid cells group 1; T_{reg}: Regulatory T cells; (Daniela F. Quail et al., 2019, *Nature reviews Endocrinology*). Permission to reuse this figure is showing in Appendix 6.2.

During the progression of obesity, the adipose tissue experiences a metabolic dysfunction. Adipocytes undergo cell death due to the hypertrophy and hyperplasia, which can trigger the innate immune responses, activate immune cells and support the switch from an anti-inflammatory immune response type to a pro-inflammatory type. This inflammatory immune response change supports the release of numerous cytokines and chemokines into the microenvironment, which will further perturb the adipose tissue homeostasis. With the expansion of the adipose tissue, the vascular and oxygen supply becomes limited, leading to additional adipocyte cell death and hypoxia area formation in the tissue. The death of the adipocytes causes the release of cellular contents, including damage-associated molecular patterns (DAMP), nucleic acids and lipids, which can act as signals for the recruitment of monocytes/macrophages and other granulocytes into the adipose tissue [30]. The newly infiltrated macrophages encircle the necrotic adipocytes forming crown like structure (CLS), which are ultimately responsible for the clearance of cellular debris. Recent studies indicated that the formation of CLS and infiltration of pro-inflammatory macrophages during obesity further promote the recruitment of pro-inflammatory immune cells (B cells, CD8⁺ T cells and T_H 1 lymphocytes) and the production of pro-inflammatory cytokines (IL-6, TNF α and IFN γ) [31]. The obese adipose tissue itself can also generate large amount of pro-inflammatory adipokines, such as leptin, resistin and retinol-binding protein 4 (RBP4) as well as other chemokines (CCL2, CXCL5), attracting additional monocytes into the adipose tissue [32]. Those pro-inflammatory factors will finally lead to the chronic low-grade systemic inflammation, which is a typical feature of obesity [33, 34]. The local inflammation in the obese adipose tissue reduces insulin sensitivity through activating the downstream signalling pathways of the pro-inflammatory responses, such as the JNK, ERK and P38 and inhibits the insulin receptor, which leads, as a consequence, to the insulin resistance and impaired lipid metabolism observed in obesity (Figure 1.3).

1.2.3 Adipose tissue inflammation and macrophage recruitment during obesity

There are few reasons for the observed low-grade inflammation and macrophage recruitment during obesity. Recent studies showed that during adipocyte necrosis,

DAMP, cellular debris and lipids are massively released, promoting local inflammation and recruitment of additional pro-inflammatory macrophages into adipose tissue. The newly recruited macrophages release specific chemokines and pro-inflammatory cytokines and further exacerbate this inflammatory responses [35]. Other studies indicated that hypoxia, generated during the expansion of adipose tissue, acts as a key factor responsible for fibrosis and the pro-inflammatory gene *Hif-1* expression in the tissue [36]. Also the dysfunctional lipid metabolism during obesity can lead to the elevated circulating FFA levels, which is able to trigger the local inflammation by indirect binding to Toll like receptor 4 (TLR4) and Toll like receptor 2 (TLR2), resulting in increased MCP-1 dependent pro-inflammatory macrophage infiltration [37]. Furthermore, the perturbed gut permeability during obesity and the increase of the intestinal bacterial derived LPS levels can also potentially contribute in initiating an inflammatory response through binding to the TLR4 expressed on adipocytes [38].

1.3 Tissue resident macrophages

1.3.1 Tissue-resident macrophages

Macrophages were first discovered as phagocytic cells by Elie Metchnikoff in the late 19th century [39], they are able to engulf cellular debris, microbes and any foreign substances. They patrol and sense for pathogens through various pathogen recognition receptors (PRRs) expressed on their surface, such as the mannose receptor (CD206), MARCO and TLRs. Some of these PRRs act as signaling pathway activators responsible for augmented cytokine and chemokine secretion and cellular activation of macrophages [40-44]. Nevertheless macrophage function is not only limited to the classical phagocytosis and immune surveillance, since these cells play important functions in tissue remodelling and wound healing [45].

The development of tissue-resident macrophages can be divided into two different steps: during the first step, macrophages are governed by the lineage-determining transcription factors to acquire common macrophages features; after the macrophages seed the organs, the signals from the tissue can further educate the macrophages to perform tissue-specific functions [46]. Furthermore, tissue-resident macrophages are extremely heterogeneous and distributed across almost all the tissues and are classified into distinct subsets according their anatomical location and functional differences [45]. Different tissue-resident macrophage subsets have specific properties and are rapidly shaped by

the local tissue microenvironment to fulfil tissue-specific functions [47]. For example, alveolar macrophages located in the pulmonary alveoli in the lung can promote the clearance the inhaled allergens; Kupffer cells located beside the sinusoidal vessels in the liver are involved in the removal of the pathogens and toxins from the venous blood system as well as the regulation of the cholesterol metabolism [45, 48]; colonic macrophages are pivotal in recognizing the foreign pathogens and releasing IL-10 to mute the possible inflammatory responses caused by gut microbiota and food allergens [49, 50]; Microglia in the brain is involved in neuronal proliferation, in the development and synapse formation and in the clearance of dead neurons or other cell debris from the central nervous system [51]; while adipose tissue macrophages are involved in clearance of dead adipocytes, regulation of adipocyte lipolysis and maintenance of tissue homeostasis [52].

Murine tissue-resident macrophages can be identified by their phenotypic cellular surface markers F4/80, CD64 and MerTK, which can also distinguish them from dendritic cells [53]. But the expression of surface markers between different tissue-resident macrophage subpopulations is similar, to better distinguish distinct tissue-resident macrophage subsets from different organs, recent studies identified additional niche specific transcriptional factors (TFs) or signals involved in the development and functional identity of distinct tissue-resident macrophage subpopulations [46, 54]. For example, in steady state spleen, red pulp macrophages continuously phagocytize senescent or damaged erythrocytes to recycle the iron and produce the metabolite heme, which can regulate the activation status of the TFs BACH1 and SPIC to control the functional maturation and survival of red pulp macrophages [55, 56]. Thus, in the case of spleen, heme and TF BACH1 and SPIC are the niche derived signals that shape the tissue-specific identity of red pulp macrophages. Taken together, the niche signals could be cytokines, cell-cell contacts, or metabolites that can initiate the tissue unique transcriptional signaling network of tissue-resident macrophages to acquire tissue-specific functions. A summary of the niche signals involved in the tissue-resident macrophage functional identity from different organs are shown in Figure 1.4.

Figure 1.4: Niche specific factors and transcription factors involved in the maintenance of tissue-resident macrophages. The development of macrophages can be separated into two different stages. The first stage is the core macrophage development program shared by all macrophages, including the regulation of the lineage-determining TFs, such as PU.1, MYB, MAZF, c-MAF and ZEB2. The second stage is the acquisition of tissue specific function, this step involve different tissue derived signals and the signal-dependent TFs, which can direct the development of unique functional identity of tissue-resident macrophages. TFs: transcriptional factors; MZ: marginal zone; IL-34: interleukin 34; RANKL: receptor activator of nuclear factor kappa-B ligand; GM-CSF: granulocyte-macrophage colony-stimulating factor; M-CSF: monocyte colony-stimulating factor; SCFAs: short chain fatty acids; OPG: osteoprotegerin;

TGF β : transforming growth factor β .(Wouter T. J et al., 2018, *Cellular Immunology*).
Permission is not required to reuse this figure.

1.3.2 Function of tissue-resident macrophages in disease conditions

Recent studies have shown that several functionally different macrophage subpopulations within the same tissue have been identified and distinct macrophage subsets have either pathogenic or protective function in disease conditions [57]. For example, during the tumor progression, there are several intratumoral macrophage subtypes, such as pro-inflammatory M1-like macrophages and anti-inflammatory M2-like macrophages. Pro-inflammatory M1-like macrophages within the tumor can promote the T_H1 response and initiate the antitumor responses, whereas anti-inflammatory M2-like macrophages display immunosuppressive properties and contribute to the tumor progression [58]. Moreover, multiple macrophage subsets exist in adipose tissue, but in the progression of obesity, anti-inflammatory M2 macrophages, which are important for the maintenance of adipocyte function and insulin sensitivity, can switch to pro-inflammatory M1 macrophages to promote local inflammation in obese adipose tissue and insulin resistance [59]. The heterogeneous function of tissue-resident macrophage subsets in different tissues is probably due to the specific niche where they reside in [60]. Taken together, these examples indicate that macrophage subsets can be both inflammatory and suppressive during disease progression and they can have opposite roles in health and disease.

1.3.3 Ontology of tissue-resident macrophages

For decades, it was believed that tissue-resident macrophages are replenished by bone marrow (BM) derived monocytes. However, recently by testing different fate mapping mouse models new insights have been gained in the ontogeny issue of tissue-resident macrophages [61-63]. Several studies showed that most tissue-resident macrophages are derived from embryonic origins (yolk sac or AGM/fetal liver) and seed the tissues before birth and can self-renew locally throughout the lifetime with minimum contribution of BM-derived progenitors. However, as shown in Figure 2.2, in some specific organs such as gut, skin and heart, macrophages are replaced after birth by circulating monocytes and the replenishment kinetics is tissue dependent [63, 64].

Currently the ontogeny of tissue-resident macrophages in mice adipose tissue is still controversial, some studies suggested that the newly recruited pro-inflammatory

macrophages generated during obesity are switched from the anti-inflammatory macrophages [65]. Some other studies showed that the newly infiltrated macrophages are from BM derived $CCR2^+$ monocytes or from both local proliferation and $CCR2$ dependent monocytes [66, 67] since $CCR2$ deficiency can attenuate the macrophage content in obese mice adipose tissue [66]. Furthermore, steady state adipose tissue-resident macrophages need little hematopoietic input and the origin of lean adipose tissue-resident macrophage subsets is still unclear [46, 67].

Figure 1.5: Most tissue-resident macrophages except microglia are derived from fetal hematopoietic stem cells. Most tissue-resident macrophages such as Langerhans cells, Kupffer cells, Alveolar macrophages, Red pulp macrophages, kidney macrophages and dermis MHCII^{low} macrophages originate from fetal progenitors and can self-maintain in situ through the lifetime. While other tissue-resident macrophages, such as dermis MHCII^{hi} macrophages, colonic macrophages and peritoneum macrophages, are also derived from fetal precursors, but they need high hematopoietic input during adulthood. Microglia is the only tissue-resident macrophages that are derived from yolk sac and can self-renew through the lifetime. Gr: group; MΦ: macrophages; hi: high expression; E: embryonic day; YS: yolk sac; AGM: aortal-gonads-

mesonephros; FL: fetal liver; BM: bone marrow. (Sheng J et al., 2015, *Immunity*). Permission to reuse the figure is showing in Appendix 6.3.

1.4 Adipose tissue macrophages

1.4.1 ATM subsets and their function

Macrophages are the most abundant CD45⁺ cells in adipose tissue. Since the discovery that more macrophages, especially the pro-inflammatory macrophages infiltrate adipose tissue during obesity [68], numerous studies had been carried out to study the function of ATMs during the progression of obesity. Lumeng CN., et al found that the newly recruited ATMs during obesity express high level of CD11c and these cells can form crown-like structure around dead adipocytes [65]. There are also several research groups used different knock out mice in the attempt to deplete ATMs during the progression of obesity. For example, CD11c-DTR mice were used to deplete the CD11c⁺ cells during obesity. The ablation of the CD11c⁺ macrophages in obese mice attenuated the obesity-related disorders such as insulin resistance and local inflammation [69]. But dendritic cells (DCs) also express high level of CD11c and were proven to play important functions during obesity as well [70, 71], therefore CD11-DTR mice is not an optimal mouse model to prove the solely contribution of CD11c⁺ ATMs in the generation of obesity-related complications. As CCR2 deficiency reduces the number of ATMs, CCR2^{-/-} and CCL2^{-/-} mice were used to investigate the function of ATMs during obesity. Both knockout mice have attenuated ATMs content and inflammation in adipose tissue and show improved insulin resistance [66, 72]. In contrast, results obtained from Inouye, K.E. et al. show that absence of CCL2, the CCR2 specific ligand, does not affect either the numbers of ATMs in the obese adipose tissue, nor it improves the insulin resistance [73]. These controversial studies may suggest that other factors are involved in the macrophage recruitment during obesity and that possible functionally distinct ATM subsets exist in the adipose tissue with different CCR2 dependency.

Applying the latest state-of-the art technologies (comprehensive flow cytometry, single-cell mRNA sequencing [scRNA-seq] and RNAseq), it is possible to distinguish and dissect multiple ATM subtypes showing different transcriptional and functional properties. CD9 and Ly6c was used to separate ATMs into Ly6c⁺, CD9⁺ and CD9⁻ subsets, the latest are very similar to the CD11c⁺ ATMs involved in lipid metabolism during obesity, while the Ly6c⁺ ATMs are clearly adipogenic [74]. Another study suggested a higher ATM complexity during obesity and dissected ATMs into four

functionally distinct subpopulations by using classical myeloid cell markers, such as CD64, CD206, MHCII, CD11c and Tim-4. Interestingly, the newly infiltrated CD206^{low/int}MHCII^{high}CD64⁺CD11c⁺ ATMs may play protective roles during the progression of obesity, which is contradiction with previous studies [75, 76]. Other studies focused on the non-inflammatory functions of ATM subsets during obesity established that HFD-induced obesity can activate the lysosome biogenesis but not the inflammatory polarization of ATMs and ATMs can buffer away the increased concentrations of lipids in a lysosomal-dependent manner, indicating that ATMs may play an important and beneficial role in lipid metabolism in adipose tissue [77]. What's more, Maril, K. et al. showed that the pro-inflammatory, metabolically activated macrophages (MMe), discovered by treating macrophages with metabolic syndrome mimic conditions, such as high glucose, insulin and palmitate, do not adopt the M1 or M2 like macrophage phenotype [78] and the MMe perform both detrimental and beneficial functions during the progression of obesity depends on the HFD duration [79]. Moreover, a recent study implied that Trem2 expressing macrophages in adipose tissue can take up lipid to actively involved in lipid metabolism and protect against the obesity-induced metabolic disorders, such as inflammation and adipocyte hypertrophy [80], highlighting the importance of Trem2 signaling pathway in the metabolic function of ATMs. Taken together, it is well established that distinct tissue-resident macrophage subsets are present in adipose tissue but their specific functional contribution has still to be clarified.

1.4.2 Localization of ATM subsets

The function of macrophages is tightly related to the location where they reside, hence the “niche” of tissue-resident macrophages is intensively investigated. It was demonstrated that the anti-inflammatory M2-like macrophages reside between interstitial spaces, while the newly recruited pro-inflammatory M1-like macrophages during obesity localize in the clusters surrounding the necrotic adipocytes [81, 82]. A recent study established that the tight association of ATMs to blood vessels [74], while another research group suggested the existence of two distinct localized ATM subsets, one ATM subset (Lyve^{hi}MHCII^{low}) attached to blood vessels and a second one (Lyve^{low}MHCII^{hi}) closely associated to the nerves [76]. In addition, Pirzgalska et al. visualized a norepinephrine-producing ATM cluster close to sympathetic neuros able to

regulate lipolysis and fat mass reduction [83]. Taken together, each distinct ATM subset has its special niche in the tissue where they can exert optimally their specific function.

1.5 Clinical meaning of studying ATM subsets during obesity

Recent studies in experimental mouse models indicated that infiltrating pro-inflammatory M1-like macrophages during obesity can release high amount of pro-inflammatory cytokines, such as IL-6, TNF α , MCP-1 and inducible nitric oxide synthase, which will contribute to the increased inflammation in obese adipose tissue and insulin resistance [77]. Or there are more evidences that ATMs can buffer away extra lipids and clean up the debris to maintain the homeostasis of the tissue [77, 78, 80].

Currently the function of ATM subpopulations in human adipose tissue is not well understood. Although augmented macrophage infiltration is observed in obese human adipose tissue [84], the newly recruited macrophages do not follow the M1: M2 polarization. As the infiltrated macrophages include both M1-like and M2-like macrophages at a similar ratio as in the lean human adipose tissue [85, 86], with the M2-like macrophages which are supposed to be anti-inflammatory supporting the adipose tissue inflammation [87]. These cumulative studies suggested that human ATM subsets are more complexed than expected and the current M1: M2 polarization “model” is over simplistic and does not sufficiently characterize the macrophage subsets present in human adipose tissue [88]. Unravelling the heterogeneity of ATMs and the mechanisms behind the different functions and localization of ATM subsets during the progression of obesity can help develop novel immune-directed therapeutic approaches for the treatment of obesity related syndromes [89].

1.6 Experimental mouse models to investigate ontogeny, maintenance and function of ATMs in health and obesity

1.6.1 CCR2^{-/-} mice

CCR2 is a chemokine receptor specific for the monocyte chemoattractant protein-1 (CCL2) and is highly expressed on the surface of circulating monocytes. Recent studies showed that the CCL2 level is increased in the obese adipose tissue, which can contribute the obesity-associated macrophage infiltration [90]. On the other hand, both CCR2 and CCL2 deficient mice exhibit decreased ATMs recruitment showing

improved insulin resistance and metabolic function [73, 91]. The CCR2-deficient mouse model used in my study, is a useful tool to investigate the CCR2-dependent monocyte derived ATM subsets in both health and obesity.

1.6.2 Diphtheria toxin receptor mice and CD169-DTR mice

Diphtheria toxin (DT)/Diphtheria toxin receptor (DTR) system is a simple and sensitive method to deplete specific cell populations in transgenic mice with the aim to dissect and elucidate the specific biological function of certain cell subsets in vivo [92]. To efficiently ablate tissue-resident macrophages in vivo, a CD169-DTR mouse was generated in our laboratory, in which a human DTR is under control of CD169 that was expressed in a transgenic mouse [93] (Figure 1.6).

Figure 1.6: Diphtheria toxin/ Diphtheria toxin receptor (DT/DTR) system. In this system, human diphtheria toxin receptor is under control of tissue specific enhancer/promoter in the transgenic mouse expression DT receptor on certain cell subsets. The expression of the diphtheria toxin receptor (DTR) on the cells itself is not harmful, but upon DT injection, DT bind to the DT receptor can lead to the cell death. The DT is very toxic, one molecular of DT is enough to kill a cell. Therefore, the DT/DTR system allows an efficient ablation of a targeted cell subset in vivo in the transgenic mouse. (Saito, M. et al., 2001, *Nat Biotechnol*). Permission to reuse this figure is showing in Appendix 6.4.

Since CD169 is expressed by F4/80^{high}CD11b⁺ adipose tissue-resident macrophages present in many organs including the adipose tissue, the DT injection can effectively ablate only this specific cell fraction. Therefore, CD169-DTR mouse is a useful animal model to study the in vivo function of ATMs during both health and obesity.

1.6.3 Inducible *Kit*^{MerCreMe/R26} fate mapping mice

In order to trace the origins of distinct myeloid cell populations in various organs at different time points of ontogeny, such as early embryonic, fetal liver or adult stages, an inducible *Kit*^{MerCreMer/R26} mouse strain was generated in our laboratory [63]. Since the *Kit* gene has shown to be active only on yolk sac (YS), fetal liver (FL) precursors and early hematopoietic stem cells (HSCs), but not the mature hematopoietic cells [94]. The *Kit*^{MerCreMer/R26} is an ideal fate mapping mouse - where YFP expression can be induced by tamoxifen injections at different developmental stages (embryonic versus adult)- to monitor the origins and turnover rates driven by the BM input in distinct VAT myeloid cell populations.

1.7 Project objectives

ATMs, one of the major leukocyte fractions in the VAT, are not only the key cells for maintenance of VAT homeostasis, but they could also play an important role in metabolic dysregulation during aging and obesity. At least three phenotypically distinct subpopulations of ATMs, with different turnover rates are present in the VAT, showing different frequencies during the progression of obesity, suggesting that the tissue microenvironment drives ATMs heterogeneity and vice versa ATMs contribute to VAT homeostasis and inflammation. Therefore, it would be important to define different ATM subpopulations and understand their potential roles in both health and disease.

The objectives of my project are:

1. to characterize of ATM subsets in lean and obese conditions.
2. to elucidate the ontogeny as well as the turnover kinetics of ATM subsets during both health and obesity.
3. to elucidate the in vivo metabolic functions of each ATM subset in steady state and during the progression of obesity by using different mouse models.

2 Materials and methods

2.1 Materials

2.1.1 Mouse strains

The C57BL/6 CD169-DTR⁺ transgenic mice were generated in our laboratory, as described previously [95], the CD169-DTR⁻ littermates were used as negative control mice. CCR2^{-/-} mice (B6.129S4-Ccr2tm1Ifc/J) were obtained from The Jackson Laboratory (USA). *Cx3cr1-gfp* mice were generated by Dr. Dan R. Littman (New York University Medical Center) and were kindly provided by Dr. Florent Ginhoux (Singapore Immunology Network, A*Star). *Kit*^{MerCreMer/R26} mice were generated in our laboratory as previously described [63]. All mice were bred and maintained in a specific pathogen-free animal facility of Nanyang Technological University. The mice studies were carried out in strict accordance with the recommendations of the NACLAR (National Advisory Committee for Laboratory Animal Research) and approved by the Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University.

2.1.2 Antibodies for FACS

Antibodies used for FACS are listed in Appendix 6.5.

2.1.3 Antibodies for immunofluorescence staining

Antibodies used for immunofluorescence staining are listed in Appendix 6.6.

2.1.4 Materials and reagents

Materials and reagents are listed in Appendix 6.7.

2.1.5 Primers

Primers used in the experiments are listed in Appendix 6.8.

2.1.6 Equipment

Equipment used in the experiments are listed in Appendix 6.9.

2.1.7 Computer software

Flow cytometry data was analysed by using the FlowJo Software (TreeStar, Ashland, OR, USA). Graphs and statistical analysis was performed by using the GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). The qPCR reactions were run by using the EcoTM Software V4.0.7.0 (Illumina, San Diego).

2.2 Methods

2.2.1 High fat diet induced obesity mouse model

High fat diet (HFD), 60 % fat, was used to induced obesity of the mice. 4-6 weeks old male mice were given HFD for 8, 12 and 16 weeks to induce different levels of obesity. The age matched control male mice were given Normal diet (ND) during the same time period. The mice body weight was monitored weekly.

2.2.2 Tamoxifen-inducible fate mapping mouse model

Kit^{MerCreMer/26} fate mapping mice were used for tracing the origin and turnover rate of ATM subsets. Tamoxifen was dissolved in corn oil, for adult labelling, 4 mg of tamoxifen was given to each 6-8 weeks old Kit^{MerCreMer/26} male adult mice through gavage for consecutive five days. For embryonic mice labelling, pregnant mice (E7.5 and E13.5, respectively) were given one dose of 2 mg tamoxifen together with 1 mg progesterone through gavage. After tamoxifen treatment, the constitutive yellow fluorescent protein (YFP⁺) labelling affects only c-kit⁺ expressing cells in particular progenitor cells from embryo or BM and, as a consequence, their progenies.

2.2.3 Turnover kinetics of ATM subsets in both lean and obese mice

6-8 weeks old male Kit^{MerCreMer/26} mice were first injected with tamoxifen. For the turnover rate analysis of ATM subsets at steady state and during obesity, four weeks after the last tamoxifen injection, Kit^{MerCreMer/26} mice were separated into two groups, one group was fed with HFD, the other group was fed with ND for 8 weeks, 12 weeks and 16 weeks, respectively.

2.2.4 Glucose Tolerance Test (GTT) measurement

Glucose tolerance test (GTT) was performed to monitor the development of diabetes in the HFD treated mice. After 6 hours fasting, the basal blood glucose levels were measured in ND as well as HFD treated mice. A challenge of 1.5 g/kg glucose (dissolved in water) was given intraperitoneally and the blood glucose level was checked at 15 minutes, 30 minutes, 60 minutes, 90 minutes and 120 minutes post glucose injection.

2.2.5 Diphtheria toxin (DT) injection

The DT stock was prepared in 20 % mouse serum in $1 \times$ PBS at a concentration of 2 ng/ μ l. CD169-DTR HFD or ND treated mice were injected intraperitoneally (i.p.) with 20 ng/g DT every 3-4 days to maintain the depletion of CD169⁺ tissue resident macrophages. Since long-term injection of DT can cause DT resistance in the mice, for the lipid metabolism analysis experiment, the DT treatment lasted for 12 days.

2.2.6 Anti-CSF1R antibody injection

The anti-CSF1R antibody was freshly diluted in $1 \times$ PBS at working concentration of 2 μ g/ μ l. HFD or ND treated mice were injected with anti-CSF1R antibody intraperitoneally. The anti-CSF1R antibody administration was twice per week at a concentration of 400 μ g per mouse. To avoid anti-CSF1R antibody resistance of the mice, for the lipid metabolism experiments, anti-CSF1R antibody treatment lasted for 2 weeks.

2.2.7 Isolation of leukocytes from adipose tissue

After the mice were sacrificed, epididymal adipose tissue (eAT) was removed and minced in 2 % FBS IMDM medium. The minced adipose tissue was transferred into a 50 ml falcon tube and incubated by shaking at 270 rpm with 5 ml IMDM medium containing Collagenase D (100 μ g/ml) and DNase I (20 U/ml) at 37 °C for one hour. Five ml cold 2 % FBS IMDM medium was added into the tube to stop the digestion and the digested samples were vortexed for 20 seconds and centrifuged at 500 g for 5 minutes. The supernatant was gently removed, the cell pellet was resuspended in 2 % FBS IMDM medium and filtered (100 μ m) into a 15 ml tube. The samples were centrifuged for another 5 minutes at 500 g, the supernatant was removed and the cell pellet was resuspended in 5 ml 0.89 % NH₄CL lysis buffer for 3 minutes at room

temperature to lyse some contaminating red blood cells. After addition of 5 ml 2 % FBS IMDM medium, the cells were centrifuged at 500 g for 5 minutes, the supernatant was discarded and the cell pellet was finally resuspended in 2 % FBS 1×PBS for future experiments.

2.2.8 Adipose tissue and serum samples preparation for lipid metabolism

After 12 hours fasting, blood was taken by retro orbital bleeding and put immediately on ice. For the serum preparation, the blood samples were first centrifuged at 1000 g for 10 minutes at 4 °C, then the supernatant was collected and centrifuged at 4 °C for another 10 minutes at 6000 g. After centrifugation, serum was transferred into a new tube and stored in -80 °C for future metabolic experiments. Adipose tissue was removed immediately upon mouse sacrifice, fresh adipose tissue was weighted, 25 mg of fresh adipose tissue was put into a 1.5 ml Eppendorf tube and snapped frozen in liquid nitrogen before stored in -80 °C for future metabolic experiments.

2.2.9 Lipid metabolites processing and analysis

Adipose tissue was obtained as described in Section 2.2.8 and thawed on ice just before analysis. 35 mg eAT was homogenized with 200 µl cold 50 % methanol and then vortexed with 200 µl chloroform followed by a centrifugation at 10,000 × g for 10 minutes at 4 °C. The aqueous and organic layers were separated and collected after the centrifugation, with the aqueous layer was re-suspended in 200 µl water: acetonitrile (5.95, V/V) and the organic layer was re-suspended in 300 µl mixture of Isopropanol: Acetonitrile: Chloroform (2:1:1, V/V/V). To quantify the metabolites in the aqueous layers, an internal standard was added at 10 % of the sample volume and the LC-MS/MS metabolite profiling was performed by using BEH HILIC column (1.7 µm; 2.1× 100 mm, Waters, Milford, MA) on ACQUITY UPLC coupled with a Xevo G2-XS QTOF/MS detector (Water Corp. Milford, MA, USA) according to the manufacturer's protocol. To quantify the metabolites in the organic layer, the internal standard was added at 20 % of the sample volume and the LC-MS/MS lipid profiling was performed by using BEH C8 column (1.7 µm; 2.1x100 mm, Waters, Milford, MA) on ACQUITY UPLC coupled with a Xevo G2-XS QTOF/MS detector (Waters Corp. Milford, MA, USA) followed by the manufacturer's instructions. The raw data was obtained by using

Masslynx 4.1 workstation UPLC-QTOF/MS Acquisition software (Waters Company, Milford, MA, USA) in centroid mode. To ensure the accuracy, the m/z values of all ions acquired in the QTOF/MS were real-time adjusted by LockSpray. Leucine-enkephalin was selected as lock mass compound for positive ion mode ($[M + H]^+ = 556.2771$) and negative ion mode ($[M - H]^- = 554.2615$).

To analyse the metabolomics, the QTOF/MS raw data were imported to Progenesis QI V2.3 software for automatic data pre-processing, which included retention time alignment, peak picking, deconvolution and identification of features. Retention time (RT)-mass/charge ratio (m/z) datasets were used to characterize the detected ions and were inputted to R V3.4.2 for data processing and statistical analysis. Data was normalized to the tissue weight. Human Metabolome Database, KEGG pathway and lipid maps were used to match the metabolites.

2.2.10 ELISA for serum adipokines

Serum was collected as described in Section 2.2.8 and quick-thawed just before starting the experiment. 1 % BAS diluted in $1 \times$ PBS was used as blocking buffer. Serum adiponectin was quantified by using Mouse Adiponectin Kit and ELISA was performed according to the manufacturer's instructions.

2.2.11 FACS staining

Single-cell suspensions were incubated with 10 mg/ml anti-Fc receptor antibody (2.4G2 clone) on ice for 20 minutes. The cells were washed with 2 % FBS $1 \times$ PBS, centrifuged at 500 g for 3 minutes and stained with corresponding extracellular antibodies diluted in 2 % FBS $1 \times$ PBS for 20 minutes at 4 °C, protected from light. After a short centrifugation at 500 g, the cell pellet was resuspended in 100 μ l 2 % FBS $1 \times$ PBS and filtered into the FACS tubes for FACS analysis or cells sorting. In case the intracellular staining was required, cells were fixed in the Foxp3 Fixation & Permeabilization (eBioscience) buffer for 20 minutes at room temperature protected from light. The cells were then washed with $1 \times$ Permeabilization buffer (eBioscience) and incubated with the intracellular antibodies (diluted in the $1 \times$ Permeabilization buffer) for another 20 minutes at room temperature in the dark. The stained cells were then washed with 2 %

FBS 1×PBS and resuspended in 100 µl 2 % FBS 1×PBS and analysed on 5-laser Fortessa.

2.2.12 Adipose tissue preparation for immunofluorescence staining

Murine adipose tissue was removed and stored in fresh 2 % PFA and 30 % sucrose solution overnight at 4 °C. Then the adipose tissue was washed 3 times in 30 % sucrose 1×PBS and transferred in 30 % sucrose 1×PBS overnight at 4 °C. The fixed adipose tissue was incubated in the sucrose-gelatin solution for 1-2 hours at 37 °C, transferred into the cassette and incubated at 4 °C for 10 to 20 minutes to solidify the sucrose-gelatin solution. To make sure the adipose tissue was submerged into the sucrose-gelatin solution, the cassette was taken out from fridge and filled full with warm sucrose-gelatin solution. Then the cassette was incubated at 4 °C for another 10-20 minutes to solidify the sucrose-gelatin solution. After solidification, the samples were stored in -80 °C overnight before cutting.

The frozen sucrose-gelatin samples were sectioned at -35 °C in a cryostat. The sample block was trimmed through 100 µm until the tissue was exposed. Then the sample was sectioned at a thickness of 14 µm and sections were slowly tapped onto the slides. The slides were air-dry for 5 minutes at RT before been stored at -20 °C for later IHC staining.

2.2.13 Immunofluorescence cell staining

The slides were air dry for 15 minutes at RT and then incubated in ice-cold 1:1 acetone:methanol for 10 minutes. After incubation, a hydrophobic circle was drawn around the sample by using a liquid blocker. Then the sections were incubated in 0.3 % 1×PBS-Triton-X-100 for 5 minutes for 3 times before the slides were incubated with 1 % BSA in 0.3 % 1×PBS-Triton-X-100 for 1 hour at room temperature to block non-specific antibody binding. After the blocking buffer was removed, the primary antibody (diluted in 1 % BSA in 0.3 % 1×PBS-Triton-X-100) was added and the sections were incubated at 4 °C overnight followed the next day by an additional 1 hour incubation with a species specific fluorochrome-conjugated secondary antibody at room temperature. As a last step, the sections were washed in 0.3 % 1×PBS-Triton-X-100 for 5 minutes for 3 times

and covered with a coverslip by using the DAKO mounting media contain DAPI. Then the slides were air-dry in room temperature for 15 to 20 minutes before fluorescent microscopy visualization.

2.2.14 BODIPY staining of ATM subsets

Different ATM subsets (MHCII^{low}, CD11c⁻MHCII⁺ and CD11c⁺MHCII⁺) were sorted from adipose tissue of the obese mice. The sorted ATM subpopulations were cytopspinned onto the slides and fixed with 4 % PFA. After washing the cells with 1 × PBS for 3 times, 5 µg/ml BODIPY was added for 30 minutes at 37 °C. The slides were washed in 1 × PBS for 3 times and mounted with DAKO and DAPI.

2.1.15 RNA isolation from adipose tissue and reverse transcription to cDNA

Fresh adipose tissue was removed from mouse and transferred into a TRIzol solution. The adipose tissue was subsequently homogenized by using a power benchtop homogenizer. For complete dissociation of the nucleoproteins, the homogenized samples were incubated at room temperature for at least 5 minutes before starting the RNA isolation. Total RNA of adipose tissue was extracted by using the RNAsimple Total RNA kit (DP419, TIANGEN Biotech) and following the manufacturer's protocol. Total 2 µg isolated RNA was used together with oligo dT, RNase free H₂O, dNTPs, RNase inhibitor, M-MLV Reverse Transcriptase and M-MLV RT reaction buffer to generate cDNA according to the manufacturer's protocol.

2.2.16 RNA isolation from FACS-sorted cells and reverse transcription to cDNA

Total RNA was isolated from FACS-sorted cells by using ArcturusTMPicoPureTM RNA Isolation Kit, then RNA was quantified by using Quant-iT™ RiboGreen® RNA Reagent and Kit, subsequently RNA was retro-transcribed and amplified by using Ovation® Pico WTA System V2 Kit. The RNA isolation, quantification and reverse transcription to cDNA were performed according to the manufacturer's instruction.

2.2.17 Quantitative polymerase chain reaction (qPCR)

Quantitative polymerase chain reaction (qPCR) was performed by using 20 ng cDNA together with 10 µl FAST 2 × qPCR Master mix, 1 µl forward and reverse primers

(10mM) and H₂O to reach a final volume of 20 µl per reaction. For the qPCR analysis, all reactions were run in triplicate with minimum 3 samples in each group. The threshold cycle (Ct) value of the tested genes were normalized to the Ct value of the housekeeping gene (*β-actin*) of each sample respectively to generate ΔCt . The value $2^{-\Delta\Delta Ct}$ was calculated to generate the gene expression fold change of the targeted between different groups.

2.2.18 Uniform Manifold Approximation and Projection (UMAP)

Single cell suspension was isolated from adipose tissue with ND or HFD for 8, 12 or 16 weeks. The cell suspension was stained with antibodies and acquired by flow cytometer. Samples from the same group were merged in one file and then the data was analysed by the UMAP plugin in the new Flowjo version.

2.2.19 Statistical analysis

Statistical significance between different groups were analysed by using the Student unpaired t-test (GraphPad Prism 6 software, two-tailed or Bonferroni two-way analysis of variance (ANOVA)). Significant differences were determined with p-value < 0.005, expressed as mean $\pm$ SEM, * <0.05, ** < 0.001, *** < 0.0001, **** < 0.00001.

3 Results:

3.1 Characterization of high fat diet (HFD) treated obese mice

3.1.1 Diet-induced obesity mouse model

Obesity is usually caused by excessive food intake and body fat accumulation. To investigate how high fat diet affect the systemic and tissue specific metabolism in mice, we treated age matched 4-6 weeks old male mice with a 60 kcal % high fat diet (HFD) or normal chow diet (ND) for 16 weeks (Figure 3.1A). By monitoring the mouse body weight during the 16 weeks' feeding, HFD treated mice increased more rapidly their body weight when compared to ND treated mice. At the end of the 16 weeks' food administration, the body weight of the HFD treated mice reached almost 50 gram (g), whereas the body weight of the ND treated mice were only around 30 g (Figure 3.1B).

Figure 3.1: Effects of HFD on weight gain, GTT and fat accumulation. (A) 4-6 weeks old male mice were treated with HFD or ND for the same time period of 16 weeks. (B) Body weight increase of HFD and ND treated mice was monitored weekly during the 16 weeks treatment (upper left, n=10 for each group). (C) glucose tolerance test (GTT) (n=6 for each group) was performed in both mouse groups at the end of the treatment. (D) Representative pictures of the epididymal adipose tissue (eAT) obtained from lean and obese mice. (E) weight of the collected eAT (n=10 for each group). ND: normal diet; HFD: high fat diet; GTT: glucose tolerance test; mg/dl: milligrams per deciliter; g: gram; WT: wild type. Statistical significance was determined by using unpaired student's t-test. * P< 0.05; ** P< 0.01; *** P< 0.001; **** P< 0.0001.

To determine the diabetes development in the HFD treated mice, glucose tolerance test (GTT) was examined. Consistent with previous studies [96], mice fed with 16 weeks HFD exhibited significant higher blood glucose levels than ND treated mice (Figure 3.1C), a clear sign for the development of a worsened glucose status. As expected, the epididymal adipose tissue (eAT) from HFD treated mice was much bigger than the ND

feed mice (Figure 3.1D), when the weight of eAT was measured at the end of the food exposure, the fat accumulation in the obese mice reached around 2.5 g and showed almost a 5-fold increase when compared to the correspondent adipose tissue of lean mice (0.5 g) (Figure 3.1E). These are typical metabolic phenotypes observed in commonly used HFD-induced obesity mouse model [96, 97].

3.1.2 Macrophage infiltration during obesity

Previous studies showed an increased number of infiltrating ATMs during obesity which form typical crown like structures (CLSs) around necrotic adipocytes [65, 68]. To confirm this phenotype in our HFD-diet induced obese mouse model, we examined the ATM infiltration by using two different methods, a haematoxylin and eosin (H&E) staining and an immunofluorescence cell staining using a macrophage specific anti-CD68 antibody in lean and obese eAT, respectively.

Figure 3.2: Effects of obesity on the macrophage infiltration in eAT. Upper part: Representative H&E staining pictures of lean (left) and obese (right) eAT. Lower part: Representative immunofluorescence staining of lean (left) and obese (right) eAT. Colour labelling: red: CD68; blue: DAPI. DAPI: 4',6-diamidino-2-pheylindole. Black arrows and white arrows indicate the crown like structures (CLS) in H&E staining and immunofluorescence staining pictures, respectively. The white bar corresponds to 100 μ m in length.

In the immunofluorescence staining, CD68 and DAPI were used to co-stain macrophages and nuclei, respectively. It is clear that only the sections of the eAT obtained from mice fed with HFD displayed obvious CLS structures (Figure 3.2, right side), as indicated by the black arrows in the H&E staining or the white arrows in the immunofluorescence staining (Figure 3.2), which indicates a higher macrophage content and higher density of necrotic adipocytes. On the other hand, lean mice showed minimal increase of these CLS structures. Notably, the hypertrophy of adipocytes was obvious in the obese mice, as the sizes of adipocytes from mice fed with HFD were much bigger than the ones from ND treated mice. In summary, after 16 weeks of HFD exposure, obese mice showed clear macrophage accumulation and adipocytes hypertrophy in the eAT.

3.1.3 Gating strategy and definition of distinct myeloid cell populations in eAT

To characterize the myeloid cell populations accurately in lean eAT, comprehensive flow cytometry and sequential gating strategy was performed. The forward scatter (FSC) and side scatter (SSC) gating was used to exclude debris and visualize the mononuclear cells based on the size and granularity of the cells. Pan-hematopoietic marker CD45 was included to identify leukocytes, while CD24 marker was utilized to distinguish macrophages from dendritic cells, as only DCs but not macrophages express high level of CD24 [98]. In steady state, at least three subpopulations of CD11c^{hi}MHCII^{hi} dendritic cells (DCs) were identified (CD103⁺CD11b⁻, CD103⁺CD11b⁺ and CD103⁻CD11b⁺), with CD103⁻CD11b⁺ DCs the most abundant DC subset; and at least three subpopulations of CD24⁻F4/80^{hi}CD11b⁺ (Fr I) tissue-resident macrophages (MHCII^{low}, MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺) present in eAT. Neutrophils (F4/80⁻CD11b⁺Ly6G⁺), monocytes (P1, Ly6C^{hi}MHCII⁻) and monocyte-derived cells (P2, Ly6C⁺MHCII⁺ and P3, Ly6c⁻MHCII⁺) cells were also clearly delineated (Figure 3.3A).

By comparing the percentage and absolute cell numbers of different myeloid cell populations in lean eAT, MHCII⁺CD11c⁻ ATMs are the most abundant cell populations, followed by the MHCII^{low} ATMs and the CD103⁻CD11b⁺ DCs. Considering all subsets (MHCII^{low}, MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺), ATMs accounted for more than 50 % of the leukocytes in lean eAT, indicating that they may play a crucial and important role for the homeostasis of the tissue (Figure 3.3B and Figure 3.3C).

Figure 3.3: Myeloid cell populations in lean eAT. (A) Representative plot figures show the gating strategy of myeloid cell populations in lean eAT. CD24⁺MHCII^{hi}CD11c^{hi} defined DCs, CD24⁺F4/80^{hi}CD11b⁺ cells were identified as ATMs (Fr I), F4/80^{int} cells (Fr II) were defined as a mixture of myeloid cells. (B) Bar charts show the percentage under CD45⁺ cells and (C) the absolute cell number of different myeloid cell populations in lean eAT (n=6), Error bars represent the $\pm$ S.E.M. value. Int: intermediate; hi: high; lo: low; +: positive; -: negative; no: number; Fr: fraction; SSC: side scatter; FSC: forward scatter; MHCII: major histocompatibility complex class II.

3.1.4 Myeloid cell heterogeneity in obese eAT

Similar myeloid cell subsets can be identified in obese mice eAT, although with different frequencies. The most dramatic change was the increase of MHCII⁺CD11c⁺ ATM subset, as its percentage raised from 2-3 % to almost 50 % of the total ATMs during the progression of obesity and became a dominant macrophage subset (Figure 3.4A). In terms of absolute cell number of different myeloid cell populations, not only the MHCII⁺CD11c⁺ ATMs but also all the other myeloid cell subsets especially the MHCII⁺CD11c⁻ ATMs remarkably increased their cell numbers during obesity, implying that there is an enhanced leukocyte infiltration into the adipose tissue during obesity. The total cell number of ATMs still accounted for more than half of the

leukocytes and were the most abundant cell population in obese eAT, suggesting that they could play a central role during the progression of obesity (Figure 3.4B).

Figure 3.4: Myeloid cell profiling in obese eAT. (A) Adipose tissue macrophage (ATM) subsets were gated according to the gating strategy showed in Figure 3.3. On the left, representative plot figures show ATM subsets and on the right pie chart shows the percentage of each ATM subset of the total ATMs (n=8). (B) Bar charts show the absolute cell numbers of total leukocytes and different myeloid cell populations in lean and obese mice eAT (n=8). Error bars represent the $\pm$ S.E.M. value. ND: normal diet; HFD: high fat diet; P1: monocytes; P2 and P3: monocyte-derived cells; Mφ: macrophage; no.: number; AT: adipose tissue. Statistical significance was determined by using unpaired student's t-test. *** P < 0.001; **** P < 0.0001.

3.1.5 Positive correlation between the number of ATM subsets and the eAT tissue mass during the progression of obesity

As our previous results showed that ATM subsets increased their cell numbers during obesity (Figure 3.2 and Figure 3.4), in order to investigate whether obesity favours certain ATM subsets, the relationship between the number of different ATM subsets and the eAT mass was performed. The spearman correlation result showed that the

absolute cell number of all ATM subsets correlated positively and significantly ($P < 0.0001$) with the fat mass, especially the $\text{MHCII}^+\text{CD11c}^+$ and $\text{MHCII}^+\text{CD11c}^-$ ATMs. On the other hand, correlation between the absolute cell number of $\text{MHCII}^{\text{low}}$ ATMs and eAT mass was not that robust as the other two ATM subsets, suggesting that obesity favours more the $\text{MHCII}^+\text{CD11c}^+$ and $\text{MHCII}^+\text{CD11c}^-$ ATMs.

Figure 3.5: The relationship between the absolute cell number of different ATM subsets and eAT mass. The absolute cell number of $\text{MHCII}^{\text{low}}$ (left, $r=0.7990$; $P < 0.0001$), $\text{MHCII}^+\text{CD11c}^-$ (middle, $r=0.7434$; $P < 0.0001$) and $\text{MHCII}^+\text{CD11c}^+$ (right, $r=0.7523$; $P < 0.0001$) ATMs positively correlated with the eAT ($n=29$). The relationship between the absolute number of ATM subsets and the eAT mass was analysed by Spearman's rank correlation coefficient and the significance was performed by using the one-way ANOVA analysis. g: gram.

3.1.6 Different morphology of ATM subsets

In order to visualize the morphology of different ATM subsets, three subpopulations of ATMs and one subset of monocyte-derived cells (P3) were purified via cell sorting from eAT.

Figure 3.6: Different Morphology of ATM subsets. (A) Representative Giemsa staining pictures of the sorted myeloid cell populations from eAT: $\text{MHCII}^{\text{low}}$, $\text{MHCII}^+\text{CD11c}^-$ and $\text{MHCII}^+\text{CD11c}^+$ ATMs and P3 (monocyte-derived cells). All the pictures were acquired on $100\times$ magnification. (B) Dot-plot graph shows the size of the cellular vacuole detected in the

tested myeloid cell populations. Statistical significance was determined by using unpaired student's t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. The black bar corresponds to 10 μm in length.

The Giemsa staining (Figure 3.6 A) showed a similar nuclear shape in all three ATM subsets, which was different from the nuclear shape of monocyte-derived cells (P3). Clear differences in vacuole size could be detected among the three ATM subsets, as the $\text{MHCII}^{\text{low}}$ and $\text{MHCII}^+\text{CD11c}^-$ ATMs contained large cytoplasmic vacuoles, especially the $\text{MHCII}^{\text{low}}$ ATMs, while the cytoplasm of $\text{MHCII}^+\text{CD11c}^+$ ATMs were full of smaller-sized vacuoles (Figure 3.6 B).

3.1.7 Fluorescent detection of lipid droplets in ATM subpopulations.

As ATMs reside in a microenvironment full of lipid and our result showed that distinct ATM subsets contain different sizes of vacuoles (Figure 3.6), we aimed to examine which ATM subpopulation contains lipid droplets.

Figure 3.7: Lipid content of ATM subsets. (A) Representative pictures of BODIPY staining of the sorted myeloid cell populations. From the left: MHCII^{low}, MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs and P3 (monocyte-derived cells). The BODIPY stained lipids (green) and the DAPI stained nucleus (blue) of the cells. All the pictures were acquired on 100× magnification. (B) Representative histogram shows the flow cytometry staining of BODIPY in ATM subsets and F4/80^{int} monocyte-derived cells (P3) in lean (left) and obese (right) eAT. (C) Bar chart shows the S.E.M. MFI of BODIPY in each myeloid cell populations (n=3 for each group). MFI: mean fluorescence intensity; ND: normal diet; HFD: high fat diet; P3: monocyte-derived cells; ATM: adipose tissue macrophage. Statistical significance was determined by using Bonferroni two-way analysis of variance (ANOVA) *** P< 0.001; **** P< 0.0001. The white bar corresponds to 10 μm in length.

BODIPY, which is a most commonly used fluorescent dye to measure neutral lipid content [99], was used to quantify the lipid content in each ATM subset. Interestingly, only the MHCII⁺CD11c⁺ ATMs showed many intracellular BODIPY-stained lipid droplets, while a BODIPY signal was hardly detectable in other ATM subsets and monocyte-derived cells (Figure 3.7 A).

Flow cytometry staining using BODIPY was performed to validate the high lipid content in MHCII⁺CD11c⁺ ATMs obtained from both lean and obese eAT. Again MHCII⁺CD11c⁺ ATMs exhibit the highest levels of BODIPY dye staining when compared to other myeloid cell populations in both lean and obese situations (Figure 3.7 B and C).

3.1.8 Phenotypic Kinetics of ATM subsets during the progression of obesity

Since the function of macrophages is closely related to the niches where they reside [47], we aimed to determine whether the change of eAT microenvironment from lean to obese situations will affect the phenotype and the numbers of ATM subsets. Phenotypic profiling of ATM subsets from lean and obese mice identified a specific expression signature for each ATM subpopulation. Lean MHCII^{low} and lean MHCII⁺CD11c⁻ ATMs expressed similar levels of macrophage specific markers when compared to the lean MHCII⁺CD11c⁺ ATMs. On the contrary, in obese mice, MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs were comparable and clearly different from MHCII^{low} ATMs. When lean and obese ATM phenotypic profiles were compared, only MHCII^{low} ATMs conserved their cellular identity, while the phenotypes of the other two ATM subsets were modified by the microenvironment during the progression of obesity. For example, Tim-4, which expressed on various macrophage subsets and a potential marker for long-lived embryonic-derived cells [100, 101], was highly expressed on MHCII^{low} ATMs but absent in MHCII⁺CD11c⁺ ATMs regardless of the

microenvironment change. This result indicates that $\text{MHCII}^{\text{low}}$ ATMs are probably long-lived cells maintaining their identity even during the development of obesity, whereas $\text{MHCII}^+\text{CD11c}^+$ ATMs do not have long-lived properties and are deriving directly from BM progenitors. Notably, in lean conditions, majority of $\text{MHCII}^+\text{CD11c}^-$ ATMs are Tim-4 positive with a smaller percentage of Tim-4 negative cells ($\text{Tim-4}^+ > \text{Tim-4}^-$). But during obesity, the ratio between Tim-4 positive and negative $\text{MHCII}^+\text{CD11c}^-$ ATMs shifts, with the Tim-4 negative cells the main fraction ($\text{Tim-4}^+ < \text{Tim-4}^-$). This result suggests that in lean conditions the embryonic-derived long lived $\text{Tim-4}^+\text{MHCII}^+\text{CD11c}^-$ ATMs are favoured and during the development of obesity they are replaced by BM derived $\text{MHCII}^+\text{CD11c}^-$ Tim-4⁻ macrophages (Figure 3.8 upper left).

Figure 3.8: Phenotypic comparison of different ATM subsets between lean and obese eAT. $\text{MHCII}^{\text{low}}$, $\text{MHCII}^+\text{CD11c}^-$ and $\text{MHCII}^+\text{CD11c}^+$ ATMs from lean and obese eAT were gated according to the gating strategy shown in Figure 3.3. All the three ATM subsets were tested for the expression of core macrophage markers, including Tim-4, CD206, CD301a/b, CCR2, CD9,

CX3CR1, Lyve-1 and VSIG4. Red, orange, purple, red violet, green and blue histograms show surface marker expression on lean MHCII^{low} ATMs, obese MHCII^{low} ATMs, lean MHCII⁺CD11c⁻ ATMs, obese MHCII⁺CD11c⁻ ATMs, lean MHCII⁺CD11c⁺ ATMs and obese MHCII⁺CD11c⁺ ATMs respectively. Negative controls are indicated in grey. Data are representative of at least 3 independent mice in each group.

Analogous to Tim-4, other markers showed a similar trend. Mannose receptor (CD206) and galactose/N-acetylgalactosamine (GalNAc) specific lectin (CD301a/b) were expressed on all MHCII^{low} ATMs independently whether obtained from lean or obese mice, whereas MHCII⁺CD11c⁻ ATMs express these molecules only in lean but not in obese conditions. (Figure 3.8 upper right). On the other hand, MHCII⁺CD11c⁺ ATMs are negative for both markers in lean as well as in obese situations.

Since recent studies used CD9 and Lyve-1 to separate macrophages in adipose tissue [74, 83], we aimed to investigate whether these markers can distinguish our ATM subsets. Interestingly, all three steady state ATM subsets did not express CD9, but the molecule was upregulated in MHCII⁺CD11c⁺ and some of the MHCII⁺CD11c⁻ ATMs during the development of obesity (Figure 3.8 middle). Of note, MHCII^{low} and MHCII⁺CD11c⁻ ATMs exhibited positive expression of Lyve-1, with this expression on MHCII⁺CD11c⁻ ATMs reduced in obese condition, but majority of the MHCII⁺CD11c⁺ ATMs were negative for Lyve-1 in both lean and obese conditions. Thus, both CD9 and Lyve-1 were not able to distinguish our ATM subsets.

On the contrary, two chemokine receptors CCR2 and CX3CR1 were absent on the MHCII^{low} ATMs but present on a fraction of both MHCII⁺CD11c⁺ and MHCII⁺CD11c⁻ ATMs in both lean and obese situations (Figure 3.8 left). What's more, a B7 family-related protein V-set and Ig domain-containing 4 (VSIG4), a well established negative regulator for T cell activation, was highly expressed on a subfraction of MHCII^{low} ATMs and was even further upregulated during obese conditions, whereas the other two ATMs were negative (Figure 3.8 lower right).

Moreover, the scavenger receptor CD163 was expressed on all three ATM subpopulations in lean condition, but during obesity the molecule was down regulated in MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs, but not in the MHCII^{low} ATMs, which still maintained high levels of CD163 on their cell surface (Figure 3.9 A left). Since CD163 can be cleaved by a metallopeptidase ADAM17 and released as a soluble molecule (Figure 3.9 A right) [102], we examined by qPCR the expression of this particular peptidase in different ATM subsets from lean and obese situations (Figure 3.9 B). Interestingly, the expression of ADAM 17 was only upregulated in MHCII⁺CD11c⁻

and MHCII⁺CD11c⁺ ATMs during obesity, which explains why the down regulation of CD163 was only restricted to these two ATM subpopulations.

Figure 3.9: CD163 expression on ATM subsets. (A) Flow cytometry histogram shows the CD163 expression on different ATM subpopulations from both lean and obese mice. ATM subsets were gated according the gating strategy showing in Figure 3.3. (B) qPCR analysis of AADAM17/TACE expression in ATM subsets from both lean and obese mice. Data was normalized to the expression levels of b-actin and shown as relative fold change to the corresponding expression level of the lean MHCII^{low} ATMs. Results are representative of at least 3 independent mice in each group. ADAM17: ADAM metallopeptidase domain 17, also named as TACE: tumour necrosis factor- α -converting enzyme.

Taken together, these results suggest that in lean conditions MHCII^{low} and MHCII⁺CD11c⁻ ATMs are phenotypically similar and the small fraction of MHCII⁺CD11c⁺ present in the lean adipose tissue is in terms of surface expression pattern a distinct subpopulation. On the other hand, during obesity development MHCII^{low} ATMs still maintain their original phenotype, whereas MHCII⁺CD11c⁻ ATMs down regulate some markers and become very similar to the newly recruited MHCII⁺CD11c⁺ ATMs.

The modified surface markers on ATM subsets especially the MHCII⁺CD11c⁻ ATMs during the progression of obesity were summarized in Table 3.1.

Cell subset	Lean situation	Obese situation	Markers modified
MHCII^{low}	Tim4 ⁺ CD206 ⁺ CD301 ⁺ CCR2 ⁺ CD9 ⁺ CD163 ^{hi} CX3CR1 ⁺ Lyve-1 ⁺ VSIG4 ⁺	Tim4 ⁺ CD206 ⁺ CD301 ⁺ CCR2 ⁺ CD9 ⁺ CD163 ^{hi} CX3CR1 ⁺ Lyve-1 ⁺ VSIG4 ⁺	No
MHCII⁺CD11c⁻	Tim4 ⁺ CD206 ⁺ CD301 ⁺ CCR2 ⁺ CD9 ⁺ CD163 ^{hi} CX3CR1 ⁺ Lyve-1 ⁺ VSIG4 ⁻	Tim4 ⁺ CD206 ^{+/-} CD301 ^{+/-} CCR2 ⁺ CD9 ^{+/-} CD163 ⁻ CX3CR1 ^{+/-} Lyve-1 ⁺ VSIG4 ⁻	Tim4↓ CD206↓ CD9↑ CD163↓ CX3CR1↑
MHCII⁺CD11c⁺	Tim4 ⁺ CD206 ⁺ CD301 ⁺ CCR2 ⁺ CD9 ⁺ CD163 ^{hi} CX3CR1 ^{+/-} Lyve-1 ^{+/-} VSIG4 ⁻	Tim4 ⁺ CD206 ⁺ CD301 ⁺ CCR2 ⁺ CD9 ⁺ CD163 ⁻ CX3CR1 ^{+/-} Lyve-1 ^{+/-} VSIG4 ⁻	CD9↑ CD163↓

Table 3.1: Surface markers of ATM subsets in lean and obese mice eAT. Blue colour labelling represents the modified cellular surface markers during the progression of obesity. ↑: upregulation; ↓: downregulation; No: no change; +/-: positive and negative cells co-exist in the same ATM subset.

3.1.9. Different CCR2 dependency of ATM subpopulations

Given that ATM subsets exhibited different expression levels of CCR2 (Figure 3.8) and circulating CCR2⁺Ly6C^{hi} monocytes are important for the replenishment of macrophage pools in various organs [103], we aimed to determine the CCR2-dependency of different ATM subsets in lean and obese eAT.

WT or CCR2^{-/-} mice were given HFD for 16 weeks to induce obesity and age-matched control WT or CCR2^{-/-} mice were maintained in ND for the same period (Figure 3.10 A). The eAT myeloid cell populations from both groups were analysed (Figure 3.10 B and C). As expected, monocytes and monocyte-derived cells (P1, P2 and P3) were severely impaired in the absence of CCR2 in both lean and obese situations. In case of ATMs, both cell numbers of MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs were reduced in CCR2^{-/-} mice. In lean mice, MHCII⁺CD11c⁻ ATMs were significantly reduced by 2-fold and the tiny detectable fraction of MHCII⁺CD11c⁺ ATMs was reduced by 1.5-fold, although not significantly. The reduction was even more pronounced in obese eAT, where both fractions diminished significantly their cell numbers by 10 -fold. In contrast, the absolute cell numbers of MHCII^{low} ATMs from both lean and obese mice were not affected. This result indicates that the MHCII⁺ ATM subsets depend highly on CCR2 especially during obesity, while the MHCII^{low} ATMs are CCR2-independent, which is consistent with our CCR2 staining result (Figure 3.8).

Figure 3.10: Different CCR2 dependency of ATM subsets. (A) 4-6 weeks old WT or CCR2^{-/-} male mice were given ND or HFD for 16 weeks. Myeloid cell populations from lean (B) and obese (C) WT (upper) and CCR2^{-/-} (lower) mice eAT (n=8 for each group) were analysed, representative dot plot figures (left) and bar charts of the absolute cell numbers of different myeloid cell populations (right) are displayed. Error bars represent the S.E.M. value. Fr I: F4/80^{hi}CD11b⁺ tissue-resident macrophages; Fr II: F4/80^{int} cells; ND: normal diet; HFD: high fat diet; P1: monocytes; P2 and P3: monocyte-derived cells. Statistical significance was determined by using unpaired student's t-test. * P< 0.05; ** P< 0.01; *** P< 0.001; **** P< 0.0001; ns: no significance.

3.1.10 Distinct adipose tissue myeloid subset clusters are distinguished by high dimensional analysis

In order to present a high-resolution and unbiased data-driven overview of myeloid cell populations in eAT, the Uniform Manifold Approximation and Projection (UMAP), which is a novel, fast and reliable non-linear dimensionality reduction technique [104], was performed. The multi-parameters analysed in this UMAP analysis are shown in Table 3.2.

FSC	CD11c
SSC	MHCII
DAPI	Ly6C
CD45	Ly6G
Lin (B220, CD90.2, NK1.1)	CD103
CD24	Tim-4
F4/80	Lyve-1
CD11b	

Table 3.2: Parameters for UMAP analysis. FSC: forward scatter; SSC: side scatter; Lin: lineage; DAPI: 4', 6-diamidino-2-phenylindole.

The dataset enriched for DAPI^{neg}CD45^{pos}Lin(B220, CD90.2, NK1.1)^{neg} myeloid cells obtained from eAT treated with HFD for 12 weeks was analysed by flow cytometry to quantify and determine the diversity and evolution of the myeloid cell populations.

Uniform Manifold Approximation and Projection- UMAP analysis

12 weeks HFD

Figure 3.11: High dimensional analysis shows distinct myeloid clusters in eAT. Left: UMAP projection of myeloid cell subsets dataset from eAT treated with HFD for 12 weeks (n=3). Events are colour-labelled according to the manual gating. Right: The evolution relationship of the tested myeloid cell populations from eAT is displayed. (A collaboration with Dr Liu Dehua from SIGN).

Based on our phenotypic staining shown in Figure 3.8, total 12 distinct clusters were generated from the UMAP projections of the tested samples. The cluster delineation revealed four different ATM subsets (MHCII^{low}Tim-4⁺, MHCII⁺Tim-4⁺CD11c⁻, MHCII⁺Tim-4⁻CD11c⁻ and MHCII⁺Tim-4⁻CD11c⁺), three subpopulations of DCs (CD103⁺CD11b⁻, CD103⁺CD11b⁺ and CD103⁻CD11b⁺) and five monocyte-related cell populations (CD24⁺Ly6C^{lo}, CD24⁻Ly6C^{lo} and CD24⁻Ly6C^{hi} monocytes, Ly6C^{hi}MHCII⁺

and Ly6C⁺MHCII⁺ monocyte-derived cells) (Figure 3.11 left). The multi-branched continuous trajectory of myeloid cell populations exhibited that a large group of clusters, including monocytes, monocyte-derived cells, DCs and ATMs are connected to the CD24⁺Ly6C^{lo} monocytes, indicating an evolution relationship between them (Figure 3.11 right). However, when considering the ATM subsets, the Tim-4⁺ ATMs especially the Tim-4⁺MHCII^{low} as well as Tim-4⁺MHCII^{hi}CD11c⁻ ATMs were the most distant cell fractions from the CD24⁺Ly6C^{lo} monocytes and other myeloid cell populations, followed by the Tim-4⁺MHCII^{hi}CD11c⁻ and Tim-4⁺MHCII^{hi}CD11c⁺ ATMs (Figure 3.11 right), implying that most likely the Tim-4⁺ ATM subpopulations do not have a differentiation connection with monocytes and they have a different origin or they can self-renewal locally.

3.12: Kinetics of myeloid cell populations in eAT during the progression of obesity. 4 weeks' old age-matched male mice were given ND (left) or HFD (right) for 8, 12, and 16 weeks. Myeloid cell populations in eAT from different time points were analysed. Nine myeloid cell subsets were displayed in the UMAP figures: P1: Ly6C^{hi} monocytes; P2 and P3: F4/80^{int} monocyte-derived cells; neutrophils; CD24⁺ monocytes; four ATM subsets (Tim-4⁺MHCII^{low}, Tim-4⁺MHCII⁺CD11c⁻, Tim-4⁺MHCII⁺CD11c⁻ and Tim-4⁺MHCII⁺CD11c⁺), n=3 for each time

point and each group. The UMAP analysis was done in collaboration with Dr Liu Dehua from SIGN.

In order to determine the unbiased data-driven overview kinetics of myeloid cell populations during the progressing of obesity, 4 weeks' old male C57BL/6 mice were given ND or HFD for different time period (8, 12 and 16 weeks) and myeloid cell populations from eAT were performed for the UMAP analysis. In lean mice, it is clear that Tim-4⁺MHCII^{low} (purple) and Tim-4⁺MHCII⁺CD11c⁻ (teal) ATMs are the main ATM subsets, with the Tim-4⁺MHCII⁺CD11c⁺ ATMs (dark red) almost not visible. However, there was a clear infiltration of Tim-4⁺MHCII⁺CD11c⁻ ATMs (light blue) in eAT from 8 weeks onwards (Figure 3.12 left).

In the HFD 8 weeks' obese mice, Tim-4⁺MHCII^{low} (purple) and Tim-4⁺MHCII⁺CD11c⁻ (teal) ATMs are still the major ATM subsets, but there was a clear cluster of Tim-4⁺MHCII⁺CD11c⁺ ATMs (dark red) when compared to the lean mice. This Tim-4⁺MHCII⁺CD11c⁺ ATM subset increased gradually during the progression of obesity (from 8 weeks to 12 and 16 weeks) and became a dominant ATM subpopulation (Figure 3.12 right), while the Tim-4⁺MHCII⁺CD11c⁻ ATMs (teal) displayed opposite trend, indicating that during obesity Tim-4⁺ ATMs are favoured, which is consistent with our Tim-4 staining result (Figure 3.8 right).

3.2 Ontogeny and turnover rate of ATM subpopulations in the fate mapping mouse

3.2.1 Embryonic and adult labelling

It has been shown in different fate mapping mouse models that some macrophages in adult tissues are established already during the fetal development and that these resident macrophages then maintain themselves in situ for the rest of animal's life without any input from adult bone marrow (BM). Because of this prenatal ontogeny of many tissue-resident macrophages, we aimed to re-address the question of the origin and turnover kinetics of distinct ATM subpopulations in lean and obese mice. In order to investigate this aspect, a novel tamoxifen-inducible *Kit*^{MerCreMer/R26} mouse strain was recently generated in our laboratory to fate map distinct cell types [63]. As the *Kit* gene is expressed on Yolk sac (YS) and Fetal liver (FL) precursors as well as the early hematopoietic stem cells (HSCs) in fetal and adult mouse, but not on the mature hematopoietic cells [94], upon the tamoxifen injection, the Kit⁺ progenitors will turn on

the yellow fluorescent protein (YFP) expression and maintain the YFP expression on their descendants in the periphery.

In this fate mapping mouse model one can ask if adult macrophages are derived from adult or from fetal precursors by simply altering the timing of tamoxifen pulses (embryonic versus adult). To determine whether ATMs are derived from adult BM or from embryonic haematopoiesis, we have exploit different experimental fate mapping protocols, schematically illustrated in Fig. 3.13. Since male mice are more affected by obesity and diabetes, we have used only this mouse gender in our diet-induced obesity studies.

Figure 3.13: Embryonic and adult fate mapping. Left panel: Single dose of tamoxifen to be given at E7.5 or E13.5 during gestation to label progenitors from primitive and definitive haematopoiesis. The E7.5 progenitors (primitive haematopoiesis) can give rise to microglia, while precursors from E13.5 (definitive haematopoiesis) in FL can differentiate into fetal monocytes, which can further give rise to different myeloid cells in other organs. Right panel: Adult *Kit^{MerCreMer/R26}* mice were given consecutive tamoxifen to induce the YFP expression of the BM-precursors and their descendants. Fetal-derived self-renewing myeloid cells will not be labelled. YS: yolk sac; FL: fetal liver; E: embryonic day; BM: bone marrow; AGM: aorta-gonad-mesonephros; HSC: hematopoietic stem cell; YFP: yellow fluorescence protein.

To label the embryonic progenitors, a single dose of tamoxifen was given at E7.5 or E13.5 to the pregnant mice to label potential precursor cells generated from the primitive or definitive haematopoiesis stage (Figure 3.13 left). To fate map the adult BM derived cells, five consecutive doses of tamoxifen were given to the adult *Kit^{MerCreMer/R26}* male mice, followed by a chase period of at least 30 days to induce the YFP labelling of the descendants. Of note, only the cells derived from BM precursors can be labelled as YFP⁺ cells, those cells that originated from embryonic progenitors maintaining themselves locally will not be tagged in the adult labelling system (Figure 3.13 right). For

normalization purposes of the adult labelling experiment we selected neutrophils as internal reference because of their BM derivation and fast turnover (6-8 hours).

As the adult labelling system can label the BM derived cells, it can also be used to investigate the turnover rate of different myeloid cell populations during a chase time period (Figure 3.15) as well as to determine the BM-dependency of a distinct myeloid cell subsets during obesity (Figure 3.17) and to monitor the replenishment efficiency of an empty macrophage niche by BM derived cells (Figure 3.20).

3.2.2 Ontogeny of ATM subsets in embryonic labelled mice

Given that the origins of steady state ATMs have not been extensively investigated [46, 48], we aimed to examine whether steady state ATM subsets are from primitive or definitive progenitors.

Figure 3.14: Fate mapping of ATM subsets in embryonic labelled mice. ATM subsets were gated according to the gating strategy (Figure 3.3). Left side: Representative plot figures show labelling efficiency of microglia and myeloid cell populations from (A) E7.5 (B) E13.5 eAT and the bar chart (right) represents the mean $\pm$ SEM percentage of YFP⁺ cells of each myeloid cell subsets (n= 5 to 10 for each time point). E: embryonic day; YFP: yellow fluorescent protein; P1: monocytes; P2 and P3: monocyte-derived cells; ATM: adipose tissue macrophage.

Two different embryonic time points E7.5 and E13.5 were chosen to label the primitive (E7.5) and definitive (E13.5) hematopoiesis, respectively. Pregnant mice were injected with TAM respectively, their progeny was sacrificed at the age of 12 weeks and microglia as well as myeloid cell populations from eAT were analysed. As expected, in the case of E7.5 labelling, microglia were highly labelled (> 40 %), which is consistent with the previous studies [63]. In contrast, myeloid cell populations from eAT, including all three ATM subsets, were minimally labelled (< 5 %) (Figure 3.14 A), suggesting that myeloid cell populations from eAT are not derived from YS precursors. When tamoxifen was given at E13.5 of gestation, the microglia was labelled poorly, while myeloid cell subsets in eAT, including ATM subpopulations, displayed similar high YFP labelling (Figure 3.14 B). Collectively, these results suggest that steady state myeloid cell populations, including ATM subsets, from eAT are the progenies of Kit⁺ definitive and not of primitive precursors.

3.2.3 Slow turnover rate of ATM subsets in steady state mice

Subsequently we aimed to define the turnover rates of ATM subsets in steady state adult mice. Adult *Kit*^{MerCreMer/R26} mice were given tamoxifen to induce the YFP expression on Kit⁺ BM progenitors and maintained on ND over a period of 8 months' (Figure 3.15 A). At different time points (1, 2, 4 and 8 months), the labelling index of ATM subsets (MHCII^{low}, MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs), P1 (monocytes), P2 and P3 (monocyte-derived cells), eosinophils and neutrophils were analysed.

Interestingly, all the tested myeloid cell subsets from eAT except ATM subsets were maximally labelled with YFP and reached a labelling plateau around 2 months after the tamoxifen treatment, indicating that these myeloid cell subsets are replenished rapidly by BM-precursors during steady state. Notably, the YFP labelling of the ATM subsets (MHCII^{low} and MHCII⁺CD11c⁻ ATMs) increased slowly over the 8 months time period reaching around 40 % at the last measured time point, especially the MHCII^{low} ATMs, whose maximal labelling reached only 30 %. In contrast, the MHCII⁺CD11c⁺ ATMs always displayed higher labelling (> 50 %) during the chased time period when compared to the other two ATM subsets, but this labelling did not increase much along the time, suggesting high turnover rate of this ATM subpopulation. Taken together, steady state ATM subsets but not the MHCII⁺CD11c⁺ ATMs had a slow turnover rate and they need minimal contribution of monocytes for their replenishment, especially the MHCII^{low} ATMs (Figure 3.15 C).

Figure 3.15: Slow turnover rate of ATM subsets in steady state mice. (A) Schematic representation of the fate mapping of myeloid cell populations in lean eAT. Eight weeks old *Kit^{MerCreMer/R26}* mice were given one dose of tamoxifen per day for consecutive five days and each group of 5-8 mice were sacrificed 1, 2, 4, 8 months after the last tamoxifen injection. (B) Representative plot figures show the labelling efficiency of myeloid cell subsets in eAT at each time point, myeloid cell subsets were gated according to the gating strategy shown in Figure 3.3. (C) Bar chart shows the mean $\pm$ SEM percentage of YFP⁺ cells in each myeloid cell subset after been normalized to the percentage of YFP⁺ neutrophils. ND: normal diet; P1: monocytes; P2 and P3: monocyte-derived cells; YFP: yellow fluorescent protein; BM: bone marrow.

Since Tim-4 expression splits the MHCII⁺CD11c⁻ ATMs into two fractions (Figure 3.16 C), we addressed the question whether these two subpopulations differ in their turnover kinetics. Anti-Tim-4 antibody was included in the antibody mix and distinct ATM

fractions were gated and analysed for the YFP labelling at 2 months post TAM treatment (Figure 3.16 A). As Tim-4 delineates long-lived embryonic derived macrophages, it is not a surprise that both Tim-4 positive ATM fractions ($\text{Tim-4}^+\text{MHCII}^{\text{low}}$ and $\text{Tim-4}^+\text{MHCII}^+\text{CD11c}^-$) show the lowest levels of FYP labelling (10 % and 15 % respectively), whereas the Tim-4 negative ATM subsets ($\text{Tim-4}^-\text{MHCII}^+\text{CD11c}^-$ and $\text{Tim-4}^-\text{MHCII}^+\text{CD11c}^+$) were higher labelled with almost 40 % for $\text{Tim-4}^-\text{MHCII}^+\text{CD11c}^-$ ATMs and 50 % $\text{Tim-4}^-\text{MHCII}^+\text{CD11c}^+$ ATMs, respectively (Figure 3.16 B). This set of data reconfirms that Tim-4 expressing cells are embryonic derived, showing a slow turnover due to the minimal replacement by BM derived cells. On the other hand, Tim-4 negative ATMs are from BM origin showing a higher turnover kinetics when compared to their Tim-4 positive counterparts.

Figure 3.16: The expression levels of Tim-4 negatively correlate with the BM labelling. (A): Adult *Kit^{MerCreMer}/R26* mice were given five consecutive doses of tamoxifen, 8 weeks after the last tamoxifen injection, the mice were sacrificed and the myeloid cell populations from eAT were analysed (n=3). (B) Bar chart shows the mean $\pm$ SEM percentage of YFP⁺ cells in each myeloid cell subset after be normalized to the percentage of YFP⁺ neutrophils. (C) Representative plot figure show the gating of $\text{Tim-4}^+\text{MHCII}^{\text{low}}$, $\text{Tim-4}^+\text{MHCII}^{\text{hi}}$ and $\text{Tim-4}^-\text{MHCII}^{\text{hi}}$ ATMs under $\text{F4/80}^{\text{hi}}\text{CD11c}^-$ ATMs. ATMs were pre-gated according to the gating strategy shown in Figure 3.3. YFP: yellow fluorescent protein; TAM: tamoxifen.

3.2.4 Obesity accelerates the turnover rate of ATM subsets

After successfully characterized the turnover rate of ATM subsets in lean eAT, we aimed to examine whether the local inflammation generated during the progression of obesity can affect the turnover kinetics of ATM subsets.

To trace the turnover kinetics of myeloid cell populations from eAT during the progression of obesity, adult *Kit^{MerCreMer}/R26* mice were given tamoxifen to induced YFP expression of BM-progenitors first, 4 weeks after the last tamoxifen injection, the mice

were given HFD for 8, 12 and 16 weeks to induce different levels of inflammation, while the age-matched control mice were maintained under ND for the same time period (Figure 3.17 A). At different time point (8, 12 and 16 weeks) the adipose tissue was harvested and cells were analysed for YFP labelling. Clearly obesity accelerates the turnover kinetics of all the three ATM subsets, as the labelling of ATM subsets in ND treated mice cannot reach the same labelling levels of the HFD treated mice. Notably, the MHCII⁺CD11c⁺ ATMs were fully labelled even after 12 weeks HFD treatment, indicating that they turnover very fast and they are derived directly from BM progenitors. The MHCII⁺CD11c⁻ ATMs had moderate turnover rate but they were not fully labelled during the progression of obesity. However, the MHCII^{low} ATMs were minimally replaced by BM-derived monocytes even under obese conditions, suggesting that the MHCII^{low} ATMs are distinct from the other two ATM subsets, they probably maintain themselves locally even during inflammation (Figure 3.17 C).

Figure 3.17: Fast turnover rate of ATM subsets during the progression of obesity. (A) Schematic representation of the fate mapping of eAT myeloid cell populations during the

progression of obesity. Eight-weeks' old *Kit^{MerCreMer/R26}* mice were given one dose of tamoxifen per day for consecutive five days, then the mice were given ND or HFD for 8, 12, 16 weeks (n=4 to 8 per group). (B) Representative plot figures show the labelling efficiency of myeloid cell subsets in eAT of the 12 weeks' time point. (C) Graphical representation of the normalized labelling efficiency of ATM subsets from ND and HFD treated mice. ND: normal diet; HFD: high fat diet; YFP: yellow fluorescence protein; TAM: tamoxifen; wk: week.

3.2.5 CCR2-deficient ATMs are BM-independent

As our previous results showed that distinct ATM subsets exhibit different expression levels of CCR2 (Figure 3.8) and that the cell numbers of several myeloid subpopulation, such as monocytes and monocyte-derived macrophages as well as MHCII⁺CD11c⁻ ATMs are reduced in lean adipose tissue of CCR2-deficient mice (Figure 3.10) indicating that CCL2 is one of the main chemokines involved in the replenishment of monocyte-derived macrophages. To investigate the origins of the still detectable macrophages and monocytes in the adipose tissue of CCR2-deficient mice, we performed fate mapping analysis of ATM subsets in *Kit^{MerCreMer/R26}* mice lacking the expression of CCR2. Therefore, CCR2^{-/-} mice were back crossed to *Kit^{MerCreMer/R26}* mice to generate a CCR2^{-/-}*Kit^{MerCreMer/R26}* mouse line. Adult CCR2^{-/-}*Kit^{MerCreMer/R26}* and WT *Kit^{MerCreMer/R26}* mice were given tamoxifen to label the BM progenitors and sacrificed 7 weeks later for YFP expression analysis (Figure 3.18 A).

All myeloid cell subsets including two ATM subsets except the minor MHCII⁺CD11c⁺ ATM fraction were analysed. Similar to the WT *Kit^{MerCreMer/R26}* mice, neutrophils and eosinophils from CCR2^{-/-}*Kit^{MerCreMer/R26}* mice were 100 % labelled, indicating that their replenishment was not affected by the impairment of CCR2-CCL2 axis. In the CCR2-deficient mouse, within the ATMs, both MHCII^{low} and MHCII⁺CD11c⁻ ATMs did show almost no YFP labelling hence they were minimally derived from BM precursors probably maintaining their numbers by self-renewal. Notably, the labelling efficiency of CCR2-deficient monocytes (P1) was the same as the WT counterpart, which indicates that they are 100 % BM-derived. In contrast, monocyte-derived cells (P2 and P3) reduced their YFP labelling significantly by approximately 30 % in the absence of CCR2 when compared to WT counterparts. This result indicates that half of the P2 and P3 cells still derive from the BM using other CCR2-independent recruitments axis (Figure 3.18 B and C), whereas the other half probably maintains their numbers by self-renewal.

Figure 3.18: CCR2-deficient ATMs are BM-independent (A) Schematic representation of the fate mapping of myeloid cell populations in CCR2^{-/-}Kit^{MerCreMer/R26} eAT. Eight-weeks old CCR2^{-/-}Kit^{MerCreMer/R26} and Kit^{MerCreMer/R26} mice were given one dose of tamoxifen per day for consecutive five days and more than 10 mice for each group were sacrificed 7 weeks after the last tamoxifen injection. (B) Representative plot figures show the labelling efficiency of myeloid cell subsets in eAT of Kit^{MerCreMer/R26} (upper) and CCR2^{-/-}Kit^{MerCreMer/R26} (lower) mice. (C) Bar chart shows the mean $\pm$ SEM percentage of YFP⁺ cells in each myeloid cell population after normalization, white bar represents Kit^{MerCreMer/R26} mice, blue bar represents CCR2^{-/-}Kit^{MerCreMer/R26} mice. P1: monocytes; P2 and P3: monocyte-derived cells; YFP: yellow fluorescent protein; BM: bone marrow. Statistical significance was determined by using unpaired student's t-test. ** P< 0.01; **** P< 0.0001; ns: no significance.

3.2.6. Depleted ATMs are replaced by BM-derived macrophages within 3 days.

Subsequently, we investigated the refilling kinetics of a completely emptied ATM “niche” as well as the origins of the newly refilled macrophages. To do this, we exploited a CD169-DTR mouse, a well-established mouse model which efficiently depletes tissue-resident macrophages in different organs inclusive the adipose tissue [93].

Figure 3.19: Replenishment kinetics of ATM subsets in the empty niche. CD169-DTR mouse, a well-established mouse model which can efficiently deplete tissue-resident macrophages in different organs inclusive the adipose tissue upon DT administration, were injected with two doses of DT and the ATM subsets were analyzed at different time points. (A) Representative plot figures show the repopulated ATM subsets (MHCII^{low}, MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺) after empty the niche (at least 3 mice were sacrificed in

each time point. ATM subsets were gated according to the gating strategy shown in Figure 3.3. (B) The kinetics of the repopulated MHCII^{low} ATMs, MHCII⁺CD11c⁻ ATMs, MHCII⁺CD11c⁺ ATMs and the mobilization of monocytes (P3) were analysed. Fold change of each myeloid cell population was normalized to its steady state counterpart respectively. Fr I: tissue-resident macrophage; Fr II: F4/80^{int} cells; Fr III: F4/80⁻CD11b⁺ cells; Mφ: macrophage; Day 0: steady state mice.

Two consecutive doses of DT was injected to the CD169-DTR mice to ablate ATM subpopulations, the repopulated ATM subpopulations were chased 1, 2, 3, 4, 7, 8, 14 and 60 days after the last DT injection. It is clear that the emptied niche was fast refilled with new ATMs within 3 days after the depletion (Figure 3.19 A and B). Interestingly, the replenishment started with the MHCII^{low} ATMs probably due to their Ly6C⁺MHCII⁻ monocyte origin, with the percentage of MHCII^{low} ATMs increased strikingly within 2 days after the depletion and reduced gradually to normal level during the refilling time period (Figure 3.19 B). Similar replenishment trend was observed in the MHCII⁺CD11c⁺ ATMs but with a more pronounced refilling kinetics, which could be due to their few cell numbers present in lean eAT (Figure 3.3 and Figure 3.19 B). In contrast, the MHCII⁺CD11c⁻ ATMs displayed opposite trend and increased their percentage gradually along the replenishment (Figure 3.19 B), suggesting that the newly repopulated MHCII⁺CD11c⁻ ATMs may come from the newly recruited MHCII^{low} ATMs. Notably, all the newly refilled ATMs exhibited clear BM dependency, as the percentage of monocytes dramatically increased (> 4000 %) within 1 day after the ablation and reduced their percentage gradually until normal levels during the ATM refilling period (Figure 3.19 B lower). Taken together, the emptied ATM niche can be fast refilled within 3 days after the ablation and the newly recruited ATMs (MHCII^{low}, MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATM subsets) are derived from BM-derived monocytes, but it takes more than 2 weeks for the ATM subsets to return to normal basal levels.

To examine whether the newly repopulated macrophages in the empty niche were from bone marrow monocytes, the original CD169-DTR mouse line was back crossed to *Kit*^{MerCreMer/R26} mice to generate CD169-DTR *Kit*^{MerCreMer/R26} mice. Tamoxifen was given to induce YFP labelling in the CD169-DTR *Kit*^{MerCreMer/R26} mice and the control *Kit*^{MerCreMer/R26} mice. Six weeks after the last tamoxifen injection, the mice were injected with 2 consecutive doses of DT (Figure 3.20 A) and analysed 7 days post the last DT injection. The analysis showed that the newly replenished ATM subsets (MHCII^{low},

MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs are fully labelled with YFP, suggesting that they are derived from BM-monocytes (Figure 3.20 B and C).

Figure 3.20: ATM emptied niches are refilled efficiently by BM-derived cells. (A) Eight-weeks old CD169-DTR *Kit*^{MerCreMer/R26} and *Kit*^{MerCreMer/R26} mice were treated with 5 doses of tamoxifen first, 6 weeks later, 2 consecutive doses of 20 ng/g DT were injected to the mice to deplete ATMs (n=6 for each group). The mice were sacrificed 7 days after the last DT injection. (B) Representative plot shows the labelling efficiency of myeloid cell subsets in WT (left) and CD169-DTR (right) eAT. (C) Bar charts represent the mean $\pm$ SEM of the normalized labelling efficiency of different myeloid cells populations from DT treated CD169-DTR *Kit*^{MerCreMer/R26} and control *Kit*^{MerCreMer/R26} mice. White and red bars represent control and CD169-DTR *Kit*^{MerCreMer/R26} mice respectively. P1: monocytes; P2 and P3: monocyte-derived cells; YFP: yellow fluorescent protein; i.p.: intraperitoneal injection; DT: diphtheria; DTR: diphtheria toxin receptor; BM: bone marrow. Statistical significance was determined by using unpaired student's t-test. **** P < 0.0001.

3.3 Transcriptomics analysis of distinct ATM subsets in lean and obese mice

3.3.1 Distinct transcriptomics of ATM subsets

In order to better understand the functional differences of the ATM subsets, three myeloid cell subsets (monocyte-derived cells [P3], MHCII^{low} and MHCII⁺CD11c⁻

ATMs) and four myeloid cell populations (monocyte-derived cells (P3), MHCII^{low}, MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs) were sorted from lean and obese eAT, respectively. Due to the limited number of MHCII⁺CD11c⁺ ATMs in lean mice, this ATM subset was not analysed for RNA sequencing. Total RNA was extracted from the sorted cells and sent for transcriptomic analysis (Genomax Technologies). The sorting efficiency of each myeloid cell population was shown in Appendix 6.10.

Figure 3.21: Distinct transcriptome of ATM subsets in lean and obese mice. (A) 2D-PCA of genes with variable expression among ATM subsets and P3 monocyte-derived cells from lean and obese mice (n=3 for each subset). (B) Heat map of mean log₂ fold change in expression levels of genes and hierarchical clustering of all the analysed ATM subsets and P3 monocyte-derived cells from lean and obese mice. Labelling colours: green: normal diet; red: high fat diet. (C) Number of transcripts differentially regulated when comparing two different myeloid cell types (≥ 2 -fold change and $P < 0.01$, t test). ND: normal diet; HFD: high fat diet; P3: monocyte-derived cells.

The two-dimensional principal component analysis (2D-PCA) and the heat map analysis of the different expressed genes (DEGs) of the tested samples revealed a striking segregation of all the sorted myeloid cell subsets (Figure 3.21 A and B) and it is clear that all ATM subsets are clustered closely and separated away from monocyte-derived cells. Interestingly, based on the 2D-PCA result ATM subsets and monocyte-derived cells from lean mice were clustered separately from their obese counterparts, indicating that the obese microenvironment can shape the transcriptomics of the adipose tissue

myeloid cells. Furthermore, in the heat map analysis lean $\text{MHCII}^{\text{low}}$, $\text{MHCII}^{\text{+}}\text{CD11c}^{-}$ and obese $\text{MHCII}^{\text{low}}$ ATMs showed remarkable similarity, but were distant from the other two obese $\text{MHCII}^{\text{+}}\text{CD11c}^{-}$ and $\text{MHCII}^{\text{+}}\text{CD11c}^{+}$ subsets, suggesting that $\text{MHCII}^{\text{low}}$ ATMs can preserve their transcriptomics during the progression of obesity, which is consistent with the phenotypic profile result (Figure 3.8). When comparing the number of DEGs between different myeloid cell populations in both lean and obese mice, nearly 2000 genes were differentially expressed (> 2.5 -fold) between different ATM subsets and monocyte-derived cells (P3), while less than 1000 genes attained the 2.5-fold threshold among the three ATM subsets, especially between the two lean $\text{MHCII}^{\text{low}}$ and $\text{MHCII}^{\text{hi}}\text{CD11c}^{-}$ ATM subsets. Numbers of significantly different expressed genes in pairwise comparison between all four myeloid cell subsets in lean and obese eAT are presented in Figure 3.21 C.

Figure 3.22: Transcriptional landscape of myeloid cell subsets in lean and obese mice. (A) Scatter plots show the DEGs among the three myeloid cell populations. $\text{MHCII}^{\text{low}}$ (black) and $\text{MHCII}^{\text{+}}\text{CD11c}^{-}$ (green) ATMs and P3 monocyte-derived cells (red) in lean mice. Blue represents the genes expressed in ATMs. (B) Fold-change plot comparing gene expression in the sorted four myeloid cell subsets from obese mice. Coloured circles represent transcripts expressed more than 2 folds change in one subset versus another. $\text{MHCII}^{\text{low}}$ ATMs: orange; $\text{MHCII}^{\text{+}}\text{CD11c}^{-}$ ATMs: blue; $\text{MHCII}^{\text{+}}\text{CD11c}^{+}$ ATMs: pink; P3 (monocyte-derived cells): yellow.

When comparing the DEGs among the three myeloid cell subsets from lean mice, the genes specialized for ATMs and monocyte-derived cells can be easily distinguished. For example, *Cbr2*, *Cd209g*, *Lyve-1* and *Cd209f* (blue colour) were the specific genes for ATMs, while the *Klr1l*, *Havcr1*, *IL-12b* and *Ccl22* (red colour) were highly expressed on monocyte-derived cells (P3) (Figure 3.22 A). However, when the DEGs were compared between the two lean ATM subsets, few genes with 2-fold change were displayed, such as *Alox15*, *saa3* and *Prg4* (black colour) were expressed specifically on

MHCII^{low} ATMs; *H2Eb1*, *H2-Ab1*, *Ehf* and *Ctnnd2* (green colour) were expressed highly on MHCII⁺CD11c⁻ ATMs, indicating that even though the two lean ATM subsets are distinct cell populations, they share similar transcriptomic profile, which is consistent to the transcriptional analysis results shown in Figure 3.21.

In addition to the overall different transcriptomics of the myeloid cell populations, 2-by-2-fold comparisons of the four tested myeloid cell populations from obese eAT identified a limited cohort of genes for each myeloid cell subset, likely reflecting their distinct cell identity (Figure 3.18 B). Notably, *Alox15*, *Mctp2*, *Fgf9* and *Cd209b* genes were specifically expressed on MHCII^{low} ATMs, the *H2-Eb1*, *Cxcl9*, *Gbp2b* and *Serpina3g* were expressed highly on MHCII⁺CD11c⁻ ATMs, and the *Shisa9* and *Trpc4* genes identified the MHCII⁺CD11c⁺ ATMs, whereas the P3 monocyte-derived cells expressed specific genes like *Klr1d1*, *Kynu* and *Mreg*. Despite their divergence from other cell subsets, each myeloid cell subset shared a small set of genes, for example, the MHCII^{low} and MHCII⁺CD11c⁺ ATMs shared the common macrophage genes of *Cd209g* and *Cd209f*, suggesting that these two macrophage subsets can respond to similar cues. Taken together, transcriptomic analysis of obese myeloid cell subsets defined cohorts of genes expressed specifically on each ATM subpopulation, reflecting the specialized roles of different ATM subsets.

3.3.2 Specific transcriptomics of MHCII^{low} ATMs

In order to investigate the specific function of each ATM subpopulation, the DEGs between different ATM subsets were analysed. The Volcano plots highlighted around 200 genes are differentially expressed between MHCII^{low} and MHCII⁺CD11c⁻ ATMs in both lean and obese situations. Of note, among the 200 DEGs, the most abundant gene expressed in both lean and obese MHCII^{low} ATMs was *Alox15* (Figure 3.23 A). Arachidonate 15-lipoxygenase (*Alox15*) is a member of the LOX family, it can oxygenate polyunsaturated fatty acids and ester lipid-protein assemblies into physiological and pathological metabolites [105]. Previous studies showed that *Alox15* has both anti-inflammatory and pro-inflammatory roles in various diseases, including osteoporosis, cancer and diabetes under certain conditions [105]. Recent studies established that *Alox15* expressing macrophages play critical anti-inflammatory functions in steady state adipose tissue remodelling and clearance of apoptotic cells to maintain immunologic tolerance [106-108]. However, there is evidence also showed that *Alox15* is involved in the promotion of local inflammation, insulin resistance and

metabolic disorders in obese adipose tissue [109]. Thus, more investigation of *Alox15* expressing macrophages is still needed.

Figure 3.23: MHCII^{low} ATMs express genes involved in Arachidonic acid metabolism pathway. (A) Volcano plots show significance and magnitude of expression changes between MHCII^{low} and MHCII⁺CD11c⁻ ATMs in lean (left) and obese (right) mice. Coloured genes highlight > 2-fold differences that are statistically significant (P < 0.01, t test). (B) qPCR analysis of *Alox15* gene expression in the sorted lean and obese ATM subsets to validate the RNA-seq data. Data was normalized to the expression levels of β -actin and shown as relative fold change to the corresponding expression level of the lean MHCII^{low} ATMs. (C) GSEA enrichment of genes involved in Arachidonic acid metabolism pathway are selectively upregulated in MHCII^{low} versus MHCII⁺CD11c⁻ ATMs. (D) Heatmap analysis shows the genes involved in the arachidonic acid metabolism pathway expressed highly in MHCII^{low} ATMs. KEGG: kyoto encyclopedia of genes and genomes; GSEA: gene set enrichment analysis; ND: normal diet; HFD: high fat diet. Statistical significance was determined by using unpaired student's t-test. *** P < 0.001.

Our real time qPCR results also indicated that only the MHCII^{low} ATM express higher levels of *Alox 15*, even though the expression level of *Alox 15* was reduced in the obese situation, the expression levels are still much higher than the other two ATM subsets (Figure 3.23 B). Further gene set enrichment analysis (GSEA) revealed that MHCII^{low} ATMs upregulated genes involved in the Arachidonic acid (the precursor of *Alox15* enzyme) metabolism pathways (Figure 3.23 C and D) [110]. Taken together, the results

indicate that Alox15 expressing MHCII^{low} ATMs may play important functions during the progression of obesity.

Notably, the heat map result also revealed that the MHCII^{low} ATMs expressed high levels of *Adrb2* gene (Figure 3.24 C lower), which encodes the neurotransmitter receptor, implying that the MHCII^{low} ATMs can respond to the signals from the nerve system.

3.3.3 MHCII⁺CD11c⁺ ATMs are specialized in lipid metabolism

Subsequently, the GSEA results of the obese ATM subsets showed that MHCII⁺CD11c⁺ ATMs exhibit a distinctly upregulated transcriptional signature of Oxidative phosphorylation, TCA cycle and electron transport chain (ETC) activity when compared to the MHCII⁺CD11c⁻ ATMs (Figure 3.24 A and B), the genes related to these pathways are shown in Figure 3.24 C (middle).

Figure 3.24: MHCII⁺CD11c⁺ ATMs are involved in the lipid metabolism. Gene set enrichment analysis identifies significant transcriptional upregulation of TCA cycle and respiratory electron transport signaling pathway (A) and oxidative phosphorylation pathway (B) in MHCII⁺CD11c⁺ ATMs when compared to the MHCII⁺CD11c⁻ ATMs (Gene Ontology [GO]/KEGG with false discovery rate [FDR] < 0.05). (C) Mean log2 fold change in expression levels of genes in fatty acid lipolysis and uptake (upper), TCA and Oxidative pathways (middle) and neurotransmitter receptors (lower) in obese ATM subsets. (D) CD36 antibody staining in ATM subsets (n=3). (E) QPCR analysis of sorted lean and obese ATM subsets to validate the relative expression of *Lpl* and *Fabp4* of the RNA-seq heat map. Data was normalized to the expression levels of β -actin and shown as relative fold change to the corresponding expression level of the lean MHCII^{low} ATMs. TCA: tricarboxylic acid cycle; ETC: electron transport chain; KEGG: kyoto encyclopedia of genes and genomes; Lpl: lipoprotein lipase; Fabp: fatty acid binding protein. Statistical significance was determined by using unpaired student's t-test. * P< 0.05; ** P< 0.01.

Furthermore, MHCII⁺CD11c⁻ ATMs also displayed upregulation of genes involved in fatty acid lipolysis and uptake, such as *Lpl*, *Cd36* and *Fabp4* (Figure 3.24 C upper). The flow cytometry staining and the real time qPCR confirmed a clear upregulation of the cellular surface marker of CD36 as well as some metabolic related genes including *Lpl* and *Fabp4* in MHCII⁺CD11c⁺ ATMs which lacked in the other two ATM subsets (Figure 3.24 D and E), thus providing support that MHCII⁺CD11c⁺ ATMs are specialized in lipid metabolism in eAT.

3.4 Functional characterization of ATM subsets

3.4.1 Mouse models to study the in vivo function of different ATM subpopulations

Since we have already demonstrated that the ATM subsets are phenotypically and most likely functionally distinct (section 3.1 and section 3.3), we aimed to investigate their physiological and metabolic role in the adipose tissue during the development of obesity. To elucidate the in vivo relevance of different ATMs, one experimental transgenic animal model (CD169-DTR) as well as an antibody-dependent macrophage depletion protocol (Anti-CSF-1R) which specifically ablate macrophage subpopulations in the adipose tissue have been utilized (Fig. 3.25).

As shown in chapter 3.1, three phenotypically distinct subsets of ATMs are present in adipose tissue (Figure 3.25 left). DT administration in CD169-DTR mice can efficiently deplete all the three CD169⁺ ATM subsets (CD169 is specifically expressed on all the three ATM subsets in both lean and obese mice is shown in appendix 6.11) (Figure 3.25 middle column). On the other hand, anti-CSF1R antibody injection in mice can selectively ablate the MHCII^{low} and MHCII⁺CD11c⁻ ATM subsets but not the obesity-dependent infiltrating MHCII⁺CD11c⁺ ATMs (Figure 3.25 right column). Therefore,

due to their different ablation profiles, these two mouse models enabled us to investigate the in vivo contribution of different ATM subsets during health and the progression of obesity.

Figure 3.25: Selective depletion of distinct ATM subsets in various mouse models. Upper: Representative adipose tissue cartoon pictures show the depletion of different ATM subsets in each mouse model. Lower left: Representative plot figures of eAT myeloid cell profiles of various mouse models from ND (upper) treated mice or HFD (lower) treated mice. Myeloid cell populations were gated according to the gating strategy showed in Figure 3.3. From left to right: WT, CD169-DTR, anti-CSF1R treated mouse models. Red stars show the subpopulations of ATMs been selectively and efficiently ablated in each mice model. ND: normal diet; HFD: high fat diet; Fr I: F4/80^{hi}CD11b⁺ ATMs; Fr II: F4/80^{int} cells.

3.4.2 Investigation of the in vivo function of CD169⁺ ATMs in health and obesity

3.4.2.1 Phenotype of adipose tissue lacking ATMs (CD169-DTR mouse)

To assess the influence of ATMs in maintaining the function of lean and obese eAT, we treated 4 weeks old CD169-DTR and age-matched WT mice with ND or HFD for 16 weeks. Two weeks before the end of the treatment, DT was administered twice per week for 12 days before sacrificing the mice (Figure 3.26 A).

By monitoring the body weight of the mice during the DT administration period, CD169-DTR mice exhibit a slight body weight reduction after one week (black arrows) of the DT injection in both lean and obese group (Figure 3.26 B). Notably, when the glucose tolerance test (GTT) was performed, CD169-DTR mice displayed significant lower basal blood glucose levels than the control mice in both groups, suggesting that depletion of the ATMs can cause the reduction of blood glucose levels (Figure 3.26 C). However, the subsequent GTT results in these two groups revealed similar trends between WT and CD169-DTR mice, except that the HFD WT mice exhibited significant higher glucose levels at the 15 minutes' time point during the glucose challenge, probably due to the lower basal glucose levels in CD169-DTR mice.

Figure 3.26: Metabolic parameters of DT injected CD169-DTR mice. (A) Schematic explanation of the experimental procedure used to study the in vivo function of CD169⁺ ATMs in lean and obese mice. 4 weeks old CD169-DTR and WT mice were given HFD or ND for 16

weeks, the DT was injected to all the mice during the last two weeks of food feeding. (B) Body weight (n=10 for each group) and (C) Glucose tolerance tests (n=8 for obese group and n=5 for lean group) of the obese and lean CD169-DTR and their corresponding control WT mice are shown. Black arrow shows the body weight of CD169-DTR mice start to reduce obviously. ND: normal diet; HFD: high fat diet; GTT: glucose tolerance test; DT: diphtheria; mg/dl: milligrams per deciliter; WT: wild type. Statistical significance was determined by using unpaired student's t-test. * P< 0.05; *** P< 0.001; ns: no significance.

3.4.2.2 Myeloid cell profiling in adipose tissue lacking ATMs

Subsequently, we aimed to quantify the remaining myeloid cell populations in eAT after long term depletion of the ATMs. eAT Myeloid cell profiles from both lean and obese mice were analysed (Figure 3.27 A). As expected, the absolute cell numbers of ATM subsets in the CD169-DTR mice were dramatically reduced when compared to the WT mice. On the other hand, monocytes (Ly6C⁺MHCII⁺), monocyte-derived cells (Ly6C⁺MHCII⁺ and Ly6C⁻MHCII⁺) and neutrophils (Ly6G⁺CD11b⁺) were slightly augmented probably due to “compensatory” infiltration of these cells into the eAT (Figure 3.27 B) in absence of ATMs. Thus, the long term depletion of ATMs changed the representation and frequencies of other myeloid cell subsets in eAT.

Figure 3.27: Skewed representation of myeloid cell subsets in CD169-DTR mice. (A) Representative plot figures show myeloid cell profiles in lean (left) and obese (right) WT (upper) and CD169-DTR (lower) mice eAT. (B) Bar chart shows the $\pm$ S.E.M absolute cell number of different myeloid cell populations in lean (left, n=4 for each group) and obese (right, n=6 for each group) WT and CD169-DTR mice. ND: normal diet; HFD: high fat diet; no.: number; Fr I: F4/80^{hi}CD11b⁺ ATMs; Fr II: F4/80^{int} cells; Ly6C^{hi}MHCII⁺: monocytes; Ly6C⁺MHCII⁺ and Ly6C⁻MHCII⁺: monocyte-derived cells. Statistical significance was determined by using unpaired student's t-test. * P< 0.05; ** P< 0.01; *** P< 0.001; **** P< 0.0001.

3.4.2.3 Depletion of ATMs leads to the hypertrophy of adipocytes

In order to determine the consequences of ATM depletion on the mass of eAT, the tissue was collected from ND and HFD treated WT and CD169-DTR mice and weighted. Clearly the weight of eAT collected from ND treated mice was significantly reduced in CD169-DTR mice when compared to WT mice (0.16 gram versus 0.36 gram) (Figure 3.28 A upper). However, during obesity and in absence of ATMs, a marked increase in fat accumulation was observed, which can be reflected by the significant higher weight of eAT from the CD169-DTR mice fed with HFD (Figure 3.28 A lower). The opposite results observed in lean and obese situations may suggest that distinct local microenvironment drives functionally different ATM subsets. Notably, when zooming into the eAT collected from obese CD169-DTR mice, oily and transparent enlarged adipocytes were visible (Figure 3.28 A and Appendix 6.12).

Figure 3.28: Depletion of CD169⁺ ATMs leads to the hypertrophy of adipocytes. (A) Left side: eAT weight of ND treated (upper panel) and HFD treated (lower panel) WT (left) and CD169-DTR (right) mice. Right side: representative eAT pictures of ND-treated (upper) and HFD-treated (lower) WT (left) and CD169-DTR (right) mice. (B) H&E staining of the eAT from ND (upper) and HFD (lower) treated WT (left) and CD169-DTR (right) mice. Lower panel: bar chart shows the $\pm$ S.E.M. diameters of the adipocytes from the lean (left) and obese (right) WT (left) and CD169-DTR (right) mice. ND-treated WT (n=15) and CD169-DTR (n=14); HFD-treated WT (n=12) and CD169-DTR (n=12) were analysed. Black bar in the representative H&E pictures is equal to 100 μ m. ND: normal diet; HFD: high fat diet; H&E: heamatoxylin and eosin; g: gram; DT: diphtheria; +: injected with DT. Statistical significance was determined by using unpaired student's t-test. ** $P < 0.01$; **** $P < 0.0001$.

Given that there was more fat accumulation in the obese CD169-DTR eAT, we aimed to explore the mechanisms responsible for this fat accumulation. A closer examination of the hematoxylin and eosin (H&E) stained eAT sections from both lean and obese mice showed that after removing ATMs, increasing numbers of enlarged adipocytes are observed in the CD169-DTR mice when compared to the WT mice, especially in the obese situation (Figure 3.28 B upper). As shown in Fig. 3.28 B, the average size of the adipocytes is significantly bigger in the absence of ATMs. Altogether, these results suggest that ablation of ATMs can cause the hypertrophy of the adipocytes and eventually lead to the fat accumulation in the eAT.

3.4.2.4 Ablation of ATMs cause the dysfunction of eAT

Since ATMs interact dynamically with other cells residing in the eAT to maintain the tissue homeostasis [111], total RNA was prepared from eAT obtained from DT-injected WT and CD169-DTR mice and used as a template for an extensive qPCR analysis. Moreover, serum was also collected from both groups to check the circulating levels of metabolic associated mediators by doing enzyme-linked immunosorbent assay (ELISA) in absence of ATMs.

Several representative key genes, including adipogenic related genes (Fabp4 and Pparg), adipokines (adiponectin, leptin and Rbp4), lipid metabolism associated genes (LPL, CD36, DGAT-1 and ABCA-1), chemokines important for maintaining the function of ATMs (CCL2, CCL5, CCL3 and CXCR3) and some anti-inflammatory and pro-inflammatory cytokines (IL-6, IL-10 and TNF α) were examined. The results revealed that ATM absence dramatically perturbs the local eAT microenvironment, as the factors involved in adipogenesis and lipid metabolism all strikingly diminished in the CD169-DTR mice from both lean and obese mouse groups (Figure 3.29 A and D). Notably, the adipokines also reduced dramatically in the absence of ATMs and the levels of adiponectin from both the tissue and serum decreased remarkably in the CD169-DTR mice when compared to the WT mice in both situations (Figure 3.29 B and C).

On the contrary, the expression levels of the tested chemokines exhibited increased expression levels in CD169-DTR mice from both lean and obese situations (Figure 3.29 E), probably due to the high demand of monocytes to differentiate into ATMs in the empty niches. Also several cytokines were upregulated, such as IL-10, IL-6 and TNF α , when ATMs were removed (Figure 3.29 F). Collectively, these results suggest that

depleting ATMs affects the ability of eAT microenvironment to support its normal functions.

Figure 3.29: Quantification of key factors in the eAT microenvironment in the absence of ATMs. QPCR analysis of the adipogenic related factors (Fabp4 and Ppar γ) (A), adipokines (Adiponectin, leptin and RBP4)(C), lipid metabolism associated genes (LPL, CD36, DGAT-1 and ABCA1) (D), chemokines (CCL2, CCL3, CCL5 and CXCR3) (E) and cytokines (IL-6, TNF α and IL-10) (F) in eAT from both lean (left) and obese (right) WT (left) and CD169-DTR (right) mice (n=3 for each group). The expression level of each targeted gene was normalized to β -actin and was displayed as relative fold-change to the corresponding gene expression level of the ND treated WT group. (B) Circulating levels of adiponectin from serum was quantified by ELISA from both lean (left) and obese (right), WT (left) and CD169-DTR (right) mice. LPL: lipoprotein lipase; CCL: C-C motif chemokine ligand; CXCR: C-X-C chemokine receptor; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor; DGAT: diacylglycerol acyltransferase; ABCA: ATP-binding cassette transporter; RBP: retinol binding protein; Fabp: fatty acid binding protein; Ppar: peroxisome proliferator activated receptor; ND: normal diet; HFD: high fat diet. Statistical significance was determined by using Bonferroni two-way analysis of variance (ANOVA). * P< 0.05; ** P< 0.01; *** P< 0.001; **** P< 0.0001; ns: no significance.

3.4.2.5 Removing ATMs affects the lipid metabolism of the eAT

ATMs can buffer away excess lipid and actively involved in the lipid metabolism in adipose tissue [77, 112]. Therefore, we aimed to examine the lipid metabolism profiles of lean and obese eAT after removing ATMs. Fresh eAT was snap frozen in liquid nitrogen and processed for lipid metabolites analysis.

Figure 3.30: Perturbed lipid metabolisms of eAT in the absence of ATMs. (A) 2D PCA plots show distinct lipid metabolite profiles of lean (square) and obese (round) WT (light green and light blue) and CD169-DTR (red and orange) mice. Relative fold changes of 10 representative upregulated (red) and downregulated (light blue) lipid metabolites in lean (B) and obese (C) CD169-DTR mice versus WT mice in eAT are displayed. ND: normal diet; HFD: high fat diet; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; DG: diacylglycerols; TG: triacylglycerols; MG: monoacylglycerols; CL: glycerophosphoglycerophosphoglycerols; Cer: ceramides; VS: versus; O: oxygen.

The 2D-PCA result showed that all samples from the same experimental group (WT lean, WT obese, CD169-DTR lean and CD169-DTR obese) were clustered together (Figure 3.30 A), indicating that each tested group shows a distinct metabolism profile and certainly ablation of ATMs perturbs lipid metabolism in eAT since WT and CD169-DTR mice samples cluster differently.

When quantifying the lipid metabolites in detail, the metabolites were remarkably changed in the CD169-DTR mice when compared to the WT mice. Ten representative

upregulated and downregulated lipid metabolites from lean and obese mice groups are shown in Figure 3.30 B and C respectively. For example, after removing ATMs, metabolites from glycerophospholipid (GP) and glycerolipids (GL) families were significantly upregulated or downregulated, including phosphatidic acid (PA), phosphatidylcholine (PC), glycerophospho-glycerophosphoglycerols (CL), phosphatidylethanolamine (PE), diacylglycerols (DG), triacylglycerols (TG) and monoacylglycerols (MG) descendants in both lean and obese situations (Figure 3.30 B and C). These results indicate that certain lipid metabolic pathways may be affected in the absence of ATMs and ATMs play crucial roles in maintaining the normal lipid metabolism of eAT.

3.4.3 Investigation of the in vivo function of ATM subsets in anti-CSF1R antibody treated mice

3.4.3.1 Metabolic phenotypes of the anti-CSF1R antibody treated mice

After having demonstrated that ATMs are required for maintaining the normal function of eAT (result part 3.4.2) and three functionally distinct ATM subsets may exist in the adipose tissue (result part 3.3), we aimed to investigate the in vivo function of each single ATM subset. As the anti-CSF1R antibody treatment in the mice displayed selective depletion of $\text{MHCII}^{\text{low}}$ and $\text{MHCII}^+ \text{CD11c}^-$ but not the $\text{MHCII}^+ \text{CD11c}^+$ ATMs (Figure 3.25), we were therefore able to study the in vivo roles of these two ATM subsets in health and obesity.

Four weeks' old WT male mice were given HFD or ND for 16 weeks and the anti-CSF1R antibody injection was performed during the last two weeks of the food exposure (Figure 3.31 A). Similar to the CD169-DTR mice experiment, body weight of anti-CSF1R antibody treated mice was monitored every day during the antibody injection period, but unlike the CD169-DTR mice, administration of anti-CSF1R antibody did not affect the weight gain of both lean (Figure 3.31 B right) and obese (Figure 3.31 B left) mice. When the GTT was examined, the anti-CSF1R antibody injected mice retained the glucose tolerance in both lean and obese situations (Figure 3.31 C). Taken together, the results implicate that differently from the depletion of all ATM subsets in the CD169-DTR mice, ablation of only two ATM subsets ($\text{MHCII}^{\text{low}}$ and $\text{MHCII}^+ \text{CD11c}^-$ ATMs) does not lead to obvious weight and glucose tolerance differences in the mice.

Figure 3.31: Metabolic parameters of the Anti-CSF1R antibody injected mice. (A) Schematic explanation of the experimental procedure used to study the in vivo function of MHCII^{low} and MHCII⁺CD11c⁻ ATMs in lean and obese mice. 4 weeks' old male WT mice were given HFD or ND for 16 weeks, during the last two weeks of the food feeding, anti-CSF1R antibody or isotype control antibody was administrated to the mice respectively. (B) Body weight (n=8 for obese group and n=6 for lean group) and (C) Glucose tolerance tests (n=8 for obese group and n=4 for lean group) of the obese and lean anti-CSF1R antibody treated mice and their corresponding control mice are shown. ND: normal diet; HFD: high fat diet; mg/dl: milligrams per deciliter; GTT: glucose tolerance test; Mins: minutes.

3.4.2.2 Myeloid cell profiling in eAT lacking MHCII^{low} and MHCII⁺CD11c⁻ ATMs

In order to quantify the remaining myeloid cell populations present in the eAT after 12-days anti-CSF1R antibody treatment, we compared the absolute cell numbers of distinct myeloid cell populations from anti-CSF-1R treated mice to their counterparts in WT mice.

In anti-CSF-1R treated mice, the absolute cell numbers of MHCII^{low} and MHCII⁺CD11c⁻ ATMs diminished remarkably, whereas MHCII⁺CD11c⁺ ATMs were only slightly affected in both lean and obese situations (Figure 3.32 A and B), indicating a selective and specific ablation of only two ATM subpopulations. Notably, monocytes, monocyte-derived cells and neutrophils were not affected by anti-CSF1R antibody

administration in both lean and obese groups, suggesting they do not need CSF1 for their maintenance. Moreover, differently to the CD169-DTR mouse, selective depletion of MHCII^{low} and MHCII⁺CD11c⁻ ATMs did not cause dramatic infiltration of other myeloid cells. Collectively, the result establishes that unlike the CD169-DTR mice, long term administration of anti-CSF1R antibody depletes specifically MHCII^{low} and MHCII⁺CD11c⁻ ATMs, but does not significantly affect other myeloid cell profiles in the eAT.

Figure 3.32: CSF-1R mediated depletion of ATMs (A) Representative plot figures show eAT myeloid cell profiles in lean (left) and obese (right) WT (upper) and anti-CSF1R treated (lower) mice. (B) Bar chart shows the absolute cell number of different myeloid cell populations in lean (left, n=4 for each group) and obese (right, n=4 for each group) WT (white bar) and anti-CSF1R treated (green bar) mice ($\pm$ S.E.M.). ND: normal diet; HFD: high fat diet; no.: number; Fr I: F4/80^{hi}CD11b⁺ ATMs; Fr II: F4/80^{int} cells; P1: monocytes; P2 and P3: monocyte-derived cells. Statistical significance was determined by using unpaired student's t-test. ** P< 0.01; *** P< 0.001; **** P< 0.0001; ns: no significance.

3.4.2.3 Anti-CSF1R injection leads to smaller adipocytes

Since depletion of total ATMs caused a hypertrophic adipocyte phenotype (Figure 3.28), we subsequently intended to assess whether removing MHCII^{low} and MHCII⁺CD11c⁻ ATMs would affect the fat accumulation in eAT as well.

We observed that anti-CSF1R treated mice exhibited similar epididymal fat mass as the control WT mice in both lean and obese groups (Figure 3.33 A). Of note, further H&E analysis revealed that adipocytes from anti-CSF1R treated lean mice were not affected,

as their size was comparable to the WT mice (Figure 3.33 B). In contrast, the adipocytes from the anti-CSF1R administrated obese mice displayed remarkable decreased sizes, which is opposite to the result of CD169-DTR mice. Therefore, ablation of obese but not lean $\text{MHCII}^{\text{low}}$ and $\text{MHCII}^+ \text{CD11c}^-$ ATMs can lead to smaller adipocytes.

Figure 3.33: Anti-CSF1R antibody treatment leads to smaller adipocytes. (A) Left side: eAT weight of ND treated (upper panel) and HFD treated (lower panel) WT (left) and anti-CSF1R injected (right) mice. Right side: representative pictures of ND-treated (upper) and HFD-treated (lower) WT (left) and anti-CSF1R treated (right) mice eAT. (B) H&E staining of eAT from ND (upper) and HFD (lower) treated WT (left) and anti-CSF1R treated (right) mice. Lower panel: bar chart shows the diameters of the adipocytes from lean (left) and obese (right) WT (left) and anti-CSF1R treated (right) mice ($\pm$ S.E.M.). ND-treated WT (n=10) and anti-CSF1R injected mice (n=9) as well as HFD-treated WT (n=11) and anti-CSF1R injected mice (n=9) were included in the analysis. Black bar in the H&E pictures is equal to 100 μm . ND: normal diet; HFD: high fat diet; H&E: heamatoxylin and eosin; Ab: antibody; d: day. Statistical significance was determined by using unpaired student's t-test. **** $P < 0.0001$; ns: no significance.

3.4.2.4 Microenvironment change of the anti-CSF1R antibody treated eAT

In order to evaluate the effects of depleting $\text{MHCII}^{\text{low}}$ and $\text{MHCII}^+ \text{CD11c}^-$ ATMs on the eAT microenvironment, total RNA was isolated from obese anti-CSF1R treated and WT eAT and extensive qPCR analysis was performed. Similar to the CD169-DTR experiment, factors related to adipogenesis (Fabp4 and Ppar γ), adipokines (Adiponectin, Leptin and RBP4), lipid metabolism (LPL, CD36, DGAT-1 and ABCA-1), chemokines

(CCL2, CCL3, CCL5 and CXCR3) and cytokines (IL-6, TNF α , IL-10 and IFN γ) as well as circulating levels of adiponectin in serum were examined.

Interestingly, qPCR analysis of anti-CSF1R antibody treated mice revealed an opposite trend to the results of CD169-DTR mice. For example, anti-CSF1R antibody treated mice displayed slight increased expression levels of Fabp4 and Ppar γ , but with no significance, suggesting that the adipogenesis may be promoted in the absence of MHCII^{low} and MHCII⁺CD11c⁻ ATMs (Figure 3.34 A). Of note, adipokines and genes associated with lipid metabolism were not significantly affected by anti-CSF1R antibody administration (Figure 3.34 C and D), implying that the eAT can still perform normal metabolic function. But the circulating levels of adiponectin was remarkably upregulated in the anti-CSF1R injected mice when compared to the WT mice (Figure 3.34 B).

Figure 3.34: Depletion of ATM subsets in anti-CSF1R antibody treated mice did not cause dramatic disorders of the tissue microenvironment. qPCR analysis of the genes involved in adipogenesis (Fabp4 and Ppar γ) (A), adipokines (Adiponectin, Leptin and RBP4) (C), lipid metabolism (LPL, CD36, DGAT-1 and ABCA-1) (D), chemokines (CCL2, CCL3, CCL5 and CX3CR1) (E) and cytokines (IL-6, TNF α and IL-10) (F) was performed in eAT from obese WT (left) and anti-CSF1R antibody treated (right) mice. (B) Circulating levels of adiponectin from serum was quantified by ELISA from obese WT (left) and anti-CSF1R antibody treated (right) mice. n=3 for each group. LPL: lipoprotein lipase; CCL: C-C motif chemokine ligand; CXCR: C-X-C chemokine receptor; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor; DGAT: diacylglycerol acyltransferase; ABCA: ATP-binding cassette transporter; RBP: retinol binding protein; Fabp: fatty acid binding protein; Ppar: peroxisome proliferator activated receptor. Statistical significance was determined by using unpaired student's t-test. * P< 0.05; ** P< 0.01; ns: no significance.

Furthermore, unlike CD169-DTR mice, chemokines involved in monocyte migration were slightly upregulated (CCL2 and CCL5) or significantly downregulated (CXCR3) or remain unchanged (CCL3) in anti-CSF1R antibody injected mice when compared to WT mice (Figure 3.34 E), which is consistent with the results that the anti-CSF1R antibody treatment does not induce striking monocyte and monocyte-derived cell infiltration in the eAT (Figure 3.32 right). Lastly, when testing cytokines, only IL-6 and IL-10 were slightly upregulated, whereas TNF α was slightly downregulated but with no significance (Figure 3.34 F). Altogether, the results suggest that differently from depleting all ATMs in CD169-DTR mice, after removing only MHCII^{low} and MHCII⁺CD11c⁻ ATM subpopulations, the adipocyte function is not as drastic impaired as in total absence of ATMs.

3.4.2.5 Lipid metabolism in eAT lacking MHCII^{low} and MHCII⁺CD11c⁻ ATMs

Given that lacking MHCII^{low} and MHCII⁺CD11c⁻ ATMs did not significantly affect the properties of epididymal adipocytes, we subsequently aimed to explore the lipid metabolism in the anti-CSF1R antibody injected mice. To test this, fresh eAT was snap frozen in liquid nitrogen and analysed for lipid metabolites.

Ablation of MHCII^{low} and MHCII⁺CD11c⁻ ATMs failed to induce striking changes of the lipid metabolic profiles in eAT when compared to WT mice, as the 2D-PCA revealed that samples from the anti-CSF1R antibody treated and non-treated mice are not well separated in both lean and obese conditions (Figure 3.35 A). A more detailed lipid metabolites analysis showed that anti-CSF1R antibody injection over 12 days still affected some metabolites when compared to the WT mice. Five representative upregulated and downregulated lipid metabolites in eAT obtained from anti-CSF1R antibody injected obese and lean mice versus corresponding WT mice are shown in

Figure 3.35 B and C , respectively. In particular, the levels of monoacylglycerols (MG), triacylglycerols (TG) and glucosylceramide (GlcCer) were significantly increased, while the dilevalol hydrochloride was strikingly reduced when comparing the obese anti-CSF1R antibody treated mice to the corresponding WT mice (Figure 3.35 B). In contrast, in the ND treated mice, anti-CSF1R antibody treatment remarkably upregulated demonomethylchorpromazine and downregulate bltoctamide semisuccinate metabolites when compared to the WT mice (Figure 3.35 C).

Figure 3.35: Lipid metabolism in eAT in absence of MHCII^{low} and MHCII⁺CD11c⁺ ATMs. (A) 2D-PCA plots show lipid metabolites profiles of lean (square) and obese (round) WT and anti-CSF1R antibody treated eAT. Fold changes of 5 representative upregulated (red) and downregulated (light blue) lipid metabolites in obese (B) and lean (C) anti-CSF1R treated versus WT mice are shown. ND: normal diet; HFD: high fat diet; PC: phosphatidylcholine; PE: phosphatidylethanolamine; TG: triacylglycerols; MG: monoacylglycerols; Cer: ceramides; PS: phosphatidylserine; SM: sphingomyelin; VS: versus.

In conclusion, combining the lipid metabolism results from CD169-DTR mice and anti-CSF-1R antibody treated mice, it is clear that the MHCII⁺CD11c⁺ ATMs are mainly involved in the lipid metabolism in eAT, which is in consistent with the RNA-seq data shown in Figure 3.24.

4 Discussion

4.1 HFD induced obesity in mouse

Rate of obesity is now increasing dramatically worldwide and becoming a major public health concerns with its many clinical related syndrome, such as type 2 diabetes, in both adults and children [113, 114]. Using animal models to investigate and discover novel therapeutics to mitigate this pandemic is urgently needed. Small rodents sharing characteristics of human body and immune system, such as rats and mice, are the most commonly used preclinical animal models to investigate obesity associated complications. Therefore, mouse models of obesity (Table 4.1), including diet-induced obesity (DIO) and the genetically modified dysfunctional leptin signalling pathways associated (ob/ob and db/db) obese mouse models, are widely utilized in various studies [115, 116]. Among these obese mouse models, DIO mice mimic better the progression of obesity and recapitulate most parts of the obesity related pathogenesis of humans, such as glucose intolerance and insulin resistance, making them the best mouse models to test the prospective therapeutics [114].

Model name	Mutation	References
ob/ob mouse	Lep ^{ob} /lep ^{ob} (Leptin deficiency)	[17, 116]
db/db mouse	Lep ^{db} /Lep ^{db} (Leptin receptor)	[117]
s/s mouse	Disrupted STAT3 signal of leptin receptor	[118]
Glucose transporter subtype 4 transgenic	GLUT4 overexpression	[119]
High-fat diet-induced obesity	Polygenic	[120, 121]
Age-related obesity in mice	Polygenic	[122]

Table 4.1 Some commonly used animal models of obesity. There are roughly two different kinds of these mouse models including diet induced and genetic modified obese mice. STAT3: Signal transducer and activator of transcription 3.

The widely used DIO model still has some limitations: It is time-consuming and costly and the progression of obesity in HFD-treated mice can be affected by sex, age and genetic background [113]. A recent work done by Ulrika S. Pettersson et al. suggested that HFD-treated male mice develop clear adipose tissue inflammation and other obesity associated disorders such as insulin resistance. On the other hand, female mice are protected from the obesity induced detriments and display an anti-inflammatory eAT microenvironment [123]. Aging is another factor that has a considerable influence on

the development of obesity and type 2 diabetes, as aging had been shown to be involved in the progressive glucose intolerance in old mice independently from other factors [114]. Furthermore, inbred mouse strains display different inherent propensities which can lead to strain-dependent differences in the susceptibility to DIO and insulin resistance. For example, most mouse strains especially the C57BL/6J mice, are prone to develop severe obesity, glucose intolerance and subsequently insulin resistance upon HFD exposure, whereas BALB/c mice are resistant to HFD induced metabolic disorders [124].

In order to avoid experimental variations that can affect the development of obesity, only young male C57BL/6J mice and male transgenic mice backcrossed to C57BL/6J background were used for our investigations to keep the results consistent. After 16 weeks of HFD exposure, we were able to observe significant body weight gain, expansion of eAT, glucose intolerance, increasing number of CLSs and clear hypertrophy of adipocytes in the obese mice.

4.2 Distinct subpopulations of ATMs in lean and obese mice

In our study, we found that mouse eAT contains at least three distinct subpopulations of ATMs with different morphology and phenotypes. We refer to these F4/80^{hi}CD11b⁺ ATMs as MHCII^{low}, MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs. In healthy lean eAT, MHCII⁺CD11c⁻ ATMs are the most abundant ATM subsets, followed by MHCII^{low} ATMs. Upon chronic HFD exposure, a BM-derived MHCII⁺CD11c⁺ ATM subset, poorly represented in lean mice, infiltrates the eAT and becomes a dominant ATM subpopulation. In aged mice as well as during the development of obesity, the expression of the embryonic-marker Tim-4 in MHCII⁺CD11c⁻ ATMs is gradually reduced which indicates that the originally embryonic-derived fraction is replaced by BM-derived macrophages during ageing and obesity. Apparently MHCII^{low} cells are independent from the adipose tissue microenvironment, since their frequency in lean and obese mice is comparable. Further UMAP analysis of ATM subpopulations revealed that each ATM subset can form distinct clusters and the MHCII^{low} ATM cluster is distant from monocyte and other myeloid cell population clusters, indicating that MHCII^{low} ATMs are most likely not derived from monocytes and can maintain themselves locally.

During the last decades, ATMs were often conceptualized into an M1 and M2 paradigm in various studies [41]. The M1/M2 definition describes two different activated states of macrophages, M1 macrophages are stimulated by Th1 mediators, including LPS and IFN γ , and are characterized with high production of proinflammatory cytokines (e.g., IL-6), reactive oxygen intermediates (ROI) and nitric oxide (NO) which can damage contiguous tissue [125]. In contrast, M2 macrophages are activated by Th2 mediators (e.g., IL-4), and the typical characteristics of M2 macrophages include upregulation of mannose receptor and scavenger receptors as well as generation of anti-inflammatory cytokines such as IL-10 that can promote the proliferation of contiguous cells and tissue repair [125].

Following this classification, it was indicated that lean mouse eAT contains mainly the M2-like macrophages that are important for maintaining the eAT homeostasis. But during the progression of obesity, local inflammation induced by dead adipocytes can recruit M1-like macrophages which form crown-like structures around necrotic adipocytes. Thus obesity shifts the balance of M1/M2 macrophages, with M1-like macrophages overwhelming the number of M2-like macrophages [126]. However, accumulating studies suggested that the M1/M2 polarization is over simplistic and does not sufficiently identify the ATM subsets [76, 88]. Recent evidences established that obesity can activate the lysosome biogenesis in ATMs to buffer away the free fatty lipids from local microenvironment suggesting that ATMs are actively involved in the lipid metabolism [77], hence ATMs undergo an “unconventional” metabolic activation (MMe) due to their exposure to high levels free fatty acids (FFAs) during adiposity rather than a classical M1/M2 polarization [78, 79]. In agreement with the proposed metabolic activation, our gene expression, flow cytometry and qPCR analysis shows significant upregulation of the key MMe genes, such as *Cd36* [78] and lipid metabolism associated genes (*Lpl*, *Fabp4* and *Fabp5*) in ATMs during obesity development. Furthermore, BODIPY staining also revealed that particular the MHCII⁺CD11c⁺ ATMs contain more cellular lipids, therefore we favour the metabolic activation pathway rather than the conventional prevailing M1/M2 paradigm.

In recent years, with the development of novel fluorochromes and sophisticated equipment, such as spectral flow cytometry and CytoF mass cytometry, as well as with the progress of single-cell mRNA sequencing (scRNA-seq), the complex network of

myeloid cells, inclusive the ATMs, has been dissected into many phenotypically different cell subpopulations. Although many ATM subsets have been characterized and proposed [74, 80, 83], their specific function in adipose tissue is still elusive and needs still to be further unravelled.

The glycoprotein, CD9 has been recently suggested to separate ATMs in eAT, showing a high similarity of CD9⁺ ATMs to the CD11c⁺ ATMs, since they both reside within CLSs and are highly involved in lipid metabolism during obesity [74]. However, the CD9 expression profile failed to segregate our defined ATM subsets, as not only the obese MHCII⁺CD11c⁺ and but also some of the obese MHCII⁺CD11c⁻ ATMs express higher levels of CD9. Another research group found that two different ATM subsets exist in steady state adipose tissue, with the Lyve-1^{hi}MHCII^{low} ATM subset attached to the blood vessels and the Lyve-1^{low}MHCII^{hi} ATMs closely related to the neuronal networks [83] without further charactering their specific function and their localization in obese eAT. Since obesity can dramatically change the frequencies and functions of the cell populations reside in the adipose tissue, the classification and localization of Lyve-1^{hi}MHCII^{low} and Lyve-1^{low}MHCII^{hi} in lean adipose tissue may not apply to the obese condition.

Another recent study used a combination of CD206, Tim-4, CD64, CD11c and MHCII to separate ATMs into four populations: Tim-4^{hi}MHCII^{int}CD206^{hi}, Tim-4^{int}MHCII^{hi}CD206^{hi}, CD64⁺CD11c⁻MHCII^{hi}CD206^{low/int} and CD11c⁺CD64⁺MHCII^{hi}CD206^{low/int} ATMs [76]. This separation of ATMs is similar to our classification of ATMs: the Tim-4^{hi}MHCII^{int}CD206^{hi} ATMs should be identical to our MHCII^{low} ATMs, the Tim-4^{int}MHCII^{hi}CD206^{hi} ATMs are similar to our MHCII⁺CD11c⁻ ATMs, while the CD64⁺CD11c⁺MHCII^{hi}CD206^{low/int} ATMs most likely to be our MHCII⁺CD11c⁺ ATMs. On the other hand, the CD64⁺CD11c⁻MHCII^{hi}CD206^{low/int} ATMs found in their study [76] could be our monocyte-derived cells (P3), as our P3 were also CD64⁺MHCII⁺CD206⁻ (Appendix 6.13). The same authors showed that ATM subpopulations, displaying high endocytic capacity in lean state, can dynamically adapt to the environment during fasting and infection, without further delineating the function of each ATMs [76].

Moreover, other studies involving an extensive single cell RNAseq analysis demonstrated that Trem2⁺ lipid-associated macrophages (LAMs) control the metabolic homeostasis during obesity and LAMs are key players of the metabolic pathologies during metabolic dysfunction [80]. Our RNAseq data revealed that although *Trem2* is upregulated during the progression of obesity, it is expressed on all the three ATM subsets with MHCII⁺CD11c⁺ ATMs showing the highest levels (Appendix 6.14), hence Trem2 cannot be used as a specific marker to delineate specific functional properties of the ATM subpopulations.

4.3 Causes for augmented macrophage activation and recruitment during obesity

Consistent with previous studies [68], our data showed that more macrophages especially the MHCII⁺CD11c⁺ ATMs infiltrate the eAT during obesity, which can be caused and supported by different factors. For example, increased frequency of adipocytes death and hypoxia during adipose tissue expansion can trigger the release of large amount of pro-inflammatory factors, which can act as possible contributors to the initiation of macrophage infiltration [127]. Macrophage accumulation can also be promoted by fatty acid flux, as saturated fatty acids enhance TLR4 mediated activation immune pathways in macrophages [37, 128]. Moreover, high levels of leptin during obesity can stimulate BM myelopoiesis and enhance their capacity to differentiate into pro-inflammatory ATMs [30, 129].

In this context, the origin of the newly recruited macrophages during obesity is still not well understood. Some studies indicated that BM derived monocytes can replace the embryonically derived macrophage populations in adipose tissue during obesity [52, 130]. While other research groups established that in addition to the monocyte recruitment, local proliferation, reduced apoptosis and prolonged survival of ATMs all account for the enhanced ATM numbers during obesity [52, 66, 67]. There is also evidence implying that the pro-inflammatory macrophages are switched from the anti-inflammatory macrophages [65]. Moreover, since pre-adipocytes share some common features to macrophages, such as phagocytosis and expression of specific macrophage markers including F4/80, Mac-1 and CD45, indicating the possibility that pre-adipocytes can adapt ATM functions in certain conditions, like obesity [131].

Our data highlighted the importance of CCR2/CCL2 axis in the recruitment of certain ATM subsets (MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs) at steady state and during obesity, as the staining of CCR2 revealed that only MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs express high levels of CCR2. Consistent to the CCR2 staining, our HFD treated CCR2^{-/-} mice results showed that MHCII⁺CD11c⁻ (lean and obese) and MHCII⁺CD11c⁺ (obese) ATMs dramatically diminished in the absence of CCR2 when compared to the HFD treated WT mice. Of note, there were still some MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs infiltrating eAT during obesity in CCR2^{-/-} mice, indicating that other chemokines, including possibly CCR5 and CX3CR1 [132], are involved in the monocyte recruitment into the tissue during obesity. Lineage tracing experiments using our Kit^{MerCreMer/R26} fate mapping mouse model corroborate that MHCII⁺CD11c⁻ as well as MHCII⁺CD11c⁺ ATMs are efficiently replenished by BM progenitors during obesity. Thus, our data showed strong support for the BM origins of the newly infiltrating ATMs (MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs) during obesity development.

4.4 ATM ontogeny and turnover rate and growth factor/chemokine requirements

Previous studies indicated that most tissue macrophages are established during the embryonic stage and are self-renewing locally through the adulthood with exception of certain organs, including intestine and skin, which need constant input from circulating Ly6C⁺ monocytes to maintain the macrophage pools in a CCR2 dependent manner [63, 133]. However, little is known about the ontogeny and maintenance of ATMs in eAT in both steady state and obese conditions [52]. Recent evidence established that monocytes are clearly able to enter the eAT where they retain their monocyte signature [76] or they can rapidly differentiate into monocyte-derived macrophages. However, under normal steady state conditions, monocytes are quite inefficient in generating ATMs in particular the so called vascular associated macrophages (VAMs) [76], which phenotypically resemble to our herein described MHCII^{low} and MHCII⁺CD11c⁻ ATMs. In disagreement with these data, recently it has been demonstrated that 50 % of lean ATMs are derived from recruited monocytes, with this frequency increases to 80 % under HFD feeding [80].

To re-address the ontogeny issue of steady state ATM subsets, we performed single dose injection of tamoxifen in the Kit^{MerCreMer/26} mice to label the primitive or definitive

progenitors and their descendants. Our results revealed that like most other tissue-resident macrophages, all ATM subsets ($\text{MHCII}^{\text{low}}$, $\text{MHCII}^+\text{CD11c}^-$ and $\text{MHCII}^+\text{CD11c}^+$) are derived from the definitive and not from primitive haematopoiesis. Furthermore, the time point tracing experiments at different time points using lean $\text{Kit}^{\text{MerCreMer/26}}$ mice displayed that ATM subpopulations, especially the $\text{MHCII}^{\text{low}}$ ATMs are characterized by a very slow turnover speed when compared to other myeloid cell populations from the same tissue, implying that they can maintain themselves locally with minimal contribution of BM-derived progenitors.

Previous studies suggested that in tissues continuously exposed to microbes, such as skin and intestine, the macrophage pools need constant replenishment by BM progenitors probably due to high demand of macrophages to fight against the antigens [133, 134], hence the turnover rate of the macrophages in these organs is very fast [103]. As eAT is not exposed to commensal microbes or foreign antigens, the replenishment kinetics of ATMs in lean mice is quite slow. In contrast, fate mapping in the obese eAT showed that metabolic remodelling of adipose tissue in obesity can accelerate the turnover rate of all ATM subpopulations, probably due to obesity-induced inflammation in the adipose tissue and recruitment of inflammatory cells like monocytes.

Since Tim-4 expressing macrophages are well characterized to be self-renewing embryonic-derived cells [101], we have used this marker to segregate long-lived foetal-derived macrophages from the short-lived BM-derived macrophages in the adipose tissue. Under lean conditions indeed all $\text{MHCII}^{\text{low}}$ and majority of $\text{MHCII}^+\text{CD11c}^-$ cells express Tim-4 pointing to their prenatal origin and their long-lived phenotype, as shown in our fate mapping experiments that the replenishment of the post-natal ATMs is minimal. With aging but even more pronounced during obesity, the landscape of Tim-4 expressing macrophages dramatically changes, as $\text{Tim-4}^+\text{MHCII}^+\text{CD11c}^-$ ATMs are rapidly lost and replaced by Tim-4^- BM-derived counterparts, which can be confirmed by Tim-4 staining and our obtained fate mapping results. Of the three ATM subpopulations, only $\text{MHCII}^+\text{CD11c}^+$ ATMs are short lived and BM-derived in both lean and obese conditions, hence it is not surprise that they are always negative for the embryonic derived Tim-4 signature.

The reasons why embryonic $\text{MHCII}^+\text{CD11c}^-$ ATMs and but not the fetal $\text{MHCII}^{\text{low}}$ ATMs are more prone to be replaced by BM-derived progenitors and lose their long-

lived property remain to be determined, in particular since both subsets are located close to the vasculature [76], therefore both subsets should be equally replaceable by infiltrating monocytes. As this is not the case, one can only speculate that the “niche” of MHCII^{low} ATMs is more protected and less accessible than the “niche” of MHCII⁺CD11c⁻ ATMs and the factors determining this difference still need to be examined in future experiments. Remarkably when their specific “niche” is empty (e.g. via DT-mediated macrophage ablation in CD169-DTR mice) and fully available, all the ATM subsets are quickly refilled in the created “vacuum” within few days. In both cases, the replenished ATMs are BM-derived, indicating that in contrast to the steady state situation, when the niche is rendered available, both ATM subsets can be efficiently refilled by BM-derived monocytes.

Similar to the colonic MHCII^{low} tissue-resident macrophages [103], the MHCII^{low} ATMs also characterized by BM and monocyte independency. In fact, the staining of the monocyte recruitment associated chemokine receptors revealed that MHCII^{low} ATMs do not express both CCR2 and CX3CR1 [132]. Consistent with the CCR2 staining, the absence of CCR2 in the CCR2^{-/-} mice did not affect the cell number of MHCII^{low} ATMs in both lean and obese situations. Interestingly, during the progression of obesity, even though MHCII^{low} ATMs increased their cell numbers significantly, they were minimal labelled with YFP in our fate mapping experiments, hence they were not efficiently replaced by BM-derived cells as the other two ATM subpopulations. Fate mapping of ATMs in lean CCR2^{-/-} Kit^{MerCreMer/26} mice further corroborated these observations, as the remaining MHCII^{low} ATMs in the absence of CCR2 exhibited very poor labelling (< 1 %) when compared to the WT mice (around 15 %), confirming their local maintenance.

The independency of BM monocytes for their replenishment also indicates that MHCII^{low} ATMs may have other ways to maintain their pool, including proliferation in situ [103]. The development and homeostasis of tissue-specific resident macrophages is controlled by several distinct factors such as CSF-2 (e.g. alveolar macrophages), IL-34 (e.g. microglia), Cx3CL1 fractalkine and CSF-1, hence mice lacking these specific molecules show pronounced alterations in the mononuclear phagocyte subpopulations [135-138]. In particular CSF-1-deficient mice display clear monocytopenia, macrophage deficiency as well as perturbed bone remodelling [139], hence CSF1-

CSF1R axis plays an important role for the survival, differentiation and function of macrophages in many tissues [103, 140].

Colony stimulating factor (CSF1) is a homodimeric cytokine, which is crucial for monocyte attraction as well as for the survival and polarization of macrophages [141]. There are three different biologically active forms of CSF1, one membrane-spanning cell surface glycoprotein (cs-CSF1), two secreted glycoprotein (sgCSF-1) and one proteoglycan (spCSF-1) isoforms [142]. CSF1 is secreted by various cell types, including fibroblasts, endothelial cells and BM stromal cells, suggesting that CSF1 can be local-derived factors that can maintain the self-renewal of BM-independent macrophages in the specific macrophage niche. For example, the lymphatic cells derived CSF1 is important for the in situ proliferation of subcapsular sinus macrophages in the lymph node, and showing both anchoring and nurturing functions for maintaining the self-renewal of this specific macrophages subset [143]. CSF1R is a receptor for CSF1 as well as IL-34, expressed highly on various myeloid cells, such as macrophages and monocytes, and upon binding to its ligands, it can undergoes oligomerization and auto-phosphorylation [141, 144]. The CSF1R responses to the CSF1 occur in a dose dependent manner, as low CSF1 concentration can promote macrophages survival due to the inhibition of total protein degradation, whereas high CSF1 concentration can lead to the protein synthesis and proliferation of macrophages [145, 146].

In the adipose tissue, we showed that anti-CSF1R antibody treatment in lean and obese mice selectively depletes both MHCII^{low} and MHCII⁺CD11c⁻ ATM subsets, but not the newly recruited MHCII⁺CD11c⁺ ATMs, suggesting that these two subpopulations need CSF1-CSF1R axis for their survival. As these two specific macrophage subsets are located close to the vasculature [76] and endothelial cells are known to secrete CSF1 [147], one can speculate that this tight macrophage/endothelium association may provide the right microenvironment and niche for macrophage self-renewal and BM-independent maintenance.

4.5 ATM subsets show distinct transcriptome and cell identity

Accumulating studies indicate that ATMs can play both detrimental and beneficial roles during the progression of obesity as they not only secrete pro-inflammatory cytokines which promote insulin resistance, but they also efficiently buffer away FFAs and clear

necrotic adipocytes maintaining a balanced homeostasis of adipose tissue [77, 79]. These diverse functions of ATMs are probably due to the heterogeneity of ATMs present in adipose tissue. In our study, we have demonstrated that at least three ATMs subsets exist eAT, RNAseq analysis revealed that they have distinct transcriptome and the ATM subsets from lean state are clustered separately from their obese counterparts, implying that the obese microenvironment can shape the transcriptomics of cells residing in it. Furthermore, the transcriptome of lean MHCII^{low} and MHCII⁺CD11c⁻ and obese MHCII^{low} ATMs was comparable, but was distant from the other two obese MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATM subsets, suggesting that MHCII^{low} ATMs can preserve their transcriptomics during the progression of obesity.

Further GSEA analysis showed that MHCII^{low} ATMs exhibit specific upregulation of Alox15 and the Arachidonic acid metabolic pathway related genes. The qPCR result further confirmed the specific expression of Alox15 in MHCII^{low} ATMs. Arachidonate 15-lipoxygenase (Alox15) is a member of the LOX family, it can oxygenate polyunsaturated fatty acids and ester lipid-protein assemblies into physiological and pathological metabolites [105]. The specific function of Alox15 expressing macrophages is still not well understood. As Alox15 had been shown to be involved in resolving inflammation, promoting the clearance of debris as well as necrotic cells, and there is evidence suggested that Alox15 expressing ATMs play important roles in the adipogenic differentiation of pre-adipocytes and adipose tissue remodelling [106, 148, 149]. At the same time, there are also several studies suggested that Alox15 plays crucial functions in the chronic inflammation and pathogenesis of the adipose tissue [150]. For example, upregulation of Alox15 in eAT can promotes the insulin resistance and pro-inflammatory cytokines secretion, whereas, ablation of Alox15 can lead to the prevention of development of obesity induced metabolic disorders [109, 150]. Therefore, further investigation of Alox15 expressing ATMs during the progression of obesity is still needed.

Another interesting gene specifically upregulated in the MHCII^{low} ATMs is the neurotransmitter receptor *Adrb2* (encoding β_2 adrenergic receptors). Previous studies showed that *Adrb2* expressing gut macrophages reside closely to the neuronal networks and can activate the neurons in intestine to perform protective function during infection [151]. The high expression of *Adrb2* in MHCII^{low} ATMs indicates that they may have a

close localization to the neuronal networks and could response to the neural signals. However, this needs to be verified and further investigated since it has been reported that MHC^{low} cells are vascular-associated and an association with nerves has not been reported yet. [76, 83].

There were also few other cellular surface makers specifically expressed on MHCII^{low} ATMs, such as the B7 family-related protein v-set and immunoglobulin domain containing 4 (VSIG4), its expression on MHCII^{low} ATMs was further upregulated during the progression of obesity. VSIG4 had been found to be expressed by resting macrophages, including peritoneal macrophages and liver Kupper cells [152]. Recent studies suggested that mice are susceptible to HFD induced obesity and insulin resistance after removing *Vsig4* gene and VSIG4 can inhibit macrophage-mediated inflammation through reprogramming the metabolism of mitochondrial pyruvate [152]. Furthermore, there was also evidence indicates that VSIG4 expressing macrophages are involved in the diabetes resistance [152, 153]. Taken together, the MHCII^{low} ATMs may play an important anti-inflammatory and diabetes resistant role during the progression of obesity.

Analysing in more details the other two ATM subsets, we observed that the MHCII⁺CD11c⁺ ATMs exhibited significant upregulation of the transcriptional signature of lipid uptake, TCA cycle and electron transport chain as well as oxidative phosphorylation pathways. The flow cytometry and qPCR analysis further confirmed the specific upregulation of CD36, LPL and Fabp4 in MHCII⁺CD11c⁺ ATMs. Moreover, the MHCII⁺CD11c⁺ ATMs also displayed high intracellular lipid content when compared to other ATM subsets. Since previous studies established that obesity can activate the lysosome biogenesis in ATMs to buffer away the free fatty lipids from local microenvironment [77], the cellular lipid found in the MHCII⁺CD11c⁺ ATMs may from the phagocytosis of necrotic adipocytes or exocytosis of triglyceride (TG) droplets or ATMs-adipocyte transfer [77]. All these evidences indicate that MHCII⁺CD11c⁺ ATMs may play a crucial and fundamental role in homeostatic handling of lipid in eAT.

4.6 Regulation of adipose tissue metabolism by distinct ATM subpopulations

It has been demonstrated that metabolites, such as fatty acids, ceramides and cholesterol crystals, can target ATMs by eliciting pro-inflammatory responses through pathogen-

sensing signalling pathways, which often leads to a state of unresolved inflammation and eventually to a series of metabolic pathologies, including insulin resistance, fatty liver, atherosclerosis and dyslipidaemia [154]. Therefore we investigated the physiological importance and metabolic roles of ATMs in the eAT during the development of obesity. Two different mouse depletion models were used to assess the contribution of specific ATM subpopulations: CD169-DTR mice for a total depletion of ATMs and anti-CSF-1R antibody treated mice for selective depletion of MHCII^{low} and MHCII⁺CD11c⁻ ATMs without affecting the presence of the newly infiltrated MHCII⁺CD11c⁺ ATMs.

When comparing the two models, DT-treated CD169-DTR mice, but not the anti-CSF1R antibody injected mice reduced their body weight over the treatment period as well as these mice showed lower blood glucose levels at the end of the 12-days depletion period. Considering that in both animal models the macrophage depletion is not only affecting the adipose tissue but all the tissue-resident macrophages in other organs as well, such as liver, lungs, skin, heart and others [93, 95], the ablation of tissue-resident macrophages using the CD169-DTR mouse model could lead to other systemic effects.

With respect to the adipose tissue, histological analysis showed dramatic adipocyte hypertrophy in absence of ATMs in lean but even more pronounced in obese CD169-DTR mice when compared to the corresponding WT mice. In contrast, selective depletion of two ATM subpopulations (MHCII^{low} and MHCII⁺CD11c⁻ ATMs) caused a reduction in adipocyte size, probably due to the upregulated hyperplasia, which can be indicated by the upregulated adipogenic related genes (*Fabp4* and *Pparγ*) in the anti-CSF1R antibody treated mice when compared to the WT mice.

Hypertrophy of adipocytes, a characteristic of dysfunctional adipose tissue, is an adaptive process to buffer away an excess nutrients protecting other organs from lipotoxicity. In obese situation, extreme hypertrophy of adipocytes positively correlated with reduced metabolic flexibility, increased cellular stress and type 2 diabetes in both human and mice [155-157]. Two possible mechanisms had been found to be related to the increased adipocyte hypertrophy during obesity: (1) reduced fibrosis during the progression of obesity and (2) a relative small pool of preadipocytes that cannot generate enough new adipocytes [157]. For the first mechanisms, it is well known that excess of

extracellular matrix (ECM) deposit as fibrosis in adipose tissue to limit adipocyte hypertrophy, promote hyperplasia and play a beneficial role during the progression of obesity [157, 158]. Recent studies also suggested that macrophages can regulate the ECM production by affecting other cell types, such as fibroblasts and myofibroblasts [159]. Therefore, the increased adipocyte hypertrophy in CD169-DTR mice may due to the reduction of fibrosis caused by ATM removal in eAT.

The adipose tissue microenvironment consists of adipocytes, leukocytes (myeloid cells and lymphocytes), blood vessels, nerves, fibroblasts, preadipocytes, biological mediators such as cytokines, adipokines and chemokines. Different cell types and biological mediators interact actively to maintain the tissue homeostasis and support the normal function of the tissue [160]. Among these biological mediators, some factors secreted by adipose tissue, including adipokines (adiponectin and leptin), several cytokines (e.g., IL-6) and chemokines (CCL2 and CCL5), play crucial role in regulating inflammation, lipid metabolism, insulin sensitivity and energy homeostasis in the tissue [18]. For example, leptin can control appetite and its circulating level is positively correlated with obesity, therefore a loss of leptin signalling pathway will lead to massively obese [18, 19]. In contrast, adiponectin is an anti-inflammatory adipokine and it can inhibit the production of IL-6 and promote the IL-10 secretion to protect against the obesity induced complications, therefore its circulating level is negatively correlated with obesity [23]. These functions make adipocytes become the key players orchestrating local changes in the microenvironment. However, more evidences also highlighted the pivotal role of macrophages in adipose tissue remodelling, as recent studies established that ATMs are involved in clearing dead adipocytes and buffer away FFAs hence actively participating in lipid metabolism [31, 79]. Not surprisingly, after removing ATMs, we observed remarkable diminished adipokines (adiponectin and leptin), upregulated chemokines and cytokines in the CD169-DTR mice when compared to the WT mice, implying the importance of ATMs for supporting a normal function of the tissue microenvironment.

Lipid metabolism is a process involving the synthesis and degradation of lipid metabolites in cells, its primary function is to deliver lipids to peripheral organs for usage or recycle the lipids in liver [161]. It mainly consists of triglyceride, cholesterol, glycolipid and phospholipid metabolism [162]. Many factors, including proteases and

transcription factors, are involved in these metabolic processes [162]. Recent studies showed that ATMs can buffer away extra free fatty lipids through the scavenger receptor such as CD36 and LOX-1 expressed on their surface and clean up the dead lipid-laden adipocytes to actively participate in the lipid metabolism in the tissue [77, 161, 163]. Given that adipokines, lipid metabolism and adipogenesis related genes all diminished in absence of ATMs, with CD169-DTR mice showing a more dramatic effect when compared to anti-CSF1R antibody treated mice, it is not surprise that the lipid metabolism was dramatically perturbed after removing all the three ATM subsets. The perturbation of lipid metabolism in the eAT, with some lipid metabolites significantly upregulated and other lipid metabolites remarkably downregulated after removing ATMs in these two mouse models indicates that ATMs (all the three subsets, especially the MHCII⁺CD11c⁺ ATMs) play crucial and fundamental roles in homeostatic lipid handling in eAT.

In summary, our study demonstrate that three phenotypically distinct ATM subsets present in eAT, showing characteristic half-lives, turnovers and different frequencies during the progression of obesity. Our findings also suggest that the MHCII^{low} ATMs have the potential of anti-inflammatory functions, whereas MHCII⁺CD11c⁺ ATMs are actively involved in the lipid metabolism. These findings support a better understanding of the ontogeny, turnover rates and mechanisms of the maintenance and functional differences between distinct ATM subpopulations, which will eventually help develop innovative therapeutic approaches targeting ATM subset to prevent obesity-related complications.

5 Conclusion

Immune cell infiltration into the adipose tissue during the progression of obesity is closely related to the local chronic inflammation and the metabolic disorders. In particular, ATMs, one of the most abundant leukocytes present in eAT, are not only the key players for the maintenance of the tissue homeostasis, but they are also involved in the control of metabolic dysregulation during aging and obesity. Here, we have shown that three phenotypically distinct F4/80^{hi}CD11b⁺ ATM subsets present in eAT (MHCII^{low}, MHCII⁺CD11c⁻ and MHCII⁺CD11c⁺ ATMs), displaying different morphology and frequencies during the progression of obesity. The newly recruited MHCII⁺CD11c⁺ ATMs during obesity exhibit high cellular lipid content and a fast turnover rate, while the MHCII^{low} ATMs display BM independency and can self-maintain locally through the CSF1-CSF1R axis. Moreover, all the three ATM subpopulations show different transcriptomic profiles, with MHCII⁺CD11c⁺ ATMs significantly characterized with lipid metabolism associated genes. Further in vivo studies show that MHCII⁺CD11c⁺ ATMs play key roles for the homeostasis and lipid metabolism of eAT and those ATM subsets are functionally distinct. We also demonstrated that the microenvironment of the adipose tissue influences the presence of distinct ATM subpopulations showing characteristic half-lives and turnovers. In lean but even more important in obese situation, the fetal-derived MHCII^{low} macrophages are maintained and not replaced by infiltrating-monocytes due to their important function as regulatory and homeostatic macrophages. On the contrary, with the progression of FFA accumulation and inflammation, a fast recruitment of specialized monocyte-derived macrophages is initiated and maintained to control the excess of adipocyte-released lipids caused during obesity.

6 Appendix

6.1 Permission to reuse Table 1.1

SPRINGER NATURE LICENSE TERMS AND CONDITIONS	
Aug 12, 2019	
<p>This Agreement between Ms. Chen Qi ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.</p>	
License Number	4646390001763
License date	Aug 12, 2019
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Nature Reviews Immunology
Licensed Content Title	Adipokines in inflammation and metabolic disease
Licensed Content Author	Noriyuki Ouchi, Jennifer L. Parker, Jesse J. Lugus, Kenneth Walsh
Licensed Content Date	Jan 21, 2011
Licensed Content Volume	11
Licensed Content Issue	2
Type of Use	Thesis/Dissertation
Requestor type	non-commercial (non-profit)
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Will you be translating?	no
Circulation/distribution	<501
Author of this Springer Nature content	no
Title	PhD
Institution name	Nanyang Technological University
Expected presentation date	Aug 2020
Order reference number	1
Portions	Table 1
Requestor Location	Ms. Chen Qi 60 Nanyang Drive School of Biological Sciences Singapore, 637551 Singapore Attn: Ms. Chen Qi
Total	0.00 USD

6.2 Permission to reuse Figure 1.3

SPRINGER NATURE LICENSE TERMS AND CONDITIONS	
Aug 12, 2019	
<p>This Agreement between Ms. Chen Qi ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.</p>	
License Number	4646390863844
License date	Aug 12, 2019
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Nature Reviews Endocrinology
Licensed Content Title	The obese adipose tissue microenvironment in cancer development and progression
Licensed Content Author	Daniela F. Quail et al
Licensed Content Date	Nov 20, 2018
Type of Use	Thesis/Dissertation
Requestor type	non-commercial (non-profit)
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Will you be translating?	no
Circulation/distribution	<501
Author of this Springer Nature content	no
Title	PhD
Institution name	Nanyang Technological University
Expected presentation date	Aug 2020
Order reference number	2
Portions	Figure 2
Requestor Location	Ms. Chen Qi 60 Nanyang Drive School of Biological Sciences Singapore, 637551 Singapore Attn: Ms. Chen Qi
Total	0.00 USD

6.3 Permission to reuse Figure 1.5

ELSEVIER LICENSE TERMS AND CONDITIONS	
Aug 12, 2019	
This Agreement between Ms. Chen Qi ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.	
License Number	4646391489620
License date	Aug 12, 2019
Licensed Content Publisher	Elsevier
Licensed Content Publication	Immunity
Licensed Content Title	Most Tissue-Resident Macrophages Except Microglia Are Derived from Fetal Hematopoietic Stem Cells
Licensed Content Author	Jianpeng Sheng, Christiane Ruedl, Klaus Karjalainen
Licensed Content Date	Aug 18, 2015
Licensed Content Volume	43
Licensed Content Issue	2
Licensed Content Pages	12
Start Page	382
End Page	393
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	3
Original figure numbers	Graphical Abstract Figure
Title of your thesis/dissertation	PhD
Publisher of new work	Nanyang Technological University
Expected completion date	Aug 2020
Estimated size (number of pages)	1
Requestor Location	Ms. Chen Qi 60 Nanyang Drive School of Biological Sciences Singapore, 637551 Singapore Attn: Ms. Chen Qi
Publisher Tax ID	GB 494 6272 12
Total	0.00 USD
Terms and Conditions	

6.4 Permission to reuse Figure 1.6

SPRINGER NATURE LICENSE TERMS AND CONDITIONS	
Aug 12, 2019	
This Agreement between Ms. Chen Qi ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.	
License Number	4646400491578
License date	Aug 12, 2019
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Nature Biotechnology
Licensed Content Title	Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice
Licensed Content Author	Michiko Saito et al
Licensed Content Date	Aug 1, 2001
Type of Use	Thesis/Dissertation
Requestor type	non-commercial (non-profit)
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Will you be translating?	no
Circulation/distribution	<501
Author of this Springer Nature content	no
Title	PhD
Institution name	Nanyang Technological University
Expected presentation date	Aug 2020
Order reference number	4
Portions	Figure 1 A
Requestor Location	Ms. Chen Qi 60 Nanyang Drive School of Biological Sciences Singapore, 637551 Singapore Attn: Ms. Chen Qi
Total	0.00 USD

6.5 Antibodies used for FACS

Anti-mouse antibody	Clone	Company	Label
Ly6-C	HK1.4	Biolegend	BV605
F4/80	BM8	eBioscience	PE
CD45.2	104	eBioscience	PE-Cy7
CD11c	N418	eBioscience	APC
MHCII	M5/114.15.2	eBioscience	eFluor 450
CCR2	---	R&D system	APC
CD11b	M1/70	BD Biosciences	BuV395
CD45	30F11	Biolegend	APCFire
Ly6G	1A8	Biolegend	FITC
Fc	2.4G2	BD Biosciences	--
CD24	M1/16	Biolegend	PE-Cy7
CD24	M1/16	Biolegend	BV510
Tim-4	RMT4-54	Biolegend	PE-Cy7
CD103	2E7	Biolegend	Percep-cy5.5
CD206	Co68C2	Biolegend	FITC
MGL1/2	LOM-14	Biolegend	Percep-cy5.5
LYVE-1	ALY7	eBiosciences	eFluor 660
LYVE-1	ALY7	eBiosciences	FITC
CD3	145-2C11	Biolegend	FITC
CD4	GK1.5	Biolegend	APC-Cy7
CD8	53-6.7	Biolegend	Percep-cy5.5
$\gamma\delta$ TCR	GL3	Biolegend	PE
Foxp3	FJK-16S	Biolegend	APC
B220	RA3-6B2	Biolegend	BV605
CD19	1D3	Bolegend	BUV395
CD169	SER-4	eBioscience	APC
MerTK	108928	R&D systems	APC

6.6 Antibody for immunofluorescence staining

Primary antibody	Clone	Company
------------------	-------	---------

Rat Anti-CD68	FA-11	Bio-rad
---------------	-------	---------

Secondary antibody	Catalog number	Company
--------------------	----------------	---------

Goat anti-hamster AF488	ab173003	Abcam
-------------------------	----------	-------

Donkey anti-rabbit AF555	ab150074	Abcam
--------------------------	----------	-------

Anti-hamster AF647	127-605-160	JacksonImmuno
--------------------	-------------	---------------

6.7 Materials and reagents

ACCU-CHEK Performa test strips	Roche, Basel, Switzerland
Acetone	Fisher Scientific UK, Loughborough, UK
Ammonium chloride	Sigma, St. Louis, Mo, USA
Anti-CSF1R blocking antibody	BioXCell, West Lebanon, USA
Arcturus TM PicoPure TM RNA	
Isolation Kit	Thermo Scientific, Waltham, MA, USA
BODIPY	Thermo Scientific, Waltham, MA, USA
Bovine Serum albumin (BSA)	Sigma, St. Louis, Mo, USA
Chloroform	Fisher Scientific UK, Loughborough, UK
Collagenase D	Roche, Basel, Switzerland
Corn oil	Sigma, St. Louis, Mo, USA
DAKO Mounting Medium	Dako, Golstrup, Denmark
DAPI	Thermo Scientific, Waltham, MA, USA
Dihydroethidium	Sigma, St. Louis, Mo, USA
Diphtheria Toxin	Sigma, St. Louis, Mo, USA
DNase I	Life Technologies, Carlsbad, CA, USA
DNase I	Fermentas, Waltham, MA, USA
DNA gel loading dye (6×)	Thermo Scientific, Waltham, MA, USA
dNTPs	Thermo Scientific, Waltham, MA, USA
Dox-Normal diet	Envigo, Huntingdon, UK
Dox-High fat diet (60 % fat)	Envigo, Huntingdon, UK
DPX	Sigma, St. Louis, Mo, USA
Dimethyl sulfoxide (DMSO)	Sigma, St. Louis, Mo, USA
D-(+)- Glucose	Sigma, St. Louis, Mo, USA
Eco 48 real time plate	Bibby Scientific, OSA, USA
Ethanol	Merck KGaA, Darmstadt, Germany
FAST 2× qPCR master mix	PrecisionFAST-SY, Primerdesign Ltd, Cambridge, UK
Fetal Bovine Serum	Biowest, Nuaieue, France
Foxp3 Transcription factor staining buffer Kit	eBioscience, San Diego, CA, USA
Gelatin	Sigma, St. Louis, Mo, USA

Giemsa	Sigma, St. Louis, Mo, USA
High fat diet (60 % fat)	Research Diets, Inc, USA
High fat diet (60 % fat)	Envigo, Huntingdon, UK
Insulin syringe	BD, San Diego, CA, USA
IMDM	Gibco, Grand Island, NY, USA
Isoflurane	Piamal Healthcare, India
Isopropanol	Merk KGaA, Darmstadt, Germany
methanol	Merk KGaA, Darmstadt, Germany
Mouse Adiponectin ELISA Kit	Crystal Chem, USA
Nail polish	Drugstores
O.C.T. compound	Sakura Finetek, Torrance, CA, USA
Oligo dT	Promega, Fitchburg, WI, USA
Ovation Pico WTA System V2	NuGEN, Technologies, San Carlos, CA, USA V2
Paraffin wax	Leica Biosystems, Wetzlar, Germany
Paraformaldehyde	Sigma, St. Louis, Mo, USA
PBS 10 ×	Gibco, Grand Island, NY, USA
Progesterone	Sigma, St. Louis, Mo, USA
Reverse Transcriptase	Promega, Fitchburg, WI, USA
Rnase free water	Ambion Thermo Fisher Scientific, Waltham, MA, USA
RNAsimple Total RNA Kit	TIANGEN Biotech Co Ltd, Beijing, China
RNaseZAP TM	Sigma, St. Louis, Mo, USA
Quant-iTRiboGreen RNA Assay kit	Life Technologies, Carlsbad, CA, USA
Rnase inhibitor	Thermo Scientific, Waltham, MA, USA
Sodium chloride	Merck KGaA, Darmstadt, Germany
Sodium citrate	Sigma, St. Louis, Mo, USA
Sucrose	Affymetrix USB, Cleveland, OH, USA
Tamoxifen	Sigma, St. Louis, Mo, USA
Trypan blue	Sigma, St. Louis, Mo, USA
TRIzol Reagent	Life technologies, Carlsbad, CA, USA
Xylene	Sigma, St. Louis, Mo, USA

1 ml RNase free tips	Molecular Bioproducts, Fisher Scientific
1.5 ml tube	Eppendorf
1.5 ml tube	Greinerbio-one, Frickenhausen, Germany
5 ml polystyrene round-bottom tube	Thermo Scientific, Waltham, MA, USA
10 µl RNase free tips	Thermo Scientific, Waltham, MA, USA
10 % Formalin	Sigma, St. Louis, Mo, USA
15 ml falcon tube	Greinerbio-one, Frickenhausen, Germany
50 ml falcon tube	Greinerbio-one, Frickenhausen, Germany
96 well flat plate	Thermo Scientific, Waltham, MA, USA
96 well V-bottom plate	Thermo Scientific, Waltham, MA, USA
200 µl RNase free tips	Thermo Scientific, Waltham, MA, USA

6.8 Primer sequences for qPCR

Primers	Sequence 5'-3'
Actin-F Actin-R	AAG GCC AAC CGT GAA AAG AT CCT GTG GTA CGA CCA GAG GCA TAC A
Adiponectin-F Adiponectin-R	CGATTGTCAGTGGATCTGACG CAACAGTAGCATCCTGAGCCCT
Fabp4-F Fabp4-R	ACAAGCTGGTGGTGGGAATGTG CCTTTGGCTCATGCCCTTT
Leptin-F Leptin-R	GAGACCCCTGTGTCGGTTC CTGCGTGTGTGAAATGTCATTG
LPL-F LPL-R	CAACAAGGTCAGAGCCAAGAGAAG GTTGTGTTGCTTGCCATCCTC
PPAR γ -F PPAR γ -R	GTGCCAGTTTCGATCCGTAGA GGCCAGCATCGTGTAGATGA
PPAR α -F PPAR α -R	AGAGCCCCATCTGTCCTCTC ACTGGTAGTCTGCAAACCAAA
Retn-F Retn-R	CCTTGTCCTGAAGTGTGCTGG TGTCCAGCAATTAAAGCCAATGTTC
Intelectin-1-F Intelectin-1-R	CAGAAGCTGCAAGGAAATCAAG TGTTGTTCTCATGCACACTAGC
INSR-F INSR-R	CACTTCAAATGCAGGAAGTACACG GTCTTCTCACCTTCGAGGATTTG
GLUT4-F GLUT4-R	GTGACTGGAACACTGGTCCTA CCAGCCAGTTGCATTGTAG
Chemerin-F Chemerin-R	CAAACAGCCACTACCAGAACAAC GGAGACCTTTCCTGTTCACC
CD36-F CD36-R	GCCAAGCTATTGCGACATGA CTGCAAATGTCAGAGGAAAAGAATC
LIPE-F LIPE-R	CCAGCCTGAGGGCTTACTG CTCCATTGACTGTGACATCTCG
ABCA-1-F ABCA-1-R	CCAAAGAGATACCAGCATTAAGGAC CAGGCTGTCCACAGTAGATC
Alox15-F Alox15-R	CAACTCTTGCCTATAGCCATCCAG GCAAAGACCTCAGCCACCAAG
mAdrb2-F mAdrb2-R	TGGTGGTGTGATGGTCTTTGTCTATTC GAACTTGAGGACCTTCGGAG
Alox12-F Alox12-R	TCTTGTCATGCTGAGGATGGACC GCCAAGAGCCAGGCAAGTG

6.9 Equipment

Cytospin TM Cytocentrifuge	Thermo Scientific, Waltham, MA, USA
Eco Real-Time PCR system	Illumina, San Diego, CA, USA
Eppendorf Centrifuge 5810R	Sigma, St. Louis, Mo, USA
Eppendorf TM Microcentriguge	Thermo Scientific, Waltham, MA, USA
Eppendorf ThermoMixer	Thermo Scientific, Waltham, MA, USA
Fortessa LSR 5 laser X-20 cell analyzer	BD Biosciences, San Jose, CA, USA
FACSAria TM cell sorter	BD Biosciences, San Jose, CA, USA
Glucose Meter	Roche, Basel, Switzerland
Incubator shaker Innova 4200	Edison, NJ, USA
Leica CM3050S-Cryostat	Leica Microsystems, Wetzlar, Germany
Leica microtome blades	Leica Microsystems, Wetzlar, Germany
Microscope Nikon Eclipse 80i	Nikon Instruments, Melville, NY, USA
Microscope coverslips	CellPath Ltd, Newton, UK
Nano Drop ONE	Thermo Scientific, Waltham, MA, USA
100 µm Falcon Cell strainer	BD Biosciences, San Jose, CA, USA

6.10 Sorting efficiency

Figure 6.1: Sorting efficiency of ATM subsets and P3 monocyte-derived cells in adipose tissue. Three subsets of ATMs and monocyte-derived cells (P3) were sorted, the percentages exhibit in the figures show the purity of the sorted cells. Fr I: F4/80^{hi} CD11b⁺ ATMs; Fr II: F4/80^{int} cells; P3: monocyte-derived cells.

6.11 CD169 expression on myeloid cell populations in adipose tissue

Figure 6.2: CD169 is specifically expressed on ATM subsets. CD169 expression was checked on different myeloid cell populations, including ATM subsets and monocytes and monocyte-derived cells. Left and right side: representative plot figures show the expression of CD169 in ND and HFD myeloid cell subsets respectively (n=3 for each group). Colour labelling: blue: control; red: CD169 staining; P1: monocytes; P2 and P3: monocyte-derived cells.

6.12 Pictures of eAT from obese WT and CD169-DTR mice

Figure 6.3: Zoom in pictures of eAT from obese WT and CD169-DTR mice. Left: eAT from WT mice. Right: eAT from CD169-DTR mice. Transparent and oily eAT can be seen in the CD169-DTR mice eAT when compared to the WT mice.

6.13 CD64 and CD206 expression on monocyte-derived cells in eAT

Figure 6.4: CD64 and CD206 expression on monocyte-derived cells (P3). P3 cells were gated according to the gating strategy shown in Figure 3.3. Left: CD64 expression on P3 cells. Right: CD206 expression on P3 cells. Colour labelling: grey: isotype control; pink: antibody staining on P3 cells (n=3 for each group).

6.14 Trem2 expression in sorted eAT myeloid cells

Figure 6.5: Gene expression of Trem-2 in different myeloid cell populations from lean and obese mice. Data was normalized to the expression of β -actin and shown as relative fold changes to the gene expression level of lean P3 cells. Trem2: triggering receptor expressed on myeloid cells 2; P3: monocyte-derived cells. Statistical significance was determined by using Bonferroni two-way analysis of variance (ANOVA). * $P < 0.05$; ns: no significance.

7. Author's publications

Sheng J., **Chen Q.**, Soncin I., Ng S.I., Karjalainen K., Ruedl C. A discrete subset of monocyte-derived cells among typical conventional type 2 dendritic cells can efficiently cross-present. *Cell Rep.* 2017 Oct 31; 21(5):1203-1214.

Soncin I., Sheng J., **Chen Q.**, Foo S., Duan K., Lum J., Poidinger M., Zolezzi F., Karjalainen K., Ruedl C. The tumor microenvironment creates a niche for the self-renewal of tumor-promoting macrophages in colon adenoma. *Nat Commun.* 2018 Feb 8; 9(1):582.

8 Posters and invited oral presentations

Year 2019:

17th International Congress of Immunology, Beijing, China, October, 2019.

Poster presentation: How obesity changes the dynamics, diversity and function of macrophages in the adipose tissue: search for culprits.

Chen Q, Ruedl C.

11th International Singapore Symposium of Immunology, Singapore, May, 2019.

Participant

8th Winter School in Advanced Immunology IFRc-SIGN, Singapore, January, 2019.

Oral and poster presentation: Developmental origins of distinct adipose tissue macrophages in health and disease.

Chen Q, Ruedl C.

Year 2018:

6th Advanced Singaporean Immunology PhD and postdoctoral Retreat, Singapore, November, 2018

Oral and poster presentation: Developmental origins of distinct adipose tissue macrophages in health and disease.

Chen Q, Ruedl C.

5th European Congress of Immunology, Amsterdam, September, 2018.

Poster presentation: Unravel of ontogeny and function of adipose tissue macrophage subpopulations in health and obesity.

Chen Q, Ruedl C.

10th International Singapore Symposium of Immunology, Singapore, May 2018

Poster presentation: Unravel of ontogeny and function of adipose tissue macrophage subpopulations in health and obesity.

Chen Q, Ruedl C.

Year 2016:

5th Advanced Singaporean Immunology PhD student Retreat, Singapore, September 2016.

Poster presentation: CD169⁺ tissue-resident macrophage during intestinal cancer.

Chen Q, Sheng J, Ruedl C.

8th International Singapore Symposium of Immunology, Singapore, May 2016.

Poster presentation: Role of intestinal myeloid cell subsets in colorectal cancer.

Soncin I, **Chen Q**, Foo HR, Sheng J, Ruedl C.

9 Awards

2018: Conference travel award, European Congress of Immunology.

10 Reference:

1. Park, A., W.K. Kim, and K.H. Bae, *Distinction of white, beige and brown adipocytes derived from mesenchymal stem cells*. World J Stem Cells, 2014. **6**(1): p. 33-42.
2. Saely, C.H., K. Geiger, and H. Drexel, *Brown versus white adipose tissue: a mini-review*. Gerontology, 2010. **58**(1): p. 15-23.
3. Timmons, J.A., et al., *Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages*. Proc Natl Acad Sci U S A, 2007. **104**(11): p. 4401-6.
4. Gesta, S., Y.H. Tseng, and C.R. Kahn, *Developmental origin of fat: tracking obesity to its source*. Cell, 2007. **131**(2): p. 242-56.
5. Otto, T.C. and M.D. Lane, *Adipose development: from stem cell to adipocyte*. Crit Rev Biochem Mol Biol, 2005. **40**(4): p. 229-42.
6. Tang, Q.Q. and M.D. Lane, *Adipogenesis: from stem cell to adipocyte*. Annu Rev Biochem, 2012. **81**: p. 715-36.
7. Rosen, E.D. and O.A. MacDougald, *Adipocyte differentiation from the inside out*. Nat Rev Mol Cell Biol, 2006. **7**(12): p. 885-96.
8. Waki, H. and P. Tontonoz, *Endocrine functions of adipose tissue*. Annu Rev Pathol, 2007. **2**: p. 31-56.
9. Luo, L. and M. Liu, *Adipose tissue in control of metabolism*. J Endocrinol, 2016. **231**(3): p. R77-R99.
10. Smith, S.J., et al., *Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat*. Nat Genet, 2000. **25**(1): p. 87-90.
11. Kolditz, C.I. and D. Langin, *Adipose tissue lipolysis*. Curr Opin Clin Nutr Metab Care, 2010. **13**(4): p. 377-81.
12. Lafontan, M. and D. Langin, *Lipolysis and lipid mobilization in human adipose tissue*. Prog Lipid Res, 2009. **48**(5): p. 275-97.
13. Zechner, R., et al., *Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores*. J Lipid Res, 2009. **50**(1): p. 3-21.
14. Zimmermann, R., et al., *Fate of fat: the role of adipose triglyceride lipase in lipolysis*. Biochim Biophys Acta, 2009. **1791**(6): p. 494-500.
15. Fantuzzi, G., *Adipose tissue, adipokines, and inflammation*. J Allergy Clin Immunol, 2005. **115**(5): p. 911-9; quiz 920.
16. Trayhurn, P. and J.H. Beattie, *Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ*. Proc Nutr Soc, 2001. **60**(3): p. 329-39.
17. Zhang, Y., et al., *Positional cloning of the mouse obese gene and its human homologue*. Nature, 1994. **372**(6505): p. 425-32.
18. Tartaglia, L.A., et al., *Identification and expression cloning of a leptin receptor, OB-R*. Cell, 1995. **83**(7): p. 1263-71.
19. Elmquist, J.K., *Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin*. Physiol Behav, 2001. **74**(4-5): p. 703-8.
20. Bjorbaek, C. and B.B. Kahn, *Leptin signaling in the central nervous system and the periphery*. Recent Prog Horm Res, 2004. **59**: p. 305-31.
21. Sivitz, W.I., B.D. Fink, and P.A. Donohoue, *Fasting and leptin modulate adipose and muscle uncoupling protein: divergent effects between messenger ribonucleic acid and protein expression*. Endocrinology, 1999. **140**(4): p. 1511-9.

22. Minokoshi, Y., et al., *Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase*. Nature, 2002. **415**(6869): p. 339-43.
23. Chandran, M., et al., *Adiponectin: more than just another fat cell hormone?* Diabetes Care, 2003. **26**(8): p. 2442-50.
24. Cottam, D.R., et al., *The chronic inflammatory hypothesis for the morbidity associated with morbid obesity: implications and effects of weight loss*. Obes Surg, 2004. **14**(5): p. 589-600.
25. Fain, J.N., et al., *Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans*. Endocrinology, 2004. **145**(5): p. 2273-82.
26. Hotamisligil, G.S., et al., *Tumor necrosis factor alpha inhibits signaling from the insulin receptor*. Proc Natl Acad Sci U S A, 1994. **91**(11): p. 4854-8.
27. Hausman, D.B., et al., *The biology of white adipocyte proliferation*. Obes Rev, 2001. **2**(4): p. 239-54.
28. Gregoire, F.M., C.M. Smas, and H.S. Sul, *Understanding adipocyte differentiation*. Physiol Rev, 1998. **78**(3): p. 783-809.
29. Castoldi, A., et al., *The Macrophage Switch in Obesity Development*. Front Immunol, 2016. **6**: p. 637.
30. Nagareddy, P.R., et al., *Adipose tissue macrophages promote myelopoiesis and monocytosis in obesity*. Cell Metab, 2014. **19**(5): p. 821-35.
31. Haka, A.S., et al., *Exocytosis of macrophage lysosomes leads to digestion of apoptotic adipocytes and foam cell formation*. J Lipid Res, 2016. **57**(6): p. 980-92.
32. Wellen, K.E. and G.S. Hotamisligil, *Inflammation, stress, and diabetes*. J Clin Invest, 2005. **115**(5): p. 1111-9.
33. McArdle, M.A., et al., *Mechanisms of obesity-induced inflammation and insulin resistance: insights into the emerging role of nutritional strategies*. Front Endocrinol (Lausanne), 2013. **4**: p. 52.
34. Youm, Y.H., et al., *The Nlrp3 inflammasome promotes age-related thymic demise and immunosenescence*. Cell Rep, 2012. **1**(1): p. 56-68.
35. Trayhurn, P., *Hypoxia and adipose tissue function and dysfunction in obesity*. Physiol Rev, 2013. **93**(1): p. 1-21.
36. Lee, Y.S., et al., *Increased adipocyte O2 consumption triggers HIF-1alpha, causing inflammation and insulin resistance in obesity*. Cell, 2014. **157**(6): p. 1339-52.
37. Shi, H., et al., *TLR4 links innate immunity and fatty acid-induced insulin resistance*. J Clin Invest, 2006. **116**(11): p. 3015-25.
38. Amar, J., et al., *Intestinal mucosal adherence and translocation of commensal bacteria at the early onset of type 2 diabetes: molecular mechanisms and probiotic treatment*. EMBO Mol Med, 2011. **3**(9): p. 559-72.
39. Steven, J.L., *Metchnikoff on the Comparative Pathology of Inflammation*. Glasgow Med J, 1892. **38**(3): p. 195-205.
40. Geissmann, F., et al., *Development of monocytes, macrophages, and dendritic cells*. Science, 2010. **327**(5966): p. 656-61.
41. Gordon, S. and P.R. Taylor, *Monocyte and macrophage heterogeneity*. Nat Rev Immunol, 2005. **5**(12): p. 953-64.
42. Elinav, E., et al., *Regulation of the antimicrobial response by NLR proteins*. Immunity, 2011. **34**(5): p. 665-79.

43. Kawai, T. and S. Akira, *Toll-like receptors and their crosstalk with other innate receptors in infection and immunity*. Immunity, 2011. **34**(5): p. 637-50.
44. Osorio, F. and C. Reis e Sousa, *Myeloid C-type lectin receptors in pathogen recognition and host defense*. Immunity, 2011. **34**(5): p. 651-64.
45. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets*. Nat Rev Immunol, 2011. **11**(11): p. 723-37.
46. T'Jonck, W., M. Guilliams, and J. Bonnardel, *Niche signals and transcription factors involved in tissue-resident macrophage development*. Cell Immunol, 2018. **330**: p. 43-53.
47. Mowat, A.M., C.L. Scott, and C.C. Bain, *Barrier-tissue macrophages: functional adaptation to environmental challenges*. Nat Med, 2017. **23**(11): p. 1258-1270.
48. Scott, C.L., et al., *Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells*. Nat Commun, 2016. **7**: p. 10321.
49. Barnes, M.J. and F. Powrie, *Regulatory T cells reinforce intestinal homeostasis*. Immunity, 2009. **31**(3): p. 401-11.
50. Varol, C., et al., *Intestinal lamina propria dendritic cell subsets have different origin and functions*. Immunity, 2009. **31**(3): p. 502-12.
51. Chen, S., et al., *CNS Macrophages Control Neurovascular Development via CD95L*. Cell Rep, 2017. **19**(7): p. 1378-1393.
52. Boutens, L. and R. Stienstra, *Adipose tissue macrophages: going off track during obesity*. Diabetologia, 2016. **59**(5): p. 879-94.
53. Guilliams, M., et al., *The function of Fcγ receptors in dendritic cells and macrophages*. Nat Rev Immunol. **14**(2): p. 94-108.
54. Gautier, E.L., et al., *Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages*. Nat Immunol, 2012. **13**(11): p. 1118-28.
55. Kurotaki, D., et al., *CSF-1-dependent red pulp macrophages regulate CD4 T cell responses*. J Immunol, 2011. **186**(4): p. 2229-37.
56. Haldar, M., et al., *Heme-mediated SPI-C induction promotes monocyte differentiation into iron-recycling macrophages*. Cell, 2014. **156**(6): p. 1223-1234.
57. Sica, A. and V. Bronte, *Altered macrophage differentiation and immune dysfunction in tumor development*. J Clin Invest, 2007. **117**(5): p. 1155-66.
58. Biswas, S.K. and A. Mantovani, *Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm*. Nat Immunol, 2010. **11**(10): p. 889-96.
59. Odegaard, J.I. and A. Chawla, *Alternative macrophage activation and metabolism*. Annu Rev Pathol, 2010. **6**: p. 275-97.
60. Guilliams, M. and C.L. Scott, *Does niche competition determine the origin of tissue-resident macrophages?* Nat Rev Immunol, 2017. **17**(7): p. 451-460.
61. Gomez Perdiguero, E., et al., *Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors*. Nature, 2014. **518**(7540): p. 547-51.
62. Hoeffel, G., et al., *C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages*. Immunity, 2015. **42**(4): p. 665-78.
63. Sheng, J., C. Ruedl, and K. Karjalainen, *Most Tissue-Resident Macrophages Except Microglia Are Derived from Fetal Hematopoietic Stem Cells*. Immunity, 2015. **43**(2): p. 382-93.

64. Ginhoux, F., et al., *Fate mapping analysis reveals that adult microglia derive from primitive macrophages*. Science, 2010. **330**(6005): p. 841-5.
65. Lumeng, C.N., J.L. Bodzin, and A.R. Saltiel, *Obesity induces a phenotypic switch in adipose tissue macrophage polarization*. J Clin Invest, 2007. **117**(1): p. 175-84.
66. Weisberg, S.P., et al., *CCR2 modulates inflammatory and metabolic effects of high-fat feeding*. J Clin Invest, 2006. **116**(1): p. 115-24.
67. Amano, S.U., et al., *Local proliferation of macrophages contributes to obesity-associated adipose tissue inflammation*. Cell Metab, 2014. **19**(1): p. 162-171.
68. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue*. J Clin Invest, 2003. **112**(12): p. 1796-808.
69. Patsouris, D., et al., *Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals*. Cell Metab, 2008. **8**(4): p. 301-9.
70. Cho, K.W., et al., *Adipose Tissue Dendritic Cells Are Independent Contributors to Obesity-Induced Inflammation and Insulin Resistance*. J Immunol, 2016. **197**(9): p. 3650-3661.
71. LaMarche, N.M. and L. Lynch, *Adipose Dendritic Cells Come Out of Hiding*. Cell Metab, 2018. **27**(3): p. 485-486.
72. Kanda, H., et al., *MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity*. J Clin Invest, 2006. **116**(6): p. 1494-505.
73. Inouye, K.E., et al., *Absence of CC chemokine ligand 2 does not limit obesity-associated infiltration of macrophages into adipose tissue*. Diabetes, 2007. **56**(9): p. 2242-50.
74. Hill, D.A., et al., *Distinct macrophage populations direct inflammatory versus physiological changes in adipose tissue*. Proc Natl Acad Sci U S A, 2018. **115**(22): p. E5096-E5105.
75. Lumeng, C.N., et al., *Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes*. Diabetes, 2008. **57**(12): p. 3239-46.
76. Silva, H.M., et al., *Vasculature-associated fat macrophages readily adapt to inflammatory and metabolic challenges*. J Exp Med, 2019. **216**(4): p. 786-806.
77. Xu, X., et al., *Obesity activates a program of lysosomal-dependent lipid metabolism in adipose tissue macrophages independently of classic activation*. Cell Metab, 2013. **18**(6): p. 816-30.
78. Kratz, M., et al., *Metabolic dysfunction drives a mechanistically distinct proinflammatory phenotype in adipose tissue macrophages*. Cell Metab, 2014. **20**(4): p. 614-25.
79. Coats, B.R., et al., *Metabolically Activated Adipose Tissue Macrophages Perform Detrimental and Beneficial Functions during Diet-Induced Obesity*. Cell Rep, 2017. **20**(13): p. 3149-3161.
80. Jaitin, D.A., et al., *Lipid-Associated Macrophages Control Metabolic Homeostasis in a Trem2-Dependent Manner*. Cell, 2019. **178**(3): p. 686-698 e14.
81. Xu, H., et al., *Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance*. J Clin Invest, 2003. **112**(12): p. 1821-30.
82. Morris, D.L., et al., *Adipose tissue macrophages function as antigen-presenting cells and regulate adipose tissue CD4⁺ T cells in mice*. Diabetes, 2013. **62**(8): p. 2762-72.

83. Chakarov, S., et al., *Two distinct interstitial macrophage populations coexist across tissues in specific subtissular niches*. Science, 2019. **363**(6432).
84. Pirzgalska, R.M., et al., *Sympathetic neuron-associated macrophages contribute to obesity by importing and metabolizing norepinephrine*. Nat Med, 2017. **23**(11): p. 1309-1318.
85. Wellen, K.E. and G.S. Hotamisligil, *Obesity-induced inflammatory changes in adipose tissue*. J Clin Invest, 2003. **112**(12): p. 1785-8.
86. Aron-Wisnewsky, J., et al., *Human adipose tissue macrophages: m1 and m2 cell surface markers in subcutaneous and omental depots and after weight loss*. J Clin Endocrinol Metab, 2009. **94**(11): p. 4619-23.
87. Wentworth, J.M., et al., *Pro-inflammatory CD11c+CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity*. Diabetes, 2010. **59**(7): p. 1648-56.
88. Zeyda, M., et al., *Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production*. Int J Obes (Lond), 2007. **31**(9): p. 1420-8.
89. Zeyda, M., et al., *Newly identified adipose tissue macrophage populations in obesity with distinct chemokine and chemokine receptor expression*. Int J Obes (Lond), 2010. **34**(12): p. 1684-94.
90. Blaszczak, A.M., et al., *Human Visceral Adipose Tissue Macrophages Are Not Adequately Defined by Standard Methods of Characterization*. J Diabetes Res, 2019. **2019**: p. 8124563.
91. Aouadi, M., et al., *Orally delivered siRNA targeting macrophage Map4k4 suppresses systemic inflammation*. Nature, 2009. **458**(7242): p. 1180-4.
92. Saito, M., et al., *Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice*. Nat Biotechnol, 2001. **19**(8): p. 746-50.
93. Gupta, P., et al., *Tissue-Resident CD169(+) Macrophages Form a Crucial Front Line against Plasmodium Infection*. Cell Rep, 2016. **16**(6): p. 1749-1761.
94. Ivanova, N.B., et al., *A stem cell molecular signature*. Science, 2002. **298**(5593): p. 601-4.
95. Purnama, C., et al., *Transient ablation of alveolar macrophages leads to massive pathology of influenza infection without affecting cellular adaptive immunity*. Eur J Immunol, 2014. **44**(7): p. 2003-12.
96. Sansbury, B.E., et al., *Overexpression of endothelial nitric oxide synthase prevents diet-induced obesity and regulates adipocyte phenotype*. Circ Res, 2012. **111**(9): p. 1176-89.
97. Cummins, T.D., et al., *Metabolic remodeling of white adipose tissue in obesity*. Am J Physiol Endocrinol Metab, 2014. **307**(3): p. E262-77.
98. Misharin, A.V., et al., *Flow cytometric analysis of macrophages and dendritic cell subsets in the mouse lung*. Am J Respir Cell Mol Biol, 2013. **49**(4): p. 503-10.
99. Gold, E.S., et al., *ATF3 protects against atherosclerosis by suppressing 25-hydroxycholesterol-induced lipid body formation*. J Exp Med, 2012. **209**(4): p. 807-17.
100. Wong, K., et al., *Phosphatidylserine receptor Tim-4 is essential for the maintenance of the homeostatic state of resident peritoneal macrophages*. Proc Natl Acad Sci U S A, 2010. **107**(19): p. 8712-7.
101. Shaw, T.N., et al., *Tissue-resident macrophages in the intestine are long lived and defined by Tim-4 and CD4 expression*. J Exp Med, 2018. **215**(6): p. 1507-1518.

102. Etzerodt, A. and S.K. Moestrup, *CD163 and inflammation: biological, diagnostic, and therapeutic aspects*. Antioxid Redox Signal, 2013. **18**(17): p. 2352-63.
103. Soncin, I., et al., *The tumour microenvironment creates a niche for the self-renewal of tumour-promoting macrophages in colon adenoma*. Nat Commun, 2018. **9**(1): p. 582.
104. Becht, E., et al., *Dimensionality reduction for visualizing single-cell data using UMAP*. Nat Biotechnol, 2018.
105. Kuhn, H. and V.B. O'Donnell, *Inflammation and immune regulation by 12/15-lipoxygenases*. Prog Lipid Res, 2006. **45**(4): p. 334-56.
106. Kwon, H.J., et al., *The contribution of arachidonate 15-lipoxygenase in tissue macrophages to adipose tissue remodeling*. Cell Death Dis, 2016. **7**(6): p. e2285.
107. Lee, Y.H., et al., *Adipogenic role of alternatively activated macrophages in beta-adrenergic remodeling of white adipose tissue*. Am J Physiol Regul Integr Comp Physiol, 2016. **310**(1): p. R55-65.
108. Uderhardt, S., et al., *12/15-lipoxygenase orchestrates the clearance of apoptotic cells and maintains immunologic tolerance*. Immunity, 2012. **36**(5): p. 834-46.
109. Sears, D.D., et al., *12/15-lipoxygenase is required for the early onset of high fat diet-induced adipose tissue inflammation and insulin resistance in mice*. PLoS One, 2009. **4**(9): p. e7250.
110. Romano, M., et al., *Lipoxins and aspirin-triggered lipoxins in resolution of inflammation*. Eur J Pharmacol, 2015. **760**: p. 49-63.
111. Martinez-Santibanez, G. and C.N. Lumeng, *Macrophages and the regulation of adipose tissue remodeling*. Annu Rev Nutr, 2014. **34**: p. 57-76.
112. Aouadi, M., et al., *Lipid storage by adipose tissue macrophages regulates systemic glucose tolerance*. Am J Physiol Endocrinol Metab, 2014. **307**(4): p. E374-83.
113. Hainer, V., H. Toplak, and A. Mitrakou, *Treatment modalities of obesity: what fits whom?* Diabetes Care, 2008. **31 Suppl 2**: p. S269-77.
114. Kleinert, M., et al., *Animal models of obesity and diabetes mellitus*. Nat Rev Endocrinol, 2018. **14**(3): p. 140-162.
115. Lutz, T.A. and S.C. Woods, *Overview of animal models of obesity*. Curr Protoc Pharmacol, 2012. **Chapter 5**: p. Unit5 61.
116. Coleman, D.L., *Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice*. Diabetologia, 1978. **14**(3): p. 141-8.
117. Halaas, J.L., et al., *Weight-reducing effects of the plasma protein encoded by the obese gene*. Science, 1995. **269**(5223): p. 543-6.
118. Bates, S.H., et al., *STAT3 signalling is required for leptin regulation of energy balance but not reproduction*. Nature, 2003. **421**(6925): p. 856-9.
119. Shepherd, P.R., et al., *Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue*. J Biol Chem, 1993. **268**(30): p. 22243-6.
120. Banks, W.A., et al., *Triglycerides induce leptin resistance at the blood-brain barrier*. Diabetes, 2004. **53**(5): p. 1253-60.
121. Benoit, S.C., et al., *Palmitic acid mediates hypothalamic insulin resistance by altering PKC-theta subcellular localization in rodents*. J Clin Invest, 2009. **119**(9): p. 2577-89.
122. Hansen, B.C., H.K. Ortmeyer, and N.L. Bodkin, *Prevention of obesity in middle-aged monkeys: food intake during body weight clamp*. Obes Res, 1995. **3 Suppl 2**: p. 199s-204s.

123. Pettersson, U.S., et al., *Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory T cell population in adipose tissue*. PLoS One, 2012. **7**(9): p. e46057.
124. Montgomery, M.K., et al., *Mouse strain-dependent variation in obesity and glucose homeostasis in response to high-fat feeding*. Diabetologia, 2013. **56**(5): p. 1129-39.
125. Murray, P.J., *Macrophage Polarization*. Annu Rev Physiol, 2017. **79**: p. 541-566.
126. Dalmas, E., K. Clement, and M. Guerre-Millo, *Defining macrophage phenotype and function in adipose tissue*. Trends Immunol, 2011. **32**(7): p. 307-14.
127. Bai, Y. and Q. Sun, *Macrophage recruitment in obese adipose tissue*. Obes Rev, 2015. **16**(2): p. 127-36.
128. Coenen, K.R., et al., *Diet-induced increases in adiposity, but not plasma lipids, promote macrophage infiltration into white adipose tissue*. Diabetes, 2007. **56**(3): p. 564-73.
129. Singer, K., et al., *Diet-induced obesity promotes myelopoiesis in hematopoietic stem cells*. Mol Metab, 2014. **3**(6): p. 664-75.
130. Kamei, N., et al., *Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance*. J Biol Chem, 2006. **281**(36): p. 26602-14.
131. Charriere, G., et al., *Preadipocyte conversion to macrophage. Evidence of plasticity*. J Biol Chem, 2003. **278**(11): p. 9850-5.
132. Tacke, F., et al., *Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques*. J Clin Invest, 2007. **117**(1): p. 185-94.
133. Bain, C.C., et al., *Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice*. Nat Immunol, 2014. **15**(10): p. 929-937.
134. Belkaid, Y. and S. Tamoutounour, *The influence of skin microorganisms on cutaneous immunity*. Nat Rev Immunol, 2016. **16**(6): p. 353-66.
135. Shibata, Y., et al., *GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1*. Immunity, 2001. **15**(4): p. 557-67.
136. Wang, Y., et al., *IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia*. Nat Immunol, 2012. **13**(8): p. 753-60.
137. Brancato, S.K. and J.E. Albina, *Wound macrophages as key regulators of repair: origin, phenotype, and function*. Am J Pathol, 2011. **178**(1): p. 19-25.
138. Davies, L.C., et al., *Tissue-resident macrophages*. Nat Immunol, 2013. **14**(10): p. 986-95.
139. Lenda, D.M., et al., *Reduced macrophage recruitment, proliferation, and activation in colony-stimulating factor-1-deficient mice results in decreased tubular apoptosis during renal inflammation*. J Immunol, 2003. **170**(6): p. 3254-62.
140. Lin, H., et al., *Discovery of a cytokine and its receptor by functional screening of the extracellular proteome*. Science, 2008. **320**(5877): p. 807-11.
141. Luo, J., et al., *Colony-stimulating factor 1 receptor (CSF1R) signaling in injured neurons facilitates protection and survival*. J Exp Med, 2013. **210**(1): p. 157-72.
142. Pixley, F.J. and E.R. Stanley, *CSF-1 regulation of the wandering macrophage: complexity in action*. Trends Cell Biol, 2004. **14**(11): p. 628-38.

143. Mondor, I., et al., *Lymphatic Endothelial Cells Are Essential Components of the Subcapsular Sinus Macrophage Niche*. Immunity, 2019. **50**(6): p. 1453-1466 e4.
144. Byrne, P.V., L.J. Guilbert, and E.R. Stanley, *Distribution of cells bearing receptors for a colony-stimulating factor (CSF-1) in murine tissues*. J Cell Biol, 1981. **91**(3 Pt 1): p. 848-53.
145. Tushinski, R.J., et al., *Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy*. Cell, 1982. **28**(1): p. 71-81.
146. Tushinski, R.J. and E.R. Stanley, *The regulation of macrophage protein turnover by a colony stimulating factor (CSF-1)*. J Cell Physiol, 1983. **116**(1): p. 67-75.
147. He, H., et al., *Endothelial cells provide an instructive niche for the differentiation and functional polarization of M2-like macrophages*. Blood, 2012. **120**(15): p. 3152-62.
148. Spite, M., J. Claria, and C.N. Serhan, *Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases*. Cell Metab, 2014. **19**(1): p. 21-36.
149. Odegaard, J.I. and A. Chawla, *The immune system as a sensor of the metabolic state*. Immunity, 2013. **38**(4): p. 644-54.
150. Nunemaker, C.S., et al., *12-Lipoxygenase-knockout mice are resistant to inflammatory effects of obesity induced by Western diet*. Am J Physiol Endocrinol Metab, 2008. **295**(5): p. E1065-75.
151. Gabanyi, I., et al., *Neuro-immune Interactions Drive Tissue Programming in Intestinal Macrophages*. Cell, 2016. **164**(3): p. 378-91.
152. Li, J., et al., *VSIG4 inhibits proinflammatory macrophage activation by reprogramming mitochondrial pyruvate metabolism*. Nat Commun, 2017. **8**(1): p. 1322.
153. Fu, W., et al., *Early window of diabetes determinism in NOD mice, dependent on the complement receptor CRIg, identified by noninvasive imaging*. Nat Immunol, 2012. **13**(4): p. 361-8.
154. D'Angelo, C.S. and C.P. Koiffmann, *Copy number variants in obesity-related syndromes: review and perspectives on novel molecular approaches*. J Obes, 2012. **2012**: p. 845480.
155. Arner, E., et al., *Adipocyte turnover: relevance to human adipose tissue morphology*. Diabetes, 2010. **59**(1): p. 105-9.
156. Veilleux, A., et al., *Visceral adipocyte hypertrophy is associated with dyslipidemia independent of body composition and fat distribution in women*. Diabetes, 2011. **60**(5): p. 1504-11.
157. Muir, L.A., et al., *Adipose tissue fibrosis, hypertrophy, and hyperplasia: Correlations with diabetes in human obesity*. Obesity (Silver Spring), 2016. **24**(3): p. 597-605.
158. Datta, R., M.J. Podolsky, and K. Atabai, *Fat fibrosis: friend or foe?* JCI Insight, 2018. **3**(19).
159. Keophiphath, M., et al., *Macrophage-secreted factors promote a profibrotic phenotype in human preadipocytes*. Mol Endocrinol, 2009. **23**(1): p. 11-24.
160. Jeffery, E., et al., *The Adipose Tissue Microenvironment Regulates Depot-Specific Adipogenesis in Obesity*. Cell Metab, 2016. **24**(1): p. 142-50.
161. Remmerie, A. and C.L. Scott, *Macrophages and lipid metabolism*. Cell Immunol, 2018. **330**: p. 27-42.

162. Khor, V.K., W.J. Shen, and F.B. Kraemer, *Lipid droplet metabolism*. Curr Opin Clin Nutr Metab Care, 2013. **16**(6): p. 632-7.
163. Muradian, K., et al., *Fucoxanthin and lipid metabolism: A minireview*. Nutr Metab Cardiovasc Dis, 2015. **25**(10): p. 891-7.