

Immunomodulatory Leptin Receptor⁺ Sympathetic Associated Perineurial Cells Protect Against Obesity



Emma Rose Haberman
Green Templeton College
University of Oxford

A thesis submitted for the degree of
Doctor of Philosophy
Michaelmas 2023

Acknowledgements

First and foremost, I owe thanks to Wellcome Trust and the IITM DPhil programme for this extraordinary exposure to the full breadth of immunology research. Without this opportunity I would not have met some of the incredible people I now know, nor diversified my scientific interests to quite the same extent. In particular, I'd like to thank Chris Tang and Dawn Gibbons, who both provided a voice of calm and reason when times became stressful. I am all the richer for this experience, and am truly indebted. I would also like to extend my heartfelt thanks to a number of people who initially helped me to realise that doing a PhD was feasible, and that scientific research is an exciting and (hopefully) viable career. At the University of Birmingham: Laura O'Neill and Peter Balfe, you were the first people to make me really see the joy in asking - and trying to answer - weird and wonderful questions. I wouldn't be here without your support, guidance and encouragement to pursue my scientific curiosity. "Can you really say that?" This question follows me around with everything I do, and will continue to haunt me for the rest of my career. Thanks for making me a better scientist. In addition, the ongoing guidance and support of Georg Holländer, Mary Deadman, Stefano Maio, Fatima Dhalla, along with past/present members of the Developmental Immunology lab have been invaluable. I'm glad to call you friends and colleagues, even if we are no longer in the same lab. I owe you many, many beers for being such excellent human beings.

The work in this thesis would not have been possible without the input and expertise of a great number of people - including but not limited to Bernardo Arús, Noelia Martínez-Sánchez, Chloe Zentai and Eliška Freibergrová, Bobby Hedley, Vasiliki Tsioligka, Alan Wainman; and collaborators Karin Ziegler, Matteo Iannaccone, Svetoslav Chakarov, Miguel López and Florent Ginhoux. Furthermore, I would like to thank my co-supervisor Irina Udalova, and Transfer/ Confirmation examiners Audrey Gerard, Katja Simon and David Ray for their advice and feedback throughout this project. Importantly, I really appreciate the help and support from staff at the BMS, Laura Thomas, Angela Curran, James Ward, Debbie Copley who - amongst others - are the reason these experiments were possible.

To members of the Domingos lab past and present, in particular Sam Malone, Noelia Martínez-Sánchez, Chelsea Larabee and Peter (Yitao) Zhu, thank you for helping me learn to speak neurometabolism, and for teaching me the crazy techniques which underlie my project. Thank you Ana, for igniting my interest in neuroimmunometabolism, and

for giving me the opportunity to go to conferences, write papers and present my work. In your lab I truly have learned resilience and independence, and have been exposed to a range of incredibly interesting scientific ideas. For this I am very grateful. During my DPhil project, I was fortunate enough to supervise and mentor two excellent students: Eliška Freibergarová and Chloe Zentai, who I think taught me just as much as I taught them. Thank you both for being curious, enthusiastic and incredibly hard working. Conan O'Brien, despite your chaotic, anxiety-inducing desk and lab bench thank you for always being a voice of reason, calm and logic. Thank you for always lending an ear when I needed it, and for your considered and insightful suggestions. Thank you for thinking it was a great idea to buy a life-sized cardboard cut out of Danny DeVito between us, and for hiding him to make me jump during late night experiments. Thank you for being my conference buddy. I am truly grateful to have such a great work colleague-turned life-long friend.

I am incredibly fortunate to be surrounded by such excellent friends and family. Nikki, you've been like a big sister to me since I moved to Oxford. Thank you for taking me under your wing, and for sharing with me far too many good times to name. Claudio and Susie, thanks for always making me laugh with your infectious humour. Tom, thank you for being my craft buddy - long may the craft sessions continue. Susann, thank you for sharing your love of science and synchro with me. Our debriefs on the way to and from training are truly unmatched, and I look forward to them every week. Mum, Dad and Luke, thanks for your unconditional love and optimism, and for cheering me up when I'm tired. I'm incredibly grateful that I can always count on your support and advice. Finally, my greatest thanks goes to my original lab partner, current partner in crime and soul mate, Dave. I don't think I can put into words how truly grateful I am for all of your love and support. You make everything better, and somehow always manage to put a smile on my face. I could not think of a better person to have by my side and in my corner, not just throughout my PhD but for the rest of my life.

Abstract

Obesity is a culmination of long-term energy imbalance, resulting in the storage of excess fats as triglycerides in adipose tissues (ATs). Sympathetically innervated ATs are key sites of both energy storage and hormone production. Leptin is one such hormone produced by adipocytes, released in proportion to AT mass. Leptin acts in the hypothalamus to diminish hunger and increase sympathetic drive onto ATs. This increase in sympathetic-derived norepinephrine stimulates adipocyte lipolysis and thermogenesis, providing a neuroendocrine negative feedback loop which broadly controls energy storage and energy expenditure. Obesity is closely associated with chronic, low-grade AT inflammation, mirrored by an increase in pro-inflammatory and a concomitant decrease in anti-inflammatory AT immune cells. Here, we reveal a novel population of Leptin receptor⁺ perineurial cells ensheathing AT sympathetic neurons, which express the sympathetic neurotransmitter receptor *Adrb2*, and produce key anti-inflammatory cytokines, including IL-33. We show that mice with a perineurial cell-specific loss of IL-33 gain more weight when fed high-fat diet despite comparable food intake - indicative of metabolic dysfunction. In the pre-obese state, a loss of perineurial cell derived IL-33 reduces the frequency of anti-inflammatory regulatory immune cells, including regulatory T cells (Tregs) and eosinophils specifically within the brown adipose tissue (BAT). Along with this shift in BAT-populating immune cells, we show that these mice have impaired adaptive BAT thermogenesis, predisposing to obesity with metabolic challenge. Furthermore, we show that SAPC-specific deletion of *Adrb2* increases IL-33 production in the brown adipose tissue, indicating that cytokine production by SAPCs may also be regulated by the sympathetic nervous system. Together, this firmly implicates Sympathetic Associated Perineurial Cells in the regulation of adipose tissue homeostasis, and highlights a novel cellular target in the prevention and treatment of obesity.

Contents

List of Figures	xi
List of Abbreviations	xiii
1 Introduction	1
1.1 Obesity	2
1.2 Adipose tissues	4
1.2.1 Brown adipocytes	6
1.2.2 White adipocytes	8
1.2.3 Beige adipocytes	8
1.3 Leptin	9
1.3.1 Hypothalamic regulation of energy homeostasis	11
1.3.2 Central leptin resistance in obesity	14
1.4 Adipose tissue innervation	17
1.4.1 Adipose tissue is sympathetically innervated	17
1.4.2 Sympathetic neurons form the efferent arm in the neuroendocrine negative feedback loop of leptin action	20
1.4.3 Non-sympathetic innervation of adipose tissue	21
1.4.4 Adipose tissue neuronal plasticity	22
1.5 Immunomodulatory effects of leptin	24
1.6 Adipose tissue immune cells	25
1.6.1 Macrophages	26
1.6.2 Sympathetic associated macrophages	32
1.6.3 Type 2 immune cells	35
1.6.4 Type 2 Innate Lymphoid Cells	35
1.6.5 Eosinophils	36
1.6.6 Other innate immune cells	37
1.6.7 Regulatory T cells	38
1.7 Adipose tissue stromal cells	41
1.8 Summary	42
1.9 Hypothesis	43
1.10 Aims	43

2	Materials and Methods	45
2.1	General laboratory reagents	46
2.2	Animal husbandry	46
2.2.1	Mouse genotypes	46
2.2.2	Metabolic phenotyping	48
2.2.3	High fat diet challenge	48
2.2.4	Temperature measurements	49
2.2.5	Cold challenge	49
2.2.6	Induction and rescue of metabolic adaptation	50
2.2.7	PBS perfusion	50
2.3	Microscopy	51
2.3.1	Sample preparation	51
2.3.2	Immuno-electron microscopy	51
2.3.3	Cryosectioning	52
2.3.4	Immunofluorescent staining	52
2.3.5	Confocal image acquisition	54
2.4	Microscopy image analysis	54
2.4.1	Removal of background fluorescence	54
2.4.2	Quantification of eYFP ⁺ coverage	56
2.4.3	Quantification of IL-33 fluorescence intensity	59
2.5	Generation of single cell suspensions	59
2.5.1	Spleen dissociation	59
2.5.2	Adipose tissue dissociation (immune cell isolation)	59
2.5.3	Adipose tissue dissociation (stromal cell isolation)	60
2.5.4	Axon bundle dissociation (stromal and immune cell isolation)	60
2.5.5	Sympathetic ganglia dissociation (stromal cell isolation)	61
2.6	Flow cytometry	61
2.7	RNA-sequencing data	63
2.7.1	Bulk RNA-sequencing of eYFP ⁺ cells from the Superior Cervical Ganglia of LepR ^{Ai32} mice	63
2.7.2	Single nuclei RNA-sequencing of human sympathetic ganglia	63
2.7.3	Analysis of published single cell RNA-sequencing data	65
2.8	Reverse transcription-quantitative Polymerase Chain Reaction (RT-qPCR)	65
2.8.1	RNA extraction	65
2.8.2	DNase treatment	66
2.8.3	Reverse transcription	66
2.8.4	Quantitative Polymerase Chain Reaction	67
2.8.5	Quantitative Polymerase Chain Reaction analysis	67
2.9	Statistical analyses	69

3	Characterisation of LepR⁺ cells within white and brown adipose tissues	71
3.1	Introduction	71
3.1.1	Leptin receptor	71
3.1.2	Peripheral nerve-associated stromal cells	73
3.1.3	Perineurial cells	74
3.2	Results	76
3.2.1	Sympathetic Associated Perineurial Cells are LepR ⁺	76
3.2.2	LepR ⁺ Sympathetic Associated Perineurial Cells produce immunomodulatory cytokine IL-33	83
3.2.3	LepR ⁺ Sympathetic Associated Perineurial Cells are distinct from IL-33 producing stromal cells within vWAT	84
3.2.4	LepR ⁺ SAPCs are a distinct subset of perineurial cells associated with sympathetic tissues	88
3.2.5	<i>LEPR⁺IL33⁺</i> cells are present in human sympathetic ganglia	88
3.2.6	LepR ⁺ Sympathetic Associated Perineurial Cells express <i>Adrb2</i>	90
3.3	Discussion	90
3.4	Updated hypothesis	96
3.5	Updated aims	96
4	Sympathetic Associated Perineurial Cell-derived IL-33 in adipose homeostasis	99
4.1	Introduction	100
4.1.1	IL-33 signalling	100
4.1.2	IL-33 producing stromal cells	103
4.1.3	Control of brown adipose tissue thermogenesis	105
4.1.4	Immune mediated thermogenesis	106
4.2	Results	109
4.2.1	<i>Lepr</i> and <i>Il33</i> co-expression is not observed in the hypothalamus, or peripheral organs of the <i>Tabula Muris</i> database	109
4.2.2	Body weight and adipose tissue weights are unaltered in the absence of SAPC-derived IL-33	110
4.2.3	LepR ^{IL33cKO} mice have an altered brown adipose tissue immune cell milieu	110
4.2.4	Unchallenged LepR ^{IL33cKO} mice have comparable metabolism to IL33 ^{fl/fl} controls	118
4.2.5	Production of IL-33 by LepR ⁺ SAPCs is protective against diet-induced obesity	118
4.2.6	Cold challenge optimisation	119
4.2.7	Adaptive BAT thermogenesis is impaired in cold challenged LepR ^{IL33cKO} mice	124

4.2.8	The onset of metabolic adaptation to fasting is delayed in LepR ^{IL33cKO} BAT	124
4.2.9	Fasted LepR ^{IL33cKO} BAT is unresponsive to leptin-induced stimu- lation	126
4.2.10	Loss of <i>Adrb2</i> signalling in SAPCs induces obesity, independently of food intake	126
4.2.11	IL-33 production by SAPCs is altered in the absence of ADR β 2 signalling	130
4.3	Discussion	133
5	Final Conclusion and Discussion	141
5.1	Summary of key findings	141
5.2	LepR ⁺ Sympathetic Associated Perineurial Cell barrier	142
5.3	Immune modulation of brown adipose tissue function	144
5.4	Therapeutic targets for the treatment of obesity	146
5.5	Limitations of this work	147
5.6	Future direction	148
5.7	Final conclusion	150
Appendices		
References		163

List of Figures

1.1	Neuroendocrine loop of leptin action	3
1.2	Adipose tissues in mice and humans	5
1.3	Brown adipocytes are thermogenic	7
1.4	Hypothalamic leptin signalling	12
1.5	Sympathetic innervation via the paravertebral chain of sympathetic ganglia	18
1.6	Adipose tissue immune cell changes in obesity	27
2.1	Table of general laboratory reagents	47
2.2	Table of antibodies used for immunofluorescent imaging	53
2.3	Image analysis pipeline for removal of background fluorescence	55
2.4	Image analysis pipeline for quantification of eYFP coverage of whole-mount imaged axon bundles	57
2.5	Image analysis pipeline for the quantification of IL-33 fluorescence intensity within eYFP ⁺ cells	58
2.6	Table of antibodies used for flow cytometry	62
2.7	Table of primers used for quantitative Polymerase Chain Reaction	68
3.1	Organisation of peripheral nerves	75
3.2	eYFP ⁺ cells surround LepR ^{Ai32} sympathetic tissues	77
3.3	eYFP ⁺ perineurial cells in LepR ^{Ai32} mice express <i>ObRb</i>	79
3.4	A multi-layer LepR ⁺ Sympathetic Associated Perineurial Cell barrier surrounds adipose tissue axon bundles	80
3.5	LepR ⁺ Sympathetic Associated Perineurial Cells are PDGFR α ⁺ ITG β 4 ⁺	82
3.6	LepR ⁺ Sympathetic Associated Perineurial Cells are IL-33 ⁺	85
3.7	LepR ⁺ IL33 ⁺ SAPCs are distinct from IL33 ⁺ mesenchymal stromal cells reported in vWAT	86
3.8	LepR ⁺ SAPCs are distinct from sensory neuron-associated IL-33 ⁺ cells reported in mouse muscle	87
3.9	<i>LEPR</i> ⁺ IL33 ⁺ cells exist in human sympathetic ganglia	89
3.10	LepR ⁺ Sympathetic Associated Perineurial Cells express <i>Adrb2</i>	91
3.11	LepR ⁺ Sympathetic Associated Perineurial Cells form a barrier between sympathetic neurons and adipose tissues	98
4.1	<i>Lepr</i> and <i>Il33</i> co-expression is not observed in the Arcuate Nucleus of the hypothalamus, or peripheral organs of the <i>Tabula Muris</i> database	111

4.2	Body weight and adipose tissue weights are unaltered in lean, unchallenged LepR ^{IL33cKO} mice	112
4.3	Brown adipose tissue CD45 ⁺ fraction is reduced in lean LepR ^{IL33cKO} mice	114
4.4	LepR ^{IL33cKO} mice have impaired BAT Treg and eosinophil recruitment .	115
4.5	Loss of SAPC-derived IL33 results in BAT inflammation	116
4.6	Unchallenged LepR ^{IL33cKO} mice have a comparable basal metabolism to IL33 ^{fl/fl} controls	117
4.7	LepR ^{IL33cKO} mice are predisposed to high fat diet-induced obesity, independently of food intake	120
4.8	HFD-fed LepR ^{IL33cKO} mice have higher <i>Ucp1</i> BAT expression than IL33 ^{fl/fl} controls	121
4.9	Cold challenge protocol optimisation	123
4.10	Impaired thermogenic capacity in cold challenged LepR ^{IL33cKO} mice . .	125
4.11	LepR ^{IL33cKO} mice exhibit delayed induction of metabolic adaptation . .	127
4.12	Fasted LepR ^{IL33cKO} mice do not respond to leptin-induced rescue from metabolic adaptation	128
4.13	Loss of <i>Adrb2</i> in SAPCs induces obesity, independently of food intake .	129
4.14	SAPC IL-33 production is regulated by ADR β 2 signalling in brown adipose tissue axon bundles	131
5.1	LepR ⁺ Sympathetic Associated Perineurial Cells regulate adaptive thermogenesis in brown adipose tissue	143
2	Macros for measurement of LepR and IL-33 background fluorescence .	153
3	Macros for measurement and normalisation of LepR coverage	154
4	Macro for segmentation and measurement of nuclear IL-33 fluorescence intensity	155
5	Full stromal cell flow cytometric gating strategies	156
6	Immunofluorescence secondary antibody only stained controls	157
7	IL33 ^{fl/fl} and LepR ^{IL33cKO} food intake over time	158
8	Full immune cell flow cytometric gating strategies	159
9	Energy Expenditure and Respiratory Quotient time course of lean, unchallenged IL33 ^{fl/fl} and LepR ^{IL33cKO} mice	160
10	Thermoneutral-cold challenge protocol significantly induces expression of thermogenic genes compared with room temperature mice	161

List of Abbreviations

α-MSH	α -melanocyte stimulating hormone
ADRβ2	Adrenergic receptor β 2
ADRβ3	Adrenergic receptor β 3
ARC	Arcuate nucleus of the hypothalamus
ASPCs	Adipose stem and progenitor cells in white adipose tissue
AT	Adipose tissue
ATP	Adenosine triphosphate
AgRP	Agouti related peptide
B3-TUB	β 3 tubulin
BAT	Brown adipose tissue
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CCL2	C-C motif chemokine ligand 2 (monocyte chemoattractant protein-1)
CCR2	C-C motif chemokine receptor 2
CD	Cluster of differentiation
CD31	Cluster of differentiation 31 (Platelet endothelial cell adhesion molecule 1)
CD45	Cluster of differentiation 45
CD68	Cluster of differentiation 68
CDH5	Cadherin-5
CGRP	Calcitonin gene-related peptide
CIDEA	Cell death inducing DFFA like effector A
CLDN1	Claudin-1

CRE	CRE-recombinase
CSF	Cerebrospinal fluid
CSFR1	Colony stimulating factor receptor 1
CTLA4	Cytotoxic T lymphocyte associated protein 4
DCs	Dendritic cells
<i>Db</i>	Leptin receptor gene
<i>Db/Db</i> mice	Leptin receptor deficient mouse model
DHA	Dorsoal hypothalamic area
DIO	Diet induced obesity
DIO2	Type 2 iodothyronine deiodinase
DISCO	3-dimensional imaging of solvent cleared organs
DMN	Dorsomedial nucleus of the hypothalamus
DNA	Deoxyribonucleic acid
DREADDs	Designer receptors exclusively activated by designer drugs
DRG	Dorsal root ganglia
EE	Energy expenditure
ELOVL3	ELOVL fatty acid elongase 3
ER	Endoplasmic reticulum
eYFP	Enhanced yellow fluorescent protein
FACS	Fluorescence activated cell sorting
FAPs	Fibroblast progenitors in muscle
FFAs	Free fatty acids
fl/fl	Floxed
FLT3	Fms related receptor tyrosine kinase 3
FOXP3	Forkhead box protein 3
g	Grams
GATA3	GATA binding protein 3

GDF3	Growth differentiation factor 3
GLP-1	Glucagon-like peptide-1
GLUT1	Glucose transporter 1 (Solute carrier family 2 member a1)
GPR3	G-protein coupled receptor 3
HFD	High fat diet
HSV129	Herpes simplex virus 129
ICOSL	Inducible T cell costimulator ligand
ICV	Intracerebroventricular
IF	Immunofluorescence
IFNγ	Interferon γ
IHC	Immunohistochemistry
IL	Interleukin
IL-1β	Interleukin-1 β
IL1RAP	Interleukin-1 receptor associated protein
IL-33	Interleukin-33
ILC2s	Type 2 innate lymphoid cells
IP	Intraperitoneal
IRS2	Insulin receptor substrate 2
ITGβ4	Integrin β 4
JAK-STAT	Janus kinase-signal transducers and activators of transcription
KLRG1	Killer cell lectin like receptor G1
LepR	Leptin receptor
L	Litre
L1-3	Lumbar spine level 1-3
Lpl	Lipoprotein lipase
LPS	Lipopolysaccharide
M	Molar
M1-MPhs	M1-like 'pro-inflammatory' macrophages

M2-MPhs	M2-like 'anti-inflammatory' macrophages
MAOA	Monoamine oxidase A
MC4-R	Melanocortin 4 receptor
MECP2	Methyl CpG binding protein 2
Met-enk	Methionine-enkephalin
mg	Milligram
MHC	Major histocompatibility complex
MPO	Medial predorsomedial optic area of the hypothalamus
MSCs	Mesenchymal stromal cells
ND	Normal diet
NE	Norepinephrine (noradrenaline)
NF-$\kappa\beta$	Nuclear factor $\kappa\beta$
NGF	Nerve growth factor
NK	Natural killer cell
NLRP3	NLR family pyrin domain containing 3
nM	Nanomolar
nm	Nanometre
NPY	Neuropeptide Y
NR4a1	Nuclear receptor subfamily 4 group a member 1
O₂	Oxygen
Ob	Leptin gene
Ob/Ob mice	Leptin deficient mouse model
ObR	Leptin receptor
ObRb	Signalling isoform of leptin receptor
OCT	Optimal cutting temperature compound
OX40L	OX40 ligand
PB	Phosphate buffer
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PDGFRα	Platelet derived growth factor α
PDPN	Podoplanin
PET	Positron emission tomography
PFA	Paraformaldehyde
PGC1α	PPAR γ coactivator 1 α
PGE2	Prostaglandin E2
PKA	Protein kinase A
POMC	Pro-opiomelanocortin
PPARγ	Peroxisome-activated receptor γ
PRDM16	PR domain containing 16
PRV	Pseudorabies virus
pSTAT3	Phosphorylated signal transducer and activator of transcription 3
PVN	Paraventricular nucleus of the hypothalamus
RER	Respiratory exchange rate
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RQ	Respiratory quotient
RT	Room temperature
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SAMs	Sympathetic associated macrophages
SAPCs	Sympathetic associated perineurial cells
SARM1	Sterile alpha and TIR motif containing 1
SCA-1	Stem cell antigen-1
SCG	Superior cervical sympathetic ganglia
scWAT	Subcutaneous white adipose tissue
SEMA6A	Semaphorin 6A
SLC6A2	Solute carrier family 6 member 2

SOCS	Suppressor of cytokine signalling 3
SPF	Specific pathogen free
ST2	Suppressor of tumourigenicity 2 (IL-1 receptor like 1)
STAT3	Signal transducer and activator of transcription 3
SVF	Stromovascular fraction
T13	Thoracic spine level 13
T3	Active thyroid hormone 3
TCR	T cell receptor
TGFβ	Transforming growth factor β
TH	Tyrosine hydroxylase
Th1	T helper 1 cell
Th2	T helper 2 cell
TJP1	Tight junction protein-1
TNF-α	Tumour necrosis factor α
Tregs	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
UCP1	Uncoupling protein 1
VCO₂	Carbon dioxide production
VO₂	Oxygen consumption
VMH	Ventromedial hypothalamus
MSCs	Mesenchymal stromal cells within visceral WAT
vWAT	Visceral white adipose tissue
WAT	White adipose tissue
WT	Wildtype
μg	Microgram
μL	Microlitre
μM	Micromolar
μm	Micrometre
$^{\circ}\text{C}$	Degrees celsius

Chapter 1

Introduction

Contents

1.1 Obesity	2
1.2 Adipose tissues	4
1.2.1 Brown adipocytes	6
1.2.2 White adipocytes	8
1.2.3 Beige adipocytes	8
1.3 Leptin	9
1.3.1 Hypothalamic regulation of energy homeostasis	11
1.3.2 Central leptin resistance in obesity	14
1.4 Adipose tissue innervation	17
1.4.1 Adipose tissue is sympathetically innervated	17
1.4.2 Sympathetic neurons form the efferent arm in the neuroendocrine negative feedback loop of leptin action	20
1.4.3 Non-sympathetic innervation of adipose tissue	21
1.4.4 Adipose tissue neuronal plasticity	22
1.5 Immunomodulatory effects of leptin	24
1.6 Adipose tissue immune cells	25
1.6.1 Macrophages	26
1.6.2 Sympathetic associated macrophages	32
1.6.3 Type 2 immune cells	35
1.6.4 Type 2 Innate Lymphoid Cells	35
1.6.5 Eosinophils	36
1.6.6 Other innate immune cells	37
1.6.7 Regulatory T cells	38
1.7 Adipose tissue stromal cells	41
1.8 Summary	42
1.9 Hypothesis	43
1.10 Aims	43

With the permission of the other authors, this introduction was adapted from a review I wrote as joint co-first author with Conan O'Brien [1], and a research paper that I wrote as first author [2]. Only sections from these two publications that I have written are included verbatim.

1.1 Obesity

Obesity is a growing epidemic. Almost half of the world's adult population are classified as overweight, and over half a billion adults worldwide are considered obese [3]. Obesity itself is characterised as an excess in body fat accumulation. Fundamentally, obesity is a disease of energy imbalance: a long-term positive energy balance, in which energy intake exceeds energy expenditure, resulting in the storage of excess energy as triglycerides in adipose tissues (ATs). Whilst excess calorie intake and/or insufficient physical activity both contribute towards organismal energy imbalance, this overly simplistic characterisation of obesity overlooks the complexity of this condition. Recent decades have brought with them the revelation that body weight homeostasis is mediated in a neuroimmunoendocrine manner (Figure 1.1A). Functional as an endocrine organ, adipocytes release the blood-borne hormone leptin, in proportion to fat mass [4]. Leptin acts on leptin receptor⁺ (LepR⁺) neurons within the hypothalamus to reduce food intake (discussed further in section 1.3), and increase energy expenditure through the increase of sympathetic drive onto adipose tissues. As a result, increased sympathetic neurotransmitter norepinephrine (NE) stimulates energy expenditure through adipocyte lipolysis and thermogenesis, in turn reducing adipose tissue mass (Figure 1.1A, discussed further in section 1.4.2) [5]. Leptin is now widely acknowledged as the afferent signal in a neuroendocrine negative feedback loop which regulates adipose tissue and body weight. Furthermore, chronic low-grade adipose tissue inflammation has been closely correlated with obesity, also highlighting a role for adipose tissue-resident immune cells in the modulation of this neuroendocrine loop regulating adipose tissue homeostasis and energy balance (discussed further in section 1.6). In the case of obesity, this leptin-mediated negative feedback loop regulating body weight homeostasis is perturbed (Figure 1.1B). Due to increased adiposity and subsequent chronic high levels of leptin, hypothalamic LepR⁺ neurons become unresponsive to leptin stimulation (discussed further in section 1.3.2). Consequently, decreased sympathetic drive onto adipose tissues and impairments in energy expenditure through lipolysis and thermogenesis results in the

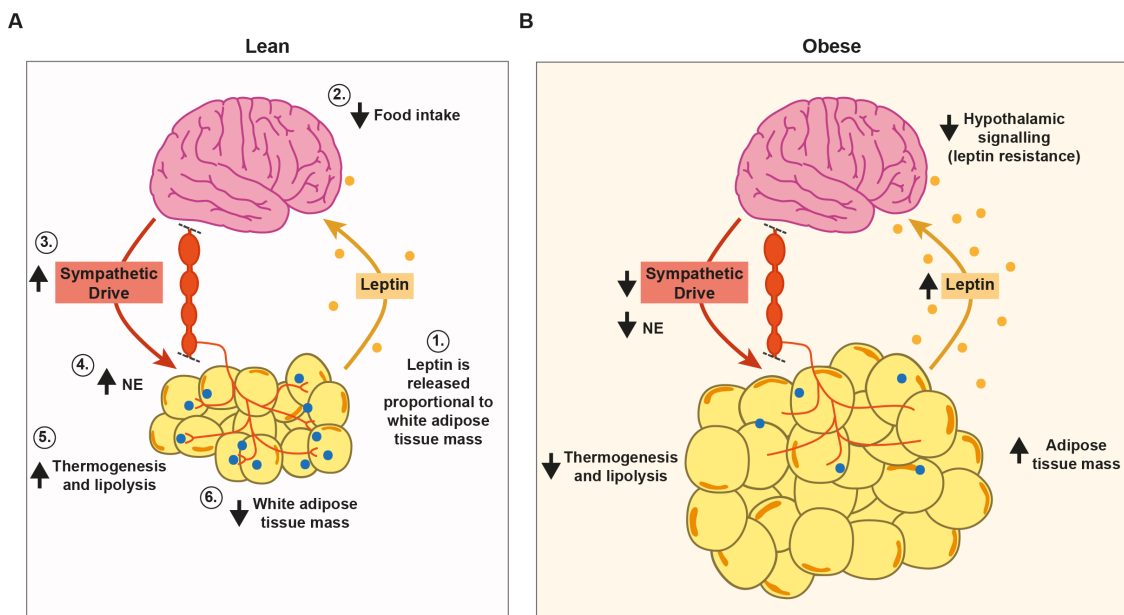


Figure 1.1: Neuroendocrine loop of leptin action

A Leptin is released by adipocytes, in proportion with white adipose tissue mass (1). Leptin signals $ObRb^+$ neurons within the hypothalamus to decrease food intake (2), and increase sympathetic drive onto the periphery (3). As a result, adipose tissue sympathetic neurons release more norepinephrine (NE) (4). Increased release of NE enhances lipolysis and thermogenesis (5), decreasing adipose tissue mass (6). **B** In obesity this negative feedback loop controlling weight is perturbed. Increased white adipose tissue mass results in higher levels of circulating leptin, which induces a state of leptin resistance within the hypothalamus, rendering the central nervous system unresponsive to leptin. As a consequence, sympathetic drive onto the periphery is reduced, adipose tissue sympathetic neurons release less NE and thermogenesis and lipolysis are also reduced - decreasing energy expenditure.

further accumulation of adipose tissue mass, propagating the obese state. Accordingly, given the multiple systems involved in the regulation of body weight, a wide range of defects in the endocrine, neuronal and immune systems can contribute towards obesity.

Obesity predisposes individuals to a plethora of life-threatening diseases, including coronary artery disease, atherosclerosis, hypertension, type 2 diabetes, cancer, and a range of autoimmune disorders [6][7]. Although multifactorial, obesity itself fulfils the criteria for consideration as a disease; it causes disorder in the structure and function of the organisms it affects, and manifests by distinguishing symptoms. Given the incidence and detrimental effects of obesity on health, it is crucial to better understand the integration and contribution of endocrine, neuronal and immune signals in the maintenance of adipose tissue and body weight homeostasis.

1.2 Adipose tissues

Adipose tissues have long been regarded solely as storage depots for excess fat, however recent decades have brought about the idea that adipose tissues are in fact *bona fide* endocrine organs, providing input signals to central neural circuits regulating peripheral energy storage and expenditure [8]. In addition to adipocytes, adipose tissues are a reservoir of stromovascular, nerve and immune cells which through engagement of paracrine and endocrine signalling have more recently been shown to mediate adipose tissue response to metabolic challenge [8][9][10][11][12].

Overwhelming evidence has implicated adipose tissues in a range of physiological processes, highlighting an active role for this endocrine organ in addition to the passive protective and insulatory roles for which it was previously considered. Collectively, adipose tissues make up one of the largest endocrine organs, and are a key site of both energy storage and expenditure. Two main types of adipose tissue exist in mammals: white adipose tissues (WAT) and brown adipose tissues (BAT), which are functionally and morphologically distinct. In newborns and hibernating animals, brown adipose tissue is crucial for the maintenance of body temperature. Anatomically, BAT is found in the

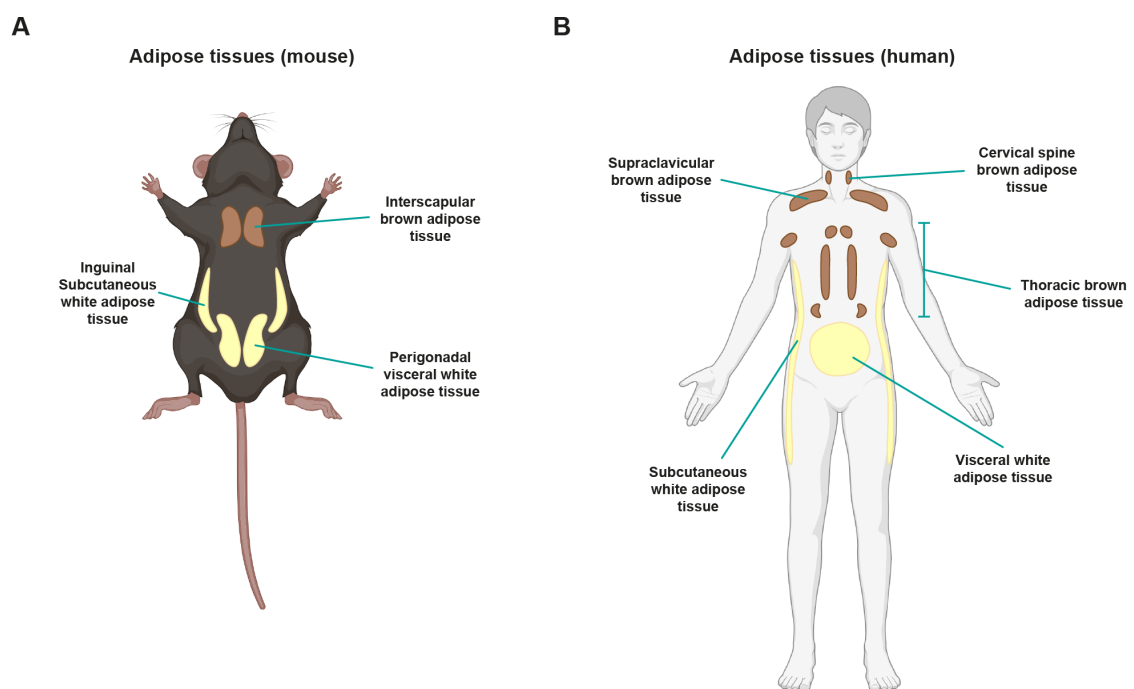


Figure 1.2: Adipose tissues in mice and humans

A-B Anatomical location of main white and brown adipose tissue depots in mice (**A**) and humans (**B**).

interscapular region of adult mice, whereas multiple, relatively smaller BAT depots can be found around the cervical spine, clavicle, thorax and abdomen of humans (Figure 1.2) [13]. Comparatively more abundant, white adipose tissues can be further subdivided into visceral WAT (vWAT) surrounding abdominal organs (e.g. mesenteric, perigonadal) and subcutaneous white adipose tissue (scWAT) which lie under the skin in both mice and humans (Figure 1.2) [14]. Increasing evidence suggests morphological and functional differences between adipose tissue depots in different locations, and is consistent with the idea that individual fat pads are distinct adipose tissue organs [1].

1.2.1 Brown adipocytes

The primary function of BAT is to produce heat. Not only is this non-shivering thermogenesis crucial for thermoregulation, the transfer of excess stored energy from food into heat is one key method of energy expenditure in metabolic homeostasis. Brown adipocytes have a low capacity for lipid storage within multiple, small lipid droplets (Figure 1.3A). In fact, the identity and thermogenic capacity of brown adipocytes is regulated by transcription factor PR domain containing 16 (PRDM16) - which is required for the expression of thermogenic gene expression [15]. Brown adipocytes, whose colour comes from their abundant mitochondria, are specialised for heat generation through the expression of *Uncoupling protein 1* (*Ucp1*). As the name suggests, UCP1 uncouples the final stages of oxidative phosphorylation and Adenosine triphosphate (ATP) generation in mitochondria during respiration [16][17][18]. Uniquely, UCP1 - a proton transporter - localises to the inner mitochondrial membrane of brown adipocytes, and renders this membrane permeable to protons [19]. The resulting dissipation of this proton gradient - established across the inner mitochondrial membrane by the electron transport chain - generates heat, as protons move from the intermembrane space down the concentration gradient and back into the mitochondrial matrix (Figure 1.3B). In cases of increased body fat percentage and obesity, brown adipose tissue thermogenic activity significantly declines, highlighting this form of thermogenic energy expenditure in propagating metabolic dysfunction [20]. In fact, a number of studies have

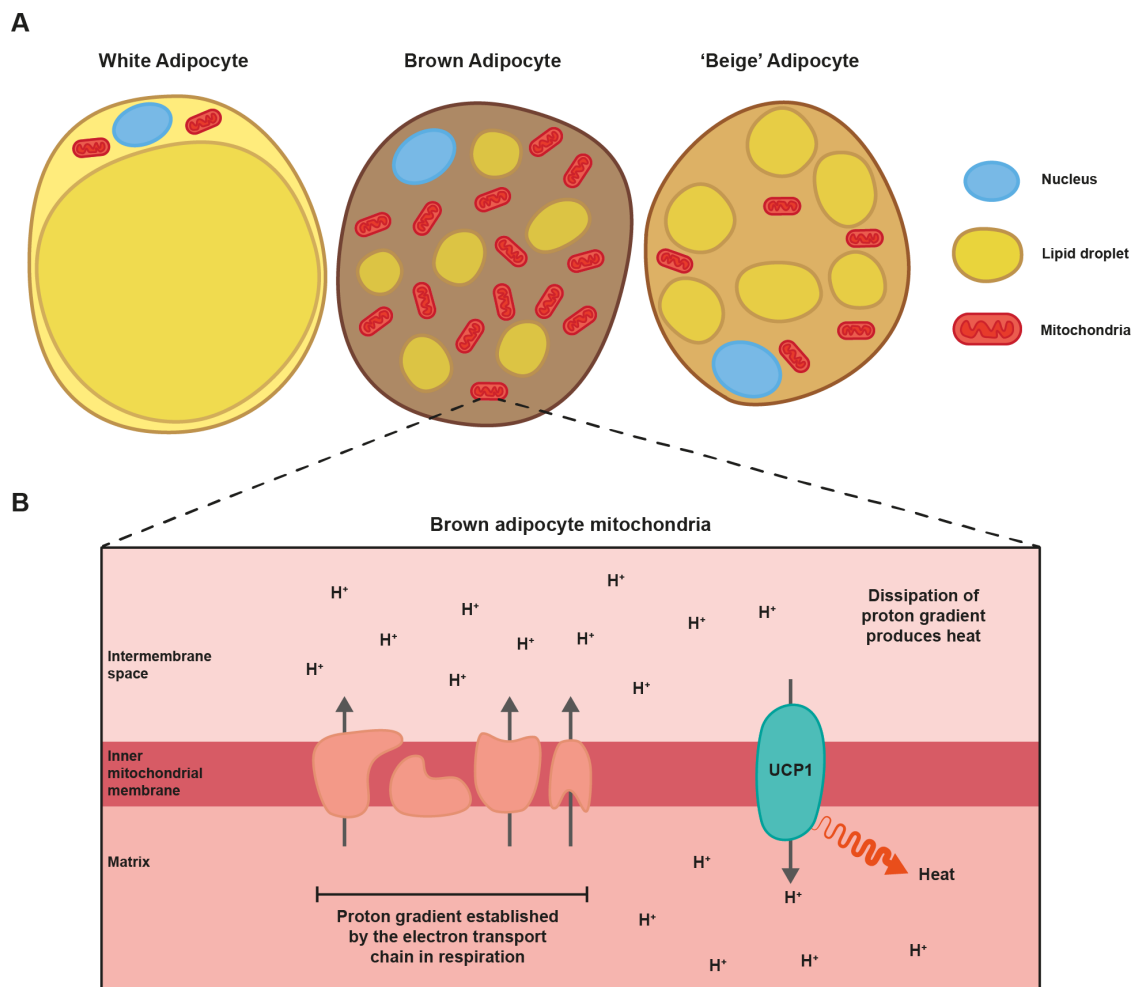


Figure 1.3: Brown adipocytes are thermogenic

A White, brown and beige adipocytes are morphologically, molecularly and functionally distinct.

B Brown and beige adipocyte-specific UCP1 generates heat through the dissipation of a proton (H^+) gradient, established across the inner mitochondrial membrane by the electron transport chain during cellular respiration.

implicated brown adipose tissue in adult human metabolic health [21][22][23], making brown adipose tissue an incredibly hot topic in obesity research.

1.2.2 White adipocytes

White adipose tissues are the primary site of excess energy storage, and mobilisation of energy-rich lipids following a deficit in food (energy) intake. Within WAT, white adipocytes possess few mitochondria, and store excess lipids as triglycerides within a single large lipid droplet (Figure 1.3A). In obesity, white adipose tissues grow in size, enabled by an increase in both the number and size of lipid-rich white adipocytes. In white adipocytes, excess storage is counteracted through lipolysis - the process in which stored triglycerides are broken down and released as free fatty acids (FFAs) for use in respiration [5][24][25][26]. White adipose tissues have also been implicated in the regulation of whole organism energy balance through the production of active signalling factors and hormones, such as leptin.

1.2.3 Beige adipocytes

More recently, an additional adipocyte subset was discovered in the WAT of mice and humans which exhibits both white and brown adipocyte features: the beige adipocyte [27]. Although their cellular origin remains unclear, beige adipocytes are phenotypically distinct from both white and brown adipocytes, with the majority found within the subcutaneous white adipose tissue. At resting state, beige adipocytes exhibit very low basal *Ucp1* expression, and therefore have a low thermogenic capacity - instead storing excess energy as triglycerides in multiple lipid droplets (Figure 1.3A). However, upon stimulation with NE, beige adipocytes upregulate expression of brown adipocyte markers *Prdm16* and *Ucp1*, vastly increasing their thermogenic capacity [28][29][30]. This dynamic adipocyte subset in which thermogenesis can be stimulated - a process termed adipose tissue 'beigeing' - is therefore also an exciting cellular target in the maintenance of energy balance and the treatment of obesity.

1.3 Leptin

Factors released by adipocytes, or 'adipokines', have been heavily implicated in a range of physiological processes, further solidifying the role of adipose tissue in whole organism metabolism and energy homeostasis. Most notably, the discovery of the *Ob* gene and its product leptin in mice [4] and humans [31] almost 30 years ago was the culmination of a decades' long search for a blood-borne factor regulating nutrient intake and metabolism [11]. Obese *Ob/Ob* mice were originally discovered around 1950, and possess a recessive, genetically heritable predisposition to extreme obesity [32]. The identification of *Ob* - the mutated gene causing extreme obesity in *Ob/Ob* mice - changed perception of adipose tissue from an energy storage depot to an endocrine organ and instigated a revolution in our understanding of obesity as a neuroendocrine condition. Mutations within the *Ob* gene result in a deficiency in the hormone leptin - so named after the Greek work 'leptos', meaning thin. Leptin is produced mainly by white adipose tissue adipocytes in proportion to the amount of fat. Therefore, serum leptin levels closely correlate with fat mass [33], and can be considered a measure of the amount of energy stored. Accordingly, leptin levels are elevated in obesity in both mice and humans, and decline with weight loss [34] [35].

Today, leptin's commonly cited mechanism of mediating energy balance is through the central regulation of appetite and food intake [11]. Leptin's role in regulating energy balance was cemented by landmark studies demonstrating that leptin administration causes weight loss and reductions in adipose tissue but not lean mass in both leptin-deficient *Ob/Ob* mice and diet-induced obese (DIO) mice. However, these effects are absent in leptin receptor-deficient *Db/Db* mice, highlighting the importance of leptin in the maintenance of whole organism energy balance [36][37][38]. The extreme obesity caused by leptin deficiency is analogous to that of animals with lesions in the ventromedial hypothalamus (VMH) [11], prompting investigation into the role of leptin within this area of the brain. Accordingly, the fully active, signalling isoform of the leptin

receptor, *ObRb*, is most highly expressed in the arcuate nucleus of the hypothalamus (ARC), proximal to the VMH [39][40]. Central intracerebroventricular (ICV) infusion of leptin into obese *Ob/Ob* and DIO mice resulted in a reduction in food intake and body weight compared with vehicle-treated controls; an effect which was lost in *Db/Db* mice, which are leptin receptor deficient [36][38][37], indicating the effects of leptin on body weight were mediated via the hypothalamus. Moreover, the deletion of the leptin receptor specifically in neurons enhanced adiposity, worsened glucose intolerance, and increased food intake in mice compared with leptin receptor-competent controls [41]. Collectively, this work established the white adipose tissue-derived hormone leptin in the control of energy balance through hypothalamic signalling (Figure 1.1A).

It is now widely acknowledged that leptin acts as the afferent signal in a negative feedback loop which regulates adipose tissue mass by reducing food intake [11][35][36][38][37]. For this reason, leptin is known as the satiety hormone. However, it is becoming increasingly apparent that the effect of leptin on weight loss goes beyond the behavioural modulation of appetite. Among other symptoms, human leptin deficiency manifests in low sympathetic tone, as measured by orthostatic hypertension and cold pressor response tests [42]; indicating that leptin's effects also extend peripherally to sympathetically innervated tissues. In experiments where food intake of vehicle-treated *Ob/Ob* mice is matched to that of leptin-treated *Ob/Ob* mice (pair-feeding), vehicle-treated mice lose only half the weight of their leptin-treated counterparts, despite equivalent food consumption [43][44]. The fact that leptin treatment causes increased weight loss compared with caloric deficit alone indicates that leptin-mediated control of body weight depends on more than just the central hypothalamic-mediated suppression of feeding, and implies peripheral mechanisms of leptin-mediated energy expenditure. However, Prieur et al. found that pair-fed *Ob/Ob* mice lost the same amount of weight as mice treated centrally or peripherally with leptin. Additionally, they showed that leptin treatment, regardless of route of administration, induced changes to hepatic and peripheral lipid metabolism indistinguishable from pair-feeding [45], suggesting that the effects are mainly attributable to leptin's effect on food intake. Of note, the last timepoint in this

study was nine day following leptin administration, whereas previous studies showing differences in body weight and feeding between pair-fed and leptin-treated groups ran for between 13 and 75 days [43][44][45]. Although the authors' interpretations differ, these findings are compatible, and demonstrate that the peripheral effects of leptin on body weight only become apparent with prolonged observation.

1.3.1 Hypothalamic regulation of energy homeostasis

The hypothalamus is a region of the brain which consists of anatomically and functionally distinct nuclei, which integrate and respond to centrally and peripherally derived autonomic and neuroendocrine feedback signals. In particular, the hypothalamus is responsible for whole organism energy homeostasis through the regulation of appetite and sympathetic drive to peripheral organs, including adipose tissues (discussed further in section 1.3.3) [46]. Whilst several hypothalamic-acting, blood-borne hormones – including the active thyroid hormone (T3), oestrogen and glucagon-like peptide-1 (GLP-1) – have been reported to promote obesity (discussed further by Larabee et al. [8]). As the key mediator of the neuroendocrine regulation of body weight homeostasis, here I will focus on the role of leptin signalling within the hypothalamus. The long, signalling, ObRb leptin receptor isoform is present throughout the brain – with particularly dense expression in the ARC, where approximately 75% of hypothalamic ObRb⁺ neurons reside [47][48]. Within the ARC, two antagonistic populations of ObRb⁺ neurons exist: pro-opiomelanocortin (POMC) anorexigenic neurons - known to reduce appetite - and agouti-related protein/ neuropeptide Y (AgRP/NPY) orexigenic neurons which stimulate appetite (Figure 1.4) [49]. POMC and AgRP/NPY neurons project onto melanocortin 4 receptor (MC4-R)⁺ pre-sympathetic neurons within the paraventricular nucleus of the hypothalamus (PVN), one of 5 distinct hypothalamic nuclei from which peripheral sympathetic nervous activity, or sympathetic drive, originates [50]. In the absence of leptin signalling, AgRP/NPY neuron release of neurotransmitter AgRP prevents activation of MC4-R neurons within the PVN through antagonism of the MC4-R receptor itself, preventing sympathetic drive onto the periphery and stimulating

A

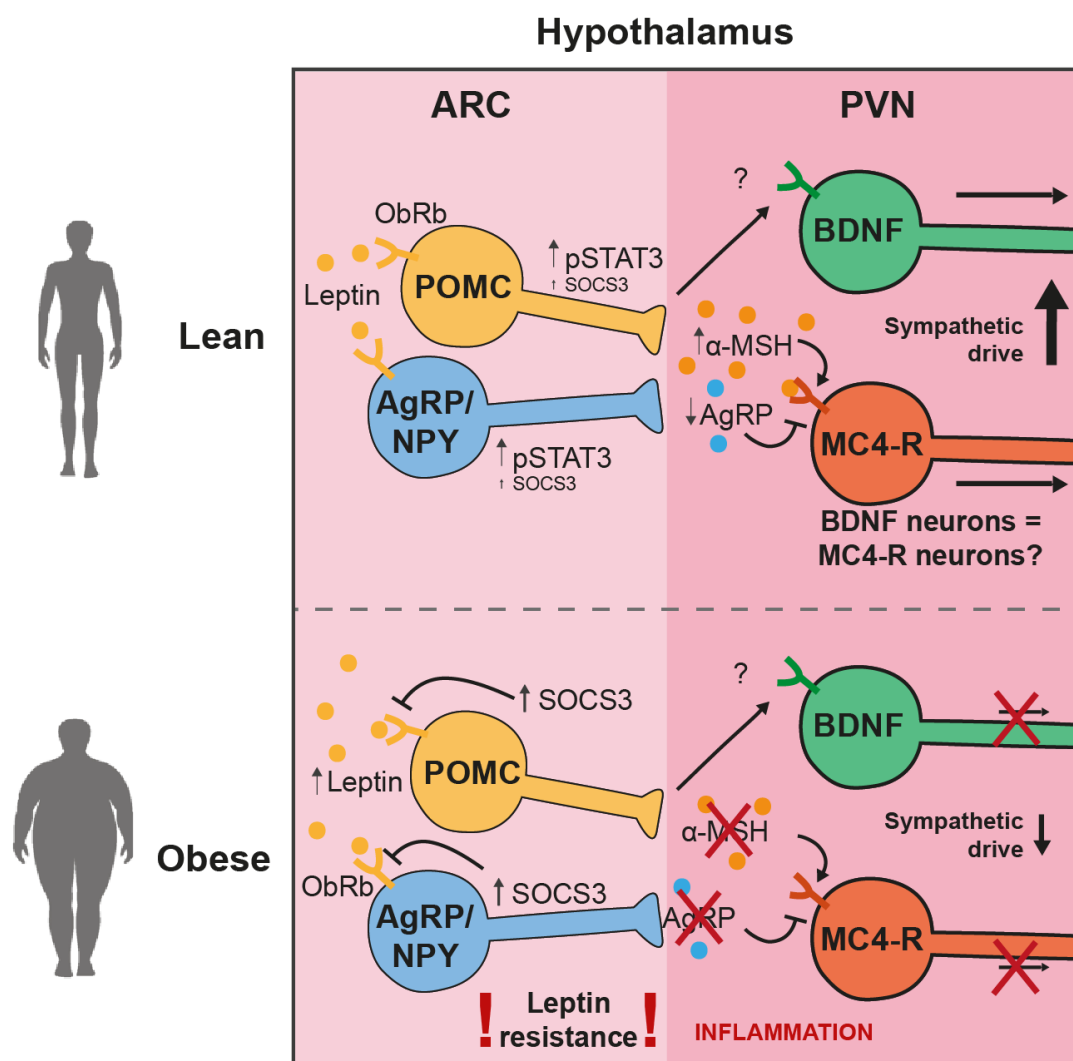


Figure 1.4: Hypothalamic leptin signalling

A Leptin receptor⁺ POMC and AgRP/NPY neurons within the arcuate nucleus of the hypothalamus (ARC) project onto MC4-R neurons in the paraventricular nucleus of the hypothalamus (PVN). Leptin signalling results in phosphorylation of STAT3 (pSTAT3) within POMC and AgRP/NPY neurons. Consequently, release of α -MSH by POMC neurons is upregulated and AgRP/NPY neuron-derived AgRP is downregulated - resulting in the activation of PVN MC4-R⁺ neurons and a subsequent increase in sympathetic drive onto peripheral adipose tissues. BDNF⁺ neurons within the PVN also act downstream of ARC leptin signalling, and are required for the regulation of adipose tissue innervation. Whether BDNF⁺ and MC4-R⁺ neurons represent one or two populations within the PVN is as yet unclear. In obesity, chronic leptin signalling induces Suppressor Of Cytokine Signaling 3 (SOCS3) - a known suppressor of leptin signalling — in ObRb⁺ POMC and AgRP neurons preventing activation of pre-sympathetic MC4-R neurons within the PVN. Coupled with hypothalamic inflammation and endoplasmic reticulum stress, this is likely causative of central leptin resistance and impaired responses downstream of ARC leptin signalling.

appetite. However, as marked by signal transducer and activator of transcription 3 (STAT3) phosphorylation, exogenous leptin treatment in lean mice activates both POMC and AgRP/NPY neurons within the ARC [51] (Figure 1.4A). Leptin-induced activation of AgRP neurons results in the downregulation of antagonistic neurotransmitter AgRP, and simultaneously enhances the release of POMC neurotransmitter and MC4-R agonist α -melanocyte-stimulating hormone (α -MSH) [52][53][54]. Collectively, this promotes MC4-R pre-sympathetic neuron activation within the PVN, inducing both satiety and sympathetic drive onto peripheral adipose tissues [55][54][56][57][58] (Figure 1.4A). Notably, an intact PVN within the hypothalamus is required for the leptin-mediated reduction in adipose tissue mass through thermogenesis and lipolysis, firmly planting the PVN as a major mediator of leptin action downstream of the ARC [59][60][61][62].

More recently, Wang and colleagues also described a direct role for brain-derived neurotrophic factor⁺ (BDNF⁺) neurons within the PVN, which also act downstream of ARC leptin signalling [63] (Figure 1.4A). A PVN-specific ablation of BDNF signalling was sufficient to blunt adipose tissue lipolysis and reduce WAT and BAT sympathetic innervation following acute leptin administration. The resulting impairment in leptin-induced weight loss highlights the importance of BDNF⁺ PVN neurons in sympathetic AT innervation [63]. Of note, it is unclear whether MC4-R⁺ and BDNF⁺ neurons downstream of ARC leptin signalling represent one or two populations within the PVN (Figure 1.4A). Furthermore, following subcutaneous (scWAT) injection of fluorescently tagged pseudorabies virus (PRV) retrograde tracing, Wang and colleagues demonstrated the presence of ObRb⁺ PRV⁺ neurons within the dorsomedial nucleus (DMN) and medial predorsomedial optic area (MPO) of the hypothalamus, which was previously undocumented [63]. Of the areas reported to mediate leptin signalling to WAT and BAT, only ablation of ARC leptin signalling was sufficient to alter activity of sympathetic neurons innervating scWAT and brown adipose tissue (BAT) [63], cementing ARC ObRb⁺ neurons as the sole central mediator of leptin-induced AT sympathetic activity. However, the inability of POMC neuron-specific ObRb deficient models to recapitulate *Ob/Ob* or *Db/Db* levels of obesity further illustrates a role for leptin signalling in energy

homeostasis outside of this described hypothalamic ARC-PVN circuit [64][65].

1.3.2 Central leptin resistance in obesity

A common feature of DIO is leptin resistance – the attenuation of hypothalamic leptin signalling thought to be due to chronic, central ObRb overstimulation [66]. Whilst short-term high-calorie intake can be countered through a centrally-mediated increase in energy expenditure, the chronic intake of highly calorific foods results in an inability to sufficiently increase energy expenditure to reinstate energy balance, culminating in weight gain. Of note, the term leptin resistance is difficult to quantify, and is used in a context-dependent manner [67]. Here I use this term to describe the inability of POMC and AgRP neurons within the ARC to phosphorylate STAT3 in response to acute increases in leptin concentration, a phenomenon accompanying chronically elevated serum leptin, which is likely a contributing factor in DIO [47]. Although exogenous leptin administration is sufficient to normalise both body weight and adiposity in leptin-deficient animals and humans [37][68], this does not represent a viable therapeutic strategy for most DIO cases. These individuals are hyperleptinaemic and unable to increase energy expenditure in response to acute leptin stimulus due to central leptin resistance [47].

Leptin-responsive cells are found both centrally and peripherally, however, DIO-induced dysregulation of leptin signalling specifically within the ARC is thought to be significant in the onset of central leptin resistance [47]. Whilst chronically high-fat diet (HFD)-fed mice have increased baseline pSTAT3 levels in comparison with normal diet (ND)-fed controls, these mice have both a significantly higher body weight and are unable to upregulate pSTAT3 in response to exogenous leptin (Figure 1.4A). This indicates a saturation of ARC leptin signalling with serum hyperleptinaemia [47]. In particular, Knight and colleagues elegantly demonstrated that chronic serum hyperleptinaemia is required for the onset of central leptin resistance by comparing leptin response in HFD-fed hyperleptinaemic WT mice and normoleptinaemic *Ob/Ob* mice chronically infused with levels of leptin comparable to ND-fed mice [69]. While both experimental

groups gained comparable body weight with HFD, only normoleptinaemic mice increased ARC pSTAT3 levels in response to exogenous leptin, indicating that leptin resistance is dependent on hyperleptinaemia, and not increased adiposity alone [69].

The induction of factors that inhibit leptin signalling in hypothalamic POMC and AgRP/NPY neurons has been suggested as one possible cause of leptin resistance within the ARC. Suppressor of cytokine signalling 3 (SOCS3) is one such inhibitor which is upregulated in a leptin signalling-dependent manner in ARC neurons of mice after only 4 weeks of HFD (Figure 1.4A) [47][70][71][72]. Upregulation of SOCS3 in ARC ObRb⁺ neurons prevents the downstream activation of pre-sympathetic neurons within the PVN, ablating the efferent sympathetic arm of the neuroendocrine loop of leptin action and further enhancing weight gain (Figure 1.1B, Figure 1.4A) [47][70][71][72]. Further corroborating an inhibitory role for SOCS3, lentiviral mediated knockdown of SOCS3 within the hypothalamus is protective against diet-induced obesity in rats [73]. These studies firmly implicate SOCS3 upregulation in POMC and AgRP/NPY neurons as a molecular mediator of ARC leptin resistance. Interestingly, SOCS3 expression is also increased in immune and stromal cells in response to inflammation in several human tissues, bringing about the possibility that hypothalamic inflammation may also be a factor in the upregulation of SOCS3 in ARC neurons in DIO [74].

Furthermore, a study by Ozcan and colleagues revealed an obesity-dependent increase in endoplasmic reticulum (ER) stress within hypothalamic neurons, and found that tunicamycin-induced ER stress in the brains of lean mice was sufficient to block hypothalamic STAT3 phosphorylation, following IP leptin administration [75]. Excitingly, they found that this impaired leptin responsiveness could be reversed in obese mice by pre-treating orally with a chemical chaperone to enhance ER function; thus giving rise to a novel class of leptin-sensitising, anti-obesity drugs targeting ER stress [75][76]. Notably, whether hypothalamic inflammation and associated ER stress and autophagy disorders, which have been extensively linked with diet-induced obesity, are causative or

a consequence of leptin resistance is yet to be determined [77][78][79].

Another possible cause of central leptin resistance was thought to be blood-brain barrier (BBB) dysfunction, which has implications in the known mechanism of leptin sensitisation [76]. Leptin itself is too large to diffuse freely through the BBB and must therefore be transported from the plasma. Whilst intraperitoneal (IP) leptin has little to no effect on pSTAT3 within the ARC following chronic HFD, ICV leptin administration induces hypothalamic STAT3 phosphorylation, albeit blunted in comparison with ND-fed, ICV leptin-treated mice; revealing saturation of leptin transport across the BBB [80][81]. Further supporting this idea, Schwartz et al. found that patients with a higher body mass index (BMI) reach a maximal cerebrospinal fluid (CSF) plasma leptin concentration, despite notably higher plasma leptin concentrations [82]. In 2005, Oh and colleagues hypothesised this may be due to impaired BBB transport mediated by unsaturated fatty acids with obesity. However using tissue clearing and light-sheet microscopy of fluorescently tagged leptin, a recent study by Harrison and colleagues provides evidence that chronic HFD does not reduce leptin accumulation within the hypothalamus, compared with ND controls [83][84]. Although, it is clear from positron emission tomography (PET) imaging that only a fraction of labelled leptin delivered peripherally enters the brain, with the majority localising to the kidneys [85]. Taken together, cell-intrinsic leptin insensitivity, caused at least in part by ER stress, appears to be a major driver of central leptin resistance in obesity. Hypothalamic responsiveness to peripheral, exogenous leptin is further impaired by the saturation of leptin transport across the BBB accompanying hyperleptinaemia [80][81].

Consistent with hyperleptinemia being causal to obesity, the group of Philipp Scherer demonstrated that normalisation of hyperleptinemia is sufficient to restore leptin sensitivity, drive weight loss, and restore body weight homeostasis [86]. This demonstration utilised neutralising antibodies against leptin and/or temporally controlled knockdown of leptin. These manipulations could seem counterintuitive, as leptin reduction would be expected to increase appetite. However, at the system level, the neuroendocrine loop

of leptin action is far from linear and may well be reset through interference with its afferent (leptin) arm. Consistent with this notion is the demonstration that facilitation of the efferent sympathetic arm in the neuroendocrine loop of leptin action also drives weight loss, independently of changes in food intake [87][88][89]. Amplification of the sympathetic efferent arm by sympathofacilitator drugs is sufficient to reset the neuroendocrine loop, and restore body weight homeostasis independently of alterations in food intake and locomotion [87][88][89]. Sympathofacilitator drugs do not enter the brain, but have an anti-obesity effect by promoting weight loss via thermogenesis, coupled to heat dissipation by peripheral vasodilation. The first in class in this category is PEGylated amphetamine, whose effect does not rely on conventional targets of amphetamine, but rather on the beta 2 adrenergic receptor - a well-known mediator of smooth muscle relaxation [87][88][89]. Taken together, interfering with either the afferent or the efferent arms in the neuroendocrine loop of leptin action can drive weight loss and shortcut leptin resistance.

1.4 Adipose tissue innervation

1.4.1 Adipose tissue is sympathetically innervated

Although not recorded in most medical textbooks, indirect evidence for adipose tissue innervation in animals and humans has been accumulating since the 1960s [24][25][26]. The sympathetic nervous system - best known for the 'fight or flight' response - relays unconscious messages from the central nervous system to organs of the periphery, in response to environmental change. Highly organised, the sympathetic nervous system innervates peripheral organs via the paravertebral sympathetic ganglia, where acetylcholinergic pre-ganglionic neurons synapse onto noradrenergic post-ganglionic sympathetic neurons, identifiable as tyrosine hydroxylase⁺ (TH⁺) (Figure 1.5A-B). A number of early studies predicted a functional role for sympathetic innervation of adipose tissue, despite a lack of direct evidence. Whilst electrical stimulation of AT nerve bundles upstream of adipose tissue induced BAT thermogenesis and WAT lipolysis, the opposite was true following ganglionic blockade [24][25][26]. Furthermore, in 1967 Galton and

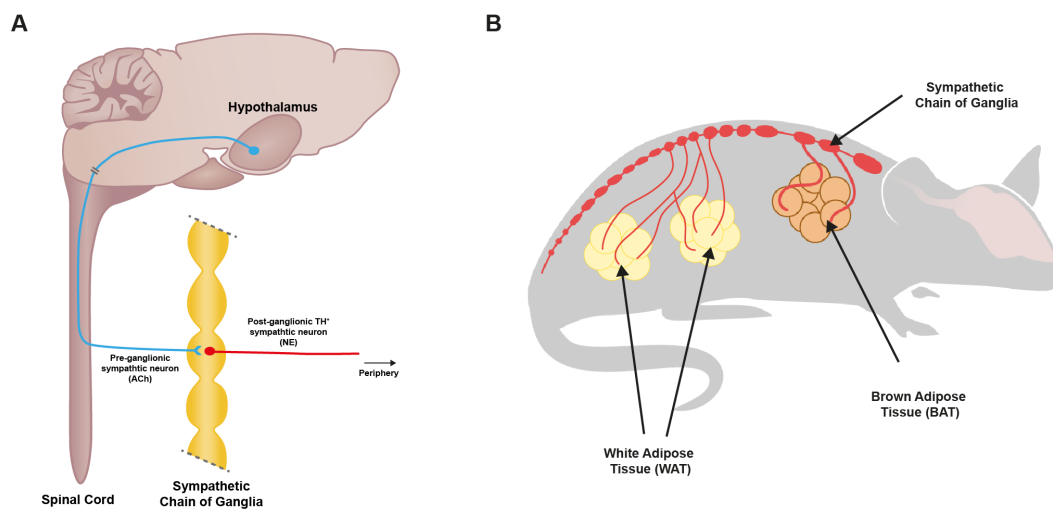


Figure 1.5: Sympathetic innervation via the paravertebral chain of sympathetic ganglia

A Acetylcholinergic (ACh) pre-ganglionic sympathetic neurons (blue) synapse with post-ganglionic noradrenergic (NE) sympathetic neurons. Peripheral tissues are sympathetically innervated by TH⁺ post-ganglionic neurons (red). **B** Schematic representation: adipose tissue is innervated by sympathetic axon neurons (red) originating from the paravertebral chain of sympathetic ganglia [5][94][95].

Bray [90] found that the addition of sympathetic neurotransmitter analogue epinephrine to *in vitro* cultures of primary white adipose tissues was sufficient to increase serum FFAs, indicative of lipolysis and an early suggestion of a role for catecholamines and the sympathetic nervous system in adipose tissue function. However, due to a lack in specificity of sympathetic neuron stimulation or inhibition, these initial efforts were unable to define a direct role for sympathetic innervation in WAT and BAT homeostasis [91][92][93].

A substantial amount of work, predominantly in hamsters and rats, has gone towards mapping the central representation of adipose tissues in the brain, through retrograde tracing of AT sympathetic neurons with detectable trans-synaptic pseudo rabies virus (PRV) [91][96][97][98][99][100][101]. Importantly, these retrograde tracing studies collectively highlighted that anatomically distinct fat pads are differentially represented in the brain, adding further weight to the idea that adipose tissues should be considered independent organs with distinctive predicted efferent pathways. Although widely used in neuroscience, one significant consideration with retrograde tracing studies is the specificity of PRV labelling. It has long been assumed that PRV transmission is exclusively trans-synaptic, however little evidence to this effect exists, and in fact, non-synaptic exocytosis has been reported along the dendrites of pyramidal neurons within the hippocampus [102][103]. Whilst it is generally accepted that BAT sympathetic innervation stems from the stellate ganglia [104][105], controversy still surrounds the innervation of scWAT and visceral WAT (vWAT). Conflicting reports have suggested that sympathetic innervation of scWAT stems from either thoracic (T13) – lumbar (L1-3) sympathetic ganglia or the coeliac ganglia [106][29][101], although these studies were performed in hamsters, rats and mice respectively, which could explain these observed differences. The previously mentioned PRV studies were revisited by Huesing and colleagues, who utilised state-of-the-art tissue clearing and 3-dimensional light sheet imaging of whole torsos, allowing for the direct visualisation of PRV transduction within in-tact paravertebral sympathetic chains *in situ* [98]. Although PRVs are polysynaptic and may be transmitted to multiple downstream neurons, these images suggest that

scWAT innervation stems from T12-L1 sympathetic ganglia in mice, with pre-ganglionic acetylcholinergic neurons arising from T7-T10 of the spinal cord [98]. Although *in situ* visualisation of the origins of sympathetic nerves has been revolutionary, more detailed ganglia-specific functional analyses remain limited, owing to the difficulty of dissecting individual sympathetic ganglia, coupled with a lack in specific targeting and the underlying complexity of neuronal circuitry.

1.4.2 Sympathetic neurons form the efferent arm in the neuroendocrine negative feedback loop of leptin action

With indirect evidence for the action of sympathetic neurons on white adipose tissue, in 2015 Zeng et al. definitively proved a direct role for sympathetic neuron-derived NE on inguinal scWAT lipolysis [5]. First, using clearing techniques which render adipose tissues optically transparent, they detected nerve axon bundles throughout the entire inguinal scWAT fat pad - the larger of which they found could be dissected. The whole axonal tree of sympathetic neurons in adipose tissue was visualised *in situ* three years later, by coupling antibody staining with 3D imaging of solvent cleared organs (DISCO) [94][95][107]. In addition, intravital two-photon microscopy of murine adipose tissue revealed direct contact between sympathetic neurons and white adipocytes – coining the term ‘sympathetic neuro-adipose junctions’ [5]. To demonstrate the functionality of the sympathetic neurons in AT homeostasis, optogenetic stimulation of sympathetic TH^{CRERosa26Chr2} expressing neurons within adipose tissues resulted in an acute increase in NE and WAT lipolysis, which translated into a reduction in fat pad size over a four-week stimulation period [5]. Moreover, leptin-mediated increases in adipose tissue NE and lipolysis were inhibited following either block of the sympathetic ganglia, nerve crush injury or diphtheria toxin-mediated ablation of adipose tissue TH⁺ sympathetic neurons, highlighting both the sufficiency and necessity of intact sympathetic AT innervation in WAT lipolysis in response to leptin (Figure 1.1A) [5]. Collectively, this study described the efferent sympathetic arm which completes the negative feedback loop of leptin action.

1.4.3 Non-sympathetic innervation of adipose tissue

Co-staining of nerve axon bundles dissected from adipose tissue with TH and a pan-neuronal marker revealed molecular heterogeneity, with scWAT nerve axon bundles containing both sympathetic TH⁺ and non-sympathetic TH⁻ axons [5]. Notably, neuron presence within an organ does not necessarily equate to a tissue-specific functional role – several neurons found within adipose tissues are thought to be *en passant*, and serve no functional role in AT homeostasis. This heterogeneous population of neurons within individual nerve axon bundles also highlights the lack of specificity of previously mentioned ‘sympathetic’ denervation or electrical stimulation/ extracellular recording methods, as non-sympathetic neurons present within axon bundles are also included in such manipulations. Interestingly, whilst reports of parasympathetic innervation of WAT have been contradictory: a series of PRV retrograde tracing and functional studies all claim the inexistence of parasympathetic innervation in the WAT of multiple species [91][106][101]. In terms of efferent innervation, conclusive evidence exists showing a role for sympathetic innervation in adipose tissue [5], whereas so far parasympathetic innervation of adipose tissue has not been reported.

The extensive PRV tracing studies performed by Bartness and colleagues also provide evidence of sensory innervation of white and brown adipose tissues. Injection of herpes-simplex virus (HSV) 129 – an anterograde trans-synaptic tracer – into the scWAT resulted in labelling 48 hours post-injection of the dorsal root ganglia (DRG), which contains most afferent sensory neurons within the spinal cord [108]. Additional evidence indicates a possible functional role for adipose tissue sensory neurons. Niiijima and colleagues [109] detected increased activity of ‘afferent’ WAT sensory neurons following intra-adipose leptin injection [109]. However, this increased activity was detected through extracellular recording of nerve axon bundles innervating white adipose tissue, which also contain leptin-stimulated sympathetic efferent neurons [5]. This increase in electrical signal may, therefore, reflect the off-target leptin-induced activation of sympathetic neurons within adipose tissues. Sensory innervation has also been linked with the indirect modulation of BAT thermogenesis. Garretson et al. [110] found that administration of

an adrenergic receptor $\beta 3$ (ADR $\beta 3$) agonist, induced lipolysis within the subcutaneous white adipose tissue of Siberian hamsters, and was sufficient to increase the expression of the neuronal activation marker C-FOS within the L1-L3 dorsal root ganglia - which contains the cell bodies of scWAT-innervating sensory neurons [106][110]. This response to ADR $\beta 3$ -induced lipolysis in scWAT indicates sensory feedback of sympathetic activity and lipolysis within the WAT. Furthermore, Garretson et al. [110] show that this stimulation of scWAT sensory neuron activity is sufficient to increase interscapular brown adipose tissue temperature - an observation which is lost following surgical denervation of the scWAT [110]. Although WAT surgical denervation is not sensory neuron-specific and likely results in damage to surrounding sympathetic neurons and vasculature, this collective finding indicates that anatomically distinct adipose tissues in the Siberian hamster are able to communicate through autonomic nerve signals. Finally, a capsaicin-induced, specific loss in unmyelinated (C) or thinly-myelinated (A- δ) sensory fibres in the WAT of hamsters has also been linked with increased adiposity, further suggestive of afferent neuronal inputs in the regulation of adipose tissue homeostasis [111]. Taken together, the presence of a functional sympathetic neuro-adipose junction regulating AT homeostasis is irrefutable, as is the presence of sensory neurons within WAT [5]. Although evidence exists for a potential sensory afferent input into the central modulation of AT homeostasis, the exact mechanism of function of these neurons - which also likely represent TH⁺ neurons within AT nerve fibres reported by Zeng and colleagues - is yet to be determined [5].

1.4.4 Adipose tissue neuronal plasticity

Adipose tissue sympathetic neurons undergo extensive morphological changes during metabolic challenge, including densification of the sympathetic axonal tree with cold shock, which is known to increase sympathetic drive onto AT, further increasing thermogenic capacity [29][94][112][113]. Conversely, adipose tissue sympathetic innervation is significantly reduced in the scWAT and BAT of severely obese *Ob/Ob* mice compared with lean controls, and also seems to be reduced in human scWAT with increased BMI

[114]. Whilst exogenous leptin treatment fully rescues this AT sympathetic neuropathy in *Ob/Ob* mice and stimulates concomitant weight loss, cold shock, and sympathetic output onto ATs, it is unable to increase BAT innervation [63][114]. Furthermore, it is thought that sustained obesity culminates in sympathetic neuropathy in both mice and humans [114] – although local players mediating such changes have not yet been identified. Young mice with higher activity levels show an increase in total scWAT innervation and in particular sympathetic innervation, compared with sedentary, age-matched controls [114]. The sympathetic drive-induced increase of BDNF in WAT stimulates an increase in neuronal – and specifically sympathetic neuron – density [63]. This observed AT sympathetic neuropathy is analogous to the reversible loss in sympathetic innervation with chronic HFD in the liver [115]. These reports suggest several potential targets which could be exploited therapeutically to restore the sympathetic efferent arm of the neuroendocrine loop of leptin action in DIO. Notably, the advancement of tissue clearing and 3D imaging techniques have been fundamental to these observations, which were not possible before due to the potential for inconsistencies in conventional 2-dimensional imaging of amorphous adipose tissues [95]. Collectively, this suggests that sustained obesity culminates in sympathetic neuropathy in both mice and humans, although local players mediating such changes remain unknown.

Morphologically, even in lean mice adipose tissue, innervation in scWAT and vWAT are not equivalent. scWAT and BAT axon bundles are thicker and dissectible, whereas it is not possible to dissect axon bundles from vWAT due to their more delicate nature (unpublished observations, Domingos lab). This is likely reflective of microenvironmental differences, and consistent with the idea that individual adipose tissue pads are distinct organs. Interestingly, in lean mice levels of transcriptional regulator PRDM16 – primarily linked with brown adipocyte differentiation and identity – are higher in scWAT than vWAT, and an adipocyte-specific ablation of PRDM16 results in the conversion of scWAT tissue towards a vWAT morphological and molecular phenotype [116][117]. Moreover, a later study in lean mice suggested a role for adipocyte-specific PRDM16 in the regulation of adipose tissue sympathetic neurite density – making this an exciting candidate in

the context of obesity-related sympathetic neuropathy, due to the spontaneous obesity accompanying an adipocyte-specific PRDM16 knockout [116]. It is also not yet clear how AT neuropathy in obesity might affect sensory innervation or function, which may also contribute to central leptin response in addition to the neuroendocrine negative feedback loop of leptin action. Another factor yet to be investigated is the intrinsic sensitivity of adipose tissue neurons to metabolic stress and proinflammatory cytokines. Both factors are enhanced during obesity and are likely also, in part, responsible for the observed peripheral adipose tissue neuropathy phenotype, as observed by Liu and colleagues in the metabolically challenged liver [115].

1.5 Immunomodulatory effects of leptin

The leptin receptor is a transmembrane receptor belonging to the class I cytokine receptor family [118]. Accordingly, leptin signalling has demonstrable immunomodulatory effects. Notably, although many studies utilise unvalidated leptin receptor antibodies to identify LepR⁺ cells, detection of transcripts by RT-qPCR or RNA *in situ* hybridisation remain the gold standard due to a lack of knockout-validated antibodies. RT-qPCR revealed that *ObRb* is functionally expressed in human peripheral blood mononuclear cells (PBMCs; lymphocytes and monocytes) and that its levels negatively correlate with BMI [119][120]. Notably, irradiated mice reconstituted with LepR-deficient *Db/Db*, rather than WT bone marrow, have a significantly lower WAT and body weight, independently of food intake [121]. Moreover, these mice show reduced vWAT macrophage infiltration, and upon feeding of high fat diet for a period of 16 weeks express pro-inflammatory cytokines at a significantly lower level [121]; indicating a role for peripheral leptin-immune cell signalling in adipose tissue homeostasis. In fact, leptin stimulation of PBMCs *in vitro* is sufficient to induce proliferation and induce secretion of potent pro-inflammatory cytokines TNF α , IL-6, and IFN γ [122]. In addition, stimulation of isolated human blood monocytes with leptin induced proliferation and activation, and also prompted the release of TNF α and IL-6 [123]. Conversely, *ex vivo* leptin treatment is sufficient to suppress both the proliferation and anti-inflammatory cytokine production of regulatory T cells

(Tregs) [124], further corroborating reports of leptin as a pro-inflammatory adipokine [125][126]. Leptin also inhibits the chemotaxis of human blood-derived neutrophils in a dose-dependent manner *in vitro* [127], and promotes the survival, chemokinesis and the release of pro-inflammatory cytokines - including IL-1 β , IL-6 and CCL2 - of human blood-derived eosinophils [128]. Furthermore, leptin is required for the full maturation of human PBMC-derived and mouse bone marrow-derived dendritic cells [129][130], and promotes the simultaneous polarisation of PBMC-derived naive T cells towards a T helper 1 (Th1) phenotype following lipopolysaccharide stimulation (LPS), whilst inhibiting the Transforming growth factor β (TGF β)-stimulated induction and proliferation of Tregs [129][130]. Whilst these *ex vivo* experiments have described the response of a range of immune cells to leptin treatment; they have utilised human PBMC and mouse bone marrow-derived immune cells, which show significant difference with those tissue-specific immune cells resident in adipose tissues. However, the contribution of leptin to immunopathologies associated with obesity should not be underestimated, and warrants further investigation.

1.6 Adipose tissue immune cells

Over recent years it has become increasingly apparent that the role of the immune system extends far beyond response to infection, with immune cells such as macrophages taking centre stage in the tissue-specific homeostatic regulation of multiple organs [131][132][133]. Similarly, immunometabolism describes the intersection of the immune system and metabolism, and can be interpreted in two ways. Firstly it relates to the cellular metabolism of immune cells, generally in the context of an immune response. Conversely, immunometabolism also describes the role of immune cells in whole organism metabolism. For this thesis, I will focus on describing the latter aspect of immunometabolism - in particular the role of adipose tissue-resident immune cells in adipose tissue homeostasis and whole-organism energy balance. Previous work has implicated adipose tissue-resident immune cells in a range of metabolic responses, including fasting-induced lipolysis [134], cold-induced thermogenesis [135][136][137],

metabolic health [138] and insulin resistance [139]. Here I will describe what is known about key adipose tissue resident immune cells, and how they are altered in obesity.

The immune system consists of a collection of cells and tissues that together defend the body against insult, and - in the context of infection defence - exists to discriminate between self and non-self. The immune system consists of two main arms: the innate and adaptive immune systems. Generally studied in response to infection, the innate and adaptive immune responses synergistically ensure the prompt eradication of pathogens, limiting damage to the body through controlled inflammation and the subsequent enhancement of tissue repair. Typically the innate immune system provides a fast, non-specific response; whereas the adaptive immune system results in an antigen-specific response and the generation of immunological memory. Fundamental components of the innate and adaptive immune systems include bone marrow derived haematopoietic cells, such as macrophages, neutrophils, eosinophils, and T cells, B cells and dendritic cells (DCs), respectively. However, more recently a role has been described for tissue-resident innate and adaptive immune cells in the context of organ homeostasis. Obesity is characterised by chronic, low-grade inflammation of adipose tissue - a consequence of adipose tissue immune dysregulation. The accumulation of pro-inflammatory immune cells coupled with a concomitant loss in anti-inflammatory leukocytes in obesity shifts adipose tissue towards a more pro-inflammatory environment. Anti-inflammatory adipose tissue immune cells prevent the induction of inflammation, indirectly regulating the function of all proximal immune cells, stromal cells and neurons. It is, therefore, clear that the balance in adipose tissue resident immune cells, and factors controlling such immune cell recruitment and activity could be important targets in the prevention and treatment of obesity.

1.6.1 Macrophages

Macrophages dominate the immune landscape of adipose tissues, comprising around 5% of visceral WAT leukocytes in the lean state and expanding up to 50% in obesity

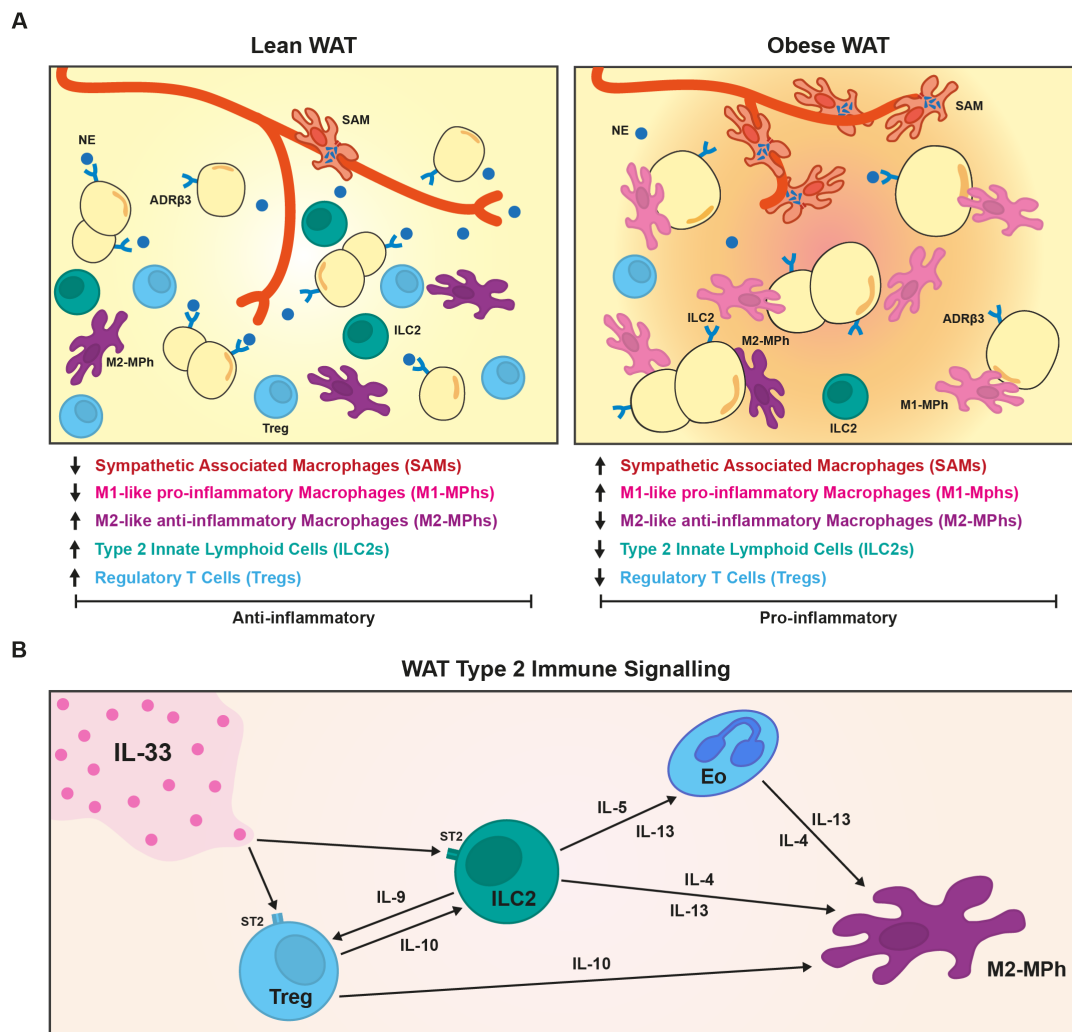


Figure 1.6: Adipose tissue immune cell changes in obesity

A White adipose tissue-resident immune cell milieu is significantly changed in obesity. Sympathetic neuron-derived norepinephrine (NE) induces adipocyte lipolysis in WAT through adrenergic receptors, including $ADR\beta3$. Sympathetic associated macrophages (SAMs) act as a sink for NE through SLC6A2-mediated uptake and MAOA degradation of NE, modulating lipolysis within adipose tissues. Few SAMs associate with sympathetic neurons in the lean state. In obesity SAMs are massively recruited to WAT sympathetic neurons, reducing NE-mediated adipocyte lipolysis. In the lean state, yolk sac-derived M2-like anti-inflammatory macrophages (M2-MPHs), type 2 innate lymphoid cells (ILC2s), eosinophils (Eo) and regulatory T cells (Tregs) maintain an anti-inflammatory state in adipose tissue. In obesity, bone marrow-derived M1-like pro-inflammatory macrophages (M1-MPHs) are highly recruited through increased release of chemotactic signals, including CCL2. Pro-inflammatory macrophages significantly contribute towards chronic, low-level adipose tissue inflammation. **B** Type 2 immune cell signalling in white adipose tissue. IL-33 = Interleukin-33, ST2 = Suppression of Tumourigenicity 2 (IL-33 receptor), IL-9 = Interleukin-9, IL-10 = Interleukin-10, IL-5 = Interleukin-5, IL-4 = Interleukin-4, IL-13 = Interleukin-13.

(Figure 1.6A) [140]. This accumulation of macrophages in white adipose tissue has been observed in both mouse models of obesity and obese humans over the last 20 years. Early immunohistochemical (IHC) staining of human adipose tissue identified cells positive for the pan-macrophage marker CD68, in both visceral and subcutaneous white adipose tissues. Of note, macrophages are present at a higher frequency in visceral than subcutaneous white adipose tissues in the lean state [141], again highlighting distinctions between different white adipose tissue depots. Given these key differences, it is likely that individual adipose tissue depots utilise tissue-specific mechanisms in AT homeostasis. Macrophages, whether recruited or tissue-resident, adopt tissue-specific characteristics dependent upon their microenvironment. They possess the receptors and machinery to detect and respond to cytokines, adipokines, insulin, free fatty acids, hormones, pathogen and danger-associated molecular patterns, and other mediators [142]. Collectively, these mediators influence macrophage phenotype and function, and lead to tissue-specific differences between macrophage populations.

In what started as an investigation into the role of macrophages in Rett syndrome, Wolf and colleagues ended up also demonstrating a central role for macrophages in energy expenditure [143]. The targeted deletion of *Methyl CpG Binding Protein 2* (*Mecp2*, loss of function mutations of which are causative of Rett syndrome) in macrophages using a Cre-Lox system resulted in spontaneous obesity in ND-fed mice, which was accelerated with HFD feeding. This was associated with significantly higher fat mass and lower lean mass in comparison to control littermates. Of note, Macrophage-restricted *Mecp2* deletion impaired innervation, NE production, and thermogenesis in brown adipose tissue [143]. In addition, another study by Rached et al. [144] reported that the macrophage-specific deletion of *Insulin Receptor Substrate 2* (*IRS2*) resulted in enhanced expression of thermogenic genes, and increased innervation within brown adipose tissue - rendering mice more resistant to obesity. Together, these two studies strongly suggest a role for macrophages in the control of brown adipose tissue thermogenic function.

Although the exact instigator of white adipose tissue inflammation in obesity is unclear, it is likely a downstream effect of cellular stress associated with remodelling of WAT for the storage of surplus energy as triglycerides. However, following the onset of obesity, highly recruited macrophages are a major source of adipose tissue inflammation (Figure 1.6A) [140]. IHC and *in situ* hybridisation experiments revealed that the macrophage genes *Cd68* and *F4/80* were upregulated in the visceral WAT of DIO, *Db/Db*, and *Ob/Ob* mice [145], an initial indication of a role for adipose tissue inflammation and macrophages in obesity. Through profiling of RNA expression in lean and obese visceral and subcutaneous fat depots, Weisberg et al. [140] also provided evidence that the expression of macrophage marker genes - including *Csfr1* and *Cd68* positively correlate with body weight and adipocyte size. Furthermore, IHC and RT-qPCR analyses have revealed that macrophages in obese white adipose tissues are pro-inflammatory (or M1-like) in nature, highly expressing pro-inflammatory mediators including *Tnfa*, *iNos* and *Il6* (Figure 1.6A). This type 1, pro-inflammatory immune response is similar to exposure of macrophages in response to infection or LPS stimulation, where the release of pro-inflammatory cytokines results in the recruitment and activation of additional immune cell subsets and pathogen eradication. However, inflammatory responses to acute infection are transient and resolve following elimination of the insult, whereas in instances of obesity this inflammation becomes chronic, more comparable to cases of autoimmune disease. Finally, bone marrow transplantation studies have suggested that the vast majority (approximately 85%) of macrophages within visceral and subcutaneous white adipose tissue in the lean state are bone marrow derived [140]. Collectively, this raised the idea that macrophage recruitment is increased in the case of obesity, and that AT inflammation is further propagated by an influx of M1-converted (pro-inflammatory) macrophages in WAT (Figure 1.6A).

Although obese brown adipose tissue is more resistant to obesogenic diet-induced inflammation than white adipose tissue [146], prolonged feeding of a high fat diet is sufficient to induce both increased BAT macrophage infiltration and subsequent inflammation - albeit to a lesser degree than that observed in visceral WAT [147][148].

Although at room temperature UCP1 levels were notably higher in high fat diet-fed mice compared with ND-fed controls (so-called diet-induced thermogenesis), high fat diet-fed mice are unable to increase UCP1 at both the mRNA and protein level following cold challenge [148]. Furthermore, thermogenic genes *Cell death inducing DFFA like effector A* (*Cidea*) and *Peroxisome proliferator-activated receptor γ* (*Ppar γ*) are expressed at a lower level in HFD-fed BAT compared with ND-fed BAT [147]. Upon histological investigation, HFD-fed BAT more closely resembles white adipose tissues, with larger and higher frequency unilocular lipid droplets than that found in normal diet BAT [147] [149]. Accordingly, interscapular brown adipose tissue expresses *Leptin* at a much higher level in obesity, reflecting a whitening of this tissue [149]. Therefore, in instances of obesity the role of BAT shifts from thermogenic towards the storing of excess lipids. Although unclear, it is likely that lipid storage itself and associated pro-inflammatory factors render brown adipose tissue less competent at thermogenesis. In fact, IP treatment with recombinant pro-inflammatory mediator $\text{TNF}\alpha$ is sufficient to compromise brown adipose tissue thermogenesis, through the impairment of *Ucp1* upregulation [148]. Although a clear correlation exists between brown adipose tissue inflammation and thermogenic capacity, an extensive investigation into the causatory immune cell infiltration has not been performed. Considering the shift of obese BAT towards a WAT phenotype it is likely that a few key players are shared, however as previously mentioned significant differences in the phenotype and regulation of anatomically distinct adipose tissue organs exist - so comparisons should be made with caution.

Though simplistic, the M1/M2 macrophage polarisation paradigm has been usefully employed to characterise the change in murine macrophage phenotype in response to obesity (Figure 1.6A). Studies have shown that diet-induced obesity induces a switch in the profile of AT macrophages from M2-like “alternatively activated” macrophages to the classically activated M1-like pro-inflammatory phenotype which contributes to insulin resistance in the visceral WAT [150][151]. Whilst M1-like macrophages are induced following exposure to type 1 pro-inflammatory cytokines including $\text{TNF-}\alpha$, type 2 anti-inflammatory cytokines - including Interleukin-4 (IL-4) and Interleukin-13 (IL-13)

promote the differentiation of M2-like macrophages. Together, M2-like macrophages along with other type 2 immune cells, including eosinophils, regulatory T cells and type 2 innate lymphoid cells (ILC2s) are described as having a homeostatic and tissue remodelling role (Figure 1.6A) [152]. Notably, AT macrophages in C-C motif chemokine receptor 2 (CCR2)-deficient mice more closely resemble M2-like macrophages from lean mice, indicating a role for bone-marrow derived macrophages in the propagation of WAT inflammation [150]. These M2-like macrophages are beneficial as PPAR γ and PPAR δ -deficient mice, which have impaired M2 polarisation, are more prone to diet-induced obesity, inflammation and insulin resistance [153][154][155]. Moreover, PPAR γ mutation in humans is associated with insulin resistance, type 2 diabetes, and lipodystrophy [156]. Collectively, this reinforces a role for anti-inflammatory, M2-like macrophages in lean adipose tissue homeostasis, which once perturbed results in conversion to a M1-like pro-inflammatory phenotype, propagating adipose tissue inflammation.

Both white and brown adipocytes secrete the primary ligand for CCR2 (expressed on circulating monocytes), the monocyte chemoattractant CCL2, and adipocyte expression of CCL2 is increased in obesity [157][158]. *In vivo* studies in which CCR2 is genetically ablated or overexpressed show that these recruited, pro-inflammatory macrophages mediate obesity, obesity-induced hepatic steatosis, adipose tissue inflammation, insulin resistance, and associated metabolic syndromes such as diabetes [159][160][161][162][163]. It is important to note that CCR2-dependent macrophages are monocyte-derived cells that are recruited to adipose tissue over time, in response to chemotactic signals such as CCL2. Additionally, evidence from Amano et al. shows that visceral AT macrophages proliferate locally in at least a partially CCL2-dependent manner, contributing to macrophage accumulation in AT independently of bone marrow-derived monocyte recruitment [164]. The ability to specifically target these monocyte-derived macrophages through CCR2 manipulations has vastly facilitated our understanding of bone marrow derived macrophages, and their role in obesity. This evidence for the involvement of bone marrow-derived macrophages supports the notion that macrophages in obesity are distinct from true adipose tissue-resident macrophages, which are yolk sac or foetal

liver-derived, seeded embryonically and undergo local self-maintenance [165]. Due to a lack of molecular tools available to specifically target true adipose tissue-resident macrophages, understanding their role in obesity has been particularly difficult - as the rise in AT macrophage frequency during obesity is not only due to the recruitment and differentiation of CCR2-dependent blood monocytes.

1.6.2 Sympathetic associated macrophages

In the context of obesity, research has primarily attributed the role of macrophages to the consequences of obesity; the associated adipo-inflammation and their contribution to insulin resistance and type 2 diabetes. The idea that macrophages could play a more direct role in organismal energy metabolism and adiposity was controversial until the latter part of the last decade. Two papers from 2017 were the first to show the presence of norepinephrine catabolising macrophage populations within adipose tissues directly regulate organismal adiposity. A study by Pirzgalska and colleagues identified Sympathetic neuron-associated macrophages (SAMs) as cellular mediators of noradrenaline clearance contributing to the obese state (Figure 1.6A) [166]. SAMs were identified in subcutaneous WAT and BAT as a unique macrophage population, distinct from AT macrophages and microglia and characterised by the functional expression of the metabolic machinery necessary for the uptake and degradation of norepinephrine: Solute carrier family 6 member 2 (SLC6A2) and Monoamine oxidase A (MAOA), respectively. Loss of SLC6A2 function in obese mice attenuated obesity, promoted lipolysis, and restored thermogenesis [166]. SAMs possessing the same machinery are also present in human sympathetic ganglia, suggesting the regulation of sympathetic neuroadipose signalling is evolutionarily conserved and, therefore, could be a viable anti-obesity therapeutic target. Simultaneously, Camell et al. reported detecting the same NE-catabolising nerve-associated macrophages in visceral WAT [134]. Here, they suggested recruitment of SAMs over time could be responsible for their observed reduction in lipolysis with age, through the degradation of norepinephrine in an NLR family pyrin domain containing 3 (NLRP3) inflammasome-dependent manner [134]. Genetic

deletion of *Nlrp3* in aged mice restored lipolysis through the Growth differentiation factor 3 (GDF3)-mediated downregulation of MAOA - which is known to break down norepinephrine [134].

Further contributing to the notion that macrophages could play more direct roles in metabolism regulation were two reports from the same lab: that M2-like 'alternatively activated' (IL-4 stimulated) AT macrophages are able to synthesise and release catecholamines (namely norepinephrine); promoting energy expenditure in response to cold exposure through non-shivering thermogenesis in BAT and lipolysis in WAT [167][168]. However, these findings were starkly refuted by Fischer and colleagues [146] who, rather than using constitutive germline-knockouts (as used in refs [167][168]), instead generated bone marrow chimeric mice in which *Th*, a gene encoding the enzyme necessary for the production of norepinephrine, could be deleted in haematopoietic cells in an inducible manner. Upon *Th* deletion, chimaeric animals did not differ from WT mice in their energy expenditure, neither at room temperature nor during cold exposure, indicating that TH is disposable in macrophage for metabolic challenge response [146]. Moreover, conditioned media from *in vitro* alternatively activated macrophages alone had no effect on thermogenesis in primary WAT or BAT, and IL-4 treatment failed to impact body weight, body composition, or energy expenditure at thermoneutrality or in cold conditions [146]. Using multiple genetic reporter approaches and RNA-seq, the group also definitively showed that adipose tissue macrophages do not express *Th* - excluding the possibility that M2-like alternatively activated macrophages in adipose tissue produce norepinephrine [146]. This finding was subsequently validated by a second group [144].

Owing to the contribution of SAMs to obesity, a local neuro-immune or neuro-adipose contribution in the dysregulation of adipose tissue innervation is highly likely, reflecting obesity-related changes within adipose tissue microenvironment. SAMs are massively recruited onto sympathetic neurons in both *Ob/Ob* and DIO mice [166]; their close involvement with sympathetic neurons in these two models of obesity may make them

an excellent candidate for the regulation of innervation, amongst other proximal immune and stromal cells. Substantiating the possibility for immune regulation of adipose tissue innervation, Wolf and colleagues described spontaneous obesity and impaired BAT innervation in a mouse model with a macrophage-specific deletion of *Mecp2* [143]. They suggest a mechanism by which the upregulation of plexin-A4 on CX3CR1⁺ *Mecp2*-knockout macrophages repels neurons in a Semaphorin 6A (SEMA6A)-dependent manner, diminishing sympathetic innervation of BAT [143][169]. Furthermore, the role of macrophage-derived TNF α in Sterile alpha and TIR motif containing 1 (SARM1)-mediated sympathetic neuropathy in the livers of mice with DIO further implicates immune cells in the modulation of innervation [115]. Besides, Wang and colleagues report impairment in the leptin-mediated enhancement of inguinal scWAT and BAT sympathetic innervation following the central disruption of BDNF neuron signalling [63]. Moreover, the presence of BDNF within the brain but not adipose tissue highlights the possibility of additional centrally-mediated regulation of sympathetic adipose tissue innervation [170]. Adipose tissue-derived Nerve growth factor (NGF) signalling has also been suggested as a mediator of sympathetic innervation, however, a mechanism detailing obesity-related perturbations of NGF in neuropathy is yet to be determined [94].

Despite seeking to answer substantially different questions, these key papers from 2017 exploring the role of adipose tissue macrophages converged at the same conclusion: nerve-associated adipose tissue macrophages regulate adipose tissue mass and energy expenditure through the modulation, not initiation, of sympathetic noradrenergic signalling. Such startling discoveries have revolutionised our understanding of adipose tissue homeostasis, and introduced an alternate paradigm of immunometabolic regulation which can be manipulated for therapeutic benefit. In this regard, the therapeutic success of immunotherapies, for diseases other than obesity, brings new hope to patients who suffer from the stigmatised condition of obesity.

1.6.3 Type 2 immune cells

Type-2 immunity is characterised by the production of Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-9 (IL-9) and Interleukin-13 (IL-13), and is generally described in response to parasitic infection or allergic inflammation. However, more recently a role type-2 immunity in tissue repair and fibrosis has been described. T helper 2 (Th2) cells, eosinophils, mast cells, basophils, type 2 innate lymphoid cells (ILC2s) along with IL-4 and IL-13 activated macrophages have all been implicated in the type-2 immune response, which is involved in the re-establishment of homeostasis following insult in a variety of tissues [171, 172].

1.6.4 Type 2 Innate Lymphoid Cells

Type 2 Innate Lymphoid Cells, or ILC2s, are considered a small-but-mighty player in the regulation of tissue homeostasis. Tissue-resident ILC2s were initially identified in the visceral WAT of mice, and their identity is governed by expression of transcription factor GATA binding protein 3 (GATA3) (Figure 1.6A) [173]. Although relatively few in number, ILC2 cells express a range of 'humoral' receptors, and are activated by Interleukin-25 (IL-25) and Thymic stromal lymphopoietin (TSLP) - although their major activator is Interleukin-33 (IL-33, Figure 1.6B) [173][174][175]. Upon activation, these cells produce high amounts of type 2 cytokines IL-4, IL-5 and IL-13, which both recruits type 2 eosinophils (IL-5 and IL-13) and promotes the induction of M2-like anti-inflammatory macrophages in adipose tissues (IL-4 and IL-13) - contributing towards an anti-inflammatory AT microenvironment (Figure 1.6B) [176][138][177][178][179]. Diminished ILC2 frequency and cytokine production are a characteristic of human and murine obesity in scWAT [135]. Furthermore ILC2 cells are almost entirely lost in the visceral WAT of aged mice, a difference which is minimised with calorie restriction [180]. Increased pro-inflammatory cytokine levels in obese visceral WAT, including Interferon γ (IFN γ), TNF α , IL-6 and Interleukin-1 β (IL-1 β) significantly stifles both the proliferation and production of type 2 cytokines IL-5 and IL-13 [181], and in particular increased pro-inflammatory cytokine IFN γ has been linked with loss of ILC2s in obese and aged

mice [182]. Furthermore, upon IL-33 stimulation ILC2s enhance the proliferation of adipose tissue Tregs, through Inducible T cell costimulator ligand (ICOSL) and OX40 ligand (OX40L) signalling [183]. Therefore, a loss in ILC2 frequency or function has a profound effect on adipose tissue, propagating a pro-inflammatory obese phenotype through a loss of anti-inflammatory (type 2) immune cells and cytokines.

1.6.5 Eosinophils

One key member of the type 2 immune response is the eosinophil, an innate immune cell which - through the production of IL-4 and IL-13 - promotes the M2-like anti-inflammatory polarisation of macrophages to prevent the onset of chronic, low-grade adipose tissue inflammation (Figure 1.6B). Reports on the effects of obesity on adipose tissue-resident eosinophil frequency have varied, with some groups citing a reduction [184][185] and others reporting an increase in frequency with diet induced obesity [186]. However, it is clear that eosinophil frequency is reduced with age [180][187]. Given that ILC2 cells are a primary source of eosinophil regulator IL-5, this could be a consequence of the reduced functionality of ILC2 cells observed with age (Figure 1.6) [180].

In diet induced obesity, Ding et al. [185], observed a reduction in CD11b⁺ Siglec F⁺ eosinophil frequency within BAT and vWAT, but not scWAT [185]. Alternatively, Lee et al. [186] described an increase in the percentage of CCR3⁺ Siglec F⁺ eosinophils within the vWAT following 12 weeks of HFD. Moreover, they identify adipocyte-differentiated 3T3 cells as an enhancer of eosinophil viability *ex vivo*, and provide evidence that selective loss of eosinophils is sufficient to reduce the expression of type 2 cytokines *Il4* and *Il13*, and adipogenic/adipocyte genes *Pparg*, *Cav1*, *Lpl* and *Leptin* in the vWAT of high fat diet-fed Δ dbIGATA mice, which specifically lack eosinophils [186]. Moreover, Δ dbIGATA mice gain significantly more weight when challenged with a high fat diet, and conversely increased eosinophil frequency through overexpression of *Il5* protected against this diet induced obesity [186]. However, IL-5-stimulated increase in vWAT CD11b^{low} Siglec F⁺ eosinophil frequency in HFD-fed mice, had no significant impact on

the metabolism of these mice - indicating that eosinophils alone are unable to affect adipose tissue metabolism in the DIO state [188]. Collectively, evidence suggests that eosinophils are positive regulators of adipose tissue homeostasis, and likely contribute to the suppression of obesity through IL-4-mediated induction and maintenance of alternatively activated M2-like anti-inflammatory macrophages.

1.6.6 Other innate immune cells

Though macrophages comprise most of the immune cell compartment in adipose tissue, dendritic cells (DCs)[189], neutrophils [190], mast cells [191], and natural killer (NK) cells [192], also accumulate in adipose tissues during weight gain. Other subsets, like innate lymphoid cells (ILCs) from sites other than the adipose tissue may also play a role in the induction of obesity [193]. The accumulation of DCs in adipose tissue and the liver during HFD-feeding is associated with macrophage recruitment to both organs by gain and loss of function experiments [194]. Mice specifically lacking functional dendritic cells, through Fms related receptor tyrosine kinase 3 (FLT3) deficiency, results in resistance to HFD-induced obesity, insulin resistance, and hepatic steatosis [194]. Despite these mice exhibiting increased caloric intake, heightened activity and metabolic rate mitigated the effects of this overnutrition [194]. Though this would suggest that DCs play a deleterious role in obesity, Macdougall and colleagues [195] revealed that conventional DCs subsets within the visceral WAT actively suppress AT inflammation and promote insulin sensitivity under homeostatic lean conditions, via PPAR γ and activation of the Wnt/ β -catenin pathway. Disruption of these pathways resulted from chronic overnutrition, and lead to dendritic cell activation, adipo-inflammation and insulin insensitivity [195]. Like DCs, neutrophils also infiltrate the liver and adipose tissue during the HFD-feeding, and help to orchestrate the infiltration of subsequent immune cells [190][196]. However, unlike DCs, neutrophils contribute towards obese adipose pathology more directly. Mice lacking neutrophil elastase, an inflammation-causing protease found in neutrophil primary granules, had a marked reduction in neutrophil and macrophage vWAT infiltration which resulted in lower levels of inflammation [196]. As

neutrophils are typically involved in the early inflammatory response following insult, this study indicates they may be initial instigators of the chronic low grade adipose tissue inflammation which characterises obesity. However, under physiological conditions it is not clear whether this adipose tissue inflammation is triggered by any one cell or signal; the chronic low grade adipose tissue inflammation characteristic of obesity likely represents an accumulation of smaller insults over time.

1.6.7 Regulatory T cells

Regulatory T cells, or Tregs, are an anti-inflammatory subset of T cells characterised by the expression of the transcription factor Forkhead Box Protein 3 (FOXP3). Whilst little is known about brown adipose tissue Tregs, vWAT Tregs were the first recognised tissue-resident Treg population. A key paper by Feurer et al. [139] describes a unique population of Tregs in lean vWAT, which is lost in obesity. Unlike lymphoid tissue-resident or circulating 'peripheral' Tregs, vWAT-resident Tregs have a distinct transcriptome mediated by the co-expression of transcription factors PPAR γ and FOXP3 [139][197][198][199]. In fact, genetic deletion of the *Pparg* gene (encoding PPAR γ) - which canonically regulates brown adipocyte differentiation - significantly reduces the frequency of vWAT Tregs [199]. Whilst they express common 'peripheral' Treg markers *Cd25* and *Cytotoxic T lymphocyte associated protein 4* (*Ctla4*) [197], and T cell activation markers *Cluster of differentiation 44* (*Cd44*) and *Nuclear receptor subfamily 4 group a member 1* (*Nr4a1*) [199], vWAT-derived Tregs uniquely express a range of cytokine receptors (*Ccr2*, *Ccr3*, *St2*, *Il9r*) [139][200][201][202] and lipid metabolism genes (*Ldlr*, *Cd36*) [198][203]. Furthermore, vWAT Tregs downregulate 'trafficking' molecules, indicating true tissue residency [204][205]. Unlike 'peripheral' Tregs, vWAT-resident Tregs are highly dependent on IL-33, both directly through IL-33-ST2 signalling (Figure 1.6B) [200][201][202] and indirectly through the ILC2 activation and ICOS-ICOSL signalling [206]. In fact, at 20-25 weeks of age approximately 80% of vWAT Tregs are positive for the IL-33 receptor Suppressor of tumourigenicity 2 (ST2) [138]. Moreover, mice with a Treg-specific conditional deletion of ST2 lack Killer

cell lectin like receptor G1 (KLRG1) - reported as a *bona fide* marker of vWAT Tregs [207].

Tregs are protective against obesity [139], likely through the maintenance of an anti-inflammatory adipose tissue environment which is lost in obesity. Diphtheria toxin-mediated depletion of FOXP3 Tregs is sufficient to increase vWAT inflammation and worsen metabolic parameters, both of which can be rescued through Treg adoptive transfer or IL-2-mediated Treg expansion [139][208][209]. Furthermore, a loss in PPAR γ Tregs is sufficient to induce metabolic dysfunction, highlighting a functional role for adipose tissue-resident Tregs in the prevention of obesity and associated metabolic disorders. Along with their expression of anti-inflammatory cytokines [139], vWAT Tregs propagate an anti-inflammatory environment through the induction of M2-like macrophages, the suppression of pro-inflammatory macrophage differentiation [200][210], and the suppression of conventional T cell activation and proliferation through the production of Prostaglandin E2 (PGE2) [211].

Tregs can be induced either in the thymus (tTregs) during thymocyte development, or peripherally following activation of naïve T cells in the presence of Treg inducer TGF β . During T cell development haematopoietic precursors enter the thymus and undergo a stereotypical step-wise development. T cell receptors (TCRs) are randomly generated through somatic gene recombination, producing a diverse pool of T cells which will eventually be able to recognise foreign peptides presented within self Major histocompatibility complex (MHC) [212]. Although TCR diversity increases the chances of T cell-pathogen recognition and response, one consequence of the random nature of somatic gene recombination is the generation of T cells which become activated in response to 'self' proteins. Therefore, the induction of tolerance to 'self' is crucial to prevent autoimmune T cell activation to proteins which may be encountered throughout the body. Before release from the thymus as naïve T cells, maturing thymocytes which recognise and react to 'self' are selectively deleted through a process called negative selection. Thymocytes recognising MHC-presented self antigens with a high affinity or avidity undergo apoptosis to prevent the development of self-reactive T cells

[213]. Thymocytes recognising self antigens with low affinity or avidity escape both 'death by neglect' and negative selection, and enter the periphery as naïve T cells [213]. TCRs inducing intermediate signalling intensities which avoid negative selection preferentially become Treg cells [213]. The incomplete nature of self-reactive T cell eradication is apparent in the lethal autoimmunity observed in FOXP3-deficient mice [214]; highlighting the importance of these cells in the prevention of autoimmune disease [215]. The generation of immunomodulatory Tregs, coupled with the clonal deletion of self-reactive thymocytes, describes the induction of central tolerance in both a dominant and recessive fashion, respectively; demonstrating the multiple mechanisms by which self-tolerance is instigated.

Extensive investigation from Kolodin et al. [200] revealed that true vWAT-resident Tregs are seeded before 3-4 weeks of age, and cannot be replenished after 8 weeks of age following Treg-specific diphtheria toxin ablation. vWAT Tregs require T cell activation for PPAR γ expression [199], namely the presentation of a cognate peptide within MHCII recognised specifically by the T cell receptor. Unlike 'peripheral' Tregs which have a diverse range of TCRs with the potential to respond to a wide range of pathogenic antigens upon infection, vWAT Tregs have restricted TCR specificity [200]. This, coupled to the close proximity of vWAT Tregs to MHCII⁺ antigen presenting cells [200] led to the question of which TCR-specific antigen could be activating and clonally expanding vWAT Tregs [200][216]. Recent work by Li et al. [199] identified a number of vWAT Treg-specific TCR from clonally expanded vWAT Treg clones. They genetically engineered a mouse line where all T cells express one of the identified vWAT TCRs (vTreg53), and found that the vWAT Tregs were specifically enriched for the vTreg53 TCR - indicative of clonal expansion in response to cognate antigen-TCR interactions [199]. Moreover, upon adoptive transfer to wildtype mice, vTreg53 Tregs accumulated specifically within the vWAT [199]. Collectively, this indicates the specific generation and induction of Tregs which are able to recognise 'self' vWAT antigens which have not yet been definitively identified. vWAT Treg activation through specific 'self' vWAT antigens therefore provides one mechanism by which vWAT Tregs maintain an anti-inflammatory

adipose tissue environment [216].

Although the relative contribution of these anti-inflammatory cells in adipose tissue homeostasis is yet to be fully determined, it is clear that each plays a role in the maintenance of adipose tissue homeostasis - a balance which is perturbed in obesity.

1.7 Adipose tissue stromal cells

When considering how intra-adipose cellular interactions mediate obesity, we must not forget that adipose tissue depots constitute *bona fide* organs, comprising heterogeneous cell subtypes, including adipocytes, immunocytes, nerves and stromal cells. Until relatively recently, the contribution of the stromal cell compartment to adipose tissue inflammation has been largely overlooked. However, two side-by-side studies published from the labs of Diane Mathis [207] and David Artis [217] have probed this gap in our knowledge in WAT. Both Spallanzani et al. [207] and Mahlaköiv et al. [217] sought to identify the cellular sources of IL-33; a cytokine which has been negatively correlated with BMI [218], and positively associated with the maintenance of an anti-inflammatory AT immune cell milieu. Local IL-33 production has been implicated in the accumulation and function of vWAT eosinophils, ILC2 cells [217] and Tregs [200][201][207][219], preventing adipose tissue inflammation and the associated metabolic dysfunction [219]. Mathis and colleagues [207] used flow cytometry and single-cell RNA sequencing to revealed substantial AT stromal cell heterogeneity, and identified $\text{PDGFR}\alpha^+\text{SCA-1}^+\text{PDPN}^+$ mesenchymal stromal cells (MSCs) as the major producers of IL-33 in vWAT. Using a $\text{PDGFR}\alpha^{\text{CRE}}$ -driven conditional IL-33 deletion model, they show that the accumulation of vWAT Tregs is dependent on MSC-derived IL-33, as FOXP3^+ Treg frequency is reduced in its absence [207]. In parallel, Artis et al. [217] used flow cytometry and RNA sequencing to attribute IL-33 production primarily to a population they call adipose stem and progenitor cells (ASPCs) in mice and humans [217], which largely overlaps with the IL-33 producing populations described by Spallanzani et al. [207]. Collectively, Spallanzani et al. and Mahlaköiv et al. identify phenotypic and functional heterogeneity

in adipose tissue stromal cells, and identify a subset which express *Il33* in vWAT and scWAT, along with mesothelial cells surrounding the vWAT [207][217].

Mahlaköiv et al. go on to describe a regulatory signalling axis in which ASPC-derived IL-33 promotes ILC2 activation, which in turn produce type 2 cytokines IL-5 and IL-13 to maintain an anti-inflammatory adipose tissue milieu [217]. Supporting the role of AT stromal cell-derived IL-33 in metabolic homeostasis, the authors show that a genetic deletion of *Il33* increases weight gain in mice fed a HFD, relative to wildtype controls [217]. However, the lack of a more specific conditional knockout mouse model in this study makes it difficult to attribute this HFD-induced weight gain to AT stromal cell-derived IL-33 alone [217], as *PDGFR α ^{CRE}*-conditional IL-33 knockout mice do not gain weight under normal diet conditions [207], and HFD metabolic challenge was not performed on these conditional knockout mice. However, supporting a role for IL-33 in adipose tissue homeostasis, obese mice injected IP with recombinant IL-33 have a higher vWAT Treg frequency, reduced inflammation and improved metabolic indices [201][219][206][220]. Finally, a more recent study by Goldberg et al. [180] highlights changes in the cellular source of vWAT IL-33 with age. Whilst young mesenchymal stromal cells are the major producer of IL-33 in young WAT, in aged vWAT IL-33 is produced almost exclusively by the surrounding mesothelium [180], likely limiting the bioavailability to type 2 IL-33 responsive immune cells within vWAT - one explanation for the age-related reduction in ILC2 cells observed with age.

1.8 Summary

Our understanding of obesity has now developed to the point where it cannot be designated a simple disease of excessive energy intake, the obese state is characterised by neuropathologies and immunopathologies which perturb energy balance and perpetuate the obese condition. Leptin, macrophages, and sympathetic neuron-derived norepinephrine take central roles in these adipo-pathologies. Leptin is more than a centrally-acting mediator of appetite; macrophages are more than just co-opted

inflammatory bystanders; and norepinephrine is a potent fat burner, constrained by central leptin resistance and the accumulation of SAMs in obesity. Our advancing understanding of the neuroimmune mechanisms underlying obesity has opened the door to therapies targeting obesity, however our incomplete understanding of 'supporting' stromal cells involved in the regulation of adipose tissue homeostasis likely leaves a number of novel cellular targets still to be discovered which could be exploited. Adipose tissue stromal cells have only recently become apparent as a key regulator of AT-resident immune cells and homeostasis, and it is clear that these 'supporting' cells are more than just structural in nature. To date IL-33 remains the main character in AT stromal-immune cell signals explored in relation to adipose tissue and obesity, but as resolution of AT immune cell dynamics and stromal cell identity increases, it is likely that additional cellular and molecular therapeutic targets will become apparent in the prevention and treatment of obesity.

1.9 Hypothesis

Sympathetic neuron-associated stromal cells within adipose tissue modulate adipose tissue immune cells through the integration of neuronal and hormonal signals.

1.10 Aims

- Assess the presence of leptin sensitive cell populations within sympathetic adipose-associated tissues.
- Determine whether sympathetic associated stromal cells within adipose tissues express immunomodulatory cytokines or sympathetic neurotransmitter receptors.

Chapter 2

Materials and Methods

Contents

2.1	General laboratory reagents	46
2.2	Animal husbandry	46
2.2.1	Mouse genotypes	46
2.2.2	Metabolic phenotyping	48
2.2.3	High fat diet challenge	48
2.2.4	Temperature measurements	49
2.2.5	Cold challenge	49
2.2.6	Induction and rescue of metabolic adaptation	50
2.2.7	PBS perfusion	50
2.3	Microscopy	51
2.3.1	Sample preparation	51
2.3.2	Immuno-electron microscopy	51
2.3.3	Cryosectioning	52
2.3.4	Immunofluorescent staining	52
2.3.5	Confocal image acquisition	54
2.4	Microscopy image analysis	54
2.4.1	Removal of background fluorescence	54
2.4.2	Quantification of eYFP ⁺ coverage	56
2.4.3	Quantification of IL-33 fluorescence intensity	59
2.5	Generation of single cell suspensions	59
2.5.1	Spleen dissociation	59
2.5.2	Adipose tissue dissociation (immune cell isolation)	59
2.5.3	Adipose tissue dissociation (stromal cell isolation)	60
2.5.4	Axon bundle dissociation (stromal and immune cell isolation)	60
2.5.5	Sympathetic ganglia dissociation (stromal cell isolation)	61
2.6	Flow cytometry	61
2.7	RNA-sequencing data	63
2.7.1	Bulk RNA-sequencing of eYFP ⁺ cells from the Superior Cervical Ganglia of LepR ^{Ai32} mice	63
2.7.2	Single nuclei RNA-sequencing of human sympathetic ganglia	63
2.7.3	Analysis of published single cell RNA-sequencing data	65
2.8	Reverse transcription-quantitative Polymerase Chain Reaction (RT-qPCR)	65
2.8.1	RNA extraction	65
2.8.2	DNase treatment	66
2.8.3	Reverse transcription	66
2.8.4	Quantitative Polymerase Chain Reaction	67

2.8.5	Quantitative Polymerase Chain Reaction analysis	67
2.9	Statistical analyses	69

2.1 General laboratory reagents

A number of standard buffers used in experiments were made in house (Figure 2.1). All concentrations listed are final concentrations.

2.2 Animal husbandry

All animal work was conducted in accordance with the operation of the Animals (Scientific Procedures) Act 1986, and approved by the relevant authorities: UK Home Office Regulations and University of Oxford (Project Licence P80EDA9F7), and University of Santiago de Compostela Ethical Committee (15012/2020/010). Animals were bred and maintained at the University of Oxford in Individually Ventilated Cages (IVCs) under Specific Pathogen Free (SPF) conditions, on a 12 hour light/dark cycle at 21°C+/-1°C, 50% humidity +/-10%. Experimental and control mice were age-matched: IL33^{fl/fl} mice were used as controls for LepR^{IL33cKO} mice, and ADR β 2^{fl/fl} mice were used as controls for LepR^{ADR β 2cKO} mice. Owing to the hormone-induced sexual dimorphism in adipose tissue physiology all mice used experimentally were male. This research provides an insight into the basic biology of Sympathetic Associated Perineurial Cells in the absence of such variables. Unless otherwise stated, experiments were performed on 10-11 week old mice with *ad libitum* access to regular chow diet (normal diet, ND, Global Diet 2916 - TekLad) and water. All unperfused mice were culled using CO₂ - an approved Schedule 1 method.

2.2.1 Mouse genotypes

LepR^{Ai32} lineage tracer, LepR^{IL33cKO} and LepR^{Adrb2cKO} mice were generated using ObRb^{CRE} mice (JAX #008320), crossed with either 'Ai32' Rosa26^{Lox-Stop-Lox-Chr2-eYFP} (JAX #012569), IL33^{fl/fl} (JAX #030619) or Adrb2^{fl/fl} mice (kindly shared by Professor

Buffer	Ingredients	Company	Catalogue Number	Concentration
PBS	-	Sigma-Aldrich	806552	1x
PFA	paraformaldehyde (16%)	Fisher Scientific	11481745	4%
	PBS	Sigma-Aldrich	806552	1x
Glycine Solution	glycine	Sigma-Aldrich	G7126	50mM
	PBS		806552	1x
Immuno-EM Permeabilisation Solution	saponin	Sigma-Aldrich	558255	0.25%
	BSA		A7030	0.10%
	PBS		806552	1x
Immuno-EM Blocking Solution	BSA	Sigma-Aldrich	A7030	0.20%
	goat serum		G9023	5%
	NH ₄ Cl		213330	50mM
	saponin		558255	0.10%
	NaCl	-	S9888	150mM
	phosphate buffer		-	20mM
Sucrose Solution	sucrose	Sigma-Aldrich	S0389	30%
	PBS		806552	1x
0.1M Phosphate Buffer, pH 7.4	sodium phosphate dibasic heptahydrate	Sigma-Aldrich	S9390	0.1M
	sodium phosphate monobasic monohydrate		S9638	0.1M
	distilled water	-	-	-
IF Blocking/ Permeabilisation Buffer (Donkey)	BSA	Sigma-Aldrich	A7030	3%
	donkey serum		D9663	2%
	triton X-100		X100	1%
	sodium azide		S2002	0.10%
	PBS		806552	1x
IF Blocking/ Permeabilisation Buffer (Goat)	BSA	Sigma-Aldrich	A7030	3%
	goat serum		G9023	2%
	triton X-100		X100	1%
	sodium azide		S2002	0.10%
	PBS		806552	1x
FACS Buffer	heat inactivated FBS	Sigma-Aldrich	F9665	2%
	sodium azide		S2002	0.02%
	PBS		806552	1x
DNase Buffer	10x DNase I Reaction Buffer	Biolabs Inc	11207	1x
	DNase I		M03032	40U/mL
	MiliQ Water	-	-	-

Figure 2.1: Table of general laboratory reagents

Gérard Karsenty, MGI:3837346). All mice used were on a C57BL/6 background. *ObRb* mice possess an IRES-CRE cassette immediately 3' to the *ObRb* stop codon, therefore CRE is only present in cells which express the fully active *ObRb* isoform of LepR [221]. Due to the lack of functional antibodies against LepR, LepR^{Ai32-Adrb2CKO} triple transgenic mice were used to identify LepR^{eYFP+} SAPCs in the LepR-specific absence of *Adrb2* expression, with LepR^{Ai32} mice used as controls.

Summary of mouse genotypes and abbreviations:

$$\text{LepR}^{\text{Ai32}} = \text{Lepr}^{\text{CRE}}::\text{Rosa26}^{\text{Lox-Stop-Lox-eYFP-Chr2}}$$

$$\text{LepR}^{\text{IL33cKO}} = \text{Lepr}^{\text{CRE}}::\text{IL33}^{\text{fl/fl}}$$

$$\text{LepR}^{\text{Adrb2cKO}} = \text{Lepr}^{\text{CRE}}::\text{Adrb2}^{\text{fl/fl}}$$

$$\text{LepR}^{\text{Ai32::Adrb2cKO}} = \text{Lepr}^{\text{CRE}}::\text{Rosa26}^{\text{Lox-Stop-Lox-eYFP-Chr2}}::\text{Adrb2}^{\text{fl/fl}}$$

2.2.2 Metabolic phenotyping

The metabolism of control and LepR^{IL33cKO} mice was measured using indirect calorimetry (LabMaster, TSE Systems) by collaborators Iara Fernández-González and Miguel López at the University of Santiago de Compostela. Mice were acclimatised for >1 week after arriving in Spain, then housed in metabolic cages for an additional week prior to measurement. This open circuit instrument determines O₂ consumption (VO₂), CO₂ production (VCO₂), Energy Expenditure (EE), and Respiratory Quotient (RQ) - using the equation $\text{RQ}=\text{RER}=\text{VCO}_2/\text{VO}_2$. Mice were measured over a period of 5 days. Nuclear Magnetic Resonance Imaging (Whole Body Composition Analyser, EchoMRI) was used to measure body composition (lean/ fat mass). RQ and EE were normalised to lean mass.

2.2.3 High fat diet challenge

At 8 weeks old, regular chow diet was removed from mice and replaced with High Fat Diet (HFD, D12492 - Research Diets, 60% fat). Mice had *ad libitum* access to water and HFD for a period of 12 weeks. Mouse body weight and food weight was measured weekly between 10am-12pm over this period. Average daily food intake for 1 mouse

was calculated as the difference in food weight over a week divided by 7.

2.2.4 Temperature measurements

Brown Adipose Tissue (BAT) and tail temperature of unanaesthetised mice were measured using a thermal camera (E76 24° - Flir) from a distance of approximately 20cm. The interscapular region of each mouse was shaved 2-3 days prior to thermal imaging to ensure fur was removed and skin temperature was measured, and to avoid stress-induced BAT activity from shaving itself. The average temperature was calculated by measuring BAT or tail temperature from >10 thermal images per mouse/condition/timepoint using Flir Tools software. All thermal imaging analysis was blinded, and performed with the help of Eliška Friebergerová. Core temperature was measured using a small (2cm) rectal probe (Precision) on unanaesthetised mice.

2.2.5 Cold challenge

To assess the thermogenic capacity of BAT in LepR^{IL33cKO} mice, I developed a cold challenge protocol which could be used to significantly and robustly stimulate BAT thermogenesis. Cold challenge enhances thermogenesis through an increase in sympathetic drive onto adipose tissues, subsequently increasing thermogenic gene expression and BAT temperature. Given that mice undergo a constant, low-level cold challenge at room temperature (21°C), I first housed mice at thermoneutrality (30°C) for a period of 10 days (HPP1060 Memmert Climate Chamber) to reduce BAT thermogenesis. After 10 days, control (thermoneutral) mice were culled at 4pm using CO₂, samples were collected and immediately snap-frozen. The following day, remaining mice underwent an 8h cold challenge (4°C) directly from 30°C, starting at 8am using the same HPP1060 Memmert Climate Chamber. Cold-challenged mice were culled using CO₂ at 4pm, samples were collected and immediately snap-frozen. Thermoneutral and cold-challenged samples were collected exactly 1 day apart at the same time of day to negate the effect of circadian rhythm on BAT activity and the result. Mice were given *ad libitum* access to

food and water throughout.

2.2.6 Induction and rescue of metabolic adaptation

To induce a state of metabolic adaptation - where energy expenditure (e.g. BAT thermogenesis) is reduced in response to decreased energy input (e.g. food restriction) - mice were fasted from 7pm-9am for a period of 14 hours with *ad libitum* access to water. To rescue from this state of metabolic adaptation, mice were injected intraperitoneal (IP) with leptin (498-OB-05M - BioTechne, 10µl/g bodyweight at a concentration of 0.5mg/ml) to stimulate thermogenesis, by increasing sympathetic drive onto adipose tissues. Control mice were instead injected IP with 1x PBS (Sigma-Aldrich). Rescue from this state of metabolic adaptation was observed by increased BAT temperature in wildtype mice following leptin injection, with no differences in BAT temperature in PBS-injected wildtype controls.

2.2.7 PBS perfusion

To remove circulating red blood cells and immune cells for immunofluorescent (IF) imaging experiments and some flow cytometry experiments, mice were first perfused. Mice were injected IP with an overdose of Pentobarbitone (Pentoject XVD133 - Animalcare, 10µl/g body weight at a concentration of 200mg/ml). Once mice lost consciousness, righting reflex and paw-pinch reflex, they underwent cardiac perfusion with PBS. The right atrium was cut open, and PBS was manually flushed through the left ventricle using a syringe with attached needle. Successful perfusion was determined by clear loss of colour in the liver due to exanguination.

2.3 Microscopy

2.3.1 Sample preparation

Following cardiac perfusion with PBS, tissues were dissected and cleaned. Tissues were fixed overnight in 4% PFA (Fisher Scientific) before being transferred to PBS (Sigma-Aldrich) with 0.01% Sodium Azide (Sigma-Aldrich) to prevent the growth of micro-organisms.

Axon bundles within adipose tissues were identified by their distinct 'stripy', unbranched nature, and dissected from the scWAT and BAT using a stereomicroscope (Zeiss Stemi508) and fine forceps. Fat and debris were removed from the outer layer of axon bundles, before samples were fixed to be stained and imaged whole-mount.

2.3.2 Immuno-electron microscopy

Immuno-Electron Microscopy (Immuno-EM) was performed by collaborators Andrea Raimondi and Matteo Iannacone at Vita-Salute San Raffaele University, on LepR^{Ai32} tissues prepared by Bernardo Arús and Noelia Martínez-Sánchez at Instituto Gulbenkian de Ciência. After fixation, axon bundles dissected from adipose tissues were washed with PBS, before incubation with 50mM glycine and Immuno-EM permeabilisation solutions for 1 hour each at room temperature (Figure 2.1). Axon bundles were then incubated with Immuno-EM blocking solution (Figure 2.1) for 20 minutes at room temperature, before incubation with anti-GFP antibody (A-11122 - Invitrogen, 1:500) for 2 hours at room temperature. Samples were washed for 10 minutes x3 with PBS, and incubated with secondary antibody conjugated to 1.4nm gold particles (#2003 Nanogold - Nanoprobes). Nanogold particles were diluted with gold particle amplification solution (GE BEEM - Nanoprobes) according to the manufacturer's instructions. Samples were wrapped with resin (Electron Microscopy Science) and cured for 48 hours at 60°C. Resin blocks were sectioned using a Leica EM UC7 ultramicrotome, resulting in cross-sections of axon bundles. Ultra-thin axon bundle slices (70-90nm) were visualised using an FEI

Talos 120kV Transmission Electron Microscope. Immuno-Electron Microscopy images were acquired using a Ceta 16M CMOS sensor camera (FEI).

2.3.3 Cryosectioning

Fixed tissues were incubated in 30% sucrose solution (Figure 2.1) overnight for tissue cryopreservation. Brains were incubated in 30% sucrose solution for 5 days. Tissues were mounted and snap-frozen (-80°C) in Optical Cutting Temperature (OCT, 00411243 - VWR) compound. Mounted, frozen tissues were then equilibrated for >1 hour in the cryostat chamber (Bright OTF5000) at -24°C, with a specimen temperature of -19°C. 15µm sections were taken and mounted directly onto Superfrost Plus Adhesion slides (10149870 - Fisher Scientific). Sections were stored at -20°C.

For brains, 30µm cryosections of the hypothalamus were transferred directly into 0.1M phosphate buffer (Figure 2.1) in a 24-well plate. 'The Mouse Brain' Book [222] was used to identify the Arcuate Nucleus of the Hypothalamus (ARC). Coronal sections identified anatomically as 'plate 45-47' in The Mouse Brain Book, containing the ARC, were used.

2.3.4 Immunofluorescent staining

Where necessary, PAP-pen (Z377821-1EA - Merck) was used to draw around samples on slides. Samples were washed for 10 minutes x3 in PBS, before being blocked in an IF blocking/ permeabilisation buffer for 1 hour at room temperature (Figure 2.1). Serum used in the IF blocking/ permeabilisation buffer from the same species as the secondary antibodies was used. Samples were incubated with primary antibodies (Figure 2.2) diluted in IF blocking/ permeabilisation buffer overnight at 4°C. Next, samples were washed for 10 minutes x3 in PBS, and incubated with secondary antibodies and DAPI (Figure 2.2) diluted in IF blocking/ permeabilisation buffer for 1 hour at room temperature. Samples were washed with PBS for 10 minutes x3, before being mounted with anti-fade medium (P36930 - Invitrogen). Nail varnish was used to seal coverslips. Wildtype tissues were stained with anti-GFP primary antibody, DAPI and all secondary antibodies to ensure staining specificity (Appendix Figure 6A).

IF Primary Antibodies

Epitope	Clone	Clonality	Host Species	Company	Catalogue Number	Concentration
DAPI	-	-	-	Invitrogen	D1306	1:1000
GFP	-	polyclonal	Chicken	Abcam	AB13970	1:1000
TH	-	polyclonal	Rabbit	Abcam	AB152	1:1000
IL-33	-	polyclonal	Goat	BioTechne	AF3626	1:50
B3-TUB	-	polyclonal	Rabbit	Abcam	AB18207	1:1000
CD31	MEC13.3	monoclonal	Rat	Biolegend	102504	1:50

IF Secondary Antibodies

Target Species	Host Species	Colour	Company	Catalogue Number	Concentration
Chicken	Donkey	AF488	Jackson ImmunoResearch	703-545-155-JIR-0.5mg	1:500
Goat	Donkey	AF647	Invitrogen	A21447	1:500
Rabbit	Donkey	AF546	Invitrogen	A10040	1:500
Chicken	Goat	AF488	Invitrogen	A11039	1:500
Rabbit	Goat	AF546	Invitrogen	A11035	1:500
Rat	Goat	AF647	Invitrogen	A21247	1:500
Rabbit	Goat	AF647	Invitrogen	A21244	1:500

Figure 2.2: Table of antibodies used for immunofluorescent imaging

DAPI = 2-(4-amidinophenyl)-1H-indole-6-carboxamide; **GFP** = Green Fluorescent Protein; **TH** = Tyrosine Hydroxylase; **IL-33** = Interleukin-33; **NG2** = Neural/Glial Antigen 2; **CD31** = PECAM-1 (Platelet Endothelial Cell Adhesion Molecule-1); **AF** = Alexa Fluor.

2.3.5 Confocal image acquisition

Z-stack images were acquired using the LSM-880 confocal microscope (Zeiss). In the case of larger samples, tiled images (with 10% overlap) were taken and stitched together using Zen Black software (Zeiss). To ensure high signal-to-noise on the confocal microscope, acquisition settings were optimised using a positive control and secondary antibody-only, negative control for each stain. Fiji was used to display confocal images. Secondary only control images acquired at identical microscope settings were used as a negative control for each stain to ensure an accurate display in experimental images (Appendix Figure 6B-D).

2.4 Microscopy image analysis

All image analysis was performed in Fiji. Macros were written by Chloe Zentai (Figure 2.3, Appendix Figure 2). All technical replicates underwent identical pre-processing and image analysis using Fiji. Experimental samples dissected from LepR^{Ai32} mice.

2.4.1 Removal of background fluorescence

We measured non-specific, 'background' fluorescence for both eYFP and IL-33 channels in secondary only-stained controls, to be subtracted from later analyses. First, maximum intensity projection images were created using secondary only-stained control images (Figure 2.3). Axon bundle area was identified using autofluorescence (autothreshold, Otsu) from an alternative channel. Holes within the axon bundle area were filled, and a mask of the axon bundle area was created.

Next, average intensity projection images were created from the original secondary only-stained control Z-stack images (Figure 2.3), and eYFP or IL-33 channel was selected. The axon bundle area mask was restored onto these single channel images, and average intensity fluorescence for eYFP and IL-33 was measured from the axon bundles. These measurements were repeated on >5 secondary only-stained control axon bundles, and an average 'background' fluorescence intensity across all secondary only-stained samples

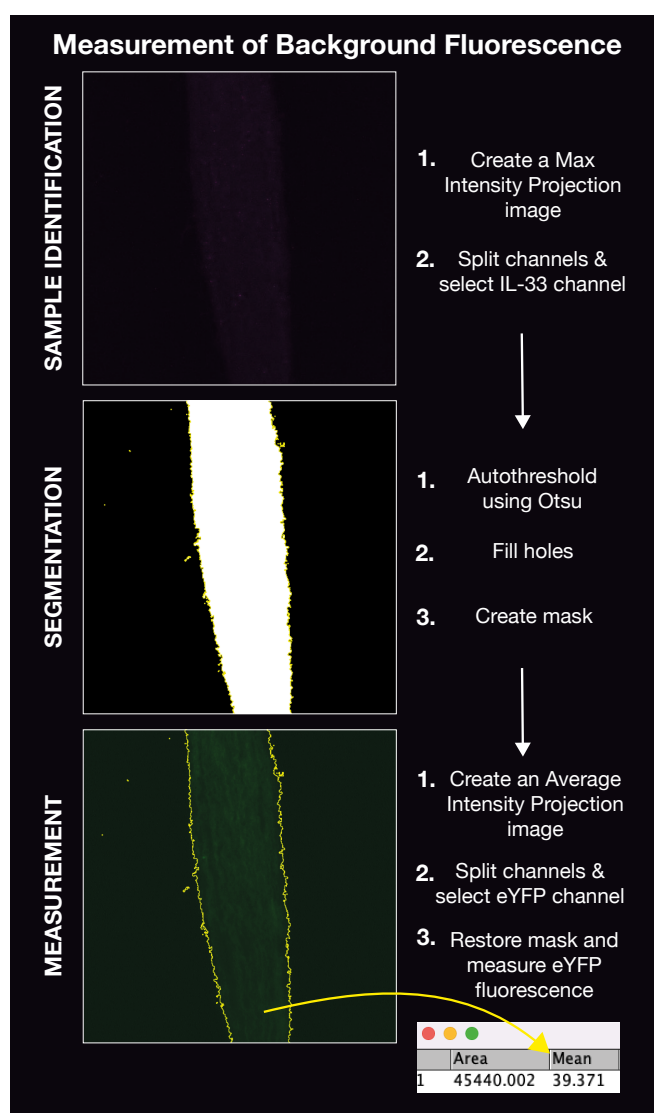


Figure 2.3: Image analysis pipeline for removal of background fluorescence

Non-specific, 'background' fluorescence was measured for each channel using secondary antibody only-stained control samples. Image analysis was performed by Chloe Zentai using Fiji.

was calculated for each channel. Whilst eYFP and IL-33 fluorescence intensity was very low in the secondary only-stained controls, this measurement allowed for the subtraction of non-specific 'background' fluorescence from all later analyses to ensure that only specific staining was included, and to prevent false positive errors in the detection and quantification of fluorescence. Macros for measurement of eYFP and IL-33 background fluorescence can be found in Appendix Figure 2.

2.4.2 Quantification of eYFP⁺ coverage

To assess the coverage of LepR⁺ cells in the outermost cell layer surrounding axon bundles, I developed an image analysis pipeline for whole-mount imaged LepR^{Ai32} axon bundles in collaboration with Chloe Zentai. First maximum intensity projection images were created using fully stained samples (Figure 2.4). After subtraction of eYFP background fluorescence, eYFP⁺ regions were identified using Otsu autothreshold, before holes were filled and a mask of eYFP⁺ regions was created. eYFP⁺ area was noted. As z-stack images of whole-mount axon bundles were taken, we next normalised the eYFP⁺ area measured to the 2D surface of the axon bundle imaged, for quantification of eYFP coverage as a % of the surface of the axon bundle (Figure 2.4). The 2D area of the axon bundle surface was calculated by multiplying the length by the cross-sectional circumference of the axon bundle surface measured. To measure the length, mean autothreshold was used on maximum projection intensity images, and the length was measured by skeletonising this area and determining the 'longest shortest path' (Figure 2.4). The cross-sectional circumference was measured by re-slicing vertical axon bundles in the orthogonal XZ axis (Figure 2.4). A maximum intensity projection of the re-sliced image was created, before using Otsu autothreshold to find the cross-sectional eYFP⁺ region surrounding the axon bundle. This area was skeletonised, and the 'longest shortest path' was determined, which was used to approximate the 2D width of the 3D axon bundle surface imaged. With these measurements, the 2D area of the imaged axon bundle surface was calculated, and % eYFP⁺ coverage was determined (Figure 2.4).

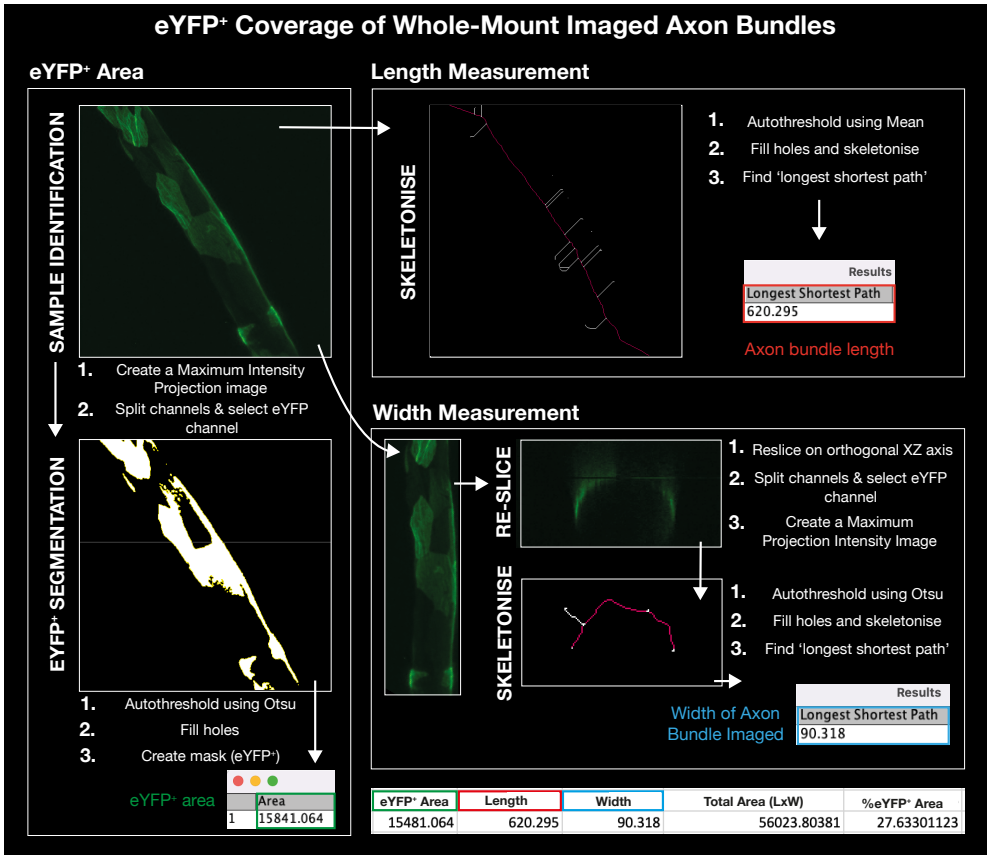
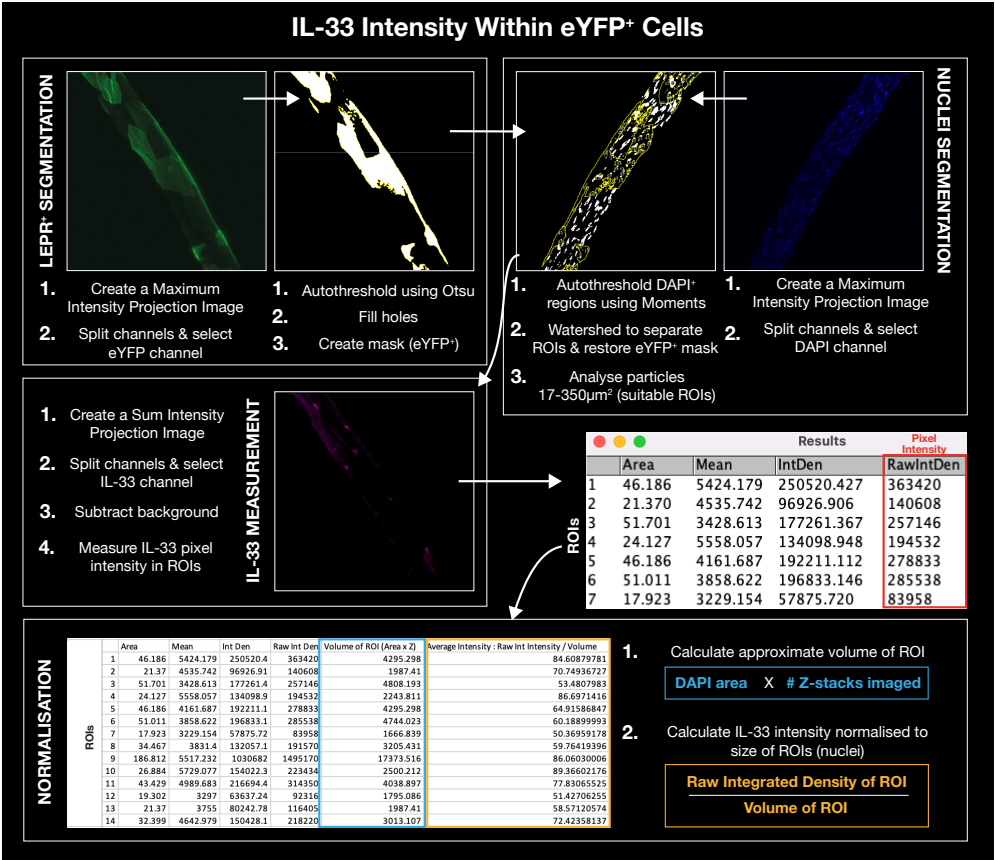


Figure 2.4: Image analysis pipeline for quantification of eYFP coverage of whole-mount imaged axon bundles

eYFP coverage was quantified as a percentage of the 2D surface area of the axon bundle outer layer. Image analysis was performed by Chloe Zentai using Fiji.



Macros for measurement of eYFP coverage can be found in Appendix Figure 3.

2.4.3 Quantification of IL-33 fluorescence intensity

Using whole-mount imaged axon bundles, I developed an image analysis pipeline to quantify IL-33 levels within eYFP⁺ cells in collaboration with Chloe Zentai. As IL-33 is a nuclear stain, we first identified DAPI⁺ regions of interest (ROIs, Figure 2.5). ROIs were watershedded to separate individual nuclei, and the eYFP mask was restored. Suitable ROIs were DAPI⁺, 17-350µm² and within eYFP⁺ cells (Figure 2.5). Having defined nuclei within eYFP⁺ cells as suitable ROIs we next created a sum projection intensity image, before splitting channels and selecting the IL-33 channel. IL-33 background fluorescence was subtracted, and IL-33 pixel intensity (raw integrated density) was measured within DAPI⁺ eYFP⁺ ROIs (Figure 2.5). We next normalised the IL-33 pixel intensity to the approximate volume of each nucleus, calculated using DAPI⁺ area x #Z-stacks imaged. Normalisation of IL-33 pixel intensity was performed by dividing the raw integrated density of an ROI by the approximate volume (Figure 2.5). Macros for measurement of IL-33 fluorescence intensity within SAPCs can be found in Appendix Figure 4.

2.5 Generation of single cell suspensions

2.5.1 Spleen dissociation

Mice were culled using CO₂, and spleens were dissected and stored in FACS buffer (Figure 2.1), before homogenisation through a wet 70µm filter using a syringe plunger. Filters were washed with FACS buffer.

2.5.2 Adipose tissue dissociation (immune cell isolation)

Mice were culled using CO₂, and whole adipose tissues (scWAT, vWAT, BAT) were dissected and chopped up into HBSS^{Mg₂₊+Ca₂₊} (14025092 - Gibco). Collagenase II (C6885

- Sigma) was added to a final concentration of 2.5mg/ml, and samples were incubated for 30 minutes at 37°C, with agitation. During this incubation, samples were mechanically disrupted using cut 1ml pipette tips of decreasing size every 10 minutes. Samples were washed with FACS buffer (Figure 2.1).

2.5.3 Adipose tissue dissociation (stromal cell isolation)

Mice were IP injected with an overdose of Pentobarbitone and perfused (Section 2.2.7) to remove circulating immune cells. Whole adipose tissues (scWAT, vWAT, BAT) were dissected and chopped up into HBSS^{Mg₂₊+Ca₂₊} (14025092 - Gibco). Collagenase II (C6885 - Sigma, final concentration of 2.5mg/ml) and 500µl hyaluronidase IV-S (H3884 - Sigma, final concentration of 4000U/ml) were added, and samples were incubated for 30 minutes at 37°C, with agitation. During incubation, samples were mechanically disrupted using cut 1ml pipette tips of decreasing size every 10 minutes. Samples were washed with FACS buffer (Figure 2.1).

2.5.4 Axon bundle dissociation (stromal and immune cell isolation)

Mice were IP injected with an overdose of Pentobarbitone and perfused (Section 2.2.7) to remove circulating immune cells. Axon bundles were dissected from the scWAT and BAT and chopped up into HBSS^{Mg₂₊+Ca₂₊} (14025092 - Gibco). Collagenase II (C6885 - Sigma, final concentration of 2.5mg/ml) and 500µl hyaluronidase IV-S (H3884 - Sigma, final concentration of 4000U/ml) were added, and samples were incubated for 30 minutes at 37°C, with agitation. Samples were washed with FACS buffer (Figure 2.1), centrifuged for 5 minutes at 1000rcf and supernatant was discarded. Samples were resuspended in FACS buffer and triturated using a 1ml syringe and needles of decreasing size (23G, 25G, 27G; 10x each) before being flushed through at 70µm wet filter.

2.5.5 Sympathetic ganglia dissociation (stromal cell isolation)

Mice were IP injected with an overdose of Pentobarbitone and perfused (Section 2.2.7) to remove circulating immune cells. Superior Cervical Ganglia (SCG) were dissected into HBSS^{Mg₂₊+Ca₂₊} (14025092 - Gibco). HBSS^{Mg₂₊+Ca₂₊} was removed, SCG were re-suspended in 2.5mg/ml collagenase II (C6885 - Sigma) and samples were incubated for 10 minutes at 37°C, with agitation. Samples were washed with FACS buffer (Figure 2.1), and centrifuged for 5 minutes at 1000 rcf. Supernatant was removed before samples were resuspended in 0.25% trypsin (25050014 - Life Tech) and incubated for 30 minutes at 37°C, with agitation. Samples were washed with FACS buffer (Figure 2.1), and centrifuged for 10 minutes at 1000 rcf. After discarding the supernatant, samples were resuspended in FACS buffer and triturated using a 1ml syringe with needles of decreasing size (23G, 25G, 27G; 10x each) before being flushed through a 70µm wet filter.

2.6 Flow cytometry

Samples were centrifuged for 5 minutes at 400rcf, supernatant was removed, and single cell suspensions were incubated with 2ml red blood cell lysis buffer (00-4333-57 - eBioscience) for 4 minutes on ice. Samples were washed with FACS buffer (Figure 2.1) and centrifuged for 5 minutes at 400 rcf. Supernatant was discarded, and samples were incubated with F_c block (Figure 2.6) for 10 minutes at 4°C. Next, Live/Dead stain (Figure 2.6) was added and samples were incubated in the dark for a further 30 minutes at 4°C, before being washed with FACS buffer and centrifuged for 5 minutes at 400 rcf. Samples were then stained with a 'surface marker' antibody mastermix, prepared using antibodies listed in Figure 2.6 for 30 minutes at 4°C. Samples were then washed with FACS buffer, and fixed/ permeabilised using the FOXP3 fix/permeabilisation kit fixation buffer (00-5523-00 - eBioscience) for 30 minutes at 4°C. Next, samples were washed with permeabilisation buffer from this kit, and centrifuged for 5 minutes at 400rcf. Supernatant was removed and samples were next incubated with an 'intracellular marker' antibody mastermix, prepared using antibodies listed in Figure 2.1 overnight at 4°C. Finally, samples were washed and flushed through a 70µm filter, before being run

Flow Cytometry Antibodies

Epitope	Colour	Clone	Company	Catalogue Number	Concentration
Live/Dead	Aqua	-	Thermofisher	L34957	1:500
Live/Dead	Near IR	-	Thermofisher	L10119	1:500
B220	FITC	RA3-6B2	Biolegend	103205	1:500
B220	ef450	RA3-6B2	Invitrogen	48-0452-82	1:500
CD3	PE-Cy5	145-2C11	Biolegend	100309	1:250
CD3	ef450	145-2C11	Invitrogen	48-0031-82	1:250
CD4	BV650	RM4-5	Biolegend	100545	1:250
CD4	PE-Cy5	RM4-5	Biolegend	100513	1:250
CD5	ef450	53-7.3	Invitrogen	48-0051-82	1:500
CD8	BV785	53-6.7	Biolegend	100749	1:500
CD11b	ef450	M1/70	Invitrogen	48-0112-82	1:500
CD11c	BV785	N418	Biolegend	117335	1:500
CD11c	ef450	N418	Invitrogen	48-0114-82	1:500
CD25	ef450	PC61.5	Invitrogen	48-0251-80	1:200
CD31	BV785	390	Invitrogen	102435	1:200
CD45	AF700	30-F11	Biolegend	103128	1:1000
CD45	BV785	30-F11	Biolegend	103149	1:1000
CD64	PE	X54-5/7.1	Biolegend	139303	1:500
CD68	APC-Cy7	FA-11	Biolegend	137024	1:500
CD90	APC-Cy7	53-2.1	Invitrogen	17-0902-81	1:500
CD127	PE	A7R34	Biolegend	135009	1:500
CDH5	PE-Cy7	B13	Biolegend	138015	1:200
FcERI	ef450	Mar-01	Invitrogen	48-5898-82	1:500
FOXP3	PE	FJK-16s	Invitrogen	12-5773-82	1:200
GATA3	BUV395	L50-823	BD	565448	1:200
ITGB4	AF405	Asn29-Ser711	R&D	FAB405V-100UG	1:250
IL-33	PE	MA5-23640	Invitrogen	IC3626P	1:200
KLRG1	PE-Cy7	2F1/KLRG1	Biolegend	138416	1:500
MHC II	PE-Cy7	M5/114.15.2	Invitrogen	25-5321-82	1:500
NK1.1	ef450	PK136	Invitrogen	48-5941-82	1:500
PDGFR α	APC	APA5	Biolegend	135907	1:250
Siglec F	BUV395	E50-2440	BD	740280	1:500
ST2	SB600	RMST2-2	Invitrogen	63-9335-80	1:250
TER119	BUV496	TER119(RUO)	BD	741079	1:250
IgG2a Isotype control	PE	eBR2a	Invitrogen	12-4321-83	1:200
IgG2a Isotype control	APC-Cy7	RTK2758	Biolegend	400523	1:500
IgG1k Isotype control	BUV395	X40	BD	563547	1:200

Figure 2.6: Table of antibodies used for flow cytometry

B220 = Protein Tyrosine Phosphatase Receptor Type C; **CD** = Cluster of Differentiation; **FcERI** = High Affinity IgE Receptor; **FOXP3** = Forkhead Box P3; **GATA3** = GATA Binding Protein 3; **ITG β B4** = Integrin Subunit β 4; **IL-33** = Interleukin-33; **KLRG1** = Killer Cell Lectin Like Receptor G1; **MHC II** = Major Histocompatibility Complex Class 2; **NK1.1** = Killer Cell Lectin Like Receptor B1C; **PDGFR α** = Platelet Derived Growth Factor Receptor α ; **Siglec F** = Sialic Acid Binding Ig Like Lectin F; **ST2** = Suppression of Tumourigenicity 2; **TER119** = Lymphocyte Antigen 76; **IgG2a** = Immunoglobulin G Type 2a; **IgG1k** = Immunoglobulin G Type 1k.

at approximately 10,000 events per second on a BD Fortessa X-20 Cell Analyser flow cytometer equipped with 355nm, 405nm, 488nm, 561nm and 640nm lasers. FlowJo was used for all flow cytometric analyses. Population gates for surface markers were set using internal negative control cell populations. To negate the possibility of non-specific intracellular antibody retention following permeabilisation, isotype control samples stained with full surface marker antibody panels plus colour-matched isotype control antibodies, instead of antibodies against intracellular markers, were used to set gates for intracellular markers.

2.7 RNA-sequencing data

2.7.1 Bulk RNA-sequencing of eYFP⁺ cells from the Superior Cervical Ganglia of LepR^{Ai32} mice

Bulk RNA-sequencing of eYFP⁺ cells was performed by Svetoslav Chakarov and Florent Ginhoux at Singapore Immunology Network (SIgN) A+STAR, on tissues prepared by Bernardo Arús and Noelia Martínez-Sánchez at Instituto Gulbenkian de Ciência. Single cell suspensions were prepared from the Superior Cervical Ganglia of LepR^{Ai32} mice (Section 2.5.5). Live, eYFP⁺ cells were sorted using a FACS Aria IIu high-speed cell separator, before cDNA libraries were prepared according to the Smart-Seq2 protocol [223] with the following adaptations: addition of 1mg/ml ultrapure BSA to lysis buffer, addition of 20µM template strand exchange nucleotide. cDNA library quality was checked (CLS760672, CLS138948) and samples were subjected to paired indexing sequencing (Illumina HiSeq 4000). RNA sequencing data were analysed by Svetoslav Chakarov and Florent Ginhoux using Seurat. Raw and analysed bulk RNA-sequencing data can be found on the GEO database (GSE227493, accessible with token ypixaiesvdinxix).

2.7.2 Single nuclei RNA-sequencing of human sympathetic ganglia

Single nuclei isolation, sequencing and analysis of human ganglia were all performed by Karin Ziegler, Sandro Meunier and Stefan Engelhardt at Technical University of Munich.

Nuclei were isolated from frozen human sympathetic ganglion tissue collected during autopsy. Details of 2 human donors are as follows:

Male, 35 years old, 1.78cm height, 107kg body weight. BMI = 33.77

Female, 50 years old, 1.67cm height, 64kg body weight. BMI = 22.95

Approval of the responsible ethics committee (reference ID: 262/19 S-SR, Ethikkommission der Fakultät für Medizin, Technical University of Munich) was obtained). Samples remained on dry ice prior to nuclei isolation, which was carried out at 4°C. Individual ganglia were placed in gentleMACS tubes (Miltenyi Biotec) containing 5ml Miltenyi Nuclei extraction buffer, supplemented with 40 U/ μ l Ambion™ cloned RNase inhibitor (ThermoFisher Scientific). Tissue dissociation was performed using the nuclei isolation programme on the gentleMACS dissociator (Miltenyi Biotec), followed by a 10 minute incubation on ice. Sample lysate was then subjected to a filter series, passing through 100, 70, 30 and 20 μ m filters (MACS SmartStrainers, Miltenyi, pluriStrainer, Pluriselect). Filter flow-through was centrifuged at 1000xg for 7 minutes at 4°C. Following centrifugation, supernatant was removed and the nuclei pellet was resuspended in 2ml suspension buffer (containing 2% biotin-free BSA and 40 U/ μ l Ambion cloned RNase inhibitor diluted in PBS). Resuspended nuclei were passed through a 20 μ m filter (pluriStrainer, Pluriselect) and centrifuged at 1000xg for 7 minutes at 4°C, before the removal of supernatant and resuspension in 200 μ l resuspension buffer. 10 μ l of the nuclei suspension was stained with NucBlue (ThermoFisher Scientific) and counted (Countess™ 3, ThermoFisher Scientific). 20,000 nuclei were loaded into one channel of the Chromium system (10x Genomics). Single Cell 3' v3 chemistry was processed according to the manufacturer's instructions (10x Genomics). Libraries were sequenced using the NovaSeq 6000 platform (Illumina).

Raw sequencing data were processed with Cellranger (v6.0.1, 10x Genomics) count including intronic reads and aligned to the human reference genome GRCh38 v39 (Genome Reference Consortium). Seurat (v5) was used for downstream analyses. Nuclei containing <500 genes, >3000 genes or >1.0% mitochondrial genes were excluded from analyses. Filtered data contained a total of 10,486 nuclei. After log normalisation, principal component analysis (PCA) was used to identify highly variably genes for linear

dimension reduction. Unsupervised clustering with a resolution of 1.55 and Uniform Manifold Approximation and Projection (UMAP) were applied for embedding and visualisation, followed by the addition of supervised cluster labelling. Density plots showing the co-expression of two genes were generated using R package Nebulosa. Single nuclei RNA-sequencing data can be found using the NCBI's Genome Expression Omnibus using the accession number GSE241386, accessible with the token crqboucapnqrhej.

2.7.3 Analysis of published single cell RNA-sequencing data

To determine whether *Lepr* and *Il33*, or *Lepr* and *Adrb2* are co-expressed in the hypothalamus or peripheral organs, published sequencing data was downloaded and analysed using Seurat by Emma Haberman, David Grainger and Gitalee Sarker. Hypothalamus dataset: GSE93374 [224]; *Tabula Muris* dataset: GSE109774 [225]. To determine whether SPC co-expression of *Lepr* and *Il33* was anatomically distinct, I compared the gene expression profile of LepR⁺ SPCs with IL-33 producing sensory neuron-associated stromal cells reported in the muscle [207, 226, 227], dataset: GSE127005 using Seurat as described [228].

2.8 Reverse transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was utilised to quantify expression of a select panel of genes. Filter pipette tips were used, and unless otherwise stated samples were kept on ice (or at 4°C) throughout.

2.8.1 RNA extraction

Snap-frozen tissues were homogenised in TRIzol (15596018 - Invitrogen), using MK28-R 2ml hard tissue homogenisation tubes (P000910-LYSK0-A.0 - Bertin) on a PreCellys 24 Tissue Homogeniser for 2 minutes. Homogenates were centrifuged at maximum speed for 3 minutes, and floating fat layer was carefully removed. Chloroform was added and incubated at room temperature for 1 minute before centrifugation for 15 minutes at 12,000g. The nucleic acid-containing aqueous phase was carefully removed

and transferred to a new tube. Isopropanol and colour paint (70748-3 - Millipore) were added, and samples were precipitated overnight at -20°C. Next, samples were centrifuged for 15 minutes at 12,000g, supernatant was removed and nucleic acid pellet was washed using 75% ethanol. Samples were centrifuged for 5 minutes at 12,000g, supernatant was removed and pellets were dried for 10 minutes at room temperature.

2.8.2 DNase treatment

DNase buffer was prepared (Figure 2.1 and used to resuspend pellets, before incubation for 30 minutes at 37°C. At room temperature samples were washed with 2.5x volume of 100% ethanol and 0.1x volume of 3M sodium acetate (10190890 - Fisher Scientific), before being stored on ice for 30 minutes and then centrifuged for 15 minutes at 12,000g. Supernatant was removed, pellets were washed with 75% ethanol and centrifuged for 10 minutes at 12,000g. Supernatant was carefully removed, and pellets were dried for 10 minutes at room temperature. 20µl DNase/ RNase-free MilliQ water was used to resuspend RNA pellet.

2.8.3 Reverse transcription

RNA concentration and purity were measured using the Nanodrop. Each sample was measured twice and an average concentration was calculated to ensure readings were as accurate as possible. A260/280 values were reliably 2-2.1, indicating reasonable purity of extracted RNA. Samples were diluted to a concentration of 0.1µg/µl. 1µg of RNA for each sample was used in reverse transcription reactions, with the SuperScript II Reverse Transcriptase kit (18064-014 - Invitrogen). First 1µl dNTPs, 0.1µl random primers and 0.9µl DNase/RNase free MilliQ water were added to each tube, and incubated for 5 minutes at 65°C. Next 4µl 5x buffer, 2µl DTT and 1µl RNaseOUT were added, and samples were incubated for 2 minutes at room temperature. Finally, 0.25µl SuperScript and 0.75µl DNase/RNase free MilliQ water were added to each tube, and samples were incubated for 10 minutes at 25°C, for 50 minutes at 42°C, and then for 15 minutes

at 70°C. 180µl of DNase/RNase free MilliQ water was added to each tube, to dilute cDNA to a concentration of approximately 5ng/µl.

2.8.4 Quantitative Polymerase Chain Reaction

Using a BioRad CFX384 Real-Time System, RT-qPCR was performed in 10µl reactions, where each well contained 4µl (20ng) of cDNA, 5µl Fast SYBR Green Master Mix (4367659, Thermofisher), 0.1µl Forward Primer, 0.1µl Reverse Primer and 0.8µl DNase/RNase free MilliQ water. Primers sequences are summarised in Figure 2.7.

qPCR reactions were performed using the protocol:

50°C: 2 minutes

95°C: 10 minutes

[40x]

95°C: 15 seconds

60°C: 1 minute

95°C: 15 seconds

60°C: 1 minute

95°C: 15 seconds

2.8.5 Quantitative Polymerase Chain Reaction analysis

All samples were loaded in technical triplicate for each gene/condition. CFX Maestro Software (BioRad) was used to check melt curves and negative control reactions, and data was exported into an Microsoft Excel format. A single melt curve was confirmed for each reaction, indicating a cDNA region of only one size was amplified. No amplification was observed in 'no-template control' reactions containing DNase/RNase free MilliQ water instead of cDNA, which ensured that no contaminating agents were being amplified. Any reactions not meeting these quality control requirements were removed from analyses

qPCR Primers

Gene	Forward Primer	Reverse Primer
Arg1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
Ccl2	GCCTGCTGTTACAGTTGC	CAGGTGAGTGGGGCGTTA
Ccl5	TGCCACGTCAAGGAGTATTT	TTCTCTGGGTTGGCACACACT
Ccl7	CCACCATGAGGATCTCTGC	TTGACATAGCAGCATGTGGAT
Cdh11	GCCAACAGCCCAATAAGGTA	CTGGGACTTTGGTTTCCTGA
Cidea	TGCTCTTCTGTATCGCCAGT	GCCGTGTTAAGGAATCTGCTG
Csf1	GCCTCCTGTTCTACAAGTGGAAG	ACTGGCAGTCCACCTGTCTGT
Dio2	CAGTGTGGTGCACGTCTCCAATC	TGAACCAAAGTTGACCACCAG
Elovl3	TTCTACGCGGGTTAAAAATGG	GAGCAACAGATAGACGACCAC
Glut1	CTTCATTGTGGGCATGTGCT	AGGTTGCGCCTTTGGTCTCAG
Gpr3	ATCACCTGAGCAACCGAGAA	AGATGGGGGTGCATTTTACA
Il1b	TGGACCTTCAGGATGAGGACA	GTTTCATCTCGGAGCCTGTAGTG
Il4	AGATCATCGGCATTTTGAACG	TTTGGCACATCCATCTCCG
Il5	CGCTACCGAGCTCTGTTG	CCAATGCATAGCTGGTGATTTTT
Il6	AAAGCCAGAGTCCTTCAGAGAGATAC	CTGTTAG GAGAGCATTGGAAATTG
Il9	CATCAGTGTCTCTCCGTCCTCACTGATG	GATTTCTGTGTGG CATTGGTCAG
Il10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Il13	GCTTATTGAGGAGCTGAGCAACA	GGCCAGGTCCACACTCCATA
Il33	TCCAACCTCAAGATTTCCTCG	CATGCAGTAGACATGGCAGAA
Il34	GGGACTCGCTGGCTATACT	CCGAAGGTAGCCTGTAAGGTC
iNos	GTTCTCAG CCAACAATACAAGA	GTGGACGGGTCGATGTCAC
Ldlr	GAGGCTTGCGAGCCAGATT	ATGAGTCTTCTGCTGCAACTCCG
ObRb	TGAAGCATCGTACTGCCACA	ATTCACAAGGGAAGCGCCGA
Lpl	CAGCTGGGCTCACTTTGAG	CCTCTCTGCAATCACACGAA
Pgc1a	CCCTGCCATTGTAAAGAC	TGCTGCTGTTCTGTTTTC
Pparg	TCAAGGGTGCCAGTTTCG	GGAGGCCAGCATCGTGT
Prdm16	CAGCACGGTGAAGCCATT	GCGTGCATCCGCTTGTG
sST2	CGTGGGTCGTCTGCAGAAAT	GCTCTCTGAGGTAGGGTCCA
ST2	TGCATTTATGGGAGAGACCTGTTA	TGTGCAGAGCAATCTCTGC
Tbp	ACCGTGAATCTTGGCTGTAAAC	GCAGCAAATCGCTTGGGATTA
Tgfb	QuantiTect RT Primer – Tgfb1_1 QT00145250	
Tnfa	ATGAGCACAGAAAGCATGATC	TACAGGCTTGCTCACTCGAATT
Ucp1	GTGAAGGTCAGAATGCAAGC	AGGGCCCCCTTCATGAGGTC

Figure 2.7: Table of primers used for quantitative Polymerase Chain Reaction

Arg1 = Arginase 1; **Ccl2** = C-C Motif Chemokine Ligand 2; **Ccl5** = C-C Motif Chemokine Ligand 5; **Ccl7** = C-C Motif Chemokine Ligand 7; **Cdh11** = Cadherin 11; **Cidea** = Cell Death Inducing DFFA Like Effector A; **Csf1** = Colony Stimulating Factor 1; **Dio2** = Iodothyronine Deiodinase 2; **Elovl3** = ELOVL Fatty Acid Elongase 3; **Glut1** = Solute Carrier Family 2 Member 1; **Gpr3** = G Protein-Coupled Receptor 3; **Il1b** = Interleukin-1b; **Il4** = Interleukin-4; **Il5** = Interleukin-5; **Il6** = Interleukin-6; **Il9** = Interleukin-9; **Il10** = Interleukin-10; **Il13** = Interleukin-13; **Il33** = Interleukin-33; **Il34** = Interleukin-34; **iNos** = Inducible Nitric Oxide Synthase; **Ldlr** = Low Density Lipoprotein Receptor; **ObRb** = Fully Active Leptin Receptor Isoform; **Lpl** = Lipoprotein Lipase; **Pgc1a** = PPARG Coactivator 1 α ; **Pparg** = Peroxisome Proliferator-Activated Receptor γ ; **Prdm16** = PR/SET Domain 16; **sST2** = Soluble Suppression of Tumourigenicity 2; **ST2** = Suppression of Tumourigenicity 2; **Tbp** = TATA-Box Binding Protein; **Tgfb** = Transforming Growth Factor β ; **Tnfa** = Tumour Necrotic Factor α ; **Ucp1** = Uncoupled Protein 1.

due to possible contamination. Cycle threshold (Ct) values were calculated using an automated threshold of fluorescence detection, set by CFX Maestro (BioRad). The mean Ct value of technical triplicates for a gene/condition was normalised against the average expression of housekeeping gene *Tbp* for each sample, and used to calculate ΔCt using the formula $\Delta\text{Ct} = 2^{-(\text{gene Ct} - \text{housekeeping Ct})}$. $\Delta\Delta\text{Ct}$ was calculated to compare the expression of a gene between 2 conditions, where the ΔCt (expression) of a gene in 'experimental' condition was plotted relative to the ΔCt (expression) of the same gene in the control condition (where the average ΔCt of control samples = 1). Any technical triplicates for a gene/condition which were significantly different from the other two replicates (with Ct values >0.5 cycles apart) were excluded from analyses.

2.9 Statistical analyses

Approximate sample sizes for planned experiments were calculated using G*Power using a p-value <0.05 and a power of >0.8. Effect size and data variability were inferred from similar published experiments/models. Although sometimes restricted by availability of mice, sample sizes were also guided by the desire to validate findings in more than 1 independent experiment, to ensure robustness and reproducibility of all results. Data are expressed as mean +/- standard error of mean (SEM). All statistical analyses were performed using GraphPad Prism Version 9. Statistical significance was defined using an alpha of 0.05, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Other than pixel intensities, all data appeared to be normally distributed. For these data, statistical significance was determined using a two-tailed student's t test (for 2 groups) or ANOVA with post hoc two-tailed Bonferroni test when more than two groups were compared. As IL-33 pixel intensities were not normally distributed, a non-parametric Mann-Whitney U test for independent groups was performed, to assess whether the mean ranks were equal between the two experimental groups.

Chapter 3

Characterisation of LepR⁺ cells within white and brown adipose tissues

Contents

3.1	Introduction	71
3.1.1	Leptin receptor	71
3.1.2	Peripheral nerve-associated stromal cells	73
3.1.3	Perineurial cells	74
3.2	Results	76
3.2.1	Sympathetic Associated Perineurial Cells are LepR ⁺ . .	76
3.2.2	LepR ⁺ Sympathetic Associated Perineurial Cells produce immunomodulatory cytokine IL-33	83
3.2.3	LepR ⁺ Sympathetic Associated Perineurial Cells are distinct from IL-33 producing stromal cells within vWAT .	84
3.2.4	LepR ⁺ SAPCs are a distinct subset of perineurial cells associated with sympathetic tissues	88
3.2.5	<i>LEPR⁺IL33⁺</i> cells are present in human sympathetic ganglia	88
3.2.6	LepR ⁺ Sympathetic Associated Perineurial Cells express <i>Adrb2</i>	90
3.3	Discussion	90
3.4	Updated hypothesis	96
3.5	Updated aims	96

3.1 Introduction

3.1.1 Leptin receptor

The main role of leptin is described in the hypothalamus, where leptin receptor positive neurons transiently reduce food intake [43] and increase sympathetic drive onto adipose tissues to induce energy expenditure [63]. Leptin receptor (LepR, also called Ob receptor or ObR) is a transmembrane receptor belonging to the class I cytokine receptor family, and is encoded by the *Db* gene [118]. *Db/Db* mice possess a spontaneous mutation within the *Db* gene resulting in a significant truncation of the LepR protein and loss

of leptin signalling [229]. These mice exhibit severe, early on-set obesity and insulin resistance, and are primarily used as a model of type 2 diabetes [229]. *Db/Db* mice phenocopy *Ob/Ob* leptin knockouts, and are similarly resistant to recombinant leptin administration [229], corroborating a key role for Leptin-LepR signalling in whole-organism metabolism. As a result of alternative splicing of the *Db* gene, six isoforms of LepR have been identified in wildtype mice; including short isoforms (ObRa, ObRb, ObRc, ObRd, ObRf), a soluble isoform (ObRe) and one fully active, long isoform (ObRb) [118]. Only the ObRb isoform possesses the full external, transmembrane and internal signalling domains required for the cellular transduction of leptin signalling, via the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway [118, 230, 231]. In *Db/Db* mice a spontaneous mutation within the *Db* gene results in the truncation of the LepR protein, and loss of leptin signalling.

The ObRb isoform of leptin receptor expressed in the central nervous system is crucial for hypothalamic leptin signalling, and the subsequent transient reduction in food intake and sympathetic drive onto peripheral tissues to stimulate energy expenditure [11][35][36][37][38][40][231][232][233][234][235]. However, LepR⁺ cells have been also been reported in the periphery. Initial identification of the leptin receptor by Tartaglia et al. [118] utilised both alkaline phosphatase-conjugated leptin and [¹²⁵I]leptin to identify LepR⁺ cells within the choroid plexus and hypothalamus of mice, and later confirmed the presence of *ObRa* and *ObRb*, respectively, at the RNA level. However, the authors revealed that both modified leptin ligands bound similarly to wildtype and *Db/Db* mice within the choroid plexus, indicating a lack in binding specificity of the modified leptin ligands, likely due to the presence of truncated leptin receptor protein in *Db/Db* mice [118]. For this reason, coupled with the absence of specific LepR antibodies, detection of *ObRb* RNA has remained the gold standard in the identification of LepR⁺ cells capable of leptin signalling. LepR⁺ cells expressing the fully active ObRb isoform have been reported outside of the brain - alluding to additional functions of the leptin hormone in the periphery of mice. *ObRb* transcripts have been detected in the mouse lung [232], kidney [232], adrenal gland [236, 237], ovary [236], bone marrow [238] and both white

and brown adipose tissues [232]. In particular, endothelial cells have been reported to express *LepR* within human scWAT and vWAT using immunohistochemistry [141], although the authors do not provide evidence confirming a lack of non-specific *LepR* antibody binding in this study. Another study by Murphy et al. [239] reports a subset of *LepR*⁺ sensory neurons within the dorsal root ganglia (DRG) of Siberian hamsters, originating in the scWAT. Using a retrograde sensory neuron-specific tracer injected into the scWAT, the authors reveal tracer⁺ pseudounipolar neurons within the DRG. However, this study on the dorsal root ganglia does not provide direct evidence of *LepR*⁺ neurons innervating adipose tissues [239].

3.1.2 Peripheral nerve-associated stromal cells

Previous research has revealed that adipose tissue is highly innervated by sympathetic neurons [95] which, upon stimulation, induce lipolysis and thermogenesis through the release of neurotransmitter norepinephrine (NE) [5]. However, little is known about the organisation of sympathetic neurons within these nerve structures found in adipose tissues - later described as nerve fascicles or axon bundles. Typically, cells associated peripheral nerves have been studied in the sciatic nerve - the largest and most accessible peripheral nerve which contains sensory and motor neurons innervating the lower legs. Dating back to the 1800s, anatomists and histologists Freidrich Henle, Ernst Key, Magnus Retzius and Louis Ranvier first described peripheral nerve structures based on their anatomy across a number of species [240][241][242]. Simultaneously, these anatomists defined and named a range of nerve-associated stromal cells based on nerve anatomy. At the lowest order of organisation, endoneurial cells are associated with individual neuronal axons (Figure 3.1). Next, perineurial cells surround bundles of axons, forming individual nerve fascicles (Figure 3.1). Finally, they describe an epineurial cell layer which surrounds and combines multiple nerve fascicles into one nerve (Figure 3.1). A study by Shanthaveerappa et al. identified a perineurial cell barrier surrounding the sympathetic ganglia and nerve trunks of rat, cat and false killer whale [243], indicating the existence of similar organisation across species of sympathetic neurons to the 'peripheral' nerve

model reported in the 1800s.

3.1.3 Perineurial cells

Historically also named the lamellated sheath and perilemma, the perineurium is an anatomically defined barrier surrounding a nerve fascicle, and is formed of concentric circles of flattened cells with flattened nuclei [240][241][242][243]. In fact, the number of perineurial cell layers constituting the perineurium directly correlates with the diameter of the nerve fascicle, where larger fascicles have a thicker perineurium [244]. Across a range of different species, perineurial cells have been described as mesenchymal [245], fibroblastic [246][247], endothelial-like [240] and epithelial [243]. Forming part of the blood-nerve barrier [248], perineurial cells are connected by tight junctions - including Claudin-1 (CLDN1) and Tight junction protein-1 (TJP1) [244] -, closely associated with a basement membrane on both sides [243], and surrounded by dense extracellular matrix proteins including type IV collagen, fibronectin, laminin, glycosaminoglycans [249]. The perineurium has a barrier function, as demonstrated in the frog sciatic nerve by Krnjević et al., who determined that incubation with an isotonic, sodium-free solution inhibited nerve transduction only when the perineurium surrounding the nerve was bypassed [250][242].

In addition, Kuswanto et al. [227] identified an immunomodulatory perineurial cell subset within the muscle [227]. These 'Fibro/Adipo Progenitors' (FAPs) are proximal to sensory neurons within the muscle, and may represent a muscle perineurial cell population. FAPs within muscle produce Interleukin-33 (IL-33) which is required for the recruitment and maintenance of reparative regulatory T cells (Tregs) within muscle following insult [227]. Furthermore, Wang et al. went on to show that IL-33 production by these neuron-associated mesenchymal stromal cells in the muscle can be 'tuned' by the sensory neuropeptide Calcitonin-gene-related peptide (CGRP); highlighting FAPs as a cellular intermediate of the neuronal regulation of muscle tissue immune cell recruitment and maintenance [226]. Comparatively, in the context of adipose tissue, two

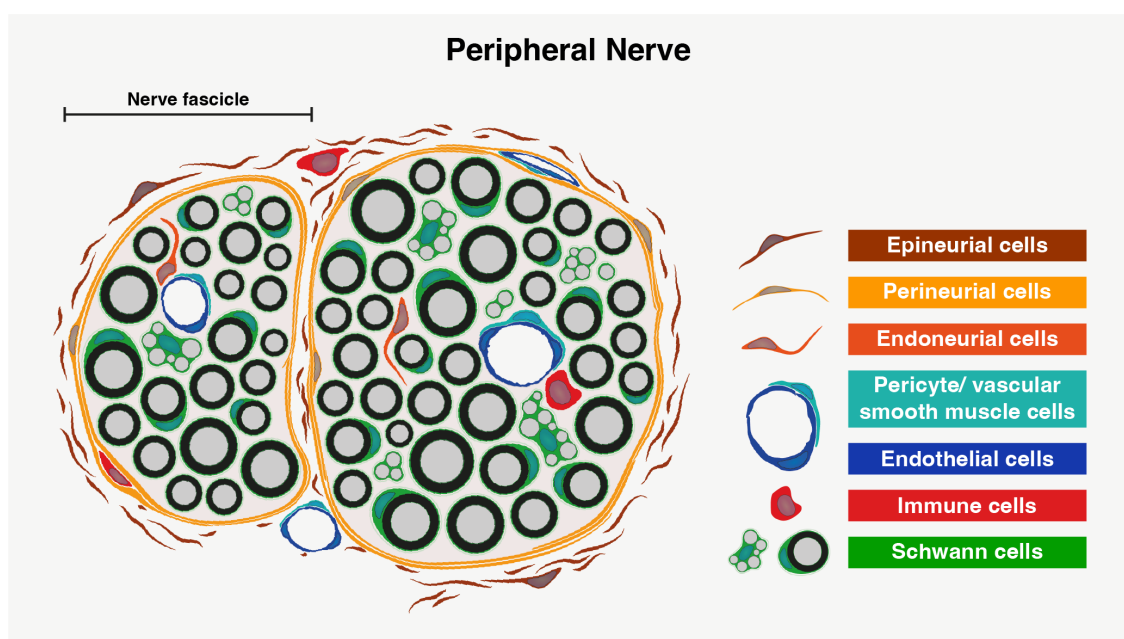


Figure 3.1: Organisation of peripheral nerves

A Schematic diagram of a transverse cross section through a peripheral nerve, adapted from [251]. Bundles of neuronal axons are organised into nerve fascicles, which are surrounded by perineurial cells. Multiple nerve fascicles are surrounded by an epineurial cell layer. Blood vessels and immune cells are also detected in association with peripheral nerves.

groundbreaking back-to-back studies have implicated IL-33 production by AT stromal populations in the maintenance of adipose tissue homeostasis.[207][217]. Spallanzani et al. [207] and Mahlaköiv et al. [217] describe a heterogeneous pool of stromal cells, constituting the major producer of immunocytokine IL-33 in visceral white adipose tissue, regulating AT immune cell recruitment and maintenance [207][217]. Focussing solely on white adipose tissues, these two studies are amongst the first to describe an immunomodulatory role of stromal cells in adipose tissue immunometabolic homostasis.

3.2 Results

3.2.1 Sympathetic Associated Perineurial Cells are LepR⁺

Given the presence of *ObRb* RNA in adipose tissues [232], reports of LepR⁺ sensory neurons [239] and established role of sympathetic neurons on energy expenditure, I aimed to determine whether ObRb⁺ cells were present in nerve fascicles innervating adipose tissue. Owing to the lack of LepR reporter mice or functional antibodies against LepR, I generated and utilised a LepR^{CRE::Rosa26^{Lox-Stop-Lox}-eYFP-Chr2} (hereafter termed LepR^{Ai32}) mouse model, where any cell which has expressed *ObRb* will become eYFP⁺. Therefore eYFP positivity does not necessarily mean that cells currently express *ObRb*, which would need to be validated at the RNA level due to the lack of functional antibodies against LepR. Of note, considering the mouse models utilised, from this point onwards LepR and ObRb are used interchangeably to refer to the fully active ObRb signalling isoform of leptin receptor.

I first isolated adipose tissue sympathetic axon bundles and the Superior Cervical Ganglia (SCG) from LepR^{Ai32} mice. As adipose tissue is functionally innervated by sympathetic axon bundles originating from the paravertebral chain of sympathetic ganglia [24][25][26], the SCG contains the cell bodies of adipose tissue-innervating sympathetic neurons (Figure 3.2A). I detected eYFP⁺ cells in the SCG, scWAT and BAT axon bundles of LepR^{Ai32} mice at similar frequencies using flow cytometry (Figure 3.2B-C). To determine the location of eYFP⁺ cells, I next imaged these LepR^{Ai32} sympathetic

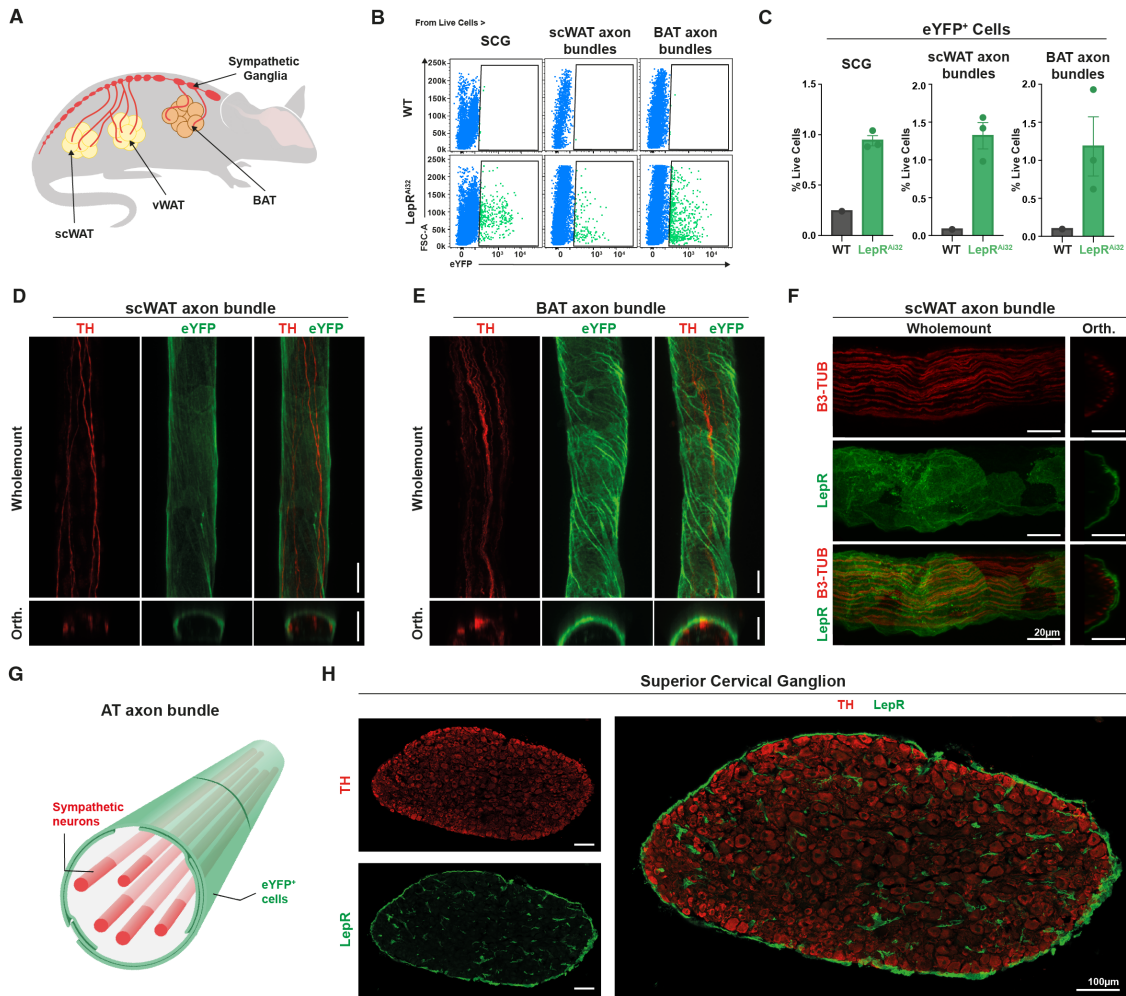


Figure 3.2: eYFP⁺ cells surround $LepR^{Ai32}$ sympathetic tissues

A Schematic representation: adipose tissue is innervated by sympathetic axon bundles originating from the paravertebral chain of sympathetic ganglia. scWAT = subcutaneous white adipose tissue, vWAT = visceral white adipose tissue, BAT = interscapular brown adipose tissue. **B-C** eYFP⁺ cells are detected in $LepR^{Ai32}$ (green) but not $LepR^{CRE}$ only control (grey) sympathetic tissues using flow cytometry. Mean $\pm$ SEM, $n=3$. **D-E** Sympathetic axon bundles dissected from the scWAT or BAT of $LepR^{Ai32}$ mice are surrounded by an eYFP⁺ cell barrier. 20x maximum intensity projection images (Wholemount) or orthogonal optical reconstruction (Orth). TH = tyrosine hydroxylase (red), eYFP (green). Scale bars = 50 μ m. **F** eYFP fluorescence (green) is not observed in B3-TUB⁺ neurons (red). $LepR^{Ai32}$ mice, 63x oil maximum intensity projection images (Wholemount) or orthogonal optical reconstruction (Orth). Scale bars = 20 μ m. **G** Schematic diagram: sympathetic neurons are surrounded by a eYFP⁺ barrier in $LepR^{Ai32}$ adipose tissue axon bundles. **H** Superior Cervical Ganglia (SCG) of $LepR^{Ai32}$ mice are surrounded by an eYFP⁺ cell barrier. eYFP (green), TH⁺ sympathetic neuron cell bodies (red). Scale bar = 100 μ m, 20x maximum intensity projection image of a ganglion cross-section. Full flow cytometric gating strategy shown in Appendix Figure 5. Secondary antibody only controls shown in Appendix Figure 6.

I next sought to find the identity of these eYFP⁺ cells closely associated with sympathetic neurons by comparing the expression profile of LepR^{eYFP+} cells with common stromal cell markers, including epineurial, perineurial, endoneurial, Schwann, endothelial, pericyte and epithelial markers (Figure 3.3D). Whilst also confirming *Lepr* expression in eYFP⁺ cells sorted from the SCG, bulk RNA-sequencing analysis reveals high expression of markers used to describe perineurial cells, including *Pdgfra*, *Cd34*, *Slc2a1*, *Itgb4*, *Cldn1*, *Cav1*, *p1C*, *Igfbbp6*, *Tjp1*, *Vcl*, *Ntng1* (Figure 3.3D). Some epineurial marker expression was detected (*Ly62c*, *Slco1a4*, *Rbp7*), but I do not see any notable endoneurial, Schwann cell, endothelial, pericyte or epithelial marker expression (Figure 3.3D). Phenotypically, I show that eYFP⁺ LepR⁺ cells in the SCG of LepR^{Ai32} mice highly express a range of

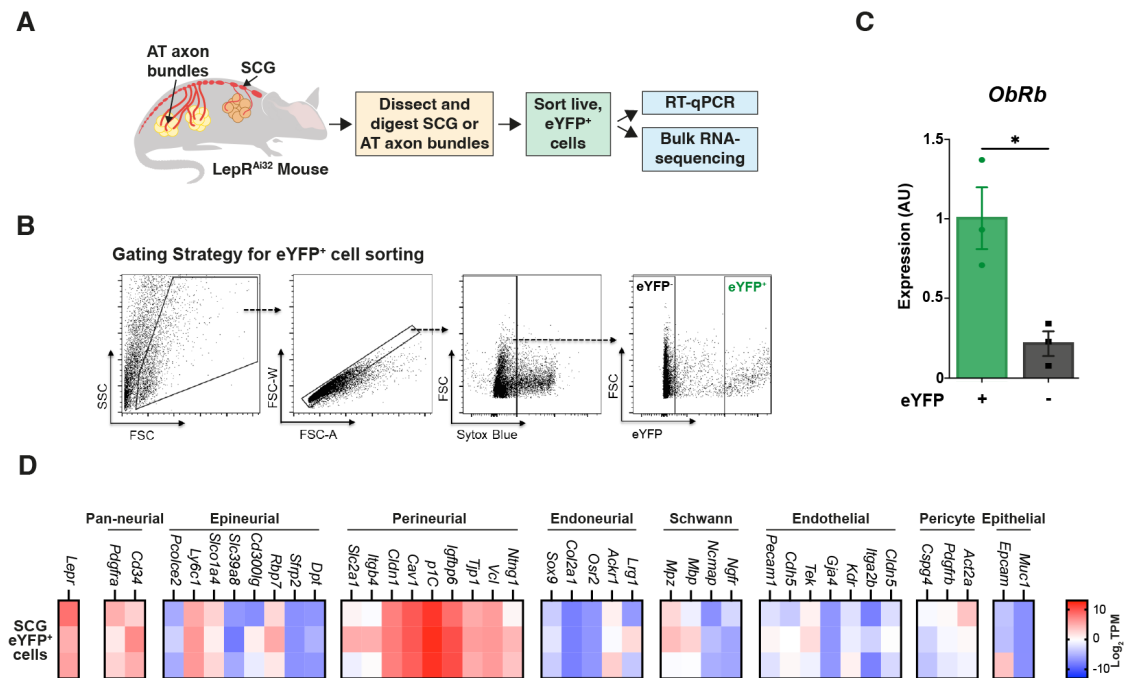


Figure 3.3: eYFP⁺ perineurial cells in *LepR^{Ai32}* mice express *ObRb*

A Schematic diagram of RT-qPCR and bulk-RNA sequencing experiments performed. Live, eYFP⁺ cells were sorted in bulk from *LepR^{Ai32}* tissues and RNA was extracted for RT-qPCR or bulk-RNA sequencing - performed by Bernardo Arús and Noelia Martínez-Sánchez in collaboration with Svetoslav Chakarov and Florent Ginhoux. **B** Gating strategy for the isolation of live, eYFP⁺ cells (SCG). **C** RT-qPCR using eYFP⁺ and eYFP⁻ cells sorted from the SCG of *LepR^{Ai32}* mice confirms that *ObRb* is expressed in eYFP⁺ cells of the SCG (n=3 biological replicates) and AT axon bundles, but not in eYFP⁻ cells (n=4 biological replicates). Gene expression is normalised to the expression of house keeping gene *Tata box binding protein* (*Tbp*). **D** Gene expression profiling of bulk RNA-sequenced live, eYFP⁺ cells sorted from the SCG of *LepR^{Ai32}* mice (n=3 biological replicates). eYFP⁺ cells express both *LepR* and a range of perineurial cell markers. Data presented as Log₂ transcripts per million (TPM).

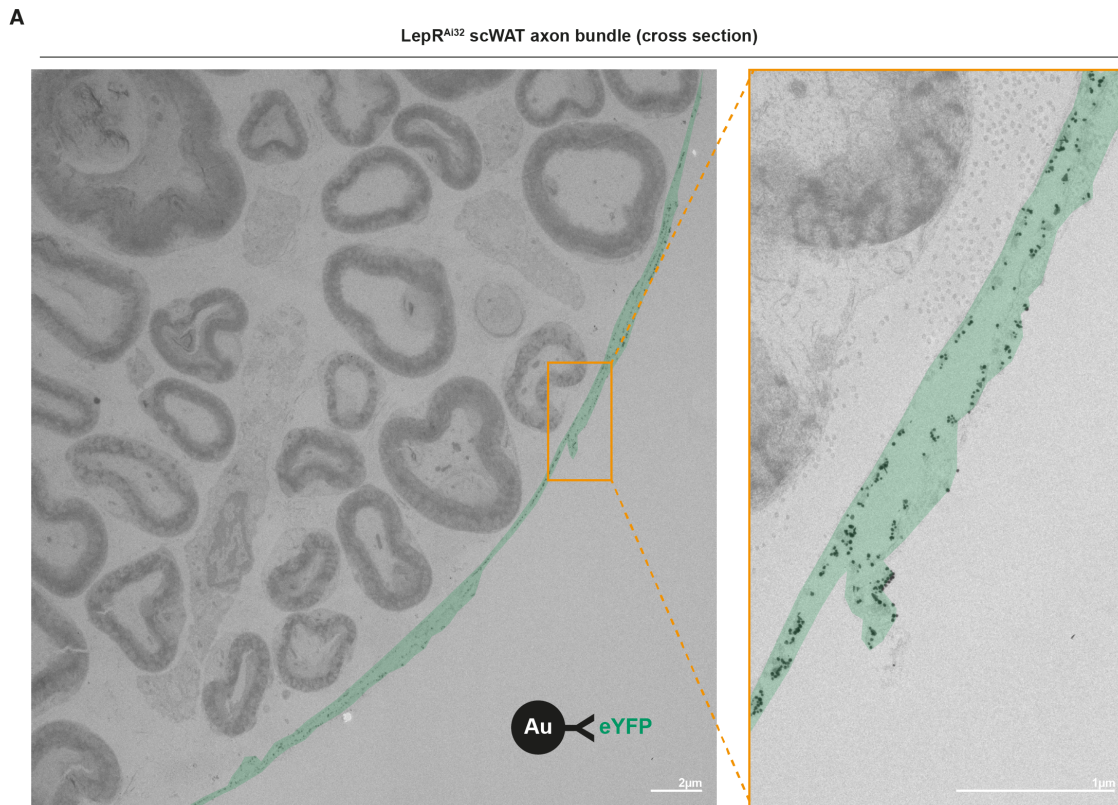


Figure 3.4: A multi-layer LepR⁺ Sympathetic Associated Perineurial Cell barrier surrounds adipose tissue axon bundles

A Transmission Electron Micrograph (TEM) of a scWAT sympathetic axon bundle cross section. Immuno-Electron Microscopy staining of LepR^{Ai32} tissues with a gold-conjugated anti-eYFP antibody reveals a multi-layer LepR⁺ Sympathetic Associated Perineurial Cell (SAPC) barrier. scWAT fibres dissected from LepR^{Ai32} mice. LepR⁺ cells false-coloured green. Scale bars = 2µm (left) / 1µm (right). Sample preparation performed by Bernardo Arús and Noelia Martínez-Sánchez, immuno-electron microscopy performed by Andrea Raimondi and Matteo Iannacone

perineurial cell markers, including *Pdgfra* which is commonly also a marker of fibroblasts.

Perineurial cells surround peripheral nerve axon bundles. To visualise the ultra-structure of the *LepR*⁺ cell barrier surrounding adipose tissue sympathetic axons, immuno-electron microscopy (immuno-EM) on cross sections of dissected *LepR*^{Ai32} scWAT axon bundles was performed. Of note, this experiment was performed by Andrea Raimondi and Matteo Iannacone on samples prepared by Bernardo Arús and Noelia Martínez-Sánchez. Staining with dense, gold-conjugated anti-eYFP antibodies to mark *LepR*⁺ cells showed that the *LepR*⁺ barrier surrounding sympathetic AT nerves is formed of multiple flattened, thin cells (Figure 3.4A), similar to the reported morphology of perineurial cells [242][248]. This morphological analysis further indicates the *ObRb*⁺ detected around sympathetic neurons are perineurial cells.

To confirm whether these *LepR*⁺ sympathetic associated cells were phenotypically perineurial at the protein level, I next performed flow cytometry on *LepR*^{Ai32} sympathetic tissues, testing for the presence of perineurial (*PDGFR* α , *ITG* β 4) and endothelial (*CD31*, *CDH5*) markers on *LepR*⁺ cells. I found that less 10 percent of cells within SCG, scWAT and BAT axon bundles were positive for endothelial markers *CD31*⁺ and/or *CDH5*⁺ (Figure 3.5A-B). Although low in frequency, this indicates the presence of some *LepR*⁺ endothelial cells, consistent with previous reports of *Lepr*-expressing endothelial cells in human adipose tissue [141]. Most notably, more than 90 percent of *LepR*⁺ cells in all sympathetic tissues measured were positive for perineurial and fibroblast marker *PDGFR* α ⁺ (Figure 3.5A-B). Approximately 50-60 percent of *LepR*⁺ cells in scWAT and BAT axon bundles were positive for perineurial marker *ITG* β 4, a frequency which was reduced in the SCG at around 15-20 percent (Figure 3.5A-B). Immunofluorescent imaging of *LepR*^{Ai32} scWAT axon bundles reveals the presence of small eYFP⁺*CD31*⁺ blood vessels running alongside the sympathetic axon bundle. In particular, from this image it is clear that *CD31* does not mark the outer *LepR*⁺ cell layer surrounding scWAT sympathetic axon bundles (Figure 3.5C), or the superior cervical ganglia of *LepR*^{Ai32} mice (Figure 3.5D). Within sympathetic scWAT and BAT axon

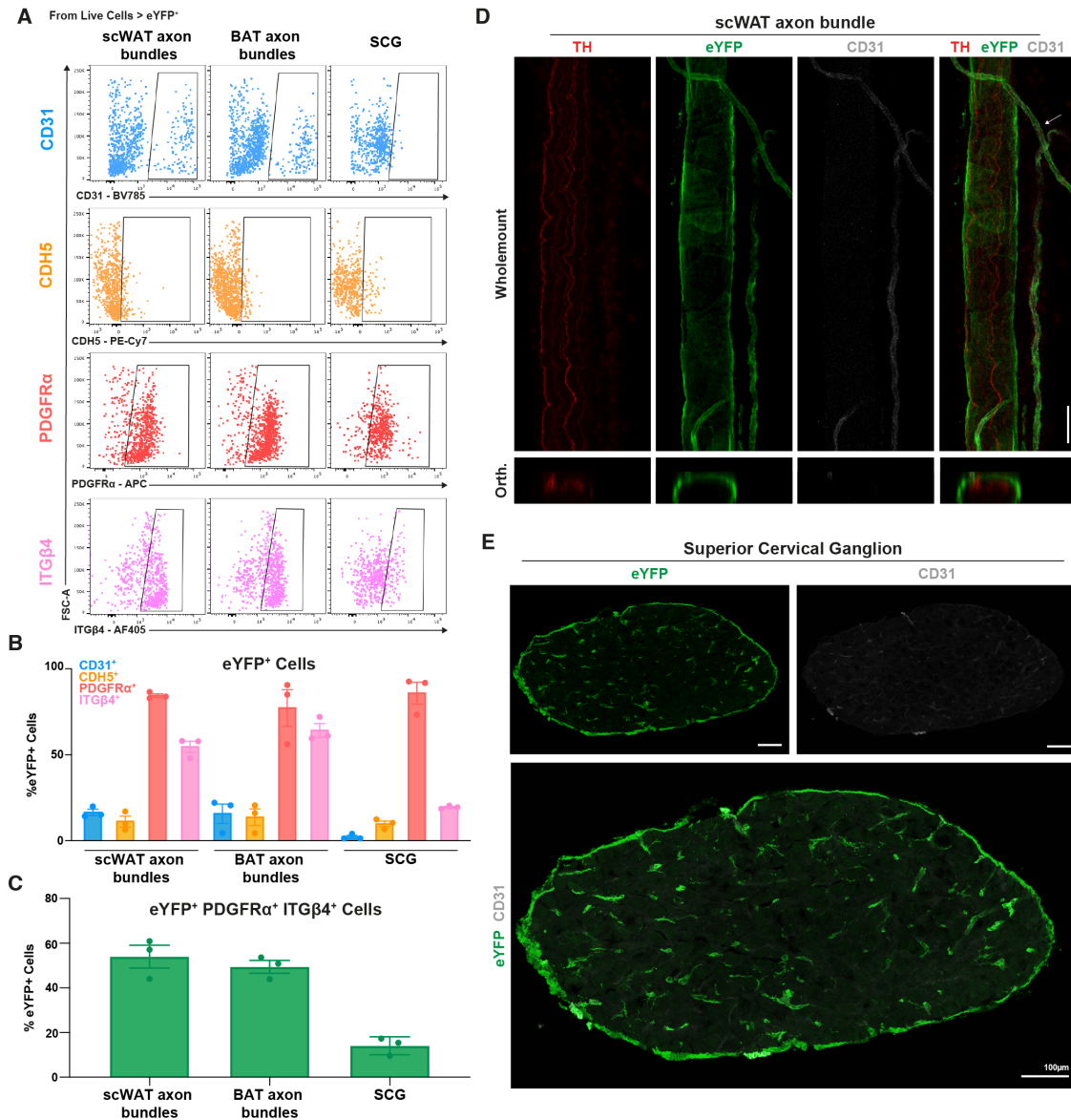


Figure 3.5: LepR⁺ Sympathetic Associated Perineurial Cells are PDGFRα⁺ITGβ4⁺

A Representative flow cytometry plots of live, LepR⁺ cells from scWAT axon bundles, BAT axon bundles and Superior Cervical Ganglia (SCG) dissected from LepR^{Ai32} mice. Full flow cytometric gating strategy for CD31, CDH5, PDGFRα and ITGβ4 shown in Appendix Figure 5. **B** Quantification of flow cytometric staining of LepR⁺ cells from scWAT axon bundles, BAT axon bundles and SCG. Most LepR⁺ cells are PDGFRα⁺ and ITGβ4⁺. n=3, mean ± SEM. **C** Immunofluorescent imaging of whole-mount scWAT axon bundles dissected from LepR^{Ai32} mice. The LepR⁺ Sympathetic Associated Perineurial Cell barrier surrounding TH⁺ sympathetic neurons is CD31⁻. White arrow highlights a small CD31⁺ LepR⁺ blood vessel running alongside the sympathetic axon bundle. 20x maximum intensity projection images (Wholemount) or orthogonal optical reconstruction (Orth). TH = tyrosine hydroxylase (red), eYFP (green), CD31 (grey). Scale bars = 50μm. **D** The LepR⁺ Sympathetic Associated Perineurial Cell barrier surrounding the Superior Cervical Ganglion (SCG) is CD31⁻. TH⁺ cell bodies (red), eYFP (green), CD31 (grey). Scale bar = 100μm, 20x maximum intensity projection image of a LepR^{Ai32} ganglion cross-section. Secondary antibody only controls shown in Appendix Figure 6 **E** Quantification of PDGFRα⁺ ITGβ4⁺ cells as a frequency of eYFP⁺ cells.

bundles *LepR*⁺ cells contribute solely to the outer barrier (Figure 3.2D-F). However, I also observe *LepR*⁺ within the SCG as well as surrounding (Figure 3.2D-G). Based on *Pdgfra* and *Itgb4* expression being characteristic of perineurial cells and in the absence of immunofluorescent imaging, I reasoned that eYFP⁺ cells positive for both PDGFR α and ITG β 4 likely form the perineurial barrier surrounding axon bundles in scWAT, BAT and the SCG (Figure 3.2D-E, H). Accordingly, I define *LepR*⁺ perineurial cells as PDGFR α ⁺ITG β 4⁺. With flow cytometric quantification, I observe similar frequencies of eYFP⁺ PDGFR α ⁺ ITG β 4⁺ perineurial cells in scWAT and BAT axon bundles, but a lower frequency in the SCG (Figure 3.5E), indicating heterogeneity in *LepR*⁺ cells within the SCG. Collectively, I confirm that whilst *LepR* marks few CD31⁺ endothelial cells, most *LepR*⁺ Sympathetic Associated Perineurial Cells surrounding adipose tissue sympathetic neurons are PDGFR α ⁺ITG β 4⁺, and not CD31⁺.

3.2.2 *LepR*⁺ Sympathetic Associated Perineurial Cells produce immunomodulatory cytokine IL-33

Having characterised *LepR*⁺eYFP⁺ cells as Sympathetic Associated Perineurial Cells (SAPCs), I hypothesised that they may play an immunometabolic role, preventing the chronic, low-grade AT inflammation closely which is closely associated with obesity [139]. Analysis of our bulk RNA-sequencing dataset revealed high expression of anti-inflammatory cytokines *Interleukin-33* (*Il33*), *Transforming growth factor β 2* (*Tgfb2*), and *Interleukin-4* (*Il4*) in *LepR*⁺ SAPCs (Figure 3.6A), along with the expression of some pro-inflammatory cytokines including *Monocyte chemoattractant protein-1* (*Ccl2*), *Interleukin-34* (*Il34*), *Colony stimulating factor-1* (*Csf1*), *Interleukin-15* (*Il15*), *Interleukin-18* (*Il18*) and *C-X-C motif chemokine ligand-10* (*Cxcl10*, Figure 3.6B). Given the recent studies which have implicated adipose tissue IL-33 and downstream IL-33-responsive immune cells in the control of AT homeostasis [207][217], I decided to explore the role of *LepR*⁺ SACP-derived IL-33 in AT homeostasis. Using immunofluorescence I confirmed the presence of nuclear localised IL-33 within *LepR*⁺ SAPCs surrounding both scWAT and BAT axon bundles (Figure 3.6C-D). By creating orthogonal reconstructions, I observed that IL-33 is present exclusively in the surrounding perineurial barrier of

scWAT and BAT axon bundles (Figure 3.6C-D). Furthermore, I observe that IL-33⁺ cells are present throughout the SCG, however a subset of LepR⁺IL-33⁺ perineurial cells surround sympathetic ganglia (Figure 3.6E). Using flow cytometry, I determine that approximately 50% of LepR⁺PDGFR α ⁺ITG β 4⁺ cells are IL-33⁺ in scWAT axon bundles, whereas around 70% are IL-33⁺ in both BAT axon bundles and the SCG (Figure 3.6F-G). Collectively, I confirm that LepR⁺ SAPCs in adipose tissue sympathetic axon bundles and in sympathetic ganglia produce IL-33.

3.2.3 LepR⁺ Sympathetic Associated Perineurial Cells are distinct from IL-33 producing stromal cells within vWAT

With the aim of utilising published datasets to further investigate LepR⁺IL-33⁺ Sympathetic Associated Perineurial Cells, I next sought to compare SAPCs with the IL-33-producing mesenchymal stromal cell populations reported in visceral WAT (vMSCs) by Spallanzani et al. [207]. Based on the common expression of *Pdgfra* and *Ly6a* between SAPCs and vMSCs, I assessed the expression of perineurial cell markers within the single cell RNA-sequencing dataset of PDGFR α ⁺SCA-1⁺ vMSCs sorted from the visceral WAT (Figure 3.7A) [207]. Whilst it was evident that all 7 clusters expressed 'pan-neurial' and mesenchymal markers *Pdgfra* and *Cd34*, I found an absence of key perineurial markers *Slc2a1*, *Itgb4* and *Cldn1*, with low expression of *Cav1*, *Tjp1* and *Vcl* (Figure 3.7B), in comparison with the generally high perineurial marker expression in bulk RNA-sequencing of LepR^{eYFP}⁺ SAPCs (Figure 3.3). SAPCs are LepR⁺IL-33⁺, so I next sought to assess the expression of *Lepr* and *Il33* within the vMSC subclusters. Whilst I observed some expression of *Il33* in clusters 0 and 4 (similar to those observed by Spallanzani et al. [207]), I detected very few *Lepr*⁺ cells (Figure 3.7C). Furthermore, I detected no vMSCs co-expressing *Lepr* and *Il33*, which would appear yellow in the single cell RNA-sequencing dot plots (Figure 3.7C). Therefore, I show that LepR⁺IL-33⁺ SAPCs are distinct from those IL-33-producing cells reported in the visceral WAT.

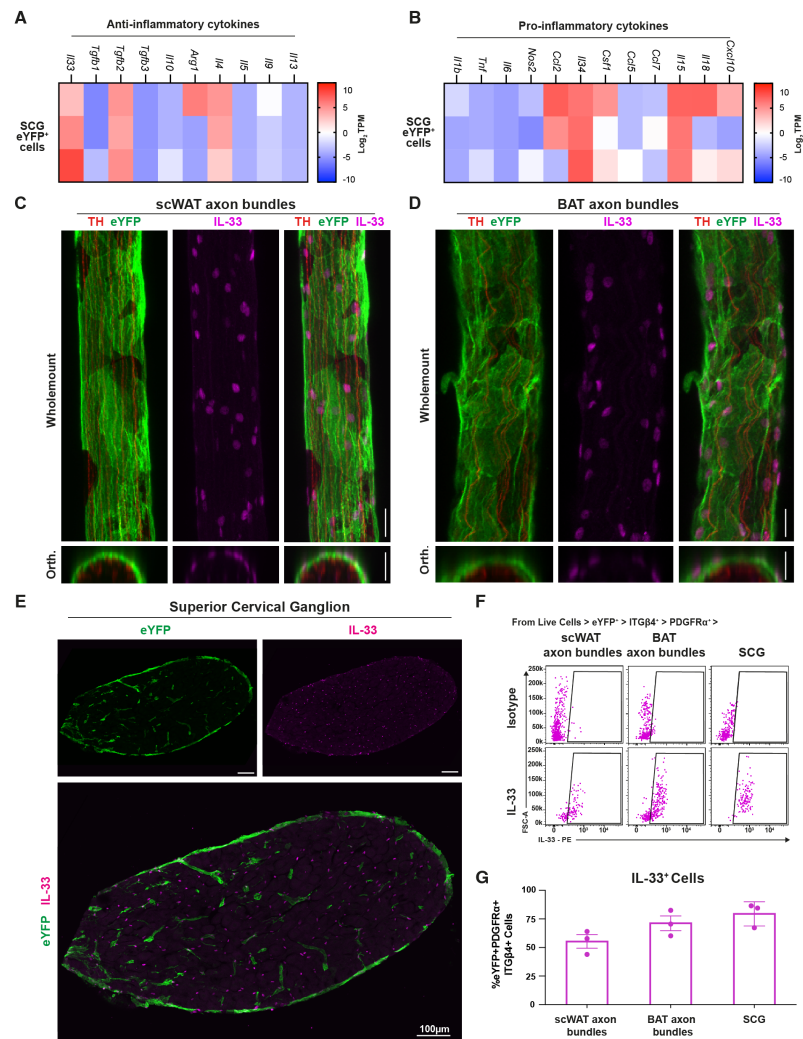


Figure 3.6: *LepR*⁺ Sympathetic Associated Perineurial Cells are IL-33⁺

A-B Gene expression from RNA-sequencing of eYFP⁺ *LepR*⁺ cells sorted from *LepR*^{Ai32} SCG (n=3 biological replicates). *LepR*⁺ cells express anti- and pro-inflammatory cytokines. Data presented as Log₂ transcripts per million (TPM). **C-D** Immunofluorescent imaging of whole-mount scWAT (**C**) and BAT (**D**) axon bundles dissected from *LepR*^{Ai32} mice. *LepR*⁺ Sympathetic Associated Perineurial Cells are IL-33⁺. 20x maximum intensity projection images, (Wholemount) or orthogonal optical reconstruction (Orth). eYFP (green), IL-33 (magenta). Scale bars = 50 μm. **E** The *LepR*⁺ cells surrounding the SCG are also IL-33⁺. eYFP (green), IL-33 (magenta). Scale bar = 100 μm, 20x maximum intensity projection image of a *LepR*^{Ai32} ganglion cross-section. Secondary antibody only controls shown in Appendix Figure 6. **F** Representative flow cytometry plots of live, *LepR*⁺ITGβ4⁺PDGFRα⁺ cells in *LepR*^{Ai32} scWAT axon bundles, BAT axon bundles and SCG. Full flow cytometric gating strategy for IL-33 shown in Appendix Figure 5. **G** Quantification of IL-33⁺ *LepR*⁺ITGβ4⁺PDGFRα⁺ cell frequencies in scWAT axon bundles, BAT axon bundles and SCG. Most *LepR*⁺ SAPCs are IL-33⁺. n=3, mean ± SEM.

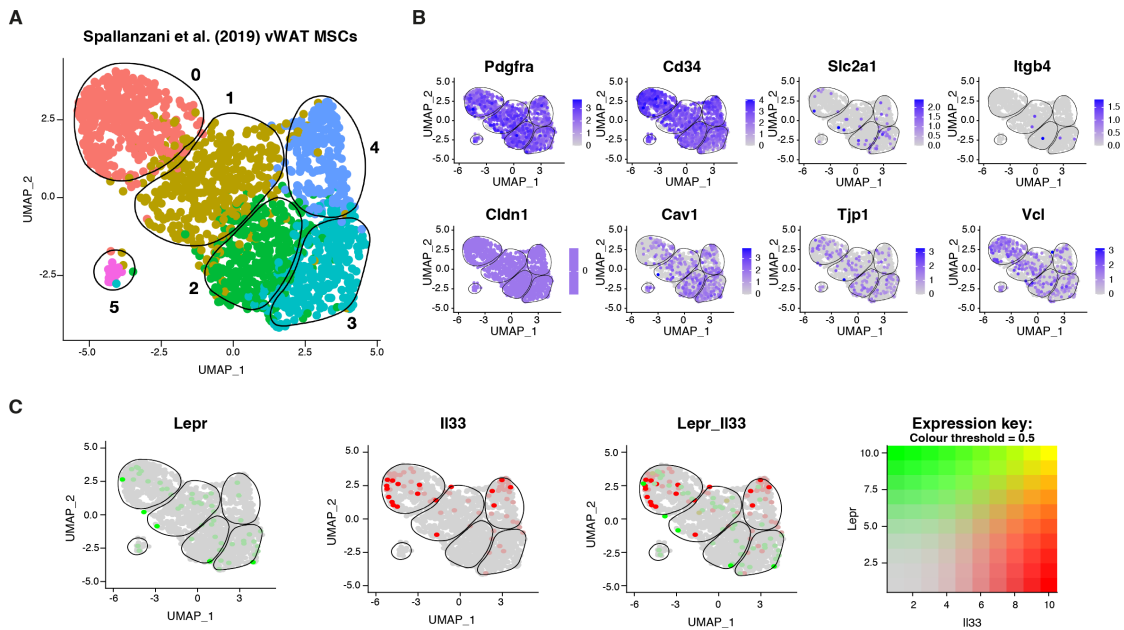


Figure 3.7: $Lepr^+IL33^+$ SAPCs are distinct from $IL33^+$ mesenchymal stromal cells reported in vWAT

A Clustering of PDGFR α ⁺SCA-1⁺ cells sorted from the visceral adipose tissue, including previously reported IL33-producing cells using the single cell RNA sequencing dataset presented in [207]. **B** All PDGFR α ⁺SCA-1⁺ cell clusters sorted from the muscle express *Pdgfra* and *Ly6a*, but show low/absent expression of perineurial markers *Slc2a1*, *Itgb4*, *Cldn1*, *Cav1*, *Tjp1* and *Vcl*. **C** Expression of *Lepr* (green) or *Il33* (red) alone, followed by co-expression plots of *Lepr* and *Il33* (Lepr $Il33$). Gene expression key (right), colour threshold = 0.5. Cells expressing both *Lepr* and *Il33* would appear yellow in sc RNA-seq dot plots. Few PDGFR α ⁺SCA-1⁺ stromal cells sorted from vWAT express *Lepr* or *Il33*. No co-expression of *Lepr* and *Il33* is observed.

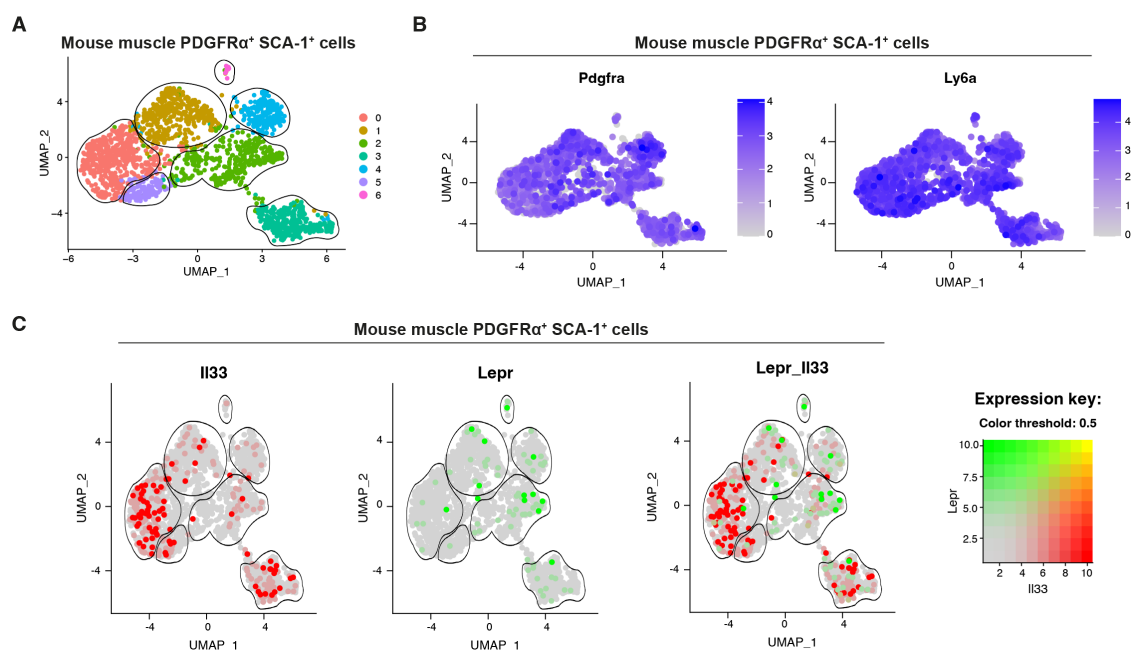


Figure 3.8: *LepR*⁺ SAPCs are distinct from sensory neuron-associated IL-33⁺ cells reported in mouse muscle

A Clustering of PDGFR α ⁺SCA-1⁺ cells sorted from the muscle, including previously reported sensory neuron-associated IL-33-producing stromal cells, using the single cell RNA-sequencing dataset presented in [207, 226, 227]. **B** All PDGFR α ⁺SCA-1⁺ cell clusters sorted from the muscle express *Pdgfra* and *Ly6a*. **C** Expression of *Lepr* (green) or *Il33* (red) alone, followed by co-expression plots of *Lepr* and *Il33* (*LeprIl33*). Gene expression key (right), colour threshold = 0.5. Cells expressing both *Lepr* and *Il33* would appear yellow in sc RNA-seq dot plots. No PDGFR α ⁺SCA-1⁺ stromal cells sorted from muscle co-express *Lepr* and *Il33*

3.2.4 **LepR⁺ SAPCs are a distinct subset of perineurial cells associated with sympathetic tissues**

To determine whether LepR⁺ SAPCs are present outside of sympathetic tissues, I compared LepR⁺ SAPCs with previously reported IL-33 producing stromal cells associated with sensory neurons in mouse muscle [226][207][227]. LepR⁺ SAPCs express both *Pdgfra* and *Ly6a* (Figure 3.3E), and are also PDGFR α ⁺ by flow cytometry staining (Figure 3.5A-B). For this reason, and the close association of PDFGR α ⁺SCA-1⁺ FAPs to sensory neurons, I compared LepR⁺ SAPCs with FAPs reported in muscle. Firstly, I reproduced the clustering of PDGFR α ⁺SCA-1⁺ FAPs and confirmed the expression of both *Pdgfra* and *Ly6a* in all stromal cell clusters (Figure 3.8A-B). I next assessed the individual expression of *Lepr* and *Il33* within all muscle stromal cell clusters (Figure 3.8C). Wang et al. [226] describe three *Il33*⁺ clusters within this muscle stromal cell dataset, which correlate with clusters 0, 3 and 5 in this analysis (Figure 3.8A,C). I observe that very few cells within this dataset express *Lepr* (Figure 3.8C). Accordingly, visualisation of the co-expression of both *Lepr* and *Il33* reveals that there are no *Lepr*⁺*Il33*⁺ cells within this muscle stromal cell dataset, which would appear yellow within the single cell RNA-sequencing dot plots (Figure 3.8C). From this comparison, I show that LepR⁺ SAPCs are a previously unreported perineurial cell subset associated with adipose tissue-innervating sympathetic neurons, which co-express *Lepr* and *Il33*.

3.2.5 **LEPR⁺IL33⁺ cells are present in human sympathetic ganglia**

To ascertain whether LepR⁺ SAPCs also exist in humans, I collaborated with Karin Ziegler, Sandro Meunier and Stefan Engelhardt, who kindly shared their human sympathetic ganglia single nuclei RNA sequencing data and analyses. Based on gene expression profiles, with unsupervised clustering ten cell clusters were identified in human sympathetic ganglia, including a number of stromal and immune cells, alongside the expected sympathetic neurons (Figure 3.9A). Interestingly, I observe no expression of mouse perineurial markers *PDGFRa*, *SLC2A1* or *CLDN1* within *LEPR*⁺ perineurial cells identified within human sympathetic ganglia. It is not surprising that human perineurial

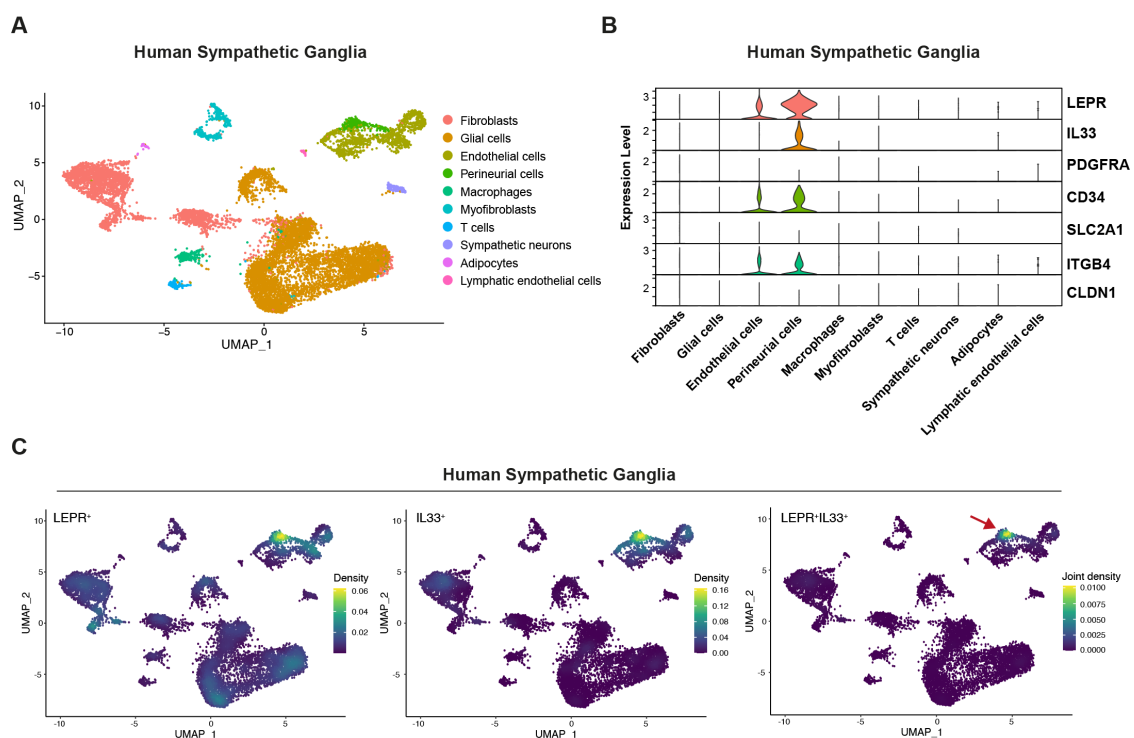


Figure 3.9: *LEPR*⁺*IL33*⁺ cells exist in human sympathetic ganglia

A A range of stromal and immune cells exist alongside sympathetic neurons in human sympathetic ganglia. **B** Cells within the cluster designated 'perineurial cells' express some, but not all, mouse perineurial cell markers. *LEPR* = leptin receptor, *IL33* = Interleukin-33, *PDGFRA* = Platelet derived growth factor α , *CD34* = Cluster of differentiation 34, *SLC2A1* = Solute carrier family 2 member 1, *ITGB4* = Integrin β 4, *CLDN1* = Claudin 1. **C** Expression of *LEPR*, *IL33* and co-expression of *LEPR* and *IL33* in human sympathetic ganglia cell clusters. *LEPR* and *IL33* are co-expressed by the perineurial cell cluster, marked by the red arrow.

cell markers do not entirely align with mouse perineurial cell markers, given the variation in perineurial cell identity observed between different species [244]. I found only one cluster with both *LEPR* and *IL33* expression, which was positive for some (*CD34*, *ITGB4*), but not all, perineurial cell markers (Figure 3.9B). We, therefore, provisionally name this the perineurial cell cluster. Importantly, within this cluster I observed that only cells within this perineurial cell cluster co-express *LEPR* and *IL33*, similarly to the perineurial barrier cells observed in mice (Figure 3.9C). From this analysis, I can conclude the presence of a *LEPR*⁺*IL33*⁺ cell subset within the human sympathetic ganglia, which may be analogous to the Sympathetic Associated Perineurial Cells observed in mice.

3.2.6 *LepR*⁺ Sympathetic Associated Perineurial Cells express *Adrb2*

Given their close proximity to sympathetic neurons, I hypothesised that *LepR*⁺ SAPCs may respond to neuron activity, and sought to determine whether they express receptors to sympathetic neurotransmitters. Using the bulk RNA-sequencing data prepared using eYFP⁺ cells sorted from *LepR*^{Ai32} SCG, I observed that *LepR*⁺ SAPCs express adrenergic receptors, cognate to the sympathetic-derived neurotransmitter norepinephrine (NE), including *Adrb2*, and to a lesser degree *Adra2c* (Figure 3.10A). *LepR*⁺ SAPCs do not express receptors to alternate sympathetic neurotransmitters Neuropeptide Y (NPY, Figure 3.10B), nor receptors to sympathetic preganglionic neurotransmitter Acetylcholine (Figure 3.10C). Together, this suggests that *LepR*⁺ SAPCs may be regulated by close-proximity post-ganglionic noradrenergic sympathetic neurons.

3.3 Discussion

Since its discovery, the primary action of leptin is thought to be on *LepR*⁺ neurons of the hypothalamus, which broadly regulate energy balance through the control of energy input (food intake) and energy expenditure (adipose tissue lipolysis and thermogenesis). Here, I show the presence of *LepR*⁺ sympathetic neuron associated perineurial barrier cells ensheathing sympathetic ganglia and axon bundles in brown and white adipose

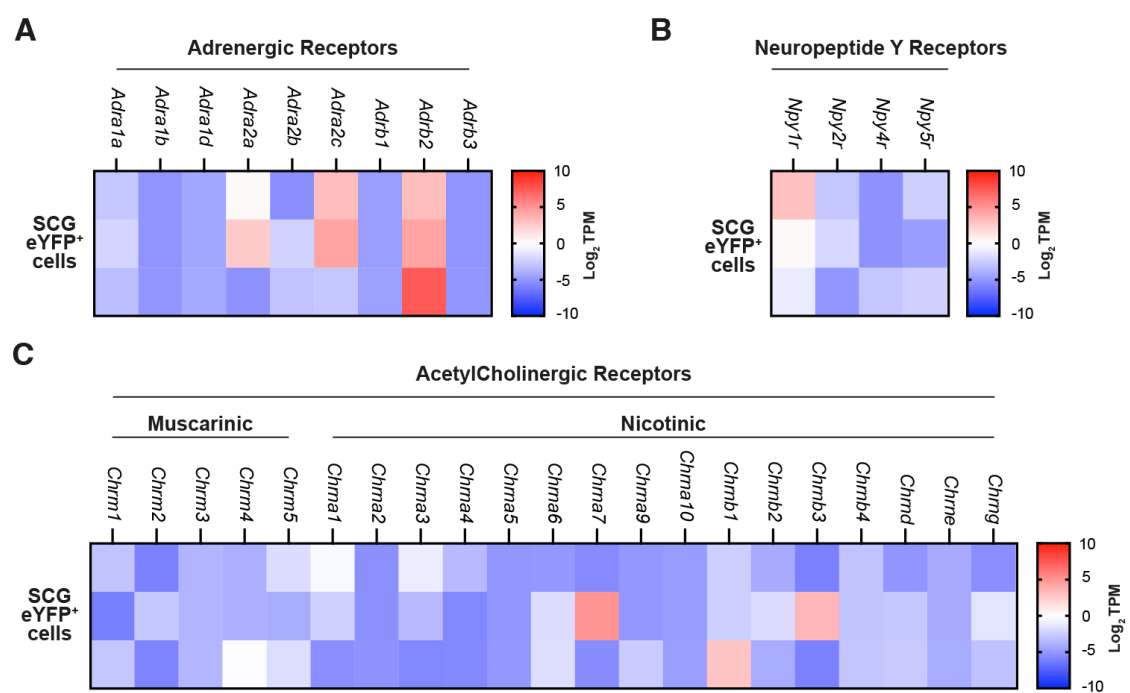


Figure 3.10: *LepR*⁺ Sympathetic Associated Perineurial Cells express *Adrb2*

A Gene expression profiling of bulk RNA-sequenced eYFP⁺ *LepR*⁺ cells, sorted from the Superior Cervical Ganglia (SCG) of *LepR*^{Ai32} mice (n=3 biological replicates). *LepR*⁺ cells express *Adrb2*, and *Adra2c* at a lower level. Data presented as Log₂ transcripts per million (TPM).

tissues, which express the full signalling *ObRb* isoform of leptin receptor. Sympathetic associated perineurial cells form a physical barrier between sympathetic neuron-derived NE and the adipocytes on which this neurotransmitter acts to induce lipolysis and thermogenesis; and therefore are well positioned to regulate the bioavailability of NE within adipose tissues through barrier permeability - similar to the reported role of SAMs [134][166]. Furthermore, in obesity this perineurial cell barrier may protect adipose tissue sympathetic neurons from adipocytes, which are known to upregulate production of pro-inflammatory cytokines in the case of obesity[157][158]. A reduction in adipose tissue sympathetic innervation, or sympathetic neuropathy, is observed in the obese state [1]. This observation could be explained by a loss in perineurial barrier integrity and increased exposure of sympathetic neurons to adipocyte-derived pro-inflammatory signals. Furthermore, it is currently unclear whether the immune cell signalling capacity or frequency of LepR⁺ SAPCs are altered in obesity. The comparison of SAPCs in the lean versus obese state would aid our understanding of their proposed function in adipose tissue and sympathetic nerve homeostasis.

Although indirect, reports of LepR⁺ sensory neurons within adipose tissue exist [239]. Following injection of a fluorescent retrograde tracer into the subcutaneous WAT of Siberian hamsters, Murphy et al. show fluorescent tracer⁺LepR⁺ sensory neurons within the DRG, indicating the presence of LepR⁺ sensory neurons which originate in the scWAT [239]. Here, I observe that LepR^{eYFP+} cells exclusively surround bundles of axons within mouse adipose tissue (Figure 3.2F), and was, therefore, able to exclude the presence of any LepR⁺ neurons within the brown and subcutaneous white adipose tissue axon bundles imaged. One explanation for this discrepancy could be the assessment of different species (Siberian hamster versus mouse) in the search for LepR⁺ adipose tissue neurons. Furthermore, as it is only possible to dissect larger nerve bundles from scWAT and BAT, I haven't excluded the presence of LepR⁺ neurons from smaller adipose tissue nerve bundles. Although widely used in neuroscience, one significant consideration with retrograde tracing studies is the specificity of the labelling. Diffusion and non-specific transmission of retrograde tracers provide the possibility of false-positive labelling of

neurons in retrograde tracing experiments. The best way to definitively prove the presence or absence of *LepR*⁺ sensory neurons within adipose tissues would be to utilise tissue clearing and whole-organ imaging techniques [95], which would also provide an insight into the possible presence of other *LepR*⁺ cells within adipose tissues.

Originally perineurial cells were named based on their anatomical location in relation to bundles of nerve axons. Using confocal and transmission electron microscopy, I show that the *LepR*^{eYFP+} barrier surrounding sympathetic AT neurons is formed of multiple, thin *LepR*⁺, reaffirming their designation as perineurial cells (Figure 3.2D-F, Figure 3.4). Across a range of different species, perineurial cells surrounding peripheral nerve bundles have been described as mesenchymal [245], fibroblastic [246][247], endothelial-like [240] and epithelial [243]. *LepR*⁺ SAPCs associated with sympathetic neurons express a range of mesenchymal and perineurial markers including, fibroblastic marker *PDGFR* α and perineurial cell marker *ITG* β 4, at both the RNA and protein level (Figure 3.3E, Figure 3.5A-C). I propose that *LepR*⁺ SAPCs may represent a specialised fibroblast population surrounding peripheral nerve bundles, which - given their proximity to sympathetic neurons and adipocytes, may serve additional functions in the control of adipose tissue homeostasis.

I show that *LepR*⁺ SAPCs also express a range of immunomodulatory cytokines including *Il33*, and demonstrate that the majority of *PDGFR* α ⁺*ITG* β 4⁺ *LepR*⁺ SAPCs are *IL-33*⁺ by flow cytometry (Figure 3.6F-G). Recent interest in *IL-33* as an immunocytokine required for the maintenance of adipose tissue immune cell maintenance and homeostasis prompted us to focus on the functional role of SAPC-derived *IL-33*, however the expression of additional anti- and pro-inflammatory genes in *eYFP*⁺ cells is likely also relevant to the role of *LepR*⁺ SAPCs in neuronal and adipose tissue homeostasis through immune cell signalling. In fact, the expression of Treg induction factor *Tgfb2* and type-2 'anti-inflammatory' cytokine *Il4*, in addition to macrophage recruitment and survival factors *Il34*, *Ccl2* and *Csf1* further suggest an immunomodulatory role for SAPCs in the maintenance of adipose tissue homeostasis through the control of resident immune

cells. In addition to IL-33, I provide additional cytokines which could be explored in relation to the immunomodulatory role of SAPCs in adipose tissue homeostasis and obesity.

Spallanzani et al. and Mahlaköiv et al. simultaneously described a number of heterogeneous IL-33-producing mesenchymal populations within visceral WAT [207][217]. Primarily, they conclude that mesenchymal stromal cells within visceral WAT are the major source of IL-33 within this tissue, with mesothelial cells also contributing to IL-33 alarmin production in defence of infection via the peritoneal cavity [207][217]. I find that LepR⁺ SAPCs detected in sympathetic ganglia, scWAT and BAT sympathetic axon bundles are transcriptionally distinct from the reported IL-33 producing mesenchymal stromal cells described in the visceral WAT (vMSCs) [207][217], as vMSCs do not express perineurial cell markers, nor do they express *Lepr* (Figure 3.7). These differences in IL-33-producing cells may relate to heterogeneity between adipose tissue depots, but are likely also due to differences in cell isolation protocols between studies. Whilst Spallanzani et al. and Mahlaköiv et al. generate a stromovascular fraction from whole adipose tissues using collagenase II enzymatic digestion, whereas I find that LepR⁺ SAPC isolation requires a stronger digestion - using both collagenase II and hyaluronidase IV-S on perfused and dissected sympathetic axon bundles. We, therefore, propose that LepR⁺ SAPCs are likely an IL-33 source distinct from those reported in scWAT and BAT by Spallanzani et al. and Mahlaköiv et al. [207][217].

Sensory neuron-associated IL-33⁺ stromal cells have also recently been reported in mouse muscle [207][226][227]. In fact, Wang et al. [226] show that these PDGFR α ⁺ SCA-1⁺ cells proximal to sensory neurons express transcripts encoding the two subunits of the CGRP receptor CALCRL and RAMP-1, and are able to increase IL-33 production in response to CGRP muscle injection [226]. Direct comparison of the transcriptional profile of LepR⁺ SAPCs and sensory neuron-associated stromal cells in muscle revealed a lack of *Lepr* expression, and an absence of *Lepr* and *Il33* co-expression, indicating that LepR⁺ SAPCs are anatomically and transcriptionally distinct from IL-33-producing sensory neuron-associated muscle stromal cells [226]. I propose that *Lepr* expression of

SAPCs in adipose tissue, but not sensory neuron-associated stromal cells in muscle may reflect a tissue-specific role of SAPCs in adipose tissue, given that white adipocytes are the primary producers of the leptin hormone. To the best of our knowledge, perineurial cells have not previously been described as *LepR⁺* within the periphery.

Importantly, I also confirm the presence of *LEPR⁺IL33⁺* cells within human sympathetic ganglia, corroborating a potential immunomodulatory role of these sympathetic associated cells in humans. However, I have not yet been able to determine where these cells are located within human sympathetic tissues, nor provide information on the morphology of these *LEPR⁺IL33⁺* cells, or proximity to sympathetic neurons. Of note, perineurial cell markers generally are not very well defined, with many species-dependent differences observed. In fact, the study of perineurial cells in species ranging from false killer whale to cats and rats [243] has likely complicated the definition of perineurial cell identity, due to the apparent lack of inter-species perineurial cell marker conservation [244]. Primarily, perineurial cells are defined anatomically, surrounding bundles of axons, and therefore the anatomical location of these *LEPR⁺IL33⁺* cells within human sympathetic ganglia is fundamental to their definitive naming as perineurial cells. Moreover, most perineurial cell studies have been performed on the sciatic nerve, as the largest and therefore most accessible peripheral nerve, where - in mice - one fascicle measures approximately 300 μ m in diameter. In comparison, mouse sympathetic axon bundles in adipose tissues measure 50-100 μ m in diameter (Figure 3.2D-F, Figure 3.5D, Figure 3.6C-D) - around 6x smaller. For this reason, coupled with reports that perineurial cell barrier cell composition and thickness correlates with fascicle size, perineurial cells of the sciatic nerve are not the best comparator for AT sympathetic axon bundles - hence our comparison with muscle axon bundles which are more similar in size, with a diameter of approximately 20-100 μ m [226].

The close proximity of *LepR⁺* SAPCs to neurons prompted us to assess the presence of neurotransmitter receptor expression within this cell barrier, where I found expression of the norepinephrine (NE) receptor *ADR β 2*, and to a lesser degree *Adra2c* (Figure 3.10). Upon stimulation sympathetic neurons release NE, which closes the neuroendocrine

loop of leptin and results in energy expenditure through adipose tissue thermogenesis and lipolysis [5]. Due to a lack of functional antibodies, I have been unable to confirm the presence of $ADR\beta 2$ or $ADR\alpha 2C$ at a protein level, however the robust expression of *Adrb2* in our bulk RNA-sequencing data indicates the presence of this NE receptor. The expression of NE receptors indicates that $LepR^+$ SAPCs within adipose tissues may have the capacity to sense both sympathetic drive and leptin, and respond accordingly to changes through the modulation of adipose tissue-resident immune cells proximal to innervating axon bundles.

3.4 Updated hypothesis

I show that SAPCs surrounding axon bundles in both subcutaneous white and brown adipose tissues express the signalling isoform of leptin receptor *Lepr*, noradrenergic receptor *Adrb2*, and immunomodulatory cytokines including *Il33* (Figure 3.11). Therefore, these cells have the capacity to sense the adipose tissue mass and sympathetic drive through leptin and NE levels, respectively; and protect adipose tissue homeostasis through the modulation of adipose tissue resident immune cells and inflammatory status.

Taken together, I hypothesise that $LepR^+$ SAPCs are able to sense leptin and/or sympathetic activity, and maintain adipose tissue homeostasis through the IL-33-dependent regulation of tissue-resident immune cells.

3.5 Updated aims

- Assess the role of SAPC-derived IL-33 on adipose tissue immune milieu
- Determine whether SAPC-derived IL-33 influences adipose tissue function

- Investigate whether SAPC-derived IL-33 is regulated by the sympathetic nervous system through ADR β 2 signalling

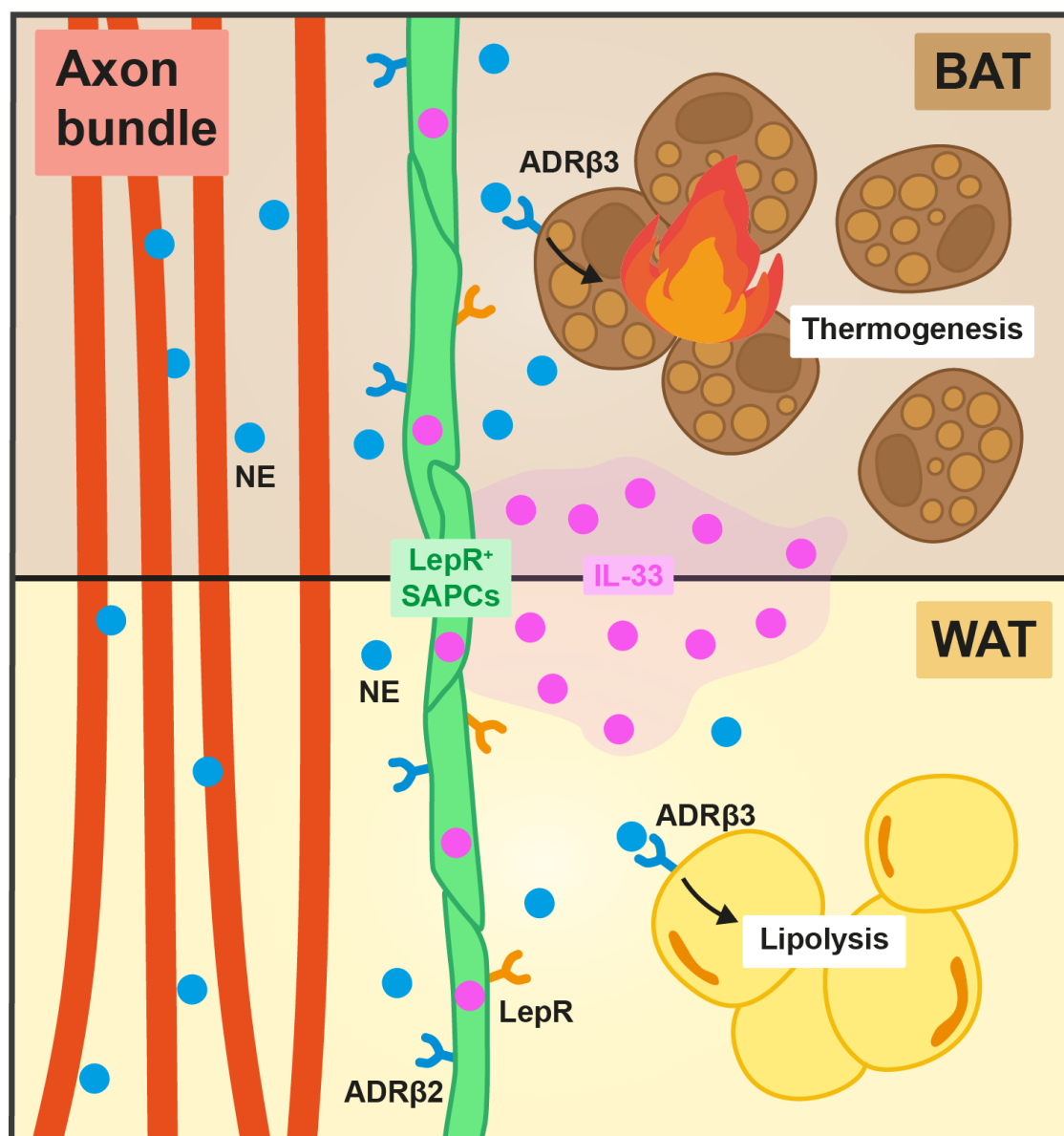


Figure 3.11: LepR⁺ Sympathetic Associated Perineurial Cells form a barrier between sympathetic neurons and adipose tissues

Sympathetic Associated Perineurial Cells (SAPCs, green) form a barrier between sympathetic neurons and adipose tissues. I show that SAPCs express the full length signalling ObRb isoform of *Lepr*, norepinephrine receptor *Adrb2* and immunomodulatory cytokines including *Il33*. I hypothesise that SAPCs orchestrate adipose tissue homeostasis through the modulation of IL-33-dependent immune cells, in response to sympathetic drive or leptin.

Chapter 4

Sympathetic Associated Perineurial Cell-derived IL-33 in adipose homeostasis

Contents

4.1	Introduction	100
4.1.1	IL-33 signalling	100
4.1.2	IL-33 producing stromal cells	103
4.1.3	Control of brown adipose tissue thermogenesis	105
4.1.4	Immune mediated thermogenesis	106
4.2	Results	109
4.2.1	<i>Lepr</i> and <i>Il33</i> co-expression is not observed in the hypothalamus, or peripheral organs of the <i>Tabula Muris</i> database	109
4.2.2	Body weight and adipose tissue weights are unaltered in the absence of SAPC-derived IL-33	110
4.2.3	LepR ^{IL33cKO} mice have an altered brown adipose tissue immune cell milieu	110
4.2.4	Unchallenged LepR ^{IL33cKO} mice have comparable metabolism to IL33 ^{fl/fl} controls	118
4.2.5	Production of IL-33 by LepR ⁺ SAPCs is protective against diet-induced obesity	118
4.2.6	Cold challenge optimisation	119
4.2.7	Adaptive BAT thermogenesis is impaired in cold challenged LepR ^{IL33cKO} mice	124
4.2.8	The onset of metabolic adaptation to fasting is delayed in LepR ^{IL33cKO} BAT	124
4.2.9	Fasted LepR ^{IL33cKO} BAT is unresponsive to leptin-induced stimulation	126
4.2.10	Loss of <i>Adrb2</i> signalling in SAPCs induces obesity, independently of food intake	126
4.2.11	IL-33 production by SAPCs is altered in the absence of ADR β 2 signalling	130
4.3	Discussion	133

4.1 Introduction

Adipose tissue (AT)-resident immune cells have been extensively implicated in obesity, which is closely linked with chronic, low-grade inflammation of the adipose tissue [1]. This is reflected in an increase of pro-inflammatory adipose tissue macrophages and a concomitant reduction in anti-inflammatory immune cells, including regulatory T cells (Tregs), ILC2 cells and eosinophils [128][134][145][139][140][166][252]. Here, I aim to investigate the role of key type 2 cytokine IL-33 derived from LepR⁺ SAPCs, in the context of adipose tissue homeostasis and obesity.

4.1.1 IL-33 signalling

IL-33 is a pleiotropic member of the IL-1 cytokine super family [253], which can act as both a pro- and anti-inflammatory cytokine. IL-33 is constitutively found in the nucleus of a range of non-haematopoietic barrier cells including endothelial, epithelial and fibroblast-like stromal cells [207][217]. Based on the high levels of IL-33 at steady state and release of IL-33 following cell damage or mechanical injury, Moussin et al. were the first to describe IL-33 as an endogenous alarmin, which alerts the immune system to cellular or tissue damage [254][255][256]. Accordingly, ischemia-reperfusion injury, mechanical injury and pollen exposure all transiently induced IL-33 release in challenged tissues, with detected IL-33 levels returning to baseline levels within 24 hours [257][258][259][260][261]. Induction of cell necrosis through hydrogen peroxide or streptolysin O treatment, but not apoptosis also resulted in increased extracellular IL-33 levels [262]. Importantly, sub-lethal cell scraping used to induce endothelial cell mechanical stress sufficiently induced an increase in extracellular IL-33, indicating that cell death is not required for IL-33 release and that cell stress may be sufficient [255]. Corroborating this study, Kakkar et al. [263] show *in vitro* that mechanical stress results in the release of IL-33 from mouse fibroblasts. They identify that nuclear-cytoplasmic

translocation of IL-33 is dependent on an in-tact microtubule network, and is ATP-dependent [263]. The exact mechanism of release remains unclear [227], however overwhelming evidence shows that cell damage and tissue injury are crucial for IL-33 release.

In addition, release of IL-33 was detected in the intraperitoneal cavity following IP infection of parasites *Nippostrongylus brasiliensis* and *Toxoplasma gondii* [217][264]. In fact, sites of parasite replication within the peritoneum inversely correlated with the presence of IL-33⁺ cells, highlighting a role for IL-33-immune cell signalling in parasite eradication [264]. Similarly, with continuous exposure of the airway to allergens, single nucleotide polymorphisms (SNPs) in *Il33* and its cognate receptor *St2* have been heavily implicated in exacerbation of type 2 allergic inflammation and asthma [265]. As one of the most highly identified genes containing SNPs in genome-wide association studies of asthma, IL-33 expression levels have been heavily correlated with increased eosinophil counts and over-reactive responses to allergen or viral exposure [266][267][268]. Accordingly, a number of the pathogenic SNPs found in asthma which have been linked with eosinophilia lie within enhancers or the promoter of the *Il33* gene, and result in increased IL-33 expression [269][268]. As an alarmin, the purpose of IL-33 is to alert the immune system in response to insults such as allergens and infections. In cases of helminth infection or allergy response in mucosal tissues IL-33 acts as a danger signal, stimulating resident mast, T-helper 2 (Th2) and NK cells [255].

IL-33 has been described as pleiotropic cytokine with a pro- and anti-inflammatory effects. This, coupled with its constitutive expression across tissues, means that it must be tightly regulated to avoid damaging effects. Firstly, IL-33 localises to the nucleus through the N-terminal nuclear localisation sequence, deletion of which results in non-resolving inflammation and early lethality in mice [270]. Nuclear localisation prevents the release of IL-33 from cells, and therefore reduces the recruitment of type-2 immune cells. Secondly, the full length, signalling-capable IL-33 protein is cleaved and inactivated by caspase-3 and caspase-7 during apoptosis [255][262][271], a programmed cell death

which does not cause inflammation, nor harm to tissues. Cleavage of IL-33 during apoptosis prevents inappropriate immune cell activation and subsequent inflammation in instances of programmed cell death. In fact, through the inactivation of IL-33, caspase-7 has also been implicated in the resolution of type-2 allergic inflammation [272][273]. Furthermore, oxidation of IL-33 cysteine residues following extracellular release results in a conformational change to a biologically inactive form of IL-33 which is incapable of signalling through ST2 [274]. In addition, bioactive, extracellular IL-33 released from cells following insult is sequestered by a soluble decoy form of ST2: soluble ST2 (sST2). sST2 is an alternatively spliced variant of the IL-33 receptor ST2 which lacks the transmembrane domain of the fully active long isoform of ST2 (ST2L), and is therefore unable to induce ST2-dependent target cell responses. Interestingly, the intestinal nematode *Heligmosomoides polygyrus bakeri* has been shown to produce the HpARI protein which modulates IL-33-ST2 signalling in infection, allowing for immune escape [275]. Of particular relevance to IL-33 and the prevention of adipose tissue inflammation, Colomb et al. identify one such isoform of immunomodulatory protein HpARI3, able to stabilise IL-33 and subsequently enhance the type 2 cytokine response during infection - which could be harnessed therapeutically in obesity [275].

ST2 is the sole receptor for IL-33 [270][276], and was originally identified as a serum-inducible receptor produced by stimulated murine fibroblasts [277]. ST2 exists in two isoforms: membrane-bound, ST2L and sST2 [278][279]. The soluble sST2 splice variant acts as a decoy receptor to limit IL-33 mediated signalling [280][281]. sST2 expression in the BAT, vWAT and scWAT is increased in obesity, where it significantly impairs the recruitment of Tregs and ILC2 cells to the vWAT [281]. sST2 is released by adipocytes in inflamed, obese visceral white adipose tissue [281]. vWAT inflammation induced by obesity further contributes towards adipose tissue immune cell regulation through the increase in sST2 release by adipocytes, resulting in impaired recruitment and maintenance of Tregs and ILC2 cells [281][139], which can be rescued with administration of exogenous IL-33 [200][217][227]. However, conflicting reports of the effect of obesity on IL-33 levels exist. Most widely studied in the vWAT, *Il33* expression in stromal cells

is decreased following short term (3 days) HFD [217].

The action of IL-33 on immune cells is very dependent on both the type of insult and the tissue in which IL-33 release has been stimulated. Importantly, IL-33-responsive immune cells express both ST2L and the shared IL-1 family co-receptor IL-1 receptor associated protein (IL1RAP). Upon IL-33 binding, ST2 and IL1RAP signalling within immune cells stimulates the canonical MyD88/ Nuclear factor kappa β (NF- κ B) signalling pathway [255] to induce the production of pro-inflammatory cytokines. In adipose tissues, IL-33 has been described as a significant regulator of 'anti-inflammatory' ILC2 cells, eosinophil and regulatory T cell recruitment and maintenance [207][217], making it an interesting target in the prevention of obesity-induced adipose tissue inflammation.

4.1.2 IL-33 producing stromal cells

Two recent complimentary studies have described the exclusive production of IL-33 by mesenchymal cells in WAT. Both Spallanzani et al. and Mahlaköiv et al. describe heterogeneous IL-33-producing PDGFR α ⁺PDPN⁺ stromal cells within vWAT [207][217]. These studies, involving either a total knockout of *Il33* [217] or a PDGFR α -dependent conditional knockout of *Il33* (PDGFR α ^{IL33cKO}) [207] have been crucial in understanding the role of IL-33 in adipose tissue, but have focussed on WAT and not BAT. A global knockout of *Il33* resulted in increased weight gain in the absence of metabolic challenge [217], whereas a PDGFR α ^{CRE} driven conditional deletion of *Il33* was not sufficient to induce obesity in the unchallenged state [207]. Furthermore, although a global knockout of *Il33* resulted comparable ILC2 recruitment to vWAT, a reduction in the frequency of eosinophils was observed, likely due to the reduction in key ILC2-derived type 2 cytokines IL-5 and IL-13 [217]. This corroborates the necessity of tonic IL-33 signalling for ILC2 function described by Goldberg et al., who show that dysregulation of IL-33 underlies loss of ILC2 survival and function with age [180]. Moreover, the frequency of pro-inflammatory macrophages in the vWAT of IL-33 knockout mice was increased concomitant with a reduction in type 2 immune cells - resulting in a pro-inflammatory

AT microenvironment and solidifying the role of IL-33 in preventing adipose tissue inflammation. In contrast, PDGFR α ^{CRE} mediated loss of IL-33 resulted in a reduction of ST2⁺ vWAT Tregs, despite no significant differences in the frequency of CD25⁺ Tregs [207].

Finally, the importance of IL-33-producing stromal cells for immune cell recruitment and maintenance has also been described in muscle. In fact, Kuswanto et al. [227] and Wang et al. [226] have recently reported an IL-33⁺ subset of stromal cells associated with both large and small nerve bundles in muscle. Collectively, they show the presence of IL-33⁺GLUT1⁺ mesenchymal stromal cells lie in close proximity to Neurofilament-200⁺ (NF200⁺) sensory neurons within muscle [227]. Following muscle injury induced by *Naja mossambica* cardiotoxin, Kuswanto et al. show that *Il33* expression is significantly increased around 12 hours after insult, resulting in an increase in the frequency of Tregs [227]. In addition, Wang et al. show that in fact IL-33⁺ mesenchymal stromal cells lying in close proximity to sensory neurons have a close association with tissue resident muscle Tregs, and express both the *Calcr1* and *Ramp1* subunits of neurotransmitter Calcitonin Gene-Related Peptide (CGRP) [226]. Following intraperitoneal injection of CGRP, Wang et observed an increase in total muscle IL-33 protein as measured by ELISA, with the expected increase in muscle Tregs despite no differences in splenic Tregs [226]. Importantly, no differences were observed in IL-33⁺ mesenchymal stromal cell viability following CGRP IP injection, adding further weight to the possibility IL-33 is released in response to cell stress and damage but not necessarily cell death [226][255]. Whilst these two studies did not investigate the possible presence of non-Treg IL-33-responsive immune cells, they collectively make a very strong case for neuron associated IL-33⁺ mesenchymal stromal cells in muscle repair following cardiotoxin injury, the capacity of which decreases with age [226][227].

4.1.3 Control of brown adipose tissue thermogenesis

Mechanistically, the effects of leptin on energy expenditure were long thought to be directly attributable to UCP1-dependent BAT thermogenesis [282], and 'beigeing': the uncoupling of respiration in white adipocytes resulting in a phenotype similar to that of brown adipocytes [27]. In particular, neuronal activation of ObRb⁺ neurons in the dorsomedial nucleus of the hypothalamus (DMN)/ dorsal hypothalamic area (DHA) using Designer receptors exclusively activated by designer drugs (DREADDS) drives energy expenditure via BAT thermogenesis and locomotor activity, independently of food intake [283] - firmly implicating the neuroendocrine loop of leptin action in the modulation of thermogenesis.

BAT thermogenesis is positively regulated by sympathetic neuron derived NE, with adrenergic β 3 receptor signalling the predominant regulator in rodent mature brown adipocytes [284]. Adrenergic β 3 receptors coupled with G_s proteins mediate activation of downstream adenylyl cyclase, cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) signalling to induce *Ucp1* expression, alongside stimulating lipolysis [284][285][286]. In particular, NE-stimulated thermogenesis in brown adipocytes is entirely dependent on *Ucp1* expression, as no thermogenesis is observed in the BAT of NE-treated *Ucp1*-ablated mice [287]. In fact, *Ucp1* expression itself is directly regulated by NE signalling [288][289]. UCP1 protein level is largely regulated by transcription [290]. Given that UCP1 protein and not *Ucp1* mRNA produces heat, *Ucp1* expression cannot be used to measure thermogenesis, only the thermogenic capacity of a tissue [291]. Furthermore, the increased frequency of thermogenic adipocytes within BAT, also referred to as BAT recruitment, is positively regulated by NE and enhances thermogenic capacity. Such *in vitro* stimulation results in the proliferation of UCP1⁻ committed pre-adipocytes isolated from the BAT stromovascular fraction, through adrenergic β 1 receptor signalling [292]. However, following differentiation of pre-adipocytes into mature brown adipocytes NE stimulation results in further differentiation, marked by an increase in mitochondrial biogenesis and upregulation of thermogenesis-enhancing genes, including *Pgc1a* [293][294][295]. Collectively sympathetic neuron-derived NE is

a major regulator of thermogenesis through the upregulation of UCP1 protein, which simultaneously enhances adipocyte lipolysis.

Furthermore, thermogenesis of brown adipocytes can be induced following the addition of free fatty acids to *ex vivo* brown adipocyte cultures [296][297] - a product of lipolysis which itself is induced by NE stimulation [298]. Similarly to NE-stimulated thermogenesis, fatty acid-induced thermogenesis is also UCP1-dependent [287], indicating that lipolysis itself is sufficient to initiate thermogenesis in UCP1⁺ brown adipocytes. In fact, free fatty acids - generated as a result of triglyceride lipolysis - are translocated to the mitochondria of brown adipocytes, where they are catabolised and release substrates used in the citric acid cycle, upstream of the heat generating, UCP1-dependent uncoupling of mitochondrial membrane proton gradient and ATP generation. Moreover, it is generally accepted that the function of mitochondrial proton transporter UCP1 is dependent on free fatty acids and their derivatives, although the exact mechanism is currently a mystery [299]. It is clear, however, that a supply of fatty acids and their derivatives is crucial to UCP1-dependent thermogenesis, indirectly implicating additional free fatty acid regulatory players in the induction of thermogenesis - including Peroxisome proliferator activated receptor gamma (PPARG), PPARG coactivator 1 α (PGC1 α), Elovl fatty acid elongase 3 (ELOVL3), Cell death inducing DFFA like effector A (CIDEA), Type II Iodothyronine deiodinase (DIO2) and Lipoprotein lipase (LPL); along with PRDM16 which itself is a key regulator of brown adipocyte differentiation and identity [15].

4.1.4 Immune mediated thermogenesis

Previous studies have implicated specialised type 2 tissue-resident immune cells in AT thermogenesis. In particular, inflammation has been strongly correlated with impaired BAT thermogenesis [147][148][149]. Given that IL-33 is a key mediator of 'anti-inflammatory' type 2 immune cells, Brestoff et al. [135] investigated the role of IL-33 on adipose tissue thermogenesis. They found that a global loss of IL-33 resulted in an absence of scWAT beige adipocytes, indicating a role for IL-33 on the induction of white

adipose tissue beiging [135]. In fact, daily intraperitoneal IL-33 administration alone for a period of 7 days was sufficient to increase whole-organism oxygen consumption (VO_2) and *Ucp1* expression in both scWAT and vWAT, but not BAT [135]. The implication that IL-33 treatment positively regulates beiging in WAT based on increased *Ucp1* expression likely overlooks the comparatively high baseline expression of *Ucp1* already observed in BAT [135]. In fact, Brestoff et al. [135] go on to describe that the IL-33-mediated activation of ILC2 cells results in the production of methionine-enkephalin (met-enk), which is sufficient to activate UCP1-mediated thermogenesis in beige - but not brown - adipocytes directly. [135]. However, the extent to which met-enk-induced beiging is functionally significant remains unclear, as the effect of met-enk-induced beiging on energy expenditure was not assessed during this study [135]. The effects of met-enk on scWAT were independent of IL-4 or IL-13, and also independent of macrophage and eosinophil frequency [135]. Both met-enk and its cognate opioid receptor are expressed in scWAT, but are present at a significantly lower amount in BAT - indicating a direct ILC2-dependent induction of beiging specific to scWAT. However, the direct action of opioid peptides such as met-enk on any type of adipose tissue (BAT or WAT) remains incompletely understood.

When delivered systemically, opioids can induce respiratory suppression and malaise, which in turn is concomitant with anorexia and weight loss; the latter of which is also associated with beiging. In addition, Lee et al. [137] provide an alternative, indirect mechanism of ILC2 action on adipocyte thermogenesis. They show that treatment of thermoneutral mice with IL-33 enhances the beiging of white adipose tissues through the IL-4 mediated proliferation and expansion of beige adipocyte progenitors, increasing WAT thermogenic capacity [137]. Although it is unclear in this study whether the IL-4 prompting adipocyte beiging is derived from ILC2s or eosinophils, collectively it is clear that IL-33 stimulation of type 2 immune cells positively regulates thermogenic capacity through the induction of beige adipocytes [135][137]. Furthermore, altered ILC2 function has been implicated in the loss of cold tolerance associated with age [180]. Goldberg et al. [180] report an almost complete loss of vWAT-resident ILC2 cells

in 18-24 month old aged mice, which have decreased cold tolerance compared with 8-12 week old controls. Whilst IL-33 administration enhanced the proliferation and rescued the frequency of ILC2s in aged vWAT, this treatment worsened cold tolerance over a 50 hour cold challenge period, indicating intrinsic dysfunction of aged ILC2 cells even with IL-33 mediated stimulation and expansion [180]. Finally, Goldberg et al. [180] show that the adoptive transfer of expanded IL-5 deficient ILC2s derived from adult vWAT protected aged mice against cold challenge, despite recipient mice still having reduced core body temperatures in the absence of ILC2-derived IL-5. Whilst this observation indicates that IL-5 is not required for cold challenge survival, it does indicate that IL-5 - and downstream AT eosinophils - may be implicated in the induction of thermogenesis, given the reduction in core temperature in the absence of IL-5 [180]. Although age related changes to adipose tissue are distinct from obesity-related dysregulation, this study provides an insight into the regulation of ILC2s, and downstream eosinophils, in AT thermogenesis. Furthermore, studies to date have focussed on WAT ILC2 cells and beigeing - owing to the proposed mechanism of met-enk in WAT. However, this effect was independent of eosinophil frequency in WAT [135], and the suggestion that IL-5-eosinophil signalling may be implicated in the initiation of thermogenesis may be explained by an alternative regulatory mechanism in anatomically, morphologically and functionally distinct brown adipose tissue.

In addition to ILC2s and eosinophils, Medrikova et al. [300] identified a $\text{PPAR}\gamma^+$ subset of specialised Tregs in the brown adipose tissue of mice. Similarly to vWAT Tregs reported by Feurer et al. [139], $\text{PPAR}\gamma^+$ Tregs in brown adipose tissue have distinct transcriptional profiles to 'peripheral' Tregs isolated from secondary lymphoid tissues [300]. Although a direct comparison of vWAT and BAT Tregs was not performed, it is likely that these anatomically distinct $\text{PPAR}\gamma$ AT Treg subsets will harbour tissue-specific identities and functions. Medrikova et al. [300] found that depleting BAT Tregs through FOXP3-specific diphtheria toxin was sufficient to increase BAT inflammation, and reduce cold challenge-induced BAT thermogenic capacity through the reduction in *Ucp1* expression [300]. Although not a specific BAT Treg depletion model, it is clear

from this research that expression of BAT inflammatory marker expression is increased in the absence of BAT Tregs [300]. Moreover, the impaired expression of BAT thermogenic genes following cold challenge-induced BAT thermogenesis indicates a role for BAT Tregs, whether direct or indirect. Comparison of room temperature and cold-challenged BAT Tregs revealed differential expression of only a few genes, indicating that the primary role of BAT Tregs may be the maintenance of an anti-inflammatory adipose tissue environment to enhance BAT function, as opposed to a direct response to cold challenge-induced stimulation of BAT thermogenesis.

A range of evidence exists describing a role for type 2 immune cells in adipose tissue thermogenesis - be it direct or indirect. Although mechanistically detail is still lacking, from this work it is clear that mechanism may not be conserved between anatomically distinct adipose tissue depots.

4.2 Results

4.2.1 *Lepr* and *Il33* co-expression is not observed in the hypothalamus, or peripheral organs of the *Tabula Muris* database

To exclude the possibility of off-target effects for a LepR^{CRE}-mediated conditional IL-33 knockout mouse model, I first assessed whether any other cell in wildtype mice is double positive for LepR and IL-33. I analysed public single-cell RNA sequencing data from the Arcuate Nucleus of the hypothalamus (ARC) [224] and found that cells in the ARC do not co-express *Lepr* and *Il33* (Figure 4.1A), which I confirmed using immunofluorescent imaging (Figure 4.1B). I next assessed *Lepr* and *Il33* co-expression in peripheral organs using the Tabula Muris single-cell RNA sequencing database [225], which revealed that the kidney, muscle, and trachea were the most likely to co-express *Lepr* and *Il33*, with correlation coefficients between 0.1–0.4 (Figure 4.1C). However, immunofluorescent imaging excluded the possibility of LepR⁺IL-33⁺ cells in these organs (Figure 4.1D). Using this method, I also excluded LepR and IL33 co-expression in the aorta, heart, and liver, with co-expression correlation coefficients of 0.08-0.1 (Figure 4.1D). The

diaphragm, pancreas, tongue, bone marrow, skin, colon, thymus, and bladder all had very low *Lepr*/*Il33* correlation coefficients (below 0.05) and hence $\text{LepR}^+\text{IL-33}^+$ cells in these organs can be safely excluded (Figure 4.1B). We, therefore, confirm that $\text{LepR}^+\text{IL-33}^+$ expressing cells are not present in the hypothalamus, nor in the peripheral tissues listed in the *Tabula Muris* database.

4.2.2 Body weight and adipose tissue weights are unaltered in the absence of SAPC-derived IL-33

To functionally assess the role of IL-33 on SAPC function, I crossed LepR^{CRE} mice with $\text{IL33}^{\text{fl/fl}}$ mice to perform a conditional knockout of IL-33 only in LepR^+ SAPCs (herein named $\text{LepR}^{\text{IL33cKO}}$ mice). Having validated that, other than the SAPCs, no cells in the hypothalamus or organs of the *Tabula Muris* co-express *Lepr* and *Il33*, I next verified using IF that IL-33 is lost in SAPCs in $\text{LepR}^{\text{IL33cKO}}$ AT axon bundles (Figure 4.3A-B). Lean $\text{LepR}^{\text{IL33cKO}}$ mice fed a normal diet (ND) have comparable body weights, adipose tissue weights and food intake to both LepR^{CRE} and $\text{IL33}^{\text{fl/fl}}$ controls (Figure 4.2 C-G, Figure 7A-B). Due to the lack of differences in weight and food intake between LepR^{CRE} and $\text{IL33}^{\text{fl/fl}}$ control mice, I can exclude any effects of LepR^{CRE} itself on body weight homeostasis. To reduce the number of mice used experimentally, I opted to compare only $\text{IL33}^{\text{fl/fl}}$ control mice with $\text{LepR}^{\text{IL33cKO}}$ mice moving forward.

4.2.3 $\text{LepR}^{\text{IL33cKO}}$ mice have an altered brown adipose tissue immune cell milieu

Considering the role of IL-33 in the type 2 immune response[180, 207, 217, 280], I next tested whether the adipose tissue immune cell milieu was altered in $\text{LepR}^{\text{IL33cKO}}$ mice. Of note, no differences were observed in total cellularity of the spleen (Figure 4.3A) nor the number of splenic CD45^+ cells (Figure 4.3E) using flow cytometry, indicating $\text{LepR}^{\text{IL33cKO}}$ mice have a comparable 'peripheral' immune system to age- and sex-matched $\text{IL33}^{\text{fl/fl}}$ control mice. In line with the similar adipose tissue weights observed between $\text{IL33}^{\text{fl/fl}}$ and $\text{LepR}^{\text{IL33cKO}}$ mice (Figure 4.2, I observed no significant difference between the total

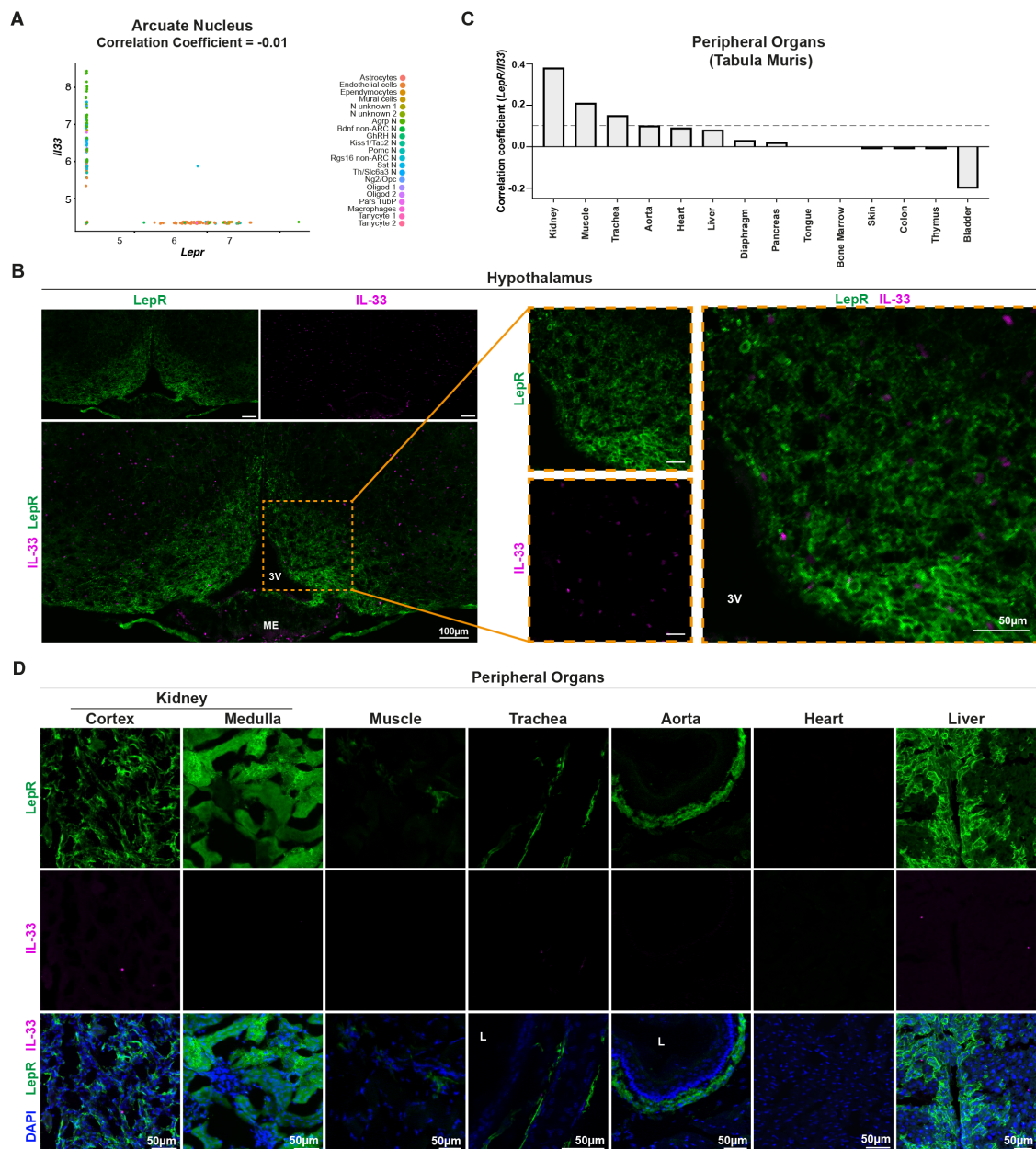


Figure 4.1: *LepR* and *Il33* co-expression is not observed in the Arcuate Nucleus of the hypothalamus, or peripheral organs of the *Tabula Muris* database

A Cells within the Arcuate Nucleus of the hypothalamus (ARC) do not co-express *LepR* and *Il33* (correlation coefficient = -0.01). Analysis of mouse ARC single-cell RNA sequencing dataset (GSE93374) [224]. N = neuron, Oligod = oligodendrocytes, Pars Tub = Pars Tuberalis. **B** Immunofluorescent staining of coronal *LepRAi32* hypothalamus section, maximum intensity projection image 10x. eYFP (green), IL-33 (magenta). Anatomical location of higher magnification image highlighted by orange box. 3V = third ventricle. Scale bar = 50µm. **C** Co-expression of *LepR* and *Il33* in peripheral mouse organs. Smart-Seq2 *Tabula Muris* dataset (GSE109774) [225]. Grey dotted line marks correlation co-efficient of 0.1. Kidney, muscle, and trachea all have a correlation coefficient between 0.4-0.1. **D** *LepR*⁺ *IL-33*⁺ cells were not detected in the kidney (medulla/ cortex), muscle, trachea, aorta, heart, and liver of lean *LepRAi32* mice, 20x maximum intensity projection images. eYFP (green), IL33 (magenta), DAPI (blue). Scale bars = 50µm. L = lumen.

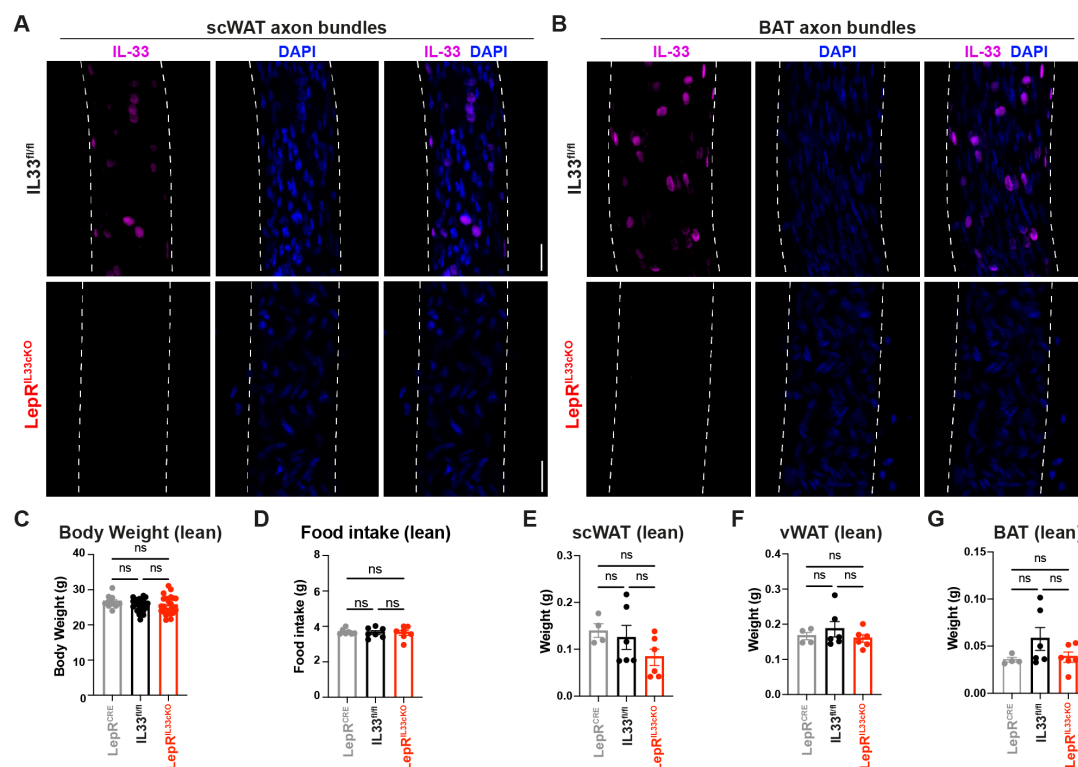


Figure 4.2: Body weight and adipose tissue weights are unaltered in lean, unchallenged $LepR^{IL33cKO}$ mice

A-B IL-33 is lost in the $LepR^{+}$ Sympathetic Associated Perineurial Cells (SAPCs) of $LepR^{IL33cKO}$ subcutaneous (scWAT, **A**) and brown (BAT, **B**) adipose tissue axon bundles. IL33^{fl/fl} and $LepR^{IL33cKO}$ mice, 20x maximum intensity projection images. IL33 (magenta), DAPI (blue). Scale bars = 25 μ m. White dotted lines mark axon bundle edges. **C** Absolute body weight of lean (normal diet-fed) $LepR^{CRE}$, IL33^{fl/fl} and $LepR^{IL33cKO}$ mice. $n=11$ ($LepR^{CRE}$, $n=24$ (IL33^{fl/fl}, $LepR^{IL33cKO}$), 3 independent experiments. **D** Average food intake per mouse per day of normal diet-fed $LepR^{CRE}$, IL33^{fl/fl} and $LepR^{IL33cKO}$ mice $n=7$, 1 independent experiment. **E-G** Comparable adipose tissue weights between lean (normal diet-fed) $LepR^{CRE}$, IL33^{fl/fl} and $LepR^{IL33cKO}$ mice. Inguinal subcutaneous white adipose tissue (scWAT, **E**), visceral white adipose tissue (vWAT, **F**) and interscapular brown adipose tissue (BAT, **G**). $n=4$ ($LepR^{CRE}$), $n=6$ (IL33^{fl/fl}, $LepR^{IL33cKO}$), 2 independent experiments. Data presented as Mean $\pm$ SEM. One-way ANOVA with Tukey's post-hoc correction, ns = non-significant, * $p<0.05$, ** $p<0.01$.

cellularity of scWAT, vWAT and BAT stromovascular fraction (SVF) in unchallenged (lean) IL33^{fl/fl} and LepR^{IL33cKO} mice (Figure 4.3B-D). Moreover, no significant difference was observed in CD45⁺ cell number in subcutaneous or visceral WAT (Figure 4.3F-G). However, in the interscapular BAT CD45⁺ cell frequency was reduced to around half of that observed in IL33^{fl/fl} BAT (Figure 4.3), indicating that SAPC-derived IL-33 is important in BAT immune cell recruitment and maintenance.

I next sought to determine whether the recruitment and maintenance of known IL-33-responsive immune cells – including Tregs, eosinophils and ILC2s – and their downstream effector cells are reliant on SAPC-derived IL-33 in adipose tissues. Whilst comparable frequencies of Tregs were observed in the spleen, scWAT and vWAT of IL33^{fl/fl} and LepR^{IL33cKO} mice (Figure 4.4A-C), I observed a significant decrease in BAT Treg frequency in ND-fed LepR^{IL33cKO} mice (Figure 4.4D). Despite no significant differences in the spleen (Figure 4.4E), eosinophil frequency was significantly decreased in the vWAT and BAT of LepR^{IL33cKO} mice, with a similar but non-significant trend observed in the scWAT (Figure 4.4F-G). Further, I found no significant differences in splenic or scWAT ILC2 frequency between IL33^{fl/fl} and LepR^{IL33cKO} mice (Figure 4.4I-J), but observed an increase in the relative frequency of ILC2 cells within both the vWAT and BAT (Figure 4.4K-L). Finally, no significant differences in splenic or AT macrophage frequency between IL33^{fl/fl} and LepR^{IL33cKO} mice were observed (Figure 4.4M-P). IL-33-responsive immune cell frequencies seem to be most significantly affected in the BAT of LepR^{IL33cKO} mice. In addition to changes in BAT-resident immune cells, using RT-qPCR I observed that the expression of *Il1b* was significantly increased in LepR^{IL33cKO} BAT, indicating some inflammation in ND-fed, lean LepR^{IL33cKO} mice (Figure 4.5A). In summary, I show that the specific loss of IL33 in SAPCs manifests in significant changes to BAT immune cell frequencies and overall BAT inflammatory status.

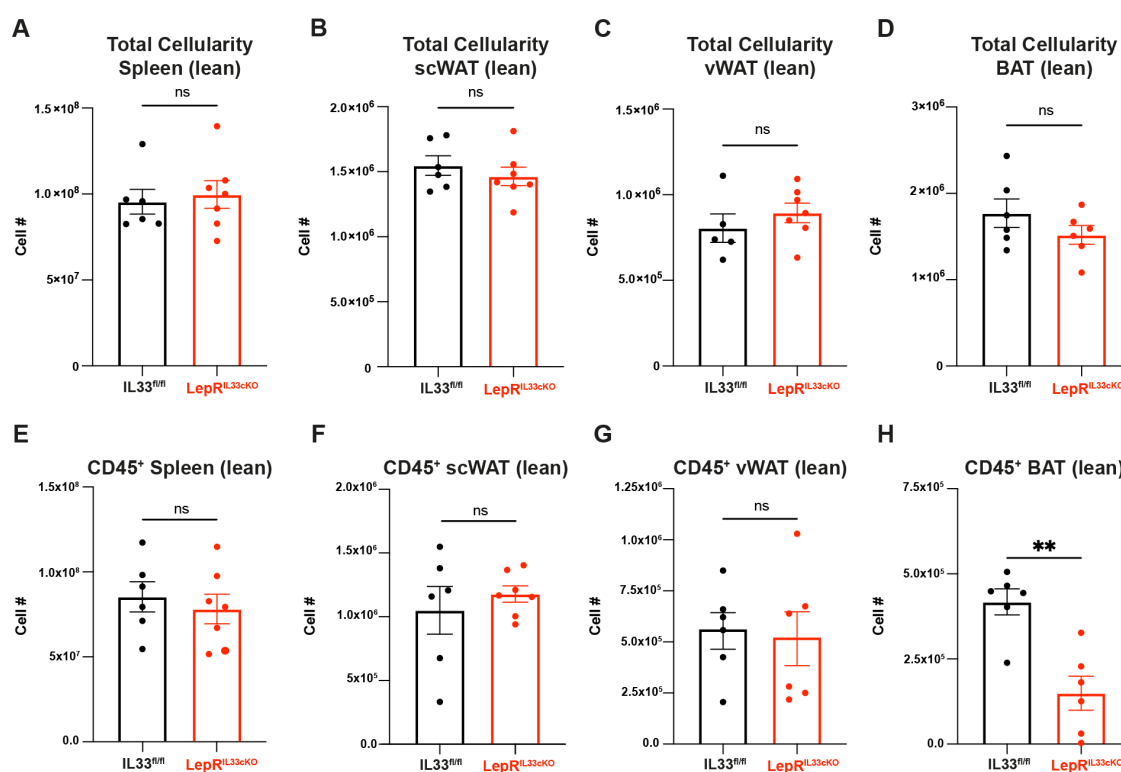


Figure 4.3: Brown adipose tissue CD45⁺ fraction is reduced in lean LepR^{IL33cKO} mice
A-D Total cellularity of Spleen (**A**), scWAT (**B**), vWAT (**C**) and BAT (**D**) of unchallenged (lean) 10 week old male IL33^{fl/fl} and LepR^{IL33cKO} mice, measured by flow cytometry. **E-H** CD45⁺ cell number in the spleen (**E**), scWAT (**F**), vWAT (**G**), and BAT (**H**) of unchallenged (lean) 10 week old male IL33^{fl/fl} and LepR^{IL33cKO} mice. 2 independent experiments, n=6-7. Data presented as Mean ± SEM. Student's t-test, ns = non-significant, **p<0.01. Full gating strategies shown in Figure 8A-C).

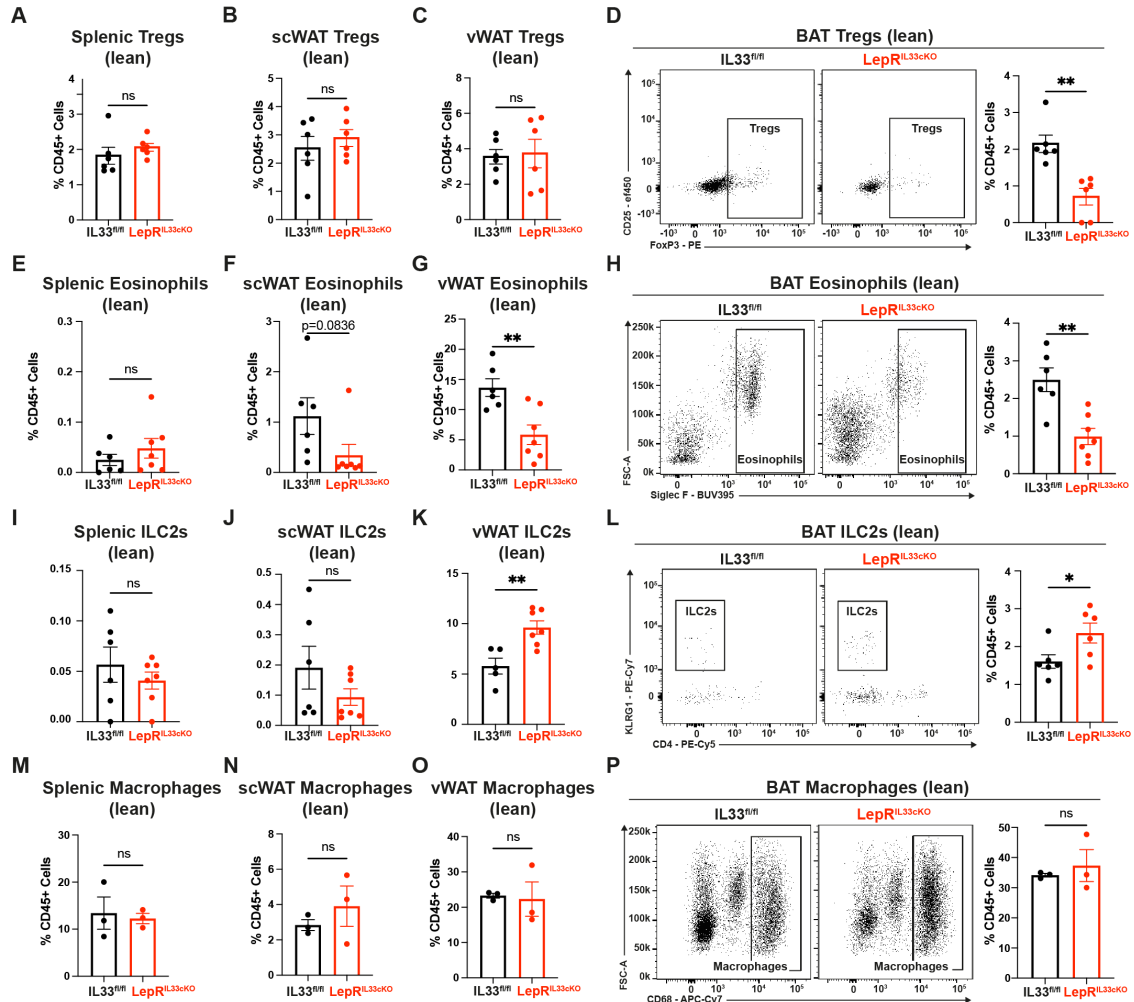


Figure 4.4: $LepR^{IL33cKO}$ mice have impaired BAT Treg and eosinophil recruitment
A-D Treg frequency as a % of $CD45^+$ cells in the spleen (**A**), scWAT (**B**), vWAT (**C**) and BAT (**D**), as measured by flow cytometry. **E-H** eosinophil frequency as a % of $CD45^+$ cells in the spleen (**E**), scWAT (**F**), vWAT (**G**) and BAT (**H**). **I-L** ILC2 frequency as a % of $CD45^+$ cells in the spleen (**I**), scWAT (**J**), vWAT (**K**) and BAT (**L**). **M-P** Macrophage frequency as a % of $CD45^+$ cells in the spleen (**M**), scWAT (**N**), vWAT (**O**) and BAT (**P**). 10 week old male ND-fed $IL33^{fl/fl}$ and $LepR^{IL33cKO}$ mice. **A-L** $n=6-7$, 2 independent experiments. **M-P** $n=3$, 1 independent experiment. Data presented as Mean $\pm$ SEM. Student's t-test, ns = non-significant, * $p < 0.05$, ** $p < 0.01$. Full gating strategies shown in Figure 8A-C).

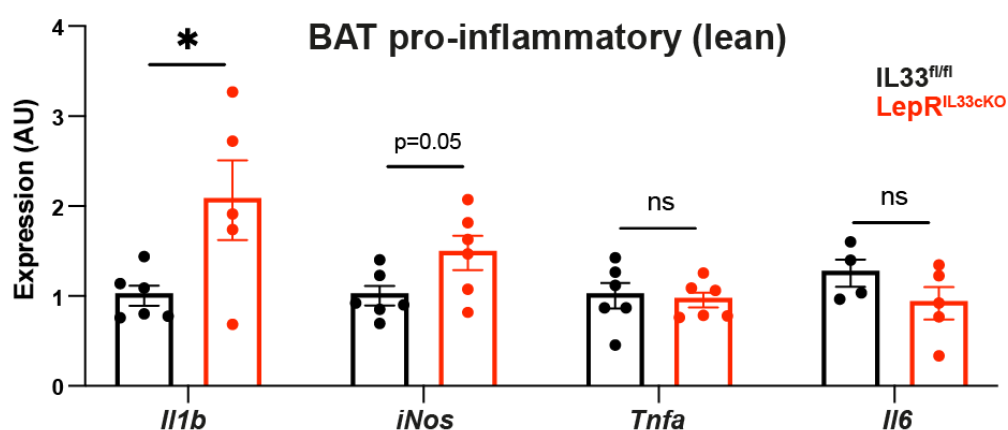
A

Figure 4.5: Loss of SAPC-derived IL33 results in BAT inflammation

A RT-qPCR of whole BAT shows increased inflammation in lean (normal diet-fed) LepR^{IL33cKO} mice compared with IL33^{fl/fl} controls. For each gene, data is presented relative to lean IL33^{fl/fl} BAT expression level (=1). *Il1b* = Interleukin-1 beta, *iNos* = Inducible nitric oxide synthase, *Tnfa* = Tumor necrosis factor alpha, *Il6* = Interleukin-6. Gene expression is normalised to the expression of house keeping gene *Tata box binding protein* (*Tbp*). n=4-6, 2 independent experiments. Data presented as Mean±/− SEM. Student's t-test, ns = non-significant, *p<0.05, **p<0.01

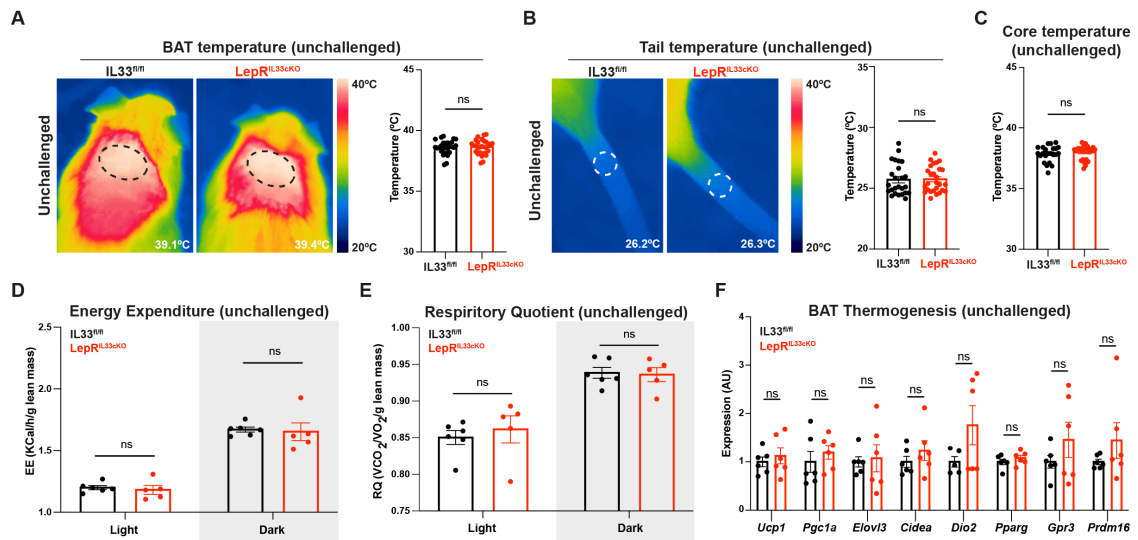


Figure 4.6: Unchallenged $LepR^{IL33cKO}$ mice have a comparable basal metabolism to $IL33^{fl/fl}$ controls

A-B BAT (**A**) and tail (**B**) temperature is comparable in lean, unchallenged $LepR^{IL33cKO}$ and $IL33^{fl/fl}$ mice. (**A-B**) Thermal imaging and quantification of BAT (**A**) and tail temperature (**B**) at room temperature (21°C). Dotted lines on indicate thermal image region from which temperature was measured, bottom right = average temperature in representative image shown. Each data point represents the average BAT (**A**) or tail temperature (**B**) per mouse, calculated from >10 thermal images. Thermal imaging analysis was blinded. $n=25-27$, 4 independent experiments. **C** Core temperature of lean, unchallenged $LepR^{IL33cKO}$ mice is comparable to $IL33^{fl/fl}$ controls at room temperature (21°C). $n=22-24$, 4 independent experiments. **D-E** Metabolic phenotyping of $LepR^{IL33cKO}$ and $IL33^{fl/fl}$ mice, performed by collaborators Iara Fernández-González and Miguel López. Energy Expenditure (EE, **D**) and Respiratory Quotient (RQ, **E**) are comparable in lean $LepR^{IL33cKO}$ and $IL33^{fl/fl}$ mice. Each data point represents an average of EE (**D**) or RQ (**E**) per mouse during either the light or dark period, measured over a 5-day period. EE and RQ normalised to lean mass. $n=5-6$, 1 independent experiment. Time course of EE and RQ shown in Appendix Figure 9. **F** RT-qPCR of whole BAT from lean $IL33^{fl/fl}$ and $LepR^{IL33cKO}$ mice shows no difference in BAT thermogenic gene expression in lean, unchallenged mice at room temperature (21°C). For each gene, data is presented relative to $IL33^{fl/fl}$ expression (=1). *Ucp1* = Uncoupled protein 1, *Pgc1a* = Peroxisome proliferator- activated receptor gamma coactivator 1-alpha, *Elovl3* = Elovl fatty acid elongase 3, *Cidea* = Cell death inducing DFFA like effector A, *Dio2* = Iodothyronine deiodinase 2, *Pparg* = Peroxisome proliferator activated receptor gamma, *Gpr3* = G-protein coupled receptor 3, *Prdm16* = PR/SET domain 16. Gene expression is normalised to the expression of house keeping gene *Tata box binding protein* (*Tbp*). $n=6$, 2 independent experiments. Data presented as Mean $\pm$ SEM. Student's t-test, ns = non-significant.

4.2.4 Unchallenged LepR^{IL33cKO} mice have comparable metabolism to IL33^{fl/fl} controls

BAT is the primary site of non-shivering thermogenesis, a major process of energy expenditure. Previous work implicating Tregs, eosinophils and ILC2s in the induction of BAT thermogenesis prompted us to investigate whether the reduction in the frequency of BAT Tregs and eosinophils observed in LepR^{IL33cKO} mice (Figure 4.4D, H, L) would impact thermogenesis. Thermal imaging and temperature measurements of lean, unchallenged LepR^{IL33cKO} and IL33^{fl/fl} mice revealed no significant differences in BAT, core, or tail temperature (Figure 4.6A-C). Further corroborating this finding, no differences were detected in the energy expenditure and respiratory quotient of lean, unchallenged LepR^{IL33cKO} and IL33^{fl/fl} mice, which underwent metabolic cage phenotyping over a period of 5 days (Figure 4.6D-E, Figure 9A-B). Of note, metabolic phenotyping was performed by collaborators Iara Fernández-González and Miguel López at the University of Santiago de Compostela. Furthermore, RT-qPCR analysis of BAT confirmed that there were no significant differences in the expression of thermogenic genes between lean, unchallenged LepR^{IL33cKO} and IL33^{fl/fl} mice (Figure 4.6F). Overall, BAT thermogenesis is comparable in LepR^{IL33cKO} and IL33^{fl/fl} mice in the unchallenged state.

4.2.5 Production of IL-33 by LepR⁺ SAPCs is protective against diet-induced obesity

Although LepR^{IL33cKO} mice did not develop obesity when fed a normal diet (Figure 4.2, I found that LepR^{IL33cKO} mice metabolically challenged with a high-fat diet (HFD) from 8 weeks old for a period of 12 weeks gained more weight than age-matched, HFD-fed IL33^{fl/fl} controls, independently of cumulative food intake (Figure 4.7A-C). Furthermore, HFD-fed LepR^{IL33cKO} mice had increased scWAT weights than control mice (Figure 4.7D), although no significant difference was observed in vWAT or BAT weights (Figure 4.7E-F). Having identified the BAT as a potential site of immune dysregulation in LepR^{IL33cKO} mice (Figure 4.4), I also found using RT-qPCR that HFD-fed LepR^{IL33cKO} BAT was more inflamed than HFD-fed control BAT, with increased expression of *Il1b*

and *Il6* (Figure 4.7G). Collectively, this further supports a role for SAPC-derived IL-33 in BAT homeostasis.

BAT thermogenesis is reduced in obesity, at least in part due to central leptin resistance and a resulting decrease in sympathetic drive onto brown adipose tissue [5]. Accordingly, I observe that obesity results in decreased thermogenic gene expression in wildtype mice fed a HFD from 8 weeks old for a period of 12 weeks, compared with age-matched, normal diet-fed control mice (Figure 4.8A). Through thermal imaging and core temperature measurements, I found no significant differences in BAT, tail or core temperature between HFD-fed IL33^{fl/fl} and LepR^{IL33cKO} mice at room temperature (21°C). RT-qPCR analysis showed that HFD-fed LepR^{IL33cKO} BAT has increased expression of key thermogenic gene *Ucp1*, compared with HFD-fed IL33^{fl/fl}, although as discussed by Nedegaard and Cannon [291] expression of *Ucp1* does not directly correlate with heat production.

4.2.6 Cold challenge optimisation

To further investigate the thermogenic capacity of LepR^{IL33cKO} mice I next sought to metabolically challenge these mice. I aimed to optimise a cold challenge protocol sufficient to induce brown adipose tissue thermogenesis - as detected by the upregulation of thermogenic gene expression. With mouse welfare and the consideration of our animal project license in mind, I aimed to optimise a minimally invasive BAT thermogenesis-inducing cold challenge protocol in wildtype mice. I first compared BAT thermogenesis of room temperature (RT) mice with mice which underwent an 8 hour cold challenge (Figure 4.9A) and found no significant differences in BAT temperature (Figure 4.9B), although cold challenged mice had lower tail temperatures and increased core temperatures relative to RT controls (Figure 4.9C-D). BAT of 8 hour cold challenged mice upregulated expression of *Pgc1a*, but no significant increase in expression of key thermogenic gene *Ucp1*, nor *Pparg*, *Prdm16* or *Cidea* was observed (Figure 4.9E). The observed increase in core temperature despite no significant difference in BAT temperature is likely a

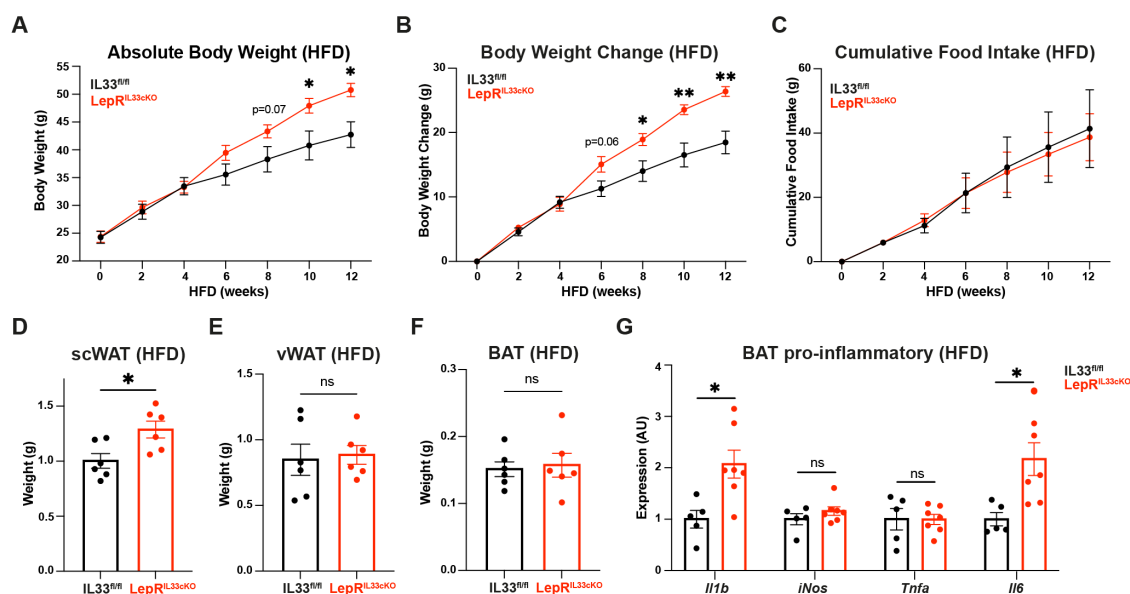


Figure 4.7: LepR^{IL33cKO} mice are predisposed to high fat diet-induced obesity, independently of food intake

A-C Absolute body weight (**A**), body weight change (**B**) and cumulative food intake (**C**) of IL33^{fl/fl} and LepR^{IL33cKO} mice fed a high fat diet (HFD) from 8 weeks old for a period of 12 weeks. LepR^{IL33cKO} mice gain more weight than IL33^{fl/fl} controls when fed a high-fat diet (HFD, **A-B**), despite no difference in cumulative food intake (**C**). $n=6$, 2 independent experiments. **D-F** IL33^{fl/fl} and LepR^{IL33cKO} mice fed a HFD have increased subcutaneous white adipose tissue (scWAT, **D**), but comparable visceral white adipose tissue (vWAT, **E**), and brown adipose tissue (BAT, **F**) weights. $n=6$, 2 independent experiments. LepR^{IL33cKO} mice fed a high fat diet from 8 weeks old for 12 weeks have increased BAT inflammation compared with HFD-fed IL33^{fl/fl} controls. RT-qPCR shows higher expression of pro-inflammatory genes *Il1b* and *Il6* in LepR^{IL33cKO} BAT. For each gene, data is presented relative to IL33^{fl/fl} HFD expression level (=1). *Il1b* = Interleukin-1 beta, *iNos* = Inducible nitric oxide synthase, *Tnfa* = Tumor necrosis factor beta, *Il6* = Interleukin-6. Gene expression is normalised to the expression of house keeping gene *Tata box binding protein* (*Tbp*). $n=5-7$, 1 independent experiment. Data presented as Mean $\pm$ SEM. Student's t-test, ns = non-significant, * $p<0.05$, ** $p<0.01$.

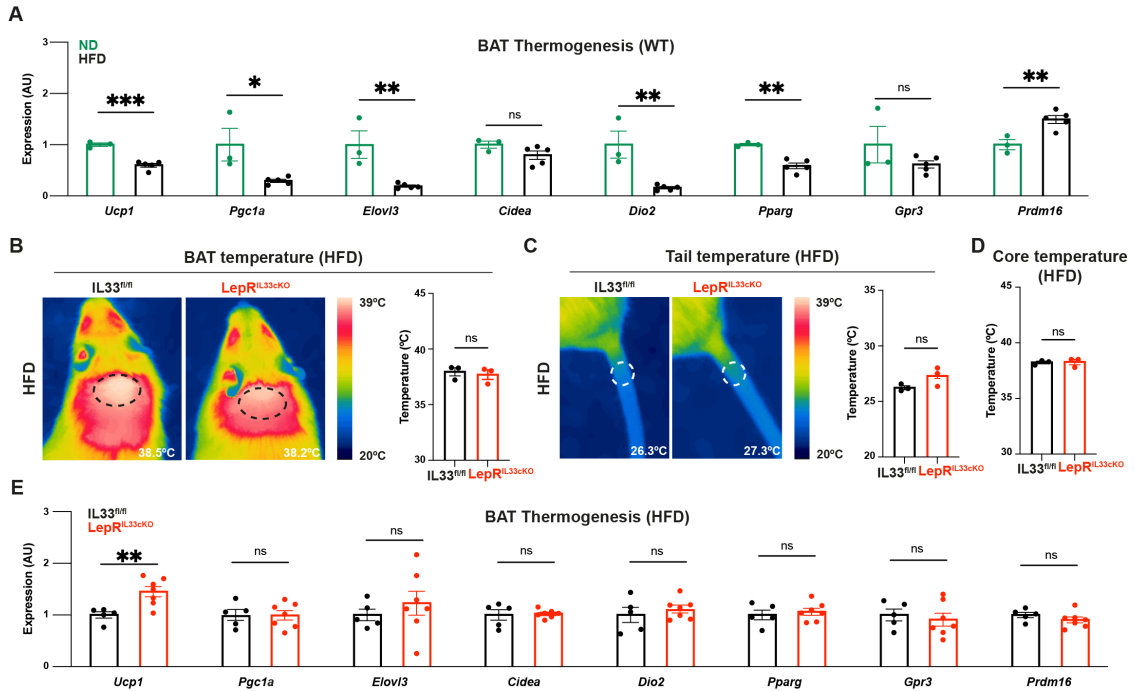


Figure 4.8: HFD-fed LepR^{IL33cKO} mice have higher *Ucp1* BAT expression than IL33^{fl/fl} controls

A BAT expression of thermogenic genes is reduced with HFD in wildtype mice. RT-qPCR of BAT from age-matched normal diet (ND, green) and high fat diet (HFD, black) fed wildtype mice. For each gene, data is presented relative to ND expression level (=1). $n=3$ (ND), $n=5$ (HFD), 2 independent experiments. **B-C** BAT (**B**) and tail (**C**) temperature is comparable in high fat diet-fed LepR^{IL33cKO} and IL33^{fl/fl} mice. (**B-C**) Thermal imaging and quantification of BAT (**B**) and tail temperature (**C**) at room temperature (21°C). Dotted lines on indicate thermal image region from which temperature was measured, bottom right = average temperature in image shown. Each data point represents the average BAT (**B**) or tail temperature (**C**) per mouse, calculated from >10 thermal images. Thermal imaging analysis was blinded. $n=3$, 1 independent experiment. **D** Core temperature of high fat diet fed LepR^{IL33cKO} mice is comparable to IL33^{fl/fl} controls at room temperature (21°C). $n=3$, 1 independent experiment. **E** RT-qPCR of BAT from age-matched high fat diet fed IL33^{fl/fl} and LepR^{IL33cKO} mice. For each gene, data is presented relative to IL33^{fl/fl} HFD expression level (=1). Student's t-test. $n=5$ (IL33^{fl/fl} HFD), $n=7$ (LepR^{IL33cKO} HFD), 2 independent experiments. *Ucp1* = Uncoupled protein 1, *Pgc1a* = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, *Elovl3* = Elovl fatty acid elongase 3, *Cidea* = Cell death inducing DFFA like effector A, *Dio2* = Iodothyronine deiodinase 2, *Pparg* = Peroxisome proliferator activated receptor gamma, *Gpr3* = G-protein coupled receptor 3, *Prdm16* = PR/SET domain 16. Gene expression is normalised to the expression of house keeping gene *Tata box binding protein* (*Tbp*). Data presented as Mean $\pm$ SEM. Student's t-test, ns = non-significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

result of shivering thermogenesis, and I saw no evidence that an 8 hour cold challenge was sufficient to stimulate BAT thermogenesis in wildtype mice.

Next, I tested a cold challenge period of 16 hours (Figure 4.9F). Alongside the expected reduction in tail temperature, I observed a trend towards a reduction in BAT and core temperature in 16 hour cold challenged mice (Figure 4.9G-I). I found no significant differences in the BAT expression of *Ucp1*, *Dio2*, *Pparg*, *Cidea*, *Pdrm16*, *Lpl* following a 16 hour cold challenge, but did observe a significant increase in the expression of *Pgc1a*, *Elovl3* and *Gpr3* (Figure 4.9J). Despite the change in the expression of some thermogenic genes, I observed no increase in the expression key gene *Ucp1* nor an increase in BAT temperature. I therefore conclude that a 16 hour cold challenge does not sufficiently induce BAT thermogenesis in wildtype mice.

In order to increase the magnitude of BAT stimulation, I next opted for an experimental protocol where I first house mice at thermoneutrality (30°C) to significantly reduce BAT thermogenesis, before an additional 8 hour cold challenge for the stimulated experimental group (Figure 4.9K). Although I observed no significant differences in BAT or core temperature alongside the expected decrease in tail temperature (Figure 4.9L-N), I did observe significant increases in the expression of *Ucp1*, *Pgc1a*, *Elovl3*, *Dio2*, *Lpl* and *Gpr2* (Figure 4.9O). Considering thermoneutrality reduces BAT activity to almost zero [301], I considered that the significant differences observed may be due to a reduction in thermogenic gene expression in mice housed at thermoneutrality. To confirm the sufficient induction of BAT thermogenesis with this protocol, I compared thermoneutral + 8 hour cold challenged mice with both thermoneutral and room temperature controls (Figure 10A). I found that relative to room temperature controls ('RT'), thermoneutral + 8 hour cold challenged mice ('Cold') had lower BAT, tail and core temperatures (Figure 10B-D). Of note, I found that expression of *Ucp1*, *Pgc1a*, *Elovl3*, *Dio2*, *Lpl* and *Gpr3* is higher in the thermoneutral + 8 hour cold challenged ('Cold') mice than unchallenged room temperature ('RT') mice, indicating that this protocol sufficiently induces BAT thermogenic gene expression above the room temperature state in which we

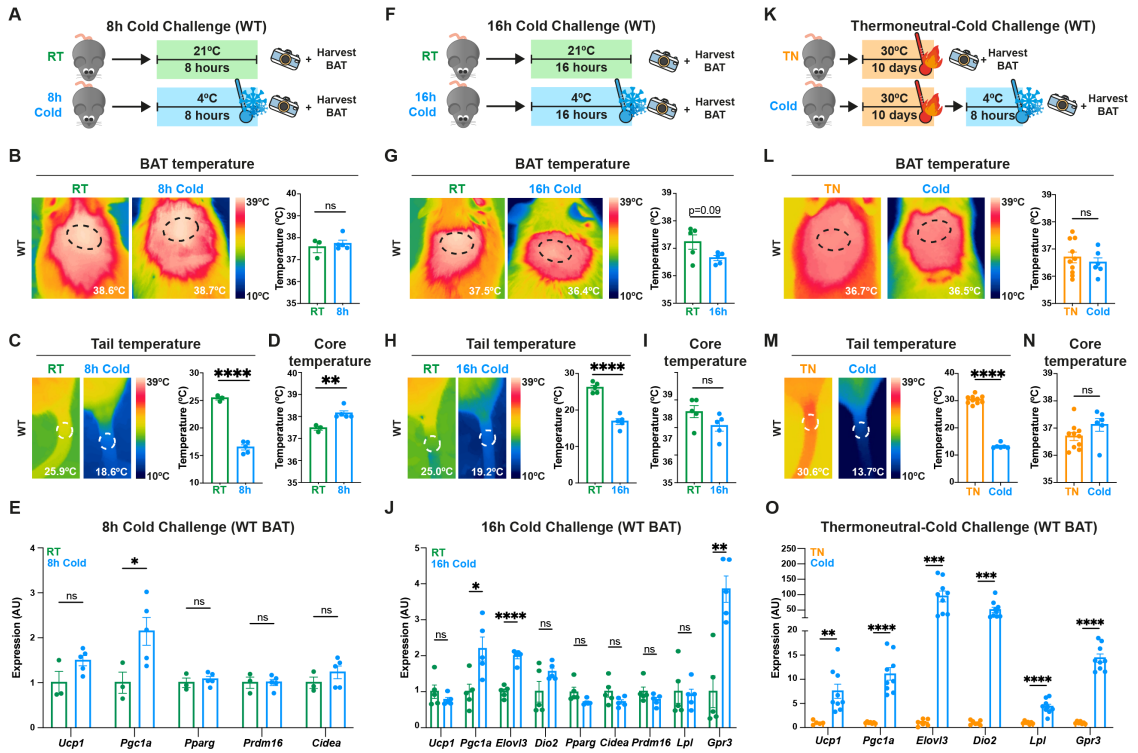


Figure 4.9: Cold challenge protocol optimisation

A Wildtype mice housed at either room temperature (green, 21°C) or 4°C (blue) for 8 hours, before thermal imaging and BAT harvest. **B-D** BAT (B), tail (C) and core temperature measurements (D) of room temperature or 8 hour cold challenged mice. **E** RT-qPCR of BAT from RT or 8 hour cold challenge wildtype mice. For each gene, data is presented relative to RT expression level (=1). n=3 (RT), n=5 (8h cold), 1 independent experiment. **F** Wildtype mice housed at either room temperature (green, 21°C) or 4°C (blue) for 16 hours, before thermal imaging and BAT harvest. **G-I** BAT (G), tail (H) and core temperature measurements (I) of room temperature or 16 hour cold challenged mice. **J** RT-qPCR of BAT from RT or 16 hour cold challenge wildtype mice. For each gene, data is presented relative to RT expression level (=1). n=5, 1 independent experiment. **K** Wildtype mice housed at either thermoneutrality for 10 days (TN, orange, 30°C) or thermoneutrality for 10 days followed by 4°C (Cold, blue) for 8 hours, before thermal imaging and BAT harvest. **L-N** BAT (L), tail (M) and core temperature measurements (N) of thermoneutral and thermoneutral + 8 hour cold challenged mice. **O** RT-qPCR of BAT from thermoneutral or thermoneutral + 8 hour cold challenge wildtype mice. For each gene, data is presented relative to thermoneutral expression level (=1). n=10 (TN) n=6 (Cold), 1 independent experiment. Thermoneutral, RT and thermoneutral + 8 hour cold challenge thermogenic gene expression compared in Appendix Figure 10. Camera denotes thermal imaging. Dotted lines indicate thermal image region from which temperature was measured, bottom right = average temperature in image shown (B-C, G-H, L-M). Each data point represents the average BAT or tail temperature per mouse, calculated from >10 thermal images. Thermal imaging analysis was blinded. *Ucp1* = Uncoupled protein 1, *Pgc1a* = Peroxisome proliferator- activated receptor gamma coactivator 1-alpha, *Pparg* = Peroxisome proliferator activated receptor gamma, *Prdm16* = PR/SET Domain 16, *Cidea* = Cell death-inducing DNA fragmentation factor alpha-like effector A, *Elovl3* = Elovl fatty acid elongase 3, *Dio2* = Iodothyronine deiodinase 2, *Lpl* = Lipoprotein lipase, *Gpr3* = G-protein coupled receptor 3. Gene expression is normalised to the expression of house keeping gene *Tata box binding protein (Tbp)*. Data presented as Mean +/- SEM. Student's t-test, ns = non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

observe no metabolic differences between IL33^{fl/fl} and LepR^{IL33cKO} mice (Figure 4.6D-E).

4.2.7 Adaptive BAT thermogenesis is impaired in cold challenged LepR^{IL33cKO} mice

Having validated the robust induction of thermogenic gene expression in wildtype mice, I next assessed the thermogenic capacity of IL33^{fl/fl} and LepR^{IL33cKO} mice following thermoneutral-cold challenge, an acute protocol which stimulates the enhancement of BAT thermogenic capacity (Figure 4.10A). I observed no differences between IL33^{fl/fl} and LepR^{IL33cKO} BAT, tail or core temperature after 10 days at thermoneutrality (Figure 4.10B-D), nor after thermoneutral-cold challenge (Figure 4.10E-G). Of note, I found that whilst the upregulation of *Ucp1*, *Pgc1a*, *Dio2* and *Lpl* were comparable following thermoneutral-cold challenge, LepR^{IL33cKO} mice were unable to upregulate the expression of *Elov13* and *Gpr3*. Together, I show that loss of IL-33 production by SAPCs impairs adaptive BAT thermogenesis in thermoneutral-cold challenged LepR^{IL33cKO} mice.

4.2.8 The onset of metabolic adaptation to fasting is delayed in LepR^{IL33cKO} BAT

I next induced a state of metabolic adaptation through fasting. Metabolic adaptation is the reduction in energy expenditure in response to fasting in order to preserve energy stores. In the absence of food, I detect a reduction in both BAT and core temperature in wildtype mice, which reach their minimal temperatures after a period of approximately 14 hours fasting (Figure 4.11A-C). When comparing IL33^{fl/fl} and LepR^{IL33cKO} mice after a 14 hour fast, I observe that LepR^{IL33cKO} BAT and core temperature are both significantly higher than IL33^{fl/fl} controls (Figure 4.11D-E). Furthermore, in response to a 14h fast I found that expression of thermogenic genes *Ucp1*, *Elov13* and *Dio2* was lower in LepR^{IL33cKO} BAT, compared with IL33^{fl/fl} controls (Figure 4.11F). Taken together, I show that LepR^{IL33cKO} mice exhibit a delayed induction of metabolic adaptation in response to fasting. Furthermore, the reduced expression of thermogenic genes in LepR^{IL33cKO} BAT after 14 hours of fasting indicates that fasted LepR^{IL33cKO} BAT has

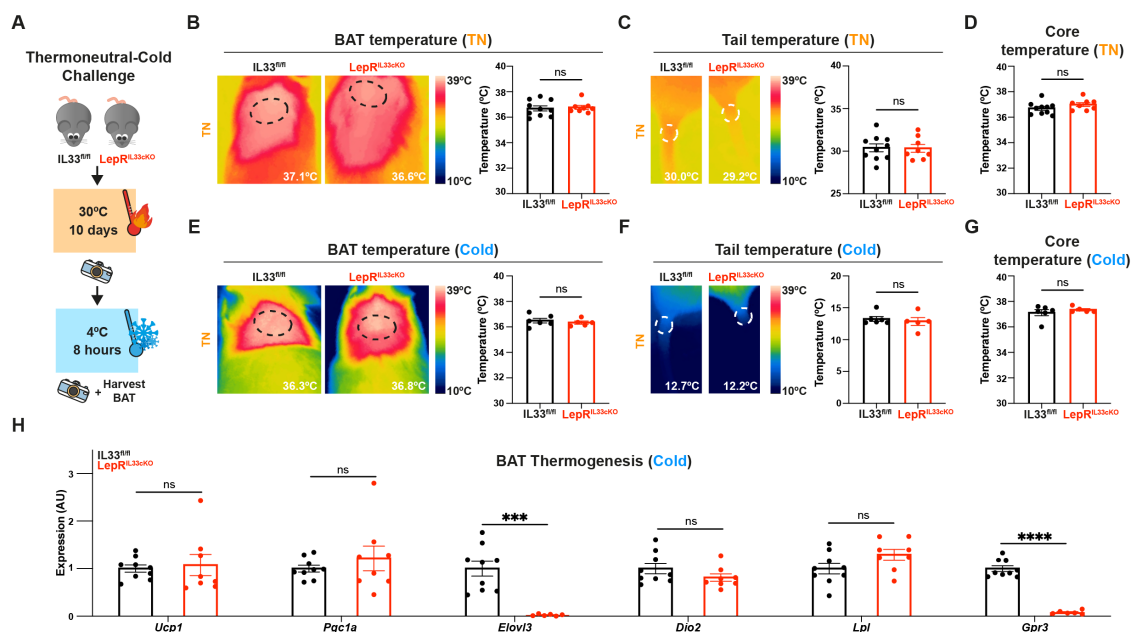


Figure 4.10: Impaired thermogenic capacity in cold challenged LepR^{IL33cKO} mice

A Schematic diagram of experimental protocol. IL33^{fl/fl} and LepR^{IL33cKO} mice were housed at thermoneutrality (30°C) for a period of 10 days, before an 8 hour cold challenge. Camera denotes points at which thermal imaging was performed. **B-C** BAT (**B**) and tail (**C**) thermal imaging and quantification of IL33^{fl/fl} and LepR^{IL33cKO} mice housed at thermoneutrality for 10 days. **D** core temperature measurements of IL33^{fl/fl} and LepR^{IL33cKO} mice housed at thermoneutrality for 10 days. **E-F** BAT (**E**) and tail (**F**) thermal imaging and quantification of IL33^{fl/fl} and LepR^{IL33cKO} mice housed at thermoneutrality for 10 days followed by an 8 hour cold challenge. **G** Core temperature measurements of IL33^{fl/fl} and LepR^{IL33cKO} mice housed at thermoneutrality for 10 days followed by an 8 hour cold challenge. **H** RT-qPCR of brown adipose tissue (BAT) shows impaired *Elovl3* and *Gpr3* upregulation in cold-challenged LepR^{IL33cKO} mice compared with cold-challenged IL33^{fl/fl} controls. For each gene data is presented relative to cold-challenged IL33^{fl/fl} expression level (=1). *Ucp1* = Uncoupled protein 1, *Pgc1a* = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, *Elovl3* = Elovl fatty acid elongase 3, *Dio2* = Iodothyronine deiodinase 2, *Lpl* = Lipoprotein Lipase, *Gpr3* = G-protein coupled receptor 3. Gene expression is normalised to the expression of house keeping gene *Tata box binding protein* (*Tbp*). n=6, 2 independent experiments. Data presented as Mean ± SEM. Student's t-test, ns = non-significant, ***p<0.001, ****p<0.0001.

an impaired capacity for thermogenesis.

4.2.9 Fasted $\text{LepR}^{\text{IL33cKO}}$ BAT is unresponsive to leptin-induced stimulation

I next used leptin to stimulate BAT thermogenesis, and rescue the fasting-induced metabolic adaptation (Figure 4.12A). Accordingly, I observed that relative to PBS-injected controls, IP leptin is sufficient to rescue the BAT temperature of 12 hour fasted wildtype mice (Figure 4.12B-C). This transient induction of BAT thermogenesis by leptin was only detected 1 hour after IP leptin injection in 12 hour fasted wildtype mice. Coupled with the comparable core temperatures observed between PBS and Leptin injected mice 3 hours post injection (Figure 4.12D), I show that leptin transiently stimulates BAT thermogenesis. Given that basal BAT temperature is reached after 14 hours of fasting (Figure 4.11), I opted to fast $\text{IL33}^{\text{fl/fl}}$ and $\text{LepR}^{\text{IL33cKO}}$ mice for 14 hours before stimulating BAT thermogenesis with IP leptin. Similarly to Figure 4.12B, I observe no significant differences in the BAT temperature of control PBS-injected $\text{IL33}^{\text{fl/fl}}$ mice after 14 hours of fasting, and show that BAT temperature is significantly increased by around 0.6 degrees 1 hour after IP leptin injection (Figure 4.12E-G). However, in $\text{LepR}^{\text{IL33cKO}}$ mice, I observed that the BAT temperature is not rescued by IP leptin injection (Figure 4.12H-J), indicating that $\text{LepR}^{\text{IL33cKO}}$ BAT is unresponsive to the leptin-induced stimulation of thermogenesis. Furthermore, $\text{LepR}^{\text{IL33cKO}}$ BAT continues to reduce in temperature regardless of leptin or PBS IP injection (Figure 4.12). Collectively, here I show that IL-33 produced by SAPCs is required for BAT response to both metabolic adaptation to fasting and leptin-induced stimulation of thermogenesis.

4.2.10 Loss of *Adrb2* signalling in SAPCs induces obesity, independently of food intake

Given the expression of noradrenergic receptor $\text{ADR}\beta 2$ by SAPCs (Figure 3.10A) and their close association with NE-releasing sympathetic neurons (Figure 3.2D-H), I finally sought to determine whether LepR^+ SAPCs, or the production of IL-33 is regulated by sympathetic neuron-derived NE. Based on mouse model availability, I planned to use

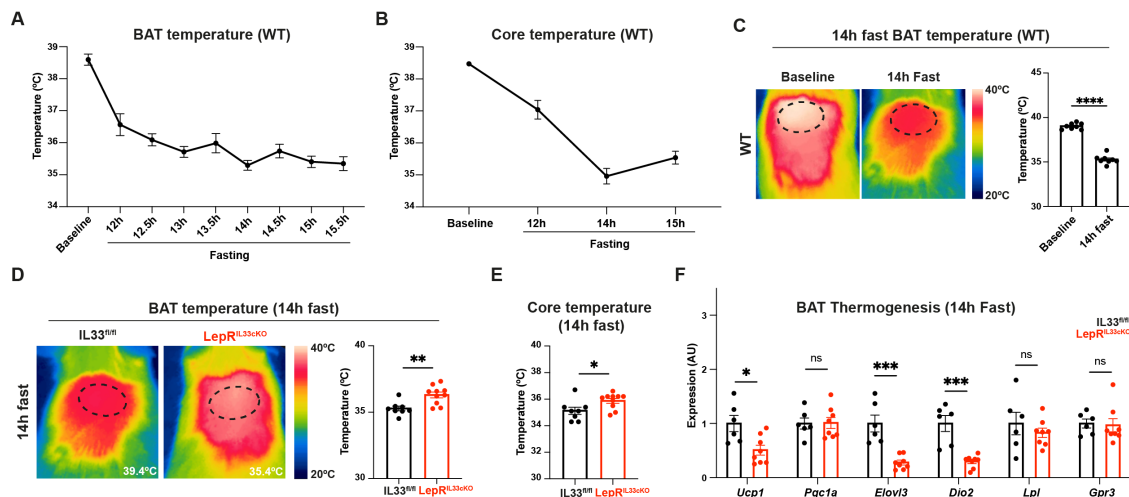


Figure 4.11: LepR^{IL33cKO} mice exhibit delayed induction of metabolic adaptation

A-B Fasting induced metabolic adaptation. Wildtype mice were fasted for a period of 15.5 hours, BAT and core temperature were measured from 12h - 15.5h fasted at room temperature (21°C). Both BAT and core temperature reach their lowest temperature at around 14h of fasting. **C** Representative thermal images of baseline and 14h fasted wildtype BAT with quantification. Dotted lines indicate region measured, bottom right = average temperature in image shown. Each data point represents average BAT temperature per mouse, calculated from >10 images. Thermal imaging analysis was blinded. **D** Thermal imaging and quantification of IL33^{fl/fl} and LepR^{IL33cKO} BAT after 14 hours of fasting. Dotted lines indicate region measured, bottom right = average temperature in image shown. Each data point represents average BAT temperature per mouse, calculated from >10 images. Thermal imaging analysis was blinded. **E** Core temperature measurements of IL33^{fl/fl} and LepR^{IL33cKO} BAT after 14 hours of fasting. **F** RT-qPCR of IL33^{fl/fl} and LepR^{IL33cKO} brown adipose tissue (BAT) after 14 hours of fasting. For each gene data is presented relative to 14h fasted IL33^{fl/fl} expression level (=1). *Ucp1* = Uncoupled protein 1, *Pgc1a* = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, *Elovl3* = Elovl fatty acid elongase 3, *Dio2* = Iodothyronine deiodinase 2, *Lpl* = Lipoprotein Lipase, *Gpr3* = G-protein coupled receptor 3. Gene expression is normalised to the expression of house keeping gene *Tata box binding protein* (*Tbp*). n=6, 2 independent experiments. Data presented as Mean±SEM. Student's t-test, ns = non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

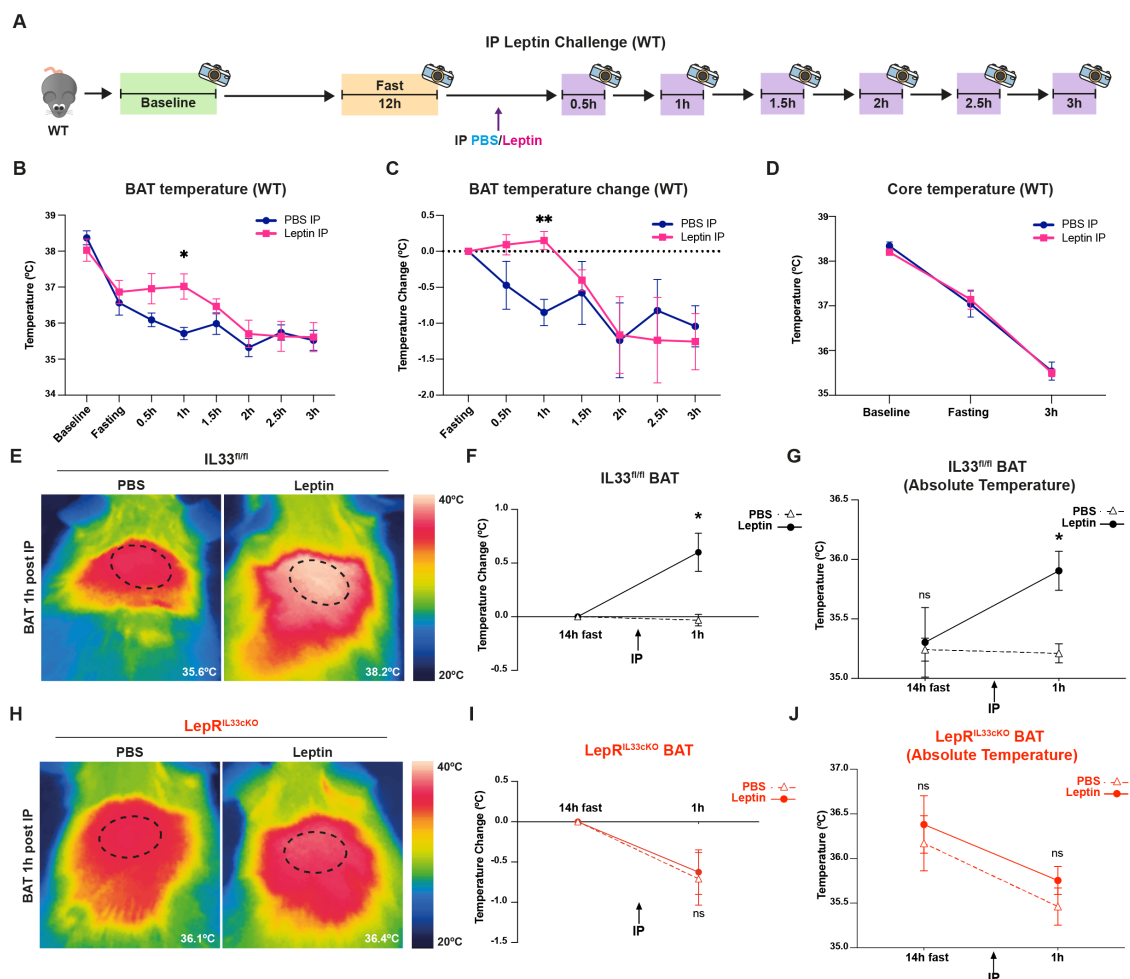


Figure 4.12: Fasted $\text{LepR}^{\text{IL33cKO}}$ mice do not respond to leptin-induced rescue from metabolic adaptation

A Schematic diagram of leptin challenge protocol. Wildtype mice were fasted for a period of 12 hours before IP injection of leptin or PBS for control mice. **B-D** Time course of BAT (**B-C**) and core temperature (**D**) of wildtype injected with either leptin or PBS. BAT temperature is significantly increased 1 hour following IP leptin injection. **E-J** Leptin rescues metabolic adaptation in IL33^{fl/fl} mice (**E-G**) but not LepR^{IL33cKO} mice (**H-J**). Representative thermal images of IL33^{fl/fl} BAT (**E**) and LepR^{IL33cKO} BAT (**H**) 1 hour following IP injection of either Leptin or PBS. Dotted lines indicate region from which the average BAT temperature was measured, bottom right = average temperature in thermal image shown. (**F-G**, **I-J**) BAT temperature was measured from mice injected with either Leptin (solid line) or PBS (dotted line). Presented relative to fasted BAT temperature (**F**, **I**) or absolute temperature (**G**, **J**). $n=5-6$, 2 independent experiments. Data presented as Mean $\pm$ SEM. Student's t-test and 2-way ANOVA with Šidak correction for multiple comparisons, ns = non-significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

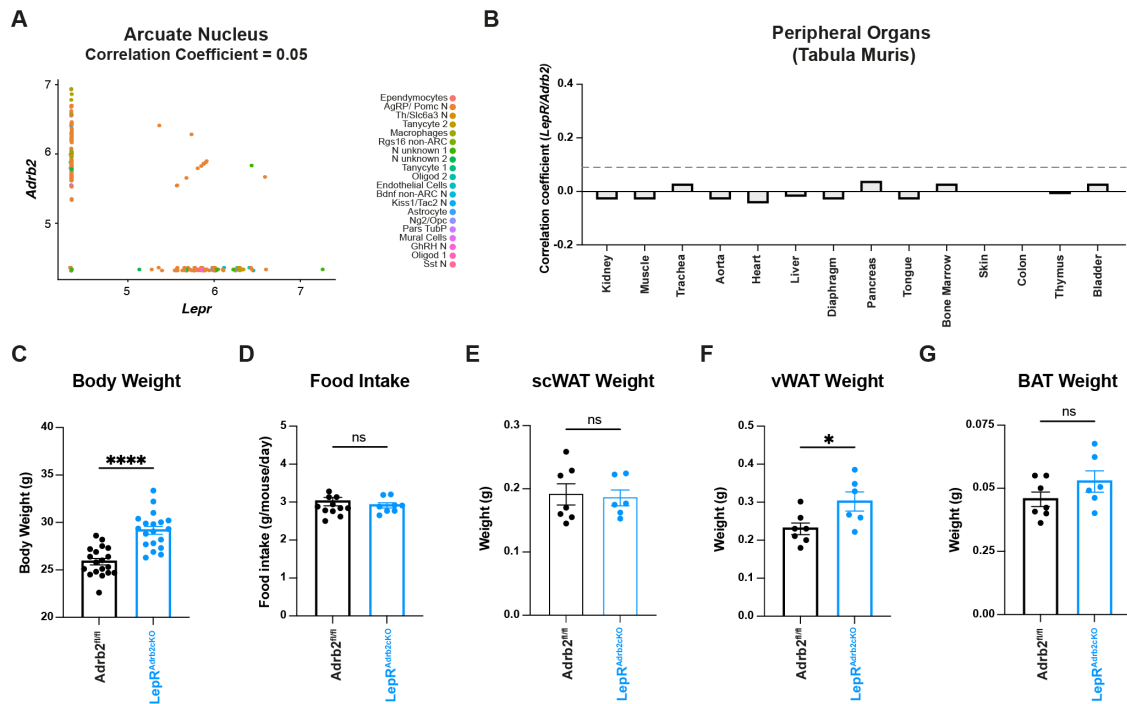


Figure 4.13: Loss of *Adrb2* in SAPCs induces obesity, independently of food intake

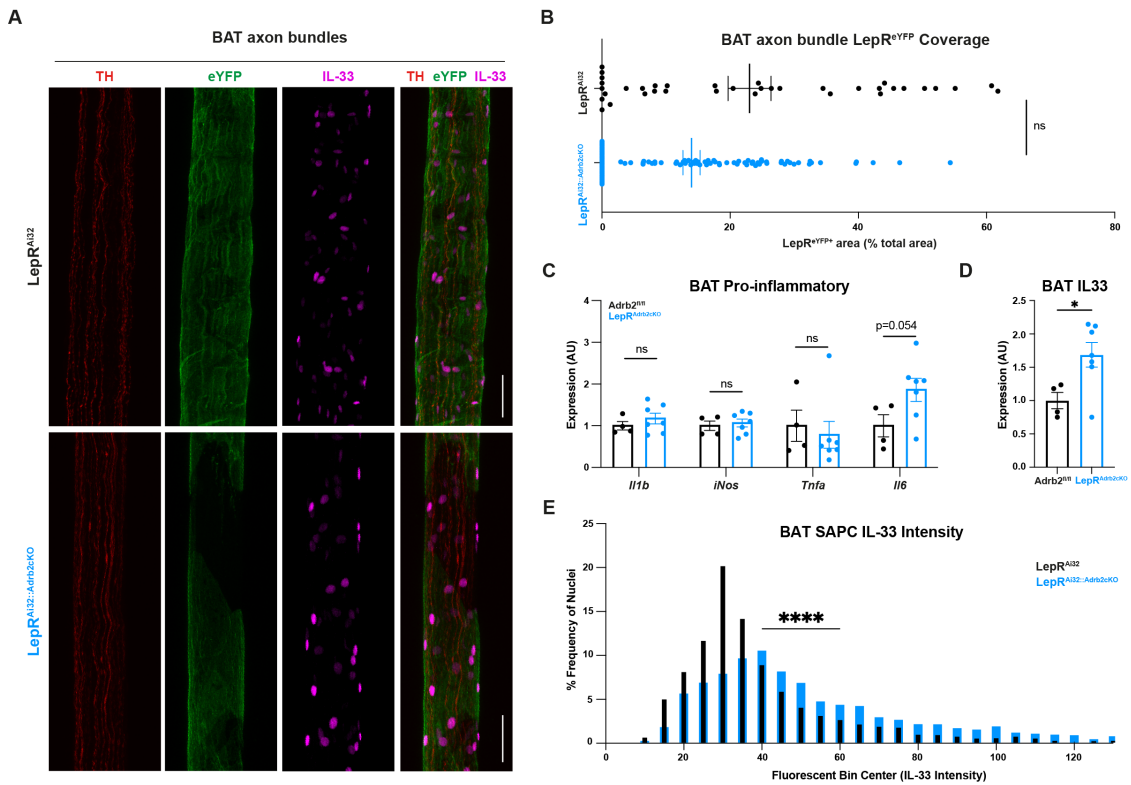
A A small proportion of AgRP/ POMC neurons within the Arcuate Nucleus of the hypothalamus (ARC) co-express *Lepr* and *Adrb2*. Analysis of mouse ARC single-cell RNA sequencing dataset (GSE93374) [224]. N = neuron, Oligod = oligodendrocytes, Pars Tub = Pars Tuberalis. **B** Co-expression of *Lepr* and *Adrb2* in peripheral mouse organs. Smart-Seq2 Tabular Muris dataset (GSE109774) [225]. Grey dotted line marks correlation co-efficient of 0.1. All organs have a correlation co-efficient <0.05, indicating that co-expression is unlikely. **C** Absolute body weight of lean (normal diet-fed) *Adrb2^{fl/fl}* and *LepR^{Adrb2cKO}* mice. *n*=19 (*Adrb2^{fl/fl}*), *n*=17 (*LepR^{Adrb2cKO}*), 3 independent experiments. **D** Average food intake of normal diet-fed *Adrb2^{fl/fl}* and *LepR^{Adrb2cKO}* mice. Each data point represents the average daily food intake of 1 mouse measured over a 4 week period. *n*=11 (*Adrb2^{fl/fl}*), *n*=8 (*LepR^{Adrb2cKO}*), 2 independent experiments. **E-G** Adipose tissue weights of normal diet-fed *Adrb2^{fl/fl}* and *LepR^{Adrb2cKO}* mice. Inguinal subcutaneous white adipose tissue (scWAT, E), visceral white adipose tissue (vWAT, F) and interscapular brown adipose tissue (BAT, G). *n*=6-7, 2 independent experiments. Data presented as Mean±SEM. Student's t-test, ns = non-significant, **p*<0.05, *****p*<0.0001.

a LepR^{CRE}-dependent conditional knockout of *Adrb2*, but first had to determine the likelihood of *Adrb2* loss in non-SAPC LepR⁺ cells. Using the single cell RNA-sequencing database published by Campbell et al. [224], I first assessed co-expression of *Lepr* and *Adrb2* within cells of the arcuate nucleus of the hypothalamus (ARC, Figure 4.13A). I found that a small proportion (<5%) of LepR⁺ AgRP/POMC neurons within the ARC co-expressed *Lepr* and *Adrb2* (Figure 4.13A). However, it is clear from analyses of the *Tabula Muris* database that there is a low probability of *Lepr* and *Adrb2* co-expression in peripheral organs (Figure 4.13B). I generated a LepR^{Adrb2cKO} mouse line by crossing LepR^{CRE} mice with *Adrb2*^{fl/fl} mice, to assess the consequences of a loss of ADR β 2 signalling in LepR⁺ SAPCs.

In the absence of ADR β 2 signalling in SAPCs, 10 week old normal diet-fed male LepR^{Adrb2cKO} mice have a significantly increased body weight, despite no significant differences in food intake (Figure 4.13C-D). Importantly, this comparable food intake between *Adrb2*^{fl/fl} and LepR^{Adrb2cKO} mice indicates that the loss of *Adrb2* expression in LepR⁺ AgRP/ POMC ARC neurons has not affected AgRP/POMC leptin signalling. Therefore, the observed metabolic dysfunction in the absence of SAPC ADR β 2 signalling is more likely due to alterations in adipose tissue homeostasis. The observed body weight gain in LepR^{Adrb2cKO} mice manifests in an increased vWAT weight, with no significant differences found in the scWAT or BAT of LepR^{Adrb2cKO} mice (Figure 4.13E-G). Collectively, I show that loss of *Adrb2* in SAPCs is sufficient to induce obesity in normal diet-fed mice, independently of food intake.

4.2.11 IL-33 production by SAPCs is altered in the absence of ADR β 2 signalling

Given the increased body weight observed in normal diet-fed LepR^{Adrb2cKO} mice and the observed role for SAPC-derived IL-33 in BAT function, I sought to determine whether loss of LepR⁺ SAPC ADR β 2 signalling impacts production of IL-33 using immunofluorescent imaging. With my help, my Masters student Chloe Zentai dissected, stained and imaged sympathetic axon bundles from the brown adipose tissue of LepR^{Ai32}



and LepR^{Ai32::Adrb2cKO} mice. Here, we relied upon the LepR^{Ai32} fate-mapping model to visualise LepR⁺ SAPCs, both in the presence (LepR^{Ai32}) and absence (LepR^{Ai32::Adrb2cKO}) of *Adrb2* expression (Figure 4.14A). We sampled the sympathetic axon bundles of 5 LepR^{Ai32} and 5 LepR^{Ai32::Adrb2cKO} normal diet-fed, 10 week old male mice. Using Fiji image analysis and macros created by Chloe, we first quantified the coverage of LepR⁺ SAPCs around sympathetic axon bundles imaged. Briefly, the total 3D area and the LepR^{eYFP+} area of the sympathetic axon bundle outer layer were measured, and LepR^{eYFP+} area was quantified as a percentage of total area. Further details of image analysis are included in materials and methods. We found that whilst LepR^{Ai32::Adrb2cKO} BAT axon bundles tended to have a lower coverage of LepR⁺ cells, this difference was not statistically significant (Figure 4.14B).

Using RT-qPCR of whole *Adrb2*^{fl/fl} and LepR^{Adrb2cKO} brown adipose tissues we found no significant differences in the expression of pro-inflammatory genes *Il1b*, *iNos*, *Tnfa* and *Il6*, although a trend of increased *Il6* expression was observed ($p=0.054$, Figure 4.14C). We did, however, detect approximately a 2-fold increase in the expression of *Il33* in whole brown adipose tissues, further indicating a possible regulatory role for SAPC ADRB β 2 signalling in IL-33 production. Of note, these RT-qPCR experiments were performed by Chloe Zentai. To determine whether SAPC IL-33 levels were altered in the absence of ADRB β 2 signalling, we quantified the intensity of nuclear-staining IL-33 within LepR^{Ai32} and LepR^{Ai32::Adrb2cKO} SAPCs using macros created by Chloe. Briefly, IL-33⁺ nuclei were detected within LepR^{eYFP+} SAPCs, intensity of IL-33 was measured and normalised to DAPI staining intensity for individual nuclei. Further details of image analysis are included in materials and methods. The positive shift of the histogram plot for LepR^{Ai32::Adrb2cKO} BAT axon bundles indicates that IL-33 fluorescence intensity within SAPCs is increased in the absence of ADRB β 2 signalling, demonstrated by the high statistical significance (**** $p < 0.0001$) between LepR^{Ai32} and LepR^{Ai32::Adrb2cKO}. Collectively, this data indicates that maintenance of LepR⁺ SAPCs, and in particular SAPC production of IL-33 are regulated by the sympathetic nervous system through

ADRB₂ signalling.

4.3 Discussion

Here, I have utilised a LepR^{CRE}-mediated conditional knockout mouse model of *Il33* to assess the role of LepR⁺ SAPC-derived IL-33 on adipose tissue homeostasis. Although confirming that LepR⁺IL-33⁺ cells were not detected in the ARC or tissues of the *Tabula Muris* database, I did identify the presence of LepR^{eYFP+} cells within the trachea, aorta liver and kidney using immunofluorescence (Figure 4.1D) - corroborating reports of *ObRb* transcript within the kidney by Fei et al. [232]. However, the LepR^{Ai32} fate mapping model used renders cells which have ever expressed *ObRb* eYFP⁺, so the true presence of functional *ObRb*⁺ cells within these peripheral tissues would need confirming at the RNA level. Nevertheless, the presence of LepR^{eYFP+} cells in the periphery again highlights the possibility of peripheral leptin signalling targets, in addition to the LepR⁺ neurons within the hypothalamus controlling food intake and sympathetic drive onto the periphery via the neuroendocrine loop of leptin action.

LepR^{CRE}-mediated conditional knockout of IL-33 was not sufficient alone to cause obesity in mice fed a normal diet (Figure 4.2C-G), nor to affect the 'peripheral' immune system - as measured in the spleen (Figure 4.3E, Figure 4.4A, E, I, M). However, assessment of the relative frequencies of vWAT CD45⁺ cell subsets revealed a significant reduction in eosinophils, and a relative increase in ILC2 frequency - with no significant differences observed in vWAT Treg or macrophage frequency (Figure 4.4C, G, K, O). I haven't directly visualised LepR⁺ SAPCs in association with vWAT sympathetic neurons due to the inability to dissect these significantly smaller and more delicate axon bundles. However, this alteration in vWAT-resident IL-33 responsive immune cells indicates that LepR⁺ SAPC-derived IL-33 is required for the regulation of vWAT eosinophil and ILC2, but not Treg populations, which may provide an insight into the proximity of these immune cells to sympathetic axon bundles and local SAPC-derived IL-33 within vWAT pads. In their seminal study on IL-33-producing stromal cells within vWAT,

Spallanzani et al. don't assess the relative frequencies of ILC2s and eosinophils [207]. However, based on the lack in reduction of vWAT Tregs in $\text{LepR}^{\text{IL33cKO}}$ mice I conclude that SAPCs are likely a minor source of IL-33 within this tissue, compared with the main $\text{PDGFR}\alpha^+$ vMSC source described [207]. In fact, whilst $\text{PDGFR}\alpha^{\text{CRE}}$ -mediated conditional knockout of IL-33 also targeted $\text{PDGFR}\alpha^+$ LepR^+ SAPCs (Figure 3.3D, Figure 3.5A-B), LepR^{CRE} -mediated conditional will not have targeted $\text{PDGFR}\alpha^+$ SCA-1^+ MSCs described by Spallanzani et al. [207], as they do not express Leptin receptor (Figure 3.7C).

Notably, I show that CD45^+ immune cell frequency is significantly reduced in the brown adipose tissue of $\text{LepR}^{\text{IL33cKO}}$ mice (Figure 4.3). This genetic deletion manifests in a reduction in BAT Tregs and eosinophils, with a concomitant increase in the relative frequency of ILC2 cells (Figure 4.4D, H, L, P). Changes in brown adipose tissue-resident immune cell frequencies in $\text{LepR}^{\text{IL33cKO}}$ mice confirms a role for IL-33 in the recruitment and/or maintenance of the BAT immune cell milieu. Although clear differences in Treg, eosinophil and ILC2 frequencies were observed (Figure 4.4D, H, L), I am unable to comment on the activity or phenotype of these BAT-resident immune cells in the absence of SAPC-derived IL-33 signalling. However, given the significant impairment in aged ILC2 cytokine licensing reported in the absence of tonic IL-33 signalling by Goldberg et al. [180] along with the relative reduction in Tregs and eosinophils, I propose that ILC2 production of Treg and eosinophil regulatory cytokines may be defective (Figure 1.6B), further propagating BAT inflammation (Figure 4.5A). Furthermore, AT macrophages were quantified using pan-macrophage marker CD68, which does not reveal the relative frequencies of 'M1-like' pro-inflammatory and 'M2-like' anti-inflammatory macrophages in BAT. However, overall the small but significant increase in expression of pro-inflammatory cytokine *Il1b* indicates dysregulation of BAT immune cell signalling in the absence of SAPC-derived IL-33 (Figure 4.5A). I speculate that although the frequency of BAT macrophages is unchanged in $\text{LepR}^{\text{IL33cKO}}$ mice that perhaps phenotypically these macrophages are more M1-like pro-inflammatory macrophages due to loss of type 2 cytokine signalling (Figure 4.4P) - although this would need to be confirmed

experimentally.

Furthermore, IL-33 is known to be the primary survival cytokine for ILC2s in visceral white, but not brown, adipose tissue - where additional ILC2 survival factors may be present. Little is known about BAT-resident ILC2 cells, however significant differences between WAT and BAT microenvironments are likely reflected in both the phenotype and function of these tissue resident immune cells. Previously ILC2s have been directly implicated in thermogenesis through the met-enk-dependent induction of *Ucp1* expression in beige but not brown adipocytes [135]. However, the implication that IL-33 positively regulates thermogenesis exclusively in white adipose tissues based on increased *Ucp1* expression overlooks the comparatively high baseline expression of *Ucp1* already observed in BAT [135]. ILC2s are key orchestrators of type 2 'anti-inflammatory' immune response in adipose tissues, and in response to IL-33 may, alongside Tregs, eosinophils and M2-like macrophages, regulate BAT function indirectly through the prevention of BAT inflammation, which itself has been closely associated with impaired thermogenesis [147][148][149].

Having identified significant changes in the brown adipose tissue immune cell milieu of lean $\text{LepR}^{\text{IL33cKO}}$ mice, I were unable to detect any differences in the temperature or thermogenic gene expression of BAT, nor the whole organism energy expenditure or respiratory quotient through metabolic cage studies (Figure 4.6A-F). This, therefore, indicates that SAPC-derived IL-33 loss - and the subsequent immune cell changes alone - are not sufficient to functionally perturb adipose tissue homeostasis and energy balance in the unchallenged state, despite some low level inflammation within the BAT (Figure 4.5A, Figure 4.6A-F). However, upon high fat diet metabolic challenge I found that $\text{LepR}^{\text{IL33cKO}}$ mice were predisposed to more severe obesity, despite comparable food intake with $\text{IL33}^{\text{fl/fl}}$ HFD-fed controls (Figure 4.7A-C). Considering the comparable energy intake of HFD-fed $\text{LepR}^{\text{IL33cKO}}$ and control mice, this weight gain is likely due to a cumulative effect of impaired energy expenditure, and is suggestive of adipose tissue dysfunction. Although HFD-fed $\text{LepR}^{\text{IL33cKO}}$ scWAT mass is higher than $\text{IL33}^{\text{fl/fl}}$,

vWAT and BAT were comparable between the two genotypes (Figure 4.7D-F). This is likely because the maximum lipid storage capacity of vWAT and BAT has been reached by HFD-fed $\text{LepR}^{\text{IL33cKO}}$ and $\text{IL33}^{\text{fl/fl}}$ at the 20 week old timepoint measured, with excess, 'unspent' energy diverted for storage within the scWAT of $\text{LepR}^{\text{IL33cKO}}$ mice. Although slightly more inflamed than normal diet-fed mice, I observe no significant differences in the BAT temperature of high fat diet fed $\text{LepR}^{\text{IL33cKO}}$ mice, compared with diet-matched $\text{IL33}^{\text{fl/fl}}$ controls (Figure 4.6A, Figure 4.8B). Counterintuitively, HFD-fed $\text{LepR}^{\text{IL33cKO}}$ mice actually had higher BAT expression of key thermogenic gene *Ucp1*, which could be indicative of increased thermogenic capacity (Figure 4.8E). However, corroborating reports by Cannon and Nedegaard [299], increased *Ucp1* expression does not directly correlate with enhanced BAT thermogenesis (Figure 4.8B, E), and may reflect a compensatory attempt to increase thermogenic BAT capacity in response to excess calorie intake. RT-qPCR measurement of thermogenic gene expression likely represents a more sensitive measurement of brown adipose tissue thermogenic capacity, unlike thermal imaging which is arguably more physiologically relevant but considerably less sensitive.

Conversely, I utilised cold challenge to assess the thermogenic capacity of $\text{LepR}^{\text{IL33cKO}}$ BAT upon stimulation. Although no differences were observed in the BAT temperature of cold challenged mice (Figure 4.10E), this metabolic challenge did reveal that $\text{LepR}^{\text{IL33cKO}}$ BAT was unable to upregulate two thermogenic genes *Elovl3* and *Gpr3* in response to cold (Figure 4.10H). ELOVL3 is a fatty acid elongase which contributes to production of <1% of the cellular BAT lipidome, providing fatty acid substrates for downstream respiration [302]. At room temperature, *Elovl3* expression is very lowly detected in WT BAT, and is dramatically upregulated following cold challenge, likely due to the increased metabolic demand of BAT thermogenesis [302]. However, Westerberg et al. [302] show that BAT lacking *Elovl3* has a reduced metabolic capacity compared with wildtype BAT, implicating this fatty acid elongase in sympathetic neuron-mediated stimulation of BAT thermogenesis. The close proximity of SAPCs to sympathetic-derived NE makes it tempting to speculate that IL-33-dependent SAPC-proximal immune cells

in BAT mediate the induction of *Elovl3* expression following acute cold challenge, as the key regulator of *Elovl3* expression within brown adipocytes is currently unknown [135]. Although loss of *Elovl3* had no obvious effect on BAT as a heat-producing organ [302], the impaired recruitment of brown adipose tissue was observed following a longer term (3 week) chronic cold challenge, limiting the maximal ability of BAT thermogenesis in these mice. In addition, GPR3 is a recently discovered, constitutively active G2-coupled receptor found in brown and beige adipocytes, which is upregulated with cold challenge [303]. GPR3 has been directly correlated with energy expenditure due to its constitutive activation - in fact, increased expression of *Gpr3* at the RNA level directly correlated with increased thermogenesis in BAT [303]. Therefore, this reduction in *Gpr3* expression likely manifests in suboptimal BAT thermogenesis. Extensive assessment by Sveidahl-Johansen et al. revealed that *Gpr3* expression is positively regulated by lipolysis within brown adipocytes, as opposed to direct stimulation by sympathetic neuron-derived NE [303]. The loss of cold-induced *Gpr3* expression in $\text{LepR}^{\text{IL33cKO}}$ BAT, therefore, indicates possible dysfunction in lipolysis - which lies downstream of sympathetic neuron-adipocyte β Adrenergic signalling. Whether the impaired $\text{LepR}^{\text{IL33cKO}}$ adaptive BAT thermogenesis in response to cold challenge is due a direct role for IL-33 or IL-33 responsive immune cells in the induction of *Elovl3* and *Gpr3* is yet to be determined, however it is more likely a result of increased BAT inflammation and subsequently impaired sympathetic neuron-adipocyte signalling. Whilst these small but robust impairments in cold-stimulated BAT thermogenesis had little physiological effect on BAT temperature with acute cold challenge (Figure 4.10E), this would likely have much more significant implications on BAT recruitment and thermogenesis which is required for longer term cold challenge and adaptation.

In response to a 14 hour fast, I found that $\text{LepR}^{\text{IL33cKO}}$ mice better preserve their body temperature than $\text{IL33}^{\text{fl/fl}}$ controls through sustained brown adipose tissue thermogenesis despite loss in caloric 'energy input' (Figure 4.11D-E). However, counter-intuitively fasted $\text{LepR}^{\text{IL33cKO}}$ BAT has reduced expression of thermogenic genes *Ucp1*, *Elovl3*, and *Dio2*, indicating that fasted $\text{LepR}^{\text{IL33cKO}}$ mice have a reduced thermogenic capacity with

calorie restriction (Figure 4.11F). This sustained BAT thermogenesis indicates a delay in the responsiveness of LepR^{IL33cKO} BAT to calorie restriction, which is reduced over time as corroborated by the continued decrease in fasted LepR^{IL33cKO} BAT temperature (Figure 4.12I, J). One explanation for the discrepancy between fasted LepR^{IL33cKO} BAT temperature and thermogenic gene expression could be a compensatory reduction in *Ucp1*, *Elovl3* and *Dio2* expression in LepR^{IL33cKO} BAT, to further minimise energy expenditure given the higher BAT temperature relative to fasted wildtype controls.

Importantly, I show that fasted LepR^{IL33cKO} BAT is unresponsive to leptin-stimulated thermogenesis (Figure 4.12H-J). This observed lack of responsiveness to leptin could be a result of loss of SAPC-derived IL-33 as an acute signal in response to leptin signalling. However, little evidence exists for this, as IL-33-stimulated production of met-enk by ILC2s was only able to induce thermogenesis in beige adipocytes and not brown adipocytes *in vitro* [135]. More likely, I propose that the increased BAT inflammation due to a loss in SAPC-derived IL-33 and IL-33-responsive immune cells indirectly perturbs sympathetic induction of BAT thermogenesis. Directly or indirectly, both *Elovl3* and *Gpr3* expression are downstream of sympathetic-drive, which rapidly mediates change in energy expenditure to meet metabolic demand. Collectively, I interpret this loss of adipose tissue homeostasis with metabolic challenge as a predisposition towards metabolic dysfunction due to loss of 'anti-inflammatory' IL-33 responsive immune cells proximal to axon bundles in brown adipose tissue. Their immunomodulatory capacity makes LepR⁺ SAPCs a novel cellular target in the treatment and prevention of obesity.

Finally, I provide evidence that production of IL-33 by SAPCs is negatively regulated by NE-ADR β 2 signalling. Although unexpectedly I find expression of *Adrb2* in LepR⁺ neurons of the ARC (Figure 4.13A), I observe no significant differences in food intake between LepR^{Adrb2cKO} and *Adrb2*^{fl/fl} mice indicating comparable central leptin signalling within the hypothalamus of these conditional *Adrb2* knockout mice. Furthermore, the significant increase in body weight gain and vWAT weight in LepR^{Adrb2cKO} mice (Figure

4.13C-G) indicates that sympathetic neuron signalling likely regulates a range of SAPC-derived factors modulating adipose tissue homeostasis, more significantly perturbing adipose tissue than in the SAPC-specific loss of IL-33 (LepR^{IL33cKO}). Although not significant, the trend towards a decrease in LepR⁺ SAPC coverage of BAT axon bundles in the absence of ADR β 2 SAPC signalling (Figure 4.14A-B) further indicates that SAPC survival and function may be directly regulated by sympathetic neuron-derived NE. Similarly to previous work on sensory neuron-associated stromal cells in muscle which revealed the modulation of IL-33 by proximal neurotransmitters, I show that the production of IL-33 by SAPCs in BAT is increased in the absence of sympathetic neuron-derived NE-ADR β 2 signalling. In fact, despite the role of IL-33 in the prevention of BAT inflammation, the increased production of alarmin IL-33 by SAPCs in the absence of ADR β 2 signalling may itself reflect increased SAPC cellular stress or death relative to ADR β 2 sufficient SAPCs [227][255][257][258][259][260][261][263], firmly implicating the sympathetic nervous system in the regulation of SAPCs - a finding which warrants significant further investigation.

Chapter 5

Final Conclusion and Discussion

Contents

5.1	Summary of key findings	141
5.2	LepR ⁺ Sympathetic Associated Perineurial Cell barrier	142
5.3	Immune modulation of brown adipose tissue function	144
5.4	Therapeutic targets for the treatment of obesity	146
5.5	Limitations of this work	147
5.6	Future direction	148
5.7	Final conclusion	150

5.1 Summary of key findings

Our understanding of obesity has now developed to the point at which it cannot be designated a simple disease of energy imbalance. The obese state is characterised by disorder of the sympathetic nervous, endocrine and immune systems which perpetuate the obese condition. For decades obesity research has been divided into neuroendocrinology and immunometabolism, but a link has been missing between these diverse fields of study [304]. Previous work has determined a role for stromal cells in the maintenance of IL-33-responsive immune cells within visceral white adipose tissues [207][217], however a link has not yet been found with the sympathetic nervous system. Similarly, although peripheral nerve structures have been described anatomically and histologically since the mid 1800s, little is known about their function.

My work set out to determine whether stromal cells associated with the sympathetic neurons which innervate adipose tissues play a role in adipose tissue and whole organism homeostasis. Here, I report a specialised, LepR⁺ sympathetic neuron-associated subset of perineurial cells which form a barrier around adipose tissue-innervating axon bundles in

scWAT and BAT. Furthermore, *LepR⁺* Sympathetic neuron-Associated Perineurial Cells (SAPCs) express immunomodulatory cytokines including IL-33, which I show is required for the prevention of brown adipose tissue inflammation and thermogenic function in response to metabolic challenge (Figure 5.1). Mice lacking IL-33 production in SAPCs gain more weight when fed a high fat diet despite no significant differences in food intake - indicative of adipose tissue metabolic dysfunction. Further, they display an impaired response to metabolic challenge-induced changes in sympathetic drive, and are unresponsive to leptin stimulation of BAT thermogenesis. Collectively, this highlights the importance of *LepR⁺* SACP-derived IL-33 in the maintenance of BAT homeostasis through immune cell modulation, and the prevention of metabolic dysfunction.

5.2 *LepR⁺* Sympathetic Associated Perineurial Cell barrier

Previous work has revealed the accumulation of neuro adipose-modulating macrophages with both age and obesity, which blunt lipolysis and thermogenesis by limiting the bioavailability of sympathetic neuron-derived NE [166][134]. The presence of a perineurial cell barrier around sympathetic neurons is one additional way in which the bioavailability of sympathetic neuron-derived NE to adipocytes may be modulated, through the regulation of barrier permeability. Perineurial cells surrounding peripheral nerves are known to highly express tight junction proteins CLDN1 and TJP1 [244], and are closely associated with a basement membrane on either side [243]. This indicates they form a true mechanical barrier and limit the passage of signals between adipocytes and sympathetic neurons. Experiments performed in the mid 1950s by Krnjević et al. showed that upon incubation with an isotonic, sodium-free solution, electrical transduction of dissected frog sciatic nerve was only preserved if the perineurial barrier remained intact [242][250], corroborating the idea of an impermeable perineurial barrier surrounding axon bundles. The hypothesis of perineurial cells forming a mechanical barrier function warrants further investigation, as *LepR⁺* SAPCs may also be responsible for the active transport of NE across the barrier, further regulating the neuroadipose junction. In addition, I propose that the surrounding perineurial cell barrier may protect sympathetic, and other, neurons

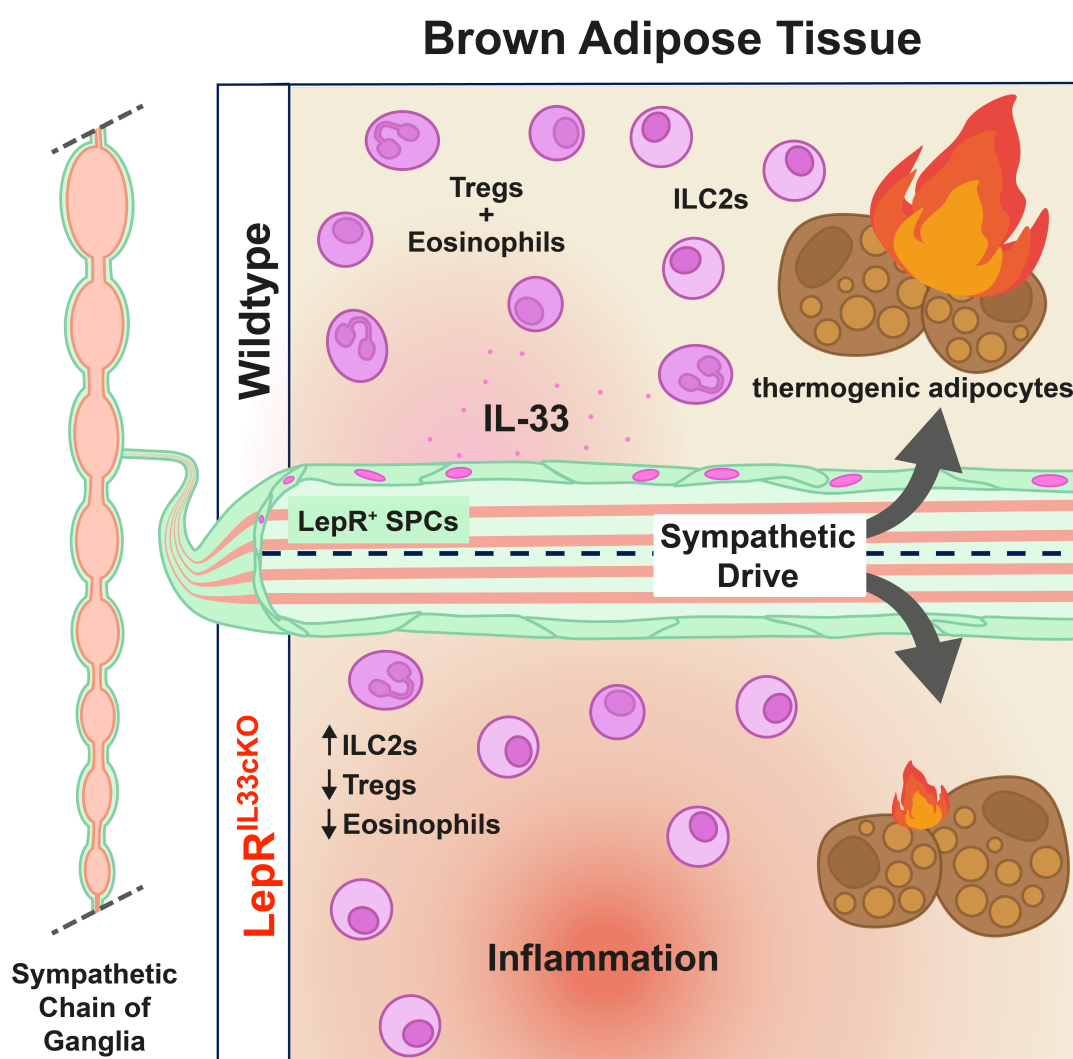


Figure 5.1: LepR⁺ Sympathetic Associated Perineurial Cells regulate adaptive thermogenesis in brown adipose tissue

LepR⁺ Sympathetic Associated Perineurial Cells (SAPCs) regulate adaptive thermogenesis in brown adipose tissue through the maintenance of resident IL-33 sensitive type 2 immune cells and the prevention of inflammation. Mice lacking IL-33 production in SAPCs (LepR^{IL33cKO}) have impaired adaptive BAT thermogenesis in response to metabolic challenge.

within axon bundles from adipose tissue inflammation in obesity; preserving sympathetic function upon stimulation. Therefore, the integrity of the perineurial cell barrier may itself be important in the modulation of the neuroadipose junction.

Loss of *Adrb2* signalling in SAPCs, which may mirror the leptin resistance and decreased sympathetic drive observed in obesity, results in an insignificant reduction in the coverage of BAT axon bundles with LepR^{eYFP+} cells (Figure 4.14A-B). Furthermore, in the absence of ADR β 2 signalling, LepR⁺ SAPCs increase production of alarmin IL-33 (Figure 4.14E), consistent with cell stress or cell death [254][255][256]. This observation indicates that NE, and therefore tonic sympathetic signalling, could be a survival signal for LepR⁺ SAPCs. Furthermore, in response to metabolic stress within adipose tissues, I propose that LepR⁺ SAPCs surrounding sympathetic neurons may themselves become stressed or necrotic, and subsequently attempt to reduce inflammation through IL-33 signalling and the recruitment or activation of type 2 immune cells to maintain AT homeostasis. Therefore, as observed in LepR^{IL33cKO}, metabolic stress-induced adipoinflammation cannot be countered in the absence of SAPC-derived IL-33, resulting in the exacerbation of adipose tissue dysfunction, including BAT adaptive thermogenesis, and increased weight gain with HFD metabolic challenge.

5.3 Immune modulation of brown adipose tissue function

I have shown that the loss of IL-33 specifically in LepR⁺ SAPCs results in impaired adaptive BAT thermogenesis, and describe how LepR⁺ SAPCs are required for the maintenance of type 2, 'anti-inflammatory' immune cells within BAT. However, it remains unclear whether the observed impairment in LepR^{IL33cKO} BAT thermogenesis is solely due to a loss in anti-inflammatory immune cells and concomitant increase in inflammation, or whether key IL-33-responsive immune cells play a direct role in the induction of adaptive thermogenesis. Little is known about the phenotype and function of immune cells within the brown adipose tissues, as AT-resident immune cells have most extensively been studied in the visceral WAT due to the correlation of dysfunction

and inflammation with diabetes mellitus [305]. Considering white and brown adipose tissues are morphologically and functionally distinct, it is similarly likely that differences will be observed in the regulation, phenotype and function of resident immune cells in BAT, scWAT and vWAT. Brown adipose tissue is known to be more resistant to high fat diet-induced inflammation than visceral white adipose tissue [306] - further highlighting the differential immune regulation between adipose tissue depots.

Induction of BAT inflammation through loss of tissue resident Tregs has been correlated with impaired BAT thermogenic capacity [300]. Furthermore, although ILC2 cells have been implicated in the stimulation of thermogenesis through the production of met-enk, Brestoff et al. [135] found that this was only possible in beige, and not brown, adipocytes *in vitro*. Therefore, evidence for the direct regulation of BAT thermogenesis by immune cells is lacking, however given the observed decrease in BAT thermogenesis with inflammation, BAT-resident immune cells may play an indirect role in the maintenance of an anti-inflammatory, thermogenically competent BAT microenvironment. Furthermore, evidence to date suggests that the key regulator of thermogenesis is the activation of BAT-innervating sympathetic neurons. In the absence of inflammation, Wolf et al. describe a role for sympathetic neuron-associated macrophages in brown adipose tissue thermogenic function, through the maintenance of BAT sympathetic innervation [143]. Considering the proximity of IL-33-producing LepR⁺ SAPCs to sympathetic neurons, I consider that the IL-33 dependent recruitment of immune cells to adipose tissue axon bundles may enhance BAT thermogenesis through the prevention of inflammation proximal to sympathetic neurons. Although an exact mechanism is not yet clear, here I show that IL-33-responsive BAT immune cells prevent inflammation proximal to BAT-innervating axon bundles, likely indirectly promoting the sympathetic-mediated modulation of adaptive BAT thermogenesis.

A full description of IL-33-producing cells within brown adipose tissues is lacking, however the significant changes observed in IL-33-responsive BAT immune cells may reflect that SAPCs are a major source of IL-33 within this tissue. More recently,

changes in the cellular source of IL-33 has been implicated in ILC2 regulation with age. Goldberg et al. [180] report that the major vWAT source of IL-33 shifts from PDGFR α ⁺ MSCs to mesothelial cells with age, which they propose results in an aged, cold-intolerant ILC2 phenotype due to a loss in tonic IL-33-AT-resident immune cell signalling. Little is known about the relative localisation of these resident immune cells within adipose tissues, a factor which is perhaps more difficult to accurately quantify but likely plays a significant role in adipose tissue immune cells and their cellular neighbourhoods. In fact, it currently remains unclear whether immune cells other than sympathetic neuron-associated macrophages SAMS [166][134] reside within or proximal to adipose tissue axon bundles. Although conjecture, I hypothesise that through the loss in local tonic type 2 immune cell signalling the specific deletion of IL-33 within LepR⁺ SAPCs promotes inflammation around adipose tissue axon bundles, and may result in impaired neuro-adipose signalling. Our understanding of a role for type 2 immune cells within ATs would be further advanced by the knowledge of their localisation relative to axon bundles, blood vessels and IL-33⁺ MSCs, which would likely allude to their function.

5.4 Therapeutic targets for the treatment of obesity

Our advances in understanding of the neuroimmune mechanisms underlying obesity have opened the door to therapies targeting these mechanisms and a number of novel anti-obesity drugs. The preclinical evidence gathered so far strongly indicates that anti-obesity drugs and immunotherapeutics that curtail the metabolic consequences of obesity will be a feature of the therapeutic landscape in years to come. Accordingly, previous work by Spallanzani et al. and Mahlaköiv et al., coupled with the data presented in this thesis, indicates that adipose tissue mesenchymal stromal cell- and LepR⁺ SAPC-derived IL-33 is protective against obesity [207][217], highlighting IL-33 as a possible therapeutic tool. Furthermore, parasite-derived protein HpARI3, which stabilises IL-33 and prolongs signalling [275], provides one such potential therapeutic tool which could be utilised to manipulate and promote tonic adipose tissue IL-33 signalling, which is required for ILC2 function in adaptive thermogenesis [180]. However, IL-33 propagates

asthma and type 2 allergic response [265], and therefore likely does not represent a viable therapeutic target in the treatment of obesity, due to the potential for significant off-target effects. Importantly, here I describe LepR⁺ SAPCs as a novel cellular target which may modulate adipose tissue homeostasis through the integration of neuro, immune and endocrine signals. LepR⁺ SAPC-derived IL-33 represents only one way in which LepR⁺ SAPCs modulate adipose tissue homeostasis. A better understanding of the role of LepR⁺ SAPCs, and additional cellular or molecular players, will likely reveal additional therapeutic targets which can be manipulated in the treatment of obesity.

5.5 Limitations of this work

This work describes a previously unreported cellular player in the regulation of adipose tissue homeostasis. However, I have identified some limitations of this work. Due the delicate nature of LepR⁺ SAPCs, it was not possible to isolate enough LepR^{eYFP+} cells from adipose tissue axon bundles for bulk-RNA sequencing analysis. Instead, we opted to isolate and sequence LepR^{eYFP+} cells from the sympathetic ganglia, with the assumption that adipose tissue axon bundles are an extension of the sympathetic ganglia in which adipose tissue-innervating post-ganglionic neuron cell bodies reside. Where possible, all findings in sympathetic ganglia have also been verified in adipose tissue axon bundles. Using immunofluorescence we identified two LepR^{eYFP+}, IL-33⁺ populations: one surrounding the ganglia, which is anatomically more comparable to LepR⁺ SAPCs in adipose tissue axon bundles, and another population within the sympathetic ganglia. Our bulk RNA-sequencing analyses are unable to differentiate between these two spatially distinct LepR^{eYFP+} populations. Furthermore, we have not excluded the possibility that loss of SAPC-derived IL-33 in LepR^{IL33cKO} mice results in the inflammation of sympathetic ganglia, which could affect sympathetic drive onto adipose tissues. Therefore, the impaired response of LepR^{IL33cKO} BAT to metabolic challenge, may also represent sub-optimal sympathetic neuron signalling due to ganglionic inflammation and dysfunction. Of note, LepR⁺ SAPCs within the sympathetic ganglia express only receptors to the post-ganglionic sympathetic neurotransmitter NE, and not

the pre-ganglionic sympathetic neurotransmitter acetylcholine, indicating a functional association of these cells with the noradrenergic post-ganglionic sympathetic neurons which innervate adipose tissues.

Moreover, we show that BAT Treg, eosinophil and ILC2 relative frequencies are altered in the absence of LepR⁺ SAPC-derived IL-33, however this does not provide information on the activity or functional capacity of these BAT-resident immune cells. Considering the reported necessity of tonic IL-33 signalling on ILC2 function and cytokine licensing in vWAT [180], it would be reasonable to suspect that a loss of SAPC-derived IL-33 may also result in BAT ILC2 dysfunction. Assessment of the functional capacity of BAT immune cells which have developed in the absence of SAPC-derived IL-33 would likely aid our understanding of their role in adaptive thermogenesis.

Owing to the role of the oestrous cycle on both feeding and sympathetic adipose tissue innervation in females [307], we opted to use only male mice experimentally, although the presence of LepR⁺IL-33⁺ SAPCs was confirmed in the adipose tissue axon bundles of female mice. In contrast, the analysed single nuclei RNA-sequencing data of human sympathetic ganglia presented in this thesis includes both male and female samples, confirming the presence of a *LEPR*⁺*IL33*⁺ population in both sexes. However, perineurial cells are identified anatomically. Therefore, without information on the anatomical location of this *LEPR*⁺*IL33*⁺ population reported in human sympathetic ganglia, we are unable to definitively confirm that these cells are SAPCs. However, the presence of *LEPR*⁺*IL33*⁺ cells within human sympathetic ganglia indicates human relevance for the work presented in this thesis.

5.6 Future direction

Here, I describe LepR⁺ SAPCs as a specialised subset of perineurial cells surrounding adipose tissue axon bundles, with the capacity to modulate adipose tissue-resident immune cells in response to sympathetic and leptin signalling. However, a number of

questions regarding their function still remain. We do not know whether sympathetic neuron-derived NE can freely diffuse across the perineurial barrier, or whether the transport of this neurotransmitter - and therefore bioavailability of NE - is actively regulated by SAPCs. To determine this, we could first assess the expression of possible transport machinery within SAPCs, and assess whether the sympathetic-dependent stimulation of adipose tissue thermogenesis or lipolysis is blunted upon the loss or antagonism of transport machinery. In addition, we are currently unaware of the effects of obesity on LepR⁺ SAPCs. Considering the dysfunction of both leptin and sympathetic neuron-derived NE in obesity, LepR⁺ SAPCs are likely altered in the obese state. The integrity of the LepR⁺ SAPC perineurial barrier in the obese state should also be assessed through the dissection, imaging and quantification of LepR⁺ SAPCs in diet-induced obese LepR^{Ai32} adipose tissue axon bundles, versus LepR^{Ai32} normal diet age-matched controls. Moreover, we know little about the direct effects of both leptin and NE on the production of immunomodulatory cytokines by LepR⁺ SAPCs. *Ex vivo* incubation of adipose tissue with leptin and/or NE analogues could be used to assess the dependence of SAPC survival, immunomodulatory cytokine production and phenotype on these proposed modulatory factors. Given the profound weight gain in unchallenged LepR^{Adrb2cKO} mice, which parallels loss of sympathetic drive due to leptin resistance in obesity, I propose that sympathetic-SAPC signalling in particular could be one important factor in the function of these specialised perineurial cells. Furthermore, given the proximity of LepR⁺ SAPCs to specialised sympathetic associated macrophages (SAMs) which regulate the neuroadipose junction, and the increased recruitment of SAMs in obesity, it is tempting to speculate that LepR⁺ SAPCs could be involved in the recruitment of SAMs; the mechanism of which still remains elusive. This could be determined through the assessment of compatible ligand-receptor expression by LepR⁺ SAPCs and SAMs respectively, followed by functional analyses if suitable recruitment signals are identified.

5.7 Final conclusion

Collectively, I have shown that LepR⁺ SAPC regulate BAT response to metabolic challenge through the recruitment of IL-33-responsive cells, establishing a role for LepR⁺ SAPCs in the regulation of energy homeostasis. We have uncovered LepR⁺ SAPCs as an upstream, immunomodulatory, sympathetic-associated stromal cell that is a missing link between leptin and immunometabolic mechanisms controlling body weight homeostasis and obesity.

Appendices


```

1 selectWindow("CCZ2022_03 WT 2only B f B4.2.2 DAPI-GFP488-IL33647-TH546 x20 280422 44Z.czi");
2 run("Z Project...", "projection=[Max Intensity]");
3 run("Split Channels");
4 selectWindow("C2-MAX_CCZ2022_03 WT 2only B f B4.2.2 DAPI-GFP488-IL33647-TH546 x20 280422 44Z.czi");
5 run("Median...", "radius=1.5");
6 run("Auto Threshold", "method=Otsu white");
7 setOption("BlackBackground", true);
8 run("Convert to Mask");
9 run("Fill Holes");
10 run("Set Measurements...", "area mean redirect=None decimal=3");
11 run("Create Selection");
12 selectWindow("CCZ2022_03 WT 2only B f B4.2.2 DAPI-GFP488-IL33647-TH546 x20 280422 44Z.czi");
13 run("Z Project...", "projection=[Average Intensity]");
14 run("Split Channels");
15 selectWindow("C1-AVG_CCZ2022_03 WT 2only B f B4.2.2 DAPI-GFP488-IL33647-TH546 x20 280422 44Z.czi");
16 run("Restore Selection");
17 run("Measure");

```

```

1 selectWindow("CCZ2022_03 WT 2only B f B4.2.2 DAPI-GFP488-IL33647-TH546 x20 280422 44Z.czi");
2 run("Z Project...", "projection=[Max Intensity]");
3 run("Split Channels");
4 selectWindow("C1-MAX_CCZ2022_03 WT 2only B f B4.2.2 DAPI-GFP488-IL33647-TH546 x20 280422 44Z.czi");
5 run("Median...", "radius=1.5");
6 run("Auto Threshold", "method=Otsu white");
7 setOption("BlackBackground", true);
8 run("Convert to Mask");
9 run("Fill Holes");
10 run("Set Measurements...", "area mean redirect=None decimal=3");
11 run("Create Selection");
12 selectWindow("CCZ2022_03 WT 2only B f B4.2.2 DAPI-GFP488-IL33647-TH546 x20 280422 44Z.czi");
13 run("Z Project...", "projection=[Sum Slices]");
14 run("Split Channels");
15 selectWindow("C2-SUM_CCZ2022_03 WT 2only B f B4.2.2 DAPI-GFP488-IL33647-TH546 x20 280422 44Z.czi");
16 run("Restore Selection");
17 run("Measure");

```

Figure 2: Macro for measurement of LepR and IL-33 background fluorescence

These Macros relate to the image analysis pipeline shown in Figure 2.3. Macros were written by Chloe Zentai.

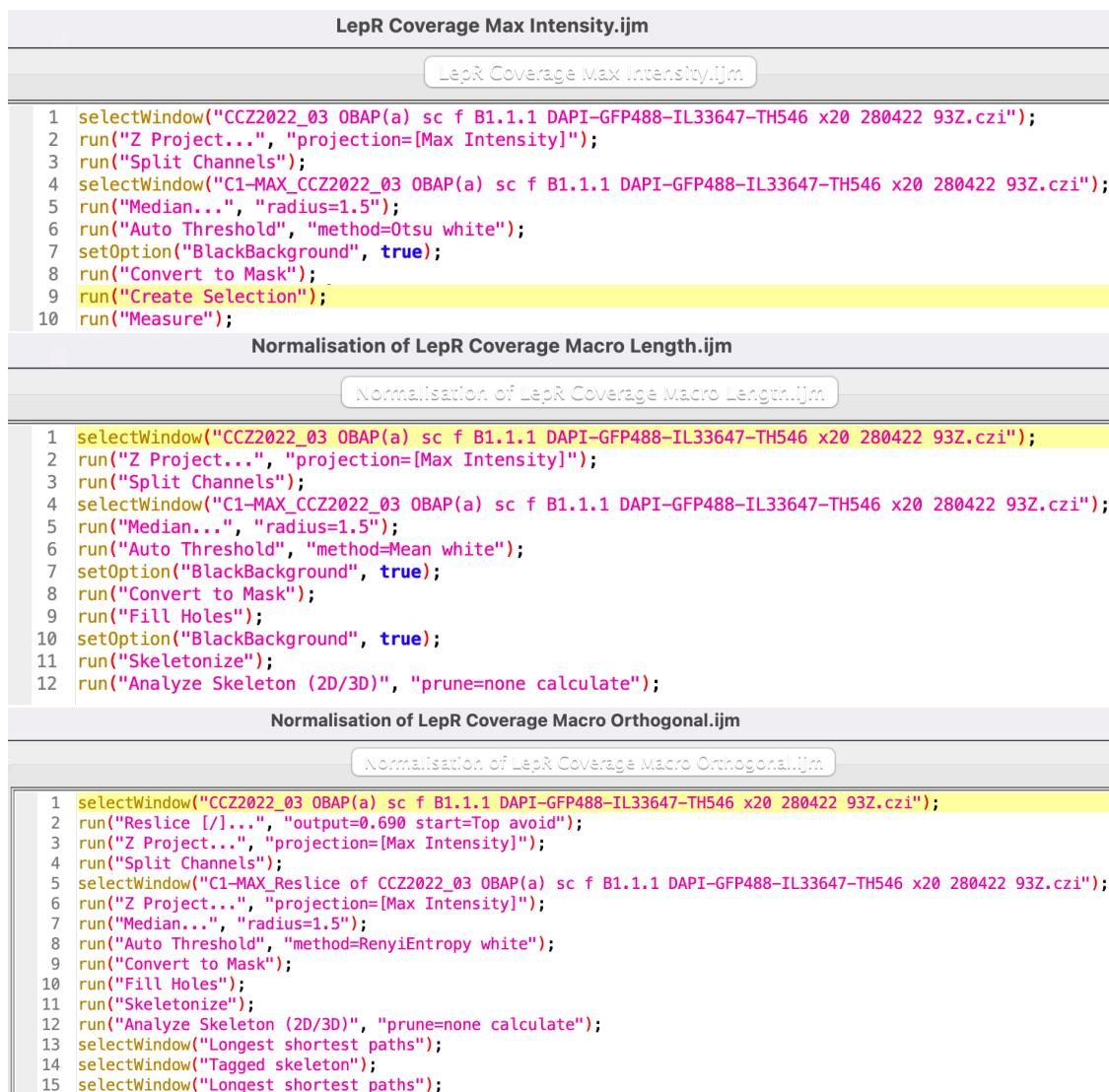


Figure 3: Macros for measurement and normalisation of LepR coverage

These Macros relate to the image analysis pipeline shown in Figure 2.4. Macros were written by Chloe Zentai.

```

1 roiManager("reset");
2
3 selectWindow("CCZ2022_03 OBAP(a) sc f B1.1.1 DAPI-GFP488-IL33647-TH546 x20 280422 93Z.czi");
4 run("Z Project...", "projection=[Max Intensity]");
5 run("Split Channels");
6 run("Set Measurements...", "area mean integrated redirect=None decimal=3");
7 selectWindow("C1-MAX_CCZ2022_03 OBAP(a) sc f B1.1.1 DAPI-GFP488-IL33647-TH546 x20 280422 93Z.czi");
8 run("Median...", "radius=0.5");
9 run("Subtract...", "value=35");
10 run("Auto Threshold", "method=Otsu white");
11 setOption("BlackBackground", true);
12 run("Convert to Mask");
13 run("Fill Holes");
14 //run("Watershed");
15
16 selectWindow("C3-MAX_CCZ2022_03 OBAP(a) sc f B1.1.1 DAPI-GFP488-IL33647-TH546 x20 280422 93Z.czi");
17 run("Median...", "radius=0.5");
18 run("Auto Threshold", "method=Moments white");
19 run("Convert to Mask");
20 //run("Dilate");
21 run("Watershed");
22
23 selectWindow("C1-MAX_CCZ2022_03 OBAP(a) sc f B1.1.1 DAPI-GFP488-IL33647-TH546 x20 280422 93Z.czi");
24 run("Create Selection");
25 selectWindow("C3-MAX_CCZ2022_03 OBAP(a) sc f B1.1.1 DAPI-GFP488-IL33647-TH546 x20 280422 93Z.czi");
26 run("Restore Selection");
27 run("Analyze Particles...", "size=17-350 display add");
28
29 selectWindow("CCZ2022_03 OBAP(a) sc f B1.1.1 DAPI-GFP488-IL33647-TH546 x20 280422 93Z.czi");
30 run("Z Project...", "projection=[Sum Slices]");
31 run("Split Channels");
32 selectWindow("C2-SUM_CCZ2022_03 OBAP(a) sc f B1.1.1 DAPI-GFP488-IL33647-TH546 x20 280422 93Z.czi");
33 run("Subtract...", "value=2167");
34 run("Clear Results");
35 roiManager("Measure");
36 //close("*");
37 //run("Close All");

```

Figure 4: Macro for segmentation and measurement of nuclear IL-33 fluorescence intensity

This Macro relates to the image analysis pipeline shown in Figure 2.5. Macros were written by Chloe Zentai.

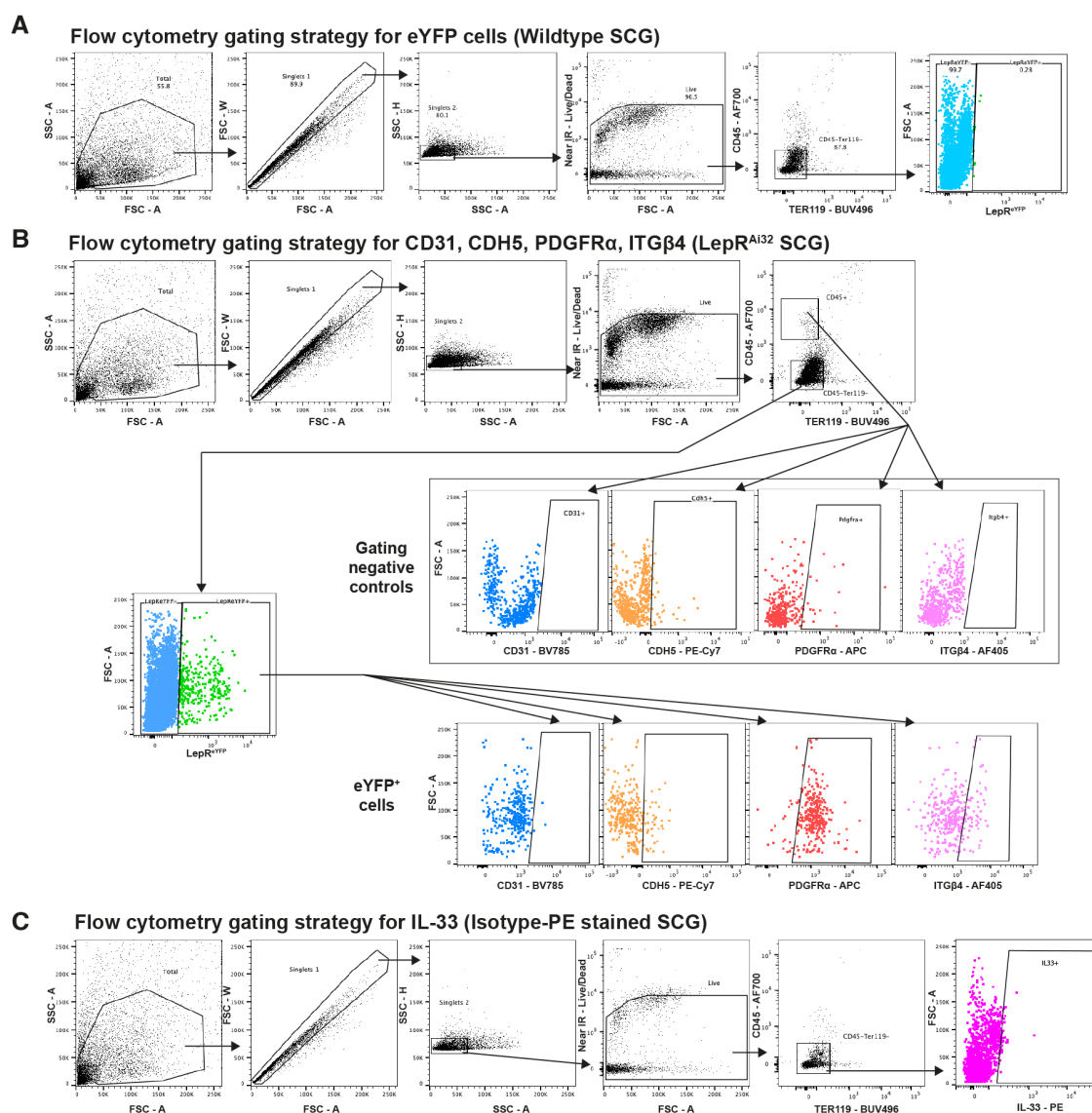


Figure 5: Full stromal cell flow cytometric gating strategies

A Full flow cytometric gating strategy for eYFP⁺ Sympathetic Associated Perineurial Cell (SAPC) phenotyping in sympathetic tissues. Fully stained wildtype Superior Cervical Ganglia (SCG) sample used to set eYFP⁺ gate. **B** Flow cytometric gating strategy for CD31, CDH5, PDGFR α and ITG β 4. CD45⁺ cell populations, negative for all markers, were used as negative controls to set gates. LepR^{AI32} SCG, fully stained. **C** Flow cytometric gating strategy for IL-33 staining of eYFP⁺ SAPCs. LepR^{AI32} SCG with full surface marker stain and isotype control-PE intracellular stain. Isotype control stained samples were used to set IL-33⁺ gates.

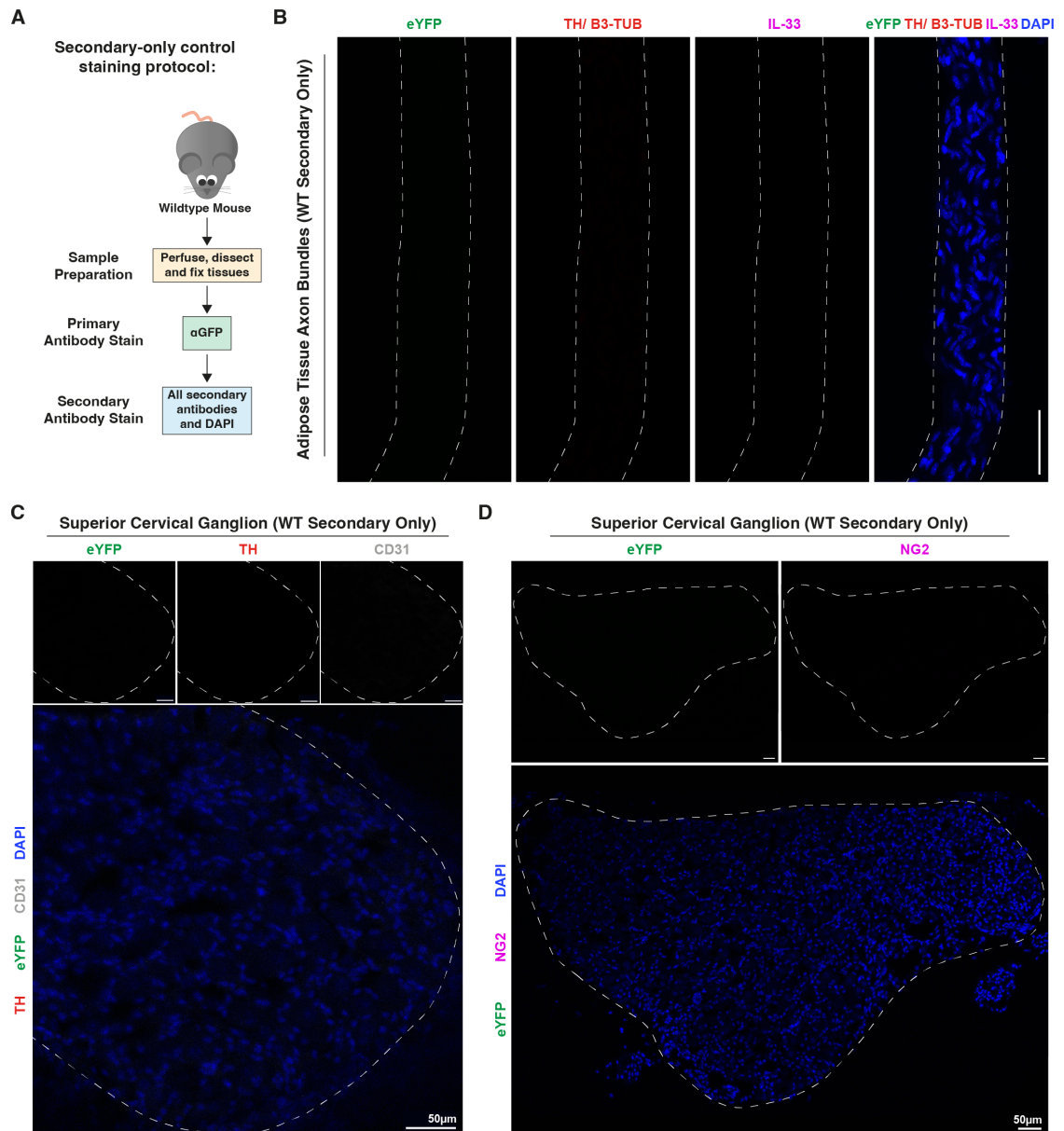


Figure 6: Immunofluorescence secondary antibody only stained controls

A Schematic diagram of secondary antibody stained controls. For the most stringent control of true eYFP staining, wildtype tissues lacking the eYFP protein were stained with an anti-GFP primary antibody (which also binds eYFP), before full DAPI and secondary antibody staining. **B** Whole-mount axon bundles dissected from the scWAT of wildtype mice, stained with anti-GFP primary antibody, DAPI and all secondary antibodies against eYFP (green), TH/B3-TUB (red) and IL-33 (magenta). **C-D** Wildtype Superior Cervical Ganglion section, stained with anti-GFP primary antibodies, DAPI and all secondary antibodies against **C**: eYFP (green), TH (red), CD31 (grey). and **D**: eYFP (green), NG2 (magenta). Scale bar represents 50 μ m.

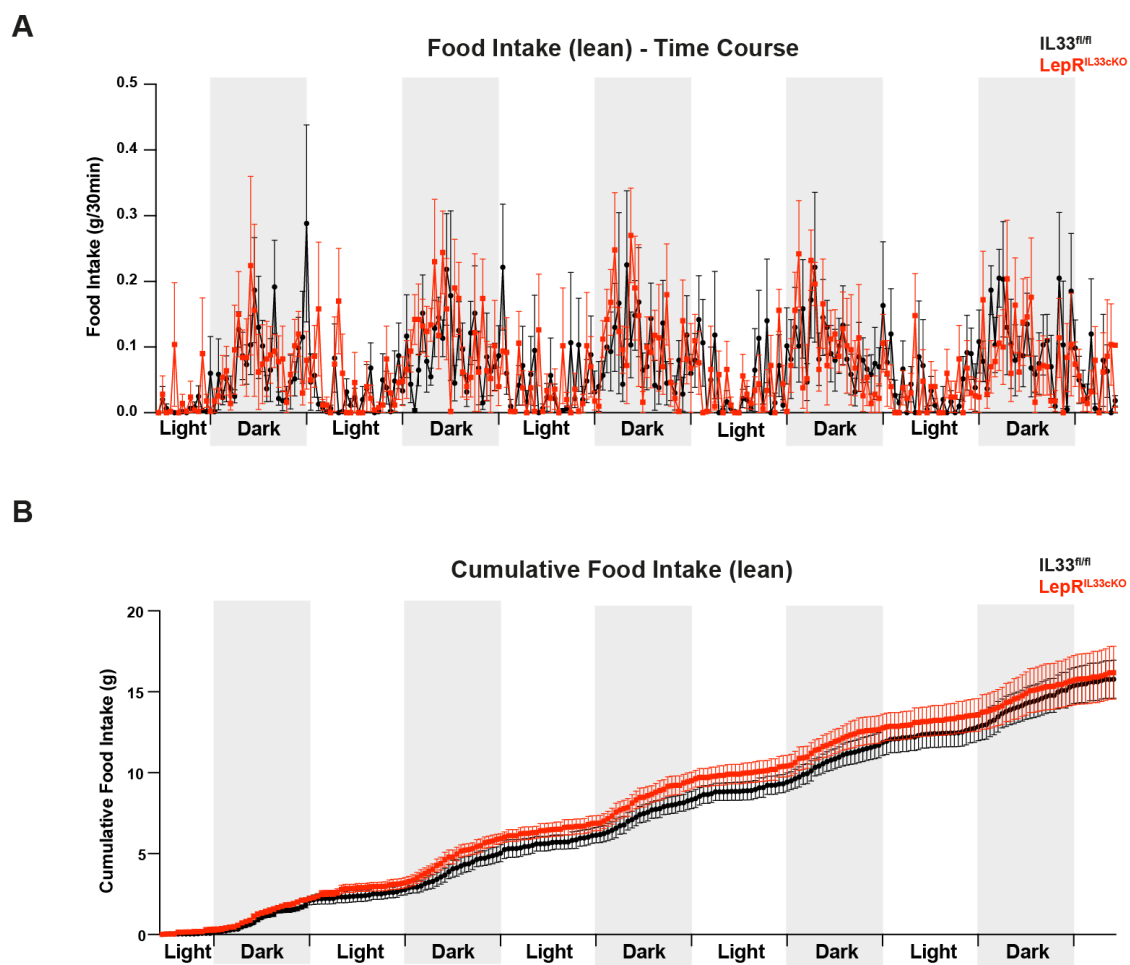


Figure 7: IL33^{fl/fl} and LepR^{IL33cKO} food intake over time

A-B Food intake (g/30min, A) and cumulative food intake (g, B) of lean (normal diet-fed) IL33^{fl/fl} and LepR^{IL33cKO} mice, measured over 5 days. n=5-6, 1 independent experiment.

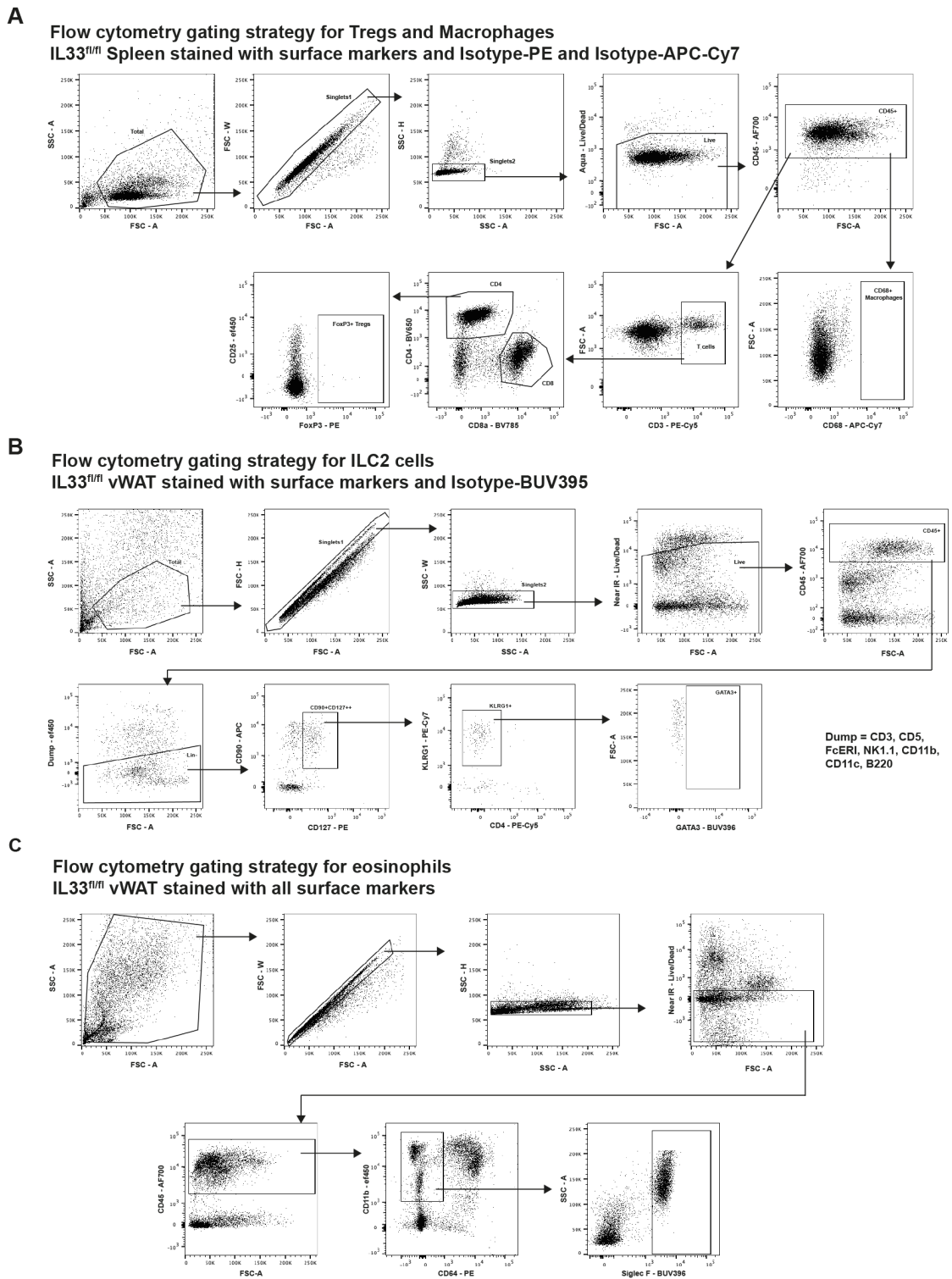


Figure 8: Full immune cell flow cytometric gating strategies

A Full flow cytometric gating strategy for regulatory T cells (Tregs) and macrophages. IL33^{fl/fl} spleen stained with all surface markers and isotype-PE and isotype-APC-Cy7 controls instead of intracellular FoxP3-PE and CD68-APC-Cy7, used to set FoxP3⁺ and CD68⁺ gates. **B** Flow cytometric gating strategy for ILC2 cells. IL33^{fl/fl} vWAT stained with all surface markers and isotype-BUV396 instead of GATA3-BUV396, used to set GATA3⁺ gate. **C** Flow cytometric gating strategy for eosinophils. IL33^{fl/fl} vWAT stained with all surface markers. Clear Siglec F⁺ population used to set Siglec F⁺ gate.

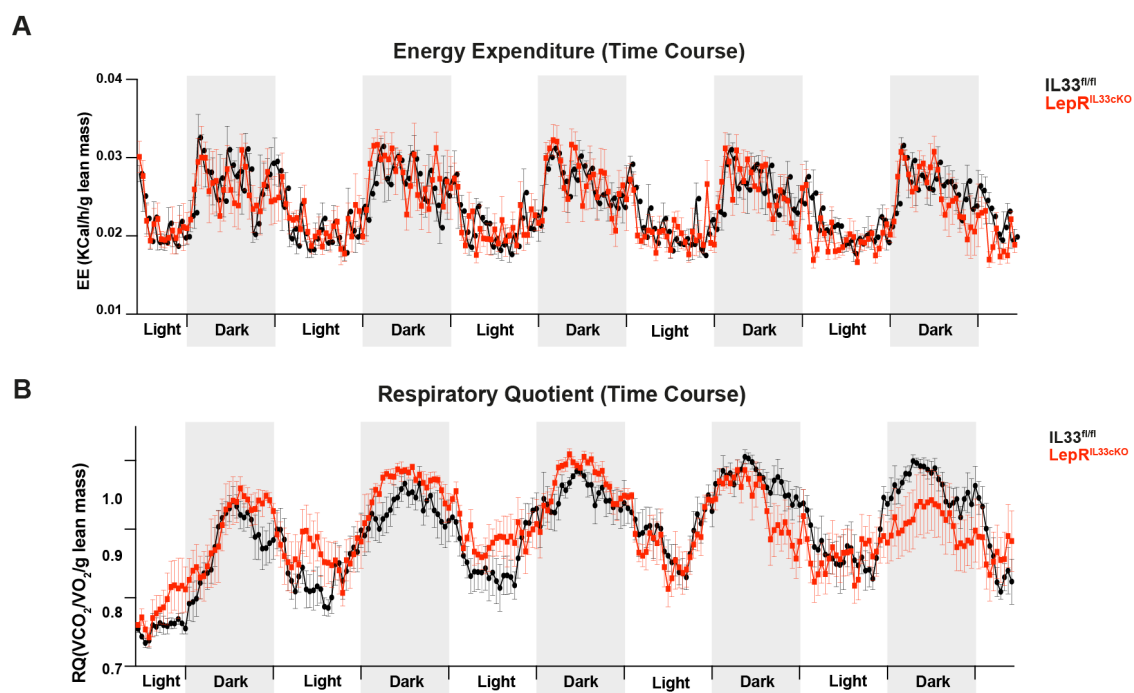


Figure 9: Energy Expenditure and Respiratory Quotient time course of lean, unchallenged IL33^{fl/fl} and LepR^{IL33cKO} mice

A-B Energy Expenditure (EE, A) and Respiratory quotient (RQ, B) are comparable between IL33^{fl/fl} and LepR^{IL33cKO} mice, measured over a 5-day period. EE and RQ normalised to lean mass. n=5-6, 1 independent experiment. Data presented as Mean+/-SEM. Student's t-test, ns = non-significant.

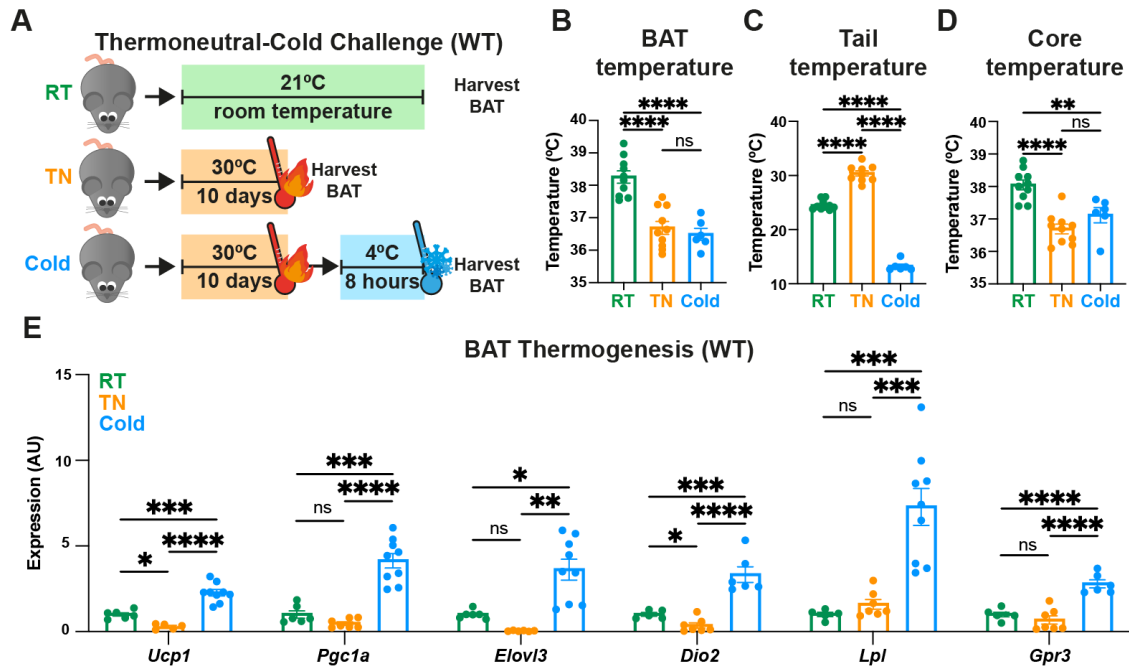


Figure 10: Thermoneutral-cold challenge protocol significantly induces expression of thermogenic genes compared with room temperature mice

A Schematic of the experimental procedure. RT = room temperature, TN = thermoneutral, Cold = thermoneutral + 8h cold challenge. **B-D** BAT (B), tail (C) and core temperature (D) are reduced in thermoneutral-cold challenged wildtype mice compared with room temperature controls (21). 2 independent experiments, n=10 (RT, TN), n=6 (cold). **E** Expression of thermogenic genes is higher in WT BAT that has undergone thermoneutral-cold challenge compared with room temperature (21) controls. qPCR of BAT from wildtype mice which have been housed at room temperature (green), exposed to thermoneutrality (orange) or have undergone a thermoneutral-cold challenge (blue). For each gene, data is presented relative to wildtype BAT room temperature expression (=1). *Ucp1* = Uncoupled protein 1, *Pgc1a* = Peroxisome proliferator- activated receptor gamma coactivator 1-alpha, *Elovl3* = Elovl3 fatty acid elongase 3, *Dio2* = Iodothyronine Deiodinase 2, *Lpl* = Lipoprotein lipase, *Gpr3* = G-protein coupled receptor 3. n=6 (RT), n=7 (TH), n=9 (cold), 2 independent experiments. Data presented as Mean+/-SEM. 2-way ANOVA with Šidak correction for multiple comparisons. ns = non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

References

- [1] Conan J.O. O'Brien, Emma R. Haberman, and Ana I. Domingos. "A Tale of Three Systems: Toward a Neuroimmunoendocrine Model of Obesity". In: *Annual review of cell and developmental biology* 37 (2021), pp. 549–573. URL: <https://pubmed.ncbi.nlm.nih.gov/34613819/>.
- [2] Emma R. Haberman et al. "Immunomodulatory leptin receptor+ sympathetic perineurial barrier cells protect against obesity by facilitating brown adipose tissue thermogenesis". In: *Immunity* (Dec. 2023). URL: <https://linkinghub.elsevier.com/retrieve/pii/S1074761323004880>.
- [3] *Obesity and overweight*. URL: <https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight>.
- [4] Yiyang Zhang et al. "Positional cloning of the mouse obese gene and its human homologue". In: *Nature* 372.6505 (Dec. 1994), pp. 425–432.
- [5] Wenwen Zeng et al. "Sympathetic Neuro-adipose Connections Mediate Leptin-Driven Lipolysis". In: *Cell* 163.1 (2015), pp. 84–94. URL: <https://pubmed.ncbi.nlm.nih.gov/26406372/>.
- [6] Dinh Toi Chu et al. *An update on physical health and economic consequences of overweight and obesity*. Nov. 2018.
- [7] Mathilde Versini et al. *Obesity in autoimmune diseases: Not a passive bystander*. 2014.
- [8] Chelsea M Larabee, Oliver C Neely, and Ana I Domingos. *Obesity: a neuroimmunometabolic perspective*. 2020. URL: www.nature.com/nrendo.
- [9] Giamila Fantuzzi. *Adiponectin in inflammatory and immune-mediated diseases*. Oct. 2013.
- [10] John N Fain 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". In: *Endocrinology* 145.5 (May 2004), pp. 2273–2282.
- [11] Jeffrey M Friedman. *Leptin and the endocrine control of energy balance*. Aug. 2019.
- [12] Erin E Kershaw and Jeffrey S Flier. "Adipose Tissue as an Endocrine Organ". In: *The Journal of Clinical Endocrinology & Metabolism* 89.6 (June 2004), pp. 2548–2556.
- [13] Brooks P. Leitner et al. "Mapping of human brown adipose tissue in lean and obese young men". In: *Proceedings of the National Academy of Sciences of the United States of America* 114.32 (Aug. 2017), pp. 8649–8654. URL: <https://www.pnas.org/doi/abs/10.1073/pnas.1705287114>.
- [14] Caroline M. Pond. "An evolutionary and functional view of mammalian adipose tissue". In: *Proceedings of the Nutrition Society* 51.3 (Dec. 1992), pp. 367–377. URL: <https://www.cambridge.org/core/journals/proceedings-of-the-nutrition-society/article/an-evolutionary-and-functional-view-of-mammalian-adipose-tissue/C5E8F054C9197E4FE828372A9E01B6EE>.

- [15] Matthew J. Harms et al. "Prdm16 is required for the maintenance of brown adipocyte identity and function in adult mice". In: *Cell metabolism* 19.4 (Apr. 2014), p. 593. URL: [/pmc/articles/PMC4012340/%20/pmc/articles/PMC4012340/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4012340/](https://pubmed.ncbi.nlm.nih.gov/24812340/).
- [16] Johannes Rafael, Ludolph HJ, and Hans-Jürgen Hohorst. "[Mitochondria from brown adipose tissue: uncoupling of respiratory chain phosphorylation by long fatty acids and recoupling by guanosine triphosphate]". In: *Hoppe-Seyler's Zeitschrift für physiologische Chemie* (1969).
- [17] Gillian M. HEATON et al. "Brown-adipose-tissue mitochondria: photoaffinity labelling of the regulatory site of energy dissipation". In: *European journal of biochemistry* 82.2 (1978), pp. 515–521. URL: <https://pubmed.ncbi.nlm.nih.gov/624284/>.
- [18] C. S. Lin and M. Klingenberg. "Isolation of the uncoupling protein from brown adipose tissue mitochondria". In: *FEBS letters* 113.2 (May 1980), pp. 299–303. URL: <https://pubmed.ncbi.nlm.nih.gov/7389900/>.
- [19] Valeria Golozoubova et al. "Only UCP1 can mediate adaptive nonshivering thermogenesis in the cold". In: *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 15.11 (Sept. 2001), pp. 2048–2050. URL: <https://pubmed.ncbi.nlm.nih.gov/11511509/>.
- [20] Luciano Pirola and José Candido Ferraz. "Role of pro- and anti-inflammatory phenomena in the physiopathology of type 2 diabetes and obesity." In: *World journal of biological chemistry* 8.2 (May 2017), pp. 120–128. URL: <http://www.ncbi.nlm.nih.gov/pubmed/28588755%20http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5439163>.
- [21] Jan Nedergaard, Tore Bengtsson, and Barbara Cannon. "Unexpected evidence for active brown adipose tissue in adult humans". In: *American Journal of Physiology - Endocrinology and Metabolism* 293.2 (Aug. 2007), pp. 444–452. URL: <https://journals.physiology.org/doi/10.1152/ajpendo.00691.2006>.
- [22] Aaron M. Cypess et al. "Identification and Importance of Brown Adipose Tissue in Adult Humans". In: *New England Journal of Medicine* 360.15 (Apr. 2009), pp. 1509–1517. URL: <https://www.nejm.org/doi/full/10.1056/NEJMoa0810780>.
- [23] Kirsi A. Virtanen et al. "Functional Brown Adipose Tissue in Healthy Adults". In: *New England Journal of Medicine* 360.15 (Apr. 2009), pp. 1518–1525. URL: <https://www.nejm.org/doi/full/10.1056/NEJMoa0808949>.
- [24] M D BOGDONOFF, A M WEISSLER, and F L MERRITT. "The effect of autonomic ganglionic blockade upon serum free fatty acid levels in man." In: *The Journal of clinical investigation* 39.6 (1960), pp. 959–965. URL: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC441843/>.
- [25] James W Correll. "Adipose tissue: Ability to respond to nerve stimulation in vitro". In: *Science* 140.3565 (1963), pp. 387–388. URL: <http://science.sciencemag.org/content/140/3565/387#BIBL>.
- [26] C J Goodner et al. "Studies of substrate regulation in fasting. I. Evidence for central regulation of lipolysis by plasma glucose mediated by the sympathetic nervous system." In: *Diabetes* 16.8 (1967), pp. 576–589. URL: <https://pubmed.ncbi.nlm.nih.gov/6041186/>.

- [27] Dragutin Lončar. "Convertible adipose tissue in mice". In: *Cell and Tissue Research* 266.1 (Oct. 1991), pp. 149–161. URL: <https://link.springer.com/article/10.1007/BF00678721>.
- [28] Qiang Cao et al. "Sympathetic nerve innervation is required for beigeing in white fat". In: *Physiological reports* 7.6 (Mar. 2019). URL: <https://pubmed.ncbi.nlm.nih.gov/30873754/>.
- [29] Haochen Jiang et al. "Dense Intra-adipose Sympathetic Arborizations Are Essential for Cold-Induced Beiging of Mouse White Adipose Tissue". In: *Cell Metabolism* 26.4 (2017), 686–692.e3. URL: <https://pubmed.ncbi.nlm.nih.gov/28918935/>.
- [30] Jun Wu et al. "Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human". In: *Cell* 150.2 (July 2012), pp. 366–376. URL: <https://pubmed.ncbi.nlm.nih.gov/22796012/>.
- [31] Eric D. Green et al. "The human obese (OB) gene: RNA expression pattern and mapping on the physical, cytogenetic, and genetic maps of chromosome 7". In: *Genome research* 5.1 (1995), pp. 5–12. URL: <https://pubmed.ncbi.nlm.nih.gov/8717050/>.
- [32] Ann M. Ingalls, Margaret M. Dickie, and G. D. Snell. "Obese, a new mutation in the house mouse". In: *The Journal of heredity* 41.12 (1950), pp. 317–318. URL: <https://pubmed.ncbi.nlm.nih.gov/14824537/>.
- [33] Noelia Martinez-Sanchez et al. "The sympathetic nervous system in the 21st century: Neuroimmune interactions in metabolic homeostasis and obesity". In: *Neuron* 110.21 (Nov. 2022), pp. 3597–3626. URL: [http://www.cell.com/article/S0896627322009461/fulltext%20http://www.cell.com/article/S0896627322009461/abstract%20https://www.cell.com/neuron/abstract/S0896-6273\(22\)00946-1](http://www.cell.com/article/S0896627322009461/fulltext%20http://www.cell.com/article/S0896627322009461/abstract%20https://www.cell.com/neuron/abstract/S0896-6273(22)00946-1).
- [34] Bo Åhrén. "Plasma leptin and insulin in C57Bl/6J mice on a high-fat diet: Relation to subsequent changes in body weight". In: *Acta Physiologica Scandinavica* 165.2 (1999), pp. 233–240.
- [35] M Maffei et al. "Leptin levels in human and rodent: Measurement of plasma leptin and ob RNA in obese and weight-reduced subjects". In: *Nature Medicine* 1.11 (Nov. 1995), pp. 1155–1161.
- [36] L Arthur Campfield et al. "Recombinant mouse OB protein: Evidence for a peripheral signal linking adiposity and central neural networks". In: *Science* 269.5223 (1995), pp. 546–549.
- [37] Mary Ann Pelleymounter et al. "Effects of the obese gene product on body weight regulation in ob/ob mice". In: *Science* 269.5223 (1995), pp. 540–543. URL: <https://pubmed.ncbi.nlm.nih.gov/7624776/>.
- [38] Jeffrey L Halaas et al. "Weight-reducing effects of the plasma protein encoded by the obese gene". In: *Science* 269.5223 (1995), pp. 543–546.
- [39] Denis G. Baskin, John F. Breininger, and Michael W. Schwartz. "Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus". In: *Diabetes* 48.4 (1999), pp. 828–833. URL: <https://pubmed.ncbi.nlm.nih.gov/10102700/>.

- [40] Julian G. Mercer et al. "Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization". In: *FEBS letters* 387.2-3 (June 1996), pp. 113–116. URL: <https://pubmed.ncbi.nlm.nih.gov/8674530/>.
- [41] Julie E McMinn et al. "Neuronal deletion of *Lepr* elicits diabetes in mice without affecting cold tolerance or fertility". In: *American Journal of Physiology-Endocrinology and Metabolism* 289.3 (Sept. 2005), E403–E411.
- [42] Metin Ozata, I Caglayan Ozdemir, and Julio Licinio. "Human Leptin Deficiency Caused by a Missense Mutation: Multiple Endocrine Defects, Decreased Sympathetic Tone, and Immune System Dysfunction Indicate New Targets for Leptin Action, Greater Central than Peripheral Resistance to the Effects of Leptin, and S". In: *The Journal of Clinical Endocrinology & Metabolism* 84.10 (Oct. 1999), pp. 3686–3695.
- [43] Nancy Levin et al. "Decreased food intake does not completely account for adiposity reduction after ob protein infusion." In: *Proceedings of the National Academy of Sciences* 93.4 (Feb. 1996), pp. 1726–1730. URL: <https://www.pnas.org/doi/abs/10.1073/pnas.93.4.1726>.
- [44] Johannes Rafael and Andreas W. Herling. "Leptin effect in ob/ob mice under thermoneutral conditions depends not necessarily on central satiation". In: *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 278.3 47-3 (2000). URL: <https://journals.physiology.org/doi/10.1152/ajpregu.2000.278.3.R790>.
- [45] Xavier Prieur et al. "Leptin Regulates Peripheral Lipid Metabolism Primarily through Central Effects on Food Intake". In: *Endocrinology* 149.11 (Nov. 2008), pp. 5432–5439.
- [46] Martin G Myers and David P Olson. "Central nervous system control of metabolism". In: *Nature* 491.7424 (2012), pp. 357–363. URL: <http://www.nature.com/articles/nature11705>.
- [47] Heike Münzberg, Jeffrey S Flier, and Christian Bjørbæk. "Region-specific leptin resistance within the hypothalamus of diet-induced obese mice". In: *Endocrinology* 145.11 (2004), pp. 4880–4889. URL: <https://pubmed.ncbi.nlm.nih.gov/15271881/>.
- [48] Michael M Scott et al. "Leptin targets in the mouse brain". In: *Journal of Comparative Neurology* 514.5 (2009), pp. 518–532. URL: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2710238/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2710238/>.
- [49] Marie Louise Håkansson et al. "Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus". In: *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18.1 (1998), pp. 559–572. URL: <https://pubmed.ncbi.nlm.nih.gov/9412531/>.
- [50] Carol F. Elias et al. "Leptin Activates Hypothalamic CART Neurons Projecting to the Spinal Cord". In: *Neuron* 21.6 (Dec. 1998), pp. 1375–1385.
- [51] Heike Münzberg et al. "Role of signal transducer and activator of transcription 3 in regulation of hypothalamic Proopiomelanocortin gene expression by leptin". In: *Endocrinology* 144.5 (2003), pp. 2121–2131. URL: <https://pubmed.ncbi.nlm.nih.gov/12697721/>.

- [52] Tadahiro Kitamura et al. "Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake". In: *Nature Medicine* 12.5 (2006), pp. 534–540. URL: <https://pubmed.ncbi.nlm.nih.gov/16604086/>.
- [53] Judith Korner et al. "Regulation of hypothalamic proopiomelanocortin by leptin in lean and obese rats". In: *Neuroendocrinology* 70.6 (1999), pp. 377–383. URL: <https://pubmed.ncbi.nlm.nih.gov/10657730/>.
- [54] Judith Korner et al. "Leptin regulation of Agrp and Npy mRNA in the rat hypothalamus". In: *Journal of Neuroendocrinology* 13.11 (2001), pp. 959–966. URL: <https://pubmed.ncbi.nlm.nih.gov/11737554/>.
- [55] Michael A Cowley et al. "Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus". In: *Nature* 411.6836 (2001), pp. 480–484. URL: <https://pubmed.ncbi.nlm.nih.gov/11373681/>.
- [56] Michael W. Schwartz et al. "Identification of targets of leptin action in rat hypothalamus". In: *Journal of Clinical Investigation* 98.5 (Sept. 1996), pp. 1101–1106. URL: <https://pubmed.ncbi.nlm.nih.gov/pmc/articles/PMC507530/>.
- [57] Michael W Schwartz et al. "Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus". In: *Diabetes* 46.12 (1997), pp. 2119–2123. URL: <https://pubmed.ncbi.nlm.nih.gov/9392508/>.
- [58] Thomas W Stephens et al. *The role of neuropeptide Y in the antiobesity action of the obese gene product*. 1995. URL: <https://www.nature.com/articles/377530a0>.
- [59] William G Haynes et al. "Receptor-mediated regional sympathetic nerve activation by leptin". In: *Journal of Clinical Investigation* 100.2 (1997), pp. 270–278. URL: <https://pubmed.ncbi.nlm.nih.gov/pmc/articles/PMC508189/>.
- [60] H Kannan, Y Hayashida, and H Yamashita. "Increase in sympathetic outflow by paraventricular nucleus stimulation in awake rats". In: *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 256.6 (1989). URL: <https://pubmed.ncbi.nlm.nih.gov/2567578/>.
- [61] Sarah F Leibowitz, Norman J Hammer, and Kevin Chang. "Hypothalamic paraventricular nucleus lesions produce overeating and obesity in the rat". In: *Physiology and Behavior* 27.6 (1981), pp. 1031–1040.
- [62] Philip J Scarpace and Michael Matheny. "Leptin induction of UCP1 gene expression is dependent on sympathetic innervation". In: *American Journal of Physiology-Endocrinology and Metabolism* 275.2 (1998), E259–E264. URL: <https://www.physiology.org/doi/10.1152/ajpendo.1998.275.2.E259>.
- [63] Putianqi Wang et al. "A leptin–BDNF pathway regulating sympathetic innervation of adipose tissue". In: *Nature* 583.7818 (2020), pp. 839–844. URL: <https://pubmed.ncbi.nlm.nih.gov/32699414/>.
- [64] Nina Balthasar et al. "Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis". In: *Neuron* 42.6 (2004), pp. 983–991. URL: <https://pubmed.ncbi.nlm.nih.gov/15207242/>.

- [65] Esther Van De Wall et al. "Collective and individual functions of leptin receptor modulated neurons controlling metabolism and ingestion". In: *Endocrinology* 149.4 (Apr. 2008), pp. 1773–1785. URL: <https://pubmed.ncbi.nlm.nih.gov/18162515/>.
- [66] C E Koch et al. "High-fat diet induces leptin resistance in leptin-deficient mice". In: *Journal of Neuroendocrinology* 26.2 (2014), pp. 58–67. URL: <https://pubmed.ncbi.nlm.nih.gov/24382295/>.
- [67] Martin G. Myers et al. *Challenges and opportunities of defining clinical leptin resistance*. Feb. 2012. URL: [/pmc/articles/PMC3281561/%20/pmc/articles/PMC3281561/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3281561/](https://pubmed.ncbi.nlm.nih.gov/22511111/).
- [68] I Sadaf Farooqi et al. "Effects of Recombinant Leptin Therapy in a Child with Congenital Leptin Deficiency". In: *New England Journal of Medicine* 341.12 (1999), pp. 879–884. URL: <http://www.nejm.org/doi/abs/10.1056/NEJM199909163411204>.
- [69] Zachary A Knight et al. "Hyperleptinemia is required for the development of leptin resistance". In: *PLoS ONE* 5.6 (2010). URL: <https://pubmed.ncbi.nlm.nih.gov/20613882/>.
- [70] Christian Bjørbæk et al. "The role of SOCS-3 in leptin signaling and leptin resistance". In: *Journal of Biological Chemistry* 274.42 (1999), pp. 30059–30065. URL: <https://pubmed.ncbi.nlm.nih.gov/10514492/>.
- [71] Kevin M Gamber et al. "Over-expression of leptin receptors in hypothalamic POMC neurons increases susceptibility to diet-induced obesity". In: *PLoS ONE* 7.1 (2012). URL: <https://pubmed.ncbi.nlm.nih.gov/22276206/>.
- [72] Louise E Olofsson et al. "Modulation of AgRP-neuronal function by SOCS3 as an initiating event in diet-induced hypothalamic leptin resistance". In: *Proceedings of the National Academy of Sciences of the United States of America* 110.8 (2013). URL: <https://pubmed.ncbi.nlm.nih.gov/23386726/>.
- [73] Zheng Juan Liu et al. "Lentiviral vector-mediated knockdown of SOCS3 in the hypothalamus protects against the development of diet-induced obesity in rats". In: *Diabetes, Obesity and Metabolism* 13.10 (2011), pp. 885–892. URL: <https://pubmed.ncbi.nlm.nih.gov/21554522/>.
- [74] Gemma E White et al. "Suppressor of cytokine signalling protein SOCS3 expression is increased at sites of acute and chronic inflammation". In: *Journal of Molecular Histology* 42.2 (2011), pp. 137–151. URL: [/pmc/articles/PMC3070874/%20/pmc/articles/PMC3070874/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3070874/](https://pubmed.ncbi.nlm.nih.gov/21554522/).
- [75] Lale Ozcan et al. "Endoplasmic Reticulum Stress Plays a Central Role in Development of Leptin Resistance". In: *Cell Metabolism* 9.1 (Jan. 2009), pp. 35–51. URL: <https://pubmed.ncbi.nlm.nih.gov/19117545/>.
- [76] Junli Liu et al. "Treatment of Obesity with Celastrol Article Treatment of Obesity with Celastrol". In: *Cell* 161 (2015), pp. 999–1011. URL: <http://dx.doi.org/10.1016/j.cell.2015.05.011><http://dx.doi.org/10.1016/j.cell.2015.05.011>.

- [77] Cláudio T. De Souza et al. "Consumption of a Fat-Rich Diet Activates a Proinflammatory Response and Induces Insulin Resistance in the Hypothalamus". In: *Endocrinology* 146.10 (Oct. 2005), pp. 4192–4199. URL: <https://academic.oup.com/endo/article-lookup/doi/10.1210/en.2004-1520>.
- [78] Joshua P Thaler et al. *Hypothalamic inflammation: Marker or mechanism of obesity pathogenesis?* 2013. URL: <https://pubmed.ncbi.nlm.nih.gov/23881189/>.
- [79] Kezhong Zhang and Randal J Kaufman. *From endoplasmic-reticulum stress to the inflammatory response*. 2008. URL: <https://pubmed.ncbi.nlm.nih.gov/18650916/>.
- [80] Karim El-Haschimi et al. "Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity". In: *Journal of Clinical Investigation* 105.12 (2000), pp. 1827–1832. URL: <https://pubmed.ncbi.nlm.nih.gov/10862798/>.
- [81] Lawrence M Maness, William A Banks, and Abba J Kastin. "Persistence of blood-to-brain transport of leptin in obese leptin-deficient and leptin receptor-deficient mice". In: *Brain Research* 873.1 (2000), pp. 165–167. URL: <https://pubmed.ncbi.nlm.nih.gov/10915826/>.
- [82] Michael W Schwartz et al. "Cerebrospinal fluid leptin levels: Relationship to plasma levels and to adiposity in humans". In: *Nature Medicine* 2.5 (1996), pp. 589–593. URL: <https://pubmed.ncbi.nlm.nih.gov/8616722/>.
- [83] Luke Harrison et al. "Fluorescent blood–brain barrier tracing shows intact leptin transport in obese mice". In: *International Journal of Obesity* 43.6 (2019), pp. 1305–1318. URL: <https://doi.org/10.1038/s41366-018-0221-z>.
- [84] Shinsuke Oh-I et al. "Molecular mechanisms associated with leptin resistance: n-3 polyunsaturated fatty acids induce alterations in the tight junction of the brain". In: *Cell Metabolism* 1.5 (2005), pp. 331–341. URL: <https://pubmed.ncbi.nlm.nih.gov/16054080/>.
- [85] Giovanni Ceccarini et al. "PET Imaging of Leptin Biodistribution and Metabolism in Rodents and Primates". In: *Cell Metabolism* 10.2 (Aug. 2009), pp. 148–159. URL: <https://pubmed.ncbi.nlm.nih.gov/19656493/>.
- [86] Shangang Zhao et al. "Partial Leptin Reduction as an Insulin Sensitization and Weight Loss Strategy". In: *Cell Metabolism* 30.4 (Oct. 2019), 706–719.e6. URL: <https://pubmed.ncbi.nlm.nih.gov/31495688/>.
- [87] Jung Dae Kim and Sabrina Diano. "A Sympathetic Treatment for Obesity". In: *Cell Metabolism* 31.6 (June 2020), pp. 1043–1045.
- [88] Inês Mahú et al. "Brain-Sparing Sympathofacilitators Mitigate Obesity without Adverse Cardiovascular Effects". In: *Cell Metabolism* 31.6 (June 2020), 1120–1135.e7.
- [89] Alan Morris. *Unravelling novel weight loss mechanisms*. July 2020. URL: <https://doi.org/10.1016/j.cmet.2020.04.013>.
- [90] D J Galton and G A Bray. "Studies on lipolysis in human adipose cells." In: *The Journal of clinical investigation* 46.4 (1967), pp. 621–629.
- [91] Robert R Bowers et al. "Sympathetic innervation of white adipose tissue and its regulation of fat cell number". In: *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 286.6 55-6 (2004). URL: <https://pubmed.ncbi.nlm.nih.gov/15142857/>.

- [92] B Fredholm and S Rosell. "Effects of adrenergic blocking agents on lipid mobilization from canine subcutaneous adipose tissue after sympathetic nerve stimulation." In: *Journal of Pharmacology and Experimental Therapeutics* 159.1 (1968), pp. 1–7.
- [93] M Klingenspor et al. "Effect of unilateral surgical denervation of brown adipose tissue on uncoupling protein mRNA level and cytochrom-c-oxidase activity in the Djungarian hamster". In: *Journal of Comparative Physiology B* 163.8 (1994), pp. 664–670. URL: <https://pubmed.ncbi.nlm.nih.gov/8195470/>.
- [94] Ying Cao, Huanhuan Wang, and Wenwen Zeng. "Whole-tissue 3D imaging reveals intra-adipose sympathetic plasticity regulated by NGF-TrkA signal in cold-induced beiging". In: *Protein and Cell* 9.6 (2018), pp. 527–539. URL: <https://pubmed.ncbi.nlm.nih.gov/29589323/>.
- [95] Jingyi Chi et al. "Adipo-Clear: A Tissue Clearing Method for Three-Dimensional Imaging of Adipose Tissue". In: *Journal of Visualized Experiments : JoVE* 2018.137 (July 2018), p. 58271. URL: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6126572/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6126572/>.
- [96] Maryam Bamshad et al. "Central nervous system origins of the sympathetic nervous system outflow to white adipose tissue". In: *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 275.1 44-1 (1998). URL: <https://pubmed.ncbi.nlm.nih.gov/9688991/>.
- [97] Maryam Bamshad, C Kay Song, and Timothy J Bartness. "CNS origins of the sympathetic nervous system outflow to brown adipose tissue". In: *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 276.6 45-6 (1999). URL: <https://pubmed.ncbi.nlm.nih.gov/10362733/>.
- [98] Clara Huesing et al. "Sympathetic innervation of inguinal white adipose tissue in the mouse". In: *Journal of Comparative Neurology* (2020), cne.25031. URL: <https://onlinelibrary.wiley.com/doi/10.1002/cne.25031>.
- [99] Ngoc Ly T Nguyen et al. "Central sympathetic innervations to visceral and subcutaneous white adipose tissue". In: *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 306.6 (2014). URL: <https://pubmed.ncbi.nlm.nih.gov/24452544/>.
- [100] Mafalda M A Pereira et al. "A brain-sparing diphtheria toxin for chemical genetic ablation of peripheral cell lineages". In: *Nature Communications* 8.1 (2017), pp. 1–11. URL: www.nature.com/naturecommunications.
- [101] T G Youngstrom and T J Bartness. "Catecholaminergic innervation of white adipose tissue in Siberian hamsters". In: *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 268.3 37-3 (1995).
- [102] Michael A Patterson, Erzsebet M Szatmari, and Ryohei Yasuda. "AMPA receptors are exocytosed in stimulated spines and adjacent dendrites in a Ras-ERK-dependent manner during long-term potentiation". In: *Proceedings of the National Academy of Sciences of the United States of America* 107.36 (2010), pp. 15951–15956. URL: www.pnas.org/cgi/doi/10.1073/pnas.0913875107.
- [103] Karel Svoboda. *Using rabies virus for tracing neural connections: caveats and limitations exposed by studies of barrel cortex circuits*. 2019.

- [104] Marie François et al. "Sympathetic innervation of the interscapular brown adipose tissue in mouse". In: *Annals of the New York Academy of Sciences* 1454.1 (2019), pp. 3–13. URL: [/pmc/articles/PMC6810755/%20/pmc/articles/PMC6810755/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6810755/](https://pmc/articles/PMC6810755/%20/pmc/articles/PMC6810755/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6810755/).
- [105] Shaun F Morrison, Sheela Ramamurthy, and James B Young. "Reduced rearing temperature augments responses in sympathetic outflow to brown adipose tissue". In: *Journal of Neuroscience* 20.24 (2000), pp. 9264–9271. URL: <https://www.jneurosci.org/content/20/24/9264%20https://www.jneurosci.org/content/20/24/9264.abstract>.
- [106] Antonio Giordano et al. "White adipose tissue lacks significant vagal innervation and immunohistochemical evidence of parasympathetic innervation". In: *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 291.5 (2006). URL: <https://pubmed.ncbi.nlm.nih.gov/16809481/>.
- [107] Jingyi Chi et al. "Three-Dimensional Adipose Tissue Imaging Reveals Regional Variation in Beige Fat Biogenesis and PRDM16-Dependent Sympathetic Neurite Density". In: *Cell Metabolism* 27.1 (2018), 226–236.e3.
- [108] C Kay Song, Gary J Schwartz, and Timothy J Bartness. "Anterograde transneuronal viral tract tracing reveals central sensory circuits from white adipose tissue". In: *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 296.3 (2009), pp. 501–511.
- [109] Akira Nijima. "Afferent signals from leptin sensors in the white adipose tissue of the epididymis, and their reflex effect in the rat". In: *Journal of the Autonomic Nervous System* 73.1 (1998), pp. 19–25. URL: <https://pubmed.ncbi.nlm.nih.gov/9808367/>.
- [110] John T Garretson et al. "Lipolysis sensation by white fat afferent nerves triggers brown fat thermogenesis". In: *Molecular Metabolism* 5.8 (2016), pp. 626–634. URL: <https://pubmed.ncbi.nlm.nih.gov/27656400/>.
- [111] Haifei Shi and Timothy J Bartness. "White adipose tissue sensory nerve denervation mimics lipectomy-induced compensatory increases in adiposity". In: *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 289.2 58-2 (2005). URL: <https://pubmed.ncbi.nlm.nih.gov/15860651/>.
- [112] Nilton A Brito, Márcia N Brito, and Timothy J Bartness. "Differential sympathetic drive to adipose tissues after food deprivation, cold exposure or glucoprivation". In: *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 294.5 (2008). URL: <https://pubmed.ncbi.nlm.nih.gov/18321949/>.
- [113] I Murano et al. "Noradrenergic parenchymal nerve fiber branching after cold acclimatisation correlates with brown adipocyte density in mouse adipose organ". In: *Journal of Anatomy* 214.1 (2009), pp. 171–178. URL: <https://pubmed.ncbi.nlm.nih.gov/19018882/>.
- [114] Magdalena Blaszkiewicz et al. "Neuropathy and neural plasticity in the subcutaneous white adipose depot". In: *PLoS ONE* 14.9 (2019), e0221766. URL: <https://doi.org/10.1371/journal.pone.0221766>.
- [115] Kaili Liu et al. "Metabolic stress drives sympathetic neuropathy within the liver". In: *Cell Metabolism* 33.3 (2021), 666–675.e4.

- [116] Paul Cohen et al. "Ablation of PRDM16 and beige adipose causes metabolic dysfunction and a subcutaneous to visceral fat switch". In: *Cell* 156.1-2 (2014), pp. 304–316. URL: <https://pubmed.ncbi.nlm.nih.gov/24439384/>.
- [117] Patrick Seale et al. "Transcriptional Control of Brown Fat Determination by PRDM16". In: *Cell Metabolism* 6.1 (2007), pp. 38–54. URL: <https://pubmed.ncbi.nlm.nih.gov/17618855/>.
- [118] Louis A Tartaglia ' et al. "Identification and Expression Cloning of a Leptin Receptor, OB-R". 1995.
- [119] Panayoula C Tsiotra et al. "Expression of the long and short leptin receptor isoforms in peripheral blood mononuclear cells: Implications for leptin's actions". In: *Metabolism: Clinical and Experimental* 49.12 (Dec. 2000), pp. 1537–1541.
- [120] Cem Gabay et al. "Leptin Directly Induces the Secretion of Interleukin 1 Receptor Antagonist in Human Monocytes ¹". In: *The Journal of Clinical Endocrinology & Metabolism* 86.2 (Feb. 2001), pp. 783–791.
- [121] Lea H Dib et al. "Bone marrow leptin signaling mediates obesity-Associated adipose tissue inflammation in male mice". In: *Endocrinology* 155.1 (Jan. 2014), pp. 40–46.
- [122] Hamid Zarkesh-Esfahani et al. "High-Dose Leptin Activates Human Leukocytes Via Receptor Expression on Monocytes". In: *The Journal of Immunology* 167.8 (Oct. 2001), pp. 4593–4599.
- [123] José Santos-Alvarez, Raimundo Goberna, and Victor Sánchez-Margalet. "Human leptin stimulates proliferation and activation of human circulating monocytes". In: *Cellular Immunology* 194.1 (May 1999), pp. 6–11.
- [124] Veronica De Rosa et al. "A Key Role of Leptin in the Control of Regulatory T Cell Proliferation". In: *Immunity* 26.2 (Feb. 2007), pp. 241–255.
- [125] Jinhua Shen et al. "Leptin enhances TNF-alpha production via p38 and JNK MAPK in LPS-stimulated Kupffer cells". In: *Life sciences* 77.13 (Aug. 2005), pp. 1502–1515. URL: <https://pubmed.ncbi.nlm.nih.gov/15979653/>.
- [126] Raffaella Faggioni et al. "Leptin-deficient (ob/ob) mice are protected from T cell-mediated hepatotoxicity: Role of tumor necrosis factor α and IL-18". In: *Proceedings of the National Academy of Sciences of the United States of America* 97.5 (Feb. 2000), p. 2367. URL: [/pmc/articles/PMC15807/](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC15807/)[20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC15807/](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC15807/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC15807/).
- [127] Fabrizio Montecucco et al. "Induction of neutrophil chemotaxis by leptin: Crucial role for p38 and Src kinases". In: *Annals of the New York Academy of Sciences*. Vol. 1069. Blackwell Publishing Inc., 2006, pp. 463–471.
- [128] Chun Kwok Wong, Phyllis F Y Cheung, and Christopher Wai Kei Lam. "Leptin-mediated cytokine release and migration of eosinophils: Implications for immunopathophysiology of allergic inflammation". In: *European Journal of Immunology* 37.8 (Aug. 2007), pp. 2337–2348.
- [129] Benedetta Mattioli et al. "Leptin Promotes Differentiation and Survival of Human Dendritic Cells and Licenses Them for Th1 Priming". In: *The Journal of Immunology* 174.11 (June 2005), pp. 6820–6828.

- [130] Pedro M M Moraes-Vieira et al. "Leptin deficiency impairs maturation of dendritic cells and enhances induction of regulatory T and Th17 cells". In: *European Journal of Immunology* 44.3 (2014), pp. 794–806.
- [131] Dorothy P. Schafer et al. "Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner". In: *Neuron* 74.4 (May 2012), pp. 691–705. URL: <https://pubmed.ncbi.nlm.nih.gov/22632727/>.
- [132] Igor Theurl et al. "On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver". In: *Nature medicine* 22.8 (Aug. 2016), pp. 945–951. URL: <https://pubmed.ncbi.nlm.nih.gov/27428900/>.
- [133] Maarten Hulsmans et al. "Macrophages Facilitate Electrical Conduction in the Heart". In: *Cell* 169.3 (Apr. 2017), pp. 510–522. URL: <https://pubmed.ncbi.nlm.nih.gov/28431249/>.
- [134] Christina D Camell et al. "Inflammasome-driven catecholamine catabolism in macrophages blunts lipolysis during ageing". In: *Nature* 550.7674 (2017), pp. 119–123. URL: <http://dx.doi.org/10.1038/nature24022>.
- [135] Jonathan R Brestoff et al. "Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity". In: *Nature* (2015).
- [136] Ayano C. Kohlgruber et al. " $\gamma\delta$ T cells producing interleukin-17A regulate adipose regulatory T cell homeostasis and thermogenesis". In: *Nature immunology* 19.5 (May 2018), pp. 464–474. URL: <https://pubmed.ncbi.nlm.nih.gov/29670241/>.
- [137] Min Woo Lee et al. "Activated type 2 innate lymphoid cells regulate beige fat biogenesis". In: *Cell* 160.1-2 (Jan. 2015), pp. 74–87. URL: <https://pubmed.ncbi.nlm.nih.gov/25543153/>.
- [138] Ari B Molofsky et al. "Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages". In: *Journal of Experimental Medicine* 210.3 (Mar. 2013), pp. 535–549. URL: www.jem.org/cgi/doi/10.1084/jem.20121964.
- [139] Markus Feuerer et al. "Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters". In: *Nature Medicine* 15.8 (2009), pp. 930–939.
- [140] Stuart P Weisberg et al. "Obesity is associated with macrophage accumulation in adipose tissue." In: *The Journal of clinical investigation* 112.12 (Dec. 2003), pp. 1796–1808.
- [141] Stefan R. Bornstein et al. "Immunohistochemical and ultrastructural localization of leptin and leptin receptor in human white adipose tissue and differentiating human adipose cells in primary culture". In: *Diabetes* 49.4 (2000), pp. 532–538. URL: <https://pubmed.ncbi.nlm.nih.gov/10871189/>.
- [142] Elvira Mass et al. "Tissue-specific macrophages: how they develop and choreograph tissue biology". In: *Nature Reviews Immunology* 2023 23:9 23.9 (Mar. 2023), pp. 563–579. URL: <https://www.nature.com/articles/s41577-023-00848-y>.
- [143] Yochai Wolf et al. "Brown-adipose-tissue macrophages control tissue innervation and homeostatic energy expenditure". In: *Nature Immunology* 18.6 (2017), pp. 665–674. URL: <https://pubmed.ncbi.nlm.nih.gov/28459435/>.

- [144] Marie Therese Rached et al. "Deletion of myeloid IRS2 enhances adipose tissue sympathetic nerve function and limits obesity". In: *Molecular Metabolism* 20 (Feb. 2019), pp. 38–50.
- [145] Haiyan Xu et al. "Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance." In: *The Journal of clinical investigation* 112.12 (Dec. 2003), pp. 1821–1830.
- [146] Katrin Fischer et al. "Alternatively activated macrophages do not synthesize catecholamines or contribute to adipose tissue adaptive thermogenesis". In: *Nature Medicine* 23.5 (May 2017), pp. 623–630.
- [147] Timothy P. Fitzgibbons et al. "Similarity of mouse perivascular and brown adipose tissues and their resistance to diet-induced inflammation". In: *American journal of physiology. Heart and circulatory physiology* 301.4 (Oct. 2011). URL: <https://pubmed.ncbi.nlm.nih.gov/21765057/>.
- [148] Tomoya Sakamoto et al. "Macrophage infiltration into obese adipose tissues suppresses the induction of UCP1 level in mice". In: *American journal of physiology. Endocrinology and metabolism* 310.8 (Apr. 2016), E676–E687. URL: <https://pubmed.ncbi.nlm.nih.gov/26884382/>.
- [149] Carla Roberts-Toler, Brian T. O'Neill, and Aaron M. Cypess. "Diet-induced obesity causes insulin resistance in mouse brown adipose tissue". In: *Obesity (Silver Spring, Md.)* 23.9 (Sept. 2015), pp. 1765–1770. URL: <https://pubmed.ncbi.nlm.nih.gov/26242777/>.
- [150] Carey N Lumeng, Jennifer L Bodzin, and Alan R Saltiel. "Obesity induces a phenotypic switch in adipose tissue macrophage polarization." In: *The Journal of clinical investigation* 117.1 (Jan. 2007), pp. 175–184.
- [151] Carey N Lumeng et al. "Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes." In: *Diabetes* 57.12 (Dec. 2008), pp. 3239–3246.
- [152] Giulia Chinetti-Gbaguidi and Bart Staels. "Macrophage polarization in metabolic disorders: functions and regulation". In: *Current opinion in lipidology* 22.5 (Oct. 2011), pp. 365–372. URL: <https://pubmed.ncbi.nlm.nih.gov/21825981/>.
- [153] Kihwa Kang et al. "Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polarization and insulin sensitivity." In: *Cell metabolism* 7.6 (June 2008), pp. 485–495.
- [154] Justin I Odegaard et al. "Macrophage-specific PPAR γ controls alternative activation and improves insulin resistance". In: *Nature* 447.7148 (June 2007), pp. 1116–1120.
- [155] Justin I Odegaard et al. "Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance." In: *Cell metabolism* 7.6 (June 2008), pp. 496–507.
- [156] Ellen H Jenning, Mark Gurnell, and Eric Kalkhoven. *Functional implications of genetic variation in human PPAR γ* . Oct. 2009.
- [157] Peter Sartipy and David J Loskutoff. "Monocyte chemoattractant protein 1 in obesity and insulin resistance". In: *Proceedings of the National Academy of Sciences of the United States of America* 100.12 (June 2003), pp. 7265–7270. URL: www.pnas.org/cgi/doi/10.1073/pnas.1133870100.

- [158] Jiyoung Bae et al. "Activation of pattern recognition receptors in brown adipocytes induces inflammation and suppresses uncoupling protein 1 expression and mitochondrial respiration". In: *American Journal of Physiology - Cell Physiology* 306.10 (May 2014). URL: www.ajpcell.org.
- [159] Ayaka Ito et al. "Role of CC chemokine receptor 2 in bone marrow cells in the recruitment of macrophages into obese adipose tissue". In: *Journal of Biological Chemistry* 283.51 (Dec. 2008), pp. 35715–35723.
- [160] Nozomu Kamei et al. "Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance". In: *Journal of Biological Chemistry* 281.36 (Sept. 2006), pp. 26602–26614.
- [161] Hajime Kanda et al. "MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity". In: *Journal of Clinical Investigation* 116.6 (June 2006), pp. 1494–1505.
- [162] Jongkil Kim et al. "Silencing CCR2 in Macrophages Alleviates Adipose Tissue Inflammation and the Associated Metabolic Syndrome in Dietary Obese Mice". In: *Molecular Therapy - Nucleic Acids* 5.1 (2016), e280.
- [163] Amrom E Obstfeld et al. "C-C Chemokine Receptor 2 (CCR2) regulates the hepatic recruitment of myeloid cells that promote obesity-induced hepatic steatosis". In: *Diabetes* 59.4 (Apr. 2010), pp. 916–925.
- [164] Shinya U Amano et al. "Local proliferation of macrophages contributes to obesity-associated adipose tissue inflammation". In: *Cell Metabolism* 19.1 (Jan. 2014), pp. 162–171.
- [165] Florent Ginhoux and Martin Williams. *Tissue-Resident Macrophage Ontogeny and Homeostasis*. Mar. 2016.
- [166] Roksana M Pirzgalska et al. "Sympathetic neuron-associated macrophages contribute to obesity by importing and metabolizing norepinephrine". In: *Nature Medicine* 23.11 (2017), pp. 1309–1318. URL: <https://www.nature.com/articles/nm.4422>.
- [167] Khoa D Nguyen et al. "Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis". In: *Nature* 480.7375 (Dec. 2011), pp. 104–108.
- [168] Yifu Qiu et al. "Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat". In: *Cell* 157.6 (June 2014), pp. 1292–1308.
- [169] Avraham Yaron et al. "Differential requirement for Plexin-A3 and -A4 in mediating responses of sensory and sympathetic neurons to distinct class 3 Semaphorins". In: *Neuron* 45.4 (2005), pp. 513–523. URL: <https://pubmed.ncbi.nlm.nih.gov/15721238/>.
- [170] Atsushi Nakagomi et al. *Role of the central nervous system and adipose tissue BDNF/TrkB axes in metabolic regulation*. 2015. URL: www.nature.com/npjamd.
- [171] Carlos M. Minutti et al. "Tissue-specific contribution of macrophages to wound healing". In: *Seminars in Cell & Developmental Biology* 61 (Jan. 2017), pp. 3–11.

- [172] Steven J. Van Dyken and Richard M. Locksley. "Interleukin-4- and Interleukin-13-Mediated Alternatively Activated Macrophages: Roles in Homeostasis and Disease". In: <https://doi.org/10.1146/annurev-immunol-032712-095906> 31 (Mar. 2013), pp. 317–343. URL: <https://www.annualreviews.org/doi/abs/10.1146/annurev-immunol-032712-095906>.
- [173] Yuki Hikichi et al. "Posttranscriptional regulation of ILC2 homeostatic function via tristetraprolin". In: (2021). URL: <https://doi.org/10.1084/jem.20210181>.
- [174] Hiroki Kabata, Kazuyo Moro, and Shigeo Koyasu. "The group 2 innate lymphoid cell (ILC2) regulatory network and its underlying mechanisms". In: *Immunological reviews* 286.1 (Nov. 2018), pp. 37–52. URL: <https://pubmed.ncbi.nlm.nih.gov/30294963/>.
- [175] Jesse C. Nussbaum et al. "Type 2 innate lymphoid cells control eosinophil homeostasis." In: *Nature* 502.7470 (Sept. 2013), pp. 245–248. URL: <https://www.ncbi.nlm.nih.gov/pmc/articles/pmid/24037376/?tool=EBI%20https://europepmc.org/article/med/24037376>.
- [176] Emily Hams et al. "Cutting edge: IL-25 elicits innate lymphoid type 2 and type II NKT cells that regulate obesity in mice". In: *Journal of immunology (Baltimore, Md. : 1950)* 191.11 (Dec. 2013), pp. 5349–5353. URL: <https://pubmed.ncbi.nlm.nih.gov/24166975/>.
- [177] Kazuyo Moro et al. "Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells". In: *Nature* 463.7280 (Jan. 2010), pp. 540–544. URL: <https://pubmed.ncbi.nlm.nih.gov/20023630/>.
- [178] Daniel R. Neill et al. "Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity". In: *Nature* 464.7293 (Apr. 2010), pp. 1367–1370. URL: <https://pubmed.ncbi.nlm.nih.gov/20200518/>.
- [179] April E. Price et al. "Systemically dispersed innate IL-13-expressing cells in type 2 immunity". In: *Proceedings of the National Academy of Sciences of the United States of America* 107.25 (June 2010), pp. 11489–11494. URL: <https://pubmed.ncbi.nlm.nih.gov/20534524/>.
- [180] Emily L. Goldberg et al. "IL-33 causes thermogenic failure in aging by expanding dysfunctional adipose ILC2". In: *Cell metabolism* 33.11 (Nov. 2021), pp. 2277–2287. URL: <https://pubmed.ncbi.nlm.nih.gov/34473956/>.
- [181] Kazuyo Moro et al. "Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses". In: *Nature immunology* 17.1 (Jan. 2016), pp. 76–86. URL: <https://pubmed.ncbi.nlm.nih.gov/26595888/>.
- [182] Ari B. Molofsky, Adam K. Savage, and Richard M. Locksley. "Interleukin-33 in Tissue Homeostasis, Injury, and Inflammation". In: *Immunity* 42.6 (June 2015), pp. 1005–1019. URL: [http://www.cell.com/article/S1074761315002289/fulltext%20http://www.cell.com/article/S1074761315002289/abstract%20https://www.cell.com/immunity/abstract/S1074-7613\(15\)00228-9](http://www.cell.com/article/S1074761315002289/fulltext%20http://www.cell.com/article/S1074761315002289/abstract%20https://www.cell.com/immunity/abstract/S1074-7613(15)00228-9).

- [183] Timotheus Y.F. Halim et al. "Tissue-Restricted Adaptive Type 2 Immunity Is Orchestrated by Expression of the Costimulatory Molecule OX40L on Group 2 Innate Lymphoid Cells". In: *Immunity* 48.6 (June 2018), pp. 1195–1207. URL: <https://pubmed.ncbi.nlm.nih.gov/29907525/>.
- [184] William Reid Bolus, Arion J Kennedy, and Alyssa H Hasty. "Obesity-induced reduction of adipose eosinophils is reversed with low-calorie dietary intervention". In: *Physiological Reports* 6.22 (Nov. 2018).
- [185] Xiaofeng Ding et al. "IL-33-driven ILC2/eosinophil axis in fat is induced by sympathetic tone and suppressed by obesity". In: *Journal of Endocrinology* 231.1 (2016), pp. 35–48.
- [186] Eun Hui Lee et al. "Eosinophils support adipocyte maturation and promote glucose tolerance in obesity". In: *Scientific Reports* 8.1 (Dec. 2018), pp. 1–12.
- [187] Daniel Brigger et al. "Eosinophils regulate adipose tissue inflammation and sustain physical and immunological fitness in old age". In: *Nature Metabolism* 2020 2:8 2.8 (July 2020), pp. 688–702. URL: <https://www.nature.com/articles/s42255-020-0228-3>.
- [188] W Reid Bolus et al. "Elevating adipose eosinophils in obese mice to physiologically normal levels does not rescue metabolic impairments". In: *Molecular Metabolism* 8 (Feb. 2018), pp. 86–95.
- [189] Adeline Bertola et al. "Identification of adipose tissue dendritic cells correlated with obesity-associated insulin-resistance and inducing Th17 responses in mice and patients". In: *Diabetes* 61.9 (Sept. 2012), pp. 2238–2247.
- [190] Vered Elgazar-Carmon et al. "Neutrophils transiently infiltrate intra-abdominal fat early in the course of high-fat feeding". In: *Journal of Lipid Research* 49.9 (Sept. 2008), pp. 1894–1903.
- [191] Jian Liu et al. "Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice". In: *Nature Medicine* 15.8 (Aug. 2009), pp. 940–945.
- [192] Byung Cheol Lee et al. "Adipose Natural Killer Cells Regulate Adipose Tissue Macrophages to Promote Insulin Resistance in Obesity". In: *Cell Metabolism* 23.4 (Apr. 2016), pp. 685–698.
- [193] Takaharu Sasaki et al. "Innate Lymphoid Cells in the Induction of Obesity". In: *Cell Reports* 28.1 (July 2019), 202–217.e7.
- [194] Maja Stefanovic-Racic et al. "Dendritic cells promote macrophage infiltration and comprise a substantial proportion of obesity-associated increases in CD11c + cells in adipose tissue and liver". In: *Diabetes* 61.9 (Sept. 2012), pp. 2330–2339.
- [195] Claire E Macdougall, Elizabeth G Wood, and Jakob Loschko. "Visceral Adipose Tissue Immune Homeostasis Is Regulated by the Crosstalk between Adipocytes and Dendritic Cell Subsets". In: *Cell Metabolism* 27 (2018), 588–601.e4.
- [196] Saswata Talukdar et al. "Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase". In: *Nature Medicine* 18.9 (Sept. 2012), pp. 1407–1412.

- [197] Daniela Cipolletta et al. "Appearance and disappearance of the mRNA signature characteristic of Treg cells in visceral adipose tissue: age, diet, and PPAR γ effects". In: *Proceedings of the National Academy of Sciences of the United States of America* 112.2 (Jan. 2015), pp. 482–487. URL: <https://pubmed.ncbi.nlm.nih.gov/25550516/>.
- [198] Daniela Cipolletta et al. "PPAR- γ is a major driver of the accumulation and phenotype of adipose tissue Treg cells". In: *Nature* 486.7404 (June 2012), pp. 549–553. URL: <https://pubmed.ncbi.nlm.nih.gov/22722857/>.
- [199] Chaoran Li et al. "TCR Transgenic Mice Reveal Stepwise, Multi-site Acquisition of the Distinctive Fat-Treg Phenotype". In: *Cell* 174.2 (July 2018), 285–299.e12.
- [200] Dmitriy Kolodin et al. "Antigen- and cytokine-driven accumulation of regulatory t cells in visceral adipose tissue of lean mice". In: *Cell Metabolism* 21.4 (Apr. 2015), pp. 543–557.
- [201] Ajithkumar Vasanthakumar et al. "The transcriptional regulators IRF4, BATF and IL-33 orchestrate development and maintenance of adipose tissue-resident regulatory T cells". In: (2015).
- [202] Jonathan M. Han et al. "IL-33 Reverses an Obesity-Induced Deficit in Visceral Adipose Tissue ST2+ T Regulatory Cells and Ameliorates Adipose Tissue Inflammation and Insulin Resistance". In: *Journal of immunology (Baltimore, Md. : 1950)* 194.10 (May 2015), pp. 4777–4783. URL: <https://pubmed.ncbi.nlm.nih.gov/25870243/>.
- [203] Joanna R. DiSpirito et al. "Molecular diversification of regulatory T cells in nonlymphoid tissues". In: *Science Immunology* 3.27 (Sept. 2018), p. 5861. URL: <https://www.science.org/doi/10.1126/sciimmunol.aat5861>.
- [204] Bi Huei Yang et al. "TCF1 and LEF1 Control Treg Competitive Survival and Tfr Development to Prevent Autoimmune Diseases". In: *Cell reports* 27.12 (June 2019), pp. 3629–3645. URL: <https://pubmed.ncbi.nlm.nih.gov/31216480/>.
- [205] Jenna M. Sullivan, Barbara Höllbacher, and Daniel J. Campbell. "Cutting Edge: Dynamic Expression of Id3 Defines the Stepwise Differentiation of Tissue-Resident Regulatory T Cells". In: *Journal of immunology (Baltimore, Md. : 1950)* 202.1 (Jan. 2019), pp. 31–36. URL: <https://pubmed.ncbi.nlm.nih.gov/30518568/>.
- [206] Ari B Molofsky et al. "Interleukin-33 And Interferon- γ Counter-Regulate Group 2 Innate Lymphoid Cell Activation During Immune Perturbation". In: *Immunity* 43.1 (July 2015), pp. 161–174.
- [207] Raul German Spallanzani et al. "Distinct immunocyte-promoting and adipocyte-generating stromal components coordinate adipose tissue immune and metabolic tenors". In: *Science Immunology* 4.35 (2019), p. 3658. URL: <http://immunology.sciencemag.org/>.
- [208] Kathrin Eller et al. "Potential role of regulatory T cells in reversing obesity-linked insulin resistance and diabetic nephropathy". In: *Diabetes* 60.11 (Nov. 2011), pp. 2954–2962. URL: <https://pubmed.ncbi.nlm.nih.gov/21911743/>.
- [209] Yaron Ilan et al. "Induction of regulatory T cells decreases adipose inflammation and alleviates insulin resistance in ob/ob mice". In: *Proceedings of the National Academy of Sciences of the United States of America* 107.21 (May 2010), pp. 9765–9770. URL: <https://pubmed.ncbi.nlm.nih.gov/20445103/>.

- [210] Lydia Lynch et al. "Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of T(reg) cells and macrophages in adipose tissue". In: *Nature immunology* 16.1 (Dec. 2015), pp. 85–95. URL: <https://pubmed.ncbi.nlm.nih.gov/25436972/>.
- [211] Lisa Schmidleithner et al. "Enzymatic Activity of HPGD in Treg Cells Suppresses Tconv Cells to Maintain Adipose Tissue Homeostasis and Prevent Metabolic Dysfunction". In: *Immunity* 50.5 (May 2019), pp. 1232–1248. URL: <https://pubmed.ncbi.nlm.nih.gov/31027998/>.
- [212] Jacques Neefjes et al. "Towards a systems understanding of MHC class I and MHC class II antigen presentation". In: *Nature Reviews Immunology* 2011 11:12 11.12 (Nov. 2011), pp. 823–836. URL: <https://www.nature.com/articles/nri3084>.
- [213] Amy E. Moran et al. "T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse". In: *The Journal of experimental medicine* 208.6 (June 2011), pp. 1279–1289. URL: <https://pubmed.ncbi.nlm.nih.gov/21606508/>.
- [214] Jason D. Fontenot, Marc A. Gavin, and Alexander Y. Rudensky. "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells". In: *Nature immunology* 4.4 (Feb. 2003), pp. 986–992. URL: <https://pubmed.ncbi.nlm.nih.gov/12612578/>.
- [215] Shimon Sakaguchi. "Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses". In: *Annual review of immunology* 22 (2004), pp. 531–562. URL: <https://pubmed.ncbi.nlm.nih.gov/15032588/>.
- [216] Chaoran Li, Raul German Spallanzani, and Diane Mathis. "Visceral adipose tissue Tregs and the cells that nurture them". In: *Immunological reviews* 295.1 (May 2020), pp. 114–125. URL: <https://pubmed.ncbi.nlm.nih.gov/32162339/>.
- [217] T. Mahlaköiv et al. "Stromal cells maintain immune cell homeostasis in adipose tissue via production of interleukin-33". In: *Science Immunology* 4.35 (May 2019), p. 416. URL: <http://immunology.sciencemag.org/>.
- [218] Amal Hasan et al. "IL-33 is negatively associated with the BMI and confers a protective lipid/metabolic profile in non-diabetic but not diabetic subjects". In: *BMC Immunology* 15.1 (May 2014).
- [219] Jonathan M Han et al. " IL-33 Reverses an Obesity-Induced Deficit in Visceral Adipose Tissue ST2 + T Regulatory Cells and Ameliorates Adipose Tissue Inflammation and Insulin Resistance ". In: *The Journal of Immunology* 194.10 (May 2015), pp. 4777–4783.
- [220] Ashley M Miller et al. "Interleukin-33 induces protective effects in adipose tissue inflammation during obesity in mice". In: *Circulation Research* 107.5 (Sept. 2010), pp. 650–658. URL: <http://circres.ahajournals.org>.
- [221] J. DeFalco et al. "Virus-assisted mapping of neural inputs to a feeding center in the hypothalamus". In: *Science (New York, N.Y.)* 291.5513 (Mar. 2001), pp. 2608–2613. URL: <https://pubmed.ncbi.nlm.nih.gov/11283374/>.

- [222] George Paxinos and Keith B.J. Franklin. "The mouse brain in stereotaxic coordinates: hard cover edition." In: *Academic Press* 2nd Editio (2001), p. 360. URL: <https://www.elsevier.com/books/paxinos-and-franklins-the-mouse-brain-in-stereotaxic-coordinates-compact/franklin/978-0-12-816159-3>.
- [223] Simone Picelli et al. "Full-length RNA-seq from single cells using Smart-seq2". In: (2013). URL: <http://www..>
- [224] John N Campbell et al. "A molecular census of arcuate hypothalamus and median eminence cell types". In: 4 (2017), p. 4. URL: <http://mouse.brain-map.org/>.
- [225] Nicholas Schaum et al. "Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris the tabula Muris consortium*". In: (2018). URL: <https://doi.org/10.1038/s41586-018-0590-4>.
- [226] Kathy Wang et al. "Neuronal, stromal, and T-regulatory cell crosstalk in murine skeletal muscle". In: *Proceedings of the National Academy of Sciences of the United States of America* 117.10 (Mar. 2020), pp. 5402–5408. URL: <https://www.pnas.org/doi/abs/10.1073/pnas.1922559117>.
- [227] Wilson Kuswanto et al. "Poor Repair of Skeletal Muscle in Aging Mice Reflects a Defect in Local, Interleukin-33-Dependent Accumulation of Regulatory T Cells". In: *Immunity* 44.2 (Feb. 2016), pp. 355–367. URL: [http://www.cell.com/article/S1074761316000340/fulltext%20http://www.cell.com/article/S1074761316000340/abstract%20https://www.cell.com/immunity/abstract/S1074-7613\(16\)00034-0](http://www.cell.com/article/S1074761316000340/fulltext%20http://www.cell.com/article/S1074761316000340/abstract%20https://www.cell.com/immunity/abstract/S1074-7613(16)00034-0).
- [228] Yuhao Hao et al. "Integrated analysis of multimodal single-cell data". In: *Cell* 184.13 (June 2021), 3573–3587.e29. URL: [http://www.cell.com/article/S0092867421005833/fulltext%20http://www.cell.com/article/S0092867421005833/abstract%20https://www.cell.com/cell/abstract/S0092-8674\(21\)00583-3](http://www.cell.com/article/S0092867421005833/fulltext%20http://www.cell.com/article/S0092867421005833/abstract%20https://www.cell.com/cell/abstract/S0092-8674(21)00583-3).
- [229] Streamson C. Chua et al. "Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor". In: *Science (New York, N.Y.)* 271.5251 (Feb. 1996), pp. 994–996. URL: <https://pubmed.ncbi.nlm.nih.gov/8584938/>.
- [230] Louis A. Tartaglia. "The leptin receptor". In: *The Journal of biological chemistry* 272.10 (Mar. 1997), pp. 6093–6096. URL: <https://pubmed.ncbi.nlm.nih.gov/9102398/>.
- [231] Christian Bjørbæk et al. "Divergent Signaling Capacities of the Long and Short Isoforms of the Leptin Receptor". In: *Journal of Biological Chemistry* 272.51 (Dec. 1997), pp. 32686–32695.
- [232] Hong Fei et al. "Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues". In: *Proceedings of the National Academy of Sciences of the United States of America* 94.13 (June 1997), pp. 7001–7005. URL: <https://pubmed.ncbi.nlm.nih.gov/9192681/>.
- [233] Angela M. Ramos-Lobo and Jose Donato. "The role of leptin in health and disease". In: *Temperature: Multidisciplinary Biomedical Journal* 4.3 (2017), p. 258. URL: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5605162/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5605162/>.

- [234] Jeffrey M Friedman and Jeffrey L Halaas. "Leptin and the regulation of body weight in mammals". In: *Nature* 1998 395:6704 395.6704 (Oct. 1998), pp. 763–770. URL: <https://www.nature.com/articles/27376>.
- [235] Denis G Baskin et al. "Leptin receptor long-form splice-variant protein expression in neuron cell bodies of the brain and co-localization with neuropeptide Y mRNA in the arcuate nucleus". In: *Journal of Histochemistry and Cytochemistry* 47.3 (1999), pp. 353–362.
- [236] R. De Matteis et al. "Localization of leptin receptor splice variants in mouse peripheral tissues by immunohistochemistry". In: *The Proceedings of the Nutrition Society* 57.3 (Aug. 1998), pp. 441–448. URL: <https://pubmed.ncbi.nlm.nih.gov/9794002/>.
- [237] Nigel Hoggard et al. "Localization of leptin receptor mRNA splice variants in murine peripheral tissues by RT-PCR and in situ hybridization". In: *Biochemical and biophysical research communications* 232.2 (Mar. 1997), pp. 383–387. URL: <https://pubmed.ncbi.nlm.nih.gov/9125186/>.
- [238] Bo O. Zhou et al. "Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow". In: *Cell Stem Cell* 15.2 (Aug. 2014), pp. 154–168. URL: [http://www.cell.com/article/S1934590914002562/fulltext%20http://www.cell.com/article/S1934590914002562/abstract%20https://www.cell.com/cell-stem-cell/abstract/S1934-5909\(14\)00256-2](http://www.cell.com/article/S1934590914002562/fulltext%20http://www.cell.com/article/S1934590914002562/abstract%20https://www.cell.com/cell-stem-cell/abstract/S1934-5909(14)00256-2).
- [239] Keegan T Murphy et al. "Leptin-sensitive sensory nerves innervate white fat." In: *American journal of physiology. Endocrinology and metabolism* 304.12 (2013), pp. 1338–1347.
- [240] Axel Key and Gustaf Retzius. *Studien in der Anatomie des Nervensystems und des Bindegewebes (Volume 2)*. 1876. URL: <https://collections.nlm.nih.gov/catalog/nlm:nlmuid-66211410RX2-mvpart>.
- [241] Louis (1835-1922). Auteur du texte Ranvier. "Leçons sur l'histologie du système nerveux. Tome 1 / par M. S. Ranvier,... ; recueillies par M. Ed. Weber,...". In: (1878).
- [242] K Krnjević. "THE CONNECTIVE TISSUE OF THE FROG SCIATIC NERVE". In: *Quarterly Journal of Experimental Physiology and Cognate Medical Sciences* 39.1 (Jan. 1954), pp. 55–72. URL: <https://onlinelibrary.wiley.com/doi/full/10.1113/expphysiol.1954.sp001048%20https://onlinelibrary.wiley.com/doi/abs/10.1113/expphysiol.1954.sp001048%20https://physoc.onlinelibrary.wiley.com/doi/10.1113/expphysiol.1954.sp001048>.
- [243] T R Shanthaveerappa and G H Bourne. "THE PERINEURAL EPITHELIUM OF SYMPATHETIC NERVES AND GANGLIA AND ITS RELATION TO THE PIA ARACHNOID OF THE CENTRAL NERVOUS SYSTEM AND PERINEURAL EPITHELIUM OF THE PERIPHERAL NERVOUS SYSTEM". In: *Zeitschrift fur Zellforschung und mikroskopische Anatomie (Vienna, Austria : 1948)* 61.5 (Sept. 1964), pp. 742–753. URL: <https://pubmed.ncbi.nlm.nih.gov/14158192/>.
- [244] Sergio Piña-Oviedo and Carlos Ortiz-Hidalgo. "The normal and neoplastic perineurium: a review". In: *Advances in anatomic pathology* 15.3 (May 2008), pp. 147–164. URL: <https://pubmed.ncbi.nlm.nih.gov/18434767/>.

- [245] Matthew J Carr et al. "Mesenchymal Precursor Cells in Adult Nerves Contribute to Mammalian Tissue Repair and Regeneration". In: *Cell stem cell* 24.2 (Feb. 2019), 240–256.e9. URL: <https://pubmed.ncbi.nlm.nih.gov/30503141/>.
- [246] P K Thomas and S Bhagat. "The effect of extraction of the intrafascicular contents of peripheral nerve trunks on perineurial structure". In: *Acta Neuropathologica* 1978 43:1 43.1 (Jan. 1978), pp. 135–141. URL: <https://link.springer.com/article/10.1007/BF00685008>.
- [247] C H Berthold et al. "Microscopic Anatomy of the Peripheral Nervous System". In: *Peripheral Neuropathy* 1 (Jan. 2005), pp. 35–91.
- [248] Douglas W Zochodne. *Neurobiology of Peripheral Nerve Regeneration*. Cambridge University Press, Oct. 2008. URL: <https://www.cambridge.org/core/product/identifier/9780511541759/type/book>.
- [249] Kumiko Takebe et al. "Histochemical demonstration of a monocarboxylate transporter in the mouse perineurium with special reference to GLUT1". In: *Biomedical research (Tokyo, Japan)* 29.6 (2008), pp. 297–306. URL: <https://pubmed.ncbi.nlm.nih.gov/19129673/>.
- [250] K. Krnjevic. "Some observations on perfused frog sciatic nerves". In: *The Journal of Physiology* 123.2 (Feb. 1954), pp. 338–356. URL: <https://onlinelibrary.wiley.com/doi/full/10.1113/jphysiol.1954.sp005055%20https://onlinelibrary.wiley.com/doi/abs/10.1113/jphysiol.1954.sp005055%20https://physoc.onlinelibrary.wiley.com/doi/10.1113/jphysiol.1954.sp005055>.
- [251] Daniel Gerber et al. "Transcriptional profiling of mouse peripheral nerves to the single-cell level to build a sciatic nerve atlas (Snat)". In: *eLife* 10 (2021).
- [252] Davina Wu et al. "Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis". In: *Science* 332.6026 (Apr. 2011), pp. 243–247.
- [253] Jochen Schmitz et al. "IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines". In: *Immunity* 23.5 (Nov. 2005), pp. 479–490. URL: <https://pubmed.ncbi.nlm.nih.gov/16286016/>.
- [254] Christine Moussion, Nathalie Ortega, and Jean Philippe Girard. "The IL-1-Like Cytokine IL-33 Is Constitutively Expressed in the Nucleus of Endothelial Cells and Epithelial Cells In Vivo: A Novel 'Alarmin'?" In: *PLOS ONE* 3.10 (Oct. 2008), e3331. URL: <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0003331>.
- [255] Corinne Cayrol and Jean-Philippe Girard. "The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1". In: *PNAS* 106.22 (2009).
- [256] Corinne Cayrol and Jean Philippe Girard. "IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy". In: *Current Opinion in Immunology* 31 (Dec. 2014), pp. 31–37.
- [257] Claire Galand et al. "IL-33 promotes food anaphylaxis in epicutaneously sensitized mice by targeting mast cells". In: *Journal of Allergy and Clinical Immunology* 138.5 (Nov. 2016), pp. 1356–1366.

- [258] Maroua Ferhat et al. "Endogenous IL-33 contributes to kidney ischemia-reperfusion injury as an alarmin". In: *Journal of the American Society of Nephrology* 29.4 (Apr. 2018), pp. 1272–1288. URL: https://journals.lww.com/jasn/fulltext/2018/04000/endogenous_il_33_contributes_to_kidney.23.aspx.
- [259] Louise Barbier et al. "Endogenous Interleukin-33 Acts as an Alarmin in Liver Ischemia-Reperfusion and Is Associated With Injury After Human Liver Transplantation". In: *Frontiers in Immunology* 12 (Sept. 2021).
- [260] Yoko Haenuki et al. "A critical role of IL-33 in experimental allergic rhinitis". In: *Journal of Allergy and Clinical Immunology* 130.1 (July 2012), pp. 184–194.
- [261] Sachin P. Gadani et al. "Characterization of meningeal type 2 innate lymphocytes and their response to CNS injury". In: *Journal of Experimental Medicine* 214.2 (Feb. 2017), pp. 285–296. URL: <https://doi.org/10.1084/jem.20161982>.
- [262] Alexander U Lü Thi et al. "Article Suppression of Interleukin-33 Bioactivity through Proteolysis by Apoptotic Caspases". In: ().
- [263] Rahul Kakkar et al. "Interleukin 33 as a mechanically responsive cytokine secreted by living cells". In: *Journal of Biological Chemistry* 287.9 (Feb. 2012), pp. 6941–6948.
- [264] Joseph T. Clark et al. "IL-33 promotes innate lymphoid cell-dependent ifn- γ production required for innate immunity to toxoplasma gondii". In: *eLife* 10 (Apr. 2021).
- [265] N Eomi et al. "Decoding asthma: Translating genetic variation in IL33 and IL1RL1 into disease pathophysiology". In: (2013). URL: <http://dx.doi.org/10.1016/j.jaci.2012.11.028>.
- [266] Daniel F. Gudbjartsson et al. "Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction". In: *Nature Genetics* 2009 41:3 41.3 (Feb. 2009), pp. 342–347. URL: <https://www.nature.com/articles/ng.323>.
- [267] Klaus Bønnelykke et al. "A genome-wide association study identifies CDHR3 as a susceptibility locus for early childhood asthma with severe exacerbations". In: *Nature Genetics* 2013 46:1 46.1 (Nov. 2013), pp. 51–55. URL: <https://www.nature.com/articles/ng.2830>.
- [268] Maria E. Ketelaar et al. "Phenotypic and functional translation of IL33 genetics in asthma". In: *Journal of Allergy and Clinical Immunology* 147.1 (Jan. 2021), pp. 144–157.
- [269] Ivy Aneas et al. "Asthma-associated genetic variants induce IL33 differential expression through an enhancer-blocking regulatory region". In: *Nature Communications* 12.1 (Dec. 2021).
- [270] Juliana Bessa et al. "Altered subcellular localization of IL-33 leads to non-resolving lethal inflammation". In: *Journal of autoimmunity* 55.1 (Dec. 2014), pp. 33–41. URL: <https://pubmed.ncbi.nlm.nih.gov/24786898/>.
- [271] Shafaqat Ali et al. "Caspase 3 inactivates biologically active full length interleukin-33 as a classical cytokine but does not prohibit nuclear translocation". In: *Biochemical and biophysical research communications* 391.3 (Jan. 2010), pp. 1512–1516. URL: <https://pubmed.ncbi.nlm.nih.gov/20035719/>.

- [272] Lark Kyun Kim et al. "AMCase is a crucial regulator of type 2 immune responses to inhaled house dust mites". In: *Proceedings of the National Academy of Sciences of the United States of America* 112.22 (June 2015), E2891–E2899. URL: <https://www.pnas.org/doi/abs/10.1073/pnas.1507393112>.
- [273] Fahima Madouri et al. "Caspase-1 activation by NLRP3 inflammasome dampens IL-33-dependent house dust mite-induced allergic lung inflammation". In: *Journal of Molecular Cell Biology* 7.4 (Aug. 2015), pp. 351–365. URL: <https://dx.doi.org/10.1093/jmcb/mjv012>.
- [274] E. Suzanne Cohen et al. "Oxidation of the alarmin IL-33 regulates ST2-dependent inflammation". In: *Nature communications* 6 (Sept. 2015). URL: <https://pubmed.ncbi.nlm.nih.gov/26365875/>.
- [275] Florent Colomb et al. "IL-33-binding HpARI family homologues with divergent effects in suppressing or enhancing Type 2 immune responses". In: *bioRxiv* (Oct. 2023), p. 2023.10.09.561567. URL: <https://www.biorxiv.org/content/10.1101/2023.10.09.561567v1%20https://www.biorxiv.org/content/10.1101/2023.10.09.561567v1.abstract>.
- [276] Dominique Talabot-Ayer et al. "Severe neutrophil-dominated inflammation and enhanced myelopoiesis in IL-33-overexpressing CMV/IL33 mice". In: *Journal of immunology (Baltimore, Md. : 1950)* 194.2 (Jan. 2015), pp. 750–760. URL: <https://pubmed.ncbi.nlm.nih.gov/25505285/>.
- [277] Akira Tominaga et al. "Establishment of IL-5-dependent early B cell lines by long-term bone marrow cultures". In: *Growth factors (Chur, Switzerland)* 1.2 (1989), pp. 135–146. URL: <https://pubmed.ncbi.nlm.nih.gov/2624778/>.
- [278] Ken Yanagisawa et al. "Presence of a novel primary response gene ST2L, encoding a product highly similar to the interleukin 1 receptor type 1". In: *FEBS letters* 318.1 (Feb. 1993), pp. 83–87. URL: <https://pubmed.ncbi.nlm.nih.gov/7916701/>.
- [279] Xi Liu et al. "Structural insights into the interaction of IL-33 with its receptors". In: *Proceedings of the National Academy of Sciences of the United States of America* 110.37 (Sept. 2013), pp. 14918–14923. URL: <https://pubmed.ncbi.nlm.nih.gov/23980170/>.
- [280] Chris Schiering et al. "The alarmin IL-33 promotes regulatory T-cell function in the intestine". In: *Nature* 2014 513:7519 513.7519 (July 2014), pp. 564–568. URL: <https://www.nature.com/articles/nature13577>.
- [281] Xu Yun Zhao et al. "The obesity-induced adipokine sST2 exacerbates adipose Treg and ILC2 depletion and promotes insulin resistance". In: *Science Advances* 6.20 (May 2020), pp. 6191–6204. URL: <https://www.science.org/doi/10.1126/sciadv.aay6191>.
- [282] Scott P Commins et al. "Leptin selectively reduces white adipose tissue in mice via a UCP1-dependent mechanism in brown adipose tissue". In: *American Journal of Physiology - Endocrinology and Metabolism* 280.2 43-2 (2001).
- [283] Kavon Rezai-Zadeh et al. "Leptin receptor neurons in the dorsomedial hypothalamus are key regulators of energy expenditure and body weight, but not food intake". In: *Molecular Metabolism* 3.7 (Oct. 2014), pp. 681–693.

- [284] J. Zhao et al. "Coexisting beta-adrenoceptor subtypes: significance for thermogenic process in brown fat cells". In: *The American journal of physiology* 267.4 Pt 1 (1994). URL: <https://pubmed.ncbi.nlm.nih.gov/7943293/>.
- [285] J. Granneman. "Norepinephrine infusions increase adenylate cyclase responsiveness in brown adipose tissue." In: *Journal of Pharmacology and Experimental Therapeutics* (1988).
- [286] Jin Zhao, Barbara Cannon, and Jan Nedergaard. "alpha1-Adrenergic stimulation potentiates the thermogenic action of beta3-adrenoreceptor-generated cAMP in brown fat cells". In: *The Journal of biological chemistry* 272.52 (Dec. 1997), pp. 32847–32856. URL: <https://pubmed.ncbi.nlm.nih.gov/9407062/>.
- [287] A. Matthias et al. "Thermogenic responses in brown fat cells are fully UCP1-dependent. UCP2 or UCP3 do not substitute for UCP1 in adrenergically or fatty acid-induced thermogenesis". In: *The Journal of biological chemistry* 275.33 (Aug. 2000), pp. 25073–25081. URL: <https://pubmed.ncbi.nlm.nih.gov/10825155/>.
- [288] Anders Jacobssons et al. "THE JOURNAL OF BIOLOGICAL CHEMISTRY Mitochondrial Uncoupling Protein from Mouse Brown Fat MOLECULAR CLONING, GENETIC MAPPING, AND mRNA EXPRESSION*". In: *Journal of Biological Chemistry* 260.30 (1985), pp. 16250–16254.
- [289] Daniel Ricquier et al. "Rapid increase of mitochondrial uncoupling protein and its mRNA in stimulated brown adipose tissue. Use of a cDNA probe". In: *FEBS letters* 178.2 (Dec. 1984), pp. 240–244. URL: <https://pubmed.ncbi.nlm.nih.gov/6548975/>.
- [290] A. Jacobsson et al. "The uncoupling protein thermogenin during acclimation: indications for pretranslational control". In: *The American journal of physiology* 267.4 Pt 2 (1994). URL: <https://pubmed.ncbi.nlm.nih.gov/7943441/>.
- [291] Jan Nedergaard and Barbara Cannon. "UCP1 mRNA does not produce heat". In: *Biochimica et biophysica acta* 1831.5 (May 2013), pp. 943–949. URL: <https://pubmed.ncbi.nlm.nih.gov/23353596/>.
- [292] Gennady Bronnikovsq et al. "Beta-adrenergic, cAMP-mediated stimulation of proliferation of brown fat cells in primary culture. Mediation via beta 1 but not via beta 3 adrenoceptors." In: *Journal of Biological Chemistry* 267.3 (1992), pp. 2006–2013.
- [293] Ronald A. Butow and El Mustapha Bahassi. "Adaptive thermogenesis: orchestrating mitochondrial biogenesis". In: *Current biology : CB* 9.20 (Oct. 1999). URL: <https://pubmed.ncbi.nlm.nih.gov/10531019/>.
- [294] Javier Gómez-Ambrosi, Gema Frühbeck, and J. Alfredo Martínez. "Rapid in vivo PGC-1 mRNA upregulation in brown adipose tissue of Wistar rats by a β 3-adrenergic agonist and lack of effect of leptin". In: *Molecular and Cellular Endocrinology* 176.1-2 (May 2001), pp. 85–90. URL: <https://pubmed.ncbi.nlm.nih.gov/11369446/>.
- [295] Pere Puigserver et al. "A Cold-Inducible Coactivator of Nuclear Receptors Linked to Adaptive Thermogenesis". In: *Cell* 92.6 (Mar. 1998), pp. 829–839.
- [296] S. B. Prusiner, B. Cannon, and O. Lindberg. "Oxidative metabolism in cells isolated from brown adipose tissue. 1. Catecholamine and fatty acid stimulation of respiration". In: *European journal of biochemistry* 6.1 (1968), pp. 15–22. URL: <https://pubmed.ncbi.nlm.nih.gov/5725810/>.

- [297] Nora Reed and John N Fain. "Potassium-dependent Stimulation of Respiration in Brown Fat Cells by Fatty Acids and Lipolytic Agents*". In: *Journal of Biological Chemistry* 243.23 (1968), pp. 6077–6083.
- [298] Qin Zeng et al. "A Unique Population: Adipose-Resident Regulatory T Cells". In: *Frontiers in Immunology* 9.September (2018), pp. 1–14.
- [299] Barbara Cannon and Jan Nedergaard. "Brown adipose tissue: function and physiological significance". In: *Physiological reviews* 84.1 (Jan. 2004), pp. 277–359. URL: <https://pubmed.ncbi.nlm.nih.gov/14715917/>.
- [300] Dasa Medrikova et al. "Brown adipose tissue harbors a distinct sub-population of regulatory T cells". In: *PLoS ONE* 10.2 (2015), pp. 1–13.
- [301] Sam Virtue and Antonio Vidal-Puig. "Assessment of brown adipose tissue function". In: *Frontiers in Physiology* 4 (2013). URL: [/pmc/articles/PMC3671177/%20/pmc/articles/PMC3671177/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3671177/](https://pubmed.ncbi.nlm.nih.gov/24811177/).
- [302] Rolf Westerberg et al. "ELOVL3 is an important component for early onset of lipid recruitment in brown adipose tissue". In: *Journal of Biological Chemistry* 281.8 (Feb. 2006), pp. 4958–4968. URL: [http://www.jbc.org/article/S0021925819767921/fulltext%20http://www.jbc.org/article/S0021925819767921/abstract%20https://www.jbc.org/article/S0021-9258\(19\)76792-1/abstract](http://www.jbc.org/article/S0021925819767921/fulltext%20http://www.jbc.org/article/S0021925819767921/abstract%20https://www.jbc.org/article/S0021-9258(19)76792-1/abstract).
- [303] Olivia Sveidahl Johansen et al. "Lipolysis drives expression of the constitutively active receptor GPR3 to induce adipose thermogenesis". In: *Cell* 184.13 (June 2021), 3502–3518.e33. URL: [http://www.cell.com/article/S0092867421005729/fulltext%20http://www.cell.com/article/S0092867421005729/abstract%20https://www.cell.com/cell/abstract/S0092-8674\(21\)00572-9](http://www.cell.com/article/S0092867421005729/fulltext%20http://www.cell.com/article/S0092867421005729/abstract%20https://www.cell.com/cell/abstract/S0092-8674(21)00572-9).
- [304] Ana I Domingos. "Leptin: a missing piece in the immunometabolism puzzle". In: *Nature reviews. Immunology* 20.1 (Jan. 2020), p. 3. URL: <https://pubmed.ncbi.nlm.nih.gov/31772343/>.
- [305] Nicholas J. Carruthers et al. "The human type 2 diabetes-specific visceral adipose tissue proteome and transcriptome in obesity". In: *Scientific Reports* 11.1 (Dec. 2021), p. 11. URL: [/pmc/articles/PMC8405693/%20/pmc/articles/PMC8405693/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8405693/](https://pubmed.ncbi.nlm.nih.gov/35405693/).
- [306] F. Villarroja et al. "Inflammation of brown/beige adipose tissues in obesity and metabolic disease". In: *Journal of Internal Medicine* 284.5 (Nov. 2018), pp. 492–504. URL: <https://onlinelibrary.wiley.com/doi/full/10.1111/joim.12803%20https://onlinelibrary.wiley.com/doi/abs/10.1111/joim.12803%20https://onlinelibrary.wiley.com/doi/10.1111/joim.12803>.
- [307] Miguel López and Manuel Tena-Sempere. "Estrogens and the control of energy homeostasis: a brain perspective". In: *Trends in Endocrinology & Metabolism* 26.8 (Aug. 2015), pp. 411–421.