

Adrenergic, Circadian, and Dietary Regulation of Interleukin-17A Expression by Adipose $\gamma\delta$ T Cells



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Declaration

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Aaron Douglas

For my Family and Friends

Abstract

Adipose tissue is home to a unique population of IL-17A expressing $\gamma\delta$ T cells ($\gamma\delta^{17}$ T cells), which are necessary for maintaining body temperature during cold environmental temperatures. However, the physiological role of adipose $\gamma\delta^{17}$ T cells in body temperature regulation and whole body metabolism during homeostatic conditions remains to be fully understood. Here, we show that wild-type adipose $\gamma\delta^{17}$ T cells display decreased effector function (IL-17A, TNF and ROR γ t expression), and cellular metabolism (mitochondrial ROS, mass, and membrane potential) in response to cold (4°C) environmental temperature. Core body temperature fluctuates over a 24-hour period in response to changes in the external environment, which is known as circadian rhythm. Interestingly, we observed that IL-17A expression by adipose $\gamma\delta^{17}$ T cells fluctuates over 24-hours in mice, similar to body temperature rhythm. IL-17A expression is highest at night when mice are active and feeding, and lowest during the day when mice are inactive. Administration of IL-17A *in vitro* and *in vivo* revealed a necessary role for IL-17A in regulating *de novo* lipogenesis gene expression in adipose tissue. Mice lacking IL-17A/F or adipocyte IL-17RC showed impaired *de novo* lipogenesis gene expression, which could be rescued upon administration of recombinant IL-17A/F. Crucially, impaired *de novo* lipogenesis in *Il17a/f^{-/-}* mice disrupted circadian whole body metabolism, and body temperature maintenance. Whole body metabolic rhythm and IL-17A circadian rhythm entrain to the light cycle, but were disrupted by a high fat diet (HFD), or reverse feeding regimen. Furthermore, disruption of circadian IL-17A expression and whole body metabolic rhythm by HFD or reverse feeding increased the onset, severity and susceptibility to a mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). However, this was counteracted when mice were fed a 50% calorically restricted diet, which maintained whole body metabolic rhythm and prevented disease onset. As shift work is becoming more prevalent in modern society and is known to disturb circadian rhythm, IL-17A circadian signalling may offer a novel therapeutic route for preventing and treating diseases associated with circadian disruption, such as metabolic syndrome.

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Publications

Distinct metabolic programs established in the thymus control effector functions of $\gamma\delta$ T cell subsets in tumour microenvironments.

Lopes N, McIntyre C, Martin S, Raverdeau M, Sumaria N, Kohlgruber AC, Fiala, GJ, Agudelo LZ, Dyck L, Kane H, **Douglas A**, Cunningham S, Prendeville H, Loftus R, Carmody C, Pierre P, Kellis M, Brenner M, Arguello RJ, Silva-Santos B, Pennington DJ, Lynch L.

Nature Immunology, 2021.

Suppressive effects of the obese tumour microenvironment on CD8 T cell infiltration and effector function.

Dyck L, Prendeville H, Raverdeau M, Wilk MW, Loftus RM, **Douglas A**, McCormack J, Moran B, Wilkinson M, Mills EL, Doughty M, Fabre A, Hogan A, Heneghan H, LeRoux C, Chouchani ET, O'Shea D, Brennan D, Lynch L.

Journal of Experimental Medicine, 2022.

The source of dietary fat, rather than obesity, influences anti-tumour immunity.

Prendeville H, Loftus RM, Dyck L, Sinclair LV, Rollings C, **Douglas A**, Kunkemoeller B, McIntyre CL, Mitchelson KAJ, Harmon C, Raverdeau M, O'Brien KL, Kane H, Roche HM, Finlay DK, Kaech S, Mills EL, Chouchani ET, Lynch L.

Revision Experiments for *Nature*, January 2022.

Circadian expression of IL-17A by adipose $\gamma\delta$ T cells maintains *de novo* lipogenesis rhythm in adipose tissue.

Douglas A, Stevens B, O'Brien K, Kane H, Prendeville H, Garza AE, Lynch L.

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Abbreviations

2-DG	2-deoxy-D-glucose
β-me	2-mercaptoethanol
ACACA	Acetyl-CoA carboxylase alpha
ACLY	ATP citrate lyase
Act1	NF-κB activator 1
Anti-A	Antimycin-A
AP-1	Activator protein 1
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
B-AR	β-adrenergic receptor
BAT	Brown Adipose Tissue
BDNF	Brain derived neurotrophic factor
BFA	Brefeldin A
BM	Bone marrow
BMAL1	Brain and muscle ARNT-like 1
BSA	Bovine Serum Albumin
BTN	Butyrophilin
BTNL	Butyrophillin-like
CACT	Carnitine acylcarnitine translocase
CC	4°C cold temperature
CD	Cluster of differentiation 3
C/EBPβ	CCAAT/enhancer-binding protein beta
C/EBPδ	CCAAT/enhancer-binding protein delta
ChREBP	Carbohydrate response element binding protein
CLOCK	Circadian locomotor output cycles kaput
CPT1α	Carnitine palmitoyltransferase 1A
cRPMI	Complete RPMI
CRY	Cryptochrome
Ct	Cycle threshold
DETC	Dendritic epidermal T cells
DN	Double negative
DNL	<i>De novo</i> lipogenesis
DMEM	Dulbecco's modified eagle's medium

DMSO	Dimethyl sulfoxide
EAE	Extracellular acidification rate
ECAR	Experimental autoimmune encephalomyelitis
EGR3	Early growth response 3
ELOVL	Elongation of long-chain fatty acids family member
epiWAT	Epididymal white adipose tissue
ETC	Electron transport chain
FACS	Fluorescent-activated cell sorting
FADH ₂	Flavin adenine dinucleotide
FASN	Fatty acid synthase
FCS	Foetal calf serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FFA	Free fatty acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HFD	High-fat diet
HSL	Hormone sensitive lipase
IKK	IκB kinase
IFN γ	Interferon gamma
IL-17	Interleukin-17
IL-17R	Interleukin-17 receptor
ILC2	Type-2 innate lymphoid cells
ILC3	Type-3 innate lymphoid cells
ILCP	Innate lymphoid progenitor cell
iNKT cell	Invariant natural killer T cells
LN	Lymph node
MAPK	Mitogen-activated protein kinase
MAIT	Mucosal-associated invariant T cell
MHC	Major histocompatibility complex
MOA	Monoamine oxidase
MR1	MHC I-related gene
MTG	Mitotracker green
NADH	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NFAT	Nuclear factor of activated T cells
NF-K β	Nuclear factor K β

NFIL3	Nuclear factor interleukin 3 regulated
NGF	Nerve growth factor
NK cell	Natural Killer cell
NKT cell	Natural killer T cells
NOTCH1	Neurogenic locus notch homolog protein 1
OCR	Oxygen consumption rate
Oligo	Oligomycin
OxPhos	Oxidative phosphorylation
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PER	Period
PGC-1 α	Peroxisome proliferation receptor- γ co-activator- α
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome proliferation receptor
PRR	Pattern recognition receptor
P/S	Penicillin-streptomycin
RER	Respiratory exchange ratio
ROS	Reactive oxygen species
ROR	Retinoic orphan receptor
ROR γ t	Retinoic acid receptor-related orphan receptor γ t
Rot	Rotenone
RPMI-1640	Roswell Park memorial institute-1640 medium
RT	Room temperature
S1P1	Sphingosine-1-phosphate-receptor-1
SAM	Sympathetic associated macrophage
SCD	Stearoyl-coenzyme A desaturase
SCN	Suprachiasmatic nucleus
scWAT	Subcutaneous white adipose tissue
SFD	Standard fat diet
SOX13	SRY-box13
SREBP	Sterol regulatory element-binding protein
T2D	Type 2 diabetes
TAG	Triacylglycerol
T-bet	T-box expressed in T cells
TCA cycle	Tricarboxylic acid cycle

TCR	T cell receptor
TdT	Deoxynucleotidyl transferase
TGF- β	Transforming growth factor beta
Th	T helper cell
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF6	TNF-associated factor 6
Treg	Regulatory T cell
TMRM	Tetramethylrhodamine, methyl ester, perchlorate
UCP-1	Uncoupling protein 1
VLRC	Variable lymphocyte receptor C

Chapter 1

Introduction

1.1 Interleukin-17A: An evolutionarily conserved cytokine

1.1.1 The discovery of the IL-17 cytokine family

In 1993, the founding member of the interleukin-17 (IL-17) family of cytokines, later known as IL-17A, was identified and mapped to the mouse and human genome¹. Subsequent screening for IL-17A homologs revealed 5 structurally similar proteins which were designated IL-17B through to IL-17F². The first receptor for IL-17A was discovered 2 years later, when it was observed that IL-17A could elicit IL-6 secretion from fibroblasts, and was termed IL-17 receptor (IL-17R)³. Similar to the IL-17 cytokine family, multiple receptors for IL-17 were identified following the discovery of IL-17R, which was later termed IL-17RA, with 4 subsequent receptors named IL-17RB through to IL-17RE⁴. Interest in the IL-17 cytokine family accelerated almost a decade after their discovery, when early papers suggested that IL-17A was produced by a novel subset of T helper cells, which were characterised based on their cytokine secretion, and later became known as Th17 cells⁵. These findings challenged the existing paradigm of a Th1 and Th2 duality which was postulated by Coffman and Mosmann in 1986⁶. More recently, IL-17A has been identified as a highly conserved cytokine among both invertebrate (worms⁷, molluscs⁸ and sea urchins⁹) and vertebrate species (fish¹⁰, mice and humans), and has been suggested to play an evolutionarily conserved innate role in epithelial immune defence¹¹.

1.1.2 IL-17 Signal Transduction

IL-17A and IL-17F are the best characterized members of the IL-17 cytokine family, and share the closest structural homology. IL-17A and IL-17F function as obligate homodimers, or a heterodimer consisting of an IL-17A and IL-17F molecule¹². The different IL-17A-IL-17F dimeric conformations signal through the same receptor, which consists of IL-17RA and IL-17RC subunits, to form a heterodimeric receptor¹³. Although IL-17A and IL-17F signal through the same receptor, it has been suggested that IL-17A homodimers have a higher affinity for the IL-17RA subunit, while IL-17F homodimers display a higher affinity for IL-17RC¹⁴. Additionally, IL-17A homodimers may signal through an alternate receptor complex consisting of IL-17RA and IL-17RD subunits¹⁵. IL-17 receptors are categorized by a conserved cytoplasmic motif known as the “similar expression of fibroblast growth factor and IL-17 receptor” or SEFIR domain, which is similar in structure to the toll/interleukin-1 receptor (TIR) domain found in toll-like receptors (TLRs), and IL-1 receptors¹⁶. The primary step in IL-17R signal transduction is the recruitment of NF-κB activator 1 (Act1) which displays E3 ubiquitin ligase activity (Figure 1.1)¹⁷. Act1 is an essential component of IL-17

receptor signal transduction, as loss of Act1 in mice mimics the phenotype of mice lacking IL-17RA¹⁸. Upon IL-17A binding, Act1 recruits and ubiquitinates TNF-associated factor 6 (TRAF6), which triggers I κ B kinase (IKK) activation and degrades I κ B α , ultimately activating the transcription factor nuclear factor κ B (NF- κ B)¹⁹. In addition to increased NF- κ B activity, TRAF6 promotes the activation of the mitogen-activated protein kinase (MAPK) intracellular pathway, which upregulates the expression of the pro-inflammatory transcription factors activator protein 1 (AP-1), CCAAT/enhancer-binding protein beta (C/EBP β) and CCAAT/enhancer-binding protein delta (C/EBP δ)²⁰. Together NF- κ B, AP-1, C/EBP β and C/EBP δ enhance the expression of IL-17A and IL-17F target genes. IL-17A mediated activation of NF- κ B also induces negative feedback loops which regulate NF- κ B activity, which includes deubiquitinating enzymes, to end the intracellular signalling cascade²¹.

1.1.3 Protective roles of IL-17A in host immunity

IL-17A plays a well-established role in mucosal barrier immunity, and in defence against a broad range of infections². Typically, naïve T helper (Th) cells are differentiated into effector Th17 cells in response to bacterial infection²². Microbial peptides from pathogens such as *Bordetella pertussis* and *Salmonella typhimurium* stimulate pattern recognition receptors (PRRs) on the surface of antigen presenting cells (APCs) which induces IL-23 production, resulting in increased Th17 differentiation²². Mice lacking IL-17A signalling display increased susceptibility to *S. typhimurium* dissemination from the gut, demonstrating a key role of IL-17A in defence against bacterial infection²³. Additionally, IL-17A signalling from Th17 cells is enhanced in response to viral antigens such as Poly(I:C). It has been demonstrated that IL-17A plays a role in the development of autism spectrum disorders due to viral infection during pregnancy²⁴. Furthermore, humans with rare genetic defects in IL-17A signalling pathways show increased development of fungal infections, particularly increased prevalence of candidiasis which manifests in the oral mucosa, and the skin²⁵. Therefore, IL-17A plays a crucial role in host immunity against bacterial, viral and fungal pathogenic infections.

1.1.4 Non-immunological roles of IL-17A

Although IL-17A plays a crucial role in host defence, the molecular structure of IL-17A displays a cysteine-knot fold which is more similar to the structures of nerve growth factor (NGF) and platelet derived growth factor (PDGF), rather than to other cytokines²⁶. Furthermore, IL-17R is lowly expressed by cells of the immune system, and is ubiquitously expressed in non-hematopoietic cells such as fibroblasts, keratinocytes and stromal cells²⁷.

Novel roles for IL-17A have been described beyond the role of a pro-inflammatory molecule in the regulation of energy homeostasis^{28,29}, embryonic development²⁴, short-term memory³⁰, and anxiety behaviour³¹. As the physiological role of homeostatic IL-17A is becoming increasingly apparent, the frequency of aberrant IL-17A expression in pathological conditions is becoming more clear. Overexpression of IL-17A can contribute to the development of disease not typically associated with autoimmunity, such as the development of obesity from consuming a high-fat diet (HFD) and Alzheimer's disease^{32,33}. IL-17A is largely synergistic in activity, working in concert with other cytokines and immune molecules such as tumour necrosis factor (TNF) and interferon gamma (IFN γ), which may fine tune the intracellular signalling of IL-17A in target cells to context-specific cues in homeostasis^{27,34,35}. Additionally, the expression of IL-17A is differentially regulated by cells of the immune system, for example CD4⁺ Th17 cells can be primed via T cell receptor stimulation for IL-17A expression, or regulated by commensal bacteria of the gut microbiota to express transient IL-17A³⁶. Moreover, CD4⁺ T cells are further defined into pathogenic and non-pathogenic subtypes, depending on the cytokine milieu used to differentiate them from naïve T cells³⁷. Non-pathogenic Th17 cells are induced by a cytokine combination of IL-6 and transforming growth factor beta (TGF- β 1)³⁸, with the addition of IL-23 signalling inducing pathogenic Th17 cells³⁹. Additionally, the cytokine IL-1 β plays an indispensable role in the differentiation and activation of Th17 cells, particularly in autoimmune disease development, reinforcing the contextual regulation of IL-17A expression. IL-17A is expressed by various subsets of innate lymphocytes including type-3 innate lymphoid cells (ILC3s), natural killer T cells (NKT cells), mucosal-associated invariant T cells (MAITs), and $\gamma\delta$ T cells which have a tendency towards tissue residency^{2,27}. Therefore, the activation state of an IL-17A producing cell, and the tissue microenvironment may also contextualise the role of IL-17A in homeostasis.

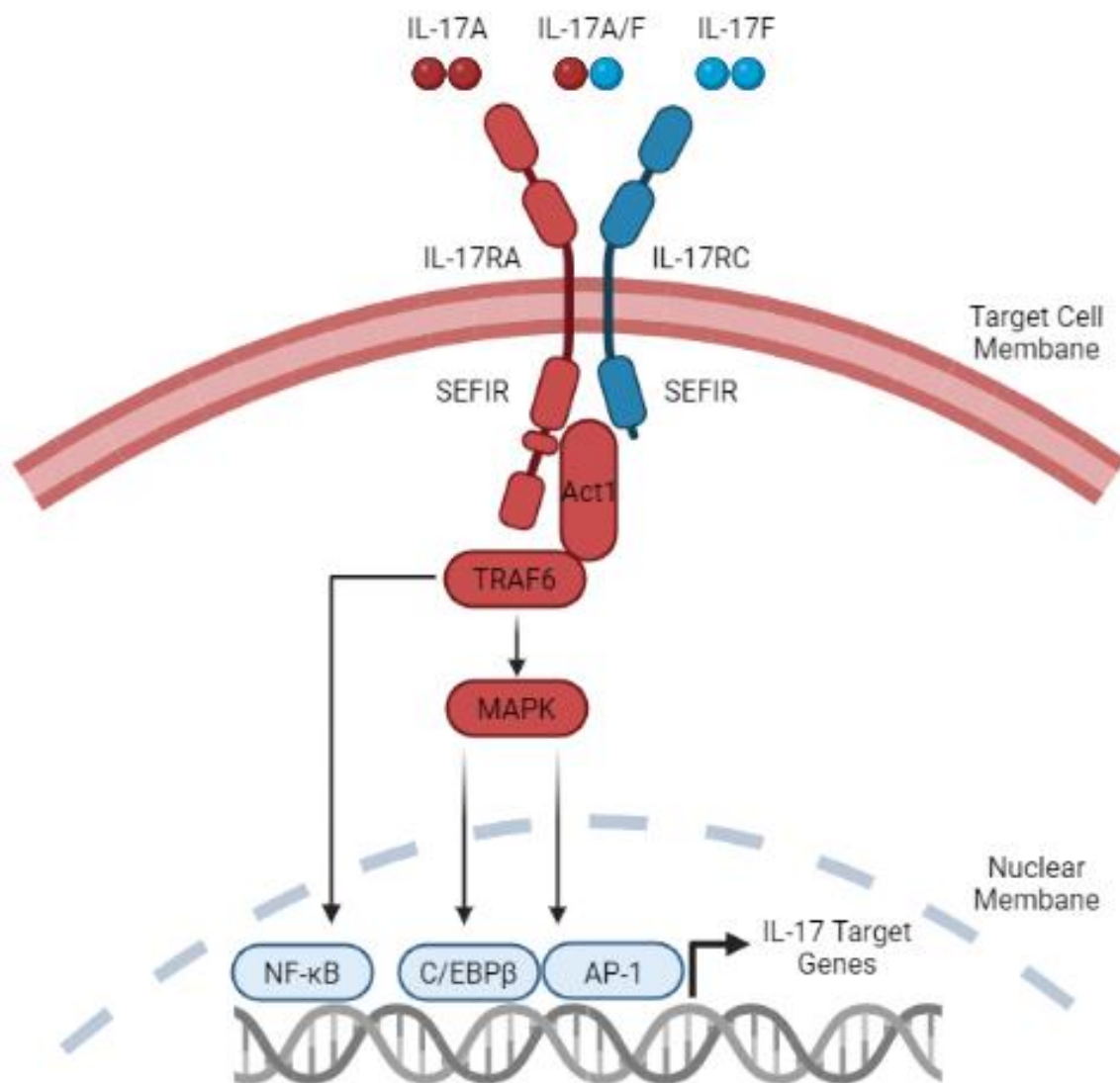


Figure 1.1: IL-17A and IL-17F signal transduction.

Binding of IL-17A and IL-17F homodimer or heterodimer complexes to their cognate receptor IL-17RA/IL-17RC recruits Act1 to the cytoplasmic tail of the receptor. Signals are transduced via the SEFIR domain of IL-17RA and IL-17RC, ubiquitinating TRAF6 through Act1, resulting in NF-κB translocation to the nucleus and transcription of IL-17 target genes. Additionally, TRAF6 induces the MAPK intracellular signalling cascade, upregulating AP-1 and C/EBPβ activity, enhancing the transcription of IL-17 associated target genes.

1.2 $\gamma\delta$ T cells: An unconventional T cell

1.2.1 $\gamma\delta$ T cell development (*Heilig and Tonegawa nomenclature*⁴⁰)

In 1986, a circulating population of human lymphocytes was identified that expressed cluster of differentiation 3 (CD3), a co-receptor for the TCR, without the traditional $\alpha\beta$ TCR⁴¹. Immunoprecipitation of chemically cross-linked CD3⁺ $\alpha\beta$ TCR⁻ lymphocytes revealed two novel CD3 associated peptides which were deemed γ and δ ⁴¹. This discovery solidified a third subset of adaptive lymphocytes outside of $\alpha\beta$ T cells and B cells. Recent studies have identified orthologous γ and δ genes in jawless invertebrates, suggesting an evolutionary conservation of $\gamma\delta$ T cells, which in lamprey were designated variable lymphocyte receptor C (VLRC⁺) lymphocytes⁴². However, the mechanism of $\gamma\delta$ T cell development remained elusive until recently. $\gamma\delta$ T cell development occurs in the thymus during embryonic development, similar to $\alpha\beta$ T cells⁴³. However, $\gamma\delta$ T cells are the first T cells to develop in every vertebrate in which T cell development has been studied⁴³. $\gamma\delta$ T cells diverge from $\alpha\beta$ T cells, and other innate T cells such as NKT cells and MAITs as early as the first double negative (DN1; CD3⁺CD4⁻CD8⁻) thymocyte stage, around the time at which the TCR δ locus opens on the chromosome⁴⁴. High expression of IL-7R α , SRY-box13 (SOX13) and strong TCR signalling, commits stage 4 double negative thymocytes to the $\gamma\delta$ T cell lineage (Figure 1.2A)⁴⁵. Thymocytes that receive weak TCR stimulation develop into $\alpha\beta$ T cells mediated by β -selection via neurogenic locus notch homolog protein 1 (NOTCH1) signalling, a TCR mediated event which rescues cells from apoptosis⁴³.

Murine $\gamma\delta$ T cells leave the thymus in concerted waves during development, expressing distinct pairs of TCR γ - and δ - chains, and home to tissues where they take residency. At embryonic day 15, the first wave of IFN γ ⁺ V γ 5V δ 1 $\gamma\delta$ T cells leave the thymus and home to the dermis, shortly followed by V γ 6V δ 1 $\gamma\delta$ T cells at embryonic day 16.5, which populate multiple peripheral tissues including the tongue, dermis, testis, adipose tissue, and meninges⁴⁶ (Figure 1.2A). Thymocytes committed to the $\gamma\delta$ T cell lineage require further TCR stimulation to develop into IFN γ - or IL-17A-producing $\gamma\delta$ T cells subsets⁴⁷. IFN γ commitment is achieved by suppression of SOX13 via strong $\gamma\delta$ TCR activation, which upregulates the transcription factors nuclear factor of activated T cells (NFAT), early growth response 3 (EGR3) and T-box expressed in T cells (T-bet)⁴⁷. Conversely, weak $\gamma\delta$ TCR stimulation maintains SOX13 expression, which increases expression of retinoic acid receptor-related orphan receptor γ t (ROR γ t), driving the commitment of IL-17-producing V γ 6⁺ and V γ 4⁺ $\gamma\delta$ T cells⁴⁷. However, distinct subsets of $\gamma\delta$ T cells also have specific TCR strength requirements, as lowering surface expression of $\gamma\delta$ TCR detrimentally impacts IFN γ -producing and V γ 6⁺ $\gamma\delta$ T cells, but not V γ 4⁺ cells⁴⁸. Additionally, SOX13 null mice

display a loss of V γ 4⁺ $\gamma\delta$ T cells, while the V γ 6⁺ compartment remains intact, highlighting the complexity of $\gamma\delta$ T cell development⁴⁹.

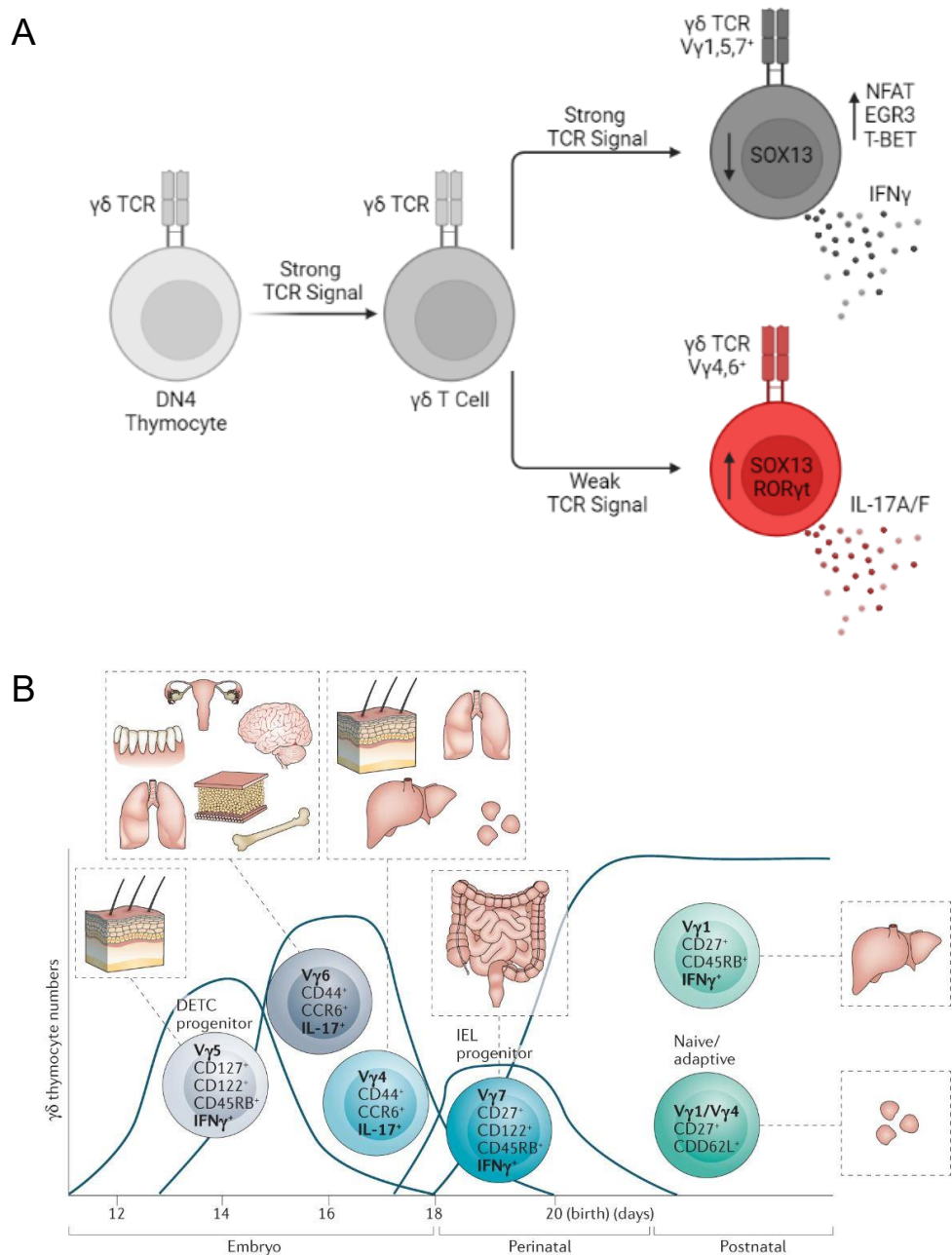


Figure 1.2: $\gamma\delta$ T cell lineage commitment and development.

(A) Strong $\gamma\delta$ TCR signalling is required for $\gamma\delta$ T cell lineage commitment, with further TCR signalling strength delineating development of $\gamma\delta$ T cell subsets into IL-17, or IFN γ , effector cells. **(B)** $\gamma\delta$ T cells leave the thymus in γ -chain subset waves during development, homing to specific tissues where they become tissue resident cells. *Adapted from Ribot, Lopes & Silva-Santos 2020⁴³.*

1.2.2 $\gamma\delta$ T cell activation and function

$\gamma\delta$ T cells are commonly referred to as the link between innate and adaptive immunity. Although $\gamma\delta$ T cells express a functional TCR, TCR activation is not essential for $\gamma\delta$ T cell effector function. During early thymic development, $\gamma\delta$ T cells lack the enzyme deoxynucleotidyl transferase (TdT), which limits diversity of variable, diversity and junction (VDJ) segment rearrangements, leading to the expression of invariant TCRs by $V\gamma 5^+$ and $V\gamma 6^+$ $\gamma\delta$ T cells⁵⁰. Unlike their $\alpha\beta$ T cell counterparts, $\gamma\delta$ T cells are not restricted by peptide antigens presented by major histocompatibility complexes (MHCs), lipid antigens presented by CD1, or metabolites presented by MHC I-related gene (MR1)⁵¹. As such, $\gamma\delta$ T cells display a rapid response kinetics to infection without the need for clonal expansion^{52,53}.

A seminal observation found that human $V\gamma 9V\delta 2$ TCR responded to a sterol metabolism intermediate of bacteria known as hydroxymethylbut-2-enyl pyrophosphate, or phosphoantigens, suggesting an adaptive role for $\gamma\delta$ T cells in host defence⁵⁴. However, it has been suggested that $V\gamma 9V\delta 2$ are innate rather than adaptive, as they display no clonal expansion dynamics after activation, as is the case with $V\delta 1$ $\gamma\delta$ T cells, which are considered more adaptive. In addition to phosphoantigens, $\gamma\delta$ TCRs respond to a family of glycoproteins known as butyrophilins, or butyrophilin-like proteins (BTNs/BTNLs), which share structural similarities with the CD80/CD86 family of costimulatory receptors for $\alpha\beta$ T cells⁵⁵. BTNs have been shown to play an important role in $\gamma\delta$ T cell thymic development, and homing of $V\gamma 5^+$ dendritic epidermal T cells (DETCs) to the skin via Skint1, a mouse specific member of the BTN family⁵⁶. Recent studies have demonstrated a crucial role for BTNs, BTNLs in $\gamma\delta$ TCR activation, as heterodimerization of human BTN3A1 and BTN2A1 binds the $V\gamma 9$ chain to induce optimal phosphoantigen sensing by $\gamma\delta$ T cells, indicating an essential role for $\gamma\delta$ TCR-BTN interactions in $\gamma\delta$ T cell immune response⁵⁷. However, it has been suggested that the $V\gamma 9V\delta 2$ TCR functions more as a TLR rather than a TCR, perceiving BTNLs as pathogen associated molecular patterns (PAMPs). Studies have also demonstrated that $\gamma\delta$ T cells can generate memory cells in response to infection, which display heightened response kinetics to reinfection⁵⁸. Memory IL-17A producing $\gamma\delta$ T cells have been shown to exaggerate inflammation in the skin in mouse models of psoriasis⁵⁸. These studies suggest complicated innate and adaptive roles of mouse and human $\gamma\delta$ T cells in host immunity.

Murine $\gamma\delta$ T cells can be separated into two distinct populations based on the expression of CD27, a member of the TNF receptor family, which demarcates murine $\gamma\delta$ T cells functionally⁵⁹. Circulating and lymphoid $\gamma\delta$ T cells are predominantly CD27⁺, express the transcription factor T-bet, and release high levels of IFN γ , TNF and granzymes upon activation⁴³. Additionally, CD27⁺ $\gamma\delta$ T cells express receptors for cytokines associated with

innate IFN γ production, such as IL-12 receptor and IL-18 receptor⁶⁰. In contrast, CD27⁻ $\gamma\delta$ T cells express the transcription factor ROR γ t and produce IL-17A, IL-17F, IL-21, IL-22 and GM-CSF in response to activation with the cytokines IL-1 β and IL-23⁶¹. Furthermore, cytokine receptor expression and cytokine production appears to be dependent on tissue residency, as adipose tissue resident V γ 6⁺ $\gamma\delta$ T cells express the IL-7 receptor, and produce IL-17A and TNF simultaneously in response to activation⁶². Rapid cytokine production in response to cytokine stimulation highlights the innate functionality of peripheral $\gamma\delta$ T cells (Figure 1.3).

1.2.3 $\gamma\delta$ T cell cellular metabolism

Immune cell function is intricately linked with cellular metabolism, the interconnection of which is known as immunometabolism. Tissue microenvironments have unique metabolomes, which in turn influence the nutrient availability to resident and circulating immune cells⁶³. This in turn regulates the effector function of immune cell populations based on their typical metabolic configuration⁶⁴. Perturbations in the local microenvironment can dramatically effect immune cell function. This is observed in obesity where a lipid rich environment inhibits IFN γ production by natural killer (NK) cells, that predominantly use glucose to fuel their effector function⁶⁵. In response to infection, immune cells increase proliferation, migration and protein synthesis of effector molecules, which are energetically demanding processes, and depend on nutrient accessibility and oxygen availability to support the increased cellular energy consumption⁶⁶. Additionally, metabolites have immunomodulatory aspects, regulating the activity of transcription factors to modulate cytokine production⁶⁷. Therefore, nutrient availability must match the metabolic demands of active immune cells to ensure an effective response to an inflammatory insult.

Similarly, $\gamma\delta$ T cells engage in specific metabolic pathways to support their effector programming and display a metabolic dichotomy between effector subsets⁶⁸. Murine CD27⁺ $\gamma\delta$ T cells engage in aerobic glycolysis, similar to the observations made in the innate production of IFN γ by NK cells⁶⁹. Glycolysis is a catabolic pathway which occurs in the cytosol in a series of enzymatic steps and converts one molecule of glucose to two molecules of pyruvate⁷⁰. The glycolytic pathway is rapid and produces two molecules of adenosine triphosphate (ATP; an energy rich molecule) for each glucose molecule metabolised. In the absence of oxygen, pyruvate is converted to lactate which is exported from the cell. Alternatively, pyruvate can be converted to acetyl-CoA in the presence of oxygen to be further metabolised in the mitochondria⁷⁰. Aerobic glycolysis occurs when oxygen is plentiful, but cells still preferentially convert glucose to lactate, rather than acetyl-

CoA. Aerobic glycolysis sustains heightened rates of glycolysis to maintain nicotinamide adenine dinucleotide (NAD^+) levels, which acts as a co-factor for the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). When not engaged in glycolysis GAPDH regulates IFN γ production by preventing the translation of IFN γ mRNA⁷¹. Therefore, sustaining NAD^+ levels through aerobic glycolysis enhances IFN γ production by occupying GAPDH in the glycolytic pathway, and may explain why CD27^+ $\gamma\delta$ T cells preferentially engage this metabolic pathway (Figure 1.3).

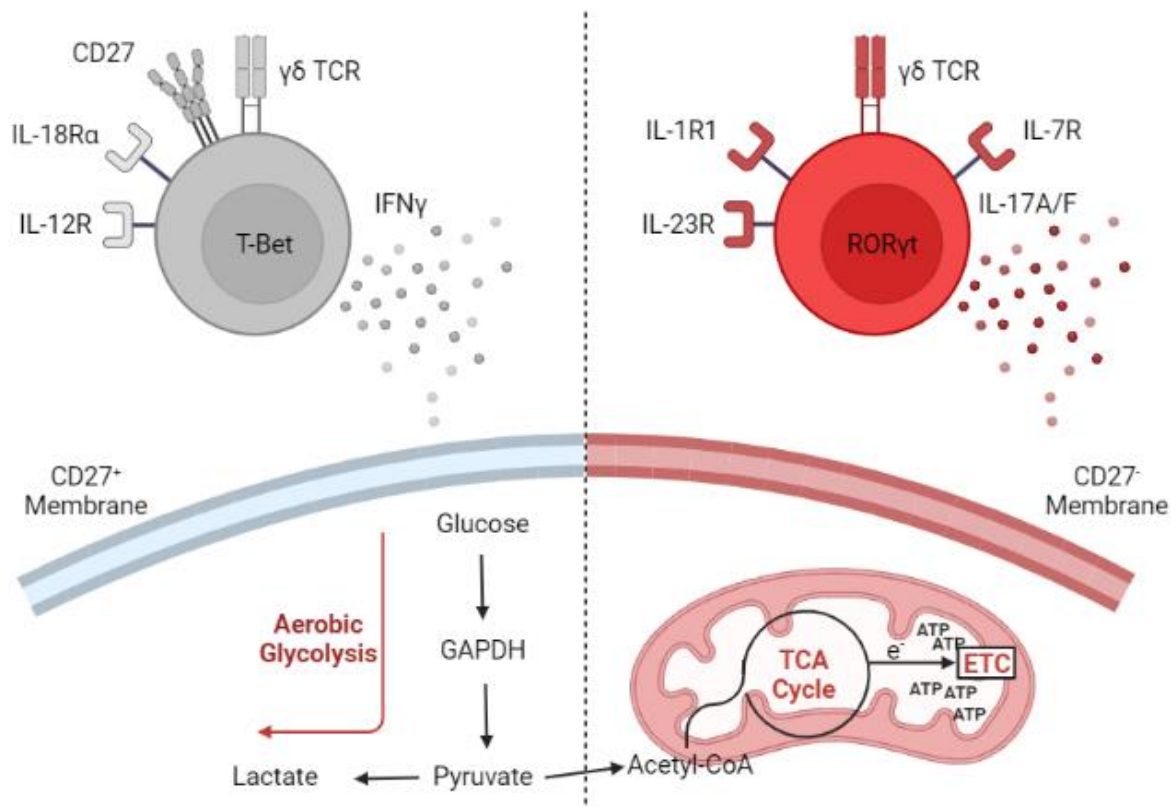


Figure 1.3: Functional and metabolic dichotomy of murine $\gamma\delta$ T cells.

The functional and metabolic dichotomy of murine $\gamma\delta$ T cells is developmentally pre-programmed in the thymus. IFN γ -producing $\gamma\delta$ T cells express the CD27 receptor, and rely on aerobic glycolysis for their effector function. Conversely, IL-17A-producing $\gamma\delta$ T cells lack expression of CD27 and utilise the TCA cycle and OxPhos for more efficient ATP generation, generating 32 molecules of ATP per glucose, compared to 2 molecules of ATP per glucose molecule for aerobic glycolysis.

Conversely, CD27^- $\gamma\delta$ T cells preferentially utilised the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OxPhos). CD27^- $\gamma\delta$ T cells displayed a higher mitochondrial mass, and membrane potential compared to CD27^+ $\gamma\delta$ T cells⁶⁸. The metabolism of glucose through the TCA cycle and OxPhos is a more energy efficient pathway than aerobic

glycolysis, in terms of ATP production, where a single molecule of glucose generates 34 molecules of ATP⁷². The TCA cycle generates reducing molecules from pyruvate such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) to feed into OxPhos to generate ATP⁷². NADH and FADH₂ donate electrons to the electron transport chain (ETC), which are ultimately accepted by oxygen and hydrogen ions (H⁺) to form water⁷². CD27⁺ $\gamma\delta$ T cells had higher expression of genes associated with the mitochondrial ETC, further supporting the utilisation of OxPhos as fuel for IL-17A production (Figure 1.3)⁶⁸. Interestingly, lipids were also identified as key metabolites for CD27⁺ $\gamma\delta$ T cells, as proliferation of $\gamma\delta^{17}$ T cells was increased when treated with cholesterol⁶⁸. Furthermore, $\gamma\delta^{17}$ T cells showed preferential uptake of palmitate and cholesterol esters in culture⁶⁸, suggesting a role for lipid metabolism in regulating $\gamma\delta^{17}$ T cell function.

1.2.4 Homeostatic and pathogenic roles of $\gamma\delta$ T cells

In recent years, the role of $\gamma\delta$ T cells in tissue homeostasis has become increasingly clear. DETCs which reside in the epidermis and dermis of the skin have been shown to play a necessary role in wound healing, where it was found that mice lacking IL-17A or $\gamma\delta$ T cells had delayed wound healing⁷³. Furthermore, TCR stimulation was required for IL-17A production, and was not elicited by cytokine stimulation alone⁷³. A subset of V γ 6⁺ $\gamma\delta$ T cells aid bone repair following a fracture by enhancing the proliferation and differentiation of osteoblasts from progenitor cells via IL-17A signalling⁷⁴. Moreover, V γ 6⁺ $\gamma\delta$ T cells are expanded in a ketogenic diet model, where they play a metabolically protective role in adipose tissue, as mice lacking $\gamma\delta$ T cells presented with higher fasting glucose levels and impaired glucose tolerance⁷⁵. Interestingly, tissue resident V γ 6⁺ $\gamma\delta$ T cells in the meninges surrounding the CNS produce IL-17A into brain parenchyma which enhances brain derived neurotrophic factor (BDNF) expression by glial cells to support excitatory glutamatergic signalling over inhibitory GABAergic signalling to form short-term memories³⁰. $\gamma\delta$ T cell derived IL-17A plays a clear role in tissue physiology, regulating response in order to maintain, or achieve, homeostasis.

Although IL-17A is necessary for maintaining tissue homeostasis, preserving an optimal concentration of IL-17A appears to be required. Although meningeal IL-17A supports short-term memory, a subsequent study observed that an over-abundance of IL-17A accelerates the onset of Alzheimer's disease and cognitive decline. Similarly, IL-17A signalling in adipocytes increases lipid uptake and storage in a diet-induced obesity model, where antagonists of ROR γ t or loss of adipocyte specific IL-17RA was sufficient to induce weight loss, or prevent weight gain, respectively³². Additionally, tumour microenvironments are

generally inhospitable to immune cells, as they are hypoxic, lipid rich environments with high levels of reactive oxygen species. However, IL-17A-producing $\gamma\delta$ T cells are enriched in tumours, where they correlate with both enhanced tumour progression and a negative prognosis⁶⁸. Furthermore, IL-17A-producing V γ 4⁺ $\gamma\delta$ T cells initiate the onset of a mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE)⁷⁶. V γ 4⁺ $\gamma\delta$ T cells are early producers of IL-17A in EAE onset. Early IL-17A production recruits 1 β -producing neutrophils and Ly6Chi inflammatory monocytes to the draining lymph nodes via enhancement of chemokine signalling. Neutrophil and monocyte recruitment to the draining lymph nodes primes pathogenic $\gamma\delta$ T cells, and Th17 cells, leading to the development of autoimmunity⁷⁶. There is a clear homeostatic window to tissue IL-17A concentration, where too little promotes infection, and too much promotes autoimmune, neurological and metabolic disorders. As such, there is a need to further understand the physiological role of homeostatic IL-17A at steady state.

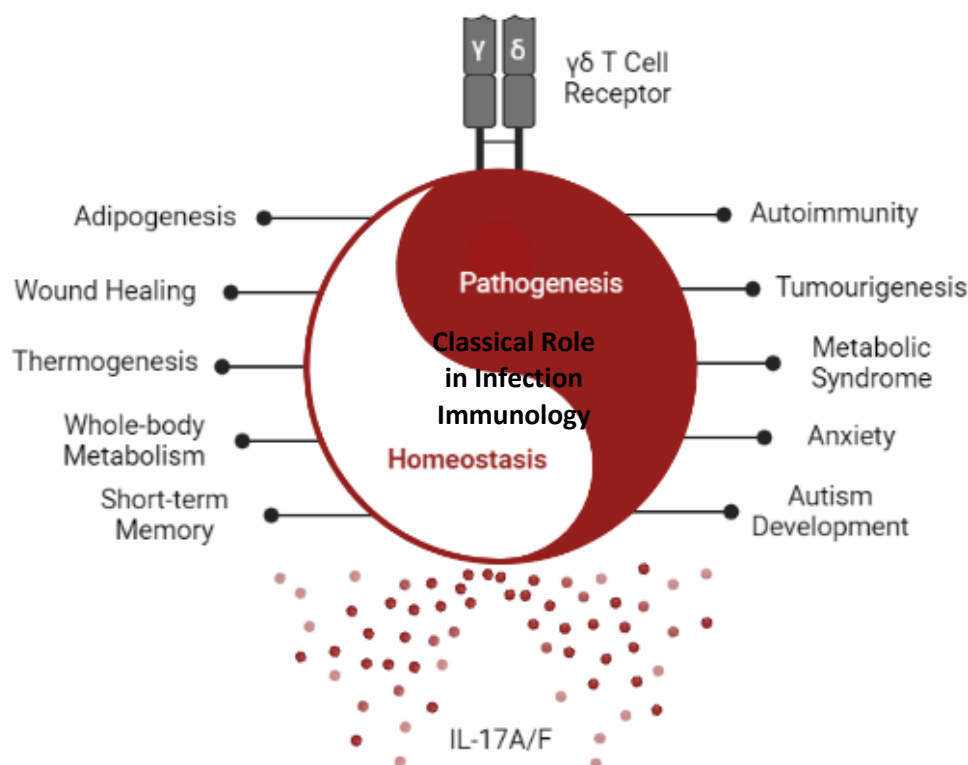


Figure 1.4: Homeostatic and pathogenic roles of $\gamma\delta$ T cell derived IL-17A.

$\gamma\delta$ T cell derived IL-17A plays an essential role in physiological processes across multiple tissues to maintain homeostasis. However, an overabundance of IL-17A signalling leads to detrimental neurological, metabolic and autoimmune disorders. It is becoming increasingly apparent that there is a temporal and spatial regulation to IL-17A signalling that exists in specific contexts to maintain physiological homeostasis.

1.3 Adipose tissue: A unique neuroimmunometabolic environment

1.3.1 *The adipose tissue immune system*

Adipose tissue is a dynamic organ which receives signals from the nervous, endocrine, and immune systems which regulate the function of the tissue. In turn a combination of these signals regulates adipose tissue function, such as adipokine secretion (leptin, adiponectin), lipid metabolism and body temperature maintenance⁷⁷. Traditionally, it was thought no crosstalk existed between adipose tissue and the immune system, with adipose tissue primarily playing a role in energy homeostasis and the immune system in host defence⁷⁷. However, it was found that TNF was elevated in an obese setting and contributed to insulin resistance in mice, which indicated a role of the immune system in adipose tissue⁷⁸. The adipose tissue immune system at steady state is an anti-inflammatory and immune-regulatory environment, biased towards a type-2 immune profile, containing M2 macrophages, type 2 innate lymphoid cells (ILC2s), T regulatory cells (Tregs) and iNKT cells⁷⁹.

The adipose immune system is predominantly tissue resident and is adapted to the lipid rich environment⁷⁷. For example, iNKT cells are the lipid sensing arm of the immune system, which respond to lipid antigens presented by CD1d molecules, and primarily express T-bet and IFN γ in the periphery⁸⁰. However, adipose tissue resident iNKT cells express the transcription factor nuclear factor interleukin 3 regulated (NFIL3), and are an important source of IL-2, IL-4 and IL-10, regulating glucose sensitivity in adipose tissue^{81–83}. Furthermore, a unique population of peroxisome proliferator-activated receptor γ (PPAR γ) expressing Tregs, which respond to the cytokine IL-33 through expression of the ST2 receptor, are fundamental to the maintenance of healthy adipose tissue. PPAR γ is a transcriptional regulator of lipid metabolism, which is important for Treg homing to adipose tissue and likely adjusts the immunometabolic profile of Tregs to survive in the lipid rich environment⁸⁴.

Adipose tissue is an immunosuppressive environment, and it is therefore unusual to find an adipose resident population of IL-17A-producing V γ 6V δ 1⁺ $\gamma\delta$ T cells²⁸. IL-17A-producing $\gamma\delta$ T cells display increased lipid and cholesterol uptake compared to CD27⁺ $\gamma\delta$ T cells⁶⁸. Furthermore, adipose $\gamma\delta$ T cells play a metabolically protective role in adipose tissue in response to a ketogenic diet, where approximately 90% of calories are derived from lipids. Additionally, lymph node $\gamma\delta$ T cells display enhanced IL-17A production in a HFD feeding model^{68,75}. Therefore, CD27⁻ $\gamma\delta$ T cell effector function may rely on lipid metabolism, which may explain the enrichment of V γ 6⁺ $\gamma\delta$ T cells specifically in adipose tissue. It has been

suggested that adipose $\gamma\delta$ T cells play a homeostatic role in adipose and systemic lipid metabolism, as loss of IL-17A signalling in adipocytes prevents the development of diet-induced obesity³². However, the homeostatic role of IL-17A production by adipose resident $\gamma\delta$ T cells in steady state remains to be fully elucidated.

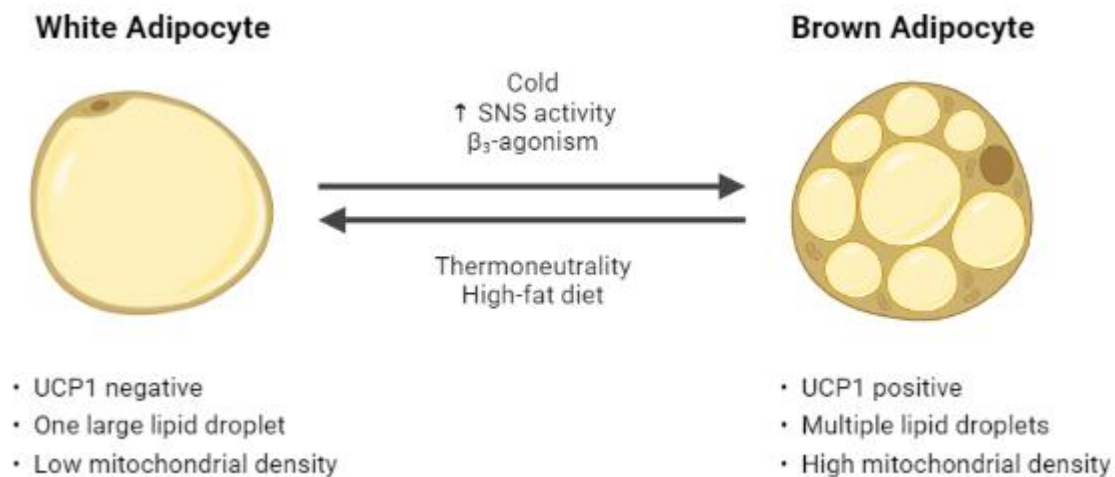


Figure 1.5: Adipose tissue heterogeneity.

Adipose tissue participates in multiple physiological functions to maintain systemic metabolic homeostasis. WAT prominently acts as an energy container and endocrine organ, while BAT participates in heat generation for the maintenance of core body temperature.

1.3.2 Adipose tissue lipolysis and thermogenesis

Adipose tissue plays an essential role in maintaining whole-body metabolism and systemic metabolic homeostasis⁸⁵. There are two major types of adipose tissue depots – white adipose tissue, which consists of epididymal (epiWAT) and subcutaneous adipose tissue (scWAT) in mice, and brown adipose tissue (BAT), which is predominantly interscapular⁸⁵. WAT plays a predominant role in energy storage in the form of triglycerides absorbed from the diet, in addition to secreting adipokines which regulate satiety, and regulating systemic glucose homeostasis. As such, the physical structure of white adipocytes consists of a single large lipid droplet, with relatively few mitochondria⁸⁶. Brown adipocytes on the other hand contain multiple small lipid droplets and have a high abundance of mitochondria⁸⁶. Brown adipocytes are enriched for expression of the β_3 -adrenergic receptor, which responds to catecholamine signalling from adipose neurons, and uncoupling protein 1 (UCP-1) for generating heat (Figure 1.45)⁸⁶. BAT is particularly enriched in infant mice and humans, as infants lack the muscle mass to generate heat through shivering, therefore BAT generates heat in a process known as non-shivering thermogenesis⁸⁷.

Thermogenesis is the ability of mammals to maintain physiological body temperature homeostasis through the generation of heat⁸⁸. Thermogenesis occurs predominantly in brown adipocytes which centre on mitochondrial respiration for heat dissipation. BAT is highly vascularized, and dissipated heat is transferred to the blood to maintain optimal body temperature⁸⁹. BAT thermogenesis is strongly induced by cold temperatures, where cutaneous thermoreceptors in the skin sense external temperature and send temperature information to the hypothalamus of the brain⁹⁰. Temperature is then relayed to adipose tissue via the sympathetic nervous system, where nerve terminals release noradrenaline into the adipose tissue environment (Figure 1.6A)⁹⁰. Noradrenaline activates the β 3-adrenergic receptor (β 3-AR) on the surface of adipocytes, which activates cyclic adenosine monophosphate (cAMP) intracellular signalling cascades to upregulate UCP-1 expression on the inner mitochondrial membrane⁸⁶. UCP-1 generates heat by uncoupling the ETC from ATP generation to heat dissipation⁹¹. This is achieved by the rapid shuttling of hydrogen ions (H^+) across the inner mitochondrial membrane into the mitochondrial matrix, and alleviates the inhibition of mitochondrial respiration caused by an increased ATP/ADP ratio⁹¹. However, heat generation for thermogenesis is an energetically demanding process, and requires a large amount of fuel for mitochondria respiration.

In addition to upregulating UCP-1 expression, activation of adipocyte β 3-AR liberates free fatty acids (FFAs) adipocyte lipid droplets to be used as fuel for mitochondrial respirations, in a process known as lipolysis⁹². Increased concentrations of intracellular adipocyte cAMP induces phosphorylation and activation of the hydrolytic enzymes adipose triglyceride lipase (ATGL), and hormone sensitive lipase (HSL), which convert lipid droplet triacylglycerides (TAGs) to FFAs and glycerol⁹². Metabolism of FFAs occurs in a process known as β -oxidation, where FFAs are converted to a fatty acyl-CoA molecule by acyl-CoA synthases. Fatty acyl-CoA molecules are converted to acylcarnitines by the enzyme carnitine palmitoyltransferase 1 (CPT1) on the surface of the outer mitochondrial membrane. Acylcarnitines are then transported into the mitochondrial matrix by the transporter carnitine acyltransferase (CACT), where it is converted back to a fatty acyl-CoA by CPT2 to be metabolised by mitochondrial β -oxidation (Figure 1.6B)⁹³. FFAs used as fuel for BAT thermogenesis are derived from lipolytic activity of WAT, as genetic deletion of ATGL from brown adipocytes had no effect on BAT thermogenesis, however ATGL deletion from both white and brown adipocytes dramatically attenuated thermogenesis^{94,95}. This study confirmed that both WAT and BAT activity during thermogenesis are essential for optimal body temperature homeostasis. Furthermore, WAT lipolysis promotes acylcarnitine production in the liver, which is actively taken up and metabolised by BAT⁹⁶.

Recent studies have confirmed a role for adipose resident IL-17A-producing $\gamma\delta$ T cells in regulating thermogenesis^{28,29}. IL-17A synergises with TNF to induce IL-33 expression by adipose tissue stromal cells, which induces thermogenesis²⁸. Loss of adipose $\gamma\delta$ T cells or IL-17A decreased expression HSL and UCP-1, which left mice susceptible to cold challenge²⁸. Furthermore, IL-17F directly influences adipose tissue thermogenesis by promoting sympathetic innervation of adipose tissue via upregulation of TGF β expression through adipocyte IL-17RC activation²⁹. Although the authors of this study suggest that IL-17F mediates adipose tissue thermogenesis and not IL-17A, both IL-17A and IL-17F are expressed by adipose tissue $\gamma\delta$ T cells simultaneously, and both signal through IL-17RC⁹⁷. Additionally, adipocytes express IL-17RA, suggesting a functional heterodimeric IL-17 receptor for IL-17A and IL-17F signalling³². These studies suggest a necessary role for homeostatic IL-17A signalling in the maintenance of whole body metabolism.

1.3.3 Adrenergic regulation of the adipose immune system

Cells of the immune system express multiple receptors which respond to neurotransmitters of the nervous system⁹⁸. The β 2-adrenergic receptor (β 2-AR; ADRB2) is the most widely expressed neurotransmitter receptor in the immune system, which modulates the differentiation and function of several immune cell populations, generally acting as an immunosuppressant⁹⁹. Adipose tissue has high sympathetic tone and noradrenergic activity, which primarily signals through adipocyte β 3-ARs to stimulate thermogenesis⁸⁶. However, adipose tissue immune cells play a bidirectional role in adrenergic activity in adipose tissue, both modulating sympathetic tone, and being modulated by noradrenaline release. Sympathetic associated macrophages (SAMs) are a subset of adipose tissue macrophages which directly impact sympathetic signalling. Adipose tissue SAMs were found to be essential for appropriate adipose tissue innervation, as loss of SAM MECP2 expression compromised sympathetic innervation of adipose tissue, impairing BAT thermogenesis¹⁰⁰. Furthermore, SAMs express the noradrenaline uptake receptor SLC6A2, and the enzyme monoamine oxidase (MOA) which degrades noradrenaline¹⁰¹. Ablation of SLC6A2 by SAMs enhanced adipose tissue thermogenesis, suggesting a homeostatic role of SAMs in noradrenaline degradation¹⁰¹. Interestingly, noradrenaline uptake shifts SAMs to a more proinflammatory phenotype, suggesting that neurotransmitter metabolism may act to fuel immune cells. In contrast, adipose tissue resident ILC2s express the β 2-AR which promotes expression of the regulatory cytokines IL-5 and IL-13, as ablation of noradrenergic neurons decreased IL-5 and IL-13 expression, but were enhanced after administration of the β 2-AR agonist clenbuterol¹⁰². Some studies have suggested that $\gamma\delta$

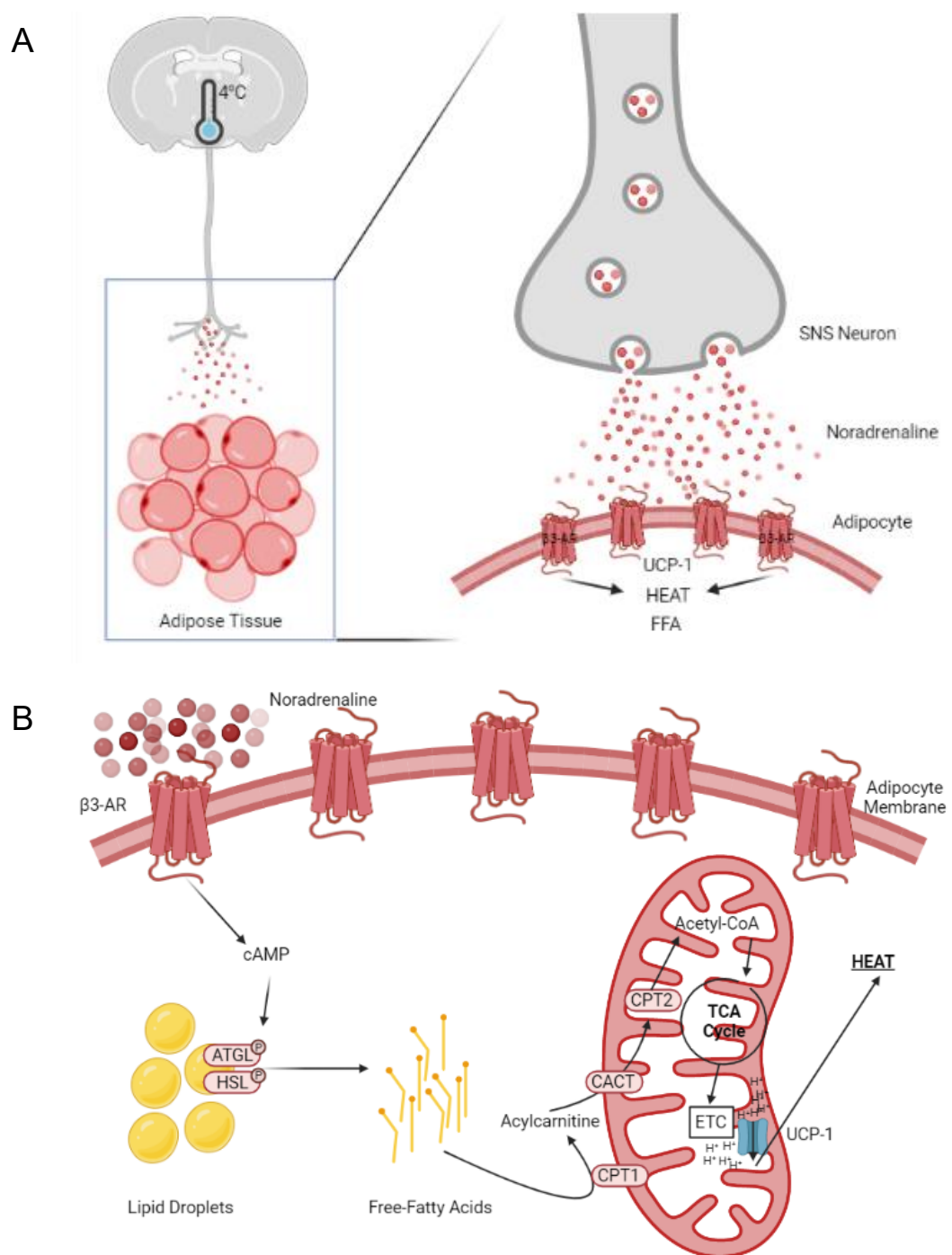


Figure 1.6: Adipose tissue thermogenesis and lipolysis.

(A) The hypothalamus of the brain sense cold temperature and release noradrenaline into the adipose tissue environment to activate β_3 -adrenergic receptors. **(B)** β_3 -adrenergic receptor activation liberates free fatty acids from lipid droplets using ATGL and HSL, which are then used to fuel mitochondrial respiration via β -oxidation of fatty acids.

T cells express the β 2-AR, and that activation of the β 2-AR inhibits IL-17A expression by Th17 cells^{103,104}. As V γ 6⁺ $\gamma\delta$ T cell are tissue resident in a highly innervated tissue, their effector function may also be regulated by sympathetic activity. However, whether adipose $\gamma\delta$ T cells express adrenergic receptors, and what effect β -AR activation has on $\gamma\delta$ T cell effector function remains to be elucidated.

1.3.4 Effect of obesity and calorie restriction on the adipose immune system

Obesity is now recognised as a healthcare crisis affecting approximately 13% of the population worldwide¹⁰⁵. Obesity leads to the development of metabolic disease, such as type-2 diabetes, which are characterised by low-grade inflammation, impaired metabolic homeostasis and dysregulated immune function¹⁰⁶. Obesity contributes to increased risk of developing at least 14 different types of cancer, including colorectal and liver cancer¹⁰⁷. The increased cancer risk in obesity is multifactorial, but is contributed to in part by excess production of circulating adipokines which promote tumour growth¹⁰⁸. Obesity reconfigures the metabolic landscape of adipose tissue from an immune-regulatory environment to a pro-inflammatory environment⁷⁷. Furthermore, it has been demonstrated that a HFD expands pro-tumoral IL-17A-producing $\gamma\delta$ T cells in the lymph nodes, adipose³² and tumours of mice⁶⁸. However, some studies suggest that IL-17A contributes to weight gain during obesity by enhancing the expression of fatty acid synthesis genes, such as carbohydrate response element binding protein (ChREBP) and sterol regulatory-element binding proteins 1c (SREBP-1c), which lead to the development of fatty liver disease¹⁰⁹. In agreement, mice lacking IL-17A signalling via genetic knock-out of IL-17A or adipocyte IL-17RC, or administering small molecule inhibitors of ROR γ t such as digoxin, GSK805, TMP778, prevented the development of obesity in mice fed a HFD^{110,111}. These studies suggest a pathological role for IL-17A in the development of obesity, and obesity associated comorbidities.

Weight loss is used as a treatment to counteract obesity associated illness and meta-inflammation caused by obesity. Weight loss approaches involve a lower consumption of calories to induce a caloric deficit to order to burn lipids from adipose tissue. However, malnutrition associated with a calorie deficit can induce starvation which is as detrimental to immunological health as obesity¹¹². Calorie restriction is associated with an overall shift to an anti-inflammatory phenotype in mouse adipose tissue⁷⁷. Furthermore, calorie restriction decreases the levels of circulating leptin, which modulates T cell differentiation and function away from a Th1 phenotype, towards a Th2 phenotype¹¹³. Crucially, calorie restriction decreased the onset and severity of EAE, suggesting calorie restriction

modulates the response of IL-17A expressing $\gamma\delta$ T cells in response to a central nervous system antigen¹¹⁴. In addition to an immunosuppressive role, the adipose tissue environment plays a protective role during calorie restriction. Calorie restriction enhanced adipogenesis in the bone marrow niche, providing nutrients for memory CD8⁺ memory T cells during energy restriction caused by a calorie deficit¹¹⁵. These studies suggest that calorie restriction promotes a healthy immune system, which supports an immunosuppressive environment preventing autoimmune and metabolic disease. However, it is unknown whether nutritional status affects the effector function of $\gamma\delta$ T cells, and how this might shape the role of $\gamma\delta$ T cells in homeostasis.

1.4 Circadian rhythm: An essential pacemaker

1.4.1 Circadian rhythm and the molecular clock

Biological processes fluctuate over a 24-hour period in response to the sleep-wake cycle, in a process known as circadian rhythm. Circadian rhythms provide an adaptive advantage for anticipating daily changes in the environment, such as light, food and temperature, and adjusting internal processes accordingly¹¹⁶. Circadian rhythms are kept in time by the master biological pacemaker in the hypothalamus of the brain, the suprachiasmatic nucleus (SCN), which is synchronised to the light cycle (Figure 1.7A)¹¹⁷. However, each individual cell in a mammalian system express their own biological pacemaker known as the molecular clock, which are kept in synchrony by the SCN¹¹⁸. The molecular clock is a system of transcriptional activators and repressors, who's own expression fluctuates in a circadian rhythm¹¹⁸. As different animals units around the world utilise different light cycles, in addition to organisms having different sleep-wake cycles between species, the term zeitgeber is used to denote the general time of a circadian day, with ZT0 denoting lights-on, and ZT12 denoting lights-off¹¹⁹. The core molecular clock proteins brain and muscle ARNT-like 1 (BMAL1) and circadian locomotor output cycles kaput (CLOCK), regulate the transcription of genes with E-box elements in the promoters¹¹⁸. BMAL1 and CLOCK form a heterodimer complex, which transcribes the expression of the transcriptional repressors period (PER1) and cryptochrome (CRY1), in addition to retinoic orphan receptors (RORs) and nuclear receptor subfamily 1 group D (NR1D1, also known as REV-ERB α)¹¹⁸. As PER-CRY complexes accumulate, they actively inhibit the transcription of BMAL1 and CLOCK. As levels of PER-CRY peak, they begin to repress their own expression in a negative feedback loop, ultimately leading to PER-CRY degradation and a release on the BMAL1

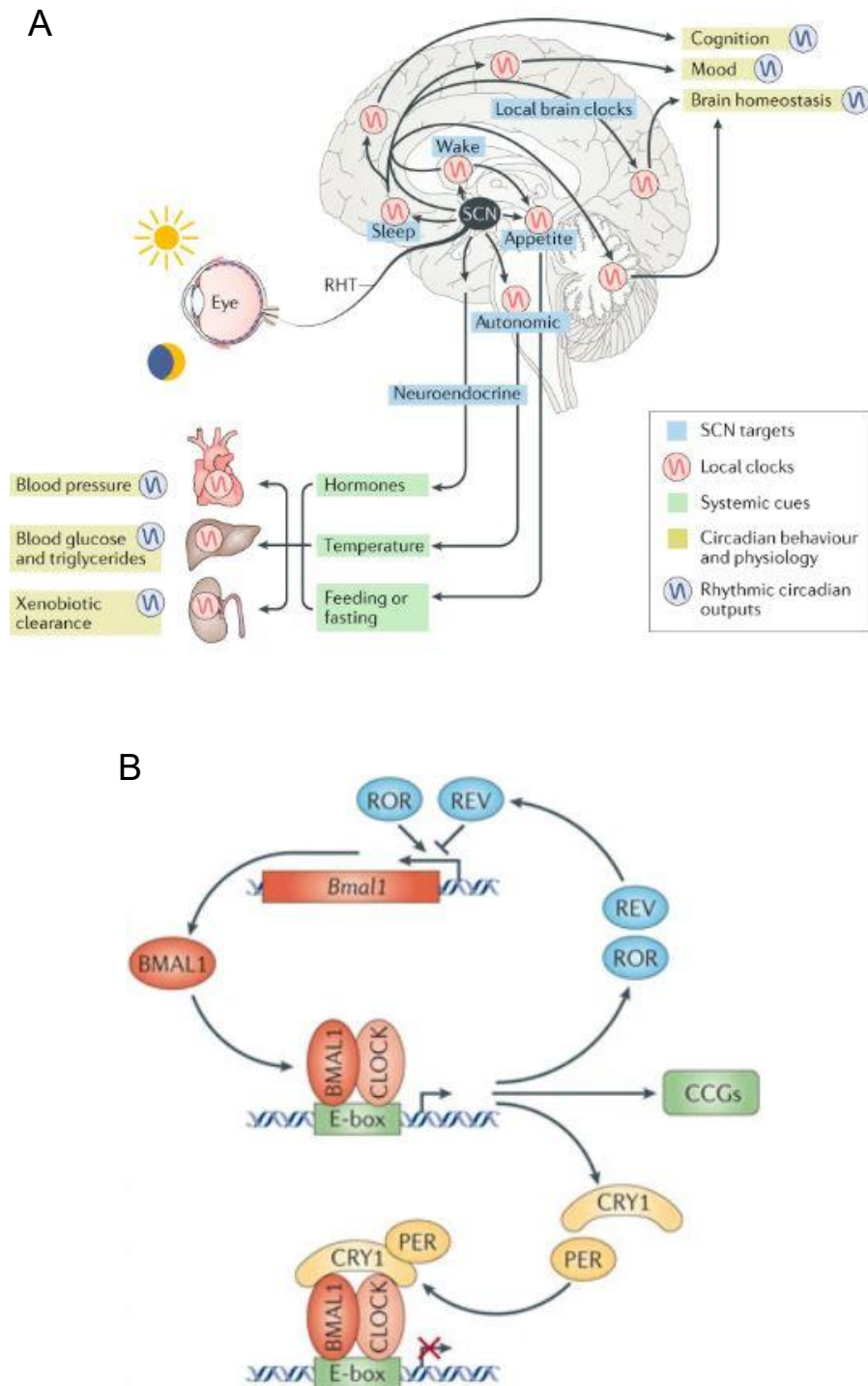


Figure 1.7: Physiological circadian rhythms and the molecular clock.

(A) The suprachiasmatic nucleus of the hypothalamus synchronises the circadian oscillations of biological functions to the light cycle. *Adapted from Hastings et al, 2018* **(B)** The molecular clock is network of transcription activators (red), and repressors (yellow), with expression of stabilising loops to maintain BMAL1 transcription (blue). *Adapted from Kondratova et al 2012.*

and CLOCK transcription site (Figure 1.7B)¹¹⁸. REV-ERB α stabilises the circadian expression of BMAL1 and CLOCK by activating BMAL1 transcription in the absence of PER-CRY, beginning the circadian loop anew, which repeats daily for the entire lifespan of an organism¹¹⁸.

1.4.2 The circadian immune system

In 1960, mice were administered high doses of an endotoxin derived from *Escherichia coli* at dawn or at dusk¹²⁰. Mice administered the endotoxin at dusk displayed increased mortality to the endotoxin compared to mice injected at dawn¹²⁰. This pioneering observation was one of the first to link circadian rhythm with immune function. It has since been observed that lymphoid tissues are equipped with functional circadian clocks, although the amplitude and phase of circadian rhythms differ between tissues¹²¹. This suggests that the expression of immune associated molecules are under circadian regulation by systemic circadian signals. Indeed, cytokine expression and cytotoxic factors produced by splenic NK cells have been shown to be modulated by noradrenergic signals in a circadian rhythm¹²². Additionally, the circadian migration of lymphoid immune cells is dependent on noradrenergic signalling from lymph node sympathetic fibres¹²³. Lymphocyte numbers peak in the blood during the day in mice, and are lowest at night. Conversely, lymphocyte numbers are highest in the lymph nodes during the day, caused by lymphocyte retention in the lymph node via β 2-AR activation¹²³. These studies show that immune function and migration is under strict circadian regulation.

In addition to innate lymphocyte function (NK cells), adaptive immune cells are also regulated by the molecular clock. Th17 cell differentiation is regulated by NFIL3, as NFIL3 inhibits ROR γ t transcription factor activity. This was shown to be specific to Th17 cells, as mice lacking NFIL3 displayed increased proportions of splenic Th17 cells, with no effect on splenic Th1 cells¹²⁴. Furthermore, it was demonstrated that NFIL3 linked ROR γ t activity and Th17 cells to the molecular clock through REV-ERB α . NFIL3 is negatively regulated by REV-ERB α , and indeed NFIL3 expression was enhanced in mice lacking REV-ERB α , demonstrating that IL-17A expression is linked to the molecular clock. Although it was shown that the molecular clock plays an essential role in immune system function, whether the molecular clock of tissue resident immune cells impacts tissue homeostasis remained to be examined.

ILC3s are gut resident innate lymphocytes which maintain gut homeostasis through interactions with gut epithelial cells, and are innate producers of IL-17A and IL-22. Recent studies have defined a role for the molecular clock in the regulation of gut resident ILC3s.

It was observed that ILC3s were enriched for the expression of molecular clock genes compared to other innate lymphoid cell subsets, and that ILC3s displayed diurnal expression of IL-17A, IL-22 and ROR γ t^{125,126}. The diurnal expression of IL-17A, ROR γ t and IL-22 was entrained by the light cycle, and not by the microbiota or the timing of food intake¹²⁵. To further investigate the role of the molecular clock in ILC3 function, mice were generated with ablated molecular clock expression specifically in ROR γ t expressing cells (BMAL1 Δ Rorc). BMAL1 Δ Rorc mice had increased adipose tissue mass compared to wild-type littermate controls, and loss of the molecular clock in ILC3s upregulated the expression of genes associated with lipid metabolism in gut epithelial cells¹²⁵. Although the researchers attributed these findings to a loss of IL-22 observed in the BMAL1 Δ Rorc mice, a similar study confirmed that BMAL1 Δ Rorc had enhanced production of IL-17A by gut ILC3s¹²⁶. Although counterintuitive, IL-17A expression is increased by gut ILCs of BMAL1 Δ Rorc, as lack of BMAL1 prevents transcription of REV-ERB α , which competes with ROR γ t for binding sites. Although there was less ROR γ t expression BMAL1 Δ Rorc ILC3s, what expression remained was impeded at transcriptional binding sites¹²⁶. These studies highlight the complexity of circadian regulation of IL-17A production, and suggest that high molecular clock expression by IL-17A producing cells may be a conserved feature of the IL-17A lineage. Studies are yet to be carried out on the role of the molecular clock in regulating tissue resident $\gamma\delta$ T cell homeostatic function.

1.4.3 Circadian regulation of adipose tissue

The coordinated expression of molecular clocks in hepatocytes and adipocytes mediates the activity of enzymes and hormone involved in carbohydrate, lipid and protein metabolism, giving rise to circadian rhythms in whole metabolic rates¹²⁷. As such, circadian disruptions alter adipose circadian rhythm, resulting in disrupted whole body metabolism¹²⁸. One of the primary functions of adipose tissue is lipid metabolism, storing energy in the form of lipids from the diet, and mobilising fatty acids in times of fasting, exercise or food scarcity. Unsurprisingly, different aspects of lipid metabolism are under regulation by the molecular clock, as different aspects of lipid metabolism must be timely regulated to meet systemic energy demands. Lipolytic activity is higher during the inactive phase of mice, while asleep, cold and fasting¹²⁹. Lipolysis is linked to the molecular clock through direct BMAL1-CLOCK mediated transcription of ATGL, and HSL which release fatty acids from lipid droplets to undergo β -oxidation to generate heat while asleep and inactive (Figure 1.8)¹³⁰. Alternatively, genes associated with lipid uptake and storage peak during the active when mice are awake, active, and feeding, in a process known as *de novo* lipogenesis¹²⁹.

Daily timing of food intake has strong effect on adipose tissue circadian rhythm and metabolic homeostasis¹³¹. Mice limited to eating during the inactive phase display increased expression of adipose lipogenic gene expression, and a loss of lipolytic gene expression, with perturbations in the circadian rhythms of leptin and insulin, predisposing mice to weight gain¹³². Furthermore, consumption of a HFD is sufficient to dampen the circadian expression of molecular clock genes in adipose tissue, disrupting whole body metabolism¹³³. Therefore, adipose tissue circadian rhythms are essential for maintaining optimal whole body metabolic homeostasis.

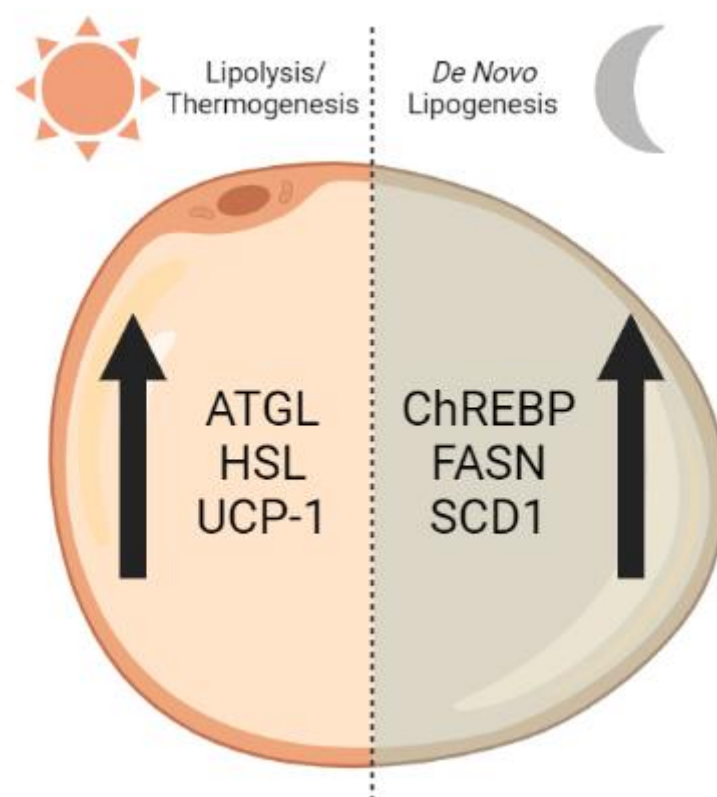


Figure 1.8: Diurnal expression of lipid metabolism genes in adipose tissue.

The molecular clock governs metabolic process in adipocytes, regulating the expression of genes involved in lipolysis during the day in mice, and lipogenesis associated genes at night, when feeding.

1.5 Thesis Aims

It is now established that IL-17A-producing $\gamma\delta$ T cells are enriched and resident in adipose tissue where they play an essential role in the regulation of lipolysis, thermogenesis, and body temperature maintenance^{28,29}. Moreover, it is clear that IL-17A expression, in addition to adipose tissue metabolism and body temperature, are under strict circadian regulation. However, little is known about the role of tissue resident innate T cells in circadian tissue physiology. In this project, **we hypothesised that circadian expression of IL-17A by adipose $\gamma\delta$ T cells would regulate adipose tissue circadian metabolic homeostasis and whole body metabolism.**

1.5.1 Specific Aims

- 1) To define a role of IL-17A expression by $\gamma\delta$ T cells in the adipose tissue of wild-type mice during thermogenesis.
- 2) To investigate the role of circadian IL-17A production by adipose $\gamma\delta$ T cells in tissue physiology and whole body metabolism.
- 3) To investigate the effects of disrupted circadian IL-17A signalling in adipose tissue homeostasis.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

2-Deoxy-D-Glucose (2-DG), 2-Mercaptoethanol (β -Me), bovine serum albumin (BSA), fatty acid free BSA, brefeldin A (BFA), bromophenol blue, dimethyl sulfoxide (DMSO), Dulbecco's phosphate buffered saline (PBS), Dulbecco's modified eagle's medium (DMEM), fluoro-carbonyl cyanide phenylhydrazone (FCCP), ionomycin, isopropanol, mowiol, non-essential amino acids, oligomycin, paraformaldehyde (PFA), penicillin-streptomycin (P/S), phorbol 12-myristate 13-acetate (PMA), rotenone, Roswell Park memorial institute (RPMI-1640), sodium pyruvate, trypan blue were purchased from **Merck**. 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), ascarite II, ammonium chloride potassium (ACK) lysis buffer, cell-tak[™] cell and tissue adhesive, drierite, eBioscience[™] intracellular (IC) fixation buffer, eBioscience[™] IC fixation & permeabilization buffer set, eBioscience[™] permeabilization buffer (10X), N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid solution (HEPES), OneComp eBeads[™] compensation beads, ultra-pure water nuclease free and TRIzol reagent were purchased from **Fisher Scientific**. Collagenase type II was purchased from **Worthington Biochemical International**. LS columns, MS columns, QuadroMACs magnet, OctoMACs magnet and TCR γ/δ^+ T cell isolation kit were purchased from **Miltenyi Biotec**. Foetal calf serum (FCS) and Glutamax was purchased from **Gibco**. Lymphoprep[™] were purchased from **Stem Cell**. Ethylenediaminetetraacetic acid (EDTA) and virkon powder were purchased from **VWR International**. 2-propanol and ethanol were purchased from **school stores**. High-capacity cDNA reverse transcription kit and nuclease-free water were purchased from **Biosciences Ltd**. PBS tablets were purchased from **Medical Supplies Company**. Precision count beads were purchased from **Biolegend**. Rodent high-fat diets (HFDs) were purchased from **Research Diets**. Seahorse XF calibrant solution, seahorse XF RPMI media (without phenol red), glucose, glutamine, Seahorse XFp T cell metabolic profiling kit and XFp Glycolytic rate assay kit (oligomycin, BAM, rotenone, antimycin-A, 2-DG) were purchased from **Agilent Technologies**. SYBR[™] Select Master Mix was purchased from **eBioscience**.

2.1.2 Equipment

Falcon tubes (15 ml, 50 ml), sterile tissue culture plates (24, 96-U bottom), sterile tissue culture flasks (T75), pipette tips (p10, p200, p1000), petri dishes (15 mm, 60 mm), syringes (2ml, 5ml), Eppendorf tubes (0.5ml, 1.5ml), 1.5ml microtubes, serological pipettes (5ml,

10ml, 25ml) and 2ml cryovials were all purchased from **Cruinn Diagnostics Ltd**. Pipette sets (p2, p10, p20, p200, p100), aerosol barrier pipette tips (10µl, 20µl, 200µl, 1000µl), transfer pipettes, pipette boy, cell strainers (70µm, 100µm), FACs tubes (without lids), PCR strip tubes, 0.2µm, 70µm and 100µm filters, needles (18g, 23g, 27g), scalpels, dissection tools (scissors, tweezers), steel razor blades, heparin/EDTA micro tubes, MicroAmp Optical 96-well and 384-well reaction plates, QuantStudio™ 5 real-time PCR system, StepOnePlus™ real time PCR system were all purchased from **Fisher Scientific**. Seahorse Analyzer (XFp, XFe96, XF-HS Mini), Seahorse cell culture plates, Seahorse cartridges were all purchased from **Agilent Technologies**. Centrifuges (5424R, 5425R, 5804R) were purchased from **Eppendorf**. Water bath, vortex, mini-fuge and multi-channel pipettes were purchased from **Medical Supplies Company**. Cell culture hood, steri-cycle CO₂ incubator and nanodrop 2000 UV spectrophotometer and ultrasonic cleaner were purchased from **Thermo Fisher Scientific**. Haemocytometer was purchased from **Hausser Scientific**. Leica SP8 scanning confocal and Leica LAS X acquisition software were purchased from **Leica**. LSR Fortessa, FACs CANTO, FACs Aria Fusion, MiniAmp Plus Thermal Cycler and MiniAmp optical adhesive film were purchased from **BD Biosciences**. 10x Chromium Controller was purchased from **10x Genomics**. NextSeq 500 System was purchased from **Illumina**. FlowJo v10 software was purchased from **Treestar INC**. TissueLyser2 was purchased from **Qiagen**. Promethion metabolic cages were purchased from **Sable Systems International**. Olympus BX51 upright microscope was purchased from **Olympus**.

2.1.3 R Package Library

Abind, annotate, annotationDbi, AnnotationHub, askpass, assertthat, babelgene, backports, base64enc, BH, Biobase, BiocFile Cache, BiocGenerics, BiocManager, BiocParallel, BiocVersion, Biostrings, bit, bit64, bitops, blob, brio, broom, bslib, cachem, Cairo, callr, car, carData, caTools, checkmate, circlize, cli, clue, colorspace, commonmark, ComplexHeatmap, corrplot, cosinor, cosinor2, cowplot, cpp11, crayon, crosstalk, curl, data.table, DBI, dbplyr, DelayedArray, deldir, desc, diffobj, digest, doParallel, dplyr, dqrng, DT, egg, ellipsis, evaluate, ExperimentHub, fansi, farver, fastmap, fastmatch, fgsea, filelock, fitdistrplus, FNN, fontawesome, foreach, formatR, Formula, fs, futile.logger, futile.options, future, future.apply, generics, GenomeInfoDb, GenomeInfoDbData, GenomicRanges, GetoptLong, ggplot2, ggpubr, ggrepel, ggribes, ggsci, ggsignif, ggtext, ggthemes, GlobalOptions, globals, glue, goftest, gplots, graph, gridExtra, gridtext, GSEABase, gtable, gtools, here, highr, Hmisc, hms, htmlTable, htmltools, htmlwidgets,

httpuv, httr, ica, igraph, interactiveDisplayBase, IRanges, irlba, isoband, iterators, jpeg, jquerylib, jsonlite, KEGGREST, knitr, labelling, lambda.r, later, latticeExtra, lazyeval, leiden, lifecycle, listenv, lme4, ltest, magrittr, maptools, markdown, MAST, MatrixGenerics, MatrixModels, matrixStats, memoise, mime, miniUI, minqa, msigdb, munsell, nloptr, numDeriv, openssl, openxlsx, org.Hs.eg.db, org.Mm.eg.db, parallelly, patchwork, pbapply, pbkrtest, pillar, pkgconfig, pkgload, plogr, plyr, png, polyclip, polynom, praise, prettyunits, process, progress, promises, ps, purrr, quantreg, R6, RANN, rappdirs, RColorBrewer, Rcpp, RcppAnnoy, RcppArmadillo, RcppEigen, RcppProgress, RcppTOML, RCurl, rematch2, reshape2, reticulate, rjson, rlang, ROCR, rprojroot, RSpectra, RSQLite, rstatix, rstudioapi, Rtsne, S4Vectors, sass, scales, Scattermore, sctransform, Seurat, SeuratObject, shape, shiny, SingleCellExperiment, sitmo, snow, sourcetools, sp, SparseM, spatstat.core, spatstat.data, spatstat.geom, spatstat.random, spatstat.sparse, spatstat.utils, stingi, stringr, SummarizedExperimentContainer, sys, tensor, testthat, tibble, tidyr, tidyselect, utf8, uwot, vctrs, viridis, viridisLite, waldo, withr, xfun, XML, xml2, xtable, XVector, yaml, zip, zlibbioc and zoo.

2.1.4 Module Scores

Table 2.1: List of genes used to generate module scores.

Module Score	Genes
<i>Polyamine Metabolism</i>	<i>Odc1, Srm, Sms, Amd1, Sat1, Paox, Smox</i>
<i>Amino Acid Metabolism</i>	<i>Slc1a5, Slc3a2, Slc7a1, Slc38a1, Glis, Glud1, Got2, Got1, Oat, Arg1, Pycr1, Ass1, Ahcyl2, Mat2a, Mt', Amd1, Apip, Mpst, Adi1</i>
<i>Pentose Phosphate Pathway</i>	<i>Aldoa, Dera, H6pd, Pfkf, Pfkp, Pgd, Pgm1, Pgm2, Prps1, Prps2, Rbks, Rpe, Rpia, Taldo1, Tkt</i>
<i>Steroid Metabolism</i>	<i>Dhcr24, Ebp, Lipa, Soat1, Sqle, Msmo1, Sc5d, Cyp51, Hsd17b7, Pmvk, Mvd, Fdps, Idi1, Hmgcr, Mvk, Gggs1</i>
<i>Fatty Acid Synthesis</i>	<i>Abcd1, Abcd2, Abcd3, Acaca, Acacb, Acly, Acot2, Acot8, Acot9, Acot13, Acsbg1, Acsf3, Acs13, Acss1, Acss2, Alox8, Anxa1, Asah2, Brca1, Cbr4, Decr2, Degs1, Eif6, Elovl1, Elovl4, Elovl5, Elovl6, Elovl7, Fabp5, Fads1, Fads6, Fasn, Gstm4, H2-Ke6, Hacd1, Hacd2, Hacd3, Hacd4, Hpgds, Lias, Lipc, Lipg, Liph, Lpgat1, Lpl, Ltc4s, Mapk9, Mcat, Mecn, Mgl1, Mid1ip1, Mlycd, Myo5a, Ndufab1, Nr1h2, Oxsm, Pecr, Pla2g12a, Pla2g4a, Plp1,</i>

	<i>Prkaa1, Prkab1, Prkag1, Prkag2, Ptges3, Ptgs1, Qk, Scap, Scd2, Srebf1, Tbxas1, Tecr</i>
<i>β-Oxidation</i>	<i>Abcd1, Abcd2, Abcd3, Abcd4, Acaa1a, Acaa2, Acad11, Acadl, Acadm, Acadvl, Acat1, Acat2, Acox1, Acox3, Acox1, Acs15, Akt2, Auh, Bdh2, Cpt1a, Cpt1a, Cpt2, Crat, Crot, Decr1, Echdc1, Echdc2, Echs1, Eci1, Eci2, Ehhadh, Etfa, Etfb, Etfdh, Gcdh, Hadha, Hadhb, Hsd17b10, Hsd17b4, Hsd17b7, Irs2, Ivd, Lonp2, Mlycd, Mtor, Pex2, Pex5, Pex7, Scp2, Sesn2, Slc25a17, Slc27a2, Tuba8, Twist1, Tysnd1</i>
<i>Glycolysis</i>	<i>Pkm, Pglk1, Hk1, Hk2, Ldha, Gapdh, Eno1, Pgam1, Pfkfb, Pfkfb, Slc2a1, Slc16a1, Pgm1, Pgm2, Tpi1.</i>
<i>Oxidative Metabolism</i>	<i>Acly, Aco1, Aco2, Cs, Dlat, Dlat, Pdha1, Pdha2, Dlat, Idh2, Idh3a, Idh3b, Idh3g, Mdh1, Mdh2, Ogdh, Sdha, Sdhb, Sdhc, Sdhc, Sucla2, Suclg1, Suclg2, Cox10, Cox11, Cox15, Cyc1, mt-Atp6, mt-Atp8, mt-Co1, mt-Co2, mt-Co3, mt-Nd1, mt-Nd2, mt-Nd3, mt-Nd4, mt-Nd5, mt-Nd6, Ndufa1, Ndufa10, Ndufa11, Ndufa2, Ndufa3, Ndufa4, Ndufa5, Ndufa6, Ndufa7, Ndufa8, Ndufa9, Ndufab1, Ndufb10, Ndufb2, Ndufb3, Ndufb4, Ndufb5, Ndufb6, Ndufb7, Ndufb8, Ndufb9, Ndufc1, Ndufc2, Ndufs1, Ndufs2, Ndufs3, Ndufs4, Ndufs5, Ndufs6, Ndufs7, Ndufs8, Ndufv1, Ndufv2, Ndufv3, Ppa1, Ppa2, Sdha, Sdhb, Sdhc, Sdhc, Tcirg1, Uqcr10, Uqcr11, Uqcrb, Uqcrc1, Uqcrc2, Uqcrcs1, Uqcrcr, Uqcrcq</i>
<i>Calcium Signalling</i>	<i>Plcb4, Camk4, Atp2b1, Adrb1, Atp2b4, Atp2a2, Gnas, Itpr1, Calm1, Gnaq, Plcg1, Adrb2, Prkx, Phkg2, Calm3, Atp2a3, Vdac1, Plcb2</i>
<i>Molecular Clock Score</i>	<i>Arntl, Clock, Per1, Cry1, Nr1d1, Nfil3</i>

2.1.5 Mice

In all experiments, male C57BL/6J-Olahsd mice between 8-12 weeks of age were used. Mice were bred in-house under specific pathogen-free conditions in accordance with Irish and European Union regulations, and *Il17a^{f/-}* mice were a kind gift from the lab of V.K.

Kuchroo and Dr Yoichiro Iwakura, *IL-17a^{GFP}* mice were purchased from Jackson Laboratory and *Plzf^{GFP}* mice were generated in the Sant'Angelo laboratory as previously described⁸². All animal work was performed in compliance with the Lynch laboratory project licence, with ethical approval from the Trinity College Dublin ethics committee and the Animal Research Ethics Committee from the Health Products Regulatory Authority (HPRA), and the Brigham and Women's Hospital Institutional Animal Care and Use Committee guidelines. Animals were euthanised by CO₂ inhalation. Organs were harvested in the comparative medicine unit (CMU) and carcasses were disposed of in accordance with the guidelines described by the TCD CMU.

2.1.6 Cell culture medium

γδ T cell culture medium

Complete RPMI (cRPMI) cell culture medium was prepared by supplementing RPMI-1640 with 1% Glutamax, 1% non-essential amino acids, 1% P/S, 10% FCS, 1mM sodium pyruvate, 10mM HEPES and 54μM β-Me.

Brown adipocyte cell culture medium

Complete DMEM (cDMEM) cell culture medium was prepared by supplementing DMEM with 10% FCS, 2.5mM L-glutamine, and 5mM glucose.

2.1.7 Cell culture treatments

Table 2.2: List of stimulants and inhibitors used during cell culture.

Reagent	Vehicle	Stock Concentration	Desired Concentration
2-DG	H ₂ O	1M	41.7mM
Anti-IFNγ	PBS	1mg/ml	1μg/ml
Antimycin A	Ethanol	5mM	0.5μM
Anti-TCRδ	PBS	2.74mg/ml	20μg/ml
BAM	H ₂ O	5mM	2.5μM
BFA	DMSO	1mg/ml	5μg/ml
FCCP	Ethanol	10.1μM	1μM
Ionomycin	DMSO	1mg/ml	1μg/ml
Oligomycin	Ethanol	2mM	1.5μM
PMA	DMSO	1mg/ml	5ng/ml
rmIL-1β	dH ₂ O + 0.1% BSA	10μg/ml	5ng/ml

<i>rmIL-7</i>	dH ₂ O + 0.1% BSA	20µg/ml	40ng/ml
<i>rmIL-17A</i>	dH ₂ O + 0.1% BSA	50µg/ml	50ng/ml
<i>rmIL-23</i>	dH ₂ O + 0.1% BSA	10µg/ml	5ng/ml
<i>Rotenone</i>	DMSO	1mM	0.5nM

2.1.8 Buffers and Solutions

Table 2.3: List of commonly used buffer solution recipes.

<i>Solution</i>	<i>Recipes</i>
<i>3% BSA Medium</i>	50ml: 1.5g BSA in cRPMI
<i>2% FACs Buffer</i>	50ml: 1ml FCS + 49ml PBS
<i>1X Permeabilization Buffer</i>	50ml: 5ml 10X perm buffer + 45ml dH ₂ O
<i>Adipose Digestion Buffer</i>	50ml: 50mg collagenase II + 50ml serum free RPMI
<i>Cell-Tak</i>	3.5µg/ml conc: 2986.1µl Sodium bicarbonate (0.1M pH 8) + 9.29µL cell tak (1.85mg/ml) + 4.56µl 1M NaOH
<i>MACs γδ Isolation Buffer</i>	50ml: 50ml PBS + 500µl FCS + 200 µl EDTA
<i>RBC Lysis Buffer</i>	1L: 1L dH ₂ O + 8.79g NH ₄ CL
<i>Seahorse Medium</i>	RPMI medium without phenol red + 10mM glucose + 2mM glutamine, warmed to 37°C, pH 7.4.

2.1.9 Flow cytometry / confocal antibodies and stains

Table 2.4: Antibodies used for flow cytometry.

<i>Target</i>	<i>Fluorophore</i>	<i>Clone</i>	<i>Company</i>	<i>Dilution</i>
<i>CD3</i>	FITC	17A2	Biolegend	1:200
	e450	17A2	eBioscience	1:200
	BV650	17A2	Biolegend	1:200
<i>CD4</i>	APCeF780	RM4-5	eBioscience	1:200
	BV785	RM4-5	Biolegend	1:200
<i>CD8</i>	APCeF780	53-6.7	eBioscience	1:200
<i>CD19</i>	PE-CF594	ID3	BD Biosciences	1:200
<i>CD27</i>	APCeFluor-780	LG.7F9	eBioscience	1:200
	APC-Cy7	LG.3A10	Biolegend	1:200
	PE	LG.7F9	eBioscience	1:200
	Pe-Cy7	LG.7F9	eBioscience	1:200
<i>CD45</i>	BV605	30-F11	Biolegend	1:200

	BV711	30-F11	Biolegend	1:200
CD69	BV785	H1.2F3	Biolegend	1:200
CD71	PE	RI7217	Biolegend	1:200
FoxP3	FITC	FJK-16s	Invitrogen	1:200
IL-17A	BV650	TC11-18H10.1	Biolegend	1:200
	PE	TC11-18H10.1	Biolegend	1:200
IL-17F	AF647	9D3.1C8	Biolegend	1:200
IFNγ	PerCpCy5.5	XMG1.2	Biolegend	1:200
	BV785	XMG1.2	Biolegend	1:200
	PE-CF594	XMG1.2	BD Bioscience	1:200
NFIL3	PE	S2M-E19	Biolegend	1:200
RORγt	APC	B2D	eBioscience	1:200
	BV421	Q31-378	BD Biosciences	1:200
	PE	600380	R&D Systems	1:200
T-Bet	PE-CF594	O4-46	eBioscience	1:200
TCRδ	APC	GL-3	Biolegend	1:200
	e450	GL-3	eBioscience	1:200
	PeCy7	GL-3	eBioscience	1:200
TNF	e450	MP6-XT22	eBioscience	1:200
	BV711	MP6-XT22	Biolegend	1:200
	BV785	MP6-XT22	Biolegend	1:200

Table 2.5: Stains used for imaging and flow cytometry.

<i>Stain</i>	Stock Conc.	Desired Conc.	Company	Dilution
Bodipy FL-C16	10mM	1 μ M	Invitrogen	1:10000
4',6-diamidino-2-phenylindole, dihydrochloride (DAPI)	3mM	300nM	Thermo Fischer Scientific	1:10000
HCS LipidTOX™ Green Neutral Lipid Stain	1mM	1 μ M	Invitrogen	1:1000
MitoTracker™ Green FM (MTG)	1000X in DMSO	Dilute 1:1000	Invitrogen	1:1000
MitoSOX™ Red	1mM	2.5 μ M	Invitrogen	1:2000
MitoSpy™ Red CMXRos	1mM	250nM	Biolegend	1:4000

<i>Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM)</i>	100µM	100nM	Invitrogen	1:1000
<i>Zombie Aqua Fixable Viability Dye</i>	1000X in DMSO	Dilute 1:1000	Biolegend	1:1000

2.1.10 PCR primers

Gene IDs were obtained from the NCBI website located at: <https://www.ncbi.nlm.nih.gov/>. and were loaded into the Harvard PrimerBank (<https://pga.mgh.harvard.edu/primerbank/>.) which generate a list of validated forward and reverse primer sequences for quantitative real time PCR (qRT-PCR).

Table 2.6: List of forward and reverse primer sequences for qPCR.

Gene	Forward Sequence	Reverse Sequence
Ppib	GGAGATGGCACAGGAGGAA	GCCCGTAGTGCTTCAGCTT
Actb	GCTCTGGCTCCTAGCACCAT	GCCACCGATCCACACCGCGT
Adrb2	ATGTCGGTTATCGTCCTGGC	GGTTTGTAGTCGCTCGAACTTG
Adrb3	AACTGAAACAGCAGACAGGGAC	CCCCCATGTACACCCTAGTT
Arntl	TGACCCCTCATGGAAGGTTAGAA	GGACATTGCATTGCATGTTGG
Clock	ATGGTGTTTACCGTAAGCTGTAG	CTCGCGTTACCAGGAAGCAT
Per1	TTCGTGGACTTGACACCTCTT	GGAACGCTTTGCTTTAGAT
Cry1	CACTGGTTCCGAAAGGGACTC	CTGAAGCAAAAATCGCCACCT
Nr1d1	TACATTGGCTCTAGTGGCTCC	CAGTAGGTGATGGTGGGAAGTA
Rorc	TTCACCCACCTCCACTG	GTGCAGGAGTAGGCCACATT
Mlxip1	GAGTGCTTGAGCCTGGCTTACA	GCTCTCCAGATGGCGTTGTTCA
Acly	AGGAAGTGCCACCTCCAACAGT	CGCTCATCACAGATGCTGGTCA
Fasn	CACAGTGCTCAAAGGACATGCC	CACCAGGTGTAGTGCCTTCCTC
Acaca	GTTCTGTTGGACAACGCCTTCAC	GGAGTCACAGAAGCAGCCCATT
Scd1	GCAAGCTCTACACCTGCCTCTT	CGTGCCTTGTAAGTTCTGTGGC
Scd2	GTCTGACCTGAAAGCCGAGAAG	GCAAGAAGGTGCTAACGCACAG
Elovl6	CGGCATCTGATGAACAAGCGAG	GTACAGCATGTAAGCACCAGTTC
Cidea	GGTGGACACAGAGGAGTTCTTTC	CGAAGGTGACTCTGGCTATTCC
Ppargc1a	GAATCAAGCCACTACAGACACCG	CATCCCTCTTGAGCCTTTTCGTG
Pnpla2	GGAACCAAAGGACCTGATGACC	ACATCAGGCAGCCACTCCAACA
Lipe	GCTCATCTCCTATGACCTACGG	TCCGTGGATGTGAACAACCAGG
Ucp1	GCTTTGCCTCACTCAGGATTGG	CCAATGAACACTGCCACACCTC

2.2 Methods

2.2.1 *In vivo* Drug Administration

For injection experiments, 100µl of PBS, BFA, FTY720, CL316,243 or recombinant IL-17A, were administered by intra-peritoneal (i.p.). Mice were administered BFA (300µg/mouse) and were euthanized 2-hours post injection. Mice were administered CL316, 243 (1mg/kg) were euthanized 1, 4, 6, or 12-hours post injection. Mice were administered FTY720 (2mg/kg) and were euthanized 12-hours post injection. Recombinant IL-17A (2µg/mouse) was injected three times over five days.

2.2.2 Diet Models

For **high-fat diet (HFD) feeding experiments** mice were fed a 60% high-fat diet for 3-6 weeks *ab libitum*, compared to control standard-fat diet (SFD) mice where 12% of calories were from fat. For **reverse feeding experiments** mice were fed a SFD *ab libitum* from 7am-7pm during the light cycle, compared to controls who were fed a SFD *ab libitum* from 7pm to 7am during the dark cycle. For **calorie restriction experiments**, mice were placed into metabolic cages, and food consumption was measured over two days. Total calories per day was calculated, and mice were then fed 50% of total calories consumed per day, for 1-3 weeks, compared to *ab libitum* fed controls who had 24-hour access to food.

2.2.3 Induction and assessment of EAE

Mice were fed either night fed (7pm-7am feeding), reverse fed (7am-7pm feeding), or 60% HFD fed for 3-weeks prior to EAE induction. Mice were fed a 50% calorically restricted diet for 1-week prior to EAE induction. EAE was induced in male mice by subcutaneous (s.c.) immunization with MOG₃₅₋₅₅ peptide (150µg/mouse; Genscript) emulsified in complete Freund's adjuvant (CFA; Condrex) containing 4mg/ml heat-killed MTB. Mice were injected intraperitoneally (i.p.) with 200ng/mouse pertussis toxin (PT; Kaketsuken) on day 0 to induced EAE development. Disease severity was monitored and assessed by clinical scores as follows: no clinical signs, 0; limp tail, 1; ataxic gait, 2; hind limb weakness, 3; hind limb paralysis, 4; tetra paralysis/moribund, 5. With more than 20% weight loss acting as an additional humane endpoint.

2.2.4 Tissue Processing

Prenatal mice were euthanised by decapitation. Infant and adult mice were euthanized by CO₂ inhalation. **Adipose tissue** was removed, minced with a razor, and digested in 1mg/ml of Collagenase Type II (Worthington) dissolved in RPMI, in a shaking incubator for 25-30 minutes at 37°C. Digested cells were filtered through a 70µM nylon mesh and centrifuged at 300g for 5 minutes to pellet the stromovascular fraction (SVF). **Lymph nodes** were disrupted through a 70µM filter and centrifuged for 5 minutes at 300g to pellet. **Livers** were pulped through a 100µm cell strainer, and were then layered over 10ml of lymphoprep. Liver samples were then centrifuged at 800g at room temperature for 25 minutes with the centrifuge accelerator and break switched off. The buffy coat was then removed from the liver samples. **Blood** was collected via cardiac puncture into an EDTA microtube. Red blood cells were lysed using 2mls of ACK lysis buffer for 5 minutes, and centrifuged at 300g for 5 minutes to pellet the remaining leucocytes. **Bone marrow** was removed from the femur by cutting each end of the bone, and flushing the marrow through using a 27G syringe filled with PBS. Post-processing, adipose SVF, lymph node, liver, blood, and bone marrow leucocytes were stimulated with phorbol 12-myristate 13-acetate (PMA; 10ng/ml; Sigma), ionomycin (500ng/ml; Sigma) and brefeldin A (BFA; 5µg/ml; Sigma) in cRPMI medium, incubated at 37°C for 3 hours.

For isolation of mononuclear cells from the central nervous system (CNS), mice were sacrificed and perfused with 20ml of PBS before removal of the brain and spinal cord. Brain and spinal cord mononuclear cells were then purified by first disrupting the tissues using a tissue lyser (Qiagen), then by density gradient centrifugation using Percoll.

For lymph node γδ T cell circadian cytokine production, all lymph nodes were harvested from mice at 7am (ZT0) and plated at 3-hour time points over 24 hours. Each time point was then stimulated with PMA and ionomycin in BFA for 3 hours, leading up to their respective time point before being harvested for fluorescent staining for flow cytometry analysis.

2.2.5 Murine Cell Culture

γδ¹⁷ T Cell Expansion

γδ¹⁷ T cells were expanded *in vitro* as previously described¹³⁴. In brief, pooled spleen and LN cells were cultured at 1 × 10⁶ cells per ml in RPMI 1640 containing 10% FCS, antibiotics, Glutamax (1x; Gibco), HEPES (10mM; Fisher), sodium pyruvate (1mM; Sigma), β-mercaptoethanol (54Pm; Sigma) and non-essential amino acids (1x; Sigma) with rIL-23

(5ng/ml; Miltenyi), rIL-1 β (5ng/ml; Immunotools) and α -IFN- γ (10 μ g/ml; Biolegend) in 96-well round-bottom plates coated with α -TCR- $\gamma\delta$ (1 μ g/ml; clone GL3; Biolegend) for 3 days. Cells were washed and re-seeded on fresh plastic at 1×10^6 cells per ml for a further 3 days as above without TCR- $\gamma\delta$ stimulation. Cells were then washed and re-seeded in rIL-7 (20ng/ml; Immunotools) and α -IFN- γ (10 μ g/ml) for a further 3 days. Expanded $\gamma\delta^{17}$ T cells were magnetically purified using a TCR γ/δ^+ T Cell Isolation Kit (Miltenyi), with LS and MS columns (Miltenyi), as per manufacturer's instructions. After culture, expanded cells were subjected to serum shock¹³⁵ in 50% horse serum for 2 hours to align molecular clocks and harvested every 6 hours over 24 hours. For cytokine stimulation, cells were cultured with rIL-1 β (10ng/ml) and IL-23 (10ng/ml) for 3 hours prior to harvesting.

Murine Adipocyte Cell Culture

Interscapular BAT was isolated, minced, and incubated in DMEM (glucose-free) supplemented with 10% FCS, 2.5 mM L-glutamine, and 5mM glucose. Tissue explants were then treated with or without recombinant IL-17A (50 ng/mL; R&D systems) for 18 hours at 37 °C. Once media was removed, explants were snap frozen in liquid nitrogen for RNA extraction.

2.2.6 Flow Cytometry Analysis

For quantification of mitochondrial mass and membrane potential, cells were co-stained with MitoTracker Green (MTG; 1 μ M; Invitrogen) and TMRM (100nm; Invitrogen) dyes. Cells were co-stained in RPMI for 30 min at 37°C. Cells stained with TMRM and MTG were not fixed and acquired live on the LSR Fortessa cytometer (BD). Lipid uptake was measured using BODIPY-FL-C₁₆ (1 μ M; Invitrogen), cells were incubated at 37°C for 10 minutes. Cells were stained for neutral lipids using lipidTOX Green FM (Invitrogen) according to manufacturer's instructions. For mitochondrial ROS staining, cells were incubated in mitoSOX (2.5 μ M; Invitrogen) at 37°C for 30 minutes.

For intracellular and intranuclear staining cells were washed with 1ml PBS and incubated in ZombieAqua Cell Viability Dye (Biolegend; 1:1000 in PBS) for 20 minutes at room temperature. Cells were incubated with an extracellular fluorochrome-labelled antibody cocktail with Fc block (1:200; BD Biosciences) in FACs buffer (PBS + 2% FCS) for 20 minutes at room temperature. Cells were washed with 2% FACs buffer resuspended in 100 μ l of Foxp3 staining kit (eBioscience) and incubated at room temperature for 20 minutes. Cells were washed with 1X permeabilization buffer (eBioscience) and incubated with an intracellular fluorochrome-labelled antibody cocktail in 1X perm buffer (eBioscience)

for 20 min at room temperature. Cells were incubated on ice for 30 minutes and subsequently washed in 2% FACS buffer. Cells were acquired on a FACS Canto or LSR Fortessa cytometer (BD). Data was analyzed with FlowJo_v10 software. Representative flow plots for gating of CD4⁺, CD8⁺, and $\gamma\delta$ T cells are provided below (Figure 2.1, 2.2).

2.2.7 Enzyme Linked Immunosorbant Assay (ELISA)

Blood was collected via cardiac puncture into Eppendorf tubes, and was centrifuged at 3000g for 10 minutes. Serum supernatant was collected and frozen at minus 80°C until use. 50 μ l of undiluted serum was used per sample, and IL-10 levels were quantified using the mouse ELISA MAX[™]IL-10 standard set (Biolegend).

2.2.8 RNA Extraction and qPCR Analysis

Tissues were snap frozen in liquid nitrogen, defrosted at RT, and transferred to a 2ml tube containing a 5mm stainless steel bead. Tissues were homogenized in 1ml trizol reagent (Fischer Scientific) in a tissue lyser for 2.5 min, 25 pulses / sec. 200 μ l chloroform was added to each tube which were inverted once and left at room temperature for 2-3 minutes, before centrifuging at 12,000g for 15 minutes. The aqueous phase containing RNA was transferred into a new Eppendorf tube and 500 μ l isopropanol was added to precipitate the RNA. Tubes were inverted 10 times and left at room temperature for 10 minutes, and then centrifuged at 12,000g for 10 min. Supernatants were discarded and RNA pellets were washed in 1ml 75% ethanol, diluted in RNase free dH₂O. Tubes were centrifuged at 12,000g for 5 minutes and supernatants were discarded by inverting the tube. The RNA pellet was left to dry at room temperature for 20-30 minutes, until transparent and the pellets were re-suspended in 50 μ l RNase free water. RNA was left on ice for 30 min, then in a heat block set 55°C for 15 minutes. RNA quality and concentration was determined using a Nanodrop 2000 UV spectrophotometer (Thermo Fischer Scientific). 20 μ l of cDNA was synthesized from 2 μ g of isolated RNA using the high-capacity cDNA reverse transcription kit (Biosciences Ltd) in a MiniAmp Thermal Cycler (BD Biosciences). To quantify the relative mRNA expression of genes of interest, qPCR was performed in a 96 or 384 well plate format (Fischer Scientific) using SYBR Green based detection (eBioscience). Relative mRNA levels were calculated using the $\Delta\Delta$ cycle threshold ($\Delta\Delta C_t$) method and normalized to corresponding endogenous controls – Ppib, β -actin or Rpl18.

2.2.9 Seahorse Metabolic Flux Analysis

A Seahorse XF-HS Mini Analyzer was used to determine the metabolic phenotype of $\gamma\delta$ T cells from spleen/lymph nodes expanded in vitro. Without stimulation, cells were harvested 5 hours (ZT5), and 17 hours (ZT17) post serum shock and plated at 8×10^4 cells per well of a PDL mini-plate (Agilent), in XF RPMI medium supplemented with glucose (10mM, Agilent) and glutamine (2mM, Agilent). Extracellular acidification (ECAR) and oxygen consumption (OCR) rates were acquired in real-time before and following the addition of inhibitors from the Seahorse XFp T Cell Metabolic Profiling Kit and XFp Glycolytic Rate Assay Kit (Agilent) – oligomycin (1.5 μ M), BAM (2.5 μ M), rotenone (0.5nM) plus antimycin-A (0.5 μ M), and 2-deoxyglucose (2DG, 41.7mM).

2.2.10 Metabolic Cage Analysis

Indirect calorimetry experiments were performed with the Promethion metabolic cage system, or the comprehensive lab animal monitoring system (CLAMs) system, essentially as described¹³⁶. Mice were singly housed and allowed at least twelve hours of acclimatisation to the new environment. O₂ consumption, CO₂ emission, energy expenditure, body weight, food and water intake, and locomotor activity were monitored throughout. For **cold challenge experiments**, mice were gradually shifted from 22°C to 4°C at a continuous rate over 2-hours, and were maintained at 4°C for 6, or 12-hours. For **experiments manipulating light cycles**, mice were placed inside a light tight cabinet at room temperature, and light was adjusted such that control animals had lights on from 7am-7pm, and test animals had lights on from 7pm-7am. For **antibiotic treatment experiments**, mice received neomycin (1g/L), ampicillin (1g/L) and vancomycin (0.5g/L) in their drinking water, supplemented with sucrose (4% w/v). Fresh antibiotics were administered every 2 days. Experimental analysis was performed using the online indirect calorimetry vignettes CalR¹³⁷, online software available at: <https://calrapp.org/>.

2.2.11 Confocal Imaging

Expanded and purified $\gamma\delta^{17}$ T cells (1.5×10^6 cells and >90% purity) were stained using Mitospy CMX Ros (250nM, Biolegend) for 30 minutes in serum free RPMI at 37°C, and fixed in 2% paraformaldehyde (PFA, Sigma) for 15 minutes at room temperature¹³⁸. Nuclear staining was performed using 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, 300nM, Thermo Fischer Scientific), before being mounted using Mowiol (Sigma). $\gamma\delta^{17}$ T cells were then imaged on a Leica SP8 scanning confocal equipped with $\times 63/1.4$ N.A. oil

objective. Z-stacks at 0.8 μ m increments were captured using an HyD detector in conjunction with Leica LAS X acquisition software.

Image analysis was performed on the maximum intensity of the z-stack. Mitochondrial morphology was determined on ImageJ Fiji (<http://fiji.sc/Fiji>) using the Mitochondrial Analyser plugin (Github; <https://github.com/AhsenChaudhry/Mitochondria-Analyzer>)¹³⁹. In brief, mitochondrial images were pre-processed using the following commands: 1) “subtract background” (radius = 1 μ m); 2) “sigma filter plus” (radius = 0.1 μ m, 2.0 sigma); 3) “enhance local contrast” (block size = 64, slope = 2.0 for 2D and 1.25 for 3D stacks) and 4) “gamma correction” (value = 0.80 for 2D and 0.90 for 3D). Mitochondria were then delineated as regions of interest (ROIs) using the adaptive thresholding method, where block size was set to an equivalent of 1.05 μ m and the optimal C value was determined for image set using the Mitochondrial Analyzer plugin. Thresholded images were post-processed using the “despeckle” and “remove outliers” (radius = 0.15 μ m) commands to removed residual noise.

Thresholded images were then input through the “analyse particles” command (size = 0.06 μ m²-infinity, circularity = 0.00–1.00), which quantifies mitochondrial area, perimeter, and shape descriptors. Form Factor (FF) was calculated as the inverse of the “circularity” output value ($P^2/(4\pi A)$, where 1 indicates a round object and increases with elongation). For network connectivity analysis, the “skeletonize 2D/3D” command was applied to the thresholded image to produce a skeleton map, and the “analyse skeleton” command was used to calculate the number of branches, branch lengths, and branch junctions in the skeletonized network¹³⁹.

2.2.12 Bulk RNA-Seq and scRNA-Seq Data Analysis

The bulk RNA-Seq dataset of BAT from wild-type and Adll17RC^{-/-} was downloaded from the GEO repository GSE144255 as cuff gene counts²⁹. Heatmaps were generated using the online platform Heatmapper¹⁴⁰, found online at: <http://heatmapper.ca/>.

scRNA-Seq was performed on single cell suspensions of sorted $\gamma\delta$ T cells and iNKT cells from the visceral adipose tissue of C57BL/6J mice using the 10x Genomics platform. A total of five visceral adipose tissue deposits from five independent biological replicates were pooled for sequencing. Single cell suspensions were loaded onto a 10x Chromium Controller to generate Gel Beads-in-emulsion (GEMS), and GEMs were processed to generate UMI-based libraries according to the 10X Genomics Chromium Single Cell 3' protocol. Libraries were sequenced using a NextSeq 500 sequencer. Raw BCL files were demultiplexed using Cell Ranger v3.0.2 mkfastq to generate fastq files with default

parameter. Fastq files were aligned to the mm10 genome (v1.2.0) and feature reads were quantified simultaneously using Cell Ranger count for feature barcoding. Filtered feature-barcode UMI count matrices containing quantification of gene expression were used for downstream analysis.

Downstream scRNA-Seq data analysis

A total of 22,748 cells murine $\gamma\delta$ T cells, iNKT cells, and MAIT cells expressing a median of 1,423 genes and 3,556 UMIs per cell were loaded from feature-barcode UMI count matrices using the Seurat v4.1.0 package¹⁴¹. Adipose and splenic iNKT cells were reanalyzed from GSE142845⁸³, thymic iNKT cells were reanalyzed from GSE141895¹⁴², thymic MAIT cells were reanalyzed from E-MTAB-7704¹⁴³, pulmonary $\gamma\delta$ T cells were reanalyzed from E-MTAB-8732¹⁴⁴, peripheral lymph node (PLN) and dermal $\gamma\delta$ T cells were reanalyzed from GSE123400¹⁴⁵, and meningeal $\gamma\delta$ T cells were reanalyzed from GSE147262³¹. Cells expressing more than 11% of mitochondrial genes as a % of total gene counts were considered to represent apoptotic or dead cells and were removed from the analysis. Cells were also filtered based on total UMI counts and total gene counts on a per sample basis to remove empty droplets, poor quality cells and doublets, with a minimum cutoff of at least 300 genes per cell across all samples. UMI counts were normalized using regularized negative binomial regression using SCTransform v0.3.3¹⁴⁶.

Dimensionality reduction was performed using principal component analysis (PCA) with $n = 100$ dimensions and 2000 or 3000 variable features, and an elbow plot was used to determine the number of PCA dimensions used as input for uniform manifold approximation and projection (UMAP)¹⁴⁷. For collective analysis of cells from different batches and cells sequenced using different scRNA-Seq technologies the harmony v1.0 package¹⁴⁸ was used with default settings to remove batch effects, and batch-corrected harmony embeddings were used for UMAP. UMAP was performed using a minimum distance of 0.3 and a spreading factor of 1. Shared nearest neighbor (SNN) graphs were calculated using $k = 20$ nearest neighbors. Graph-based clustering was performed using the louvain algorithm. In some cases, overclustering was performed and clusters were manually collapsed, and/or the first two dimensions of the UMAP reduction were used as input for graph-based clustering instead of PCA or harmony embeddings. Adipose $\gamma\delta$ T cells were distinguished from adipose iNKT cells by expression of *Trdc* and graph-based clustering, and analyzed separately for $\gamma\delta 1$ versus $\gamma\delta 17$ cell identification. Type 1 and type 17 innate T cells were individually identified in each dataset using graph-based clustering and gene expression analysis, and cycling cells were excluded. For thymic innate T cells, CD44⁺ progenitor populations were also excluded. Raw counts from type 1 and type 17 cell populations were then merged and normalized together into a single file for comparative analysis.

Gene expression analysis was performed using the FindMarkers() or FindAllMarkers() Seurat functions and the wilcoxon rank sum test. Heatmaps were generated using the Complex Heatmap v2.7.11 and circlize v0.4.14 packages¹⁴⁹, and module scores were calculated using the AddModuleScore() Seurat function with n = 10 control features. Density plots were produced using the Nebulosa v1.1.1 package¹⁵⁰. Log-normalized RNA counts were used for all gene expression analysis and visualization. All computational analysis was performed using R v4.1.2 and RStudio Desktop v1.4.1712 on an Ubuntu 20.04 Linux GNU (64 bit) system.

2.2.13 Statistical Analysis

GraphPad Prism 9.3.0 was used for statistical analysis. For all experiments, a 95% confidence interval was used and a p-value ≤ 0.05 was considered as statistically significant. A D'Agostino-Pearson omnibus normality test was first performed to test if the data were normally distributed (Gaussian distribution). If data was normally distributed, parametric testing was performed. If data were not normally distributed, non-parametric testing was performed. When comparing 2 groups, an unpaired or paired two-tailed student's t-test was used. When comparing more than 2 groups, an ordinary one-way ANOVA with Dunnett test was used. When comparing data with two variables, a two-way ANOVA with Bonferroni test was used. Cosine curves were fitted in GraphPad Prism and circadian rhythmicity was evaluated using the cosinor regression model using the cosinor, and cosinor2 R packages in Rstudio. The circadian period was assumed to be 24 h for all analysis and the significance of the circadian fit was assessed by a zero-amplitude test with 95% confidence. Amplitude and acrophase were extracted from the cosinor model. In all figures, * p ≤ 0.05 , ** p ≤ 0.01 , *** p ≤ 0.001 , **** p ≤ 0.0001 .

2.2.14 Gating Strategies

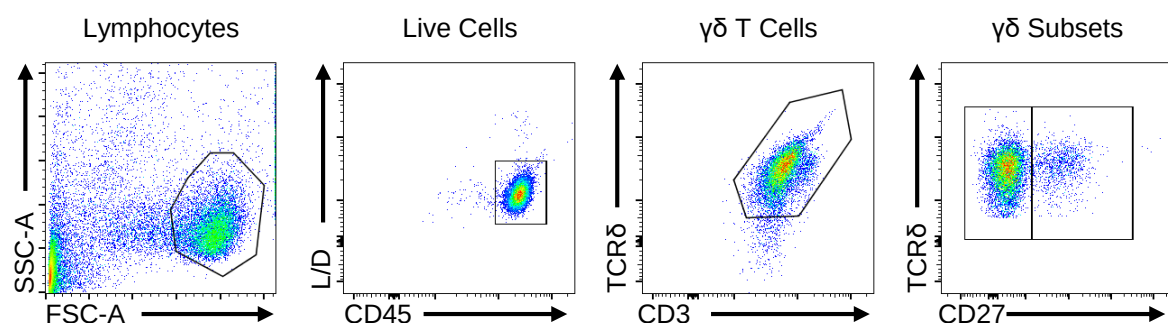


Figure 2.1: *In vitro* expanded $\gamma\delta^{17}$ T cell gating strategy.

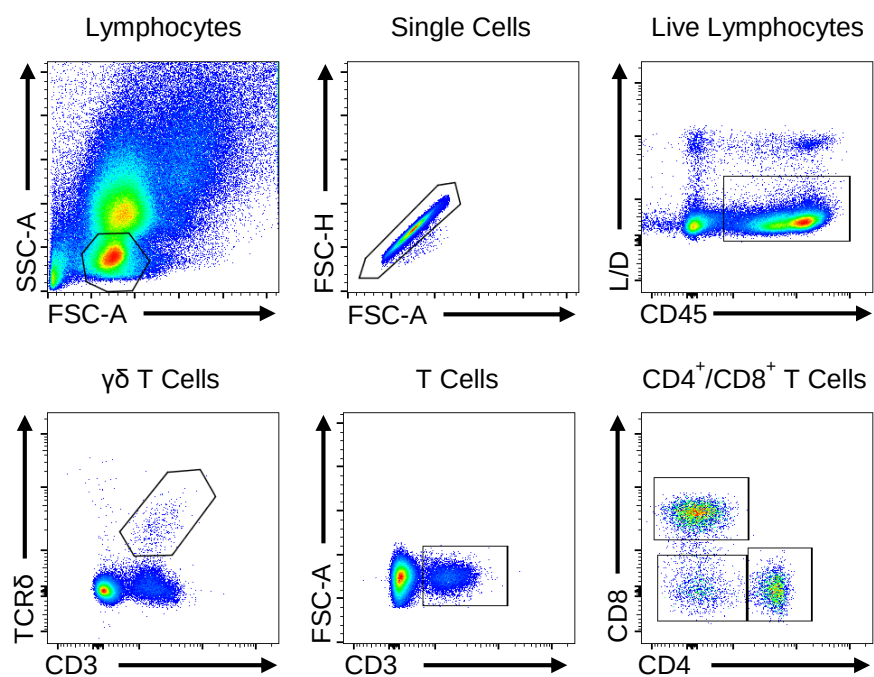


Figure 2.2: *In Vivo* gating strategy to identify CD4⁺, CD8⁺ and γδ T cells.

Representative gating strategy for tissue CD4⁺, CD8⁺ and γδ T cells using bone marrow as an example tissue. CD4⁺, CD8⁺ and γδ T cells were identified as CD45⁺CD3⁺ populations.

Chapter 3

Adipose adrenergic activity indirectly inhibits $\gamma\delta$ T cell cellular metabolism and function.

3.1 Introduction

$\gamma\delta$ T cells are pre-programmed during their development in the thymus. $\gamma\delta$ T cells migrate from the thymus in waves based on their TCR expression, which identifies their functionality¹⁵¹. In contrast to $CD4^+$ T cells which differentiate in the periphery, $\gamma\delta$ T cells develop their effector repertoire before leaving the thymus, fully committed to IL-17, or IFN γ , production¹⁵². On embryonic day 15.5, the $V\gamma6^+V\delta1^+$ subpopulation of $\gamma\delta$ T cells leave the thymus to seed mucosal tissues, including lung, tongue, gut, and uterus¹⁵³. In addition to mucosal tissues, adipose tissue has a significant $\gamma\delta$ T cell population, consisting mainly of $V\gamma6^+V\delta1^+$ $\gamma\delta$ T cells²⁸. A recent study has described a role for IL-17 produced by adipose $V\gamma6^+V\delta1^+$ $\gamma\delta$ T cells in adipose tissue nerve development, as mice lacking $V\gamma6^+V\delta1^+$ $\gamma\delta$ T cells had less innervation of BAT²⁹. Human infants have a large amount of BAT to maintain body temperature, as they lack sufficient skeletal muscle to generate enough heat through shivering⁸⁷. Although adipose $V\gamma6^+V\delta1^+$ $\gamma\delta$ T cells have been well characterised²⁸, their development, homing, and homeostatic role in the adipose tissue of foetal, and infant mice, remains to be fully elucidated.

Adipose tissue has a unique immune environment that is enriched for innate T cells, including $\gamma\delta$ T cells^{154,155}, MAIT cells and iNKT cells. It has recently been realised that the adipose tissue immune system holds potential for treating metabolic disease^{75,81}. In particular, innate T cells that are tissue resident appear to be critical for maintaining homeostasis. Cytokines including TNF¹⁵⁶, IL-6¹⁵⁷, IL-17A⁶² and IL-17F²⁹ from adipose immune, and non-immune, cells regulate local homeostasis and systemic metabolic processes. We have previously found that clonal $V\gamma6^+$ $\gamma\delta$ T cells reside in adipose tissue and produce IL-17, which is important for maintaining body temperature during cold challenge, while mice lacking $\gamma\delta$ T cells or IL-17A are susceptible to hypothermia⁶². A subsequent study demonstrated that IL-17RC signalling in adipocytes was important for this pathway, as adipocyte specific deletion of IL-17RC led to impaired thermogenesis²⁹. These studies highlight the IL-17 family as important mediators of adipose thermogenesis, however the regulation of IL-17 production by $\gamma\delta$ T cells during thermogenesis is incompletely understood.

Here we investigated if $\gamma\delta$ T cells migrate to adipose tissue prenatally. We identified that the foetal BAT lymphocyte compartment consists predominantly of $\gamma\delta$ T cells, specifically $\gamma\delta^{17}$ T cells. Moreover, $\gamma\delta^{17}$ T cells are the largest population of lymphocytes in the BAT and spleen of infant mice, as this population is decreased in the BAT of $V\gamma4/6^{-/-}$ mice. As $\gamma\delta$ T cells play a homeostatic role in adipose tissue, we examined the functionality of $\gamma\delta$ T

cells by inducing thermogenesis via cold challenge, or administration of CL316,243, which induces adipose tissue lipolysis. We observed that the proportion, but not cell number, of adipose $\gamma\delta$ T cells increased during cold challenge but not in response to CL316,243. Surprisingly, the numbers of non- $\gamma\delta$ T cells, such as CD4⁺, CD8⁺ and Treg, decreased in the adipose tissue, inguinal lymph node (inLN), and liver of cold challenged mice, but increased in the blood and bone marrow. To further investigate this we used FTY720, a lymph node egress inhibitor, to prevent T cell circulation during cold challenge. These experiments revealed that circulating Treg proportions increased during cold challenge, in addition to increased serum IL-10 during cold challenge and after CL316,243 administration. We show that thermogenesis inhibits $\gamma\delta$ T cell function (ROR γ t, IL-17A and TNF expression), and alters cellular metabolism (mitochondrial ROS, mass, and membrane potential) in a time dependent manner. We identify the β -adrenergic receptors, ADRB1 and ADRB2, as potential novel regulators of IL-17A production by adipose $\gamma\delta$ T cells. These mechanisms of cold environmental temperature-induced immunosuppression may offer novel therapeutic approaches for inflammatory disease.

Results

3.2 Foetal and infant mouse BAT are enriched for $\gamma\delta^{17}$ T Cells.

Previous work from our lab has described an essential role for $\gamma\delta^{17}$ T cells in adipose tissue homeostasis, body temperature regulation, and whole body metabolism²⁸. As adipose tissue of adult mice is enriched for tissue resident $V\gamma 6^+$ $\gamma\delta$ T cells, which are prenatally derived, we first investigated whether $V\gamma 6^+$ $\gamma\delta$ T cells migrated to BAT before birth. Lymphocytes were isolated from maternal and foetal BAT, in addition to placental tissue. We found that foetal BAT was enriched for $\gamma\delta$ T cells compared to maternal BAT and placental tissues (Figure 3.1A). Foetal $\gamma\delta$ T cells were primarily $CD27^-$ $\gamma\delta$ T cells, in contrast to placental $\gamma\delta$ T cells which were predominantly the $CD27^+$ subset (Figure 3.1B, C). Moreover, foetal BAT $\gamma\delta$ T cells had higher production of IL-17A compared to maternal BAT, and placental tissue (Figure 3.1D).

We next investigated if the increased percentage of $\gamma\delta^{17}$ T cells was maintained postnatally in the BAT of infant mice. Indeed, BAT of infant mice was further enriched for $\gamma\delta$ T cells compared to parental, and foetal, BAT $\gamma\delta$ T cells (Figure 3.2A). Furthermore, using infant $V\gamma 4/6^{-/-}$ mice resulted in a significantly reduced percentage of BAT $\gamma\delta$ T cells, further demonstrating the $\gamma\delta^{17}$ T cell phenotype of wild-type infant BAT (Figure 3.2A). In addition to adipose tissue, we found that splenic T cells were predominantly $\gamma\delta$ T cells in the infant spleen (Figure 3.2B). Using $PLZF^{GFP}$ mice, we found that infant $\gamma\delta$ T cells of BAT and spleen had higher expression of $PLZF$, a transcription factor which promotes the expression of *Rorc*¹⁵⁸, compared to BAT and splenic $\gamma\delta$ T cells of their parent (Figure 3.2C). Together, these data suggest that prenatal, and postnatal infant BAT are enriched specifically for $\gamma\delta^{17}$ T cells. As $V\gamma 6^+$ $\gamma\delta$ T cells home to tissues early in development (E15.5), $V\gamma 6^+$ $\gamma\delta$ T cells may play a role in adipose tissue during this stage of life.

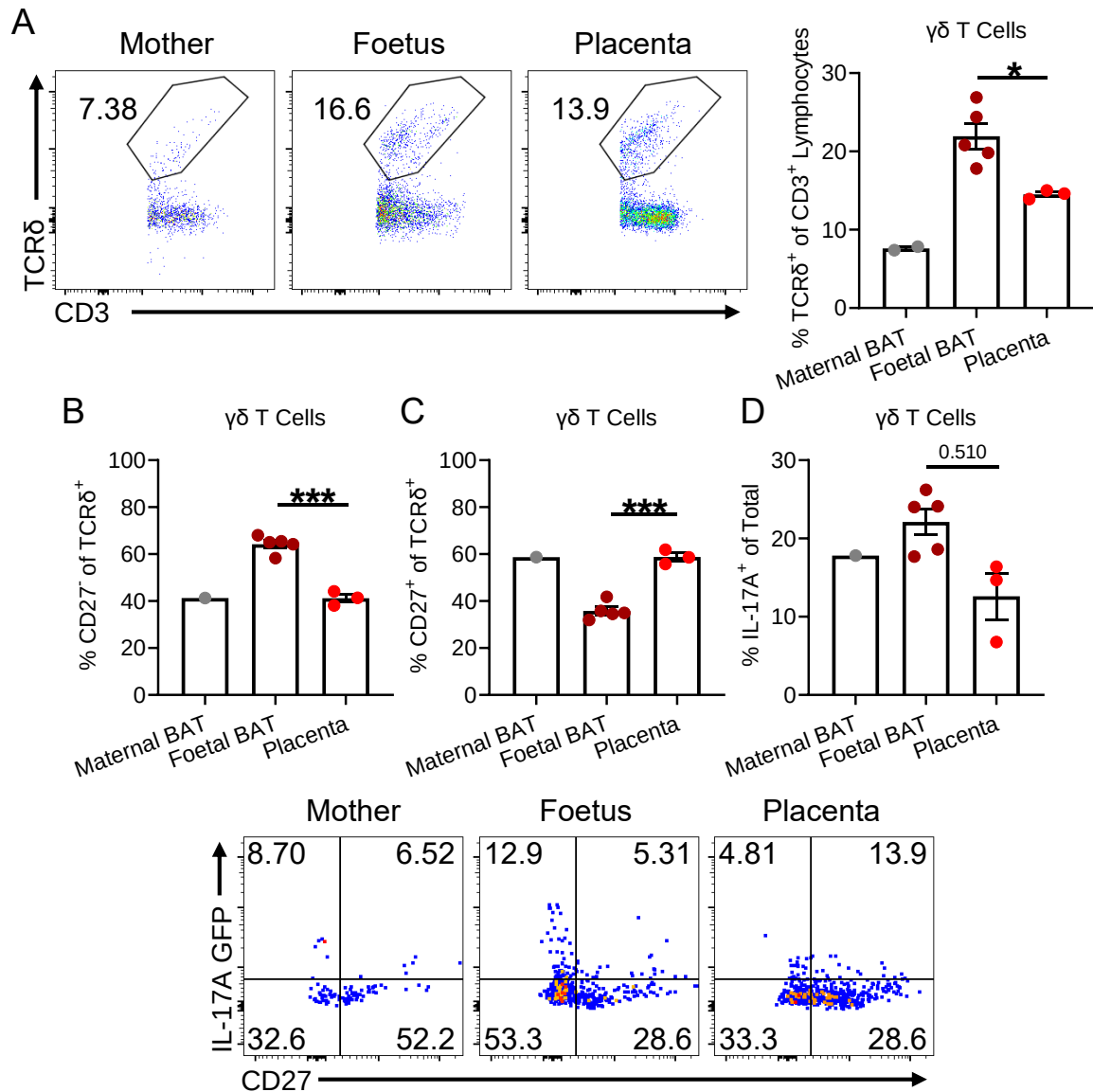


Figure 3.1: Foetal BAT is enriched for $\gamma\delta^{17}$ T cells.

(A) Representative flow plots of T cells from maternal BAT, foetal BAT, and placental tissue (left); Percentage of $\gamma\delta$ T cells across tissues (right). **(B)** Percentage of CD27 $^+$, **(C)** and CD27 $^+$ $\gamma\delta$ T cells from maternal BAT, foetal BAT, and placental tissue (top), with representative flow plots (below). **(D)** IL-17A expression by maternal BAT, foetal BAT, and placental tissue $\gamma\delta$ T cells stimulated *ex vivo* for 3-hours with PMA/Ionomycin. Data are representative of two independent experiments. **(A-D)** Data shown as mean $\pm$ SEM, $n = 1-5$ mice per group. **(A-D)** Significance was calculated using two-tailed unpaired students *t*-test. * $p < 0.05$, *** $p < 0.001$.

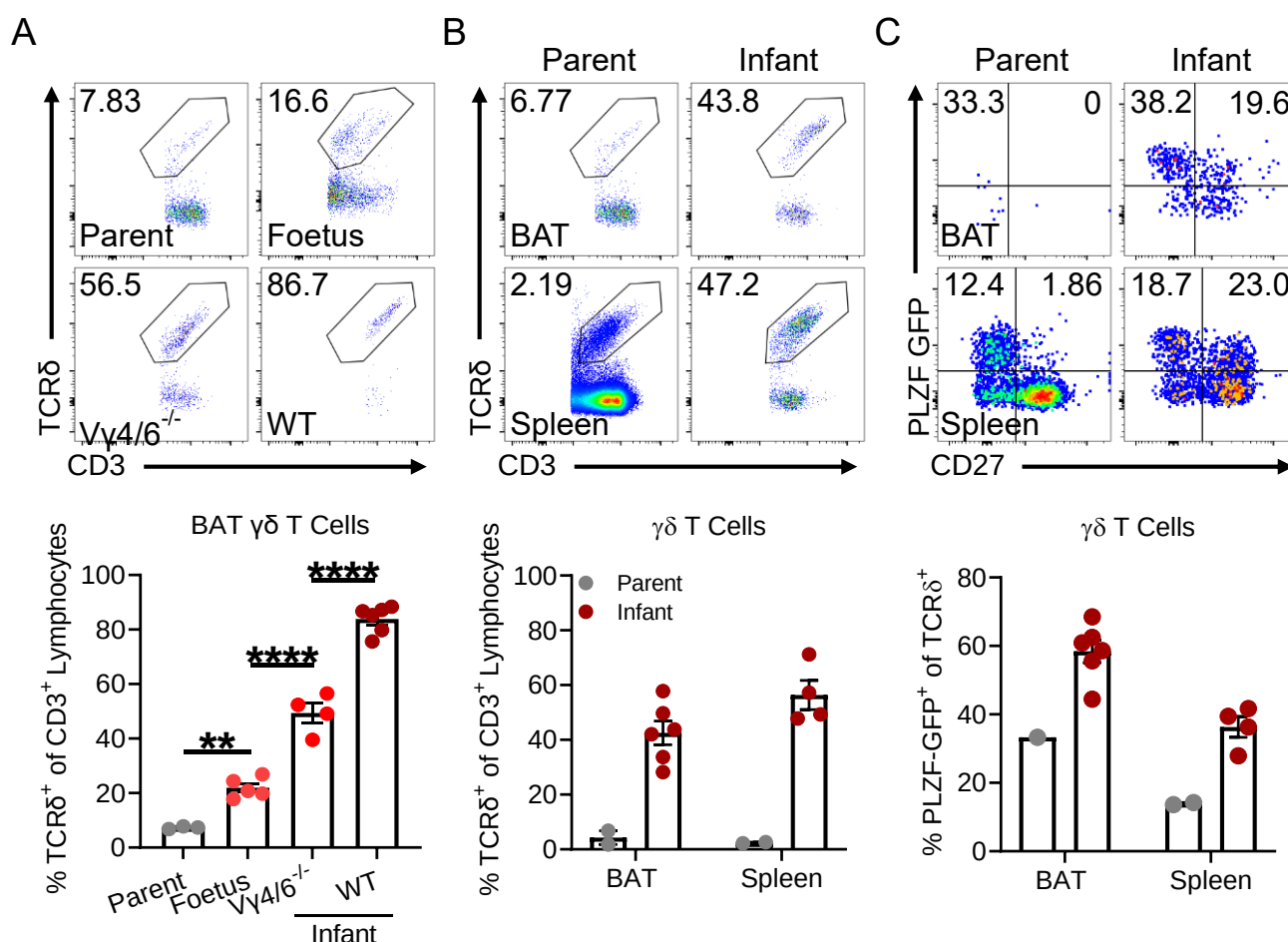


Figure 3.2: Infant BAT is enriched for $\gamma\delta^{17}$ T cells.

(A) Representative flow plots of T cells from parental, foetal, *Vγ4/6*^{-/-} and wild-type BAT (top); percentage of γδ T cells across tissues (bottom). **(B)** Representative flow plots of BAT and splenic T cells from parent and infant (top); percentage of BAT and splenic γδ T cells from parent and infant (bottom). **(C)** Representative flow plots of BAT and splenic γδ T cells (top); percentage of PLZF expression of BAT and splenic γδ T cells from parent and infant (bottom). Data are representative of two independent experiments. Data shown as mean ± SEM, n = 1-6 mice per group. **(A)** Significance was calculated using one-way ANOVA. ** p < 0.01, **** p < 0.0001.

3.3 Adipose, inguinal lymph node, and liver T cell numbers are decreased after cold challenge, but increased in blood and bone marrow.

Adipose tissue $\gamma\delta$ T cells play a crucial role in tissue homeostasis. We have previously described that epiWAT $\gamma\delta$ T cells are essential for the accumulation of homeostatic Treg cells in adipose tissue²⁸. Adipose Treg cells play a crucial role in maintaining healthy adipose physiology by preserving an immunosuppressive environment via secretion of IL-10¹⁵⁹. Therefore, we first investigated $\gamma\delta$ T cell dynamics in adipose tissue between room temperature (22°C), and cold challenged wild-type mice (12-hours at 4°C). There was a significant increase in the percentage of $\gamma\delta$ T cells in the scWAT, epiWAT and BAT of cold challenged mice (Figure 3.3A). Surprisingly however, there were no differences in the absolute number of $\gamma\delta$ T cells between conditions (Figure 3.3A), suggesting a loss of an alternative lymphocyte population. Although we observed no differences in the percentage of T cells in adipose tissue depots (Figure 3.3B), there was a significant decrease in T cell numbers in epiWAT and BAT depots (Figure 3.3B). The β 3-adrenergic agonist CL316,243 is used to mimic the lipolysis-inducing effects of cold temperatures in adipose tissue. Adipose $\gamma\delta$ T cell percentages were not increased, and T cell numbers were not decreased, after CL316,243 administration (Figure 3.4A, B), suggesting that the differences in the adipose lymphocyte populations during cold challenge, are independent of adipocyte lipolysis¹⁶⁰. We next investigated if there was an increase in T cells in the inguinal lymph node (inLN), or the liver, as liver-adipose cross communication is crucial for homeostatic whole body metabolism¹⁶¹. Similar to adipose tissue, there were no differences in T cell percentages in the inLN or liver between room temperature, and cold challenged conditions (Figure 3.5A). Remarkably, the absolute T cell number decreased in both liver and inLN, with a 50% reduction in T cell numbers in the inLN after cold challenge (Figure 3.5A).

Cold challenge induces a heavy metabolic demand for energy generation to maintain core body temperature¹⁶². During cold challenge at 4°C, up to 60% of total energy expenditure is directed towards heat generation to fit the energy demand¹⁶², which may draw energy away from other physiological processes for survival. During dietary restriction, circulating memory immune cells migrate to the bone marrow, downregulating their cellular metabolism for energy conservation¹¹⁵. We investigated whether this may also be occurring to conserve energy during cold challenge. As such, we examined whether T cells may be migrating to the bone marrow, by quantifying T cell populations in the blood and bone marrow of mice. We found that during cold challenge, T cell percentages and numbers were significantly increased in the blood and bone marrow of mice (Figure 3.5B), suggesting T

cells relocate to the bone marrow during cold challenge. We found that loss of T cells in the inLN was not specific to a particular subset, as both CD8⁺, and CD4⁺, T cell numbers were decreased in response to cold, but increased in the bone marrow (Figure 3.6A-C). Although CL316,243 administration decreased T cell numbers in the inLN, which was similar to what occurred during cold challenge (Figure 3.7A), no differences were observed in T cell percentages or numbers of mice administered CL316,243 (Figure 3.7B). These data recapitulate that the effects observed during cold challenge are independent of adipocyte lipolysis.

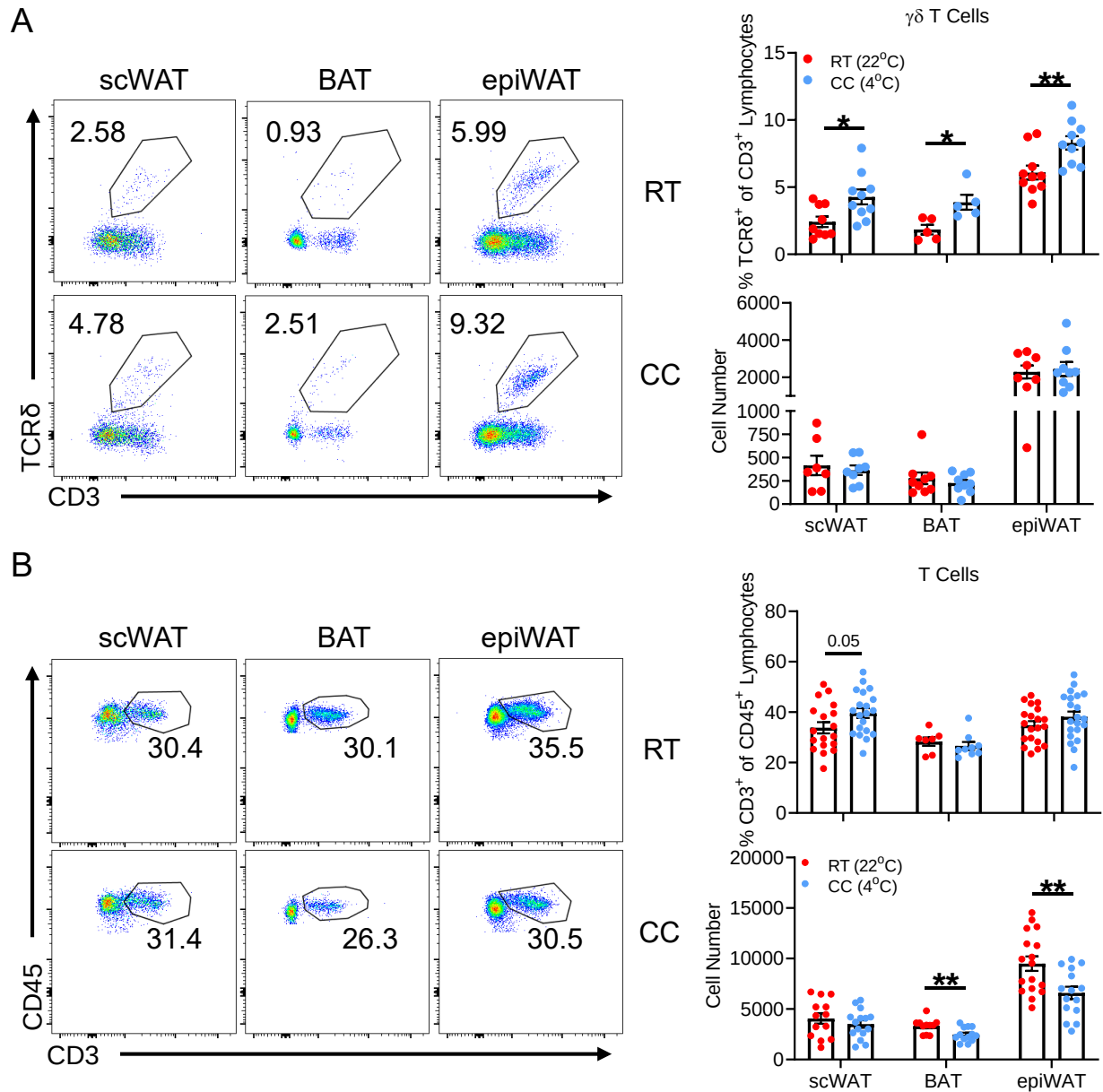


Figure 3.3: Adipose tissue T cell numbers are decreased in response to cold challenge.

(A) Representative flow plots of $\gamma\delta$ T cells from scWAT, epiWAT, and BAT of room temperature or 12-hour cold challenged mice (left); $\gamma\delta$ T cells percentage and absolute cell numbers per tissue (right). **(B)** Representative flow plots of T cells from scWAT, epiWAT, and BAT of room temperature or 12-hour cold challenged mice (left); T cell percentage and absolute cell numbers per tissue (right). Data are representative of six independent experiments, four experiments combined. Data shown as mean $\pm$ SEM, $n = 7-18$ mice per group. Significance was calculated using multiple two-tailed unpaired students t-tests. * $p < 0.05$, ** $p < 0.01$.

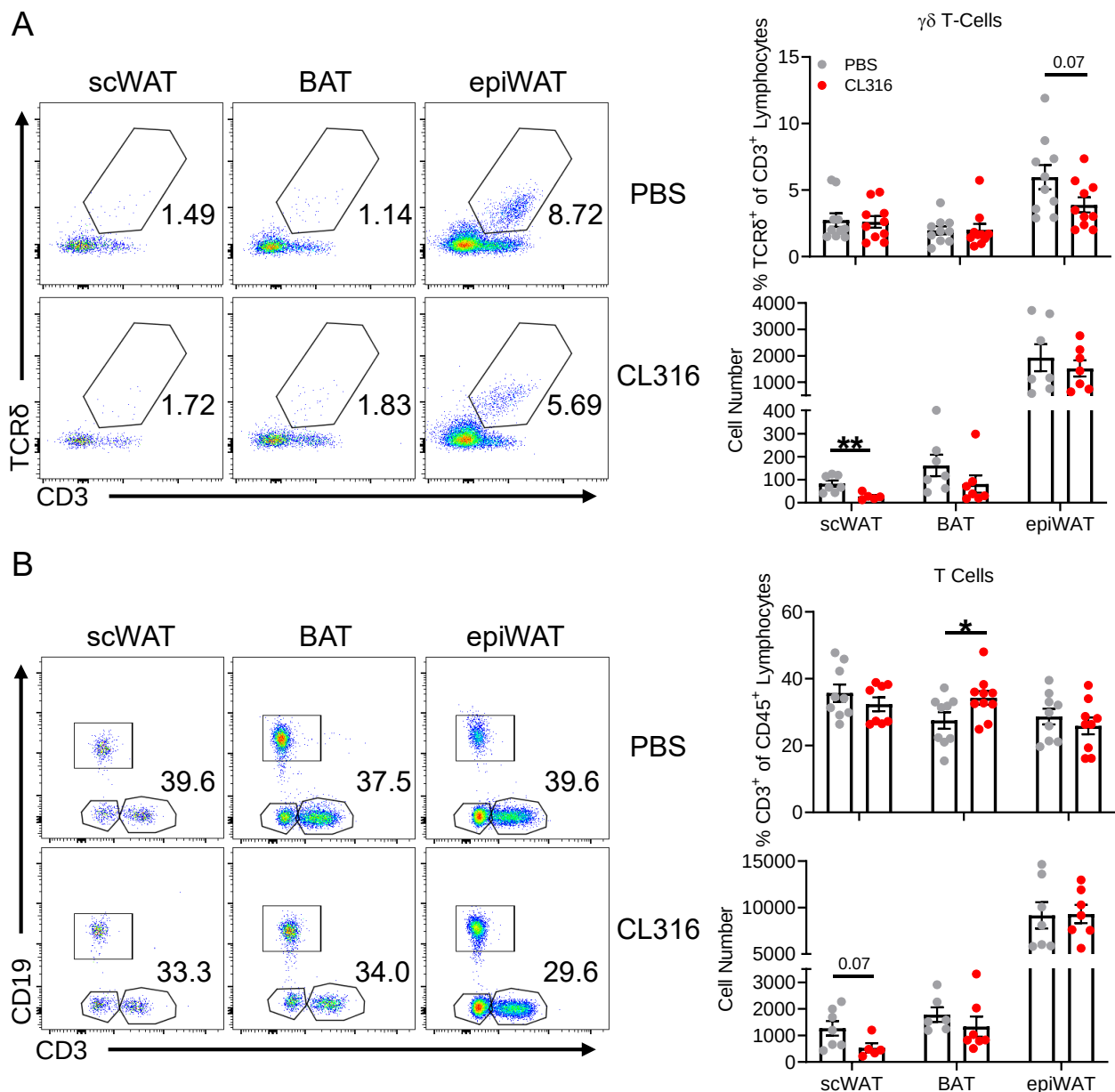


Figure 3.4: Adipose tissue T cell numbers are unaffected by CL316,243 administration.

(A) Representative flow plots of $\gamma\delta$ T cells from scWAT, epiWAT, and BAT of mice euthanized 4-hours post i.p. administration of PBS or CL316,243 (left; 1mg/kg); $\gamma\delta$ T cells percentage and absolute cell numbers per tissue (right). **(B)** Representative flow plots of T cells from scWAT, epiWAT, and BAT of mice euthanized 4-hours post i.p. administration of PBS or CL316,243 (left; 1mg/kg); T cell percentage and absolute cell numbers per tissue (right). Data are representative of five independent experiments, three experiments combined. Data shown as mean $\pm$ SEM, $n = 7-9$ mice per group. Significance was calculated using multiple two-tailed unpaired students t-tests. * $p < 0.05$, ** $p < 0.01$.

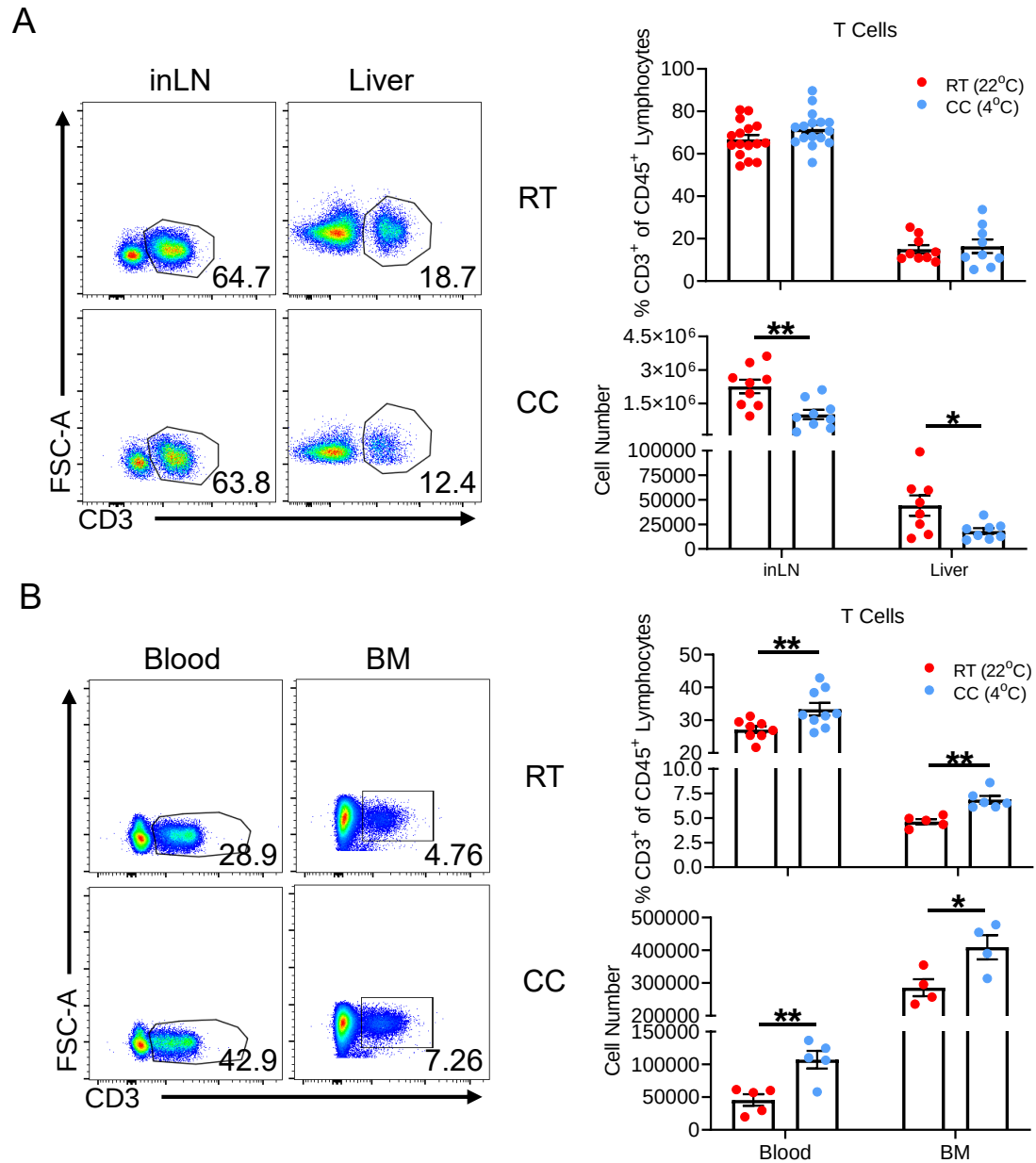


Figure 3.5: T cell numbers are increased in bone marrow in response to cold challenge.

(A) Representative flow plots of T cells from the inLN and liver of room temperature or 12-hour cold challenged mice (left); T cell percentage and absolute cell numbers per tissue (right). **(B)** Representative flow plots of T cells from blood (100 μ L) and bone marrow (a single femur) of room temperature or 12-hour cold challenged mice (left); T cell percentage and absolute cell numbers per 100 μ L of blood, and per femur (right). **(A)** Data are representative of six independent experiments, four experiments combined, **(B)** representative of three independent experiments, two experiments combined. Data shown as mean $\pm$ SEM, $n = 4$ -16 mice per group. Significance was calculated using multiple two-tailed unpaired students t-tests. * $p < 0.05$, ** $p < 0.01$.

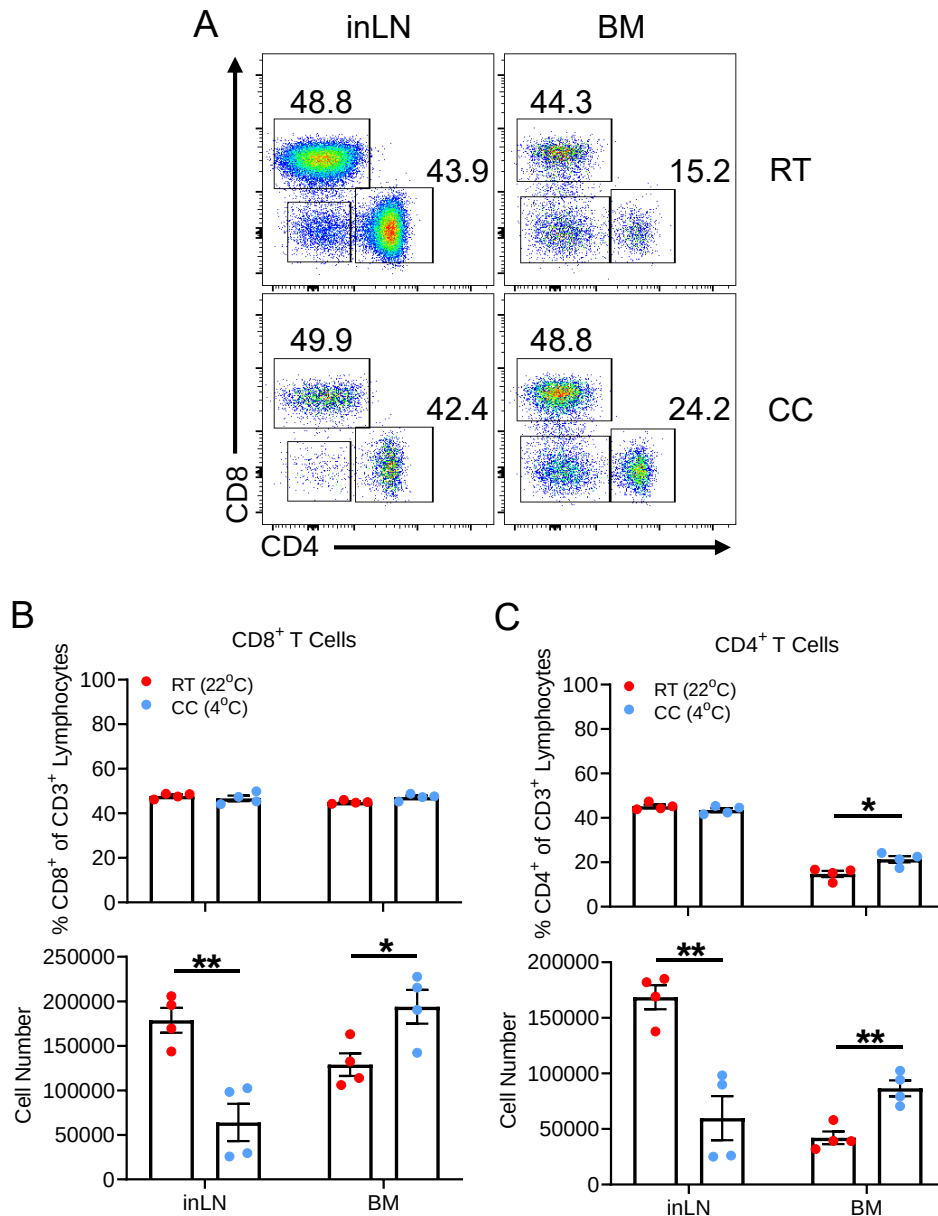


Figure 3.6: CD8⁺ and CD4⁺ T cell numbers are increased in bone marrow in response to cold challenge.

(A) Representative flow plots of CD8⁺ and CD4⁺ T cells harvested from the inLN and bone marrow (single femur) of room temperature or 12-hour cold challenged mice **(B)** CD8⁺ T cell percentages (top) and absolute cell numbers per tissue (bottom). **(C)** CD4⁺ T cell percentages (top) and absolute cell numbers per tissue (bottom). Representative of three independent experiments. Data shown as mean $\pm$ SEM, $n = 4$ mice per group. Significance was calculated using multiple two-tailed unpaired students t-tests. * $p < 0.05$, ** $p < 0.01$.

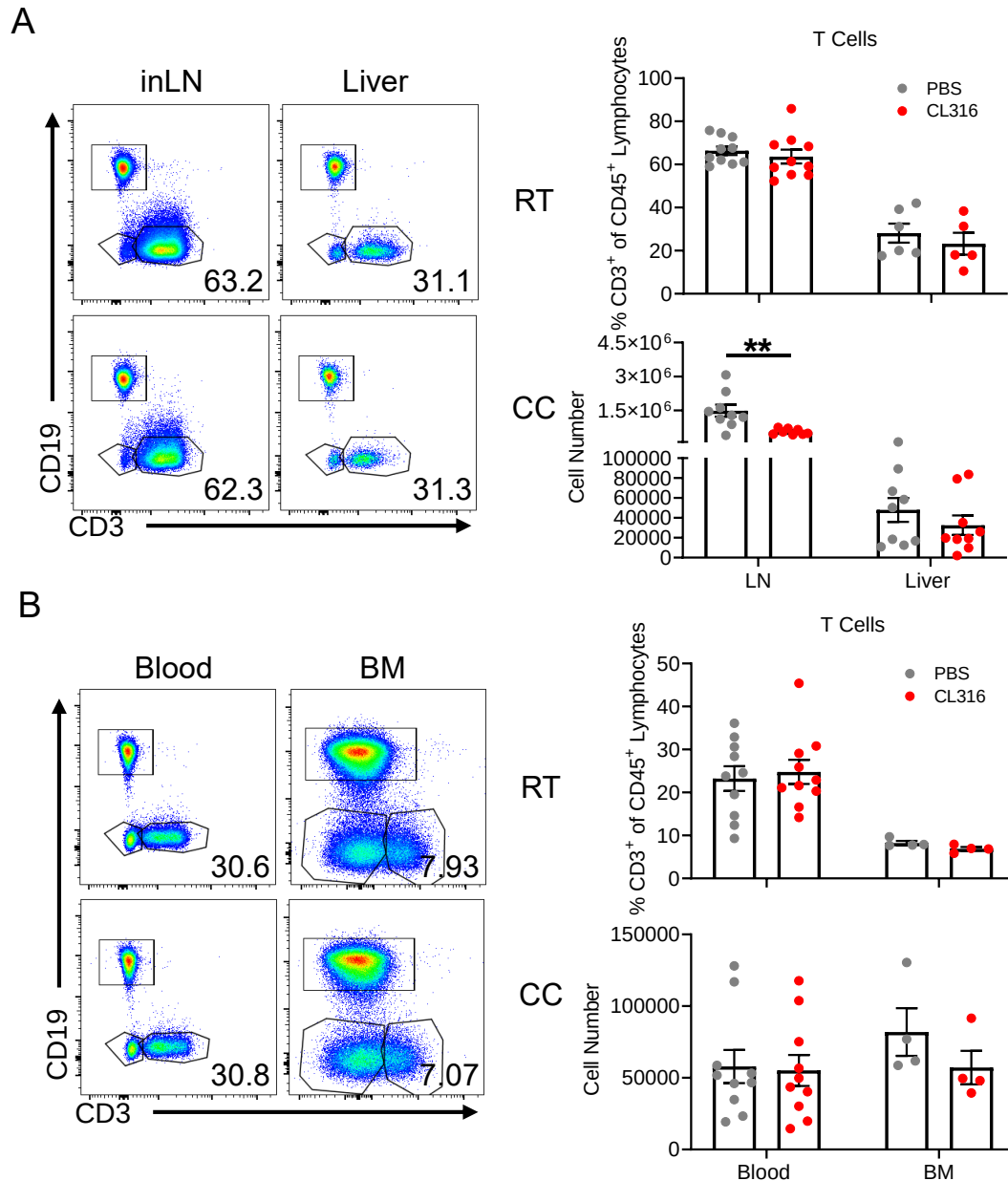


Figure 3.7: Liver, blood, and bone marrow T cell numbers are unaffected by CL316,243 administration.

(A) Representative flow plots of T cells from the inLN and liver of mice euthanized 4-hours post i.p. administration of PBS or CL316,243 (left; 1mg/kg); T cells percentage and absolute cell number per tissue (right). **(B)** Representative flow plots of T cells from the blood (100 μ L) and bone marrow (single femur) of mice euthanized 4-hours post i.p. administration of PBS or CL316,243 (left; 1mg/kg); T cell percentage and absolute cell numbers per 100 μ L of blood, and per femur (right). **(A)** Data are representative of five independent experiments, three experiments combined, **(B)** representative of three independent experiments, two experiments combined. Data shown as mean $\pm$ SEM, n = 4-10 mice per group. Significance was calculated using multiple two-tailed unpaired students t-tests. ** p < 0.01.

3.4 Cold challenge creates a circulating immunosuppressive environment.

Naïve T cells display a circadian pattern to trafficking over a 24-hour period, with highest numbers in the LN during the day, and lowest at night in mice¹⁶³. Egress of naïve T cells from the LN is mediated by the sphingosine-1-phosphate-receptor-1 (S1P1) expressed on the surface of T cells¹⁶⁴. The concentration of the chemoattractant S1P is high in the blood, but low in tissues, thus guiding T cells to sites of infection upon activation in the LN¹⁶⁵. S1P1 signalling can be manipulated by the antagonist FTY720, which inhibits LN egress by T cells¹⁶⁶. Having observed that T cell numbers decrease in the LN, and increase in the blood, we hypothesised that this may be due to cell egress. As T cells generally migrate from the lymph node after activation, we utilised FTY720 to investigate if cold challenge causes differentiation of naïve T cells into effector subsets. We first confirmed the efficacy of FTY720 by quantifying the T cell population in the LN and blood of mice administered PBS or FTY720, at room temperature or after cold challenge. We found that FTY720 sufficiently decreased T cell, and B cell, populations in the blood, while increasing these populations in the LN (Figure 3.8A-C). We observed no difference in T cell or B cell populations between room temperature and cold challenged mice administered FTY720 (Figure 3.8 A-C).

Subsequently, we characterised the activation state of CD4⁺ T cells between PBS or FTY720 administered mice. Using CD69 and CD71 as markers of T cell activation revealed no differences in the activation of CD4⁺ T cells in the circulation after cold challenge, as there was no difference in FTY720 administered mice (Figure 3.9A, B). Next, we investigated if T cells polarised towards a specific phenotype in response to cold challenge. We used the lineage specific transcription factors T-bet and ROR γ t to investigate changes in Th1, and Th17 cells respectively. The percentage of T-bet⁺ CD4⁺ T cells was increased in cold mice administered FTY720 compared to room temperature treated mice, indicating a polarisation towards a Th1 phenotype due to cold (Figure 3.10A). The proportion of Th17 T cells trended towards a decrease after cold challenge in FTY720 administered mice (Figure 3.10B). Strikingly however, there was an increased percentage of Foxp3⁺ CD4⁺ Treg cells in the LN of mice administered FTY720 compared to PBS controls (Figure 3.11A). Furthermore, there was a significant increase in the number of Treg cells in mice administered FTY720 after cold challenge (Figure 3.11B), suggesting an increased polarisation to Treg cells in cold temperatures. To support this, we performed an ELISA on the serum of mice collected from room temperature or cold challenged animals. We observed an increased concentration of serum IL-10 in cold challenged animals (Figure

3.12A). The concentration of serum IL-10 also increased after CL316 injection, suggesting that circulating IL-10 increases in response to adipocyte lipolysis (Figure 3.12B).

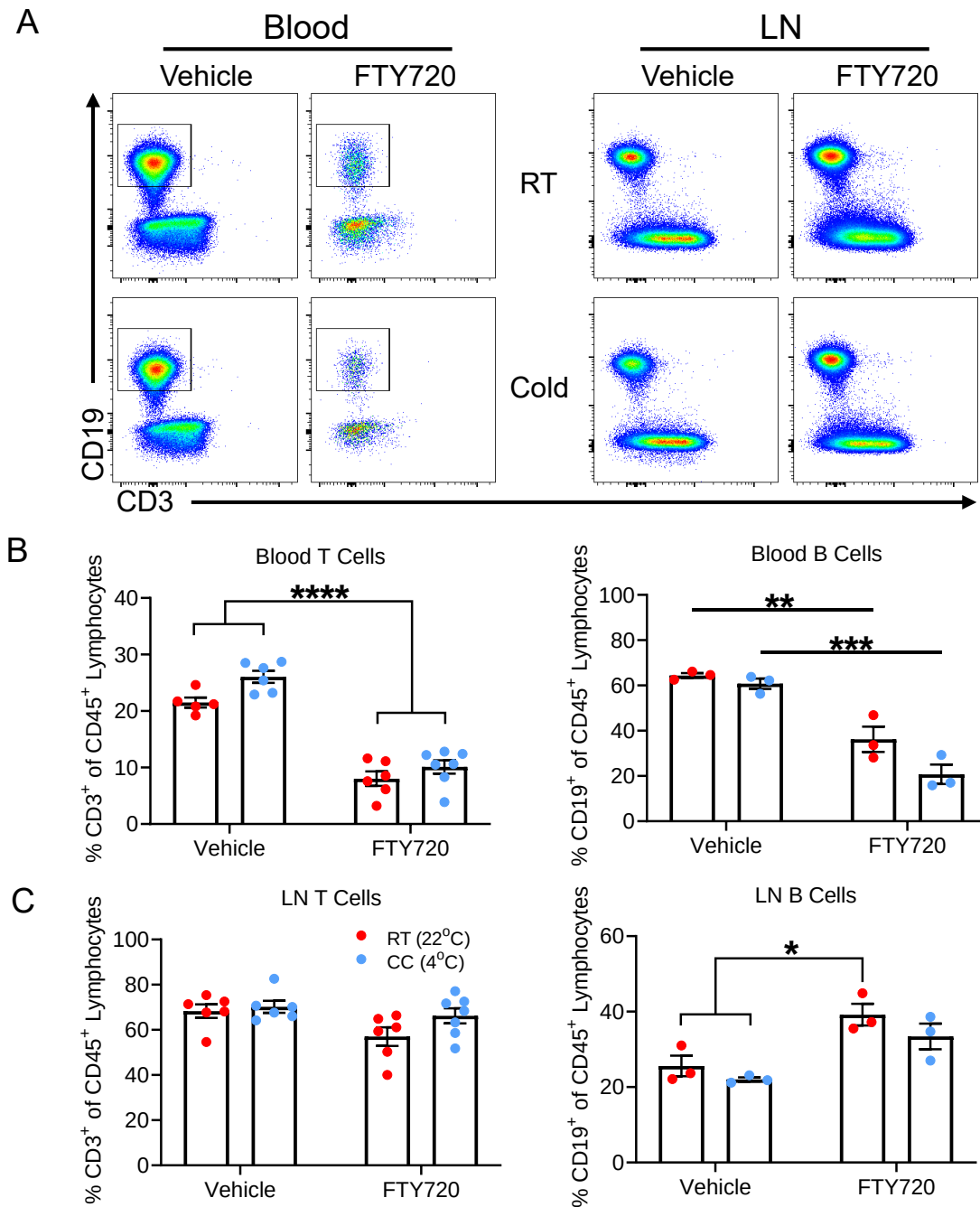


Figure 3.8: FTY720 prevents T cell and B cell egress from the lymph node.

(A) Representative flow plots of T cells from the blood and LN of room temperature and 12-hour cold challenged mice administered PBS or FTY720 (2mg/kg) i.p. prior to cold challenge.

(B) Blood T cell percentages (left) and B cell percentages (right) of room temperature and 12-hour cold challenged mice administered PBS or FTY720 (2mg/kg) i.p. prior to cold challenge.

(C) LN T cell percentages (left) and B cell percentages (right) of room temperature and 12-hour cold challenged mice administered PBS or FTY720 (2mg/kg) i.p. prior to cold challenge. Data are representative of two independent experiments, two experiments combined (T cell plots). Data shown as mean $\pm$ SEM, $n = 3-6$ mice per group. Significance was calculated using a two-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

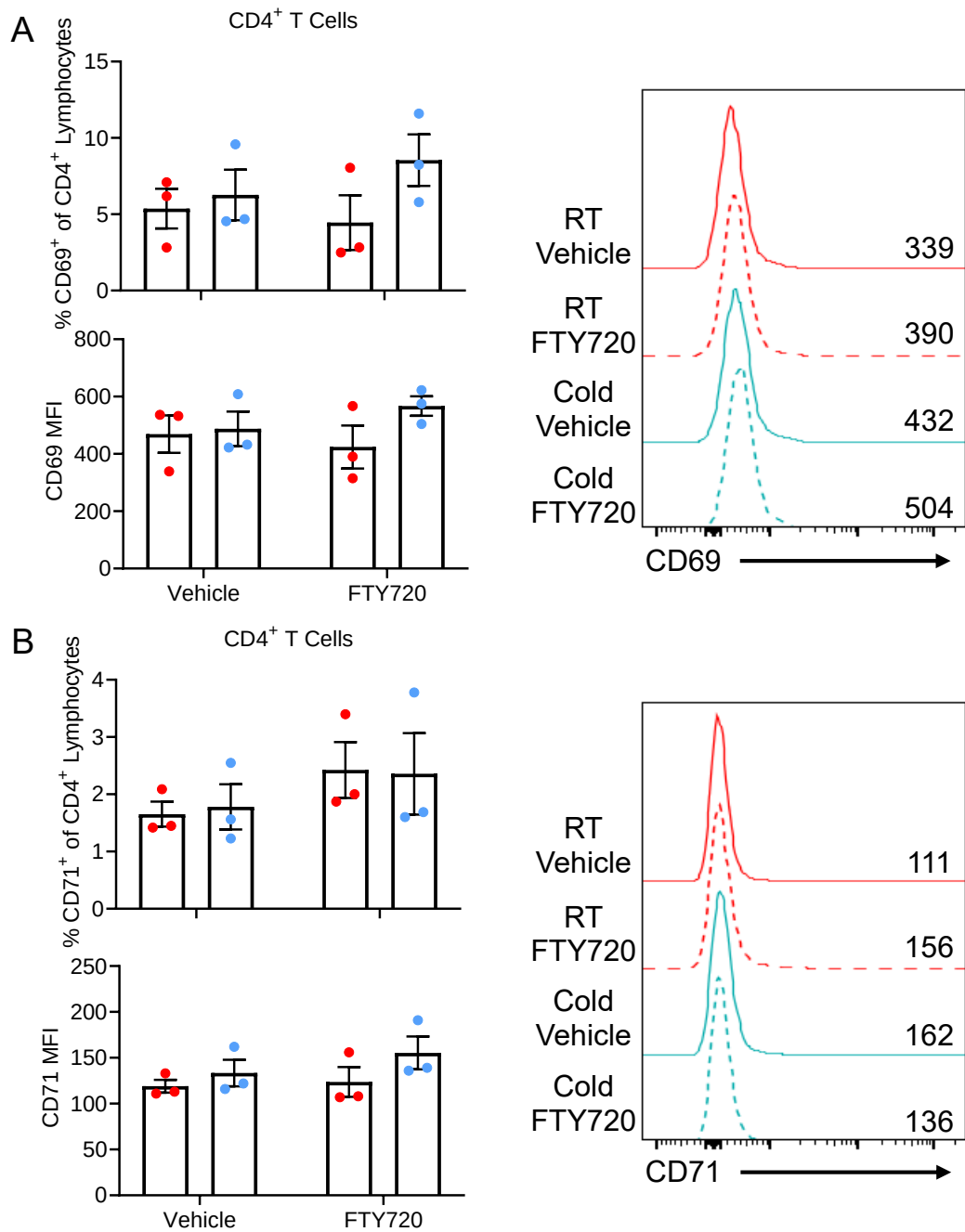


Figure 3.9: Cold challenge does not activate CD4⁺ T cells.

(A) Percentage (top), and mean fluorescence (bottom), of CD69 expression by LN CD4⁺ T cells of room temperature and 12-hour cold challenged mice administered PBS or FTY720 i.p. prior to cold challenge, with representative histograms (right; 2mg/kg). **(B)** Percentage (top), and mean fluorescence (bottom), of CD71 expression by inLN CD4⁺ T cells of room temperature and 12-hour cold challenged mice administered PBS or FTY720 i.p. prior to cold challenge, with representative histograms (right; 2mg/kg). Data are representative of one independent experiment. Data shown as mean $\pm$ SEM, n = 3 mice per group. Significance was calculated using a two-way ANOVA.

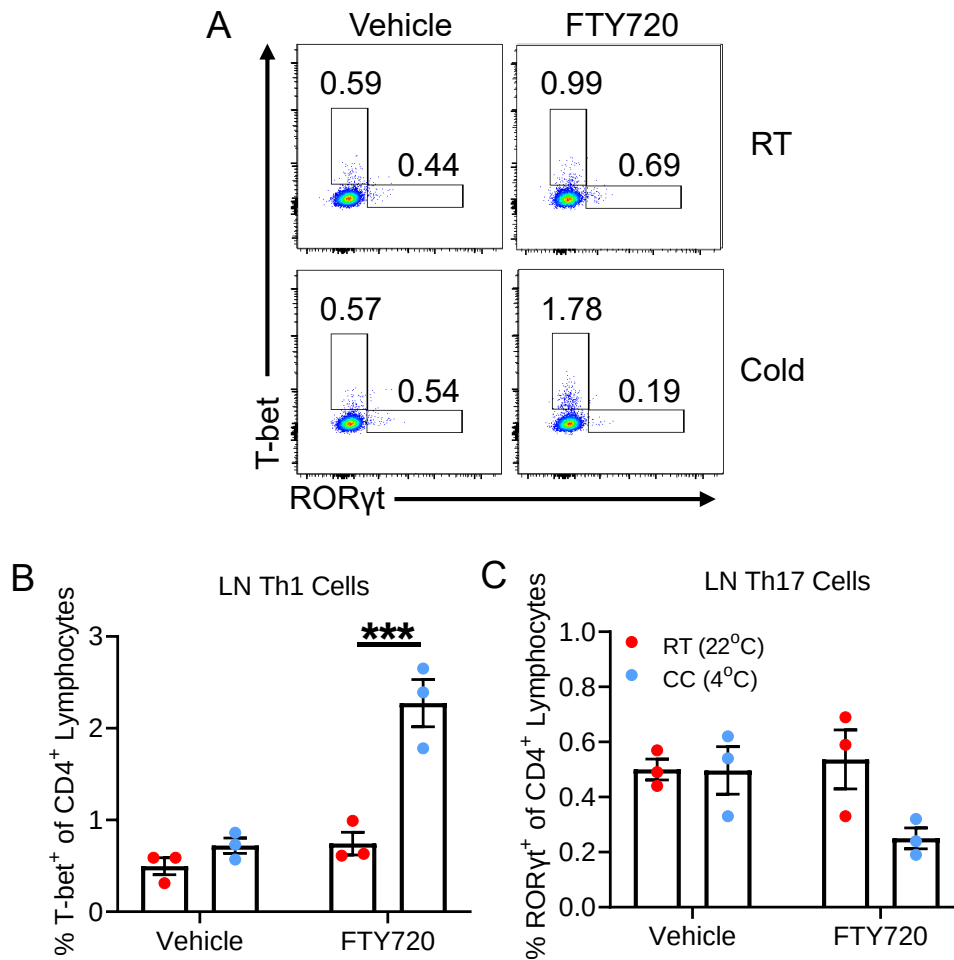


Figure 3.10: Cold challenge induces a Th1, but not Th17, phenotype.

(A) Representative flow plots of T-bet⁺ and RORγt⁺ T cells from the LN of room temperature and 12-hour cold challenged mice administered PBS or FTY720 (2mg/kg) i.p. prior to cold challenge. **(B)** Percentage of T-bet⁺ LN CD4⁺ T Cells of room temperature and 12-hour cold challenged mice administered PBS or FTY720 (2mg/kg) i.p. prior to cold challenge. **(C)** Percentage of T-bet⁺ LN CD4⁺ T Cells of room temperature and 12-hour cold challenged mice administered PBS or FTY720 (2mg/kg) i.p. prior to cold challenge. Data are representative of two independent experiments. Data shown as mean ± SEM, n = 3 mice per group. Significance was calculated using a two-way ANOVA. ** p < 0.01.

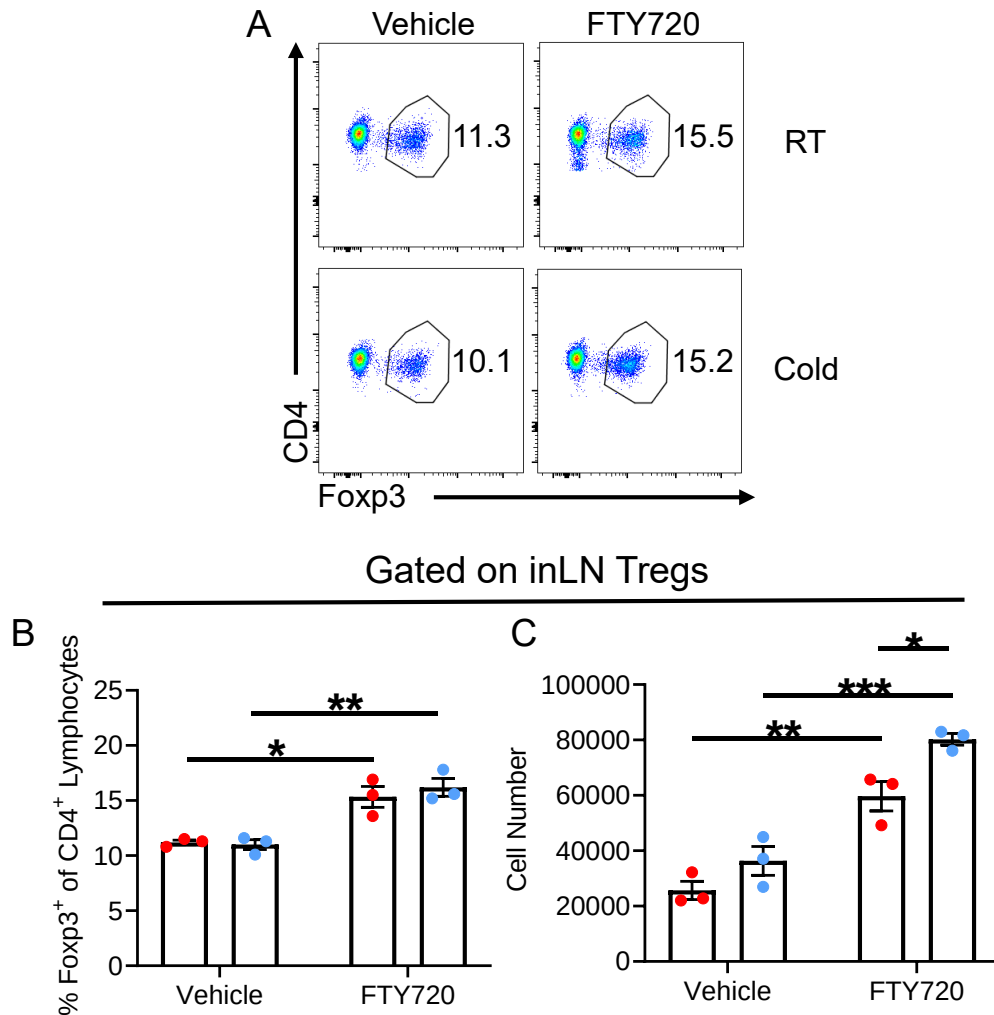


Figure 3.11: Cold challenge increases Treg numbers.

(A) Representative flow plots of Foxp3⁺ T cells from the LN of room temperature and 12-hour cold challenged mice administered PBS or FTY720 (2mg/kg) i.p. prior to cold challenge. **(B)** Percentage of inLN Foxp3⁺CD4⁺ T cells (left); absolute cell numbers of inLN Foxp3⁺CD4⁺ T cells of room temperature and 12-hour cold challenged mice administered PBS or FTY720 (2mg/kg) i.p. prior to cold challenge. Data are representative of two independent experiments. Data shown as mean $\pm$ SEM, n = 3 mice per group. Significance was calculated using a two-way ANOVA. * p < 0.05, ** p < 0.01, *** p < 0.001.

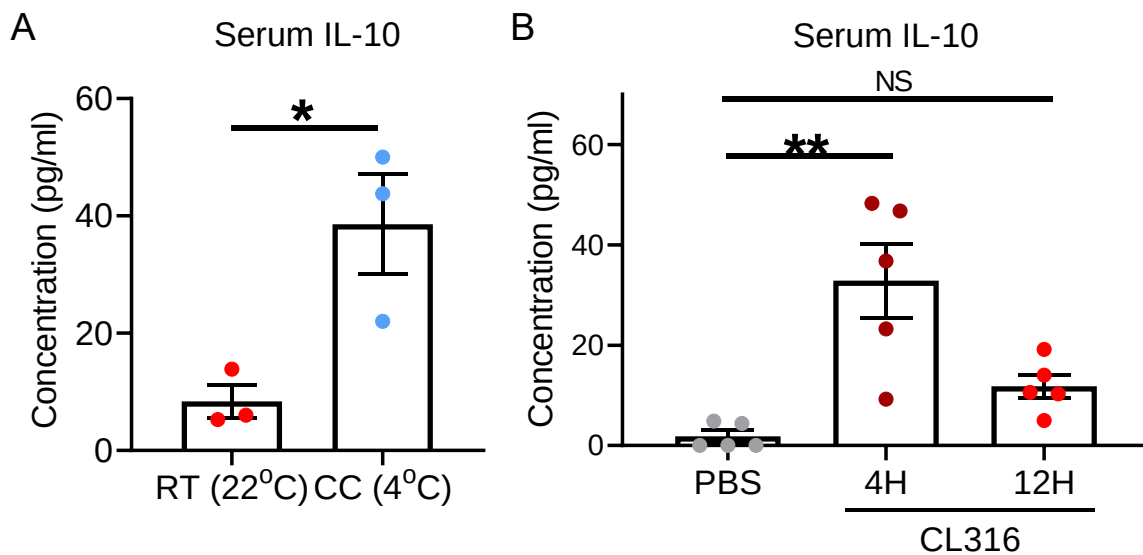


Figure 3.12: Cold challenge, and CL316,243 administration, increase serum IL-10.

(A) Concentration of IL-10 from the blood serum of mice housed at room temperature or cold challenged for 12-hours. **(B)** Concentration of IL-10 from the blood serum of mice administered PBS, or euthanised 4, or 12-hours post CL316,243 i.p. administration. **(A)** Data are representative of one independent experiment, **(B)** two independent experiments, two experiments combined. Data shown as mean $\pm$ SEM, $n = 3-5$ mice per group. **(A)** Significance was calculated using an unpaired two-tailed students t-test, **(B)** one-way ANOVA. * $p < 0.05$, ** $p < 0.01$.

3.5 IL-17A, TNF and ROR γ t expression by epiWAT $\gamma\delta$ T cells are decreased after cold challenge.

Our lab and the Spiegelman lab have previously described an important role for IL-17A and IL-17F producing $\gamma\delta$ T cells in the process of thermogenesis^{28,29}. However, the majority of this work was performed in mice lacking $\gamma\delta$ T cells, IL-17A, IL-17F, or IL-17 receptor. Therefore, we examined if cold challenge upregulated IL-17A expression in the adipose tissue of wild-type mice. We first investigated if adipose $\gamma\delta$ T cells produce IL-17A constitutively. Mice were injected with BFA two hours prior to sacrifice at room temperature, or after a 12-hour cold challenge. We found that adipose $\gamma\delta$ T cells, but not lymphoid $\gamma\delta$ T cells, expressed IL-17A constitutively (Figure 3.13A). Furthermore, adipose $\gamma\delta$ T cells were the main lymphocyte population producing IL-17A in the tissue (Figure 3.13A). Surprisingly, BAT and epiWAT $\gamma\delta$ T cells produced less IL-17A in response to cold challenge (Figure 3.13C). To further validate this finding, mice underwent a time course cold challenge, which revealed an inverse relationship between IL-17A, and the amount of time spent in the cold, with epiWAT $\gamma\delta$ T cells producing the least IL-17A after a 12-hour cold challenge (Figure 3.14A). Moreover, ROR γ t expression, and TNF production by epiWAT $\gamma\delta$ T cells were also decreased after 6, and 12-hours of cold challenge (Figure 4.14 B-D).

To investigate if inhibition of IL-17A production was driven by lipolysis in adipose tissue, mice were administered CL316,243 and sacrificed 6, or 12-hours later. Similar to the observations in the cold, CL316,243 decreased IL-17A, ROR γ t and TNF expression 6-hours post administration (Figure 3.15A-D). There was a slight decrease in IL-17A, ROR γ t and TNF expression compared to PBS controls 12-hours post administration, due to body clearance of the compound (Figure 3.15A-D). To ensure that IL-17A production by epiWAT $\gamma\delta$ T cells did not peak at an earlier time point, we sacrificed animals 1-hour, and 4-hours post CL316,243 administration (Figure 3.16A). Epididymal adipose $\gamma\delta$ T cells stimulated with PMA/Ionomycin, or with cytokines, had decreased IL-17A expression one-hour post CL316,243 administration (Figure 3.16B). Similarly, $\gamma\delta$ T cells stimulated with PMA/Ionomycin also displayed decreased IL-17A expression 4-hours post CL316,243 administration (Figure 3.16C). Finally, using IL-17A^{GFP} mice, we further confirmed that CL316,243 administration decreases IL-17A-GFP (Figure 3.16C). Together these data suggest that lipolysis induced by cold challenge, or CL316,243 injection, inhibits IL-17A and ROR γ t expression by adipose $\gamma\delta$ T cells.

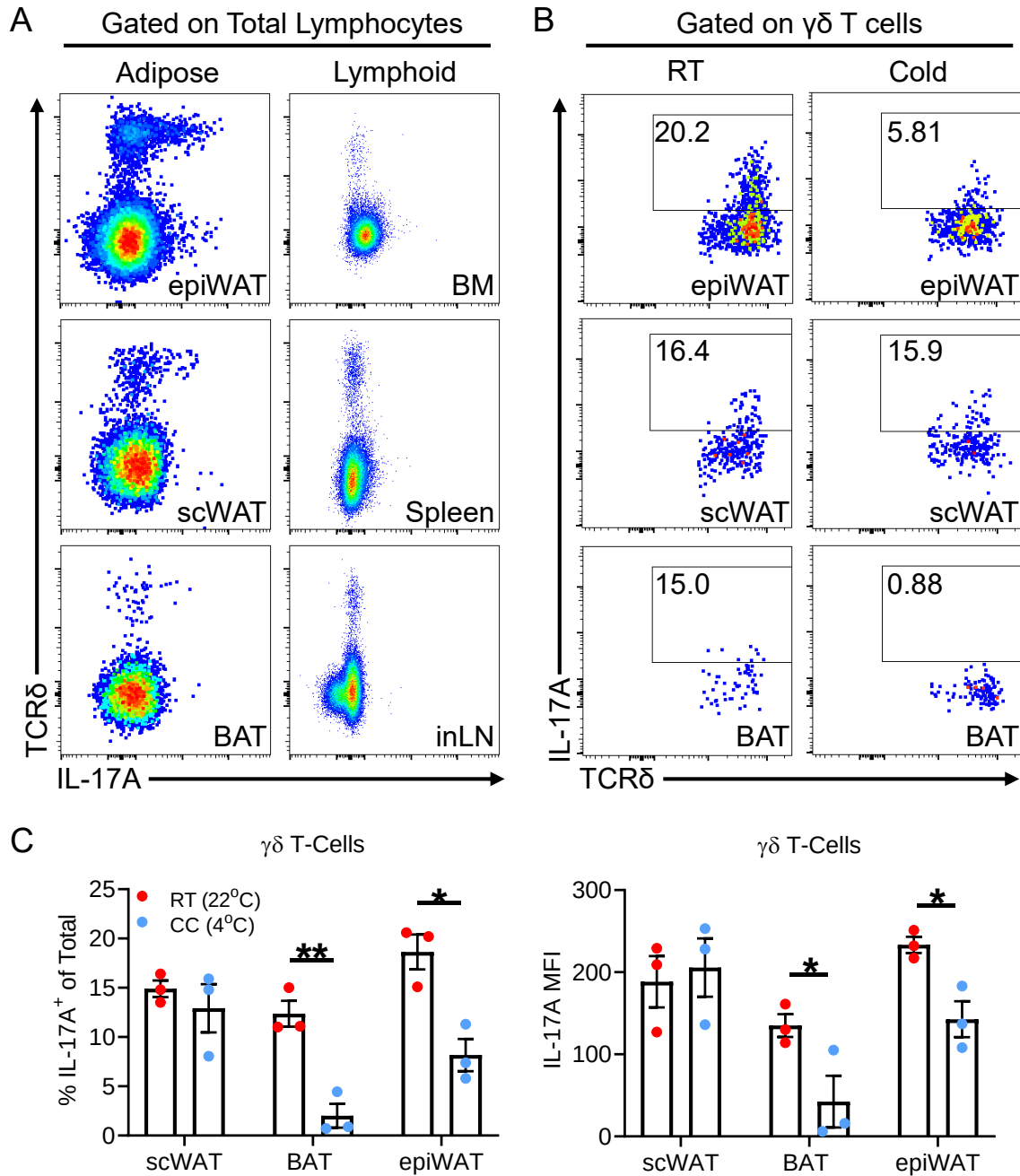


Figure 3.13: Adipose $\gamma\delta^{17}$ T cells are the main source of IL-17A, which is inhibited following cold challenge.

(A) Representative flow plots of T cells from adipose tissue and lymphoid organs of room temperature and 12-hour cold challenged mice following BFA injection (300 μ g; i.p.) for final 3-hours of cold challenge. **(B)** Representative flow plots of $\gamma\delta$ T cells from adipose tissue of room temperature and 12-hour cold challenged mice following BFA injection (300 μ g, i.p.) for final 3-hours of cold challenge. **(C)** Percentage (left), and mean fluorescence (right), of IL-17A by adipose $\gamma\delta$ T cells. Data are representative of two independent experiments. Data shown as mean $\pm$ SEM, n = 3 mice per group. Significance was calculated using multiple unpaired students t-tests. * p < 0.05, ** p < 0.01.

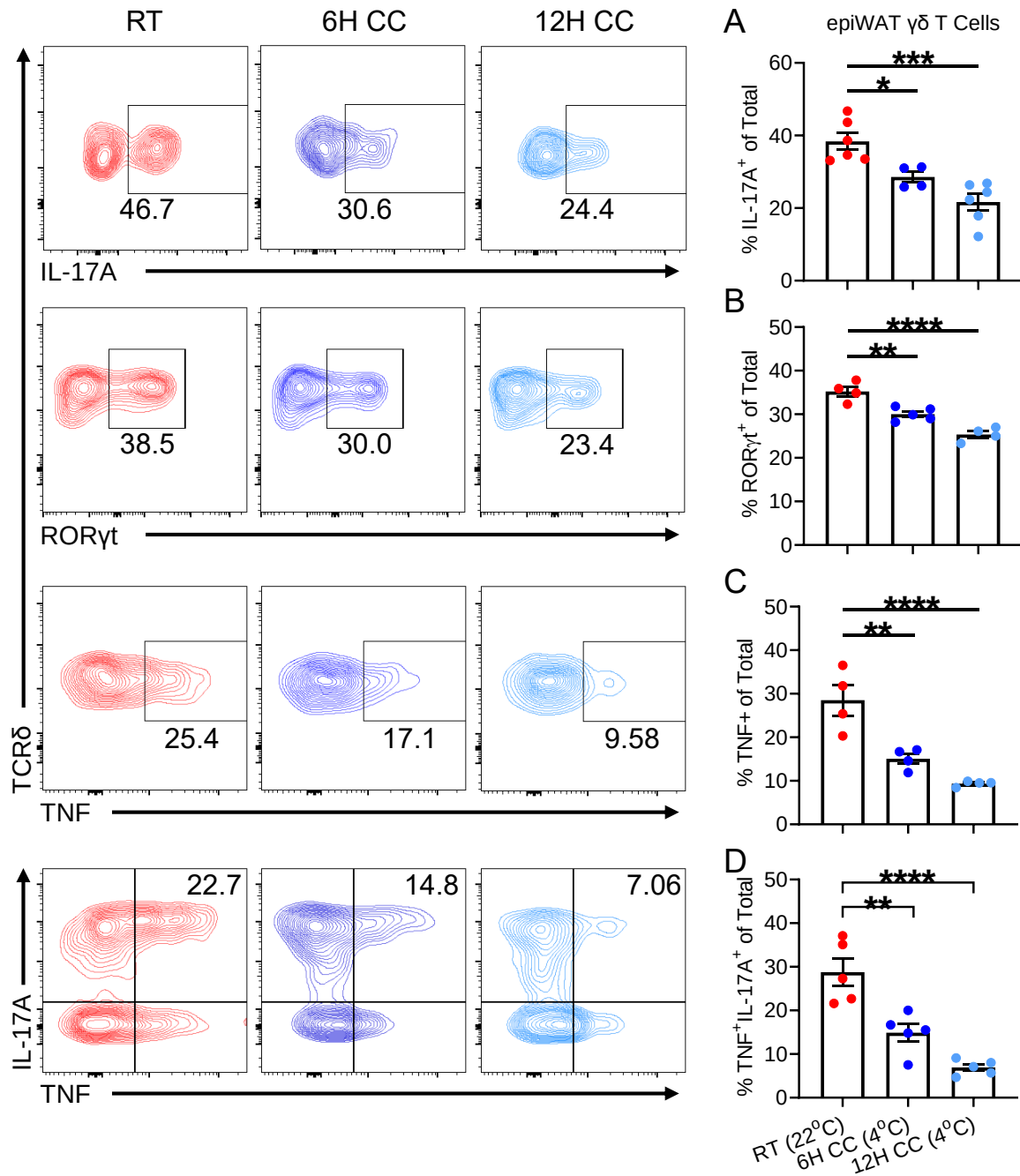


Figure 3.14: $\gamma\delta^{17}$ T cells downregulate IL-17A, ROR γ t, and TNF expression following cold challenge.

Representative flow plots of **(A)** IL-17A, **(B)** ROR γ t, and **(C)** TNF expression by epiWAT $\gamma\delta^{17}$ T cells (left), from 6-hour, and 12-hour cold challenged mice (right). **(D)** Representative flow plots of IL-17A, TNF double expression by epiWAT $\gamma\delta^{17}$ T cells (left), from 6-hour, and 12-hour cold challenged mice (right). Data are representative of 3 independent experiments. Data shown as mean $\pm$ SEM, $n = 4-6$ mice per group. Significance was calculated using a one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

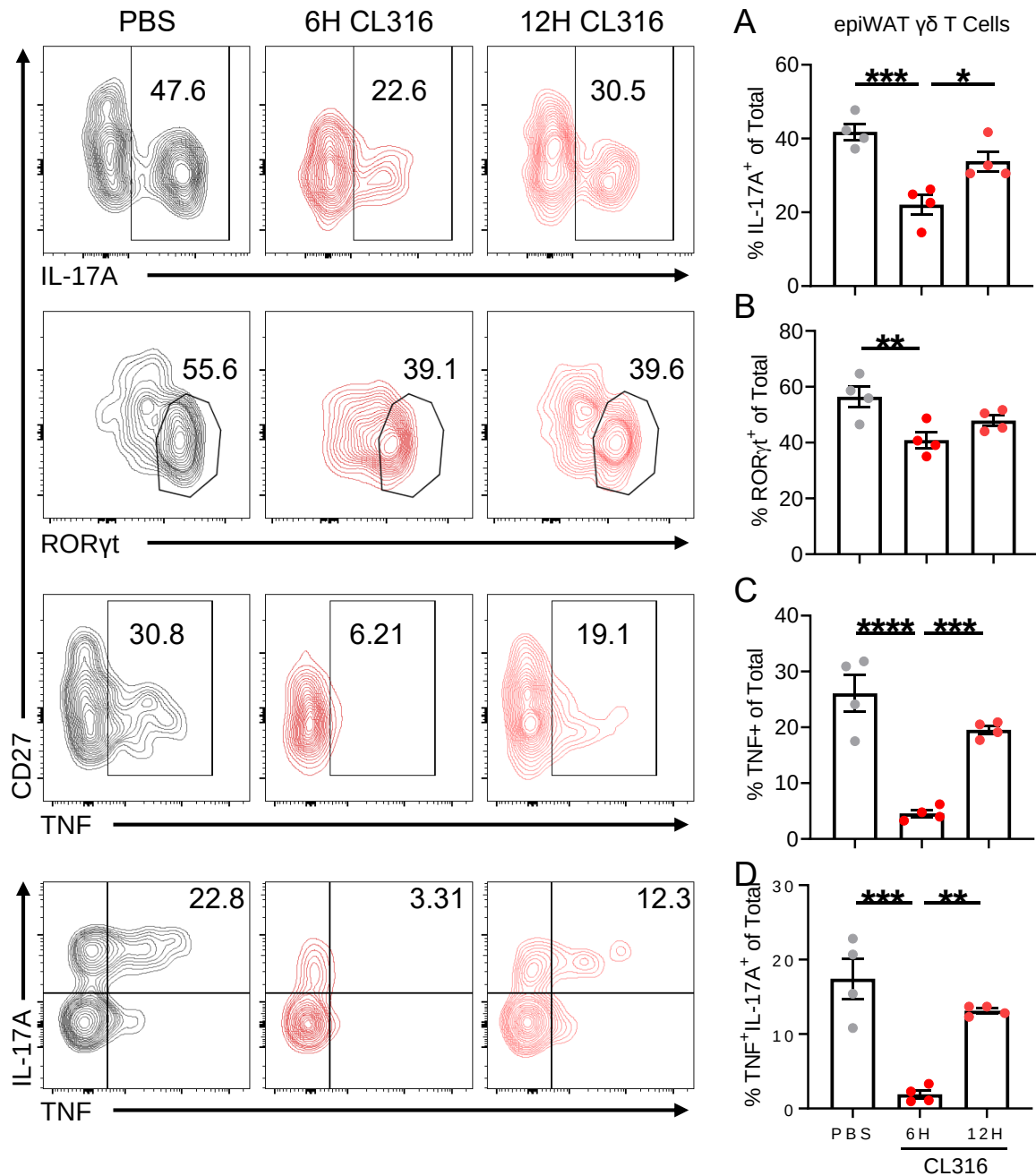


Figure 3.15: $\gamma\delta^{17}$ T cells downregulate IL-17A, ROR γ t, and TNF expression following CL316,243 administration.

Representative flow plots of **(A)** IL-17A, **(B)** ROR γ t, and **(C)** TNF expression by epiWAT $\gamma\delta^{17}$ T cells (left), from mice injected i.p. with CL316,243, and euthanized 6 and 12-hours post-administration (right). **(D)** Representative flow plots of IL-17A, TNF double expression by epiWAT $\gamma\delta^{17}$ T cells (left), from mice injected i.p. with CL316,243, and euthanized 6 and 12-hours post-administration (right). Data are representative of 2 independent experiments. Data shown as mean $\pm$ SEM, $n = 4$ mice per group. Significance was calculated using a one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

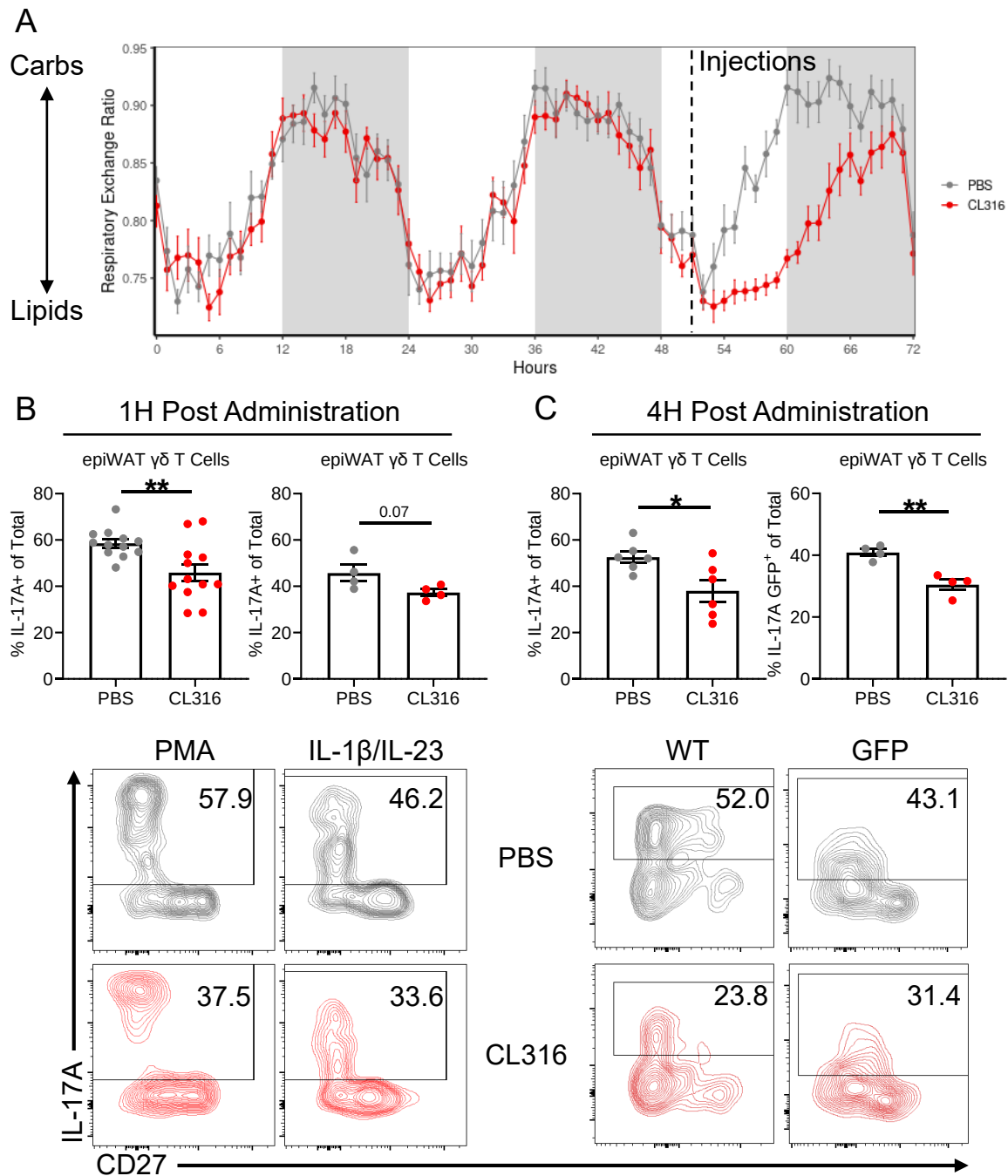


Figure 3.16: $\gamma\delta^{17}$ T cells downregulate IL-17A, ROR γ t, and TNF expression 1-hour and 4-hours post CL316,243 administration.

(A) Time plot of whole body metabolism by RER of mice injected with PBS (grey), versus mice injected with CL316,243 (red; 1mg/kg; i.p.). **(B)** Percentage of IL-17A expression by epiWAT $\gamma\delta$ T cells stimulated with PMA/Ionomycin or IL-1 β /IL-23 1-hour post CL316,243 administration. **(C)** Percentage of IL-17A expression by epiWAT $\gamma\delta$ T cells stimulated with PMA/Ionomycin or unstimulated 4-hours post CL316,243 administration. **(B, C)** Data are representative of three independent experiments, **(B, left)** three experiments combined. Data shown as mean $\pm$ SEM, n = 3-12 mice per group. Significance was calculated using an unpaired students t-test. * p < 0.05, ** p < 0.01.

3.6 Cold challenge decreases epiWAT $\gamma\delta^{17}$ T cell cellular metabolism.

The utilisation of specific metabolic pathways by T cells is closely linked to their effector functions¹⁶⁷. $\gamma\delta$ T cells rely on specific metabolic pathways depending on whether they express ROR γ t or T-bet. $\gamma\delta^{IFN\gamma}$ T cells rely on glycolysis, while $\gamma\delta^{17}$ T cells rely on oxidative phosphorylation⁶⁸. As IL-17A expression by epiWAT $\gamma\delta$ T cells was decreased following cold challenge, we next investigated if there were changes to epiWAT $\gamma\delta^{17}$ T cell metabolism. epiWAT is a lipid rich environment which upregulates FFA and glycerol release into the local environment and circulation during cold challenge¹⁶⁸. Therefore, we examined lipid uptake dynamics using the fluorescent palmitate BODIPY FL-C16. After a 6 or 12-hour cold challenge, epiWAT $\gamma\delta^{17}$ T cells took up less palmitate compared to their room temperature controls (Figure 3.17A). However, epiWAT $\gamma\delta^{17}$ T cells had a higher lipid content following cold challenge (Figure 3.17B). As $\gamma\delta^{17}$ T cells rely on oxidative phosphorylation, we investigated their mitochondrial dynamics. epiWAT $\gamma\delta^{17}$ T cells had a lower percentage, and mean expression of mitochondrial ROS (Figure 3.18A-C), in addition to a decrease in their mitochondrial membrane potential (Figure 3.19B), and no change in mitochondrial mass (Figure 3.19A). Induction of adipose tissue lipolysis using CL316,243 decrease lipid uptake by epiWAT $\gamma\delta^{17}$ T cells, but increased their cellular lipid content similar to that of cold challenge epiWAT $\gamma\delta^{17}$ T cells (Figure 3.20A, B). Furthermore, CL316,243 administration significantly decreased the mitochondrial mass and membrane potential of epiWAT $\gamma\delta^{17}$ T cells (Figure 3.21). These data suggest that the cold induced decrease in IL-17A expression by epiWAT $\gamma\delta$ T cells may be mediated by changes in mitochondrial metabolism during the cold, induced by adipocyte lipolysis.

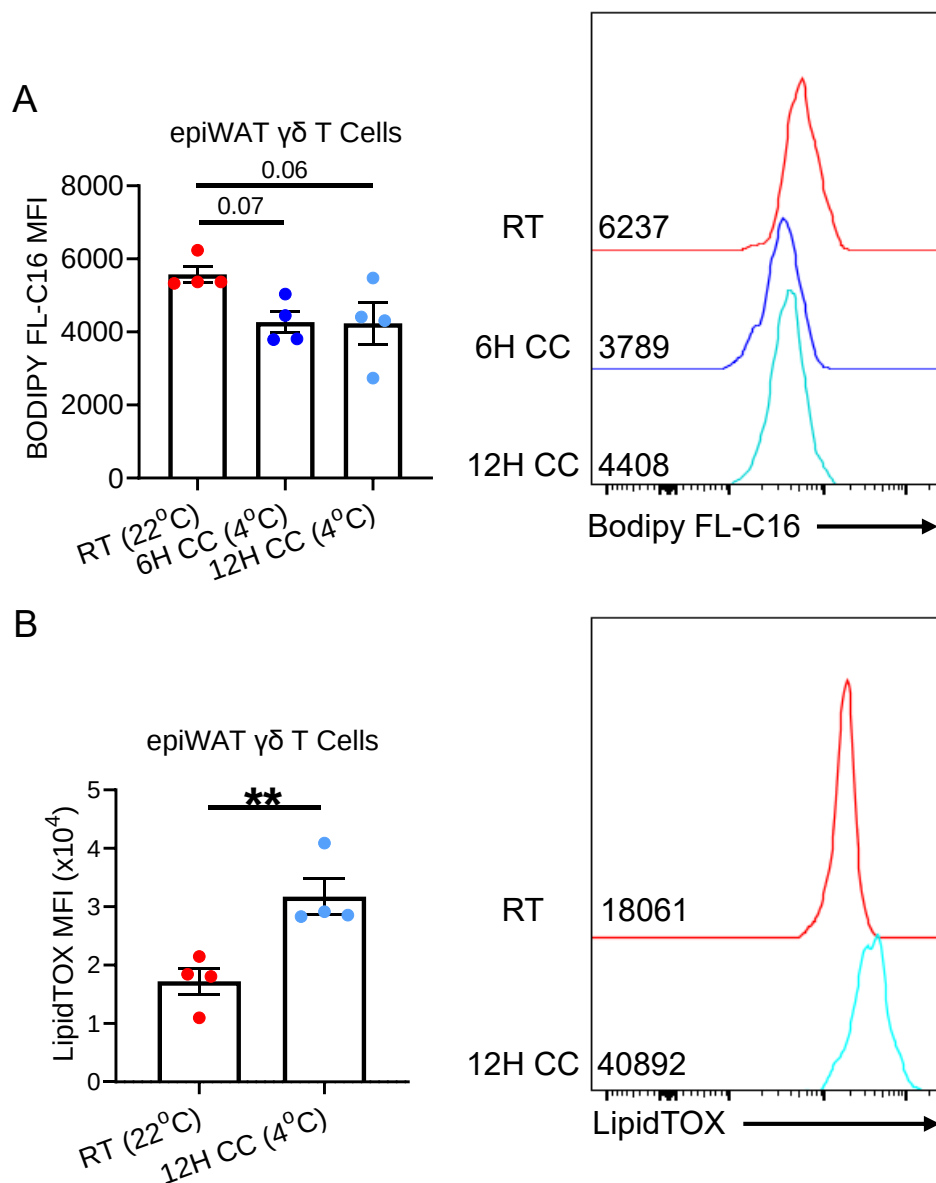


Figure 3.17: Cold challenge increases lipid content in epiWAT $\gamma\delta$ T Cells.

(A) Lipid uptake (MFI) of fluorescent palmitate BODIPY FL-C16 in epiWAT $\gamma\delta$ T cells of mice subjected to room temperature, 6-hours, or 12-hours of cold challenge, with representative histograms (right). **(B)** Neutral lipid content (MFI) by lipidTOX in epiWAT $\gamma\delta$ T cells of mice subjected to room temperature, or 12-hours of cold challenge, with representative histograms (right). Data are representative of two independent experiments. Data shown as mean $\pm$ SEM, $n = 4$ mice per group. **(A)** Significance was calculated using a one-way ANOVA, **(B)** Two-tailed unpaired student's t-test. ** $p < 0.01$.

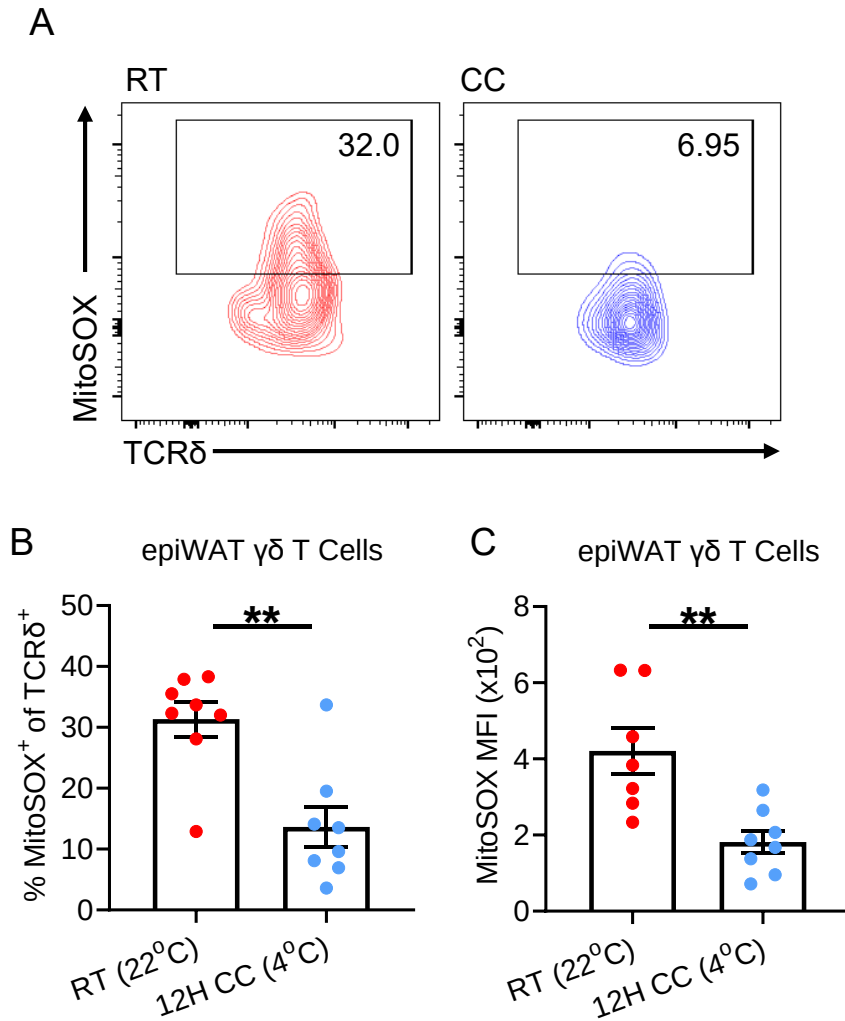


Figure 3.18: Cold challenge decrease mitochondrial ROS in epiWAT $\gamma\delta$ T cells.

(A) Representative histograms of mitochondrial ROS production. **(B)** Percentage of epiWAT $\gamma\delta$ T cells producing mitochondrial ROS from room temperature and 12-hour cold challenged mice. **(C)** Mean fluorescence of mitochondrial ROS production by epiWAT $\gamma\delta$ T cells from room temperature and 12-hour cold challenged mice. Data are representative of two independent experiments, two experiments combined. Data shown as mean $\pm$ SEM, $n = 7-8$ mice per group. **(A)** Significance was calculated using a two-tailed unpaired student's t-test. ** $p < 0.01$.

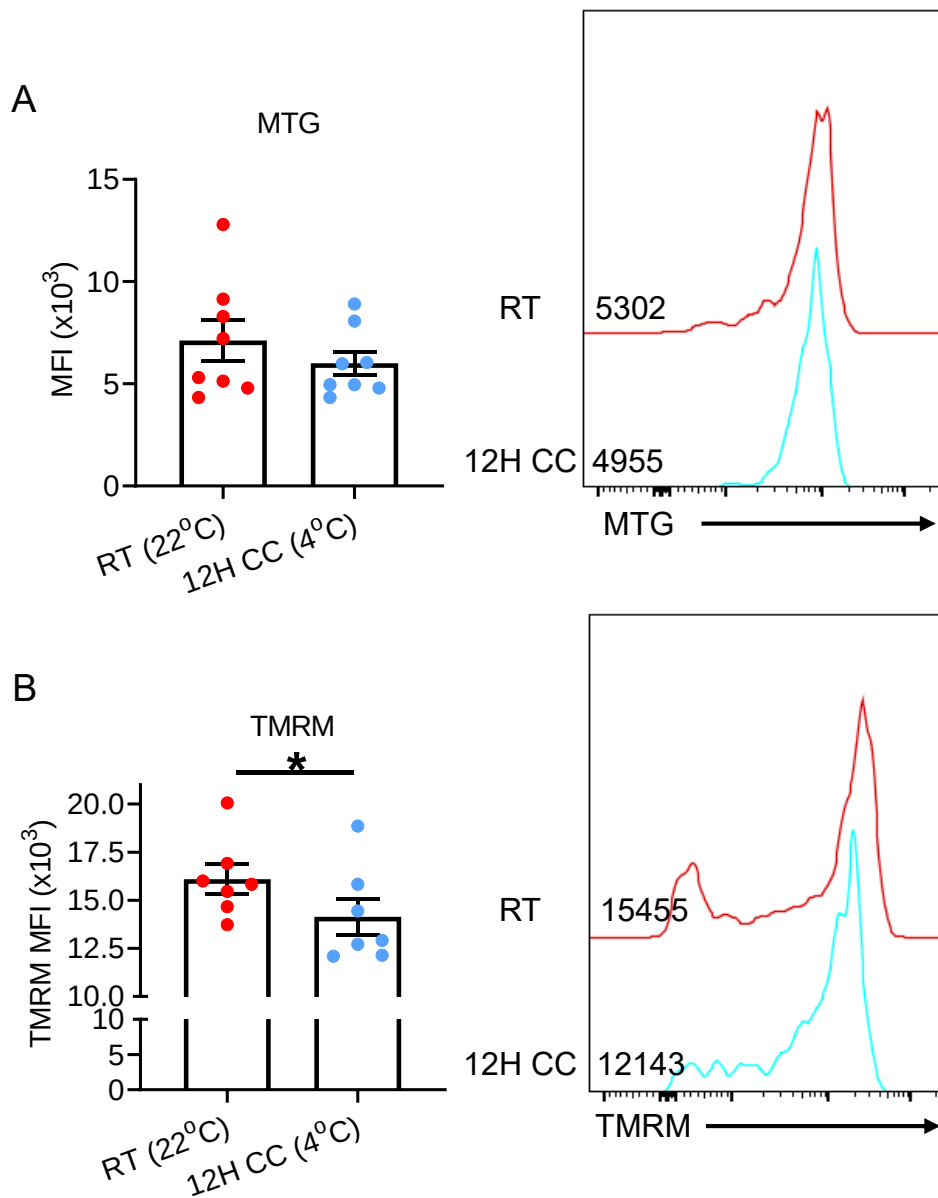


Figure 3.19: Cold challenge decreases the mitochondrial membrane potential of epiWAT $\gamma\delta$ T cells.

(A) Mean fluorescence of mitochondrial mass (MTG) from epiWAT $\gamma\delta$ T cells of room temperature or 12-hour cold challenged mice, with representative histograms (right). **(B)** Mean fluorescence of mitochondrial membrane potential (TMRM) from epiWAT $\gamma\delta$ T cells of room temperature or 12-hour cold challenged mice, with representative histograms (right). Data are representative of two independent experiments, two experiments combined. Data shown as mean $\pm$ SEM, $n = 7-8$ mice per group. **(A)** Significance was calculated using a two-tailed unpaired student's t-test. * $p < 0.05$.

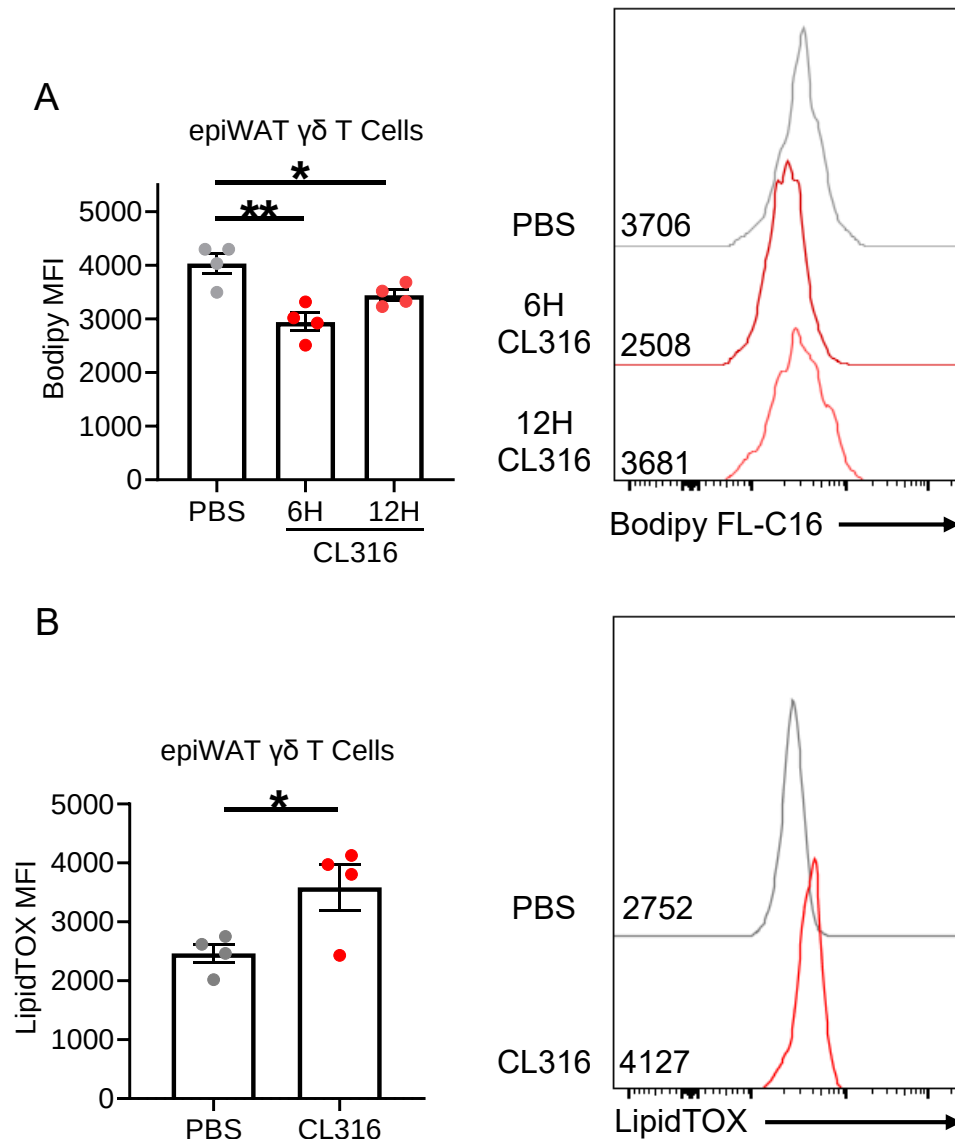


Figure 3.20: CL316,243 administration increases lipid content in epiWAT $\gamma\delta$ T Cells.

(A) Lipid uptake (MFI) of fluorescent palmitate BODIPY FL-C16 in epiWAT $\gamma\delta$ T cells of mice euthanized 4-hours post PBS or CL316,243 administration i.p., with representative histograms (right). **(B)** Neutral lipid content (MFI) by lipidTOX in epiWAT $\gamma\delta$ T cells of mice euthanized 4-hours post PBS or CL316,243 administration i.p., with representative histograms (right). Data are representative of two independent experiments. Data shown as mean $\pm$ SEM, $n = 4$ mice per group. **(A)** Significance was calculated using a one-way ANOVA, **(B)** Two-tailed unpaired student's t-test. * $p < 0.05$, ** $p < 0.01$.

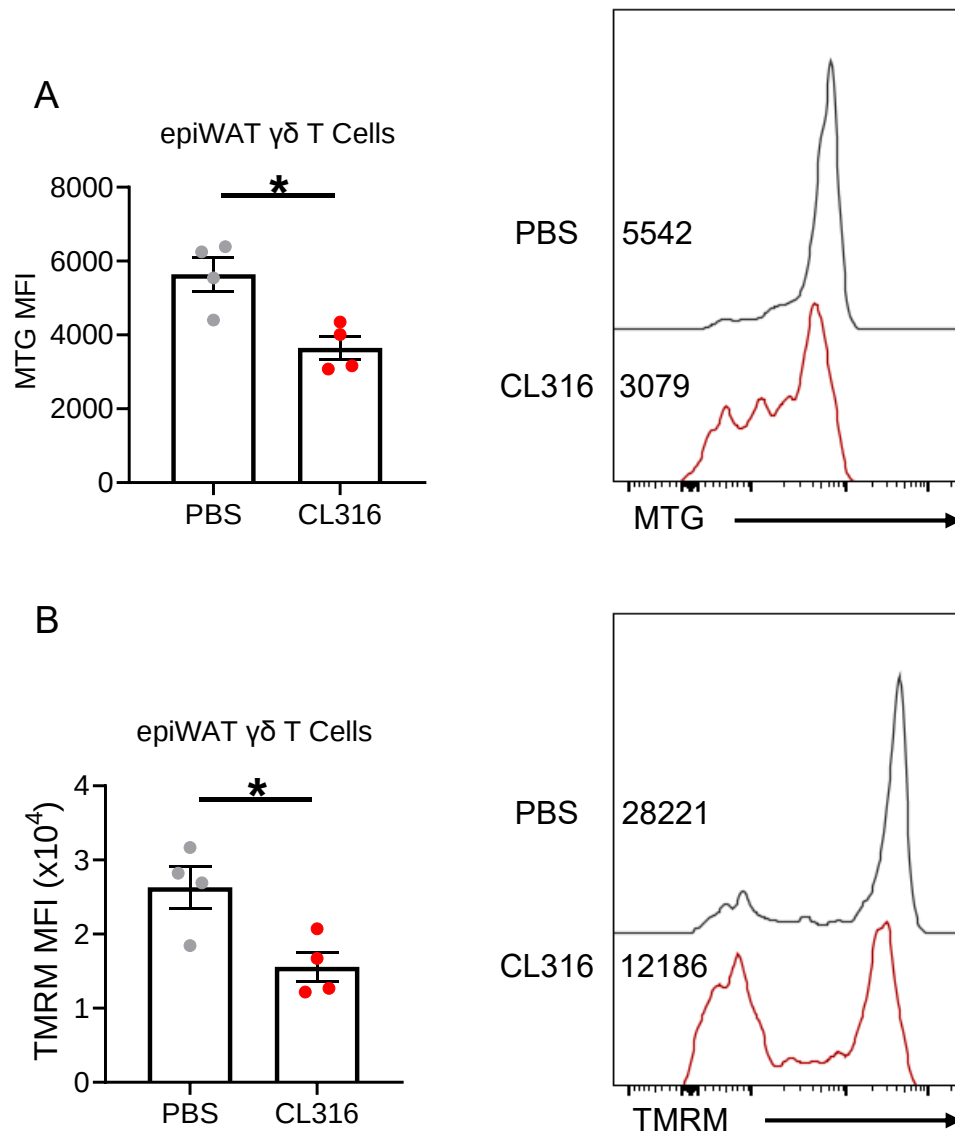


Figure 3.21: CL316,243 administration decreases the mitochondrial mass, and membrane potential of epiWAT $\gamma\delta$ T cells.

(A) Mean fluorescence of mitochondrial mass (MTG) from epiWAT $\gamma\delta$ T cells of mice euthanized 4-hours post PBS or CL316,243 administration i.p., with representative histograms (right). **(B)** Mean fluorescence of mitochondrial membrane potential (TMRM) from epiWAT $\gamma\delta$ T cells of mice euthanized 4-hours post PBS or CL316,243 administration i.p., with representative histograms (right). Data are representative of two independent experiments, two experiments combined. Data shown as mean $\pm$ SEM, $n = 7-8$ mice per group. **(A)** Significance was calculated using a two-tailed unpaired student's t-test. * $p <$

3.7 scRNA-Seq analysis reveals cold challenge decreases $\gamma\delta^{17}$ T cell glycolysis, oxidative phosphorylation, and steroid metabolism.

To further investigate the effect of cold challenge and thermogenesis on epiWAT $\gamma\delta$ T cells, we isolated innate T cells from epiWAT to perform scRNA-seq between room temperature and cold challenged animals (Figure 3.22A). scRNA-seq analysis revealed 6 lymphocyte populations which clustered based on effector function (Figure 3.22B). Using cell type specific markers such as expression of *Trac* and *Trdc* for TCR expression, in addition to *Rorc* and *Tbx21* for functionality, we confirmed the presence of IFN γ expressing, and IL-17A expressing, iNKT cells, and $\gamma\delta$ T cells in epiWAT (Figure 3.22C). We next pulled epiWAT $\gamma\delta$ T cells from the dataset specifically (Figure 3.23A). In addition to glycolysis and oxidative phosphorylation which have previously been described to be used primarily by $\gamma\delta^{\text{IFN}\gamma}$ T cells, and $\gamma\delta^{17}$ T cells respectively⁶⁸, epiWAT $\gamma\delta$ T cells could be further defined by metabolism. By creating module scores associated with the genes involved in specific metabolic pathways, we observed that epiWAT V γ 6⁺ $\gamma\delta$ T cells had higher expression of genes involved in polyamine metabolism, amino acid metabolism, steroid metabolism, glycolysis and oxidative phosphorylation compared to epiWAT CD27⁺ $\gamma\delta$ T cells (Figure 3.23B-C, E, H-I, respectively). No differences were observed in fatty acid synthesis or β -oxidation related-genes between V γ 6⁺ and CD27⁺ $\gamma\delta$ T cells (Figure 3.23F, G). Interestingly, V γ 4⁺ $\gamma\delta$ T cells had the highest expression of steroid metabolism and oxidative phosphorylation genes (Figure 3.23 E, I).

Having observed that cold challenge decreased mitochondrial metabolism in epiWAT $\gamma\delta^{17}$ T cells (Figure 3.18A-C; Figure 3.19B), we next pulled V γ 6⁺ $\gamma\delta$ T cells specifically from the dataset and separated them based on room temperature or cold challenge treatment (Figure 3.24A). KEGG pathway analysis on differentially expressed genes between room temperature and cold challenged epiWAT V γ 6⁺ $\gamma\delta$ T cells revealed that pathways involved in cellular metabolism were the most decreased pathways at 4°C (Figure 3.24B). Specifically, oxidative phosphorylation was the most decreased pathway in cold challenged epiWAT V γ 6⁺ $\gamma\delta$ T cells (Figure 3.24B, C, K). Additionally, pathways involving steroid metabolism, specifically terpenoid and steroid biosynthesis, were also decreased at 4°C (Figure 3.24B, C, G). The results further suggest that cold challenge induced thermogenesis inhibits epiWAT $\gamma\delta^{17}$ T cell metabolism.

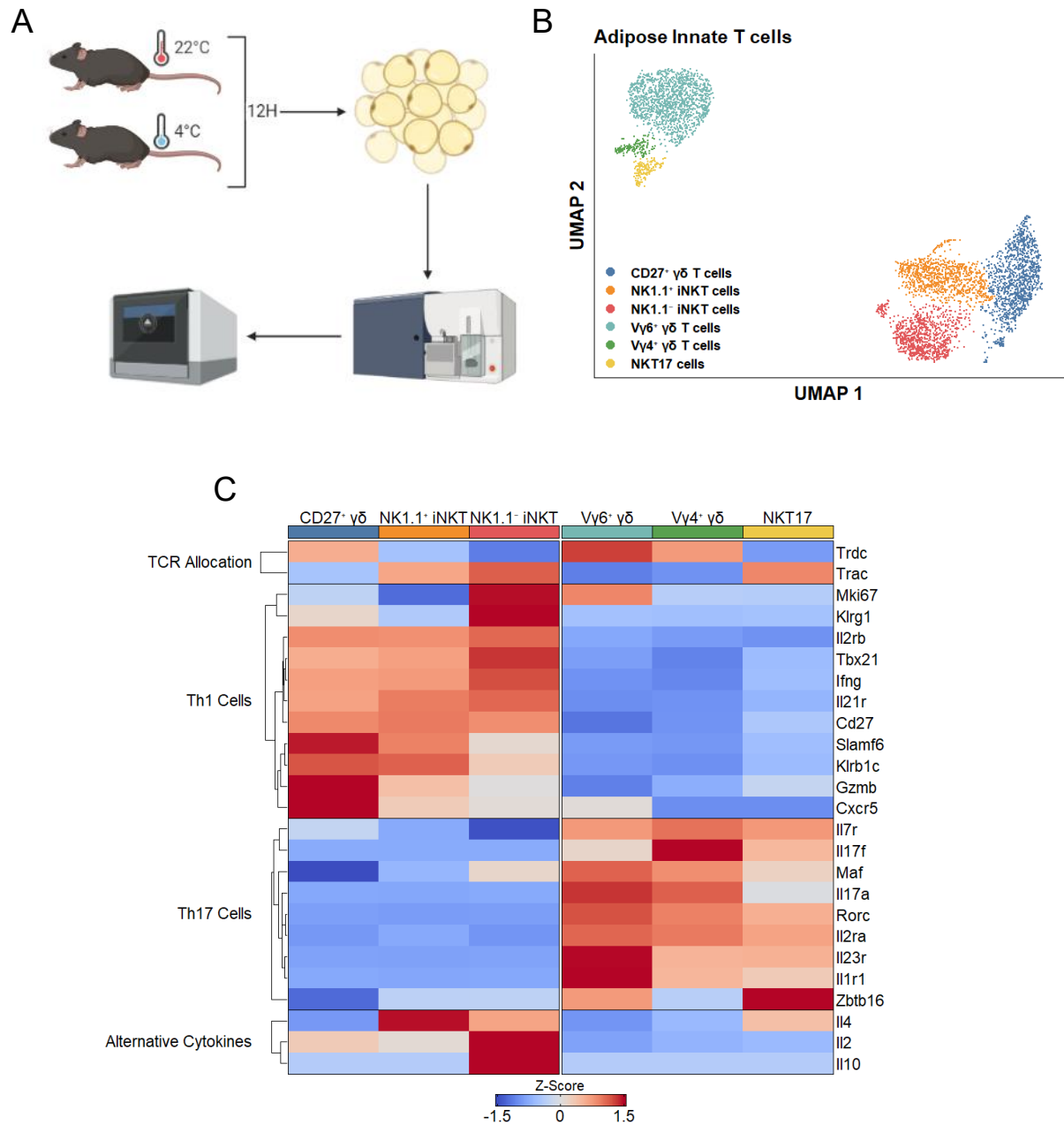


Figure 3.22: scRNA-seq characterisation of epiWAT γδ T cells, and iNKT cells.

(A) Diagrammatic representation of scRNA-seq experimental design. **(B)** UMAP of adipose innate T cell populations. **(C)** Scaled average heatmap with hierarchical clustering of genes associated with cell population phenotype. Data are representative of one independent experiment. *Experiment performed by Harry Kane and Hannah Prendeville.*

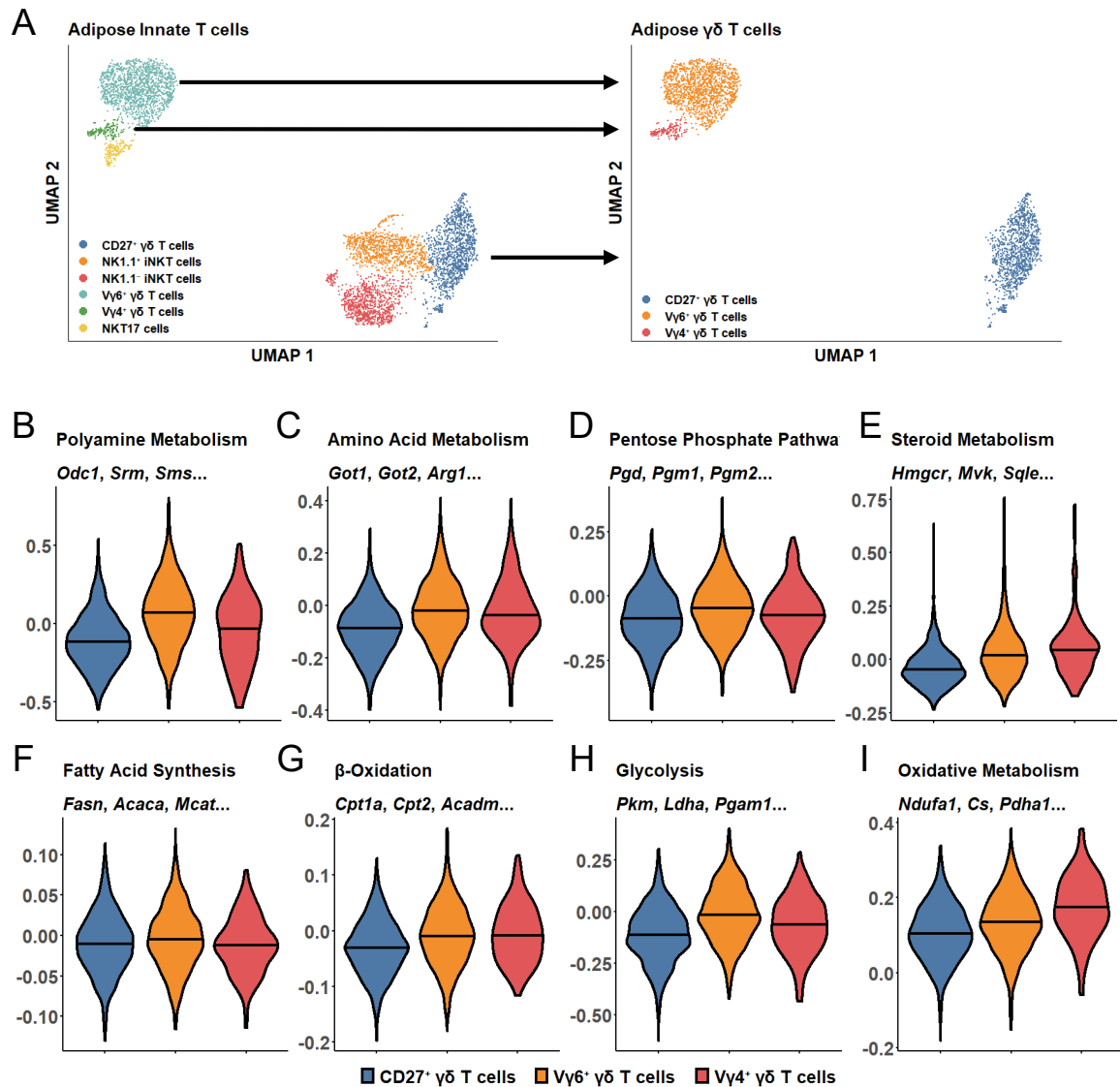


Figure 3.23: Adipose V γ 6⁺ $\gamma\delta$ T cells are enriched for metabolic genes.

(A) UMAP subset of adipose $\gamma\delta$ T cell populations. (B-I) Violin plots showing expression of metabolic gene module scores of adipose $\gamma\delta$ T cell populations; (B) Polyamine metabolism module score, (C) Amino acid metabolism module score, (D) Pentose phosphate pathway module score, (E) Steroid metabolism module score, (F) Fatty acid synthesis module score, (G) β -oxidative metabolism module score, (H) Glycolytic metabolism module score, (I) Oxidative metabolism module score. Data are representative of one independent experiments.

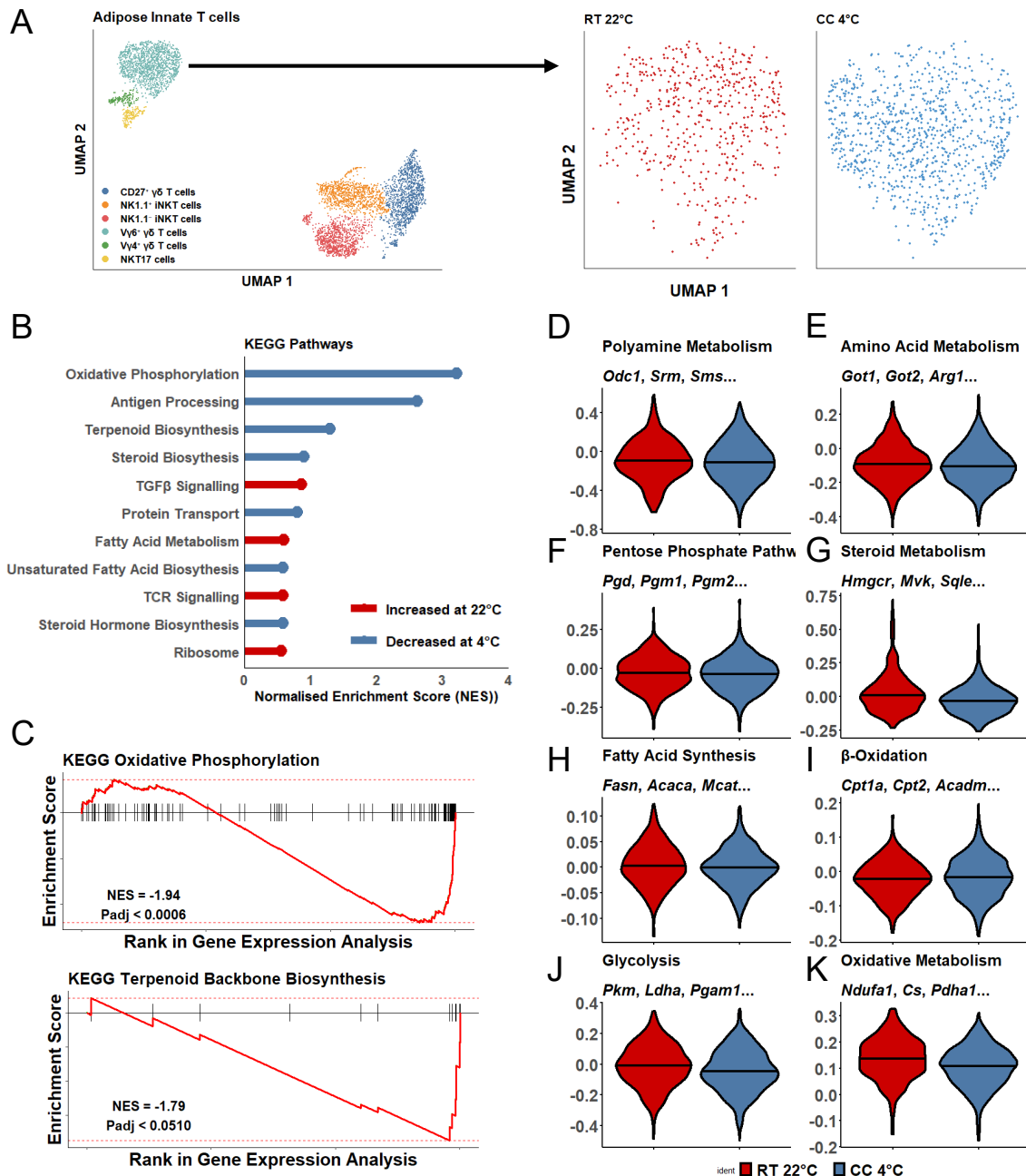


Figure 3.24: Cold challenge decreases epiWAT Vγ6⁺ γδ T cell metabolism.

(A) UMAP subset of adipose Vγ6⁺ γδ T cell populations separated by room temperature or 12-hour cold challenged conditions. (B) Lollipop plot of KEGG pathway enrichment between room temperature and 12-hour cold challenged Vγ6⁺ γδ T cells. (C) GSEA plot showing decrease expression of the KEGG oxidative phosphorylation pathway in 12-hour cold challenged Vγ6⁺ γδ T cells. (D) GSEA plot showing decrease expression of the KEGG terpenoid backbone synthesis pathway in 12-hour cold challenged Vγ6⁺ γδ T cells. (D-K) Violin plots showing expression of metabolic gene module scores of adipose Vγ6⁺ γδ T cell populations between room temperature and 12-hour cold challenged conditions Data are representative of one independent experiments.

3.8 V γ 6⁺ $\gamma\delta$ T cells express adrenergic receptors *Adrb1*, and *Adrb2* mRNA, which is upregulated at 4°C.

Signals from the brain to adipose tissue via the sympathetic nervous system, release noradrenaline into the adipose environment to induce thermogenesis¹⁶⁹. During cold exposure, adipose sympathetic activity and noradrenaline release are increased activating the β 3-adrenergic receptor on the surface of adipocytes, which increases lipolysis and UCP-1 expression^{170,171}. As adrenergic activity in adipose tissue is high during cold challenge, we investigated if adrenergic activity may play role in the inhibition of epiWAT V γ 6⁺ $\gamma\delta$ T cell function and mitochondrial metabolism. Sympathetic activity peaks when murine body temperature and RER is lowest in a 24-hour period (Figure 3.25A)¹⁷². We found that BAT displays a circadian rhythmic expression to adrenergic receptor expression, however *Adrb2*, and *Adrb3*, oscillated at different times of day (Figure 3.25B). There are an increasing amount of studies demonstrating that innate and adaptive immune cells express adrenergic receptors¹⁷³, which modulate immune function¹⁷⁴. Therefore, we characterised adrenergic receptor expression by epiWAT $\gamma\delta$ T cells, and found that V γ 6⁺ $\gamma\delta$ T cell had the highest expression of *Adrb2* compared to CD27⁺, and V γ 4⁺ $\gamma\delta$ T cells (Figure 3.25D). Interestingly, V γ 6⁺ $\gamma\delta$ T cells were the sole expressers of *Adrb1* (Figure 3.25D). Intracellular calcium release is a downstream target of β -adrenergic signalling^{175,176}, in addition to V γ 6⁺ $\gamma\delta$ T cells having the highest β -adrenergic receptor expression, the also had the highest expression of intracellular calcium signalling genes (Figure 3.25E). We next investigated if cold challenge upregulated the expression of adrenergic receptors in epiWAT $\gamma\delta$ T cells. Indeed, cold challenge increased expression of *Adrb1*, and *Adrb2* by V γ 6⁺ $\gamma\delta$ T cells (Figure 3.25G). However, there was no change in the expression of genes associated with calcium signalling in V γ 6⁺ $\gamma\delta$ T cells after cold challenge (Figure 3.25H). These results suggest that adrenergic signalling may play an important role in adipose $\gamma\delta$ ¹⁷ T cells during cold challenge.

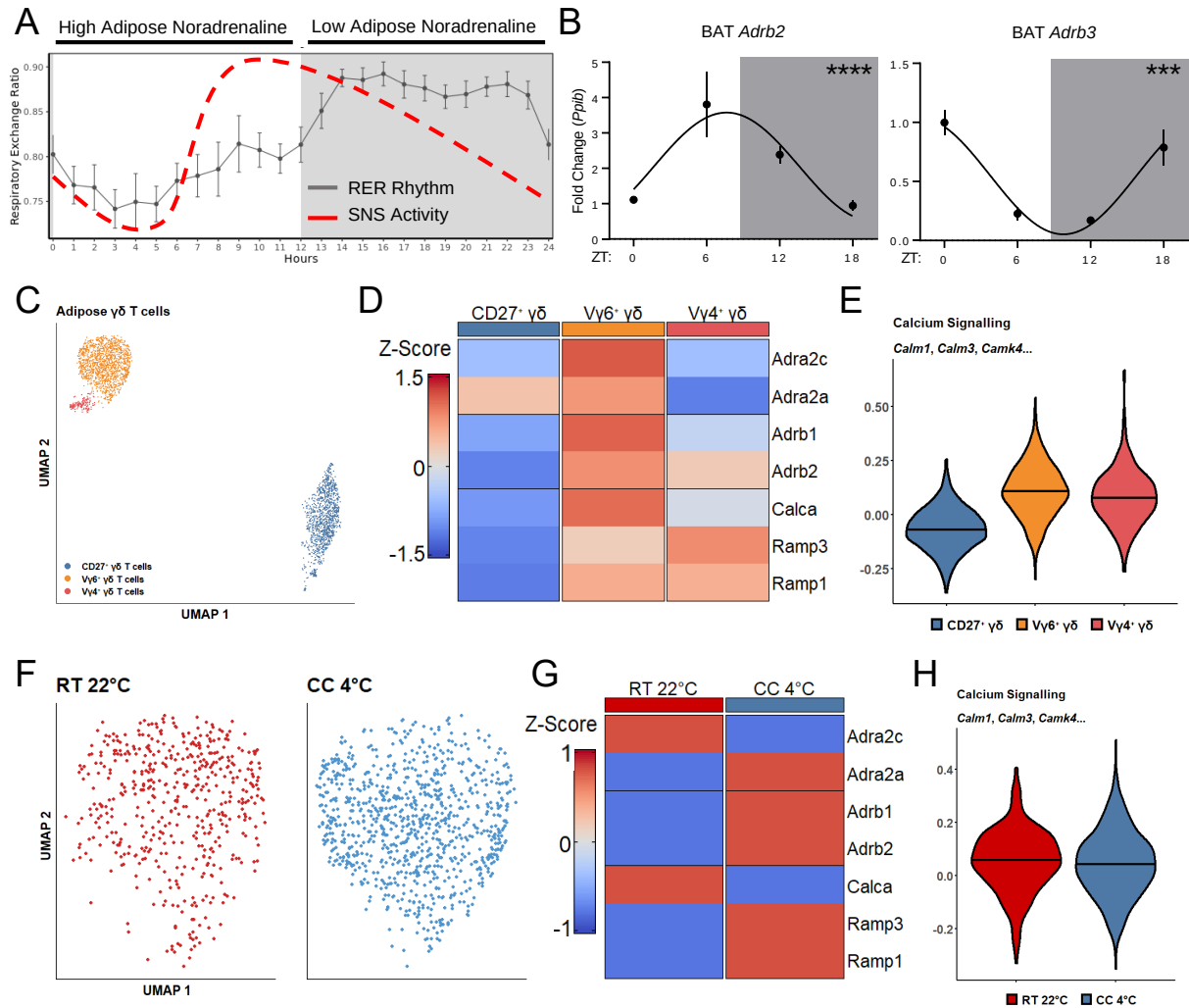


Figure 3.25: Cold challenge increases epiWAT Vγ6⁺ γδ T cell expression of adrenergic receptors.

(A) Representative circadian time plot of RER indicating time of day with lowest and highest adipose adrenergic activity. (B) Circadian plots of BAT *Adrb2* and *Adrb3* receptor expression. (C) UMAP subset of adipose γδ T cell populations. (D) Scaled average heatmap of genes associated with neuronal signalling by cell population. (E) Violin plots showing module score of genes associated with calcium signalling in adipose γδ T cell populations. (F) UMAP subset of adipose Vγ6⁺ γδ T cell populations separated by room temperature or cold challenged conditions. (G) Scaled average heatmap of genes associated with neuronal signalling by room temperature or cold challenged conditions. (H) Violin plots showing module score of genes associated with calcium signalling in adipose Vγ6⁺ γδ T cells between room temperature or cold challenged conditions. Data are representative of one independent experiment. (B) Data shown as mean ± SEM, n = 5 mice per group. Significance was calculated using cosinor analysis, with cosine fitted curves. ** p < 0.001, **** p < 0.0001.

3.9 Discussion

Here, we show that $\gamma\delta$ T cells are present and enriched in the BAT of prenatal mice, which were predominantly $\gamma\delta^{17}$ T cells, compared to placental tissue which were predominantly $\gamma\delta^{\text{IFN}\gamma}$ T cells. These data suggest that adipose tissue is seeded by $V\gamma 6^+$ $\gamma\delta$ T cells derived from the thymus during embryonic development¹⁷⁷. Human infants are born with a large amount of BAT and rely on non-shivering thermogenesis to maintain body temperature, as they lack the muscle mass to generate heat through shivering⁸⁷. We show that BAT of infant mice was also enriched for $\gamma\delta^{17}$ T cells, compared to parental BAT. It has been shown that IL-17A and IL-17F are necessary for thermogenesis, as mice lacking IL-17A or IL-17F are susceptible to hypothermia^{28,29}, therefore $\gamma\delta^{17}$ T cells may play a role in infant BAT thermogenesis. Furthermore, mice lacking expression of IL-17F, and IL-17RC in adipose tissue have less innervation, therefore causing a defect in thermogenesis²⁹. Overexpression of $V\gamma 6^+$ $\gamma\delta$ T cells in adipose tissue induced overgrowth of innervating neurons in the tissue, suggesting an essential role of IL-17 signalling in neurogenesis²⁹. As BAT innervation is crucial for its thermogenic function, adipose $\gamma\delta^{17}$ T cells may be necessary for the innervation of adipose tissue during development.

We observed that T cell numbers from adipose tissue, lymph nodes, and liver were decreased after cold challenge, but increased in the blood and bone marrow. This was not specific to any particular subset of T cells, as $CD4^+$ and $CD8^+$ T cells were decreased in the lymph nodes, and increased in the bone marrow following cold challenge. Thermogenesis in response to cold challenge results in a considerable upregulation of energy expenditure, with 60% of total energy production being spent on heat generation¹⁶². The activation of an immune response can be energetically costly, and reprograms peripheral metabolism in order to fulfil the metabolic demand of an immune response¹⁷⁸. Therefore, overlapping situations of high metabolic demand will result in trade-offs between immunity and homeothermy¹⁷⁹. For example, mice housed at thermoneutrality (30°C) do not spend additional energy on body temperature maintenance, while mice housed at 22°C require BAT thermogenesis to maintain core body temperature¹⁸⁰. LPS administration decreases metabolic rate in mice housed at both 22°C and 30°C, however thermoneutral mice lower their locomotor activity to conserve energy, with no change in body temperature¹⁸¹. Mice housed at room temperature however, decrease their locomotor activity, basal metabolic rate, and abandon homeothermy in order to fuel the immune response, which results in a dramatic drop in body temperature¹⁸¹. As such, the observation that circulating T cells relocate to the bone marrow during cold challenge, may be the result of an energetic trade off, redirecting energy to homeothermy, rather than to fighting infection. Additionally, this is

observed in times of food scarcity, as during calorie restriction CD8⁺ memory T cells migrate to the bone marrow and downregulate their cellular metabolism for energy conservation¹¹⁵. Furthermore, circulating lymphocytes decrease during bouts of hibernation, where the body temperature of hibernating animals can drop to as low as 2-10°C¹⁸².

In addition to redirecting peripheral energetic needs, skewing of the immune system towards an immunosuppressive phenotype may also contribute to energy conservation. Here, we found that cold challenge increases the number of circulating Treg cells, by using the lymph node egress inhibitor FTY720 to capture immune cells during cold challenge. In addition to an increase in Treg numbers, cold challenge also increases the concentration of circulating IL-10, providing an immunosuppressive environment. The increase in circulating Treg cells during cold challenge may be due to naïve T cells polarising to a Treg phenotype, or possibly due to Treg cells migrating from tissues during cold challenge. It has been shown that human Treg cells, cultured in mild hypothermic conditions (33°C) stimulates Treg proliferation, increases Treg expression of FoxP3, CD25 and Helios, and are more efficient inhibitors of T effector cell proliferation¹⁸³. Furthermore, short-term cold exposure increased the percentage of circulating Treg cells in human peripheral blood, and their induction from naïve T cells *in vivo*¹⁸⁴. Finally, cold challenge changes the abundance of circulating nutrients in peripheral blood, increasing the concentration of free fatty acids, triglycerides and glycerol¹⁸⁵. As Treg cells rely more on fatty acid oxidation for their effector function compared to other effector T cells¹⁸⁶, increased circulating lipids during cold challenge may contribute to their increased immunosuppressive activity. Together these data suggest that cold challenge creates an immunosuppressive environment, through a combination of metabolic trade-offs to dampen an immune response, and an enhancement of regulatory T cells. Therefore, therapeutic hypothermia may provide value in treating metabolic or autoimmune diseases, such as cachexia or multiple sclerosis where there is over activation of the immune system.

Previous studies have described a necessary role of IL-17 signalling in thermogenesis and the maintenance of body temperature, as mice lacking IL-17 signalling are susceptible to hypothermia^{28,29}. Counterintuitively, we show that wild-type mice that experience a cold challenge have decreased expression of IL-17A and ROR γ t compared to room temperature control mice by adipose resident $\gamma\delta$ T cells. Adipose tissue is a highly immunosuppressive environment that is enriched for IL-10 producing cell populations such as iNKT cells^{83,187}, M2 macrophages and Tregs^{84,188}. Induction of thermogenesis via cold challenge or CL316,243 administration induces Treg cells in adipose tissue¹⁸⁹, which may inhibit the production of IL-17A by adipose $\gamma\delta$ T cells. Furthermore, adipose $\gamma\delta$ ¹⁷ T cells had increased lipid content, and decreased mitochondrial metabolism after cold challenge, which likely

aided the decreased expression of IL-17A. Recent work by our lab has demonstrated that $\gamma\delta^{17}$ T cells rely on oxidative metabolism for their cellular function⁶⁸. Lipolysis induced during thermogenesis releases lipids into the adipose tissue environment and peripheral blood, and the increased lipid content during cold challenge may overload adipose $\gamma\delta^{17}$ T cells, similar to what has been described in NK cells⁶⁵. Moreover, the biochemical structure of lipids release by adipocytes during thermogenesis may inhibit $\gamma\delta^{17}$ T cells, as a higher saturated fatty acid dependence leads to increased IL-17A pathogenicity in Th17, whereas a polyunsaturated fatty acid dependence did not³⁷. Furthermore, it has been shown that $\gamma\delta^{17}$ T cells preferentially take up cholesterol which can act as an agonist for ROR γ t⁶⁸. We found that cholesterol biosynthesis pathways were down regulated after cold challenge, suggesting that availability of cholesterol metabolites may be reduced during cold challenge, limiting adipose $\gamma\delta^{17}$ T cell function.

Finally, we find that adipose $\gamma\delta^{17}$ T cells have the highest expression of the adrenergic receptor *Adrb2* compared to other adipose $\gamma\delta$ T cell subsets, and are the only adipose innate T cell to express the *Adrb1* receptor. We show that adipose $\gamma\delta^{17}$ T cells upregulate *Adrb1* and *Adrb2* receptor expression during cold challenge. Cold temperatures increase sympathetic activity and noradrenaline release in adipose tissue, which can bind to and activate the *Adrb1*, *Adrb2*, and *Adrb3* receptors simultaneously¹⁹⁰. Generally, adrenergic signalling in the immune system is immunosuppressive, with activation of *Adrb2* enhancing IL-10 production by Treg cells, and inhibiting TNF and IFN γ production by M1 macrophages, CD8⁺ T cells and Th1 cells¹⁷⁴. However, adrenergic signalling in $\gamma\delta$ T cells remains to be investigated, although it stands to reason that the inhibition of IL-17A expression by adipose $\gamma\delta$ T cells during cold challenge may be imparted by *Adrb1*, or *Adrb2* adrenergic receptor activation. Collectively, these data may present novel strategies to limit IL-17A associated pathologies in health and disease.

Chapter 4

Circadian expression of IL-17A by adipose $\gamma\delta$ T cells maintains *de novo* lipogenesis rhythm in adipose tissue.

4.1 Introduction

Circadian rhythms allow organisms to anticipate and adjust to predictable changes in the external environment, such as light¹⁹¹, temperature¹⁹², and feeding behaviour¹⁹³. These environmental signals converge on the suprachiasmatic nucleus, which is the master biological pacemaker in the hypothalamus, to coordinate circadian rhythms with the external environment¹⁹¹. Circadian rhythms are dictated at the cellular level by the molecular clock, an auto-regulatory system of transcriptional activators, BMAL1 and CLOCK, and repressors, PER1-3 and CRY1-2, acting in a negative feedback loop, expressed by every mammalian cell¹⁹⁴. Cellular circadian rhythms within a tissue are kept in synchrony by a complex combination of neuronal, hormonal and metabolic cues. These in turn give rise to circadian rhythms in a variety of physiological processes such as endocrine signalling¹⁹⁵, immune cell function¹⁹⁶ and systemic metabolism¹⁹⁷. How the circadian rhythms of these biological processes influence one another remains an active area of research as it is important to understand how these interactions maintain homeostasis.

Maintenance of core body temperature is one such physiological process with a circadian rhythm. It is governed by metabolic rate, which oscillates daily over 24-hours. Body temperature is highest at night during the active phase, lowest during the resting inactive phase, and is crucial for optimal whole body homeostasis¹⁹⁸. White adipose tissue (WAT), and brown adipose tissue (BAT) processes are critical for whole body metabolic homeostasis, and are heavily influenced by daily fluctuations in energy demands¹⁹⁹. Light and feeding govern adipose circadian rhythm in lipid metabolism including lipolysis, lipogenesis, and thermogenesis. Lipolysis and thermogenesis are highest during the inactive phase, to maintain body temperature during rest, while lipogenesis peaks during feeding for lipid uptake and storage from the diet^{129,200}. Lipid metabolism in adipose tissue is tightly regulated by the circadian clock, and circadian disruption can lead to impaired lipid homeostasis. For example, disrupted circadian rhythms are common for shift workers, where increased risk of developing metabolic disease is prevalent²⁰¹. Therefore, it is becoming increasingly important to understand how circadian rhythms interact, especially in the setting of metabolic disease.

The molecular clock regulates both T helper 17 (Th17) cells, and type 3 innate lymphoid cells (ILC3s) in the gut^{124,125}. Here we investigated if type 17 innate T cells, particularly in adipose tissue, were controlled by the molecular clock, and if this played a role in adipose tissue metabolic homeostasis. We show that the circadian production of IL-17A and IL-17F

from innate T cells is important for adipose and whole body metabolic rhythm. Adipose $\gamma\delta^{17}$ T cells have the highest expression of molecular clock components across multiple organs, which mediates circadian expression of ROR γ t, IL-17A, IL-17F, and their cellular metabolism. ROR γ t and IL-17 expression peak during the active (night) phase during feeding. We identify adipose *de novo* lipogenesis (DNL) as the main target of diurnal IL-17 production across adipose tissue depots. Loss of IL-17 signalling in adipocytes leads to a downregulation of circadian DNL, ultimately impairing systemic metabolic rhythm and circadian body temperature regulation. We demonstrate a crucial role for IL-17 which is strongly regulated by the molecular clock and in turn, regulates circadian physiological processes to support whole-body metabolism. This pathway may be a target for metabolic disease

Results

4.2 Innate IL-17⁺ cells are enriched for molecular clock genes.

Previous studies have shown that adaptive CD4⁺ T cells, and innate gut resident ILC3s express a rhythmic molecular clock^{124,125,202,203}. It is unknown whether this is a feature of all IL-17 producing cells, and also whether innate T cells express a rhythmic molecular clock. To investigate this, we performed single cell RNA sequencing (scRNA-seq) of innate T cells ($\gamma\delta$ T cells and iNKT cells) isolated from epididymal adipose tissue (epiWAT), and compiled publicly available scRNA-seq data on innate T cells from lungs, spleen, thymus, skin and lymph nodes of wild-type mice, including iNKT cells, MAIT cells and $\gamma\delta$ T cells (Figure 4.1A). UMAP analysis for dimensional reduction of innate T cells revealed 14 clusters, which first separated cell types based on IL-17 producing innate T cells (Type 17) compared to IFN γ producing innate T cells (Type 1), and then based on tissue origin and cell subtype (Figure 4.1A, B). This highlights the distinct transcriptional program of Th17-like cells, which cluster together regardless of tissue or cell type. We then generated a “clock gene score” based on expression of molecular clock genes *Arntl*, *Clock*, *Nr1d1*, *Cry1*, *Per1*, and *Nfil3* (Figure 4.2A, B). The clock gene score revealed that molecular clock genes are enriched in Type 17 innate T cells compared to their Type 1 producing counterparts (Figure 4.3A) We also observed that tissue resident innate T cells (adipose, lung), had higher expression of molecular clock genes than innate T cells in lymphoid organs (spleen, LN, thymus). In particular, adipose $\gamma\delta$ T cells had the highest expression of all clock genes when compared to other Type 17 innate T cells (Figure 4.3A). To confirm that $\gamma\delta$ T cells have rhythmic expression of circadian clock genes, we isolated and expanded $\gamma\delta^{17}$ T cells from the spleen and lymph nodes, and using an *in vitro* serum shock to synchronise the molecular clock, we found a robust circadian rhythm in the expression of *Arntl*, *Clock*, *Nr1d1*, *Cry1*, *Per1*, and *Nfil3* in $\gamma\delta^{17}$ T cells (Figure 4.3B-H).

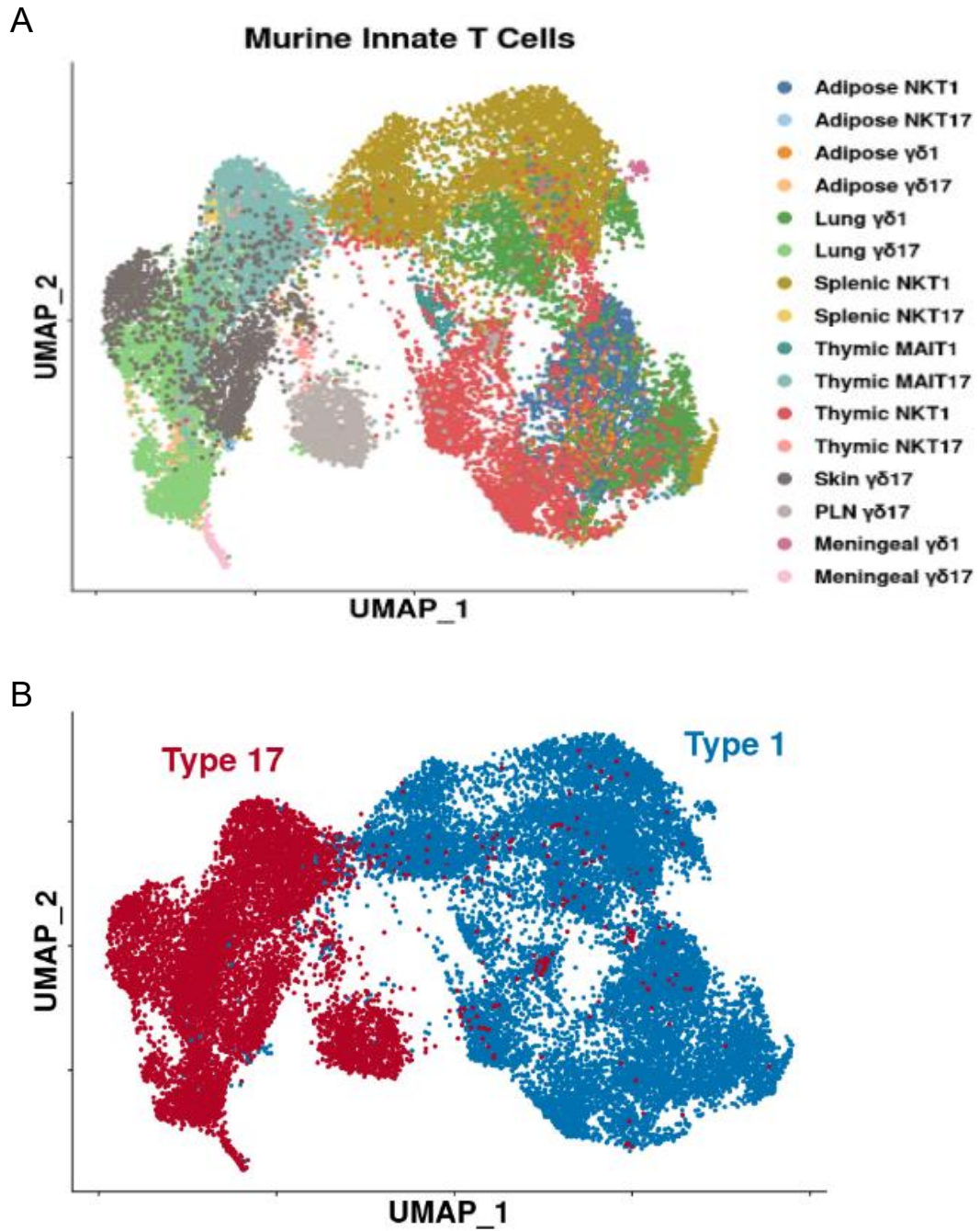


Figure 4.1: Murine innate T cell heterogeneity across tissues by scRNA-seq.

(A) UMAP of murine innate T cells across multiple organs reanalysed from GSE142845, GSE141895, E-MTAB-7704, E-MTAB-8732, GSE123400, and GSE147262 publicly available scRNA-Seq datasets. (B) UMAP of murine innate T cells subclustered based on functionality, Type 1 (Th1-like) or Type 17 (Th17-like).

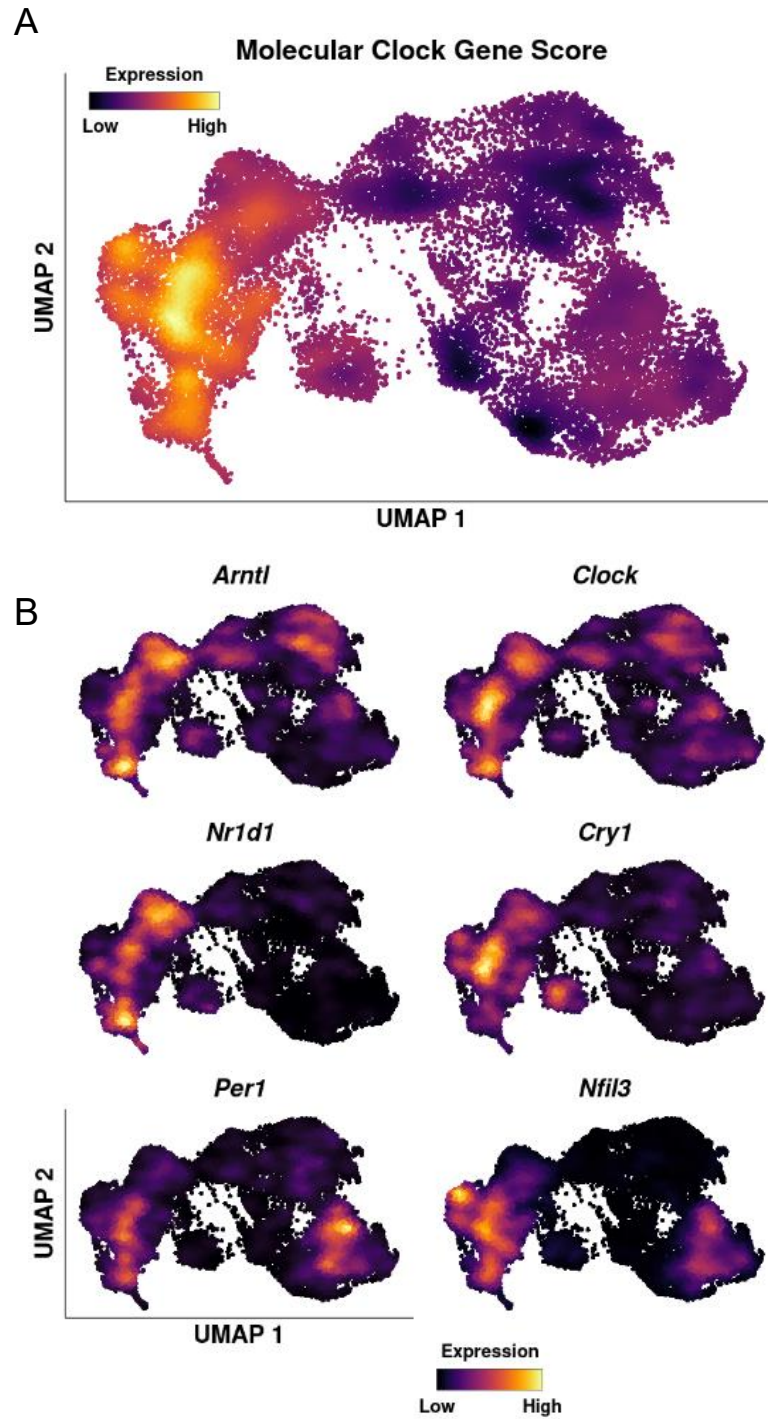


Figure 4.2: Molecular clock gene expression between IL-17⁺ and IFN γ ⁺ innate cells.

(A) Density plot of cumulative expression of all molecular clock genes across innate T cell subsets, designated molecular clock gene score. **(B)** Density plot expression of individual molecular clock genes across innate T cell subsets.

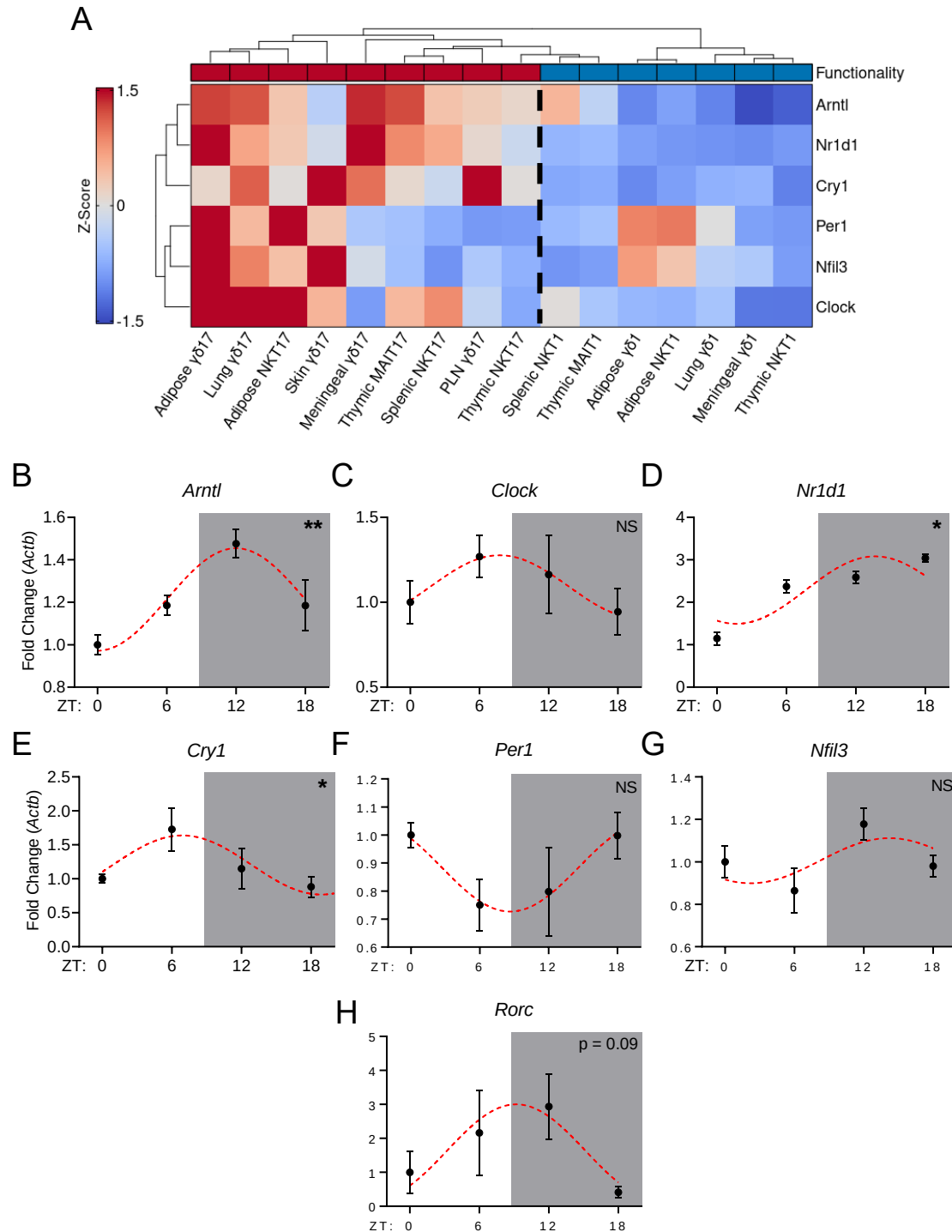


Figure 4.3: Molecular clock gene expression between IL-17⁺ and IFN γ ⁺ innate cells.

(A) Heatmap of averaged gene expression of molecular clock genes with hierarchal clustering of data from figure 4.1A. **(B-H)** Relative expression of circadian genes in expanded and serum shocked $\gamma\delta^{17}$ T cells. White and grey panels represent light and dark periods respectively. Data are representative of two independent experiments. **(B-H)** Data shown as mean $\pm$ SD, n = 4 technical replicates. Significance was calculated using cosinor analysis, with cosine fitted curves. NS = non-significant, * p < 0.05, ** p < 0.01.

4.3 $\gamma\delta$ T cells express IL-17A, IL-17F & ROR γ t in a circadian manner.

Having established that innate type 17 cells are enriched for clock genes, we investigated whether the production of IL-17A and IL-17F, and their transcriptional regulator ROR γ t, also follow a circadian rhythm over a 24-hour period in $\gamma\delta$ T cells. $\gamma\delta^{17}$ T cells were exposed to a serum shock at 6-hour intervals to stimulate cells at all-time points at the same time (ZT0, ZT6, ZT12, and ZT18; Figure 4.4A), with IL-1 β and IL-23. We found that the level of ROR γ t expression, and the percentage of $\gamma\delta$ T cells expressing IL-17A and IL-17F, had a robust circadian rhythm (Figure 4.4B, C; Figure 4.5A, B). The rhythm of ROR γ t, IL-17A and IL-17F expression peaked at ZT18 (Figure 4.4B, C; Figure 4.5A, B) translating to approximately 1am in the middle of the animals active (dark) phase.

We next measured the circadian expression of IFN γ and IL-17A produced by $\gamma\delta$ T cells *ex vivo*. Lymph node cells were harvested at ZT0 and split into 8 groups for stimulation at 3-hour intervals over 24-hours (Figure 4.6A). Similar to the *in vitro* serum shock model, *ex vivo* stimulated $\gamma\delta^{17}$ T cells displayed a robust circadian rhythm, which had an opposing diurnal rhythm compared to IFN γ , with IL-17A production displaying a nadir at ZT3 and a zenith at ZT15, and overall higher production at night (Figure 4.6B-E). $\gamma\delta^{\text{IFN}\gamma}$ T cells displayed overall higher production of IFN γ during the day (Figure 4.7B, D) displaying a weak circadian rhythm for IFN γ production, with percentage of IFN γ positive cells highest at ZT6, lowest at ZT18 (Figure 4.7E). Previous circadian rhythm studies in CD4 $^{+}$ T cells described an antagonistic role of NFIL3 to IL-17 circadian rhythm through direct inhibition of ROR γ t by NFIL3¹²⁴. NFIL3 was expressed almost exclusively in the $\gamma\delta^{17}$ T cell subset, compared to $\gamma\delta^{\text{IFN}\gamma}$ T cells, and displayed a circadian rhythm in $\gamma\delta^{17}$ T cells (Figure 4.8B-E). We observed a significant difference in the acrophase, the time of day at which the peak occurs, between NFIL3 expression (acrophase ZT0) and IL-17A expression (acrophase ZT15). The peak onset of IL-17A precedes peak onset of NFIL3, suggesting NFIL3 may inhibit IL-17A in a circadian manner in $\gamma\delta$ T cells, similar to CD4 $^{+}$ T cells (Figure 4.8E). Thus, $\gamma\delta^{17}$ T cells have a strong circadian rhythm for IL-17 production which they maintain intrinsically for at least 24-hours *ex vivo*.

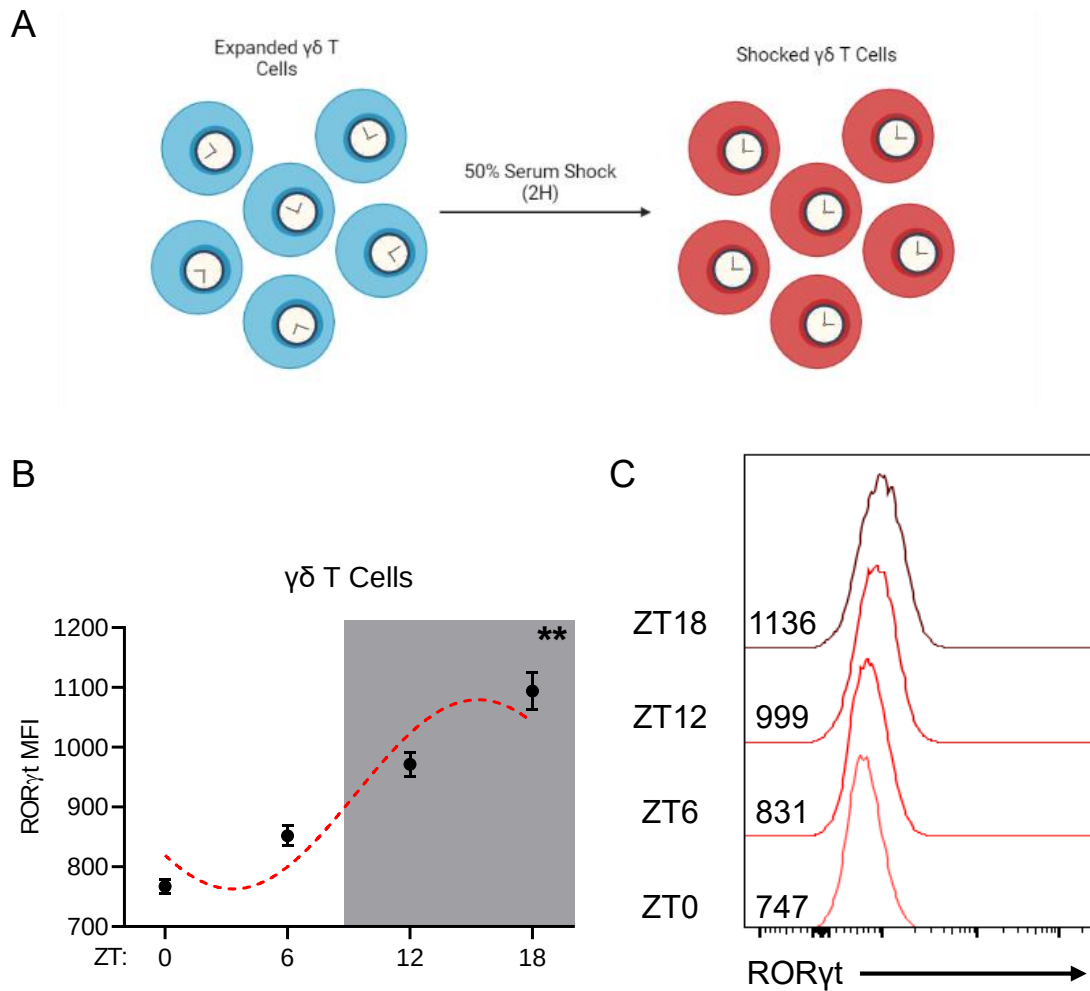


Figure 4.4: $\gamma\delta^{17}$ T cells display circadian expression of ROR γ t.

(A) Diagrammatic representation of the serum shock model to align the molecular clocks of multiple cells. **(B)** Circadian expression of ROR γ t (MFI), from expanded and serum shocked $\gamma\delta^{17}$ T cells after 3-hour stimulation with IL-1 β and IL-23. **(C)** Representative histograms of ROR γ t MFI. White and grey panels represent light and dark periods respectively. Data are representative of two independent experiments. **(B)** Data shown as mean $\pm$ SD, $n = 4$ technical replicates. Significance was calculated using cosinor analysis, with cosine fitted curves. ** $p < 0.01$.

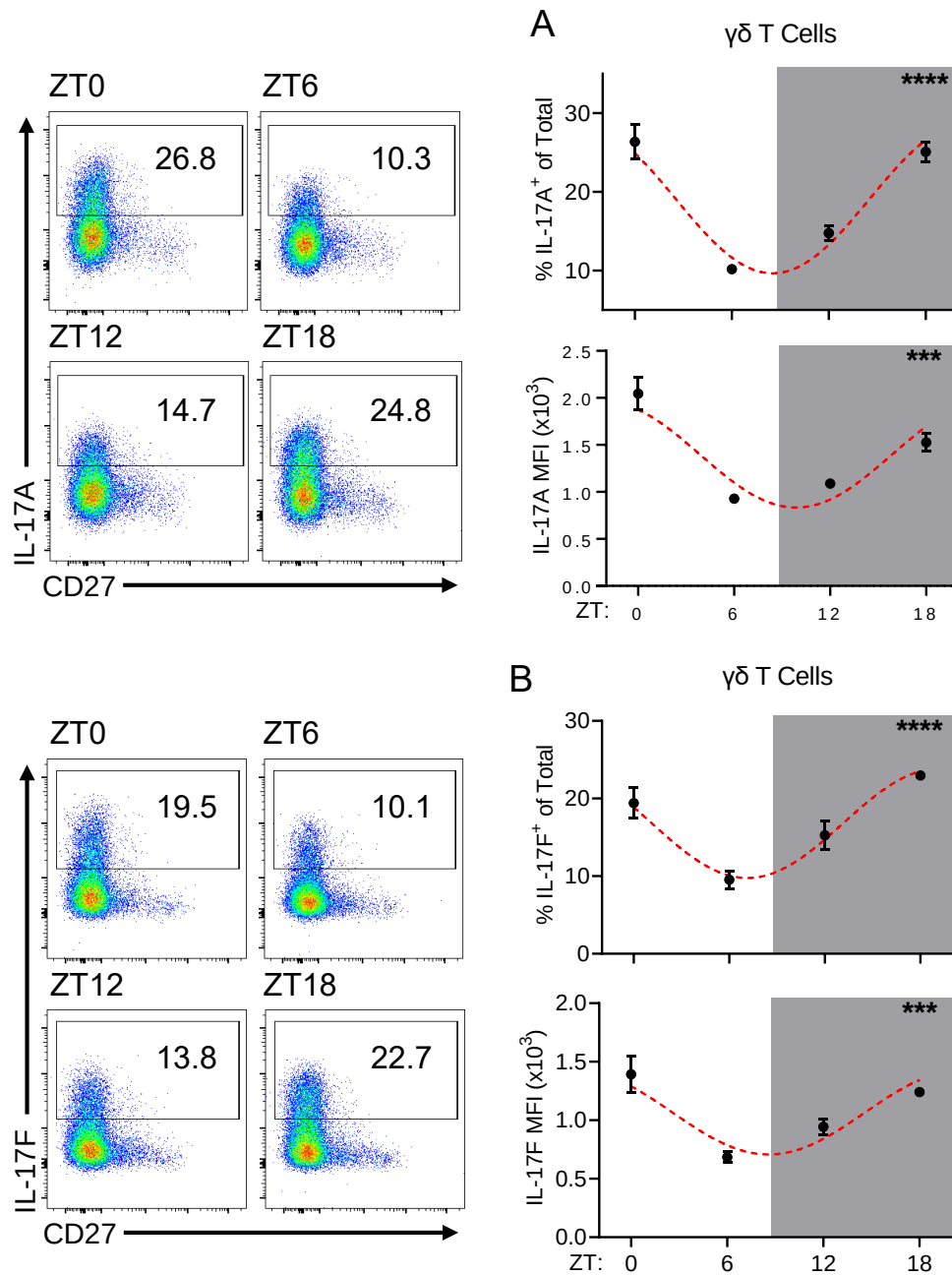


Figure 4.5: $\gamma\delta^{17}$ T cells display circadian production of IL-17A and IL-17F.

(A) Circadian expression of IL-17A, from expanded and serum shocked $\gamma\delta^{17}$ T cells after 3-hour stimulation with IL-1 β and IL-23, with representative flow plots (right). **(B)** Circadian expression of IL-17A, IL-17F, and ROR γ t (MFI), from expanded and serum shocked $\gamma\delta^{17}$ T cells after 3-hour stimulation with IL-1 β and IL-23, with representative flow plots (right). White and grey panels represent light and dark periods respectively. Data are representative of two independent experiments. **(A, B)** Data shown as mean $\pm$ SD, $n = 4$ technical replicates. Significance was calculated using cosinor analysis, with cosine fitted curves. *** $p < 0.001$, **** $p < 0.0001$.

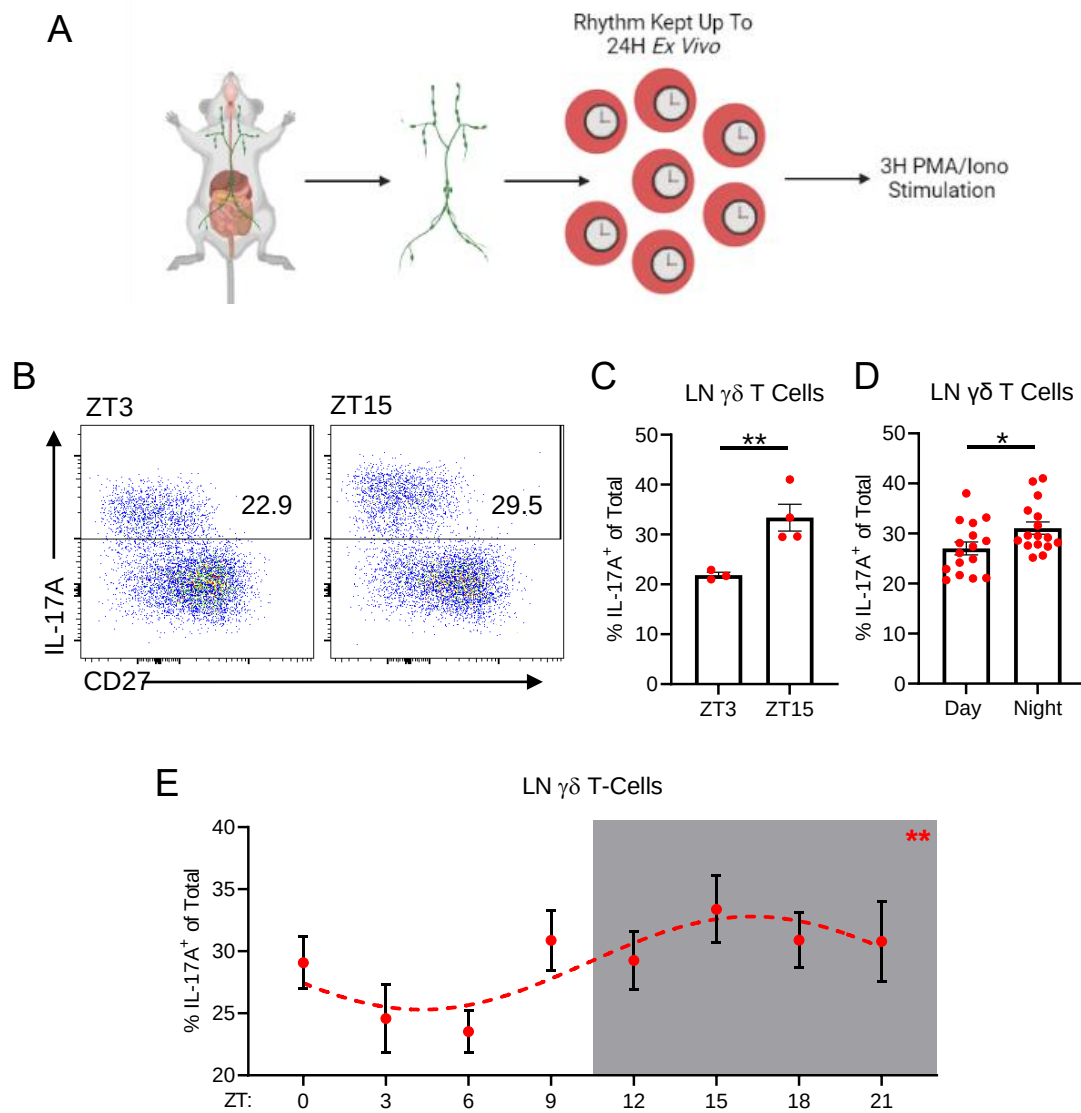


Figure 4.6: $\gamma\delta^{17}$ T cells display circadian production of IL-17A *ex vivo*.

(A) Diagrammatic representation of experimental setup. (B) Representative flow plots of IL-17A expression at nadir (ZT3), and peak (ZT15). (C) IL-17A expression by LN $\gamma\delta$ T cells stimulated *ex vivo* for 3-hours with PMA/Ionomycin, (D) displaying diurnal rhythm. (E) Circadian fluctuation of IL-17A production by LN $\gamma\delta$ T cells over 24-hours. White and grey panels represent light and dark periods respectively. Data are representative of two independent experiments. (C-E) Data shown as mean $\pm$ SEM, $n = 4$ mice per group. (C, D) Significance was calculated using two-tailed unpaired students t-test, (E) Cosinor analysis, with cosine fitted curves. * $p < 0.05$, ** $p < 0.01$.

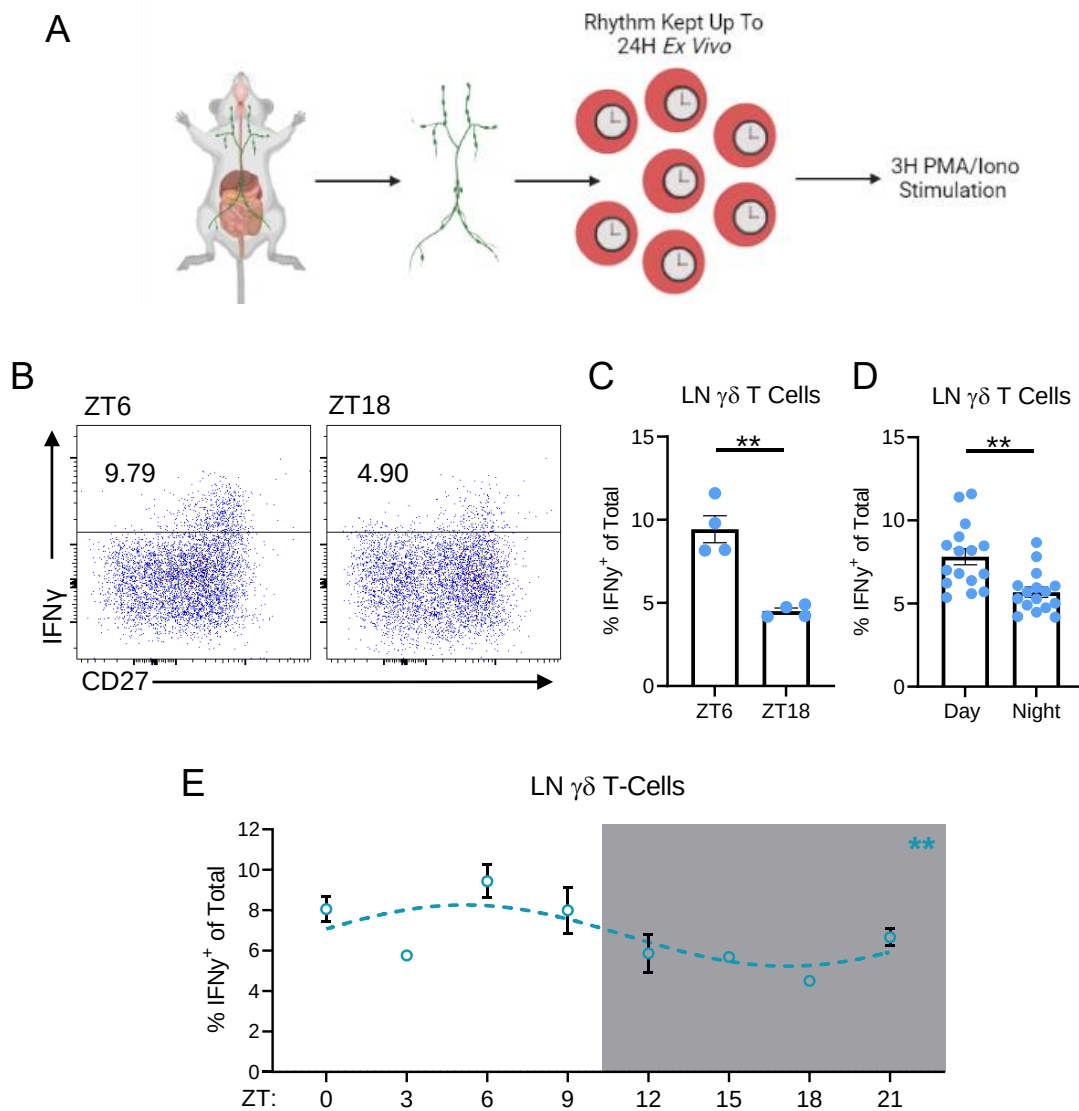


Figure 4.7: $\gamma\delta^{\text{IFN}\gamma}$ T cells display circadian production of IFN γ ex vivo.

(A) Diagrammatic representation of experimental setup. **(B)** Representative flow plots of IFN γ expression at nadir (ZT6), and peak (ZT18). **(C)** IFN γ expression by LN $\gamma\delta$ T cells stimulated ex vivo for 3-hours with PMA/Ionomycin, **(D)** displaying diurnal rhythm. **(E)** Circadian fluctuation of IFN γ production by LN $\gamma\delta$ T cells over 24-hours. Data are representative of two independent experiments. White and grey panels represent light and dark periods respectively. **(C-E)** Data shown as mean $\pm$ SEM, n = 4 mice per group. **(C, D)** Significance was calculated using two-tailed unpaired students t-test, **(E)** Cosinor analysis, with cosine fitted curves. ** p < 0.01.

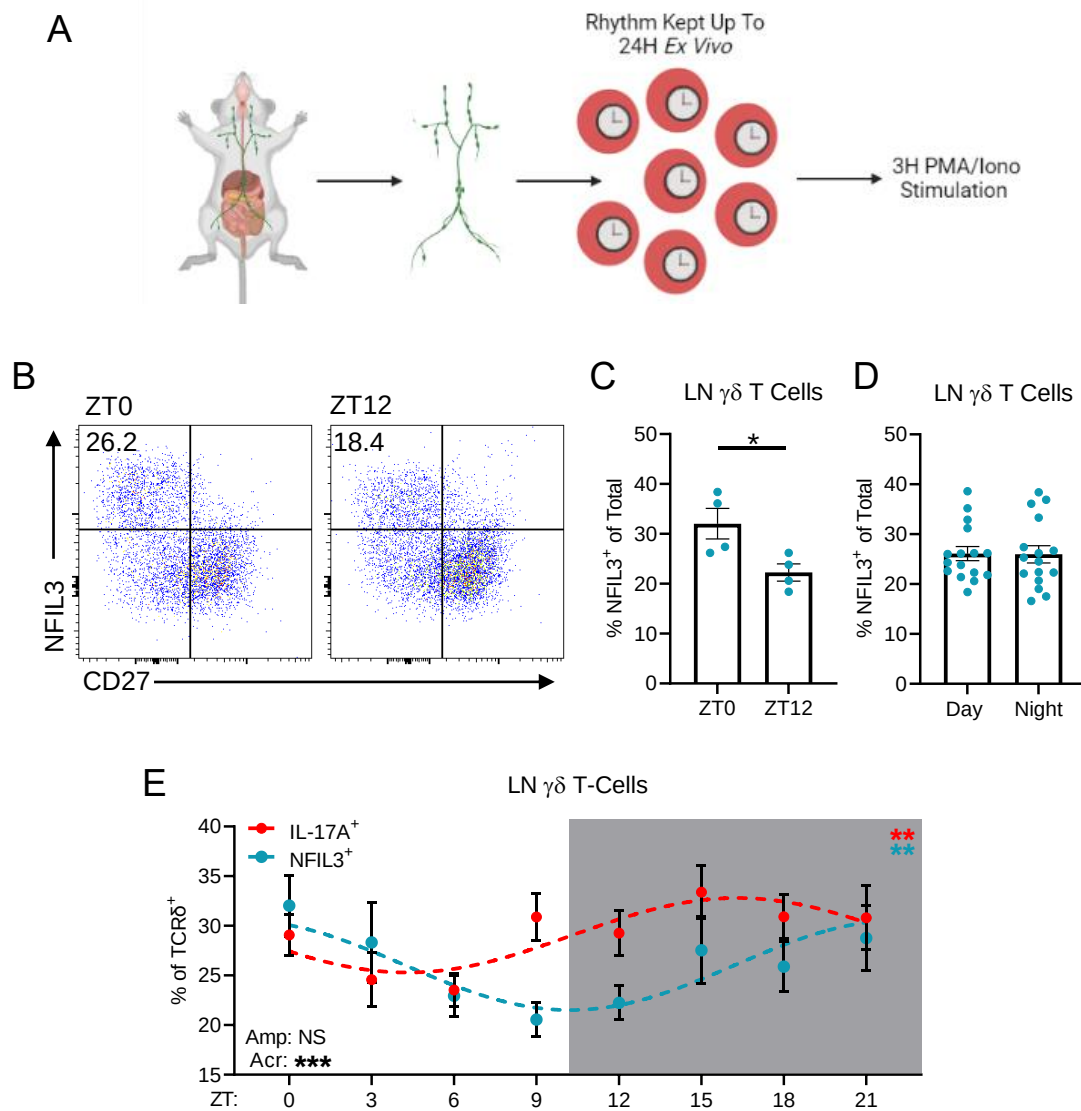


Figure 4.8: $\gamma\delta^{17}$ T cells display circadian expression of NFIL3 *ex vivo*.

(A) Diagrammatic representation of experimental setup. **(B)** Representative flow plots of NFIL3 expression at nadir (ZT12), and peak (ZT0). **(C)** NFIL3 expression by LN $\gamma\delta$ T cells stimulated *ex vivo* for 3-hours with PMA/Ionomycin, **(D)** with no diurnal rhythm. **(E)** Circadian fluctuation of NFIL3 expression (blue), overlaid with IL-17A production (red), by LN $\gamma\delta$ T cells over 24-hours. Data are representative of two independent experiments. White and grey panels represent light and dark periods respectively. **(C-E)** Data shown as mean $\pm$ SEM, $n = 4$ mice per group. **(C, D)** Significance was calculated using two-tailed unpaired students t-test, **(E)** Cosinor analysis, with cosine fitted curves. * $p < 0.05$, ** $p < 0.01$.

4.4 $\gamma\delta$ T cells display a circadian rhythm to cellular metabolism.

The metabolic pathways that immune cells use are critical for their function. We recently found that $\gamma\delta^{17}$ T cells and $\gamma\delta^{\text{IFN}\gamma}$ T cells use distinct metabolic programs. $\gamma\delta^{17}$ T cells have high lipid uptake, contain many lipid droplets, and have increased mitochondrial oxidative metabolism compared to $\gamma\delta^{\text{IFN}\gamma}$ T cells⁶⁸. We next investigated if $\gamma\delta^{17}$ T cells also had an intrinsic circadian rhythm to their metabolism. Using fluorescent palmitate BODIPY FL-C16 to measure lipid uptake, we confirmed that $\gamma\delta^{17}$ T cells (CD27⁻) were the primary $\gamma\delta$ subset taking up lipids, and that lipid uptake differed between night and day (Figure 4.9A). Indeed, lipid uptake had a peak at ZT21 and a nadir at ZT9, and overall higher uptake at night (Figure 4.9 B-D), and displayed a circadian rhythm which was similar to that of IL-17A production (Figure 4.9E; Figure 4.6E respectively).

We next measured if $\gamma\delta^{17}$ T cell cellular metabolism had a diurnal rhythm. $\gamma\delta^{17}$ T cells isolated from spleen and lymph nodes were synchronised by serum shock before performing Seahorse metabolic flux analysis. We found that $\gamma\delta^{17}$ T cells had similar levels of glycolysis, but higher levels of oxidative phosphorylation at ZT5, correlating with midday, compared to ZT17, correlating with midnight (Figure 4.10A-F). Confocal imaging of $\gamma\delta^{17}$ T cells at ZT5 and ZT17 found that there were diurnal changes to $\gamma\delta^{17}$ mitochondrial morphology. We found that mitochondria had a more fused structure at ZT5 compared to ZT17. At ZT5 there were fewer individual mitochondria per cell, but the mean area, form factor (a higher form factor indicates more elongated mitochondria), and branch length were higher, indicating a more fused phenotype (Figure 4.11A-F; Figure 4.12A-F). This phenotype has been linked with more active or efficient cellular metabolism^{139,204} and matches with the increased mitochondrial metabolism we measured at this time point (Figure 4.10A-F).

To investigate if there were circadian changes to cellular metabolism *in vivo*, lymph nodes were harvested from wild-type mice over 24-hours to analyse mitochondrial metabolism. There was a robust circadian rhythm to mitochondrial mass and mitochondrial membrane potential of lymph node $\gamma\delta^{17}$ T cells, with higher mitochondrial mass and potential during the day (Figure 4.13A, B). In summary, like IL-17 cytokine production, the intrinsic metabolism of $\gamma\delta^{17}$ T cells follows a circadian rhythm.

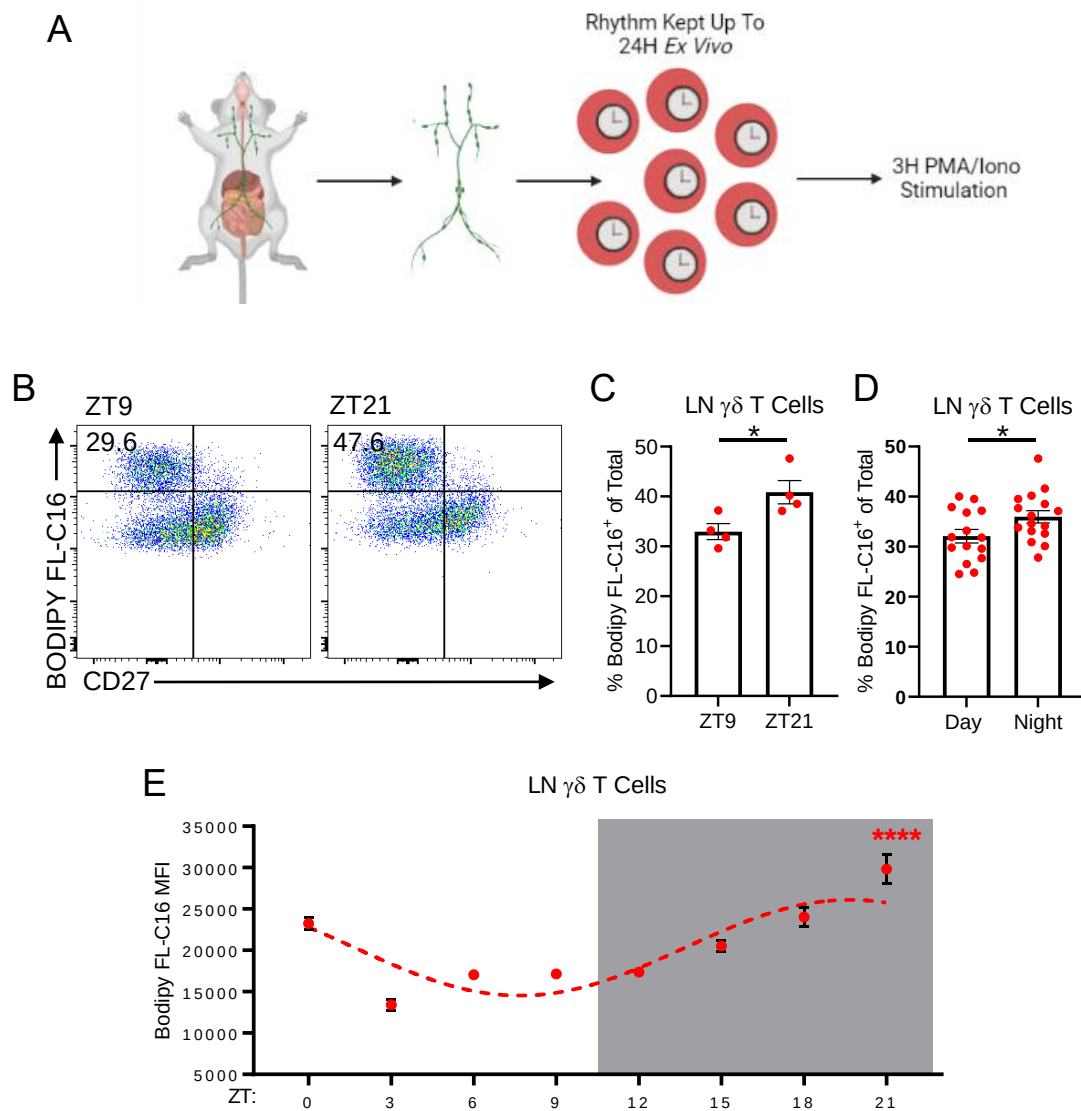


Figure 4.9: $\gamma\delta^{17}$ T cells display a circadian rhythm to lipid uptake.

(A) Diagrammatic representation of experimental setup. **(B)** Representative flow plots of BODIPY FL-C16 uptake at nadir (ZT9), and peak (ZT21). **(C)** BODIPY FL-C16 uptake by LN $\gamma\delta$ T cells stimulated *ex vivo* for 3-hours with PMA/Ionomycin, **(D)** displaying diurnal rhythm. **(E)** Circadian fluctuation of BODIPY FL-C16 uptake by LN $\gamma\delta$ T cells over 24-hours. Data are representative of two independent experiments. White and grey panels represent light and dark periods respectively. **(C-E)** Data shown as mean $\pm$ SEM, $n = 4$ mice per group. **(C, D)** Significance was calculated using two-tailed unpaired students t-test, **(E)** Cosinor analysis, with cosine fitted curves. * $p < 0.05$, **** $p < 0.0001$.

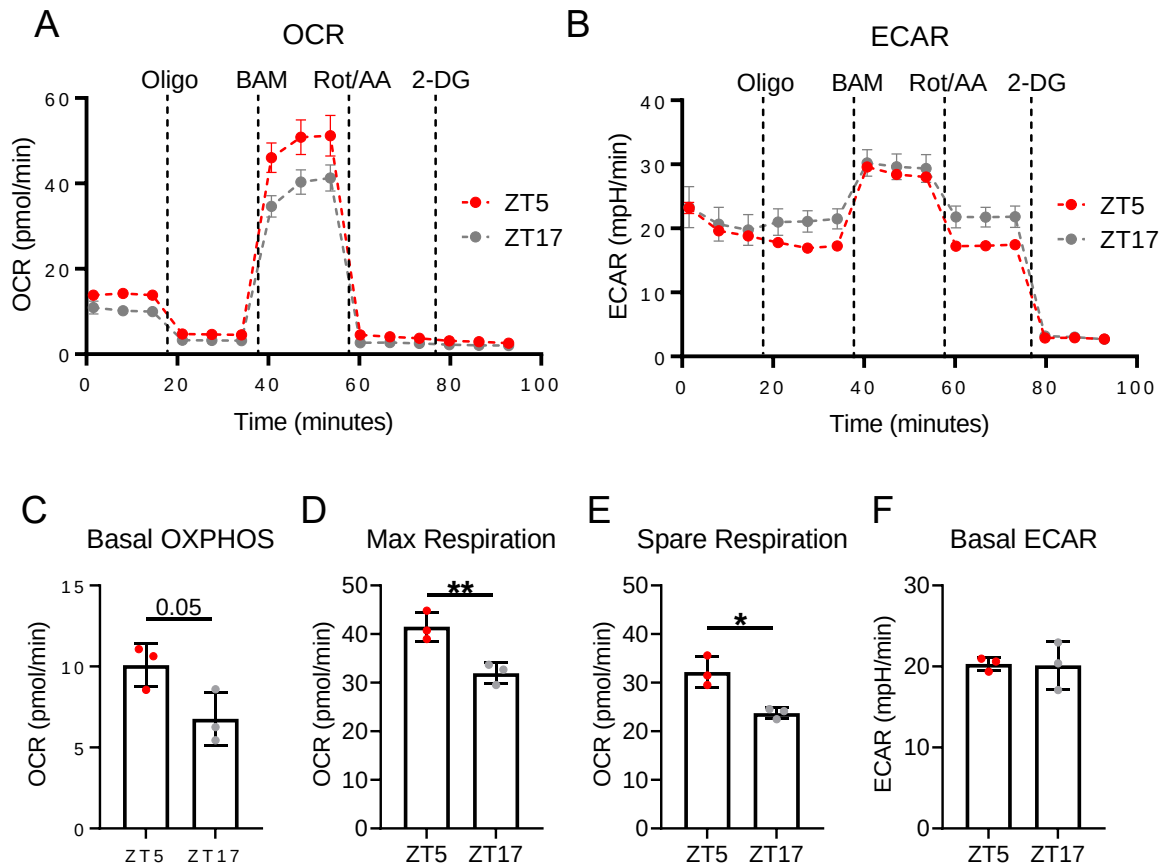


Figure 4.10: $\gamma\delta^{17}$ T cells display a diurnal rhythm to their cellular metabolism.

(A) Representative OCR trace from expanded and serum shocked $\gamma\delta^{17}$ T cells measured in real time and in response to indicated inhibitors. **(B)** Representative ECAR trace from expanded and serum shocked $\gamma\delta^{17}$ T cells measured in real-time and in response to indicated inhibitors. **(C-E)** Representative bar charts for basal, maximum, and spare respiratory capability. **(F)** Representative bar chart for basal ECAR. Data are representative of three independent experiments. **(C-E)** Data shown as mean $\pm$ SD, $n = 3$ technical replicates. **(C, D)** Significance was calculated using two-tailed paired students t-test. * $p < 0.05$, ** $p < 0.01$. *Experiment performed in collaboration with Katie O'Brien.*

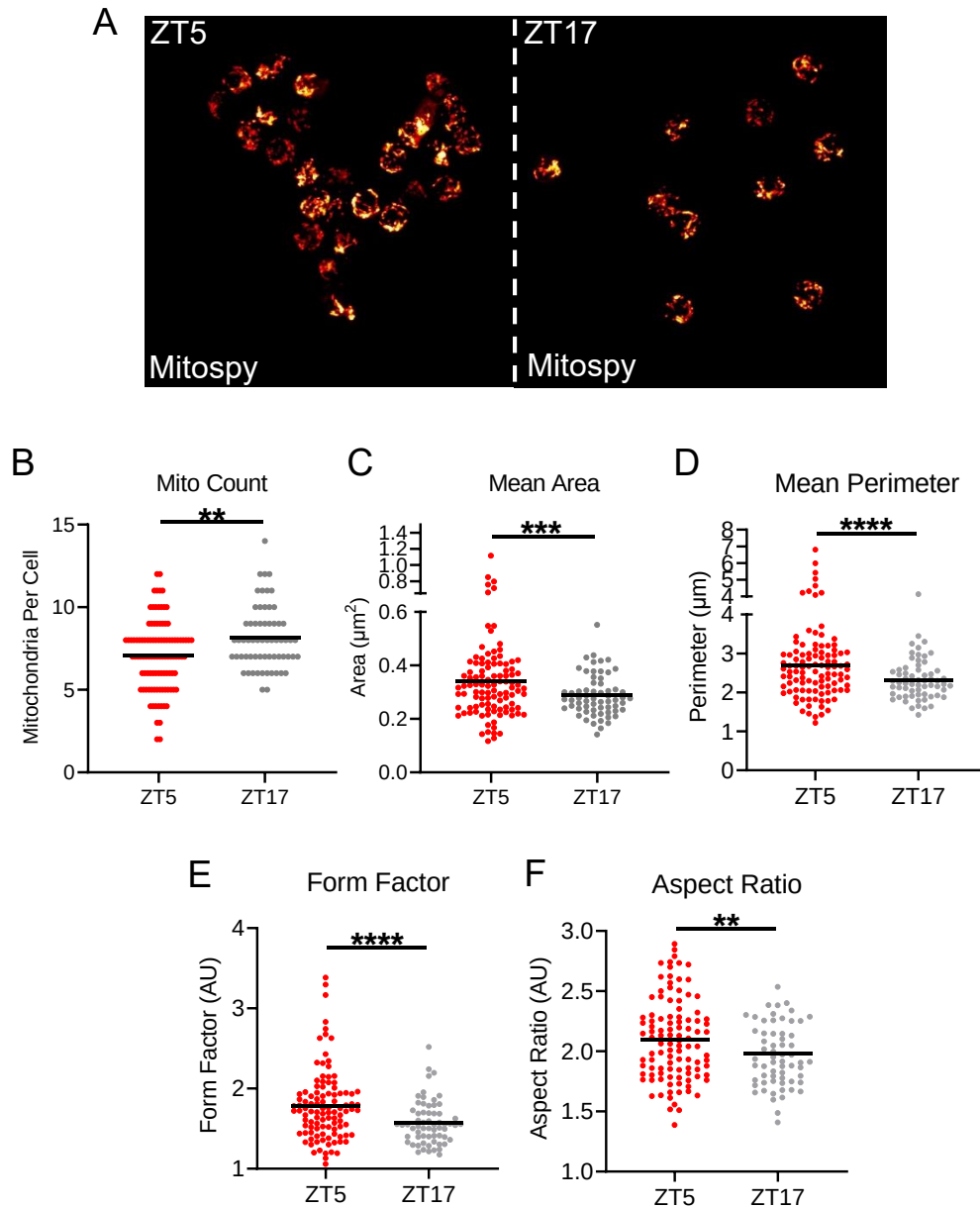


Figure 4.11: $\gamma\delta^{17}$ T cells display a diurnal rhythm to their mitochondria morphology.

(A) Representative confocal images of mitochondria from expanded and serum shocked $\gamma\delta^{17}$ T cells. **(B-D)** Morphological parameters of each time point, such as mitochondrial number, area, and perimeter. **(E, F)** Morphological parameters of each time point indicating mitochondrial circularity, such as form factor and aspect ratio. Data are representative of two independent experiments. **(B-F)** Data shown as mean, $n = 70 - 100$ cells per condition. **(B-F)** Significance was calculated using two-tailed paired students t-test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

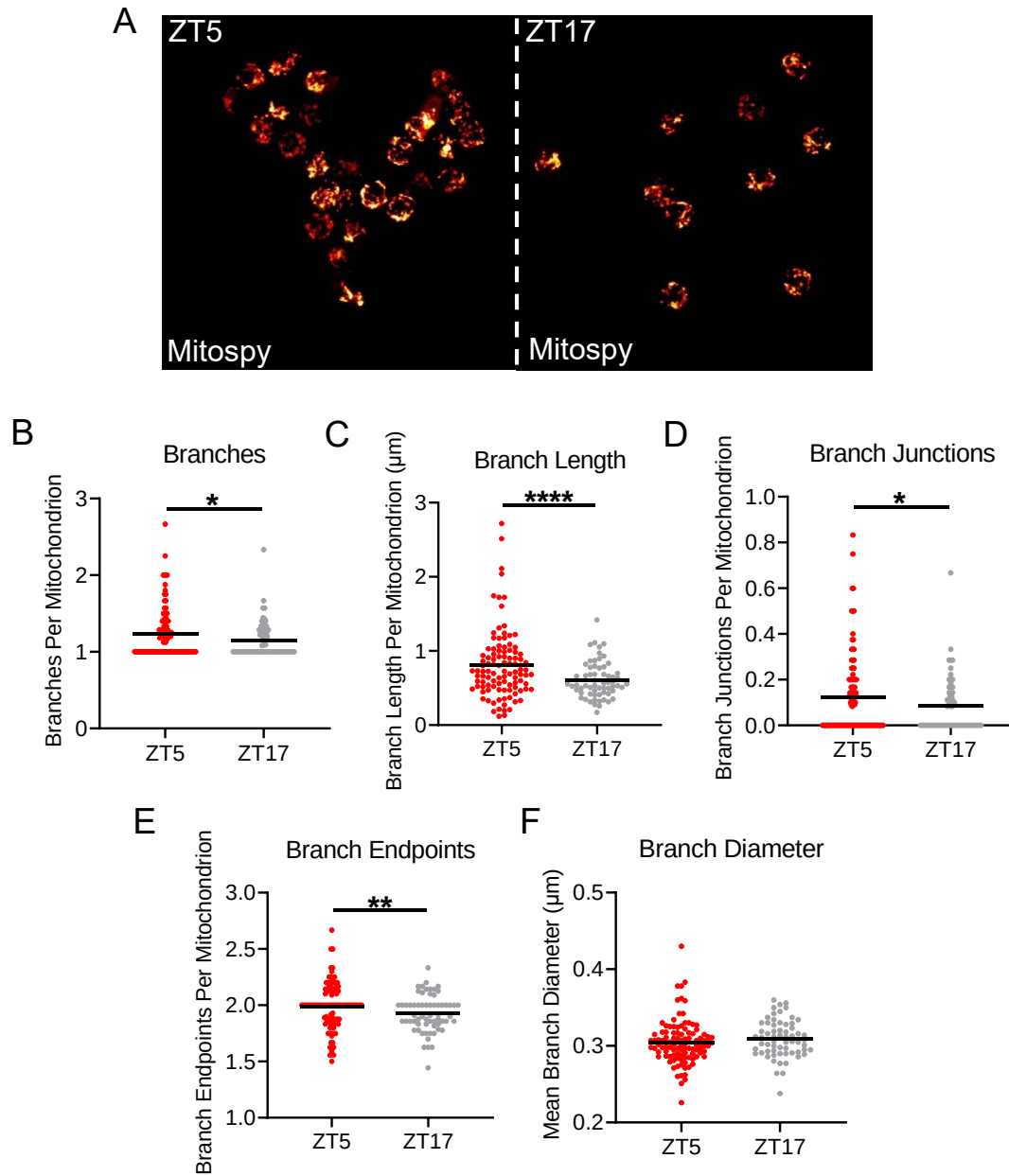


Figure 4.12: $\gamma\delta^{17}$ T cells display diurnal rhythm to their mitochondrial branch morphology.

(A) Representative confocal images of mitochondria from expanded and serum shocked $\gamma\delta^{17}$ T cells. **(B-F)** Morphological parameters of mitochondrial branches of each time point, such as branch number, length, and endpoints. Data are representative of two independent experiments. **(B-F)** Data shown as mean, $n = 70 - 100$ cells per condition. **(B-F)** Significance was calculated using two-tailed paired students t-test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

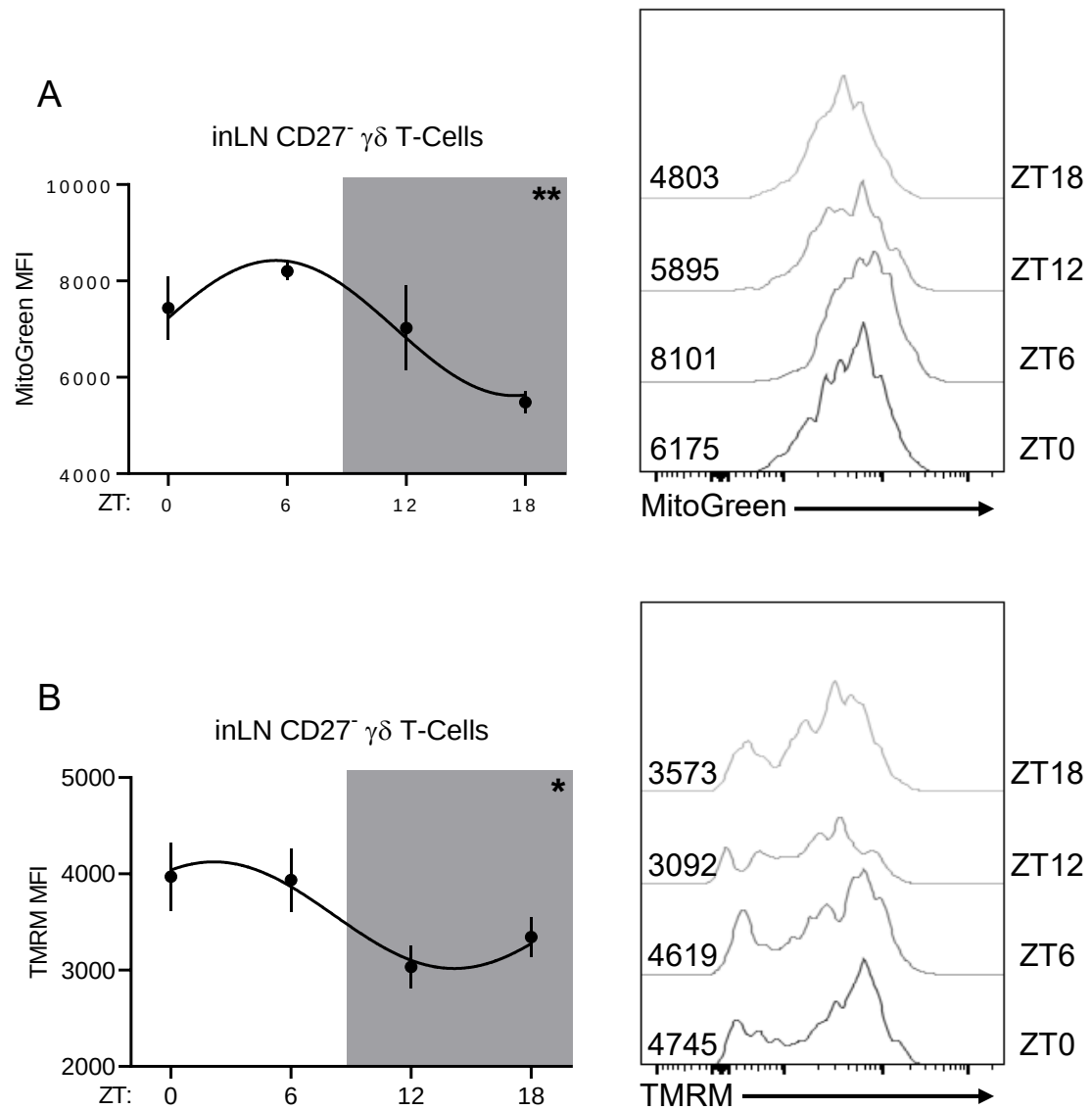


Figure 4.13: γδ¹⁷ T cells display a circadian rhythm to their cellular metabolism *in vivo*.

(A) Circadian time plots of mitochondrial mass by MFI (MTG; left), with representative histograms (right). **(B)** Circadian time plots of mitochondrial membrane potential by MFI (TMRM; left), with representative histograms (right). White and grey panels represent light and dark periods respectively. Data are representative of three independent experiments. **(A, B)** Data shown as mean ± SEM, n = 4 mice per group. **(A, B)** Significance was calculated using cosinor analysis, with cosine fitted curves. * p < 0.05, ** p < 0.01.

4.5 Light entrains whole body metabolism and IL-17A production by adipose $\gamma\delta$ T cells.

Adipose tissue is a dynamic organ with a unique immune composition that responds to many physiological signals including feeding/fasting and regulation of body temperature, both of which display strong circadian rhythms. While IL-17 production by lymph node $\gamma\delta$ T cells is regulated by the molecular clock, we found that adipose tissue resident $\gamma\delta$ T cells have the highest expression of molecular clock genes (Figure 4.14A). Firstly, we found that adipose $\gamma\delta^{17}$ T cells expressed a robust circadian rhythm for IL-17A production, in addition to their ROR γ t expression (Figure 4.14B, C). Having found that epiWAT $\gamma\delta^{17}$ T cells have a circadian rhythm to their IL-17A expression, we next investigated if other adipose depots also had diurnal expression of IL-17A by $\gamma\delta^{17}$ T cells. Using IL-17A GFP mice, we found diurnal expression of IL-17A across BAT, epiWAT and subcutaneous (scWAT) adipose tissue depots peaking at night during the active phase (Figure 4.15A). We also examined if adipose $\gamma\delta^{17}$ T cells had a circadian rhythm to their mitochondrial metabolism. Adipose $\gamma\delta^{17}$ T cells had a more striking and significant circadian rhythm to both their mitochondrial mass and membrane potential compared to lymph node $\gamma\delta^{17}$ T cells (Figure 4.16A, B).

As light is a major entrainment signal for circadian regulation of whole-body metabolic rhythm, we next asked if light is an entrainment signal for circadian IL-17 expression by adipose $\gamma\delta$ T cells. Using metabolic cages to examine the effect of reversed light-dark cycles on whole body metabolism, mice were recorded under normal light conditions, lights on 7am-7pm (control), and also after 3 weeks of reversed light-dark cycle, lights on 7pm-7am (Figure 4.17A). After 3 weeks of inverted light cycle, the circadian rhythm of the metabolic rate and energy expenditure parameters were completely reversed (Figure 4.17B-D). There was no difference in the total day average between the control and light inverted cycle, indicating complete reversal (Figure 4.17C, E). Adipose tissue was then harvested at 7am from both groups, corresponding to 7am (ZT0) in the control group, and 7pm (ZT12) in the light inverted group. IL-17A production by adipose $\gamma\delta^{17}$ T cells had diurnal expression, which was now reversed in the light inverted group, again peaking at night during the active phase (Figure 4.18A). Similarly, TNF expression by adipose $\gamma\delta^{17}$ T cells was higher in the light inverted group, peaking at night (Figure 4.18C). Together these results suggest that the circadian production of IL-17 by adipose $\gamma\delta^{17}$ T cells is entrained by light.

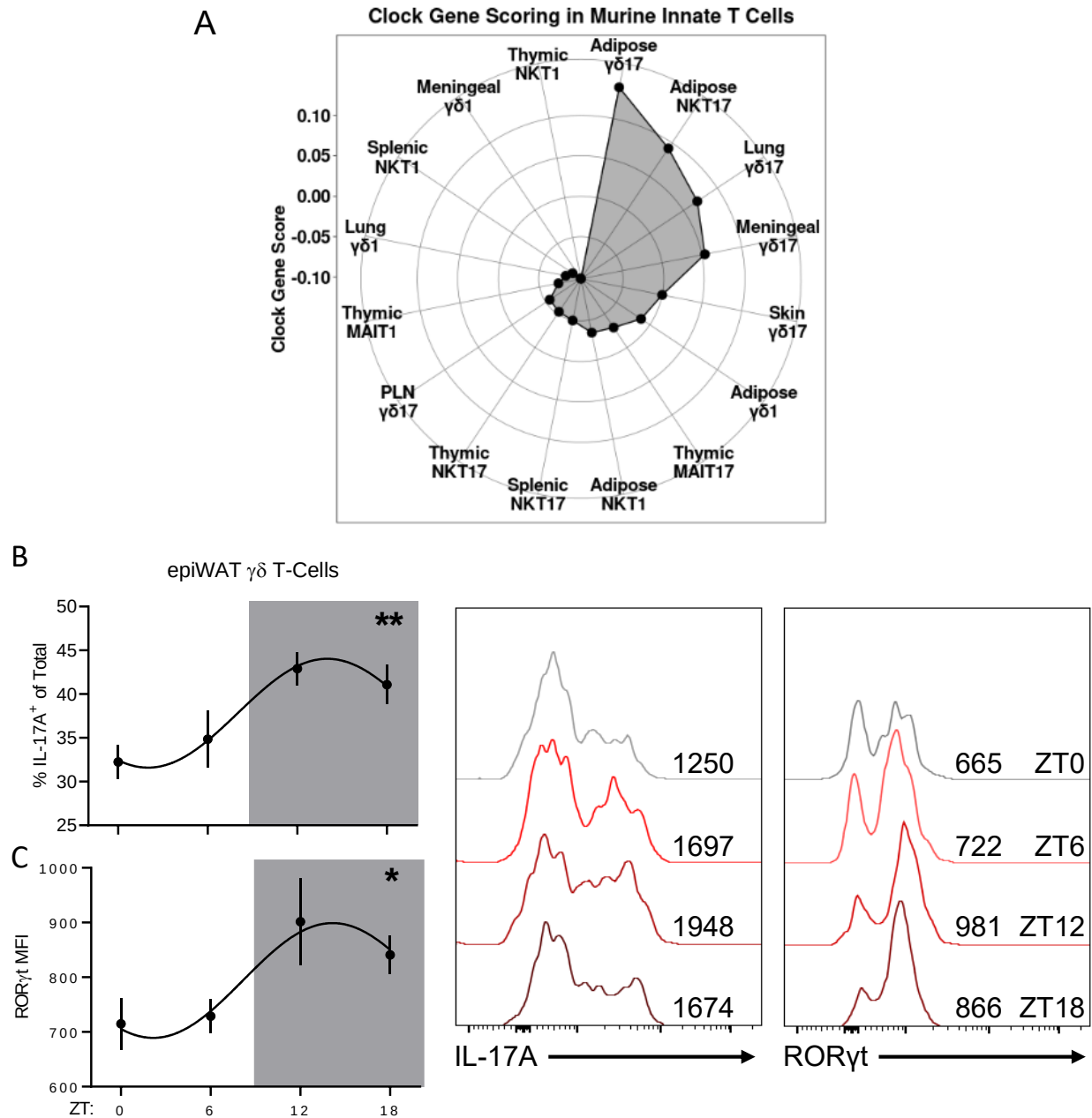


Figure 4.14: epiWAT $\gamma\delta^{17}$ T cells display a circadian rhythm to IL-17A production and ROR γ t expression.

(A) Spider plot showing adipose $\gamma\delta^{17}$ T cells have the highest cumulative expression of molecular clock genes from figure 1A **(B)** Circadian time plot of IL-17A expression by adipose $\gamma\delta$ T cells (left), with representative histograms (right). **(C)** Circadian time plot of ROR γ t expression by adipose $\gamma\delta$ T cells (left), with representative histograms (right). White and grey panels represent light and dark periods respectively. Data are representative of three independent experiments. **(B, C)** Data shown as mean $\pm$ SEM, $n = 3-4$ mice per group. **(B, C)** Significance was calculated using cosinor analysis, with cosine fitted curves. * $p < 0.05$, ** $p < 0.01$.

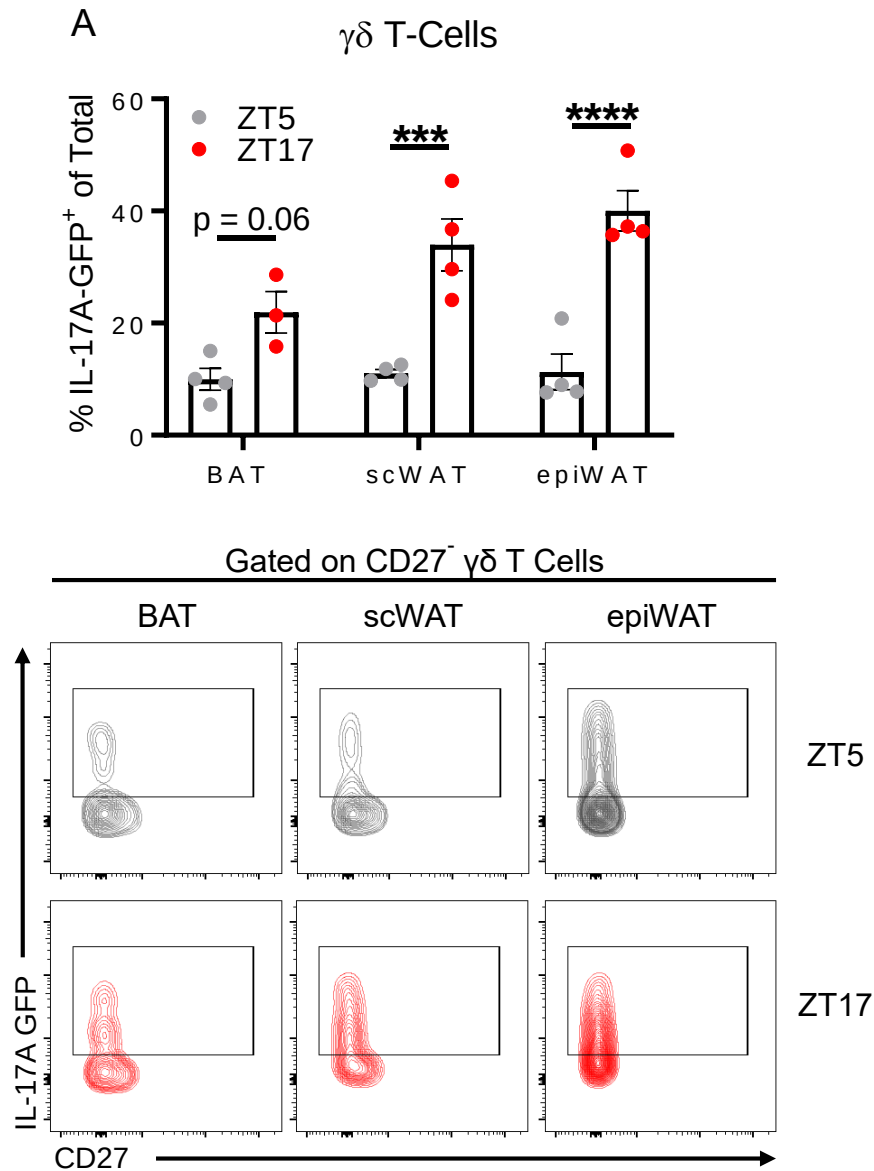


Figure 4.15: Adipose $\gamma\delta^{17}$ T cells display a diurnal rhythm to IL-17A-GFP expression.

(A) Diurnal expression of IL-17A-GFP by $\gamma\delta^{17}$ T cells across adipose tissue depots BAT, scWAT and epiWAT (above), with representative flow plots (bottom). Data are representative of two independent experiments. **(A)** Data shown as mean $\pm$ SEM, n = 3-4 mice per group. **(A)** Significance was calculated using two-tailed unpaired students t-test. *** p < 0.001, **** p < 0.0001.

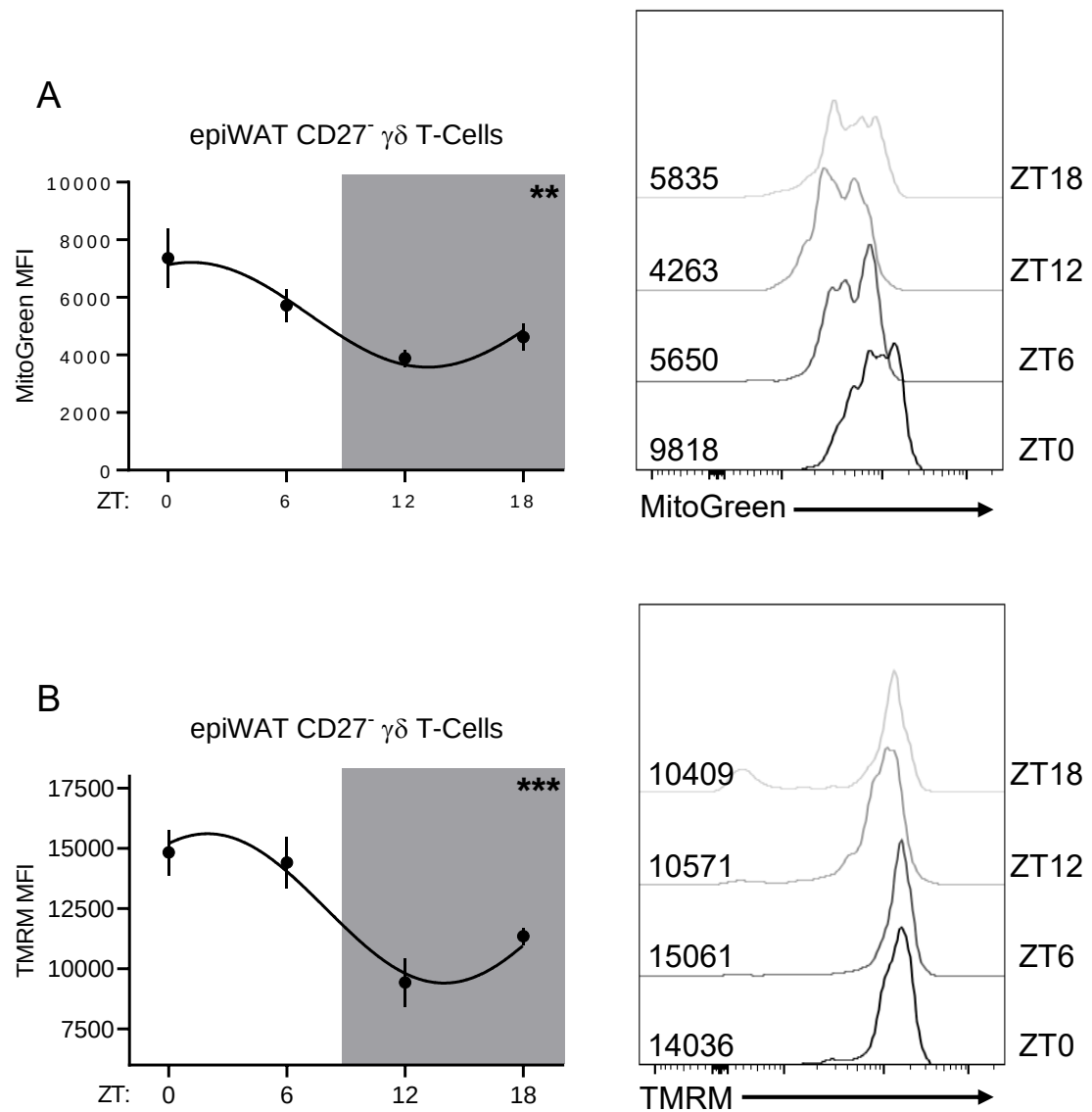


Figure 4.16: epiWAT $\gamma\delta^{17}$ T cells display a circadian rhythm to their cellular metabolism *in vivo*.

(A) Circadian time plots of mitochondrial mass by MFI (MTG; left), with representative histograms (right). **(B)** Circadian time plots of mitochondrial membrane potential by MFI (TMRM; left), with representative histograms (right). White and grey panels represent light and dark periods respectively. Data are representative of three independent experiments. **(A, B)** Data shown as mean $\pm$ SEM, $n = 4$ mice per group. **(A, B)** Significance was calculated using cosinor analysis, with cosine fitted curves. ** $p < 0.01$, *** $p < 0.001$.

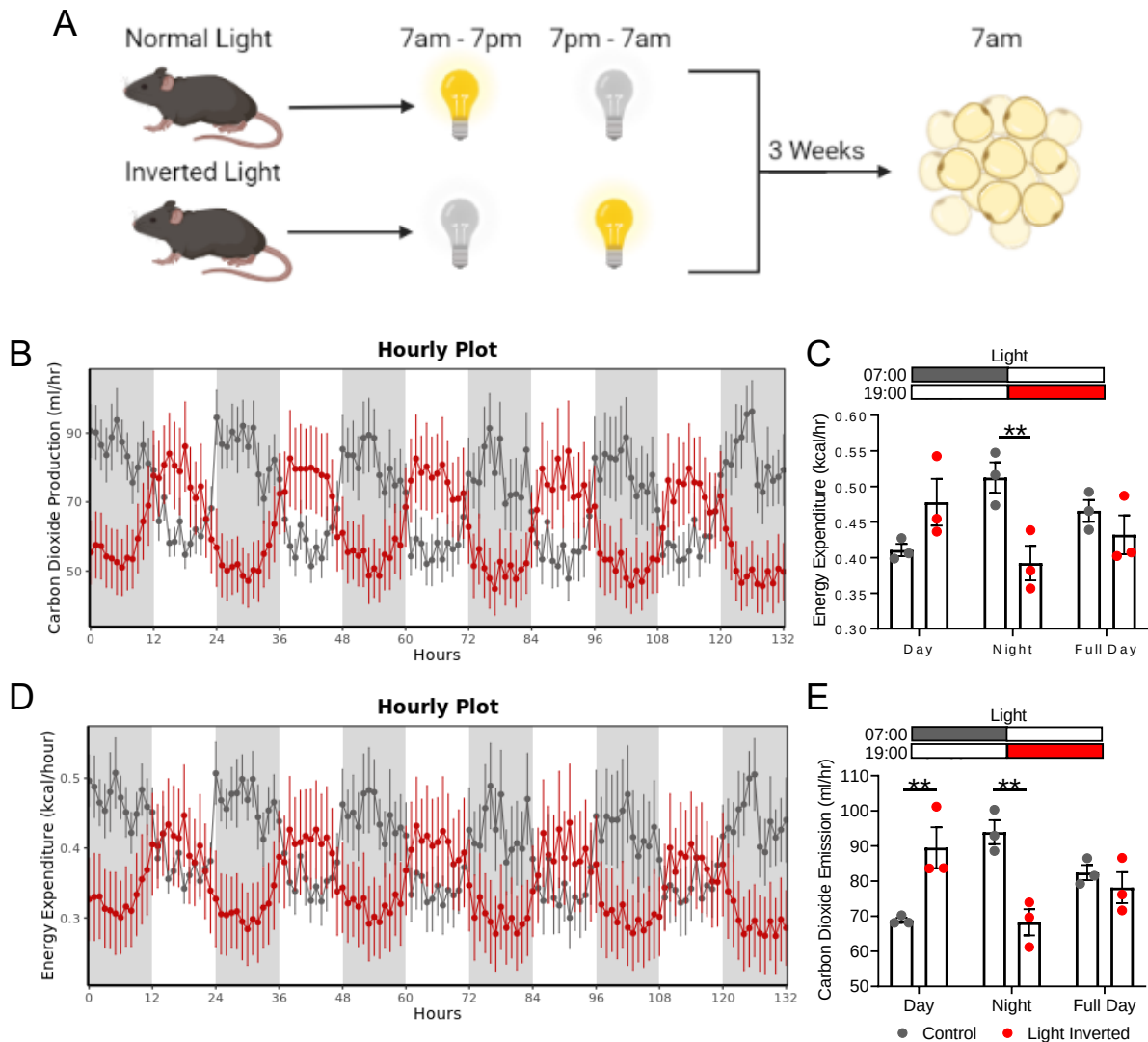


Figure 4.17: Whole body circadian metabolic rhythm entrains to light cycle.

(A) Diagrammatic representation of experimental setup. **(B)** Time plot of metabolic rate parameter CO₂ production, and **(D)** energy expenditure of mice on a regular light cycle (grey; lights on 7am - 7pm), versus mice on an inverted light cycle (red; lights on 7pm – 7am) after 3 weeks on these light cycles. **(C, E)** Bar plots of CO₂ emission and energy expenditure averaged by day, night, and whole day of regular, and inverted, light cycle mice. White and grey panels represent light and dark periods respectively. Data are representative of three independent experiments. **(B-E)** Data shown as mean $\pm$ SEM, $n = 4$ mice per group. **(C, E)** Significance was calculated using two group analysis with ANCOVA. ** $p < 0.01$.

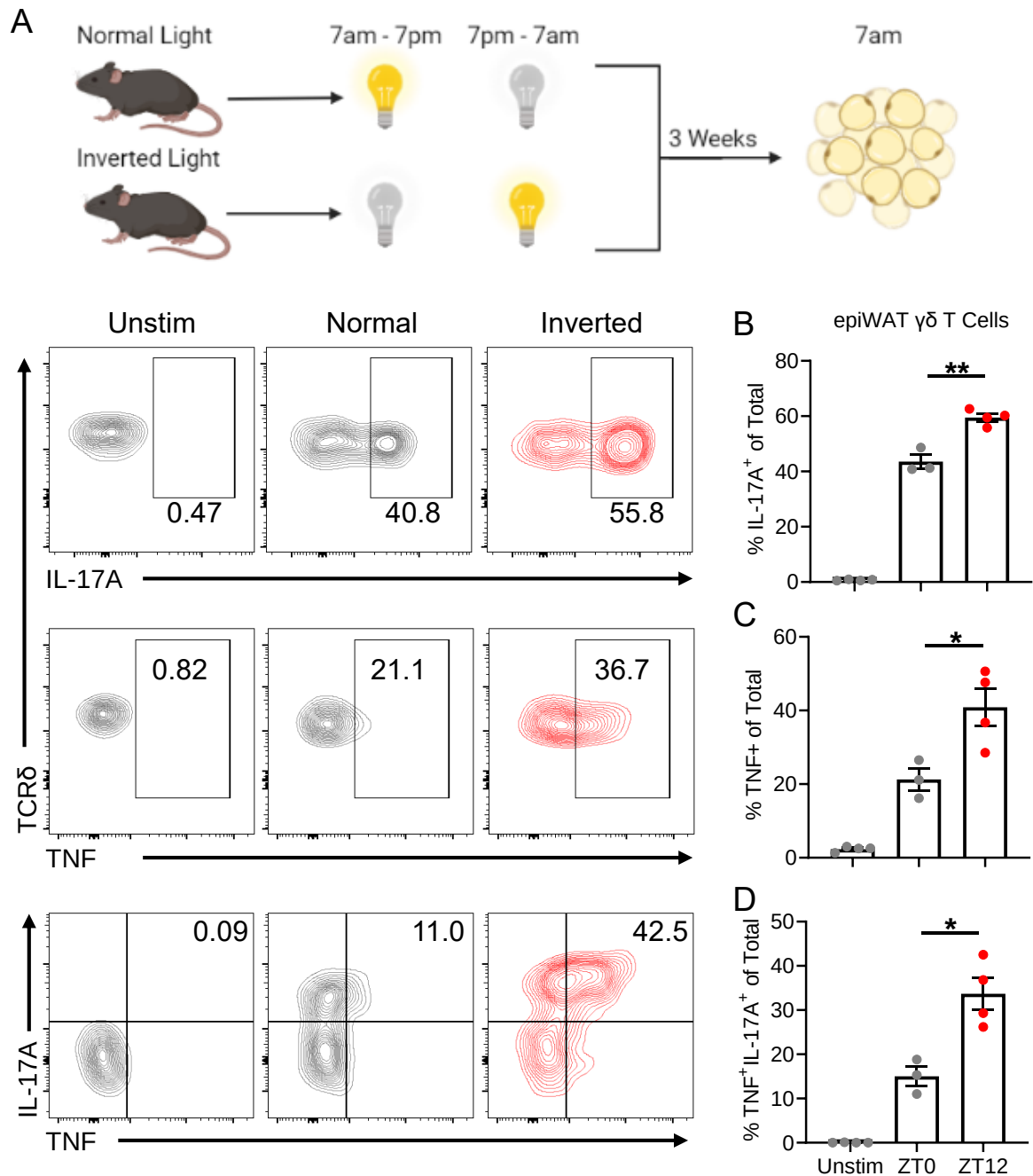


Figure 4.18: IL-17A, and TNF, circadian rhythm entrains to light cycle.

(A) Diagrammatic representation of experimental setup. **(B)** Representative flow plots of IL-17A production by adipose $\gamma\delta^{17}$ T cells (left), from regular (grey) and light inverted (red) mice. **(C)** Representative flow plots of TNF production by adipose $\gamma\delta^{17}$ T cells (left), from regular (grey) and light inverted (red) mice. **(D)** Representative flow plots of IL-17A⁺TNF⁺ double production by adipose $\gamma\delta^{17}$ T cells (left), from regular (grey) and light inverted (red) mice. Data are representative of three independent experiments. **(B-D)** Data shown as mean $\pm$ SEM, $n = 3-4$ mice per group. **(B-D)** Significance was calculated using one-way ANOVA. * $p < 0.05$, ** $p < 0.01$.

4.6 IL-17A signalling is necessary for adipocyte *de novo* lipogenesis.

Recent studies from our lab and others have shown that IL-17 production by adipose $\gamma\delta^{17}$ T cells plays a role in maintaining body temperature, as IL-17 is required for sufficient thermogenesis by brown or beige adipose tissue (BAT)^{29,62}. We next asked why there is a peak in IL-17 levels by adipose $\gamma\delta^{17}$ T cells during the active, dark phase in all adipose depots. Adipose lipolysis and lipogenesis is under strict circadian control to achieve the energy needs of the body, with lipolysis occurring during the day when the mice are inactive, and lipogenesis occurring at night when the mice are active and feeding (Figure 4.19A)¹⁹⁹. As IL-17 expression peaks when lipogenesis peaks (Figure 4.19A), we hypothesised that adipose IL-17A might play a role in *de novo* lipogenesis (Figure 4.19B). We cultured primary brown adipocytes in the presence of IL-17A or vehicle, and found that the expression of a transcriptional regulator of *de novo* lipogenesis genes, *Mlxipl* (Chrebp), and the fatty acid synthesis gene *Fasn*, were increased in IL-17A treated adipocytes (Figure 4.19C, D). Next, we administered IL-17A *in vivo* over 3 days. This resulted in a robust increase in expression of all genes involved in the *de novo* lipogenesis pathway in BAT *in vivo* (*Mlxipl*, *Acaca*, *Fasn*, *Scd1*, *Scd2* and *Elovl6*) with IL-17A treatment (Figure 4.19E). We next took advantage of publicly available transcriptomic data of brown adipocyte specific deletion of the IL-17 receptor, IL-17RC, and we found that DNL genes *Mlxipl*, *Acaca*, *Fasn*, *Scd1*, *Scd2* and *Srebf1* were significantly downregulated in BAT lacking IL-17RC compared to wild-type BAT (Figure 4.20A, B). Combined, this data shows that IL-17 signalling plays a major role in the regulation of *de novo* lipogenesis in BAT

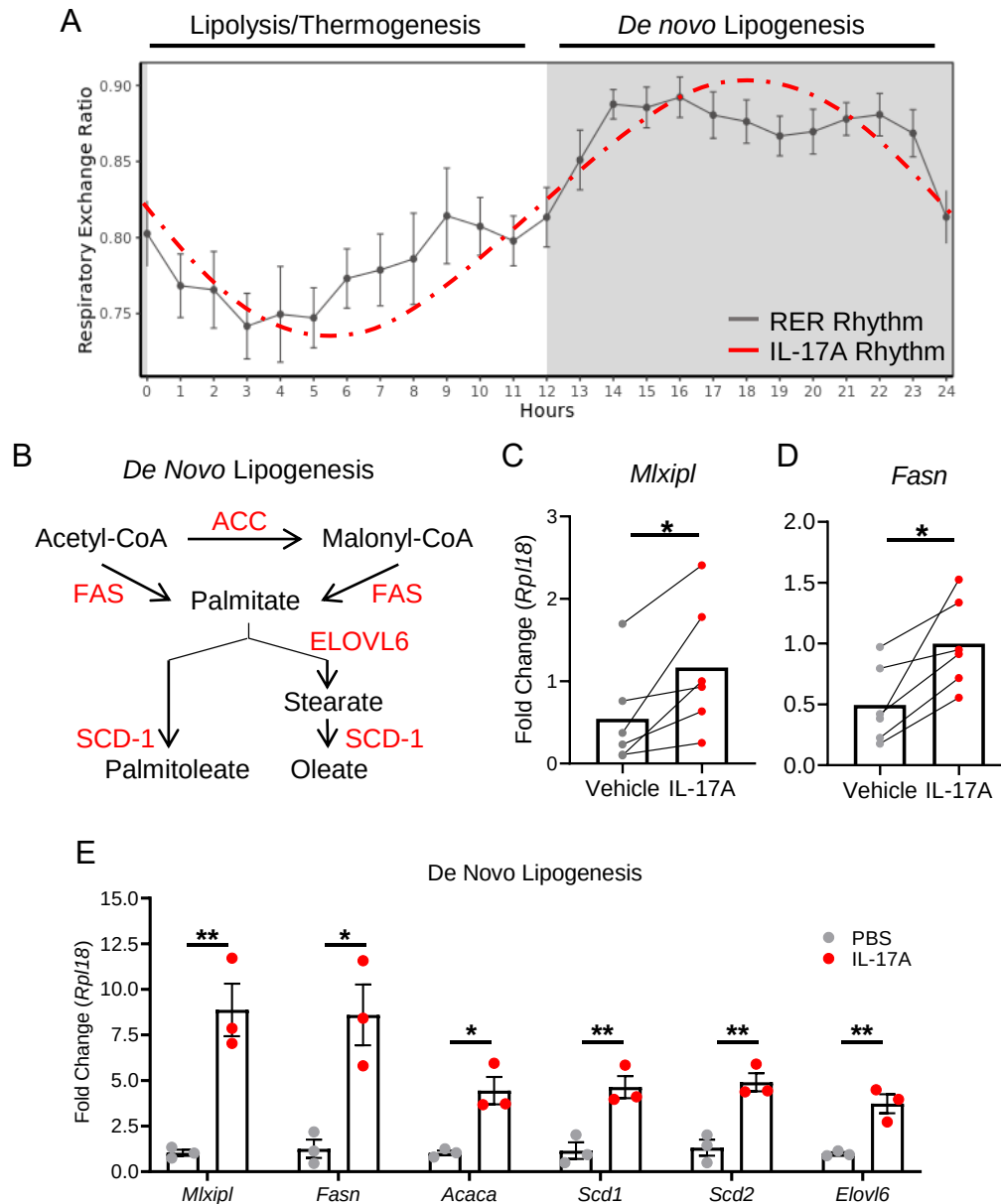


Figure 4.19: IL-17A upregulates *de novo* lipogenesis genes in adipocytes.

(A) Diagrammatic representation of circadian whole body metabolism by RER showing most active metabolic processes during the day and night, with overlay of IL-17A circadian rhythm. (B) Representation of *de novo* lipogenesis pathway. (C, D) Relative expression of *de novo* lipogenesis associated genes *Mlxipl* and *Fasn* in brown adipocytes treated ex vivo with IL-17A (2 μ g/ml) compared to vehicle. (E) Relative expression of *de novo* lipogenesis genes in BAT of mice administered IL-17A, compared to animals administered PBS. Data are representative of two independent experiments. (C, D) Data shown as mean $\pm$ SD, n = 6 mice per group, (E) Data shown as mean $\pm$ SEM, n = 3 mice per group. (C, D) Significance was calculated using Two-tailed paired student's t-test, (E) Two-tailed unpaired student's t-test. * p < 0.05, ** p < 0.01. In collaboration with Brenneeth Stevens.

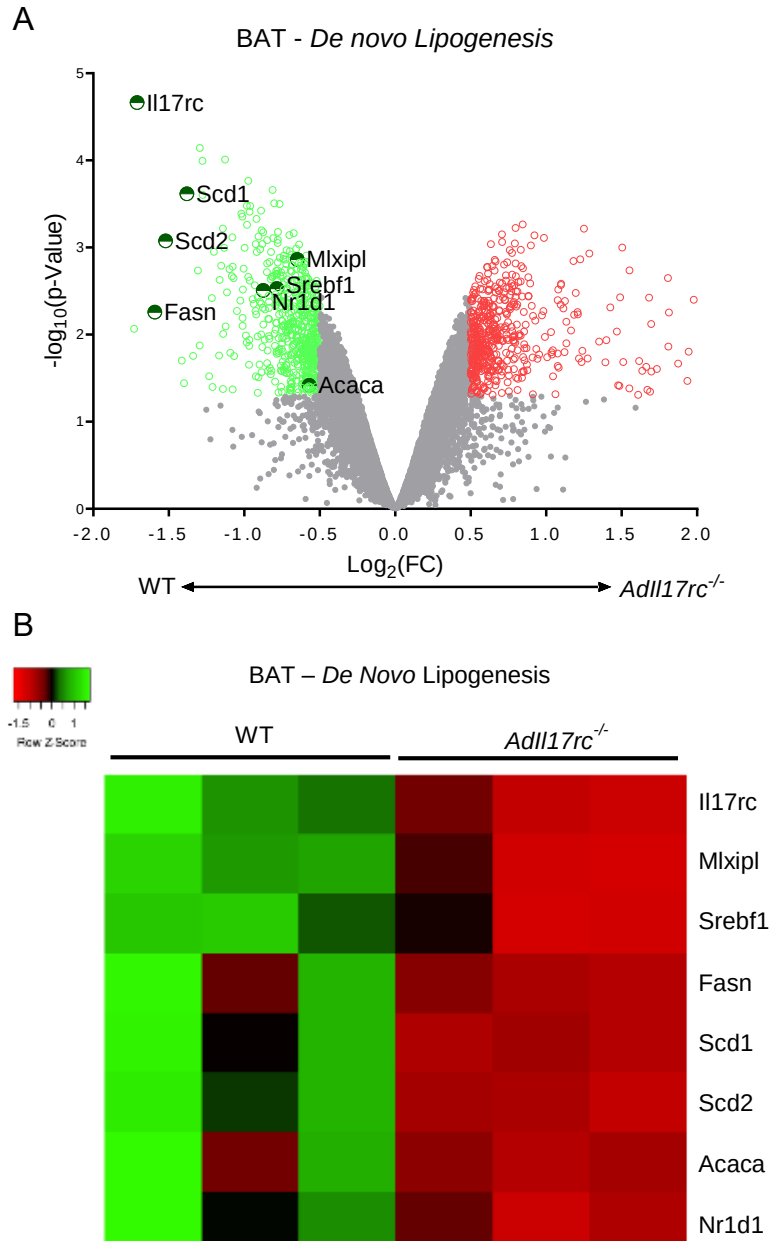


Figure 4.20: Adipocyte IL-17RC signalling is necessary for *de novo* lipogenesis.

(A) Volcano plot of reanalysed publicly available bulk RNA-Seq dataset of BAT from wild-type and *Adll17rc*^{-/-} mice from GSE144255. **(B)** Heatmap of differential expression of *de novo* lipogenesis associated genes between BAT from wild-type and *Adll17rc*^{-/-} mice. **(A, B)** Data are representative of one independent experiment.

4.7 Circadian *de novo* lipogenesis & whole-body metabolism is impaired in *Il17a/f^{-/-}* animals.

Given the diurnal rhythm to adipose IL-17A, and the role of IL-17 in *de novo* lipogenesis, we next investigated if absence of circadian IL-17 signalling impacted the circadian rhythm of *de novo* lipogenesis. We have shown that *Il17a^{-/-}* mice have impaired thermogenesis, and the Spiegelman lab has shown that *Il17f^{-/-}* mice have impaired thermogenesis^{29,62}. As adipose $\gamma\delta$ T cells produce both IL-17A and IL-17F, and both cytokines can compensate for each other, we used IL-17A and IL-17F double knock-out mice (*Il17a/f^{-/-}*). BAT of *Il17a/f^{-/-}* mice was harvested at ZT5 and ZT17, in the middle of the inactive and active phase respectively. Wild-type BAT showed higher expression of multiple *de novo* lipogenesis genes, including *Fasn*, *Acaca* and *Scd1*, at ZT17 during the active phase, compared to *Il17a/f^{-/-}* animals (Figure 4.21B). There were no differences *de novo* lipogenesis gene expression between wild-type and *Il17a/f^{-/-}* animals at ZT5 (Figure 4.21A). In addition, we found that *Il17a/f^{-/-}* animals had decreased expression of genes associated with thermogenesis at ZT5 and ZT17, compared to wild-type animals (Figure 4.22A, B). However, when wild-type, and particularly *Il17a/f^{-/-}* mice, were administered recombinant IL-17A and IL-17F, they robustly upregulated *de novo* lipogenesis genes (Figure 4.23 A-D). However, no differences were observed between wild-type and *Il17a/f^{-/-}* group that were administered PBS, as injections were performed during the day, when the mice are inactive and *de novo* lipogenesis is not occurring (Figure 4.23A-D).

We next performed whole body metabolic analysis on wild-type and *Il17a/f^{-/-}* mice at thermoneutrality (30°C). Thermoneutrality was chosen to remove any influence from adaptive thermogenesis, as mice housed at room temperature are cold^{205,206}. A recent study by Adlanmerini *et al* suggests that BAT *de novo* lipogenesis is crucial for the maintenance of murine body temperature²⁰⁷. Strikingly, the body temperature of *Il17a/f^{-/-}* mice was decreased at all times of day, but particularly during the active phase (Figure 4.24A, B). Circadian analysis found that *Il17a/f^{-/-}* mice had a greatly blunted circadian fluctuation of body temperature over the course of a day (Figure 4.24C). Additionally, we found that *Il17a/f^{-/-}* mice have altered respiratory exchange ratio (RER) especially at night (Figure 4.25A, B), and show a blunted circadian RER rhythm (Figure 4.25C). Thus, loss of IL-17A and IL-17F impairs circadian *de novo* lipogenesis, whole-body metabolism, and maintenance of body temperature. Combined our results show that circadian IL-17A from adipose tissue resident $\gamma\delta$ T cells, is essential for physiological whole-body metabolism and

body temperature, through the maintenance of adipose tissue *de novo* lipogenesis (Figure 4.26A).

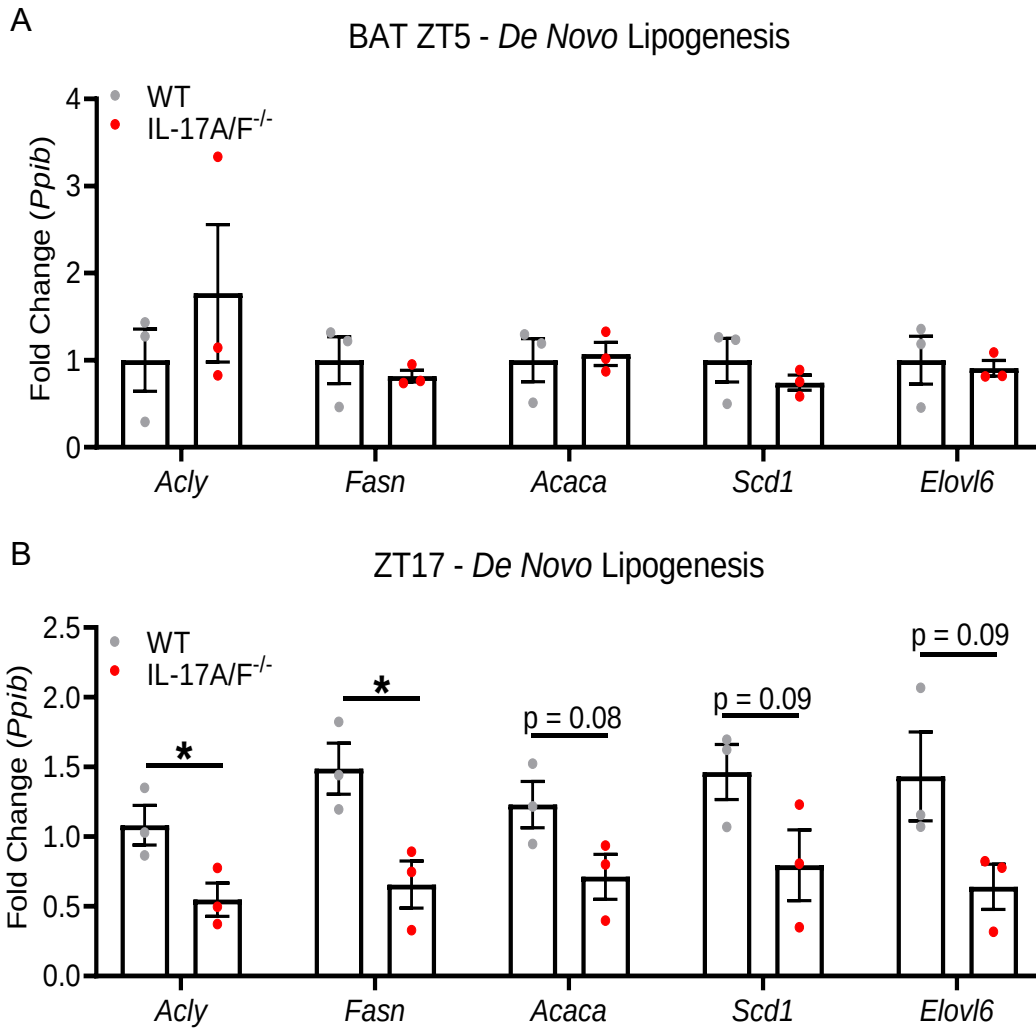


Figure 4.21: *Il17a/f^{-/-}* mice lack *de novo* lipogenesis gene circadian rhythm in BAT.

(A) Relative expression of *de novo* lipogenesis genes *Acly*, *Fasn*, *Acaca*, *Scd1* and *Elovl6* in the BAT of wild-type mice compared to BAT of *Il17a/f^{-/-}* mice at ZT5. **(B)** Relative expression of *de novo* lipogenesis genes *Acly*, *Fasn*, *Acaca*, *Scd1* and *Elovl6* in the BAT of wild-type mice compared to BAT of *Il17a/f^{-/-}* mice. Data are representative of two independent experiments. **(A, B)** Data shown as mean $\pm$ SEM, n = 3 mice per group. **(A, B)** Significance was calculated using Two-tailed paired student's t-test. * p < 0.05

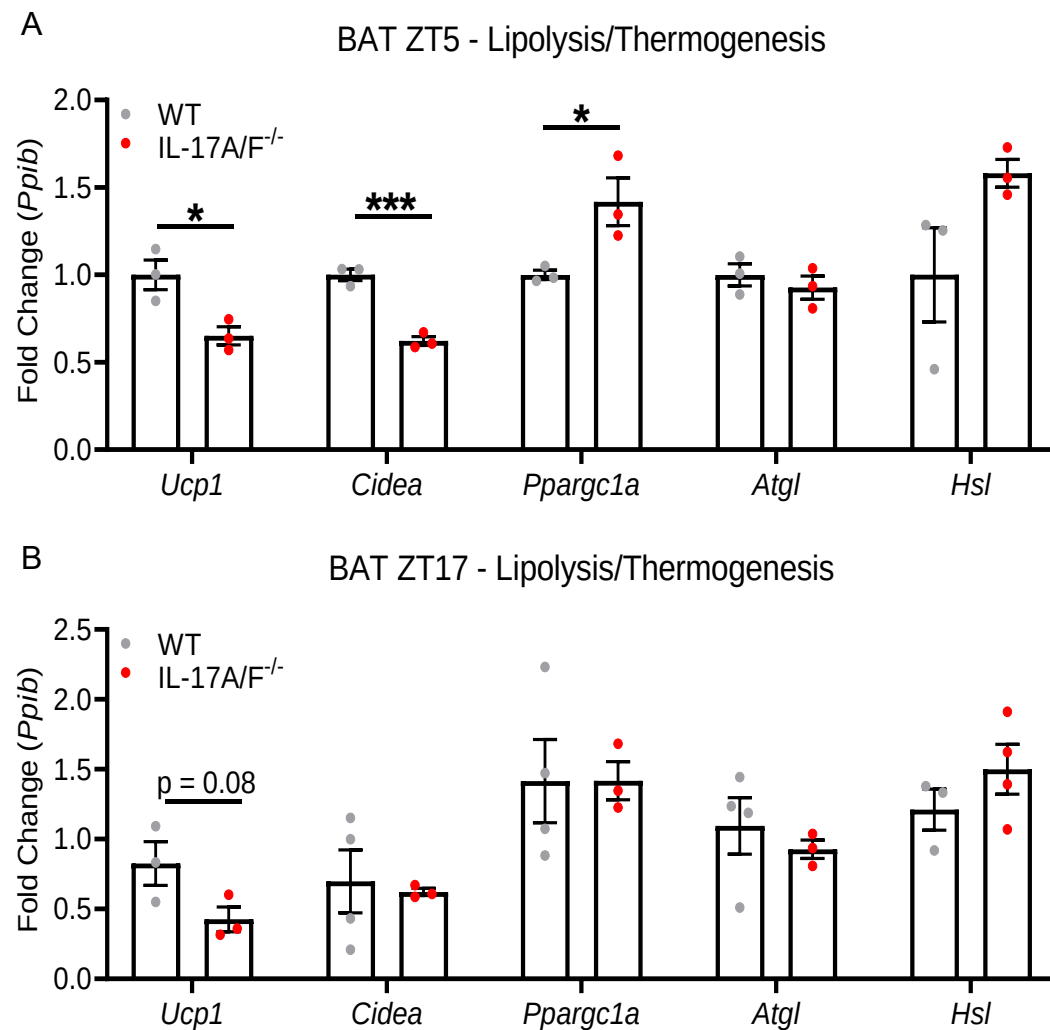


Figure 4.22: *Il17a/f^{-/-}* mice lack lipolysis gene circadian expression in BAT.

(A) Relative expression of lipolysis/thermogenesis genes *Ucp1*, *Cidea*, *Ppargc1a*, *Atgl* and *Hsl* in the BAT of wild-type mice compared to BAT of *Il17a/f^{-/-}* mice at ZT5. **(B)** Relative expression of lipolysis/thermogenesis genes *Ucp1*, *Cidea*, *Ppargc1a*, *Atgl* and *Hsl* in the BAT of wild-type mice compared to BAT of *Il17a/f^{-/-}* mice at ZT17. Data are representative of two independent experiments. **(A, B)** Data shown as mean $\pm$ SEM, n = 3-4 mice per group. **(A, B)** Significance was calculated using Two-tailed paired student's t-test. * p < 0.05, *** p < 0.001

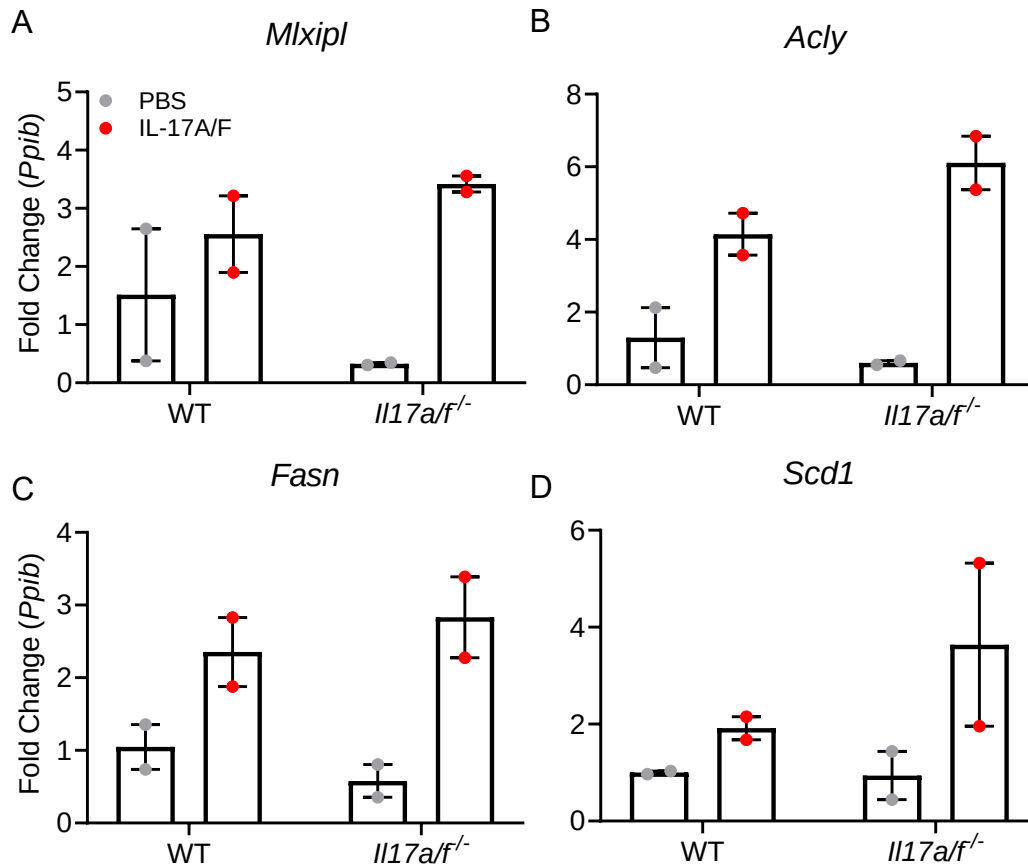
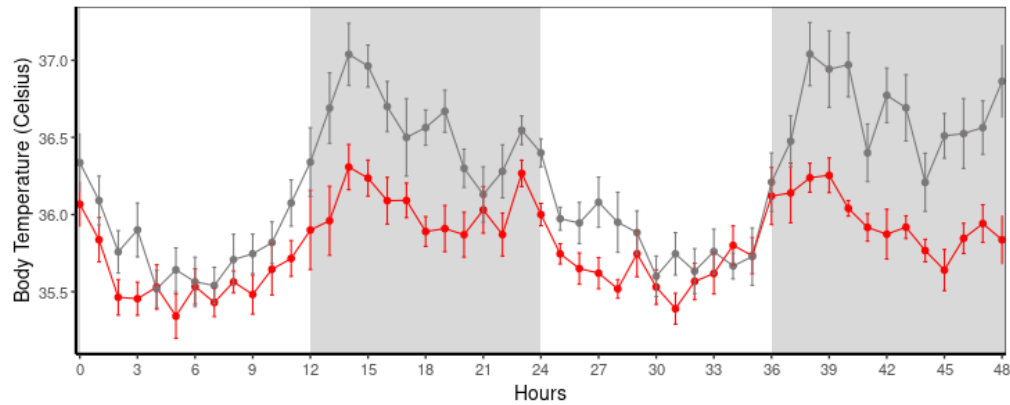


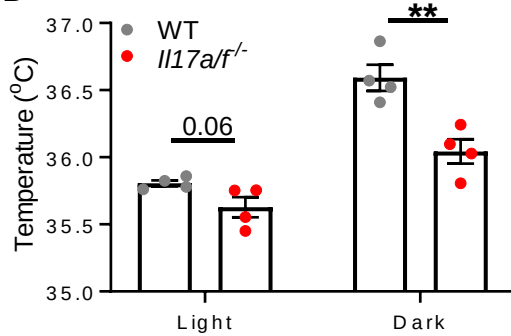
Figure 4.23: Administration of rIL-17A/F rescues *de novo* lipogenesis in BAT.

(A-D) Relative expression of *de novo* lipogenesis genes after administration of IL-17A (1μg/mouse; i.p.) and IL-17F (1μg/mouse; i.p.) to wild-type and *Il17a/f^{-/-}* mice compared to PBS administered controls. Data are representative of one independent experiments. **(A, B)** Data shown as mean ± SEM, n = 2 mice per group. *Experiment performed in collaboration with Brenneeth Stevens.*

A



B



C

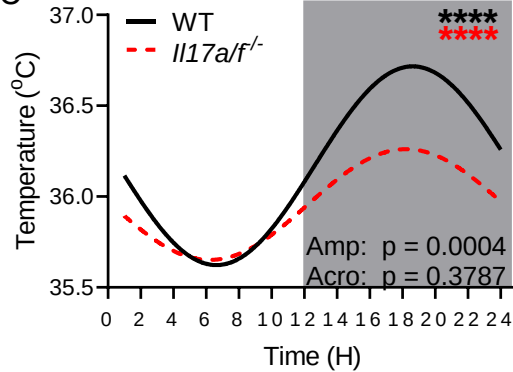


Figure 4.24: *Il17a/f^{-/-}* mice display a disrupted core body temperature rhythm.

(A) Time plot of core body temperature of wild-type (grey) and *Il17a/f^{-/-}* mice. **(B)** Core body temperature with data averaged by day, night, or total day. **(C)** Circadian rhythmicity analysis of core body temperature of *Il17a/f^{-/-}* mice compared to wild-type controls. **(A, C)** White and grey panels represent light and dark periods respectively. Data are representative of four independent experiments, two experiments pooled. **(A-C)** Data shown as mean $\pm$ SEM, $n = 4$ mice per group. **(B)** Significance was calculated using two group analysis with ANCOVA, **(C)** Cosinor analysis, with cosine fitted curves. ** $p < 0.01$, **** $p < 0.0001$. Experiment performed in collaboration with Amanda E Garza.

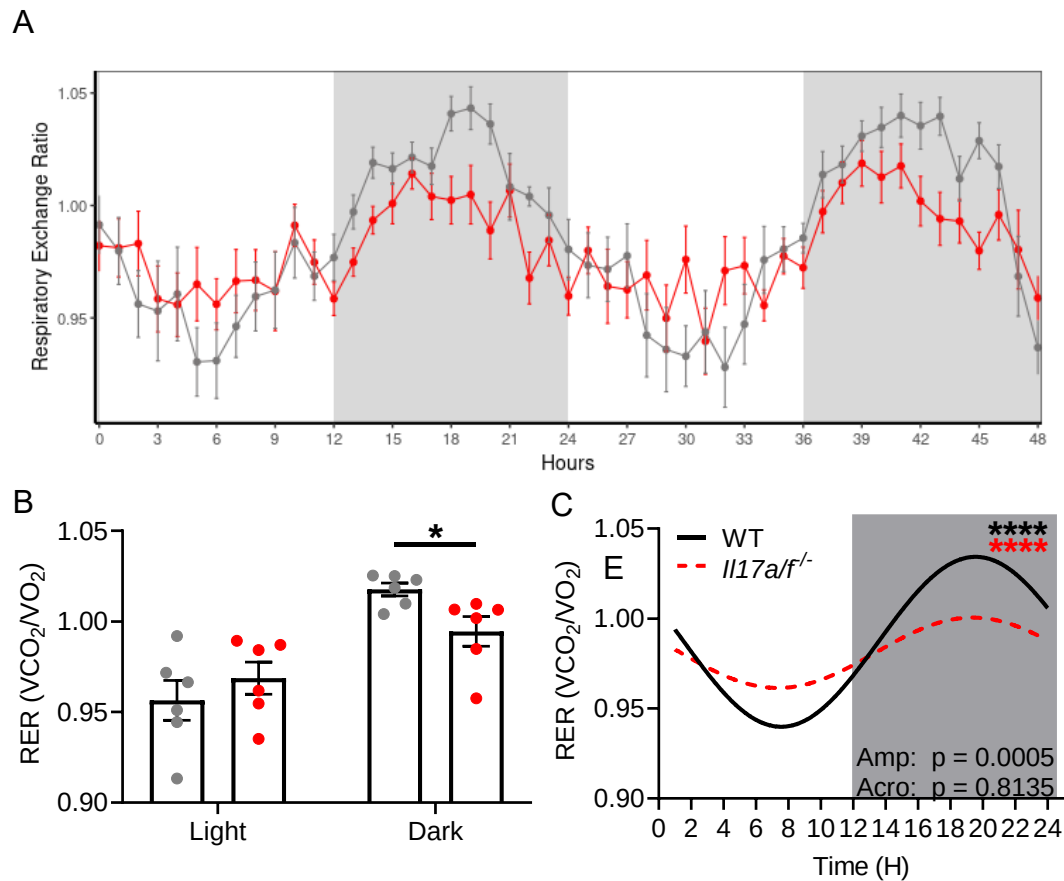


Figure 4.25: *Il17a/f^{-/-}* mice display a disrupted whole body metabolic rhythm.

(A) Time plot of RER of wild-type (grey) and *Il17a/f^{-/-}* mice. **(B)** RER with data averaged by day, night, or total day. **(C)** Circadian rhythmicity analysis of RER of *Il17a/f^{-/-}* mice compared to wild-type controls. **(A, C)** White and grey panels represent light and dark periods respectively. Data are representative of four independent experiments, two experiments pooled. **(A-C)** Data shown as mean $\pm$ SEM, $n = 4$ mice per group. **(B)** Significance was calculated using two group analysis with ANCOVA, **(C)** Cosinor analysis, with cosine fitted curves. ** $p < 0.01$, **** $p < 0.0001$. Experiment performed in collaboration with Amanda E Garza..

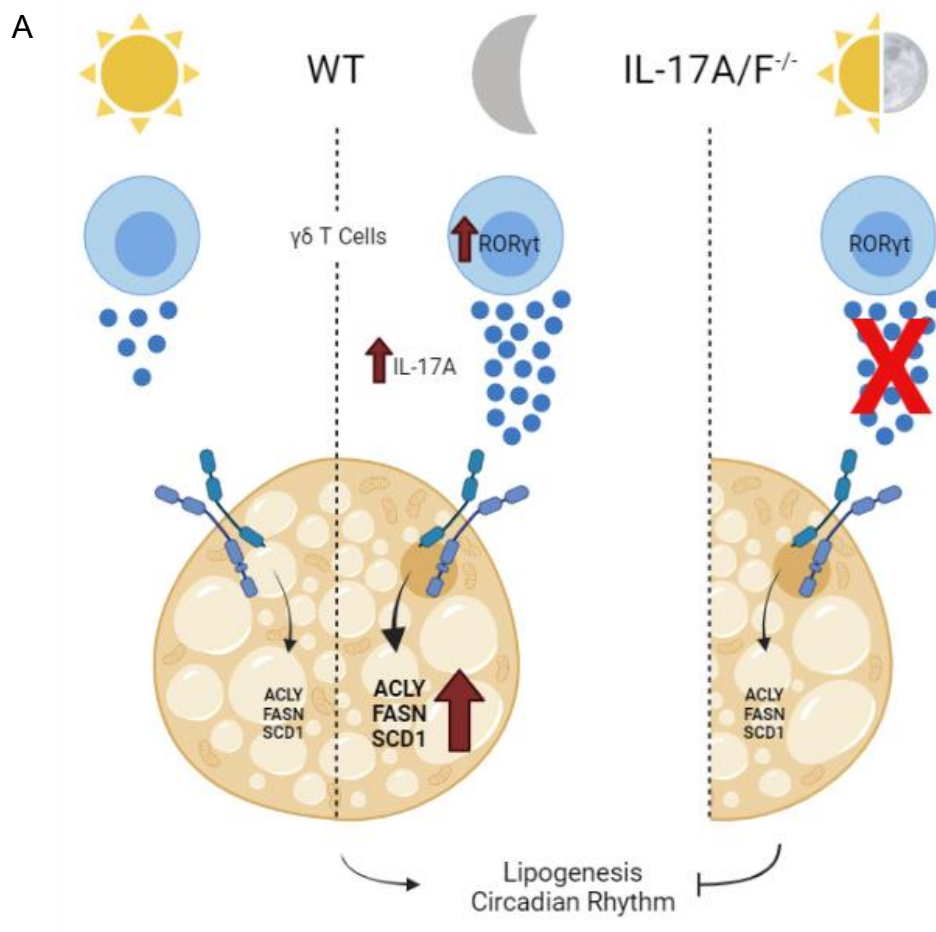


Figure 4.26: IL-17A signalling is necessary for *de novo* lipogenesis circadian rhythm, and whole body metabolic homeostasis.

IL-17A production by adipose tissue $\gamma\delta$ T cells (blue) peaks at night during the active, feeding phase of mice. IL-17A signalling in adipocytes (beige) at night is necessary for the expression *de novo* lipogenesis genes in adipose tissue. Loss of IL-17A, or IL-17 receptor c (IL-17RC; blue) expression in adipose tissue disrupts circadian expression of *de novo* lipogenesis in adipose tissue. Loss of lipogenic circadian rhythm has a detrimental effect on whole body metabolic homeostasis, decreasing the circadian amplitude of the RER, and core body temperature of mice.

4.8 Discussion

Here we show that $\gamma\delta^{17}$ T cells, as well as all other type 17 innate T cells are enriched for the molecular clock. Furthermore, adipose $\gamma\delta^{17}$ T cells are particularly enriched for clock genes, and molecular clock regulation of IL-17A expression is essential for adipose tissue circadian homeostasis, whole body metabolism, and body temperature regulation, via circadian regulation of *de novo* lipogenesis. Tissue resident innate lymphocytes produce effector cytokines ($\gamma\delta$ T cells⁶², iNKTs⁸¹ and ILCs²⁰⁸), which are important for optimal tissue physiology. Here, we show that tissue resident innate T cells express a molecular clock, which is more prominent in type 17 cells compared to type 1 cells, and this is important for maintaining normal physiological responses which change between night and day. The molecular clock has been shown to regulate IL-17A responses in ILC3s²⁰³ and Th17¹²⁴ lymphocytes, although circadian rhythm of innate T cells has not been described. Genetic deletion of *Nfil3*, or *Rev-erba*, disrupted IL-17A expression in the small intestine, while having no impact on IFN γ expression by Th1 cells¹²⁴. Furthermore, IL-17A-producing ILC3s also displayed higher expression of molecular clock genes compared to ILC1s²⁰³, ILC2s²⁰², and ILCPs¹²⁵, suggesting that enriched expression of molecular clock components may be a conserved feature of IL-17A-producing lymphocytes.

Our data show that $\gamma\delta^{17}$ T cells have a daily rhythm to IL-17A and ROR γ t expression, which may be negatively regulated by NFIL3, similar to what is observed in Th17 cells¹²⁴. Immune cell function is intricately linked with cellular metabolism, and previous work from our lab has shown that $\gamma\delta^{17}$ T cells are enriched for mitochondria and lipid metabolism genes, which aide their effector function⁶⁸. Here, we show that $\gamma\delta^{17}$ T cells also have a circadian rhythm to their mitochondrial metabolism and lipid uptake, particularly in adipose tissue. We found that multiple adipose depots, including BAT, displayed a diurnal rhythm to IL-17A and IL-17F expression by $\gamma\delta^{17}$ T cells. Previous work by our lab and the Spiegelman lab described an important thermogenic role for IL-17A, or IL-17F, signalling in adipose tissue^{29,62}. However, it was at first puzzling that we found that IL-17A and IL-17F production by adipose $\gamma\delta^{17}$ T cells peaks at night during feeding, rather than during the day when thermogenesis occurs in mice. While feeding at night, energy is stored in adipose tissue as lipids through the process of *de novo* lipogenesis²⁰⁹, where lipids are absorbed from the diet and stored as triacylglycerides (TAGs). However, we found that circadian *de novo* lipogenesis is impaired which occurs at the opposite time of day to lipolysis. A recent study by the Lazar lab found that *de novo* lipogenesis in adipose tissue is linked to the molecular clock, and that the processes of *de novo* lipogenesis and lipolysis are intricately linked. Loss of circadian rhythm of *de novo* lipogenesis in *Il17a/f^{-/-}* mice likely blunts lipolysis due to a lack

of lipid stores to act as fuel for circadian thermogenesis in body temperature regulation. In support of this, the Lazar lab found that loss of *de novo* lipogenesis, through BAT specific disruption of SREBP activity, was enough to disrupt the circadian body temperature of mice, demonstrating an essential role of BAT *de novo* lipogenesis in the maintenance of body temperature²⁰⁷. It has been previously described that *Il17a*^{-/-} and *Il17f*^{-/-} mice have susceptibility to cold challenge^{29,62} and here we link this to upstream defects in *de novo* lipogenesis. We show that disrupted *de novo* lipogenesis could be restored by IL-17A and IL-17F administration *in vitro* and *in vivo*. Analysis of the published dataset by Hu *et al*, found that loss of IL-17RC specifically on adipocytes significantly reduced *de novo* lipogenesis gene expression in BAT²⁹. In that study, the authors suggest that IL-17F, rather than IL-17A, mediates the pro-thermogenic effect on adipose tissue. However, both IL-17A and IL-17F signal through IL-17RC, highlighting that both cytokines may act in synergy to promote thermogenesis, and maintain body temperature^{210,211}. Our data show that mice lacking both IL-17F and IL-17A have a striking defect in body temperature maintenance and daily fluctuations in body temperature rhythm.

A recent study found that BMAL1 deficiency specifically in ROR γ t expressing cells (BMAL1 ^{Δ Rorc}) had enhanced lipid metabolism gene expression in gut epithelial cells¹²⁵. Although in this study, this observation was attributed to a loss of IL-22 in ILC3s, a similar study found that IL-17A expression was enhanced in BMAL1 ^{Δ Rorc} mice, suggesting the molecular clock suppresses IL-17 expression diurnally and that loss of this clock control enhances IL-17A expression, but diminishes IL-22 expression²⁰². Furthermore, this study found that BMAL1 ^{Δ Rorc} mice had increased gonadal and subcutaneous adipose tissue mass. This would also link IL-17 to both the molecular clock and lipid metabolism¹²⁵ and supports that IL-17 boosts lipid synthesis/storage. In this instance, genes associated with lipid metabolism are upregulated by epithelial cells in the gut of BMAL1 ^{Δ Rorc}, when IL-17A is increased. Thus, the IL-17 family of cytokines appear to be important for lipid metabolism in both adipocytes as shown by our study, and gut epithelial cells, where innate T cells and ILCs reside. Finally, we show that *Il17a/f*^{-/-} animals have disrupted circadian rhythm to their respiratory exchange ratio and body temperature.

Recent studies have described that a high fat diet, in addition to a ketogenic diet, expanded $\gamma\delta$ ¹⁷ T cells in both lymph nodes and visceral adipose tissue, in addition to increasing IL-17A production^{68,75}. Moreover, loss of IL-17A signalling through inhibition of ROR γ t using digoxin, GSK805, and TMP778 prevented weight gain in a high-fat diet induced obesity model³². While digoxin has other metabolic targets that could affect whole body metabolism, the authors also found that genetic deletion of adipocyte IL17RA resulted in decreased body and fat mass in standard fat diet fed mice, in addition to preventing obesity, further

suggesting a role for IL-17A signalling in energy storage³². Our data demonstrates that a major target of IL-17 in adipose tissue is *de novo* lipogenesis, and in the absence of IL-17A and IL-17F signalling, a defect in *de novo* lipogenesis may explain the inability to store fat, as seen the mice with an adipocyte specific deletion of *Il17ra*. This collection of recent studies show it is becoming increasingly clear that immune cells play a crucial role in regulating local tissue homeostasis, which impacts whole body circadian metabolism. With the ever increasing burden of metabolic disease, it will become essential to better understand the link between immune function, circadian rhythm, and whole body metabolic health for combating obesity, cachexia, and metabolic syndromes caused in shift workers. Here we provide evidence for the IL-17 family as potent metabolic modulators, impacting at the adipose tissue and whole body level, which could be a therapeutic target in metabolic disease.

Chapter 5

Timing and caloric content of food intake
differentially regulate IL-17A expression
by $\gamma\delta$ T cells.

5.1 Introduction

Circadian rhythms are essential for whole body homeostatic physiology, by predicating and adjusting to changes in the external environment. Modern society has placed an emphasis on an indoor work environment with artificial lighting, which mainly consists of melatonin-suppressing blue light, causing circadian rhythm disruption²¹². In addition, non-standard working schedules, such as shift work and night work have become more prevalent, with up to 35% of the population participating in shift work in certain countries²¹³. Both artificial light, and shift work are associated with disturbed sleep and circadian desynchronization, leading to detrimental effects on metabolic, endocrine and immune functions²¹⁴. Particularly, shift work increases the risk of developing metabolic syndrome^{215–217}, and increases peripheral inflammation²¹³. Furthermore, shift work alters the time of day at which feeding occurs, and enhances preference for high-fat, high caloric foods^{218,219}, which in turn further disrupts circadian metabolic homeostasis¹³³. As the global levels of metabolic disease continue to rise due to modern lifestyle, it is becoming pertinent to find novel therapeutic interventions.

IL-17A is emerging as a major systemic metabolic messenger, which can modulate feeding behaviour^{220,221}, lipid metabolism¹⁰⁹, and homeothermy^{28,29}. IL-17A signalling also plays a detrimental role in the development of obesity and metabolic disease. During a high fat diet (HFD), IL-17A expression increases in the lymph nodes²²², liver¹⁰⁹, blood¹¹⁰ and fat¹¹⁰ of mice. IL-17A signalling was found to increase fatty acid accumulation in the liver of mice¹⁰⁹. Fatty acid accumulation also increases in human hepatocytes treated with IL-17A, via induction of the fatty acid synthesis genes *Chrebp* and *Srebp1c*¹⁰⁹. In agreement, HFD fed mice lacking IL-17A had fewer hepatocyte lipid droplets compared to their wild-type controls¹¹¹. Moreover, mice lacking IL-17A were slower to gain weight on a HFD after 14 weeks¹¹¹. Inhibition of IL-17A signalling using small molecule inhibitors of ROR γ t, or deletion of adipocyte IL-17RA prevented HFD induced obesity¹¹⁰. These studies suggest a role for IL-17A signalling in lipid metabolism, and metabolic disease, thus presenting IL-17A signalling as a novel therapeutic target in the treatment of metabolic disease caused by circadian disruption.

In chapter 4, we described a necessary role for IL-17A in the regulation of *de novo* lipogenesis. Here, we investigated if HFD feeding, or misaligned timing of feeding, would detrimentally impact the circadian rhythm of IL-17A production by epiWAT $\gamma\delta$ T cells, and circadian whole body metabolism. We observed that a 3-week HFD was sufficient to enhance IL-17A production by epiWAT $\gamma\delta$ T cells, completely disrupting IL-17A circadian

rhythm. Additionally, reversing the time of day at which food is available to mice, disrupted whole body circadian metabolism, and decreased IL-17A expression by adipose $\gamma\delta$ T cells. Surprisingly, mice fed only during the day (inactive phase), consumed 50% less calories, but displayed no weight loss. To further investigate this, we placed mice on a 50% calorie restricted diet, and found decreased expression of IL-17A, and IL-17F, by epiWAT $\gamma\delta$ T cells. Having observed that HFD increased IL-17A expression, while reverse feeding and calorie restriction decreased IL-17A expression by adipose $\gamma\delta$ T cells, we used a mouse model of multiple sclerosis, EAE, to investigate the pathology of IL-17A in these diet experiments. Interestingly, we found that HFD fed mice had the quickest disease progression, while calorie restricted mice did not develop disease. Calorie restricted mice were protected from the development of disease, due to a migration of cells from the peripheral lymph nodes to the bone marrow, which prevented T cell infiltration into the brain and spinal cord. As such, dietary intervention may be considered in the treatment of inflammation observed in metabolic, and autoimmune disease pathologies.

5.2 Circadian metabolism, and circadian IL-17A expression are disrupted by HFD feeding.

Mice display a circadian rhythm to their whole body metabolism, with higher metabolic rate at night during the active phase, and lower during the day when mice are inactive²²³. This is reflected in the respiratory exchange ratio (RER), whereby a value closer to 0.7 indicates lipid burning during the inactive phase, and a value closer to 1 indicates carbohydrate burning during the active phase. Previous work from our lab has shown that a HFD disrupts RER circadian rhythm, pushing the ratio towards that indicative of lipid utilisation²²². Furthermore, HFD enhanced IL-17A expression by lymph node $\gamma\delta$ T cells²²². Therefore, we investigated whether a 3-week HFD was sufficient to disrupt circadian whole body metabolism. We chose 3 weeks as 3 weeks of inverted light was sufficient to invert circadian metabolic rate (Figure 5.1A; Figure 4.17B-E). Carbon dioxide production was increased during the day and decreased at night in HFD fed mice (Figure 5.1B, C), while HFD increased energy expenditure compared to SFD fed controls (Figure 5.1D, E). HFD feeding disrupted RER circadian rhythm as early as one day after feeding, pushing whole body metabolism towards constant lipid burning (Figure 5.2A, B). Additionally, HFD fed mice ate less food compared to SFD fed controls (Figure 5.2C, D), with no differences in activity between groups (Figure 5.2E, F).

Having observed that adipose $\gamma\delta^{17}$ T cells have the highest molecular clock expression (Figure 4.14A), and display a circadian rhythm to their cellular metabolism, IL-17A and ROR γ t expression (Figure 4.14-4.16), we investigated if HFD feeding disrupted the circadian rhythm of adipose $\gamma\delta^{17}$ T cells. After a 3-week 60% HFD feeding regimen, inLN and epiWAT were harvested over 24-hours (Figure 5.1A). We found that a 3-week HFD had no significant effect on inLN or adipose $\gamma\delta$ T cell numbers over 24-hours (Figure 5.3A, B). Previous work from our lab has described the importance of mitochondrial metabolism for IL-17A production by $\gamma\delta$ T cells²²². Therefore, we examined the circadian mitochondrial metabolism of adipose $\gamma\delta$ T cells over a 24-hour period after HFD feeding. A 3-week HFD was sufficient to completely ablate the mitochondrial mass circadian rhythm of adipose $\gamma\delta^{17}$ T cells, remaining constantly low across a 24-hour period (Figure 5.4A), in addition to decreasing mitochondrial membrane potential, particularly during the day (Figure 5.4B). As cellular metabolism is intricately linked to cell function, we examined if HFD feeding disrupted IL-17A expression. We found that HFD feeding enhanced IL-17A expression by adipose $\gamma\delta$ T cells at all times of day, disrupting IL-17A circadian rhythm (Figure 5.5A).

Additionally, HFD disrupted ROR γ t circadian rhythm, heightening expression in adipose $\gamma\delta$ T cells, particularly during the day (Figure 5.5B).

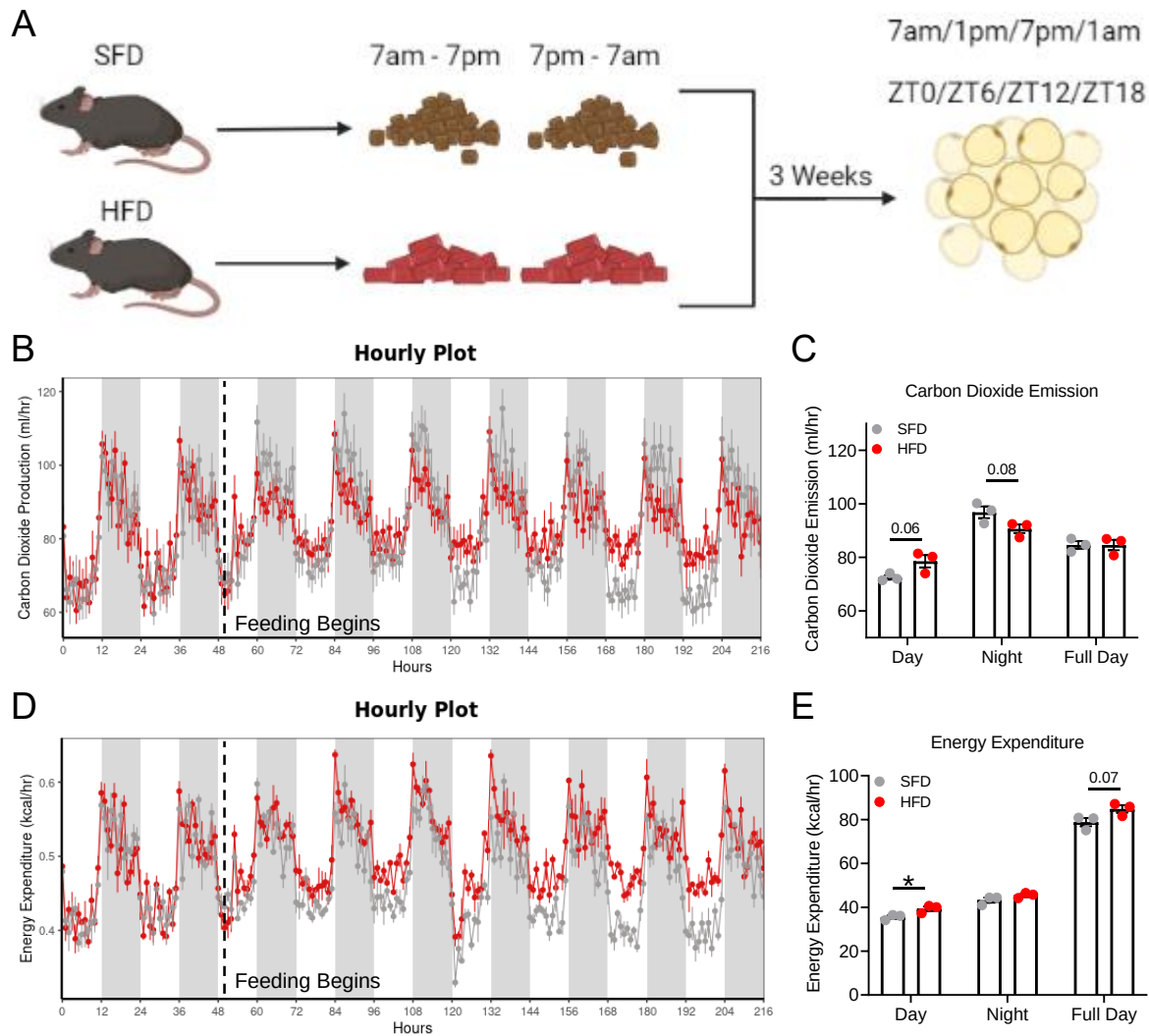


Figure 5.1: 3-Week HFD feeding increases energy expenditure.

(A) Diagrammatic representation of experimental setup. **(B)** Time plot of metabolic rate parameter CO₂ production, and **(D)** energy expenditure of mice fed a SFD (grey), versus fed 60% HFD (red). **(C, E)** Bar plots of CO₂ emission and energy expenditure averaged by day, night, and whole day of SFD and HFD fed mice. White and grey panels represent light and dark periods respectively. Data are representative of two independent experiments. **(B-E)** Data shown as mean $\pm$ SEM, n = 3 mice per group. **(C, E)** Significance was calculated using two group analysis with ANCOVA. * p < 0.05.

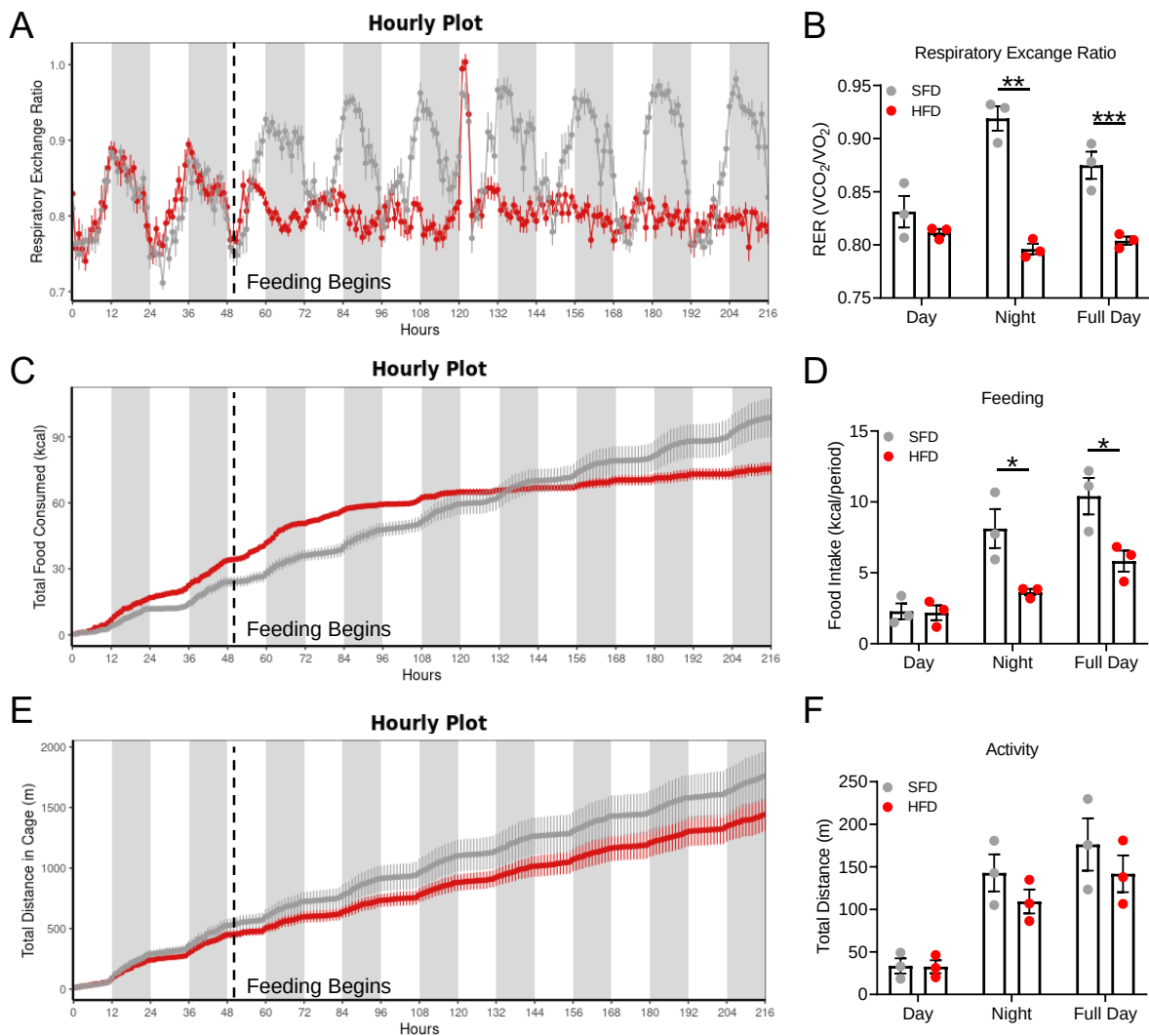


Figure 5.2: 3-Week HFD feeding disrupts circadian RER, inducing lipid metabolism.

(A) Time plot of metabolic parameter RER (VCO₂/VO₂), (C) food consumption and (E) activity of mice fed a SFD (grey), versus fed 60% HFD (red). (B, D, F) Bar plots of RER, food intake and activity averaged by day, night, and whole day of SFD and HFD fed mice. White and grey panels represent light and dark periods respectively. Data are representative of two independent experiments. (B, D, F) Data shown as mean $\pm$ SEM, $n = 3$ mice per group. (A-F) Significance was calculated using two group analysis with ANCOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

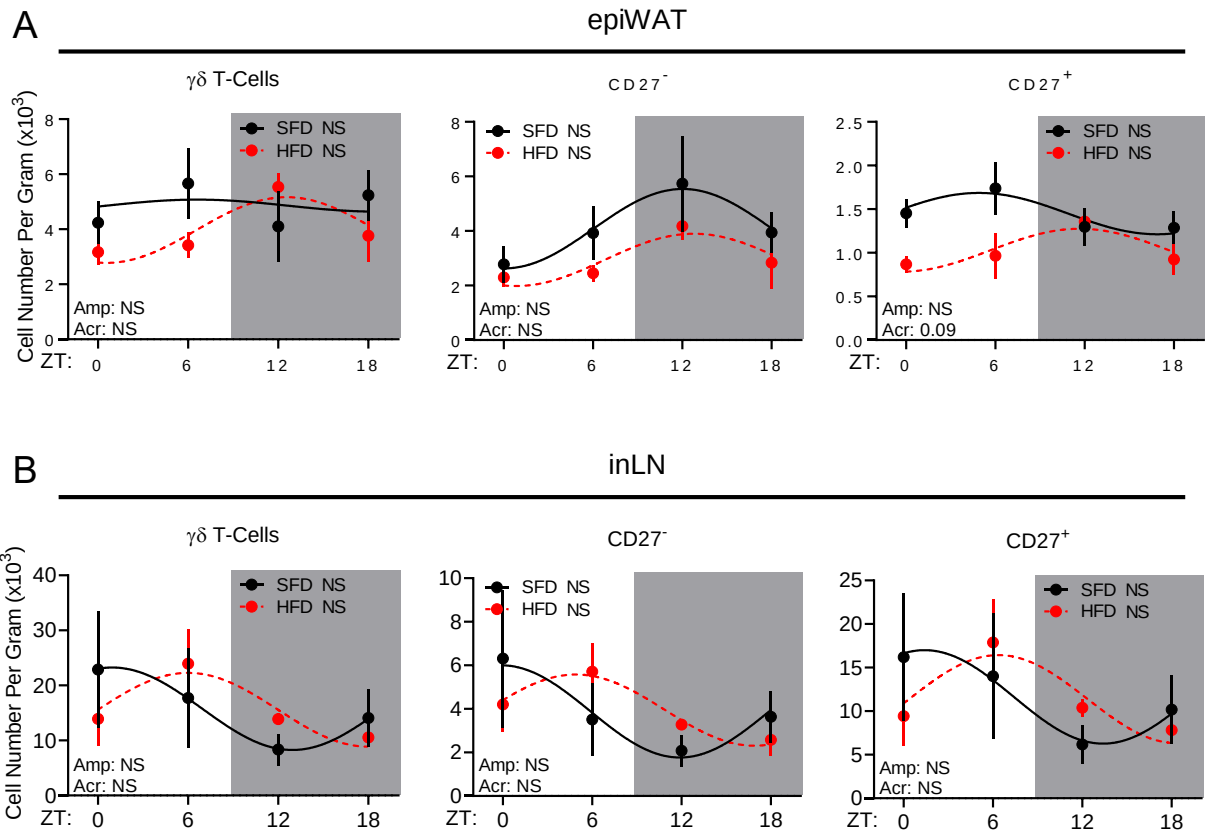


Figure 5.3: 3-Week HFD feeding does not affect inLN, or epiWAT, circadian $\gamma\delta$ T cell numbers.

(A) Circadian time plot of adipose $\gamma\delta$ T cell numbers (left), CD27⁻ $\gamma\delta$ T cell numbers (middle), and CD27⁺ $\gamma\delta$ T cell numbers (right) from SFD (black) or HFD (red) fed mice. **(B)** Circadian time plot of inLN $\gamma\delta$ T cell numbers (left), CD27⁻ $\gamma\delta$ T cell numbers (middle), and CD27⁺ $\gamma\delta$ T cell numbers (right) from SFD (black) or HFD (red) fed mice. White and grey panels represent light and dark periods respectively. Data are representative of two independent experiments. **(A, B)** Data shown as mean $\pm$ SEM, $n = 3-4$ mice per group. **(A, B)** Significance was calculated using cosinor analysis, with cosine fitted curves; amplitude (Amp) and acrophase (Acr) were extracted from the cosinor model.

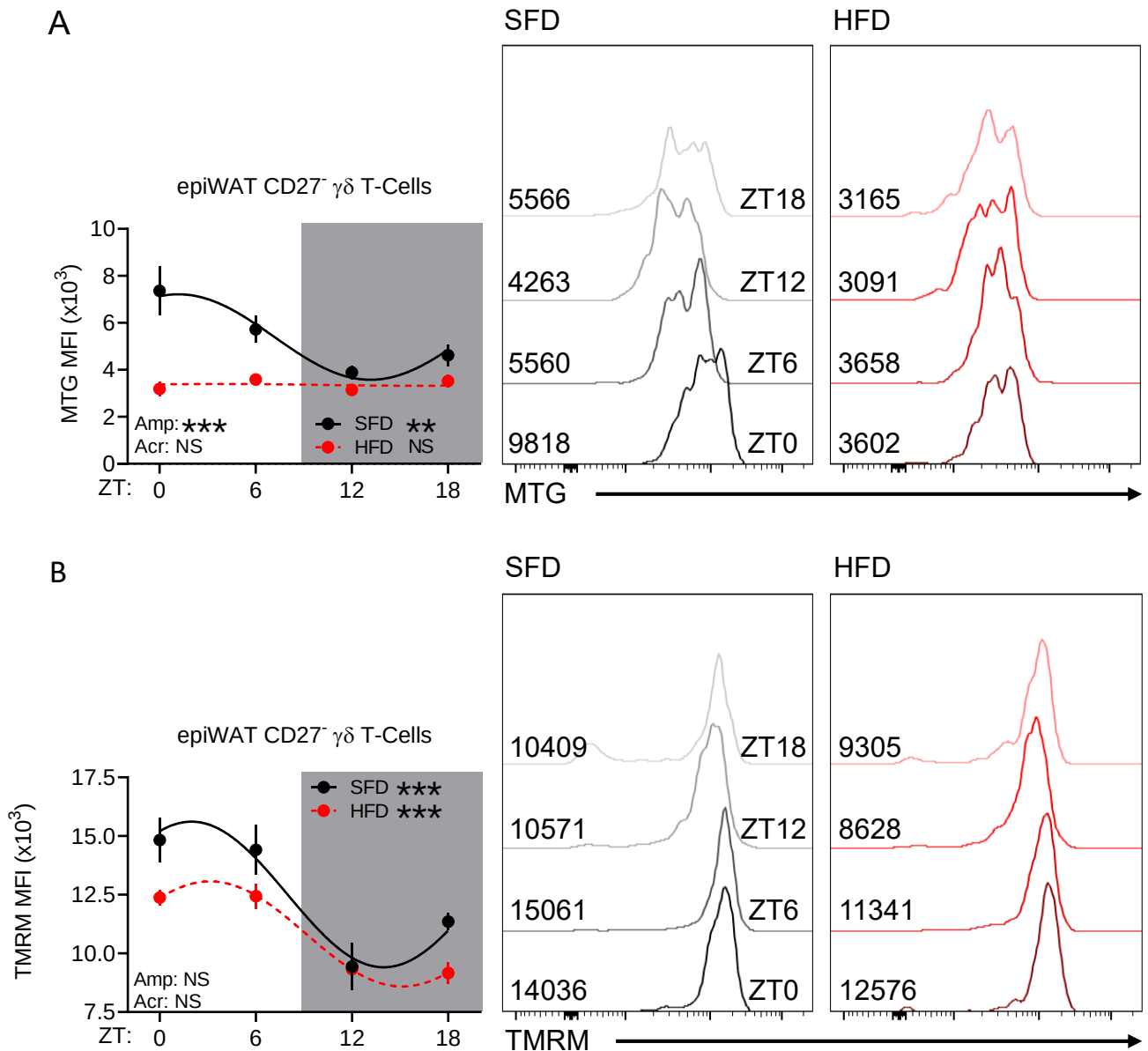


Figure 5.4: 3-Week HFD feeding disrupts adipose $\gamma\delta^{17}$ T cell mitochondrial metabolic rhythm.

(A) Circadian time plots of mitochondrial mass by MFI (MTG; left), with representative histograms (right), of adipose $\gamma\delta^{17}$ T cell from SFD (black) or HFD (red) fed mice. **(B)** Circadian time plots of mitochondrial membrane potential by MFI (TMRM; left), with representative histograms (right), of adipose $\gamma\delta^{17}$ T cell from SFD (black) or HFD (red) fed mice. White and grey panels represent light and dark periods respectively. Data are representative of two independent experiments. **(A, B)** Data shown as mean $\pm$ SEM, $n = 4$ mice per group. **(A, B)** Significance was calculated using cosinor analysis, with cosine fitted curves; amplitude (Amp) and acrophase (Acro) were extracted from the cosinor model. ** $p < 0.01$, *** $p < 0.001$.

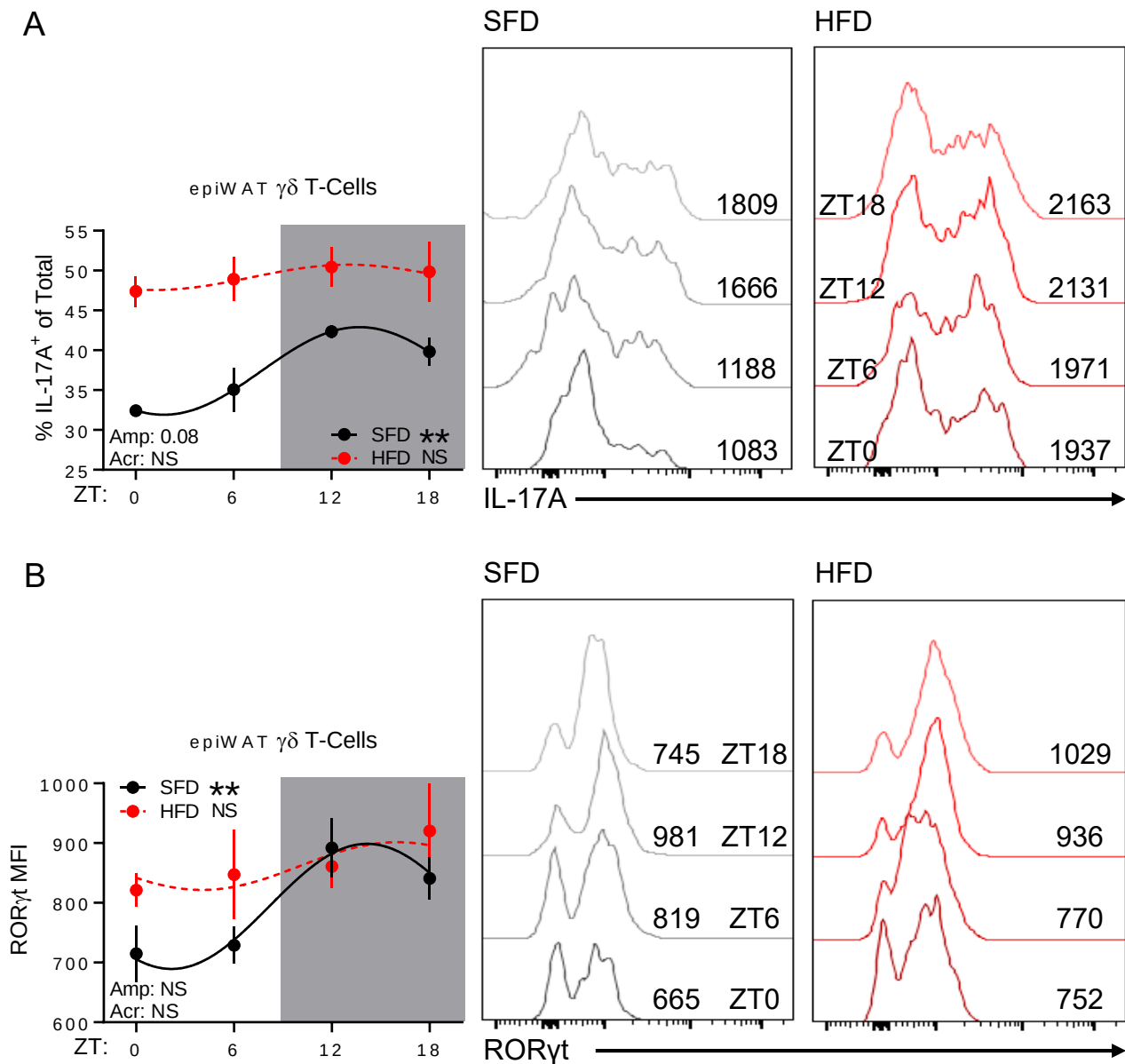


Figure 5.5: 3-Week HFD feeding disrupts adipose $\gamma\delta$ T cell IL-17A and RORyt circadian expression.

(A) Circadian time plots of IL-17A expression (percentage; left), with representative histograms (right), of adipose $\gamma\delta^{17}$ T cell from SFD (black) or HFD (red) fed mice. **(B)** Circadian time plots of RORyt expression (MFI; left), with representative histograms (right), of adipose $\gamma\delta^{17}$ T cell from SFD (black) or HFD (red) fed mice. White and grey panels represent light and dark periods respectively. Data are representative of two independent experiments. **(A, B)** Data shown as mean $\pm$ SEM, $n = 6$ mice per group, two experiments combined. **(A, B)** Significance was calculated using cosinor analysis, with cosine fitted curves; amplitude (Amp) and acrophase (Acro) were extracted from the cosinor model. ** $p < 0.01$.

5.3 Reverse feeding does not entrain whole body metabolism or IL-17A production by adipose $\gamma\delta$ T cells.

Adipose tissue is a dynamic organ that responds to a multitude of physiological signals from metabolic²²⁴, hormonal²²⁵, immune²⁸, and neuronal¹⁹⁰ signalling cues. Adipose tissue is particularly sensitive to circadian feeding behaviour, expanding and contracting during feeding (active) and fasting (inactive) periods respectively²²⁶. We have demonstrated previously that IL-17A expression by adipose $\gamma\delta$ T cells entrains to light (Figure 4.18). As adipose tissue responds to nutritional cues from feeding behaviour, we next investigated if whole body metabolic rhythm and adipose IL-17A expression is entrained by the timing of feeding. Using metabolic cages to examine the effect of reversed feeding cycles on whole body metabolism, mice were recorded under normal light conditions, fed from 7am-7pm (day fed), or from 7pm-7am (night fed) for 3-weeks (Figure 5.6A). Surprisingly, carbon dioxide production, in addition to energy expenditure, did not completely reverse (Figure 5.6B-E). Carbon dioxide production and energy expenditure decreased over 24-hours in day-fed mice compared to night fed controls (Figure 5.6B-E). Interestingly, there was no difference in the activity of day-fed mice compared to controls (Figure 5.7B, C), however day fed mice consumed up to 50% less calories than night fed controls (Figure 5.7D, E), but lost no body or adipose mass (Figure 5.8 A, B). These data suggest that reverse feeding disrupts, rather than entrains, whole body circadian metabolism.

As inverted feeding severely disrupted whole body metabolism, we next investigated the effect of reverse feeding on adipose $\gamma\delta^{17}$ T cell homeostasis. We found there was a decreased number of $\gamma\delta$ T cells in the inLN and adipose tissue of mice fed during the inactive (day) period (Figure 5.8C, D). Specifically, there was decreased CD27⁻ $\gamma\delta$ T cells in adipose tissue, but a decrease of both CD27⁺ and CD27⁻ $\gamma\delta$ T cell subsets in the inLN of day fed mice (Figure 5.8C, D). Furthermore, we found a decreased percentage, and mean fluorescence intensity, of IL-17A and IL-17F expression by $\gamma\delta$ T cells in both the inLN and adipose tissue of day fed mice (Figure 5.9 A-F). Additionally, reverse feeding decreased TNF expression by $\gamma\delta$ T cells in the inLN and adipose tissue (Figure 5.10A-C). There was also decreased expression of IFN γ by inLN $\gamma\delta$ T cells only, but no difference in IFN γ expression by adipose $\gamma\delta$ T cells (Figure 5.10D-F). These results suggest that reverse feeding suppresses $\gamma\delta$ T cell immune function, which may be due to disrupted whole body metabolic rhythm.

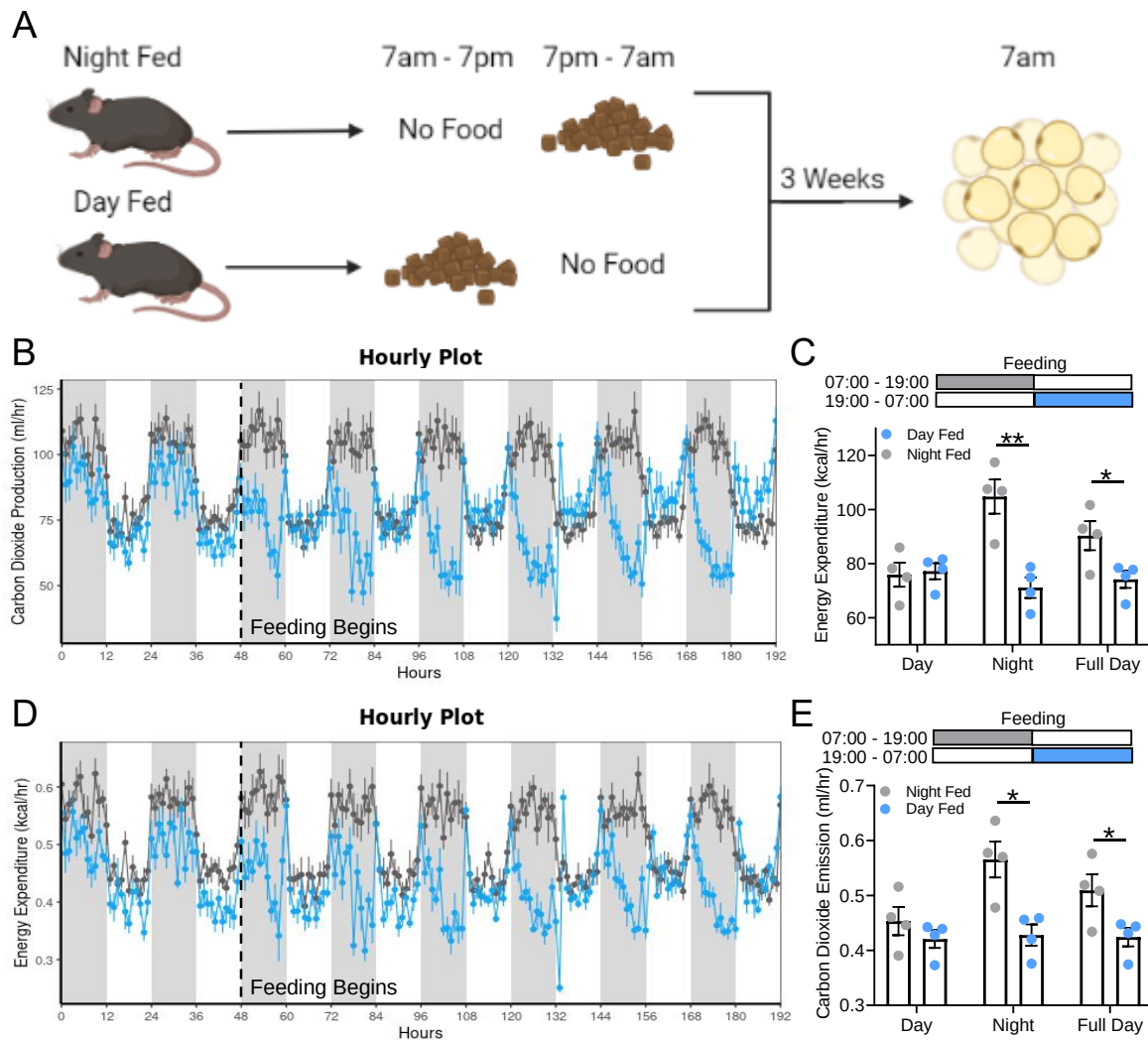


Figure 5.6: Reverse feeding disrupts whole body metabolic circadian rhythm.

(A) Diagrammatic representation of experimental setup. **(B)** Time plot of metabolic rate parameter CO₂ production, and **(D)** energy expenditure of night fed mice (grey), versus day fed mice (blue). **(C, E)** Bar plots of CO₂ emission and energy expenditure averaged by day, night, and whole day of night and day fed mice. White and grey panels represent light and dark periods respectively. Data are representative of three independent experiments. **(B-E)** Data shown as mean $\pm$ SEM, n = 4 mice per group. **(C, E)** Significance was calculated using two group analysis with ANCOVA. * p < 0.05, ** p < 0.01.

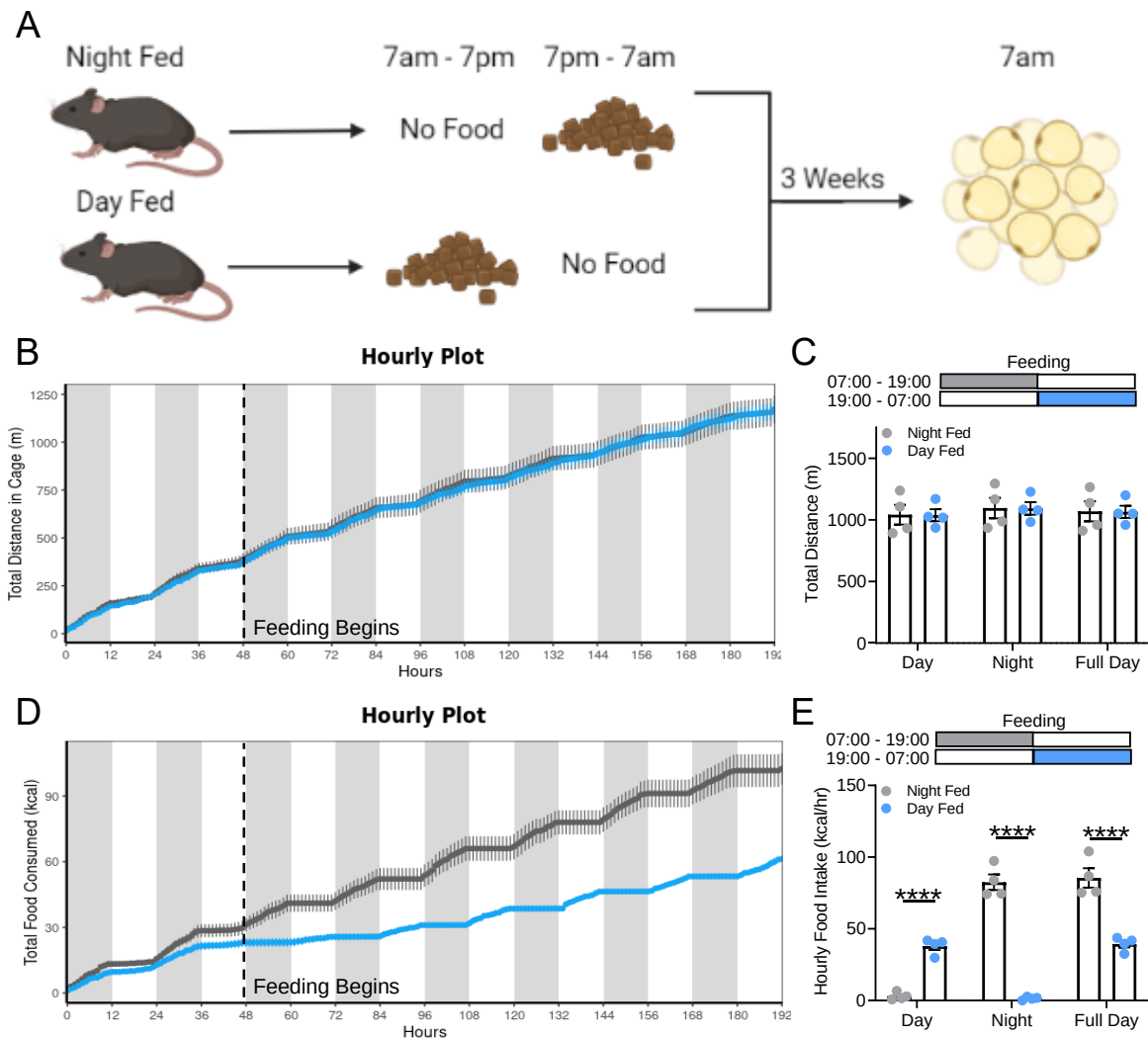


Figure 5.7: Reverse fed mice consume less calories.

(A) Diagrammatic representation of experimental setup. **(B)** Time plot of activity and **(D)** food consumption of night fed mice (grey), versus day fed mice (blue). **(C, E)** Bar plots of activity and food consumption averaged by day, night, and whole day of night and day fed mice. White and grey panels represent light and dark periods respectively. Data are representative of three independent experiments. **(B-E)** Data shown as mean $\pm$ SEM, $n = 4$ mice per group. **(C, E)** Significance was calculated using two group analysis with ANCOVA. **** $p < 0.0001$.

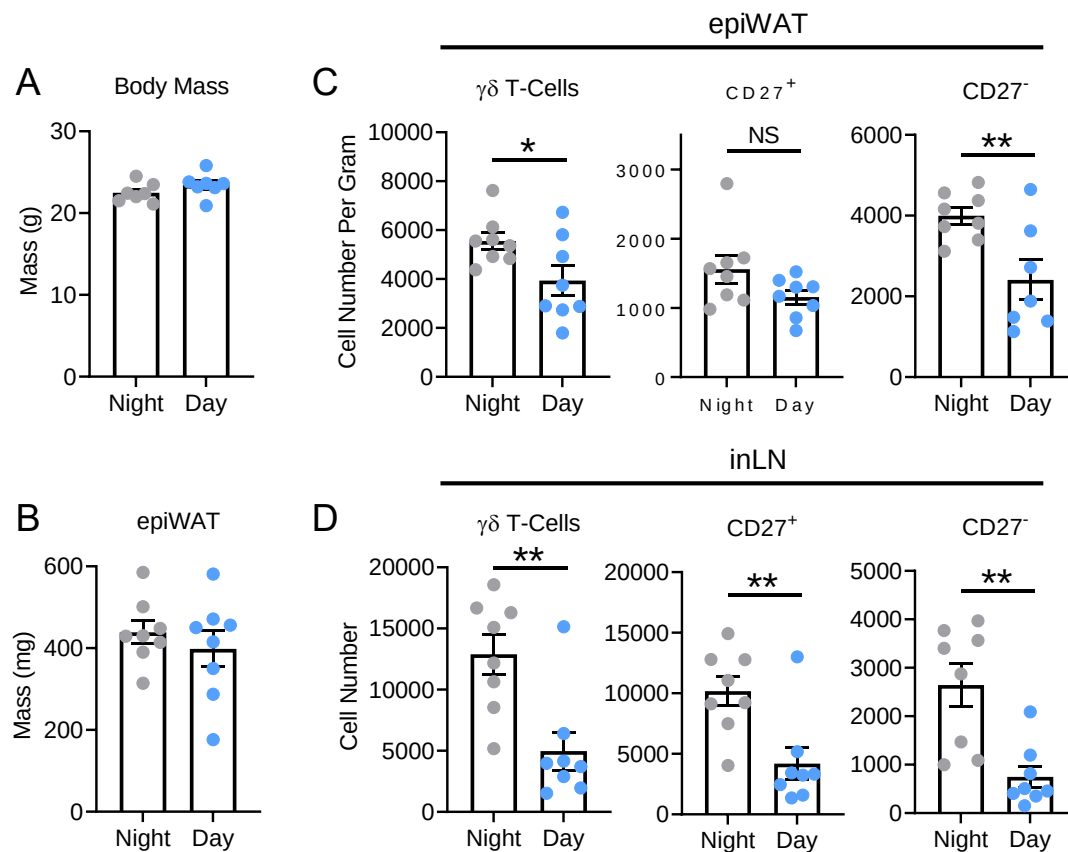


Figure 5.8: Reverse feeding decreases adipose and inLN $\gamma\delta$ T cell numbers.

(A) Body mass of day (grey) or night (blue) fed mice. **(B)** Mass of epiWAT of day or night fed mice. **(C)** Adipose $\gamma\delta$ T cell numbers (left), $CD27^+$ $\gamma\delta$ T cell numbers (middle), and $CD27^-$ $\gamma\delta$ T cell numbers (right) from SFD (black) or HFD (red) fed mice. **(D)** inLN $\gamma\delta$ T cell numbers (left), $CD27^+$ $\gamma\delta$ T cell numbers (middle), and $CD27^-$ $\gamma\delta$ T cell numbers (right) from SFD (black) or HFD (red) fed mice. Data are representative of three independent experiments, two experiments combined. **(A-D)** Data shown as mean $\pm$ SEM, $n = 7-8$ mice per group. **(A, B)** Significance was calculated using a two-tailed unpaired students t-test. NS = Non-Significant, * $p < 0.05$, ** $p < 0.01$.

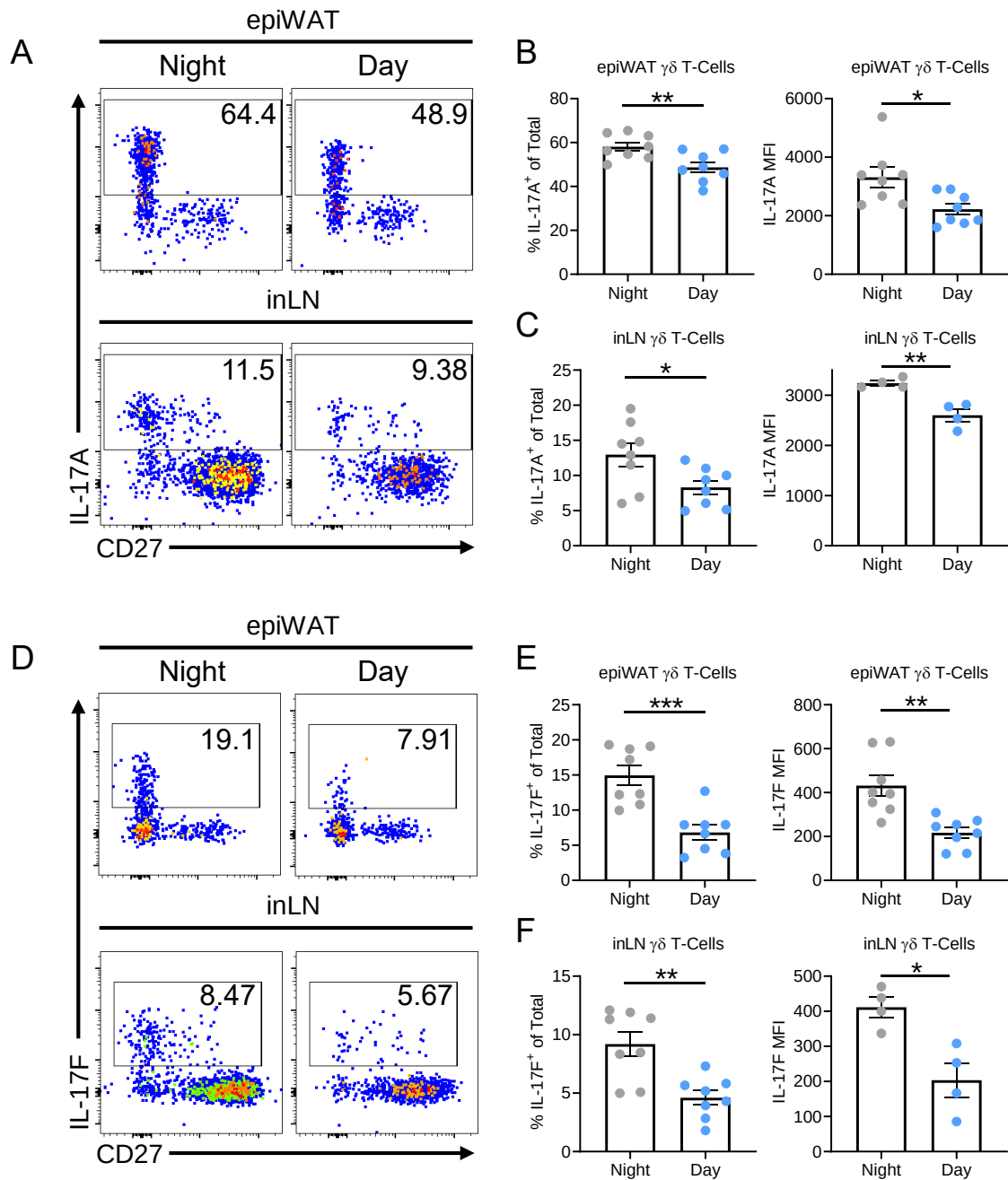


Figure 5.9: Reverse feeding decreases IL-17A and IL-17F expression by $\gamma\delta$ T cells.

(A) Representative flow plots of IL-17A expression by epiWAT and inLN $\gamma\delta$ T cells of night (grey) and day (blue) fed mice. **(B)** Percentage (left), and mean fluorescence (right) of IL-17A expression by adipose and **(C)** inLN $\gamma\delta$ T cells. **(D)** Representative flow plots of IL-17F expression by epiWAT and inLN $\gamma\delta$ T cells of night (grey) and day (blue) fed mice. **(E)** Percentage (left), and mean fluorescence (right) of IL-17F expression by adipose and **(F)** inLN $\gamma\delta$ T cells. Data are representative of three independent experiments, two experiments combined. Data shown as mean $\pm$ SEM, $n = 4-8$ mice per group. Significance was calculated using a two-tailed unpaired students t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

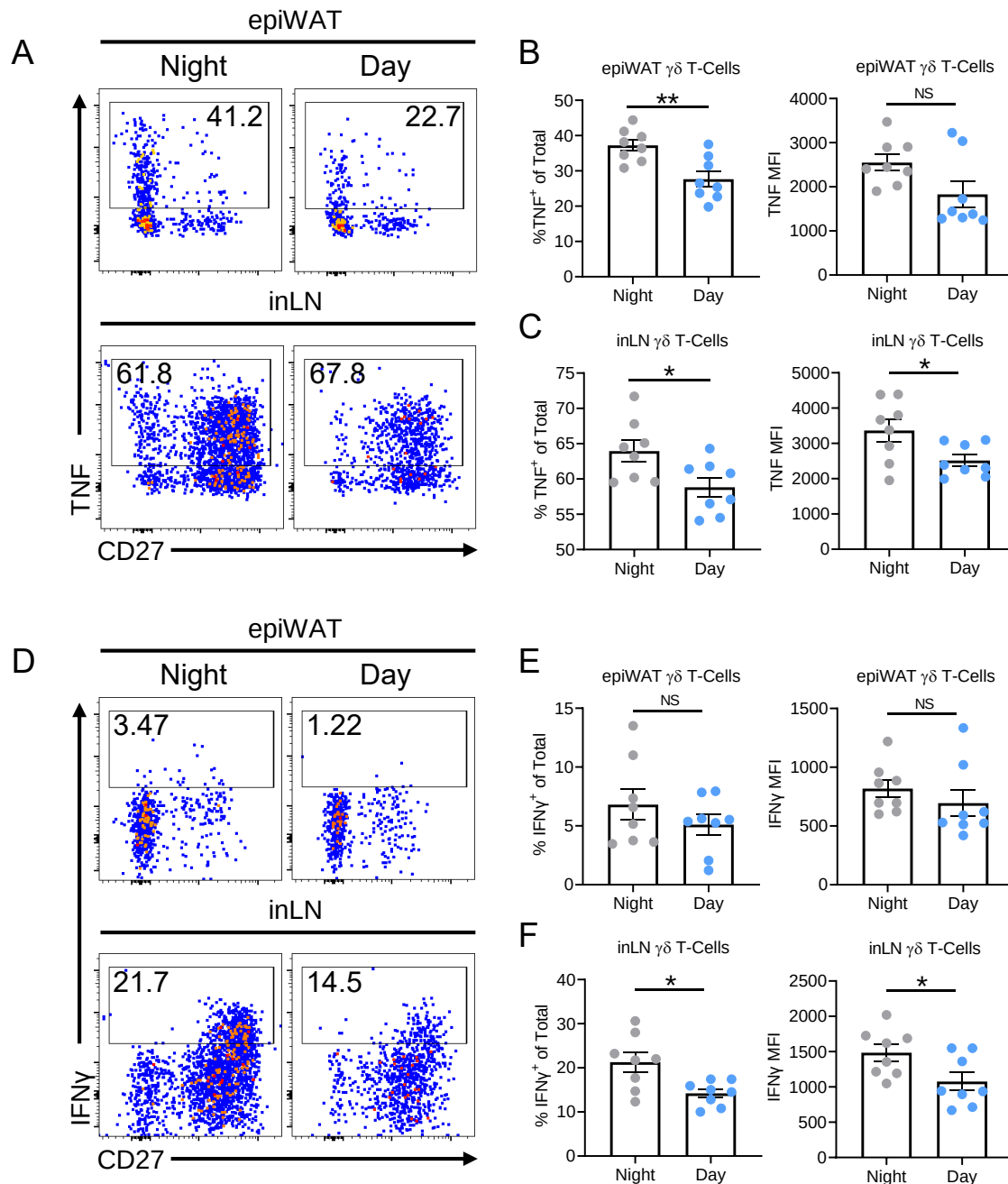


Figure 5.10: Reverse feeding decreases TNF and IFN γ expression by $\gamma\delta$ T cells.

(A) Representative flow plots of TNF expression by epiWAT and inLN $\gamma\delta$ T cells of night (grey) and day (blue) fed mice. **(B)** Percentage (left), and mean fluorescence (right) of TNF expression by adipose and **(C)** inLN $\gamma\delta$ T cells. **(D)** Representative flow plots of IFN γ expression by epiWAT and inLN $\gamma\delta$ T cells of night (grey) and day (blue) fed mice. **(E)** Percentage (left), and mean fluorescence (right) of IFN γ expression by adipose and **(F)** inLN $\gamma\delta$ T cells. Data are representative of three independent experiments, two experiments combined. Data shown as mean $\pm$ SEM, $n = 4-8$ mice per group. Significance was calculated using a two-tailed unpaired students t-test. NS = Non-Significant, * $p < 0.05$, ** $p < 0.01$.

5.4 50% calorie restriction decreases cellular metabolism and function of adipose $\gamma\delta^{17}$ T cells.

We have shown that reverse fed mice consumed 50% less calories, and displayed decreased expression of IL-17A by adipose and lymph node $\gamma\delta$ T cells. As such, we next considered if decreased IL-17A expression in day fed mice was mediated by a self-imposed calorie restriction. Therefore, we placed mice into the metabolic cage system, giving one group 24-hour access to food, while the other group were 50% calorically restricted for 1-week (Figure 5.11A). Calorically restricted mice were fed at the beginning of the active phase (7pm) each day, to avoid circadian disruption as observed by reverse feeding. We found that calorically restricted mice dramatically decreased body mass, which plateaued after a week of calorie restriction (Figure 5.11B, C), similar to what is reported in the literature²²⁷. Calorically restricted mice also lost a significant amount of epiWAT mass compared with *ab libitum* fed control mice (Figure 5.11D, E). Similar to the circadian metabolism of day-fed mice, calorically restricted mice had a disrupted carbon dioxide production, and energy expenditure circadian rhythm, as there was a significant reduction in their full day average compared to *ab libitum* fed controls (Figure 5.12A-D). Surprisingly, calorie restricted mice were as active as *ab libitum* fed mice (Figure 5.12E, F).

As the metabolic rate of calorically restricted mice was decreased, we next examined the effect of calorie restriction on the cellular metabolism and function of adipose $\gamma\delta$ T cells. Surprisingly, there was a significant increase the numbers of both CD27⁺ and CD27⁻ epiWAT $\gamma\delta$ T cells of calorie restricted mice, while calorie restriction caused a decrease in inLN $\gamma\delta$ T cells (Figure 5.13A, B). Calorie restriction decreased the mitochondrial mass, and membrane potential of adipose $\gamma\delta^{17}$ T cells compared to *ab libitum* fed controls. suggesting decreased cellular metabolism (Figure 5.14A, B). Interestingly, calorie restriction had no effect on the mitochondrial mass or membrane potential of CD27⁺ $\gamma\delta$ T cells (Figure 5.14A, B). In agreement with the effect of calorie restriction on $\gamma\delta$ T cell metabolism, IL-17A, IL-17F and TNF expression were decreased by epiWAT $\gamma\delta$ T cells (Figure 5.15A-C). However, calorie restriction had no impact on epiWAT $\gamma\delta$ T cell IFN γ expression (Figure 5.15D). These results suggest that calorie restriction inhibits the function of $\gamma\delta^{17}$ T cells, while preserving the function of $\gamma\delta^{\text{IFN}\gamma}$ T cells in adipose tissue.

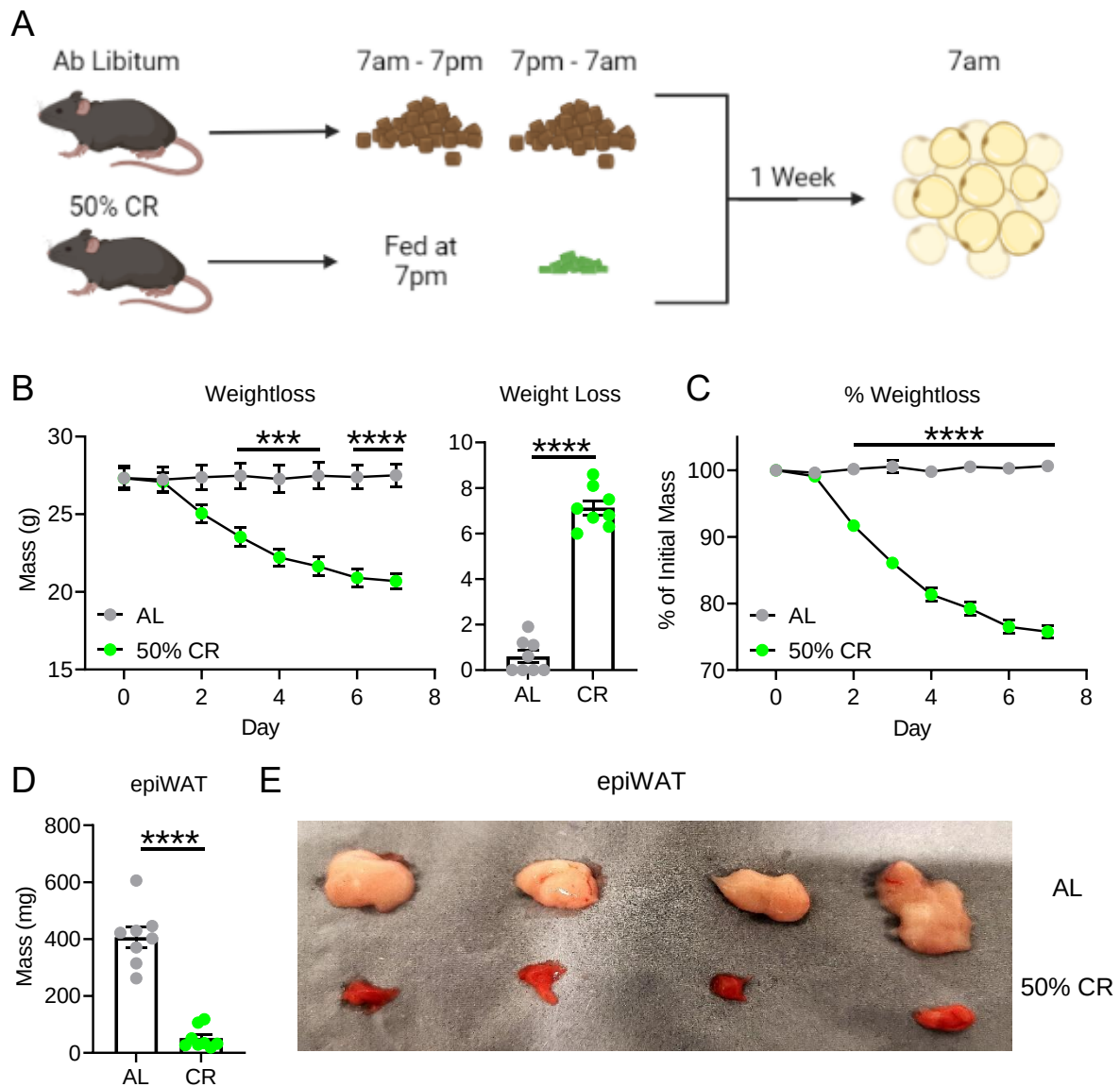


Figure 5.11: Calorie restricted mice lose body and adipose tissue mass.

(A) Diagrammatic representation of experimental setup. **(B)** Body mass of *ab libitum* (grey) and calorically restricted (green) mice over 1 week. **(C)** Percentage weight loss of calorically restricted mice. **(D)** epiWAT mass of *ab libitum* and calorically restricted mice. **(E)** Representative image of epiWAT from *ab libitum* (top), and calorically restricted (bottom) mice. Data are representative of three independent experiments, two experiments combined. Data shown as mean $\pm$ SEM, $n = 8$ mice per group. **(B, C)** Significance was calculated using multiple comparison two-way ANOVA, **(B, D)** two-tailed unpaired students t-tests. *** $p < 0.001$, **** $p < 0.0001$.

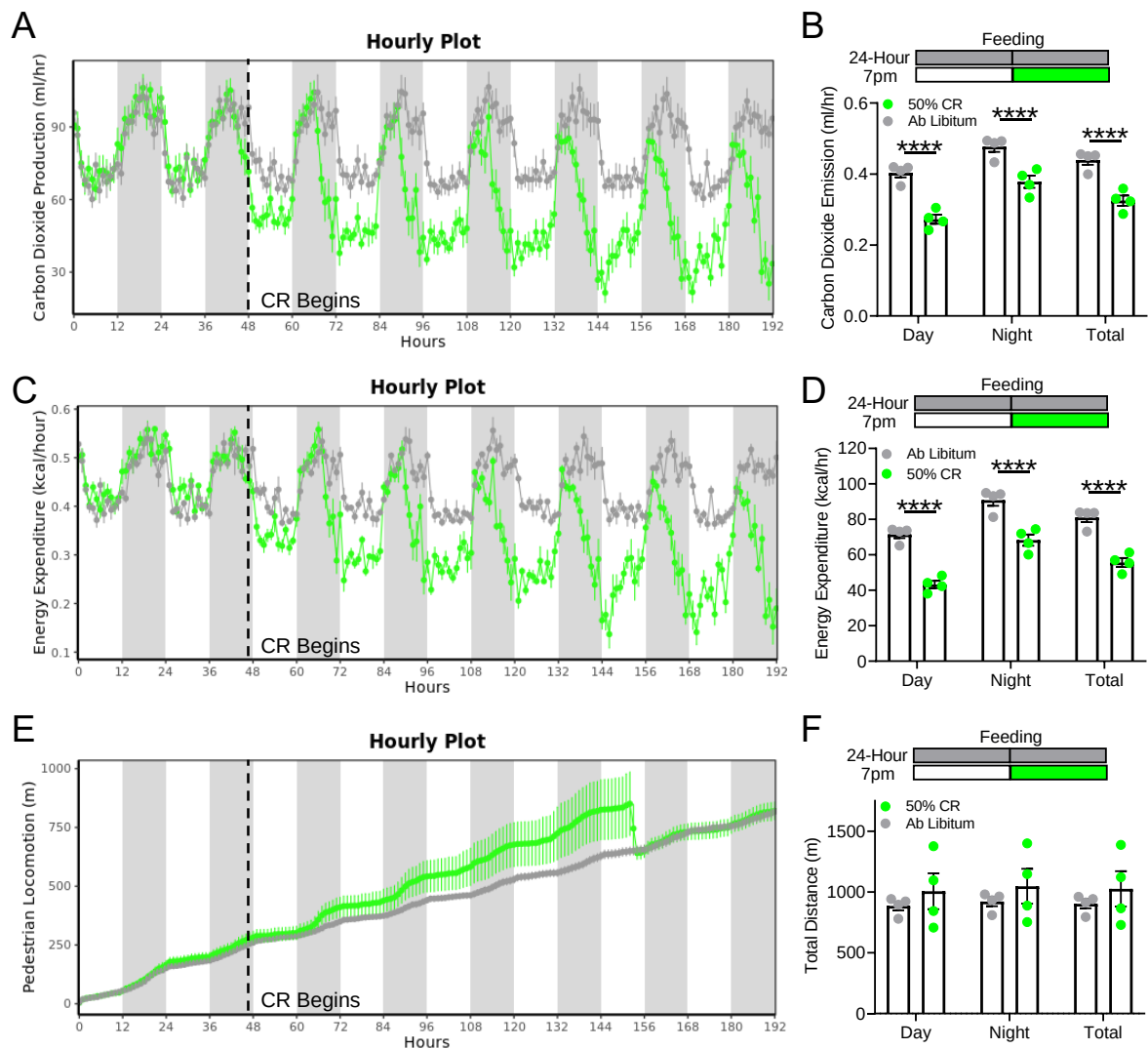


Figure 5.12: Calorie restriction decreases whole body metabolic rate.

(A) Time plot of metabolic rate parameter CO₂ production, **(C)** energy expenditure, **(E)** and activity of *ab libitum* fed mice (grey), versus calorie restricted mice (green). **(B, D, F)** Bar plots of CO₂ emission, energy expenditure, and activity averaged by day, night, and whole day of *ab libitum* and calorically restricted mice. White and grey panels represent light and dark periods respectively. Data are representative of three independent experiments. **(A-F)** Data shown as mean $\pm$ SEM, $n = 4$ mice per group. **(A-F)** Significance was calculated using two group analysis with ANCOVA. *** $p < 0.001$, **** $p < 0.0001$.

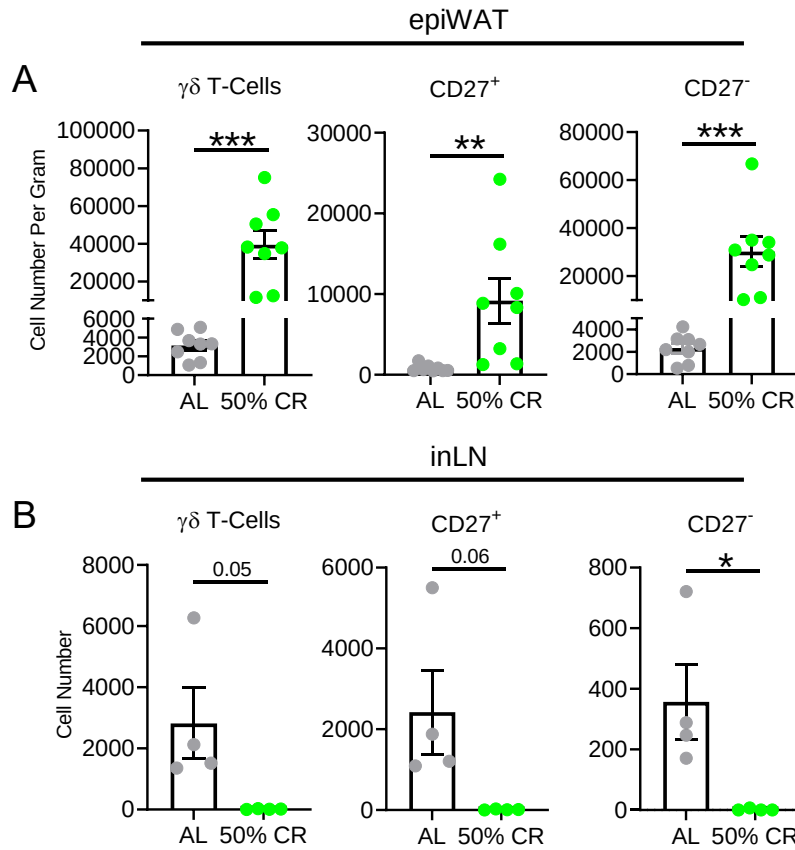


Figure 5.13: Calorie restriction increases adipose, but decreases inLN, $\gamma\delta$ T cell numbers.

(A) Body mass of day (grey) or night (blue) fed mice. **(B)** Mass of epiWAT of day or night fed mice. **(C)** Adipose $\gamma\delta$ T cell numbers (left), CD27⁺ $\gamma\delta$ T cell numbers (middle), and CD27⁻ $\gamma\delta$ T cell numbers (right) from SFD (black) or HFD (red) fed mice. **(D)** inLN $\gamma\delta$ T cell numbers (left), CD27⁺ $\gamma\delta$ T cell numbers (middle), and CD27⁻ $\gamma\delta$ T cell numbers (right) from SFD (black) or HFD (red) fed mice. Data are representative of three independent experiments, two experiments combined. **(A-D)** Data shown as mean $\pm$ SEM, n = 7-8 mice per group. **(A, B)** Significance was calculated using a two-tailed unpaired students t-test. NS = Non-Significant, ** p < 0.05, *** p < 0.01.

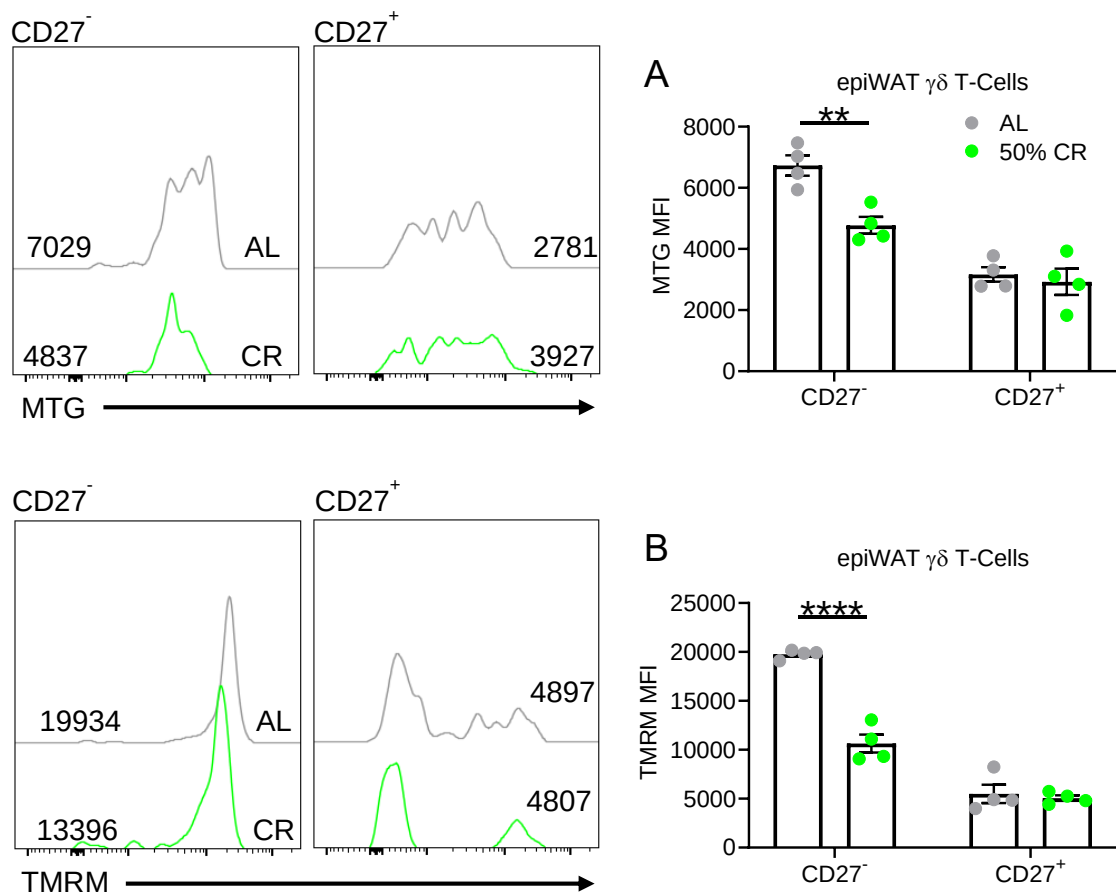


Figure 5.14: Calorie restriction decreases adipose $\gamma\delta^{17}$ T cell mitochondrial metabolism.

(A) Representative histograms of mitochondrial mass (MTG; left), with quantified MFI (right), of adipose $\gamma\delta$ T cells from *ab libitum* (grey) or calorie restricted (green) mice. **(B)** Representative histograms of mitochondrial membrane potential (TMRM; left), with quantified MFI (right), of adipose $\gamma\delta$ T cells from *ab libitum* (grey) or calorie restricted (green) mice. Data are representative of one independent experiment. Data shown as mean $\pm$ SEM, n = 4 mice per group. **(A, B)** Significance was calculated using a two tailed unpaired students t-test. ** p < 0.01, **** p < 0.0001.

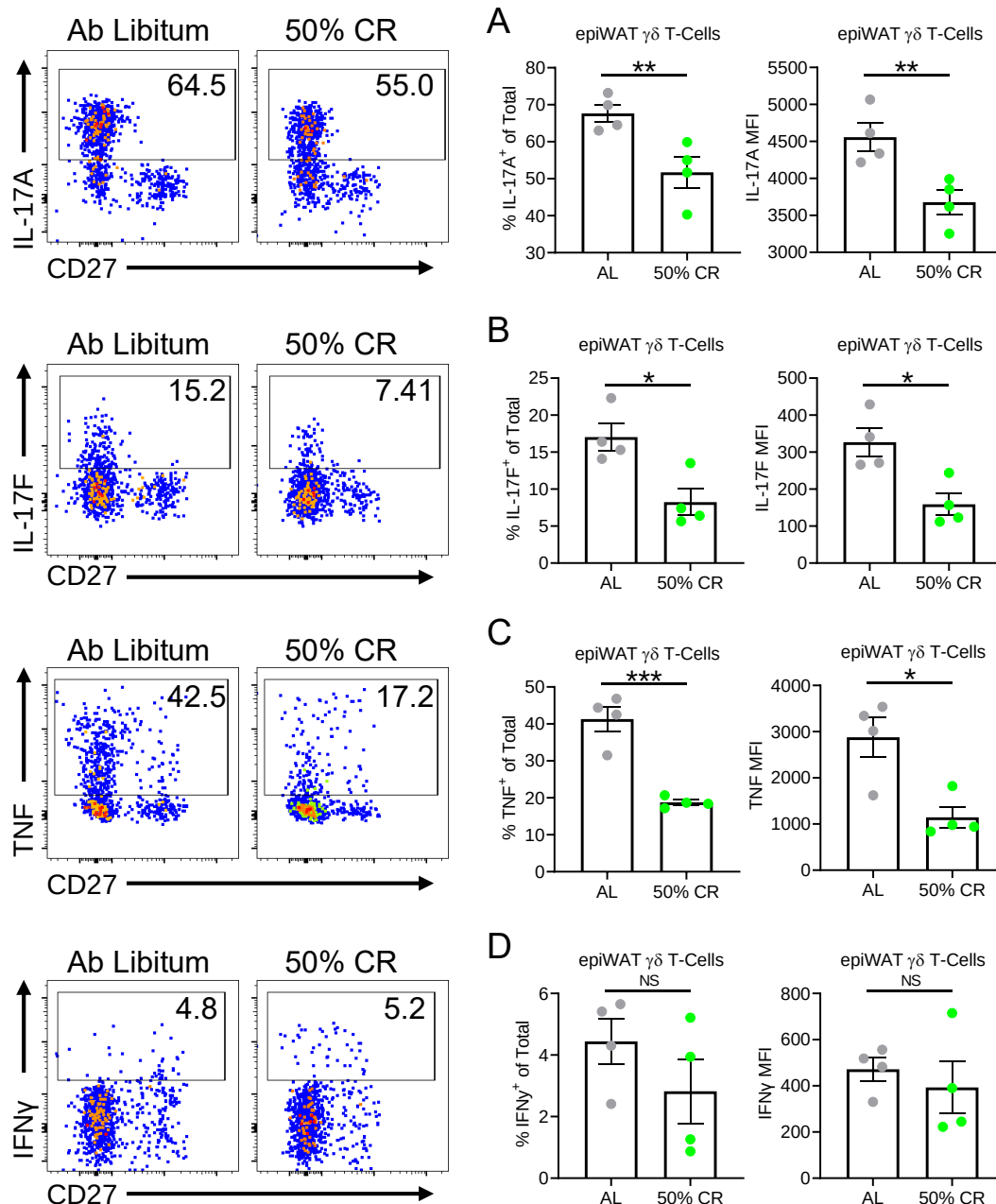


Figure 5.15: Calorie restriction decreases IL-17A, IL-17F, and TNF expression by adipose $\gamma\delta$ T cells.

Representative flow plots of **(A)** IL-17A, **(B)** IL-17F, **(C)** TNF, and **(D)** IFN γ expression by epiWAT $\gamma\delta$ T cells of *ab libitum* (grey) or calorie restricted (green) mice (left). **(A)** Percentage and mean fluorescence of IL-17A (right). **(B)** Percentage and mean fluorescence of IL-17F (right). **(C)** Percentage and mean fluorescence of TNF (right). **(D)** Percentage and mean fluorescence of IFN γ (right). Data are representative of three independent experiments. Data shown as mean $\pm$ SEM, $n = 4$ mice per group. Significance was calculated using a two-tailed unpaired students t-test. NS = Non-Significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.5 HFD enhances EAE progression, while calorie restriction is protective.

EAE is a mouse model of multiple sclerosis, where IL-17A produced by $\gamma\delta$ T cells plays a pathological role in the development and onset of the disease⁷⁶. Having observed that HFD increases IL-17A expression by epiWAT $\gamma\delta$ T cells (Figure 5.5A), while reverse feeding and calorie restriction decrease IL-17A expression (Figure 5.9A; Figure 5.15A), we next examined the pathological role of IL-17A in these feeding regimes. Mice were fed during the night only (control mice), the day only (reverse feeding), or a HFD for 3 weeks, with a final group placed on a 50% calorie restricted diet for one week before the induction of EAE (Figure 5.16A). HFD fed mice had the highest disease severity, in addition to the most weight loss (Figure 5.16B, C). HFD fed mice also had the fastest disease progression, with the least probability of survival (Figure 5.17 D, E). Surprisingly, although day fed mice had the earliest onset of disease (Figure 5.17C), there were no differences in disease severity between the night fed and day fed groups (Figure 5.16B, C; Figure 5.17C-E). Interestingly, calorically restricted mice were completely protected from EAE development (Figure 5.16B, C), with no calorie restricted mouse reaching the severity endpoint (Figure 5.17 B-E). These data suggest that IL-17A regulation by dietary intake plays a role in the pathogenesis of EAE, with calorie restriction playing a protective role.

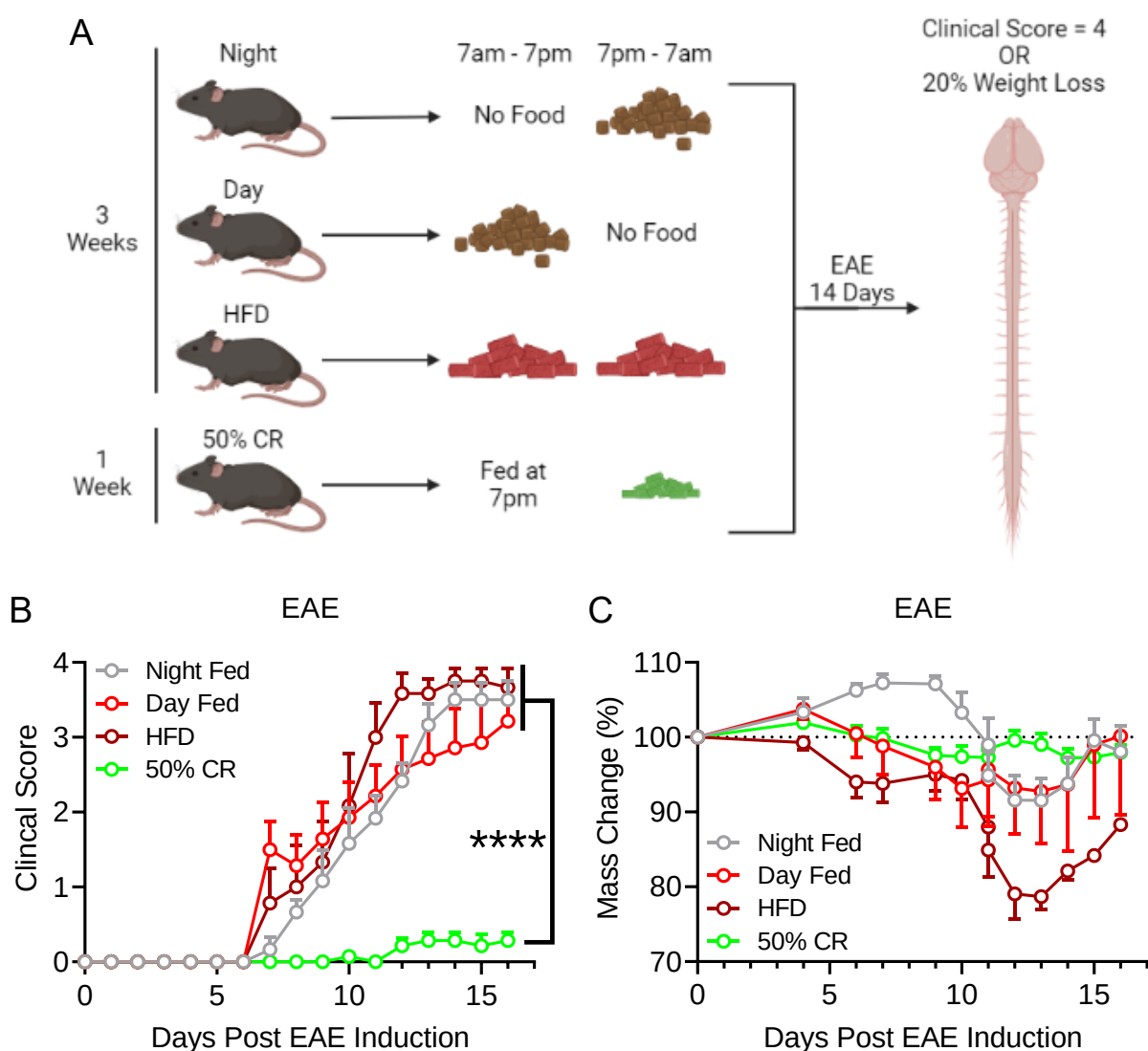


Figure 5.16: HFD enhances EAE progression, while calorie restriction is protective.

(A) Diagrammatic representation of experimental setup. **(B)** EAE scores of night fed (grey), day fed (red), HFD fed (crimson), and calorie restricted (green) mice. **(C)** Body weight of night fed (grey), day fed (red), HFD fed (crimson), and calorie restricted (green) mice post EAE induction. Data are representative of one independent experiment. Data shown as mean $\pm$ SEM, $n = 7$ mice per group. **(B)** Significance was calculated using a two-way ANOVA with Turkey post hoc analysis. **** $p < 0.0001$. *Experiment performed in collaboration with Caroline Sutton.*

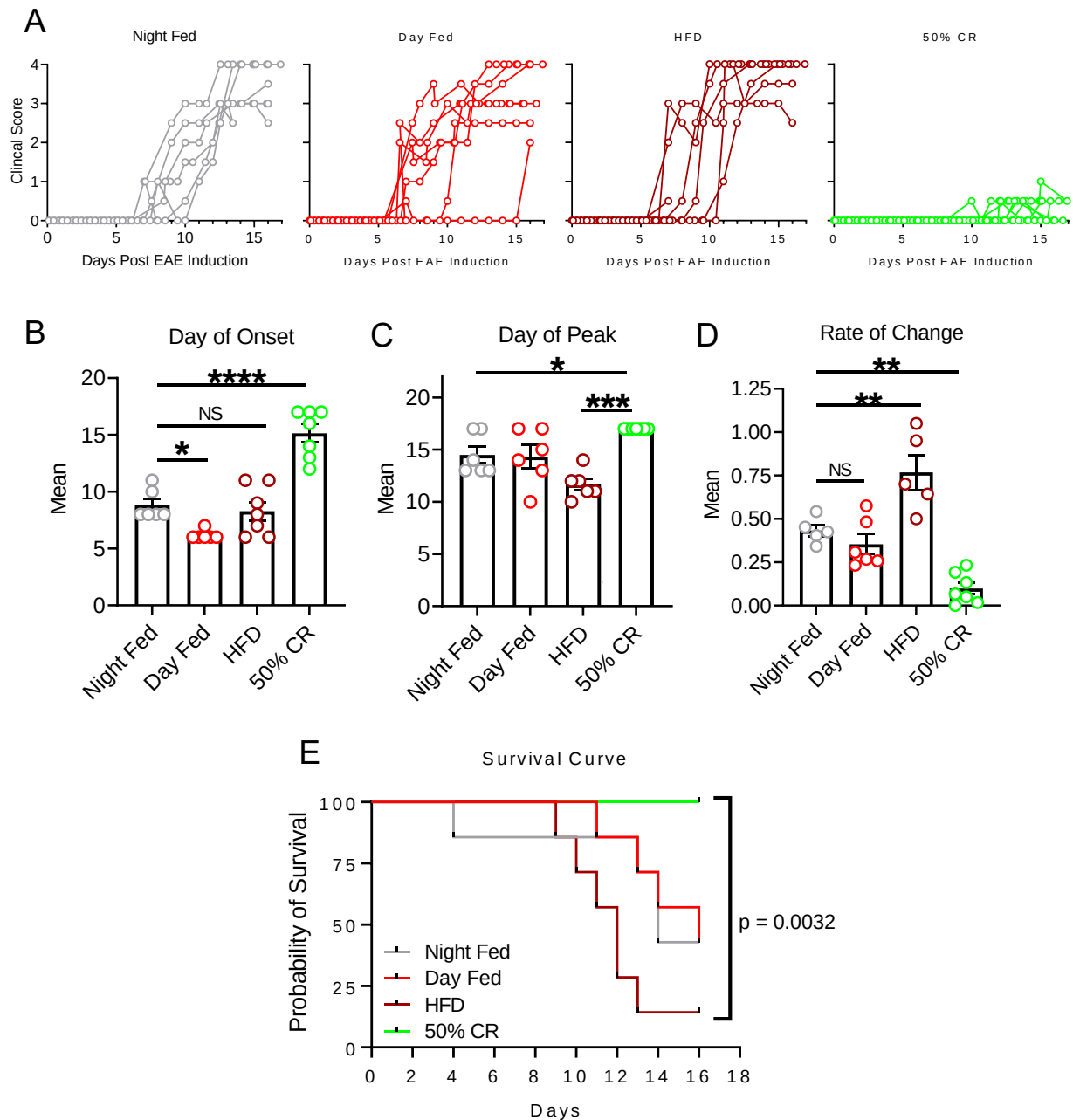


Figure 5.17: HFD enhances EAE susceptibility, while calorie restriction promotes survival.

(A) EAE scores of night fed (grey), day fed (red), HFD fed (crimson), and calorie restricted (green) mice. **(B)** Mean day of disease onset between feeding regimes. **(C)** Mean peak day of disease progression between feeding regimes. **(D)** Disease progression measured by rate of change (disease onset to disease peak) between feeding regimes. **(E)** Kaplan-Meier survival curve post EAE induction between feeding regimes. Data are representative of one independent experiment. Data shown as mean $\pm$ SEM, $n = 6-7$ mice per group. **(B-D)** Significance was calculated using a one-way ANOVA, **(E)** Simple survival analysis with Gehan-Breslow-Wilcoxon test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.6 T cells migrate to the bone marrow during calorie restriction, which is protective against EAE.

Upon immunization with an antigen for myelin, T cells are activated in the periphery, and migrate to the CNS where they damage the brain and spinal cord²²⁸. Having observed that calorie restricted mice have less lymph node $\gamma\delta$ T cells (Figure 5.13B), in addition to protection against the development of EAE (Figure 5.16B), we next investigated why calorie restricted mice are protected. We induced EAE *ab libitum* and calorically restricted mice, then harvested draining lymph nodes (dLN) and peritoneal lymphocytes (PEC) 3-days post EAE induction to investigate cell migratory dynamics (Figure 3.18A). Again, we observed that calorie restricted mice were protected against the development of EAE (Figure 5.18A-D). We found no significant difference in the percentage of dLN or PEC T cells between *ab libitum* and calorically restricted mice 3-days post EAE induction (Figure 5.19A, B). However, T cells numbers were dramatically decreased in the dLNs and PEC of calorie restricted mice (Figure 5.19A, B). This was not limited to any single lymphocyte population, as both CD4⁺ T cell, and $\gamma\delta$ T cell numbers were decreased in the dLNs and PEC of calorie restricted mice 3-days after the induction of EAE (Figure 5.19C, D; Figure 5.20A, B). Lymph node $\gamma\delta^{17}$ T cells are the propagators of EAE onset⁷⁶. We found that both $\gamma\delta^{17}$ T cells, and $\gamma\delta^{\text{IFN}\gamma}$ T cells, were decreased in the dLNs of calorie restricted mice, but only $\gamma\delta^{\text{IFN}\gamma}$ T cells were decreased in the PEC.

Upon translocation to the CNS, activated T cells cause damage to neuronal axons, ultimately leading to neurological symptoms²²⁸. Therefore, we harvested the brain and spinal cord 10-days post EAE induction from *ab libitum* and calorically restricted mice. Calorically restricted mice displayed decreased infiltration of T cells into the brain and spinal cord 10-days post EAE induction, compared to *ab libitum* fed controls (Figure 5.21A, B). This was not limited to a specific population of cells, as both CD4⁺ and $\gamma\delta$ T cell numbers were decreased in the brain and spinal cord of calorie restricted mice (Figure 5.21C, D; Figure 5.22A, B). Both $\gamma\delta^{17}$ T cells, and $\gamma\delta^{\text{IFN}\gamma}$ T cells showed decreased infiltration in the brain of calorie restricted mice, however $\gamma\delta^{17}$ T cell numbers were specifically decreased in the spinal cord 10-days post EAE induction (Figure 5.22C, D). The clinical score of EAE displays a strong correlation with the number of infiltrating T cells into the brain and spinal cord (Figure 5.23A-F). Interestingly, we observed little infiltration of immune cells into these regions in the calorie restricted mice which showed no development of EAE. These data suggest that calorie restriction prevents induction and development of EAE due to a decrease in dLN and peritoneal T cell numbers.

Calorie restriction causes a heavy metabolic burden due to a lack of energy from food consumption. It has been previously described that the bone marrow harbours a unique immunological niche¹¹⁵. Calorie restriction promotes the survival of memory CD8⁺ T cells through migration to the bone marrow where they downregulate their cellular metabolism¹¹⁵. We therefore investigated if there was an increase in the proportion and numbers of lymphocytes in the bone marrow of calorically restricted mice, 3-days post EAE induction (Figure 3.18A). We found that T cell, CD4⁺ T cell, and $\gamma\delta$ T cell numbers were all increased in the bone marrow of calorically restricted mice compared to *ab libitum* fed controls (Figure 5.24 A-D). These data suggest that T cells migrate to the bone marrow during calorie restriction, which prevents migration to the CNS during EAE.

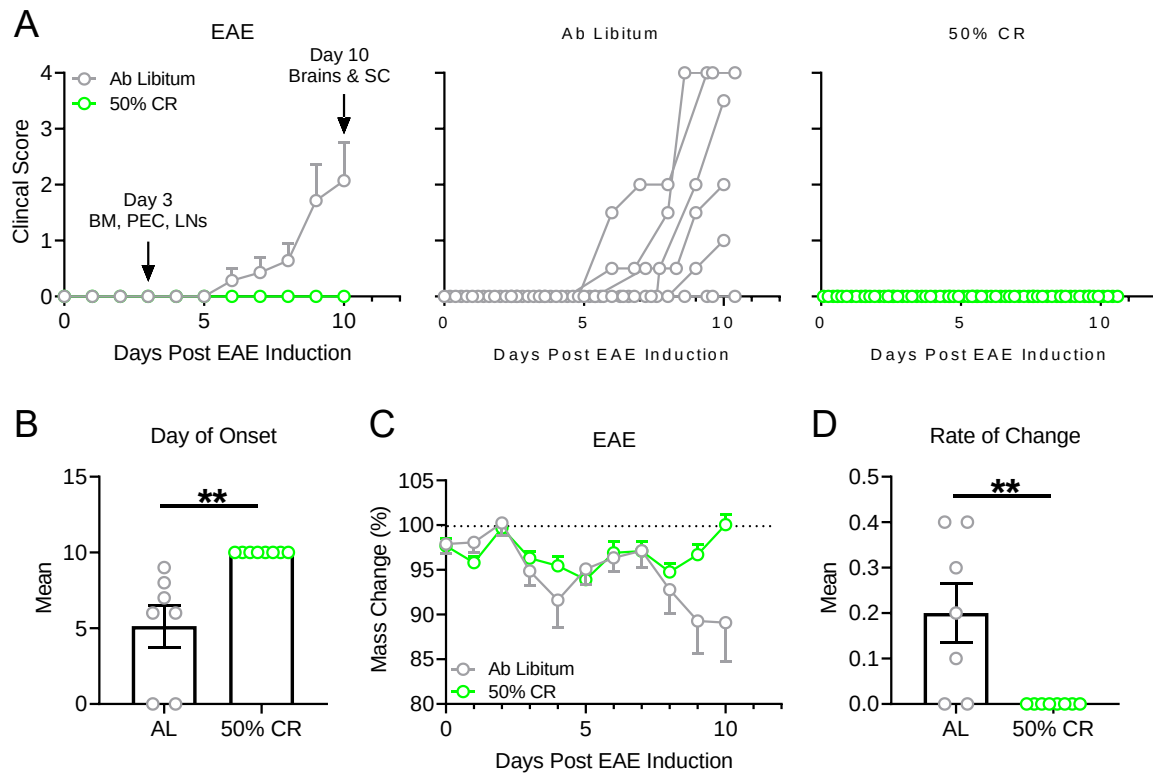


Figure 5.18: Calorie restriction is protective against the pathogenesis of EAE.

(A) EAE scores of *ab libitum* (grey) and calorie restricted (green) mice, representing timing of harvested tissues. **(B)** Mean peak day of disease progression between feeding regimes. **(C)** Body weight of *ab libitum* (grey) and calorie restricted (green) mice post EAE induction. **(D)** Disease progression measured by rate of change (disease onset to disease peak) between feeding regimes. Data are representative of two independent experiments. Data shown as mean $\pm$ SEM, $n = 7$ mice per group. **(B, C)** Significance was calculated using a two-tailed students t-test. ** $p < 0.01$. Experiment performed in collaboration with Caroline Sutton.

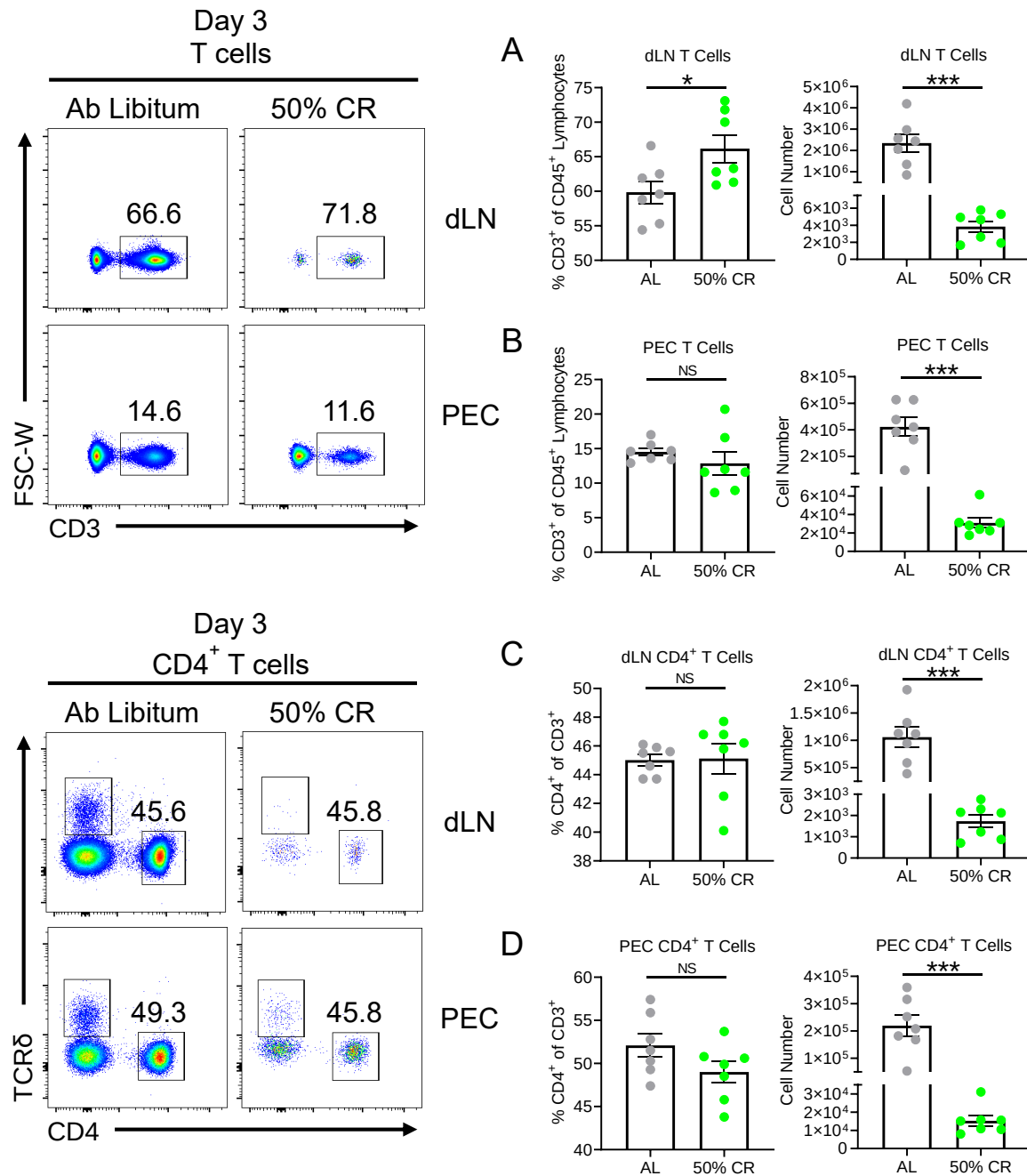


Figure 5.19: Calorie restriction decreases dLN and PEC T cell numbers.

(A, B) Representative flow plots of T cells from dLNs and PEC of *ab libitum* fed, or calorie restricted mice 3-days post EAE induction (left); (A) dLN and (B) PEC T cell percentages and absolute numbers (right). (C, D) Representative flow plots of CD4⁺ T cells from dLNs and PEC of *ab libitum* fed, or calorie restricted mice 3-days post EAE induction (left); (C) dLN and (D) PEC CD4⁺ T cell percentages and absolute numbers (right). Data are representative of three independent experiments. Data shown as mean ± SEM, n = 7 mice per group. Significance was calculated using multiple two-tailed unpaired students t-tests. NS = Non-Significant, * p < 0.05, *** p < 0.001.

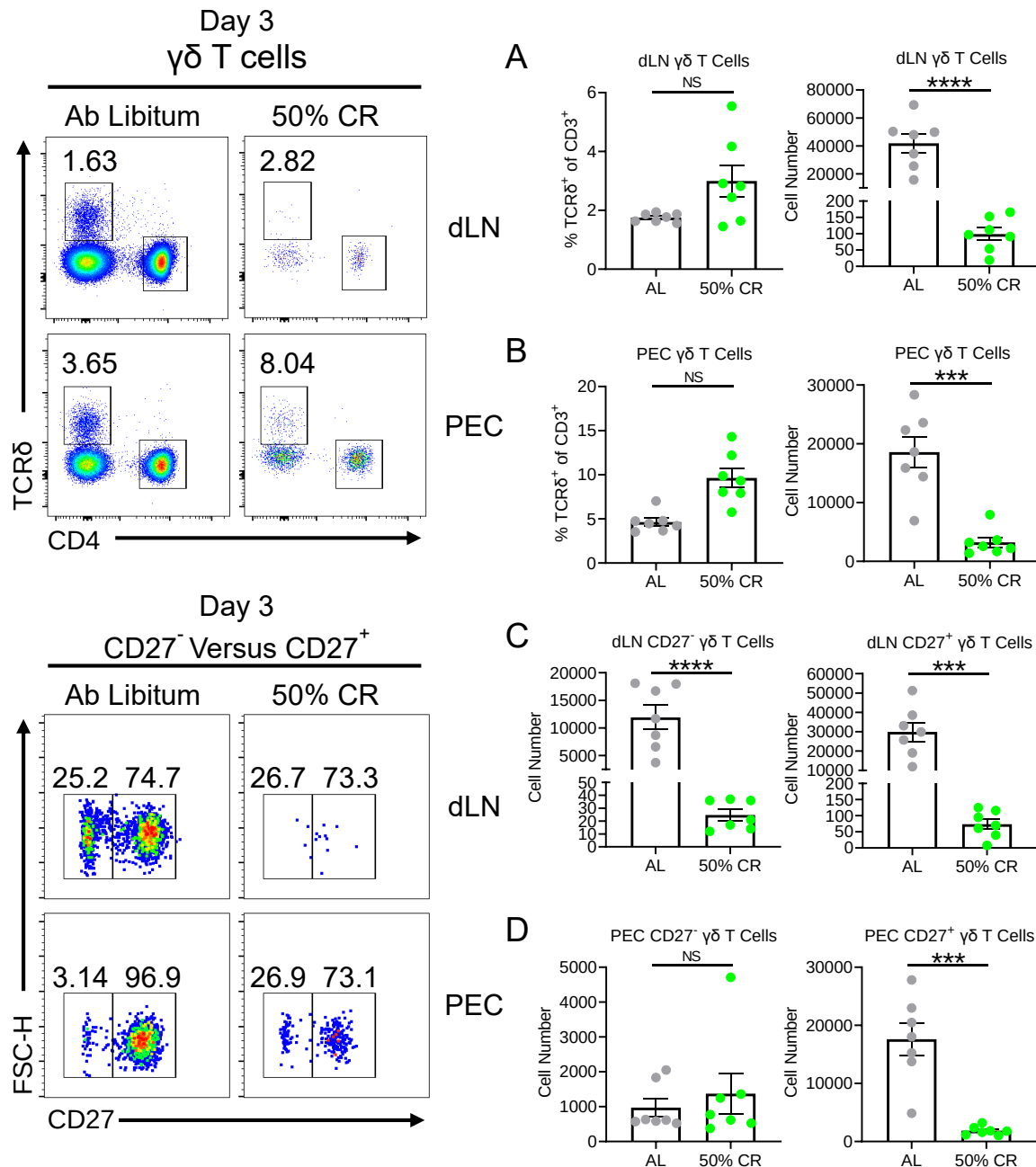


Figure 5.20: Calorie restriction decreases dLN and PEC $\gamma\delta$ T cell numbers.

(A, B) Representative flow plots of $\gamma\delta$ T cells from dLNs and PEC of *ab libitum* fed, or calorie restricted mice 3-days post EAE induction (left); (A) dLN and (B) PEC $\gamma\delta$ T cell percentages and absolute numbers (right). (C, D) Representative flow plots of $\gamma\delta$ T cell subsets from dLNs and PEC of *ab libitum* fed, or calorie restricted mice 3-days post EAE induction (left); (C) dLN and (D) PEC $\gamma\delta$ T cell subset percentages and absolute numbers (right). Data are representative of three independent experiments. Data shown as mean $\pm$ SEM, n = 7 mice per group. Significance was calculated using multiple two-tailed unpaired students t-tests. NS = Non-Significant, *** p < 0.001, **** p < 0.0001.

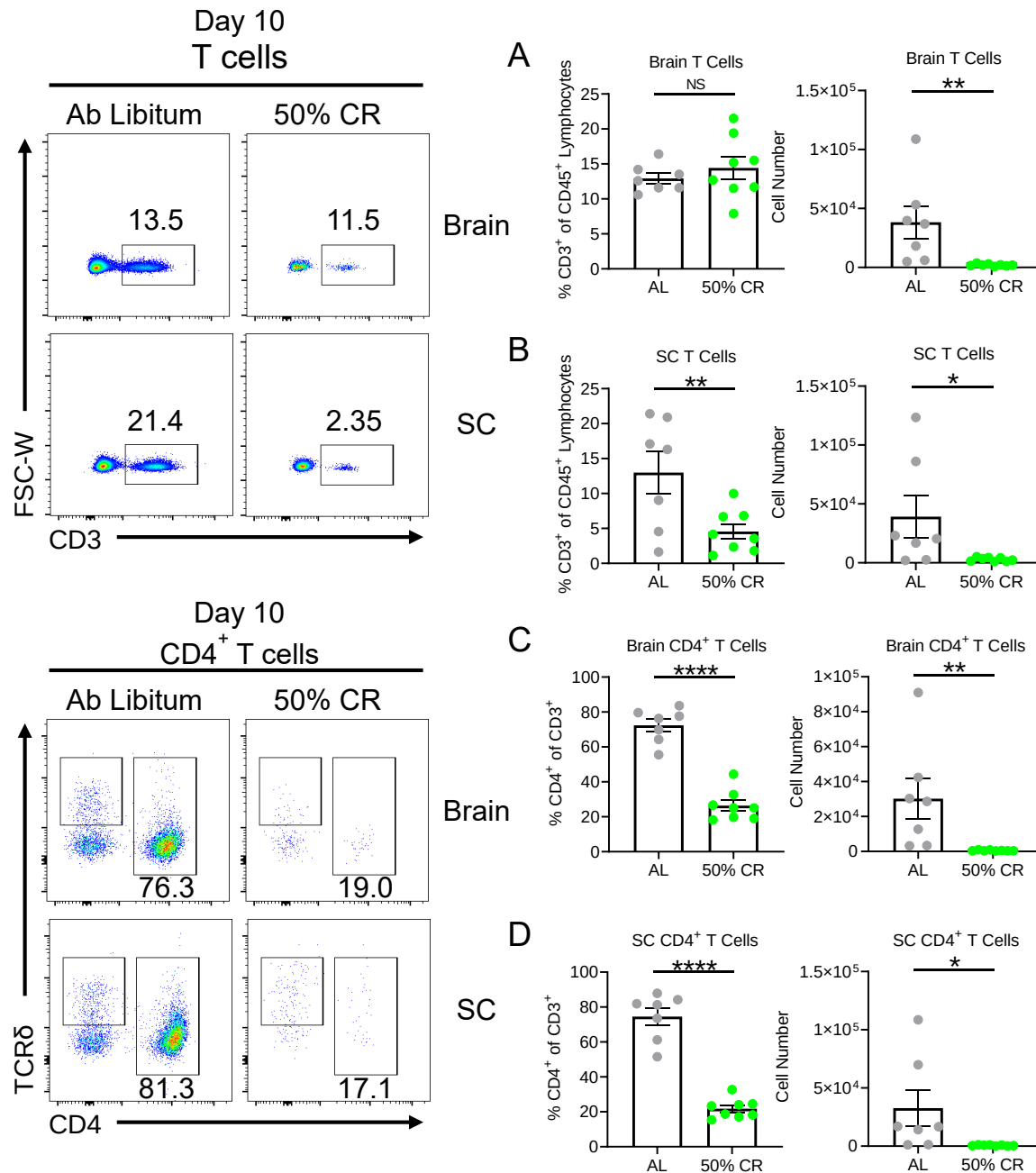


Figure 5.21: Calorie restriction decreases brain and SC T cell infiltration.

(A, B) Representative flow plots of T cells from brain and SC of *ab libitum* fed, or calorie restricted mice 10-days post EAE induction (left); (A) Brain and (B) SC T cell percentages and absolute numbers (right). (C, D) Representative flow plots of CD4⁺ T cells from brain and SC of *ab libitum* fed, or calorie restricted mice 10-days post EAE induction (left); (C) Brain and (D) SC CD4⁺ T cell percentages and absolute numbers (right). Data are representative of three independent experiments. Data shown as mean $\pm$ SEM, n = 7 mice per group. Significance was calculated using multiple two-tailed unpaired students t-tests. NS = Non-Significant, * p < 0.05, ** p < 0.01, **** p < 0.0001.

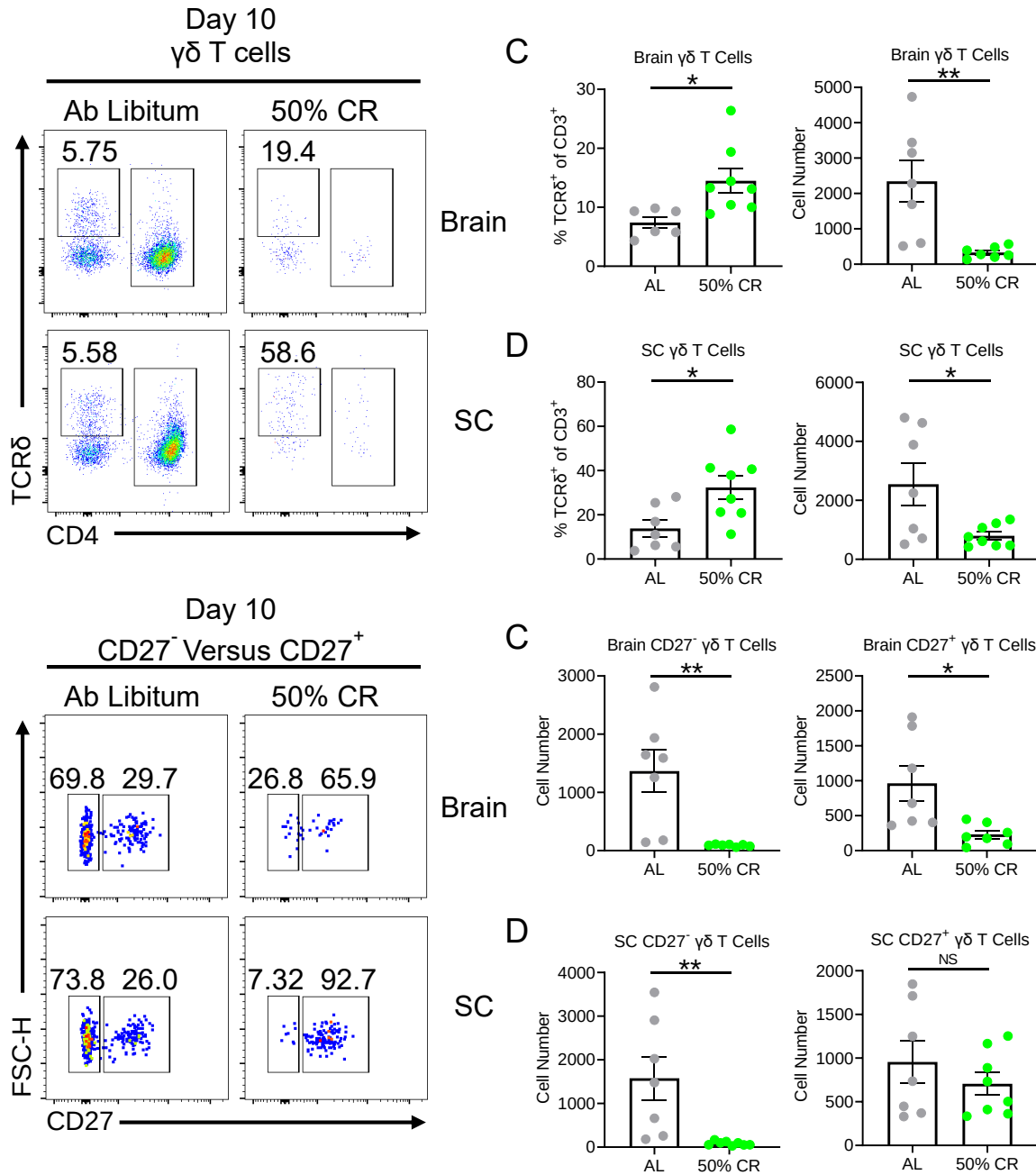


Figure 5.22: Calorie restriction decreases brain and SC $\gamma\delta$ T cell infiltration.

(A, B) Representative flow plots of $\gamma\delta$ T cells from brain and SC of *ab libitum* fed, or calorie restricted mice 10-days post EAE induction (left); (A) Brain and (B) SC $\gamma\delta$ T cell percentages and absolute numbers (right). (C, D) Representative flow plots of $\gamma\delta$ T cell subsets from brain and SC of *ab libitum* fed, or calorie restricted mice 10-days post EAE induction (left); (C) Brain and (D) SC $\gamma\delta$ T cell subset percentages and absolute numbers (right). Data are representative of three independent experiments. Data shown as mean $\pm$ SEM, $n = 7$ mice per group. Significance was calculated using multiple two-tailed unpaired students t-tests. NS = Non-Significant, * $p < 0.05$, ** $p < 0.01$.

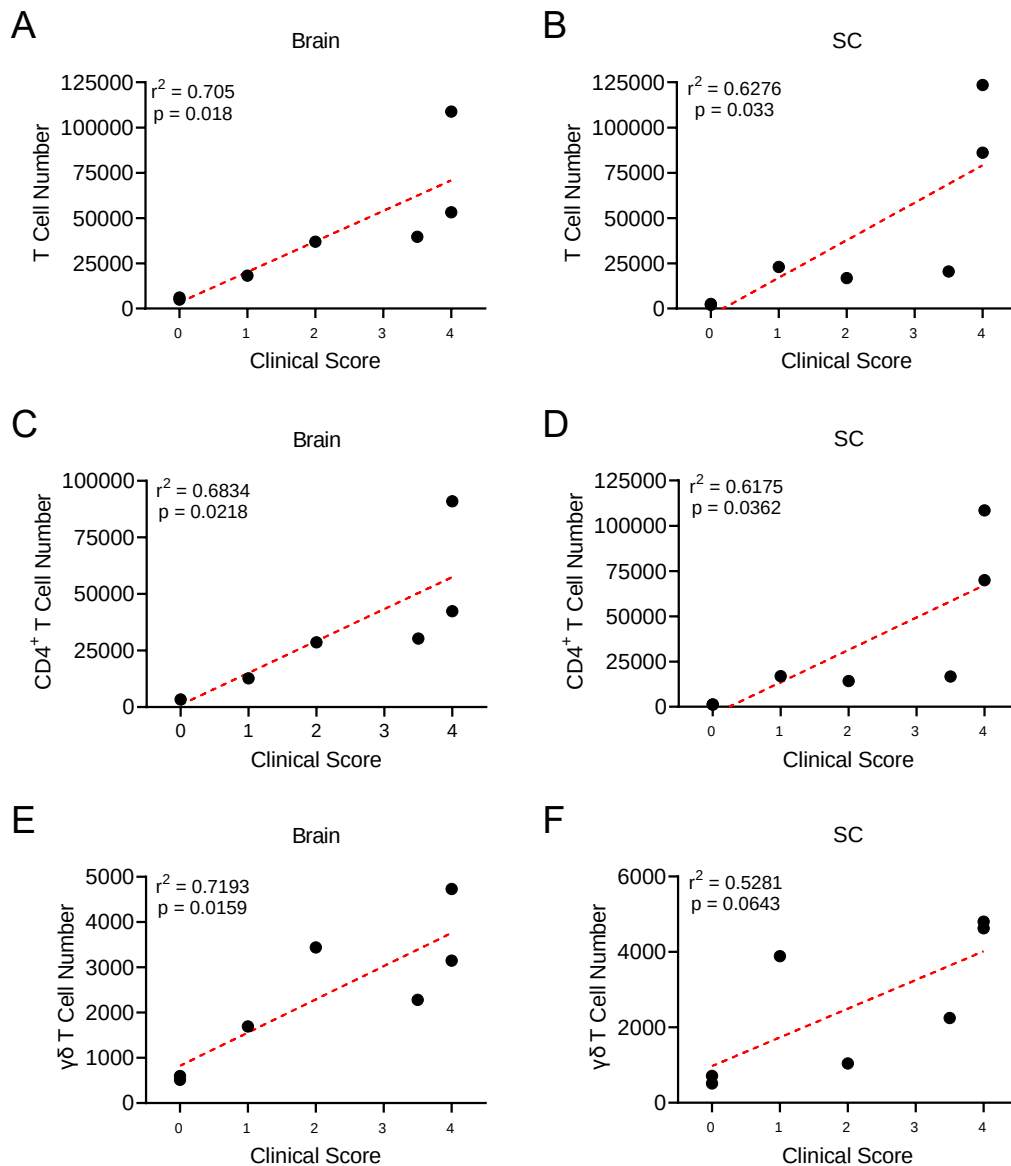


Figure 5.23: Brain and SC T cell infiltration directly correlates with disease severity.

(A, B) Correlation analysis between EAE clinical score and T cell infiltration of the **(A)** Brain and **(B)** SC of *ab libitum* fed mice. **(C, D)** Correlation analysis between EAE clinical score and CD4⁺ T cell infiltration of the **(C)** Brain and **(D)** SC of *ab libitum* fed mice. **(E, F)** Correlation analysis between EAE clinical score and $\gamma\delta$ T cell infiltration of the **(E)** Brain and **(F)** SC of *ab libitum* fed mice. Data are representative of two independent experiments. Data shown as mean $\pm$ SEM, $n = 7$ mice per group. Significance was calculated using a simple linear regression model.

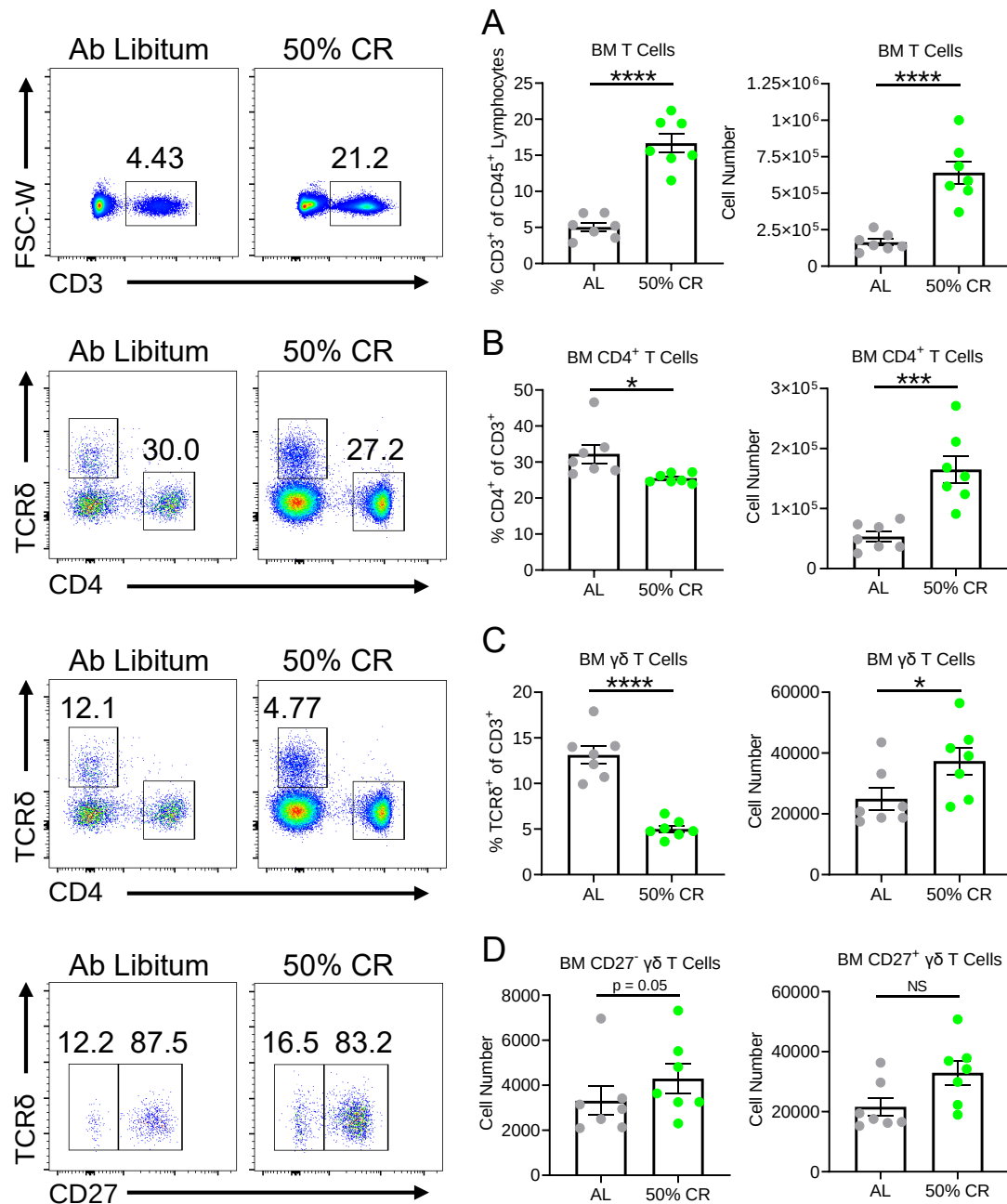


Figure 5.24: Calorie restriction increases γδ T cell numbers in the bone marrow.

Representative flow plots of **(A)** T cells, **(B)** CD4⁺ T cells, **(C)** γδ T cells, and **(D)** γδ T cell subsets from BM of *ab libitum* fed, or calorie restricted mice 3-days post EAE induction (left). **(A)** BM T cell percentages and absolute numbers (right). **(B)** BM CD4⁺ T cell percentages and absolute numbers (right). **(C)** γδ T cell percentages and absolute numbers (right). **(D)** γδ T cell subset absolute numbers (right). Data are representative of three independent experiments. Data shown as mean ± SEM, n = 7 mice per group. Significance was calculated using multiple two-tailed unpaired students t-tests. NS = Non-Significant, * p < 0.05, *** p < 0.001, **** p < 0.0001.

5.7 Antibiotic treatment decreases whole body metabolism.

Dietary intake influences the distribution of microbes in the gut, and a change in diet can rapidly restructure the gut microbiome²²⁹. Commensal microbiota play a homeostatic role in the differentiation and regulation of IL-17A producing cells in the gut²³⁰. Accumulation of Th17 T cells by the gut microbiota has been implicated in the development of EAE²³¹. Considering that antibiotic treatment prevents EAE disease onset²³², we next investigated if disruption of the microbiome decreases the metabolic rate of mice, similar to a calorie restricted diet, and if a decrease in metabolic rate aides EAE prevention. We ablated the gut microbiome by administering antibiotics in the drinking water of mice for 6-days, before returning them to normal drinking water (Figure 5.25A). We found that antibiotic treatment decreased oxygen consumption, indicating decreased metabolic rate (Figure 5.25B, C). Additionally, returning antibiotic treated mice to normal drinking water recovered the oxygen consumption to levels similar to that of control mice (Figure 5.25B, C). These data suggest that the decreased metabolic rate observed in calorie restricted mice, may in part be due to a restructure of the gut microbiome.

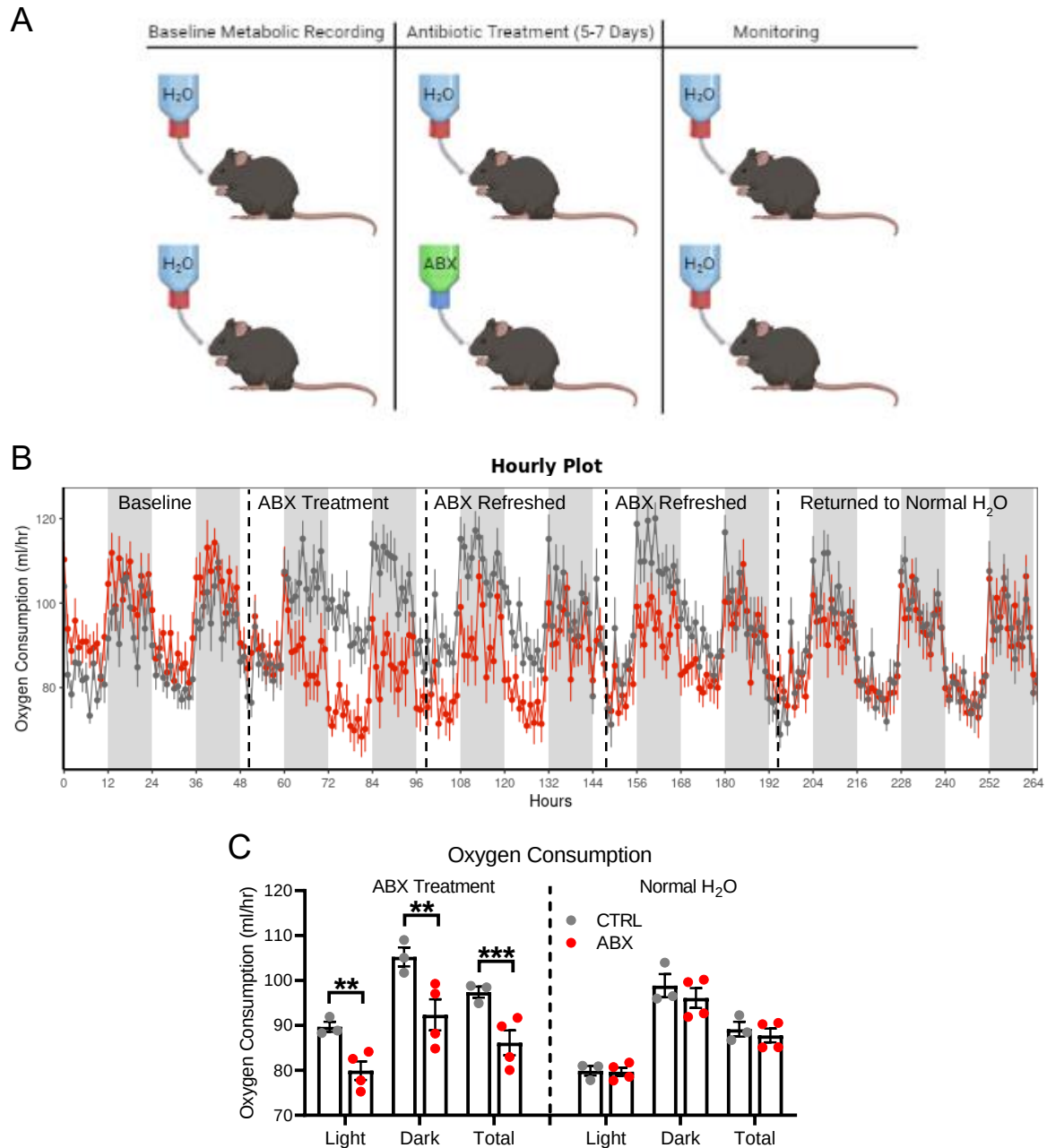


Figure 5.25: Antibiotic treatment decreases whole-body metabolism of mice.

(A) Diagrammatic representation of experimental design. **(B)** Time plot of metabolic rate parameter O_2 consumption of normal water (grey), versus antibiotic treated mice (ABX; red). **(C)** Bar plots of O_2 consumption averaged by day, night, and whole day of normal water and antibiotic treated mice, during and after antibiotic treatment. Data are representative of two independent experiments. Data shown as mean $\pm$ SEM, $n = 4$ mice per group. Significance was calculated using two group analysis with ANCOVA. ** $p < 0.01$, *** $p < 0.001$.

5.8 Discussion

Modern day lifestyle contributes to a multitude of factors which disrupt circadian rhythm. These include high-fat high-sugar diets, excess light at night, and physical inactivity, which lead to the development of obesity and metabolic disease^{233,234}. The rate of global obesity is at an all-time high²³⁵, and this increases the risk of developing cancer⁶⁵, cardiovascular²³⁶, neurological^{237,238}, and metabolic disease²³⁹. As such, it is becoming increasingly important to find therapeutic interventions in the treatment and prevention of obesity and associated comorbidities. Here we show that HFD feeding disrupts circadian nutrient utilisation. Typically, mice fed a SFD alternate between using carbohydrate metabolism at night while active, and fatty acid metabolism during the day when inactive, as measured by RER. HFD feeding abolishes the circadian rhythm of RER, causing a whole body metabolic switch to constant 24-hour lipid metabolism²⁴⁰. The molecular switch to lipid metabolism is driven in part by the disruption of molecular clock gene expression in adipocytes¹³³. Moreover, HFD feeding disrupts adipocyte precursor cell diurnal proliferation, enhancing adipocyte proliferation and expanding WAT²⁴¹.

HFD feeding enhances IL-17A expression by $\gamma\delta$ T cells⁶⁸. Furthermore, we found that HFD disrupted IL-17A and ROR γ t circadian rhythm of adipose $\gamma\delta$ T cells, which may impact adipocyte cellular metabolism. IL-17A has been implicated in the development of diet induced obesity, as administration of ROR γ t small molecule inhibitors induced weight loss in obese mice³². Moreover, specific deletion of adipocyte IL-17RA was sufficient to prevent the development of diet-induced obesity³². However, the source of dietary lipids, rather than obesity alone, appears to impact adipose $\gamma\delta$ T cell function, as a ketogenic diet expands a metabolically protective population of adipose $\gamma\delta$ T cells⁷⁵. IL-17A derived from $\gamma\delta$ T cells plays a pathological role in the initiation of EAE⁷⁶. Additionally, HFD exacerbates EAE pathology, and increases brain infiltration by Th17 cells, enhancing Th17 cell IL-17A expression. We found that a 3-week HFD feeding regimen was detrimental in a mouse model of multiple sclerosis, where HFD fed mice were more susceptible to disease, which may be caused by a disrupted IL-17A circadian rhythm induced by HFD feeding²⁴².

Shift workers display an increased preference for high-fat, high-calorie foods, in addition to irregular meal timings^{218,219,243}. Here, we show that reversing the timing of food availability of mice, detrimentally impacted their circadian whole body metabolism. The majority of metabolic health studies focus on the caloric content of food. However, the importance of the timing of food consumption is becoming more clear. HFD feeding disrupts the time of day at which mice eat, increasing the tendency to eat during the inactive phase²⁴⁴.

Interestingly, forcing HFD fed mice to adhere to a normal eating schedule was sufficient to limit excess weight gain, and adiposity, normally observed in a HFD²⁴⁵. Furthermore, SFD fed mice forced to eat during the day (inactive phase) gained weight over time²⁴⁶. In contrast, we observed that reverse feeding did not cause body or fat mass loss, however reverse fed mice consumed 50% less calories. Reverse fed mice decreased their whole body metabolism, which may prevent weight loss which is usually associated with lower food consumption. In contrast to a HFD feeding regimen which enhanced IL-17A expression by adipose $\gamma\delta$ T cells, reverse feeding decreased IL-17A expression. Although reverse fed mice displayed decreased IL-17A expression, they showed a similar EAE disease progression compared to night fed controls, but earlier disease onset. This suggests that although reverse feeding decreases IL-17A expression, reverse feeding disrupts circadian homeostasis sufficiently enough to promote auto-inflammatory disease. Shift work has been associated with increased risk of developing multiple sclerosis²⁴⁷. Considering the global number of people participating in shift work is increasing²¹³, dietary interventions to align the timing of feeding may become crucial to reduce the incidence of disease associated with shift work.

Although reverse fed mice had similar EAE disease progression compared to night fed control mice, they ate 50% less calories and had a decreased metabolic rate. As such, we investigated whether decreased IL-17A expression by adipose $\gamma\delta$ T cells was mediated by a calorie deficit. Indeed, we found that mice fed a 50% calorie restricted diet during their active phase displayed decreased IL-17A expression by adipose $\gamma\delta$ T cells. Obesity expands IL-17A producing cells in adipose tissue, which propagates systemic inflammation caused by a high fat diet²⁴⁸, and may be used as a potential biomarker to characterise metabolic disease risk²⁴⁹. In contrast, many studies have demonstrated the anti-inflammatory²⁵⁰, and metabolically protective effects of calorie restriction²⁵¹. A 6-month calorie restricted diet in male participants presenting with metabolic syndrome, attenuated inflammatory markers and decreased circulating pro-inflammatory cytokines, including levels of IL-17A²⁵⁰. Furthermore, decreased caloric intake was sufficient to decrease IL-17A levels in the adipose tissue of mice genetically modified to be obese (*ob/ob* mice)²⁵¹. Together these studies suggest that calorie restriction enhances a systemic immunosuppressive environment.

Unsurprisingly, the immunosuppressive role of calorie restriction has been investigated for the treatment of autoimmune diseases, such as multiple sclerosis¹¹⁴. We found that calorie restricted mice were protected from the onset and development of EAE. In agreement with our observations, a previous study has shown that calorie restriction attenuates EAE in mice by decreasing inflammation and demyelination in the brain¹¹⁴. This was associated

with decreased levels of circulating IL-6, however there was no difference in the priming of T cells during EAE induction¹¹⁴. Furthermore, lymphocytes from calorie restricted mice that were activated with a myelin antigen *ex vivo*, could transfer EAE to recipient mice¹¹⁴. We found that calorie restriction dramatically decreased the numbers of lymphocytes in the peripheral lymph nodes, with decreased infiltration of lymphocytes into the CNS of calorie restricted mice. Similarly, mice subjected to a fasting-mimicking diet, which also decreases calorie intake, had a decreased number of T cells infiltrating the spinal in a model of EAE²⁵². The authors suggest that a fasting-mimicking diet induces apoptosis of autoreactive cells, which limits disease progression²⁵². Here, we found that auto-inflammation during EAE may be limited by a migration of peripheral lymph node cells to the bone marrow, as we observed increased T cell numbers in the bone marrow of calorie restricted mice. A randomised-controlled study placed patients with multiple sclerosis on different calorie restrictive diets, and found that calorie restriction altered circulating T cell subsets, decreasing effector memory CD4⁺ and CD8⁺ T cell subsets, and increasing the frequency of circulating naïve T cells²⁵³. Although we did not observe a switch in circulating T cells, a previous study has demonstrated that calorie restriction in mice reduces circulating memory CD8⁺ T cells, which migrate to the bone marrow during calorie restriction¹¹⁵. Although calorie restriction provides immunosuppressive therapeutic value in an autoimmune setting, whether this would be beneficial or detrimental in an infection setting remains to be seen.

Taken together, this work and previous studies highlight the importance of dietary intake, and timing, in the regulation of IL-17A production by $\gamma\delta$ T cells in both a homeostatic, and pathogenic setting. Dietary interventions may be a relevant therapeutic strategy for metabolic and autoimmune disease, in order to correct for the maladaptation of circadian rhythms to the modern world. This will become increasingly important as societal pressures continue to encourage nocturnal work and activity.

Chapter 6

Overall Discussion

6.1 Introduction

In recent years, an important role for innate lymphocytes beyond host defence, in tissue homeostasis, has become increasingly evident. Specifically, tissue resident $\gamma\delta$ T cells play a vast range of homeostatic functions in the gut, adipose tissue, and meninges, where they coordinate responses which modulate dietary regulation²⁵⁴, metabolic homeostasis^{28,255} and behaviour^{30,256} respectively. Here we describe multiple environmental factors which influence circulating immune cell populations, and more specifically adipose $\gamma\delta$ T cell function. These environmental factors such as temperature, light and diet, may unveil novel mechanisms by which IL-17A expression by $\gamma\delta$ T cells can be modulated to maintain homeostasis in health and disease.

6.2 Metabolically demanding environments create an immunosuppressive phenotype.

We have described how environmental factors such as temperature and food abundance have a major impact on whole body metabolic processes. A change in external temperature or food availability requires appropriate metabolic adaptation to promote survival. Here we found that both cold temperatures, and calorie restriction decrease the number of lymphocytes in circulation, by redirecting them to the bone marrow. Thermogenesis, fasting (induced by calorie restriction), and immune activation are metabolically costly programs, which result in metabolic trade-offs when simultaneously active^{178,180,181}. Our data suggest that in the absence of an immune challenge, the immune system may be dispensable for energy conservation during cold environmental temperature or food scarcity. However, during an infection mice have alternate means of conserving energy to promote inflammation depending on the environmental temperature. For example, mice housed at 30°C decrease their locomotor activity in favour of energy conservation, but display decreased tolerance to inflammation. Conversely, mice housed at 22°C sacrifice core body temperature maintenance to conserve energy, and show increased survival to infection¹⁸¹. Mice housed at 30°C while fed a calorically restricted diet increased their tolerance to infection by decreasing their metabolic rate and body temperature¹⁸¹. In addition to environmental temperature, threatening situations programme whole-body metabolism to a catabolic state, as seen in a fight or flight response²⁵⁷. Psychological stress in mice, for example during predation, induces a hyperglycaemic state via hepatic gluconeogenesis,

which is beneficial for anticipating the increased energy demand for escape^{157,258,259}. However, inflammation during a fight or flight response was detrimental, due to a misdirection of metabolic resources¹⁵⁷. Therefore, metabolic trade-off is a valuable and evolutionarily conserved function for mediating survival.

Metabolic trade-offs have been shown to be beneficial during infection and autoimmune disease. During cold challenge, up to 60% of total energy expenditure is redirected to maintaining core body temperature¹⁶², as such, the activation of immune responses, which are also energetically demanding, may be detrimental to survival. However, utilising the redirection of energy resources may be beneficial in autoimmune disease. Observations in a rat model of EAE reveal that there is an increase in body temperature during EAE induction, possibly reflecting immune system priming, which turns hypothermic at the onset of EAE symptoms²⁶⁰. This may be similar to what is observed in an LPS induced endotoxemic shock model, where mice downregulate their metabolism in order to fuel the immune response to LPS¹⁸¹. Therefore, metabolically demanding environments which favour an immunosuppressive immune system may be beneficial in treating autoimmunity. Studies with therapeutic cold temperatures have shown that cold environmental temperature suppresses monocyte MHCII expression in the blood, peritoneum, and bone marrow of mice²⁶¹. This results in decreased Th17 T cell priming and attenuates the development of EAE²⁶¹. This may be in part due to the redirection of energy towards thermogenesis, and away from immune system priming. Furthermore, calorie restriction also attenuated the development of EAE in a similar manner to cold challenge¹¹⁴. As the development of EAE is primarily driven by IL-17A producing $\gamma\delta$ T cells⁷⁶, our observations show that cold temperatures and calorie restriction decrease IL-17A expression by $\gamma\delta$ T cells further support these findings. Taken together, these studies suggest that dietary and thermoregulatory interventions may be beneficial in treating autoimmune disease, without increasing susceptibility to infections or decreasing vaccine efficacy²⁶².

6.3 The adipose tissue microenvironment regulates IL-17A expression by $\gamma\delta$ T cells.

Cellular Metabolism

Immune cell function is intricately linked with the use of specific intracellular metabolic pathways²⁶³. $\gamma\delta$ T cell subsets rely on distinct metabolic pathways to fuel either IL-17A, or IFN γ -producing effector function. $\gamma\delta^{17}$ T cells primarily rely on mitochondrial oxidative metabolism while $\gamma\delta^{\text{IFN}\gamma}$ T cells are dependent on the glycolytic pathway⁶⁸. This cellular

metabolic dichotomy is established during development in the thymus⁶⁸. However, the reliance of $\gamma\delta^{17}$ T cells on other metabolic pathways cannot be completely ruled out. Here, we have found that circadian IL-17A expression peaks when mitochondrial metabolism is lowest. In agreement, a 3-week HFD feeding regiment disrupted the circadian rhythm of IL-17A, and mitochondrial mass, maintaining high IL-17A over 24-hours, and mitochondrial mass consistently low. However, calorie restriction and cold challenge decreased mitochondrial metabolism and IL-17A expression, indicating that mitochondrial metabolism may not be the sole regulator of IL-17A expression in $\gamma\delta$ T cells. Performing scRNA-seq on adipose $\gamma\delta$ T cells revealed that adipose $\gamma\delta^{17}$ T cells are enriched for the expression of genes involved in polyamine and amino acid metabolism compared to their $\gamma\delta^{\text{IFN}\gamma}$ T cell counterparts. Polyamine metabolism is essential for IL-17A expression by Th17 cells, and loss of polyamine metabolism prevents naïve T cell differentiation into Th17 cells²⁶⁴. Furthermore, pathogenic Th17 cells in autoimmune disease overexpress genes involved in polyamine metabolism. Knock out of polyamine synthesis genes limited pathogenic Th17 cell IL-17A expression which attenuated the development of EAE²⁶⁵. Additionally, amino acid metabolism regulates IL-17A expression by $\gamma\delta$ T cells. The amino acid transporter LAT1 plays a pathogenic role in psoriasis, by enhancing IL-17A production by $\gamma\delta$ T cells²⁶⁶, while glutamine uptake alleviates dextran sodium sulphate (DSS)-induced colitis through inhibition of IL-17A production by gut $\gamma\delta$ T cells²⁶⁷. These studies suggest that differential dietary and temporal regulation of adipose IL-17A expression by $\gamma\delta$ T cells may be regulated by metabolic pathways other than oxidative phosphorylation.

Lipid and Steroid Metabolism

Adipose tissue is enriched for IL-17A producing $\gamma\delta$ T cells compared to lymphoid and other non-lymphoid organs²⁸. Adipose tissue is a lipid-rich environment, and some studies suggest that $\gamma\delta^{17}$ T cells may utilise lipid metabolism to generate ATP²²². Indeed, high fat diet environments increase the expression of IL-17A by $\gamma\delta$ T cells in the lymph nodes²²², liver¹⁰⁹, blood¹¹⁰ and fat¹¹⁰ of mice. Furthermore, high fat diet and cholesterol supplementation can enhance the proliferation of $\gamma\delta^{17}$ T cells, particularly in the tumour microenvironment²²². We have shown that adipose $\gamma\delta^{17}$ T cells have a circadian rhythm to lipid uptake, which aligns with the circadian rhythm of IL-17A. Furthermore, a 3-week HFD feeding regimen enhanced IL-17A expression across 24-hours. Additionally, we have shown by scRNA-seq of adipose tissue innate T cells, that $\gamma\delta^{17}$ T cells are enriched for genes associated with steroid metabolism compared to $\gamma\delta^{\text{IFN}\gamma}$ T cells, although no differences were observed in genes associated with β -oxidation.

During cold challenge, calorie restriction, and sleep (inactive phase), WAT releases FFAs and glycerol into the adipose tissue microenvironment, and the circulation to be used as fuel to generate heat^{268–271}. Although we observed that a 3-week HFD enhanced IL-17A expression by adipose $\gamma\delta$ T cells; cold challenge, calorie restriction, and fasting during the inactive phase decreased IL-17A expression in adipose tissue. During cold challenge, adipose $\gamma\delta^{17}$ T cells had increased lipidTOX staining, suggesting increased intracellular lipids after cold challenge. Additionally, circadian IL-17A expression by adipose $\gamma\delta$ T cells is lowest during the inactive phase, when adipose lipolysis peaks. Considering that lipolysis occurs in adipose tissue during cold challenge, calorie restriction, and during sleep, our data indicates that there is an inverse relationship between adipose IL-17A expression by $\gamma\delta$ T cells, and adipose tissue lipolysis. The differences observed in IL-17A expression may be due to the lipid species generated in adipose tissue during HFD feeding versus lipolysis. For example, Th17 and $\gamma\delta$ T cell IL-17A expression is modulated by polyunsaturated fatty acids (PUFAs), which inhibit IL-17A production to limit inflammation in the skin, gut and synovial joints preventing development of psoriasis, DSS-induced colitis, and arthritis respectively^{272–274}. Additionally, production of ketone bodies such as β -hydroxybutyrate during a ketogenic diet expands a metabolically protective population of adipose $\gamma\delta^{17}$ T cells^{75,275}. Therefore, lipid species generated during specific environmental contexts may differentially regulate IL-17A expression by adipose $\gamma\delta$ T cells.

Adrenergic Signalling

The sympathetic nervous system is essential for maintaining circadian homeostasis in adipose tissue in response to light and feeding environmental cues²⁷⁶. Noradrenaline release is increased in adipose tissue in response to cold temperatures which induces lipolysis to generate heat¹⁸⁵. Furthermore, sympathetic induction of lipolysis occurs during calorie restriction, and during the inactive phase of mice, in order to increase circulating FFAs to maintain body temperature²⁷⁷. We have found that adipose innate T cells express the β 2-adrenergic receptor, and that adipose $\gamma\delta^{17}$ T cells specifically express the β 1-adrenergic receptor. Noradrenaline non-specifically binds to and activates β -adrenergic receptors¹⁹⁰. Therefore, during high sympathetic tone, noradrenaline can bind to the *Adrb1*, *Adrb2*, and *Adrb3* receptors simultaneously¹⁹⁰. We observed that during cold challenge, calorie restriction, and during the inactive phase of mice, which are all times of high adrenergic activity in adipose tissue, that IL-17A expression by adipose $\gamma\delta$ T cells is decreased. Additionally, HFD feeding can decrease adrenergic signalling to adipose tissue²⁷⁸, which may explain the enhancement and circadian disruption of IL-17A expression by adipose $\gamma\delta$ T cells during HFD feeding. Typically, adrenergic signalling

inhibits immune cell function either through enhancement of immunosuppressive Treg cell numbers or their inhibitory function¹⁷⁴. Adrenergic signalling can also directly inhibit pro-inflammatory cytokine expression by NK cells, CD4⁺ and CD8⁺ T cells¹⁷⁴. However, as adrenergic activity is essential for adipose tissue lipolysis, unravelling the effect that lipolysis or direct adrenergic activity has on adipose $\gamma\delta$ T cells may prove challenging. Currently, our results suggest a potential role for the adrenergic regulation of IL-17A production by adipose $\gamma\delta$ T cells.

6.4 $\gamma\delta$ T cells as metabolic sensors, IL-17A as a metabolic messenger.

Feeding behaviour plays an essential role in energy homeostasis, and recent studies have described a role for IL-17A signalling in behaviour^{24,31}. Studies in the nematode *C. elegans* first indicated that IL-17A signalling plays a role in feeding behaviour. Nematode feeding behaviour is mediated by the neuropeptide receptor 1 (*npr-1*) gene, where worms aggregate during feeding²⁷⁹. Loss of aggregative feeding behaviour in *npr-1* null worms was due to mutations in a homolog of the IL-17 receptor (termed *ilcr-1*), expressed on neurons, suggesting a role for IL-17A in neuronal signalling and feeding behaviour²⁸⁰. We have found that IL-17A expression by adipose $\gamma\delta$ T cells is enhanced during times of feeding, or high calorie availability. IL-17A circadian expression peaks at night when mice are active and feeding, and is enhanced in a HFD feeding model. Additionally, IL-17A expression is lowest during the day when mice are inactive, and is decreased during low calorie availability such as calorie restriction, which suggests a role for IL-17A as a nutrient, or energy sensor. In mammals feeding behaviour is regulated by both pro-opiomelanocortin (POMC) and agouti-related protein (AgRP) expressing neurons of the hypothalamus²⁸¹. POMC neurons reduce food intake, while AgRP neurons enhance food intake²⁸¹. A recent study described that IL-17A expression was increased in the gut after feeding, with no IL-17A expression detected in the hypothalamus²²¹. However, hypothalamic cells such as neurons²⁸², astrocytes and microglia expressed IL-17RA. Administration of IL-17A systemically, induced a temporal increase in both POMC and AgRP expression, with an early increase in POMC expression (1-hour post injection), and a late increase in AgRP (4 hours post injection) similar to what is observed after feeding. Interestingly, IL-17A administration decreased food consumption similar to the effects of leptin administration²²¹. Additionally, circulating serum IL-17A levels were increased in human participants who consumed food 2 hours prior to donating blood, after overnight fasting²²¹. Together these studies in worms, mice and humans suggest a role for IL-17A in regulating feeding behaviour.

In addition to feeding behaviour, there is mounting evidence that IL-17A regulates nutrient absorption in the gut. By studying the Japanese medaka fish (*Oryzias latipes*), researchers found that IL-17RA^{-/-} fish had a lower body mass compared to their wild-type controls²⁸³. Furthermore, the intestinal tract was shorter in IL-17RA^{-/-} compared to wild-type fish, with decreased expression of genes associated with lipid metabolism, lipid biosynthesis and steroid metabolism in their intestine²⁸³. Similarly, IL-17A may also regulate lipid metabolism in gut epithelial cells of mice. Molecular clock ablation specifically in ROR γ t expressing cells (*Arntl* ^{Δ ROR γ t}), increases the expression of IL-17A by gut ILC3s compared to wild-type littermate controls¹²⁶. Furthermore, gut epithelial cells of *Arntl* ^{Δ ROR γ t} mice had increased expression of genes associated with lipid uptake and metabolism¹²⁶. We have described a necessary role for IL-17A circadian expression in maintaining adipose tissue *de novo* lipogenesis, with IL-17A circadian expression peaking in the active phase of mice during feeding. Taken together, our results and previous studies suggest a link between IL-17A signalling and lipid metabolism.

Adipose tissue has a unique immune environment, including a subset of tissue resident $\gamma\delta$ ¹⁷ T cells^{62,255}. A loss of $\gamma\delta$ T cells, or IL-17A in adipose tissue causes decreased expression of adipocyte thermogenic genes *Ucp1*, *Dio2*, and *Ppargc1a*, which results in susceptibility to cold challenge⁶². Co-signalling of TNF and IL-17A from adipose $\gamma\delta$ T cells through Pdpn⁺PDGFR α ⁺ stromal cells induces IL-33 expression, which is necessary for thermogenesis and body temperature maintenance²⁸⁴. Additionally, activation of adipocyte IL-17RC by IL-17F produced by adipose $\gamma\delta$ T cells induces expression of TGF β , which enhances neurogenesis in adipose tissue²⁹. Loss of IL-17F, or IL-17RC, leaves mice susceptible to cold challenge due to a loss of neuronal signalling in BAT²⁹. Conversely however, loss of IL-17RA on the surface of adipocytes enhances adipose tissue browning, thermogenesis and prevents diet induced obesity¹¹⁰. As both IL-17A and IL-17F can signal through IL-17RA and IL-17RC these papers highlight the complexity of IL-17 signalling in whole body metabolism². We have described an important role for adipose IL-17A in maintaining whole body metabolic homeostasis. Loss of IL-17A, or adipocyte IL-17RC expression resulted in decreased expression of *de novo* lipogenesis in adipose tissue. This loss of circadian *de novo* lipogenesis disrupted whole body metabolic homeostasis, resulting in decreased body temperature circadian rhythm. Previous studies have shown that loss of IL-17A signalling disrupts adipose tissue lipolysis^{28,29}, however decreased *de novo* lipogenesis in the absence of IL-17A signalling may prevent the accumulation of lipids in adipose tissue, leading to a loss of fuel to generate heat through lipolysis.

Here we have highlighted the critical role for IL-17A signalling in maintaining circadian whole body metabolism. IL-17A expression by adipose $\gamma\delta$ T cells is largely modulated by

environmental factors, such as light, temperature and nutritional status. Although the mechanistic immunometabolic regulation of adipose IL-17A remains complex, there are multiple clear environmental strategies which could be utilised in regulating IL-17A signalling in human health and disease.

Chapter 7

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