

UNIVERSITY OF BIRMINGHAM

Investigating potential epigenetic mechanisms underpinning altered phenotypic responses of obese CD4^{+} T cells

by

Atrayee Gope

A thesis submitted to

the University of Birmingham

for the degree of

MSc by Research in Inflammation and Ageing

Institute of Inflammation and Ageing

College of Medical and Dental Sciences

University of Birmingham

July 2023

UNIVERSITY OF
BIRMINGHAM

University of Birmingham Research Archive

e-theses repository

This unpublished thesis/dissertation is copyright of the author and/or third parties. The intellectual property rights of the author or third parties in respect of this work are as defined by The Copyright Designs and Patents Act 1988 or as modified by any successor legislation.

Any use made of information contained in this thesis/dissertation must be in accordance with that legislation and must be properly acknowledged. Further distribution or reproduction in any format is prohibited without the permission of the copyright holder.

DECLARATION

Figure 3.1. Gene expression changes in mice lymph nodes (bulk) and splenic CD4$^+$ T cells.

The *in vivo* (murine) work including processing of murine tissues was done by Dr Jennifer Niven, post-doctoral researcher in Mauro lab and animal license holder, prior to me joining the lab. RNA extraction, cDNA synthesis and RT-qPCR were done by me.

Figure 3.2. Protein expression changes in the peripheral lymph node of mice.

The *in vivo* work as earlier mentioned was done by Dr Niven. I isolated proteins from the samples and ran the western blot.

Figure 3.3. Protein expression changes in the mesenteric lymph node of mice.

The *in vivo* work as earlier mentioned was done by Dr Jennifer Niven. I isolated proteins from the samples and ran the western blot.

Figure 3.4. Protein expression changes in CD4$^+$ T cells isolated from mice splenocytes.

The *in vivo* work, including isolation of splenic CD4$^+$ T cells was done by Dr Niven. Protein extraction and western blot were done by me.

Figure 3.5. NanoString nCounter analysis of mRNA expression in CD, HFD and HFD-RE mice.

All murine work at the animal facility were done by Dr Niven and PhD student Mr Salih Kucuk, animal license holders. I participated in the processing of murine tissues. Isolation of splenic memory CD4$^+$ T cells was done by Dr Niven. I isolated RNA from the samples. Quality control check of RNA was done by the Genomics facility at the University of Birmingham. Samples on the Nanostring nCounter were

run by Dr Joe Flint, Birmingham Tissue Analytics (BTA), University of Birmingham. I did the quality control check of raw data using nSolver. Downstream analysis including normalisation to house-keeping genes, and generation of PCA plot and heatmaps were done by bioinformatician Dr Christopher Mahony, Institute of Inflammation & Ageing, University of Birmingham.

Figure 3.6. Expression of candidate genes on co-culturing human CD4$^+$ T cells with adipose-conditioned media.

Adipose-conditioned media were obtained from Jones lab, Institute of Inflammation & Ageing, University of Birmingham. Rest of the work was done by me.

Figure 3.7. Gene expression analysis after co-culturing human CD4$^+$ T cells with different fatty acids.

Fatty acid preparations were done by Mr Kucuk. I did human PBMC and CD4$^+$ T cell isolation, culture and activation for two of the replicates, rest was done by Mr Kucuk. I ran test RT-qPCRs for selecting and standardising housekeeping genes. The main RT-qPCR experiments, analysis candidate gene expression changes were done by Mr Kucuk.

Figure 3.8. Comparing protein expression levels in human CD4$^+$ T cells treated with different fatty acids.

Out of the four biological replicates, I did CD4$^+$ T cell isolation and culture for two, rest was done by Mr Kucuk. Protein isolation and western blots were done by me.

Figure 3.9. Comparing the expression levels of autophagy markers in human $CD4^+$ T cells treated with different fatty acids.

Human PBMC and $CD4^+$ T cell isolation, fatty acid and Chloroquine treatments were done by Mr Kucuk. I did everything else.

ABSTRACT

Obesity, one of the leading causes of preventable death worldwide poses a huge burden on public health. It has a long-lasting negative effect on the immune system which can drive the development of chronic inflammatory disorders, contributing to high mortality rates. Essentially, a majority of obese people who lose weight go on to regain it and T lymphocytes play an important role in driving this weight re-gain. As we live longer and follow lifestyle that promotes obesity, it becomes increasingly important to understand the mechanisms that drive long-lasting immune dysregulation in obese individuals which persists even after weight loss. Research led by Prof. Claudio Mauro showed that obese $CD4^+$ T lymphocytes preferentially differentiate into a pro-inflammatory effector memory like phenotype. Subsequent studies done in the lab found that this pro-inflammatory phenotype of obese $CD4^+$ T cells is not rescued even after mice go on to lose weight after high-fat feeding. Subsequently, genome-wide DNA methylation studies identified 93 differentially methylated (70 hypomethylated and 23 hypermethylated) genes in the high-fat diet and high-fat recovery mice compared to mice on control (chow) diet. Out of the 70 hypomethylated genes, the following three were chosen for follow up studies - *STK26*, *CDKN1C* and *IDH3G* because of their relevance to autophagy, senescence, and TCA cycle, respectively. Our study made use of *in vivo* (murine) and *in vitro* (human) models of obesity and confirmed STK26 gene and protein upregulation as well as *CDKN1C* upregulation, while IDH3G gene or protein levels were not found to increase with obesity. Importantly, beside palmitic acid, stearic acid was also found to play a pro-inflammatory effect leading to altered obese $CD4^+$ T cell responses. There was also seen a trend of autophagy induction with palmitic and stearic acid

treatment, however, this would need to be repeated to establish statistical significance. Additionally, analysis of mRNA expression analysis by NanoString nCounter indicated altered senescence in the high-fat diet and high-fat recovery mice relative to chow diet. We thereby propose that STK26-induced autophagy and CDKN1C-induced senescence maintain pro-inflammatory effector memory $CD4^+$ T cell population upon weight loss. Future studies involving analysis of autophagy markers by western blotting and investigation of $CD4^+$ T lymphocytes, autophagy and senescence markers in *STK26*-knockout mice would provide deeper insights into the mechanisms by which autophagy, senescence, and the crosstalk between the two processes maintain $CD4^+$ T cell inflammatory programs upon weight loss, which shall help develop therapeutic means/practices to ensure restoration of immune homeostasis upon weight loss and ensure long-term health.

ACKNOWLEDGEMENTS

On the onset, I would like to extend my sincere gratitude to my supervisor Prof. Claudio Mauro for his continued support and patience throughout the tenure of this project. Thank you, Prof. Mauro, for your invaluable comments, suggestions, and encouragement. I have developed an immense interest in areas of metabolism and inflammation because of you.

I would like to thank Dr Michelangelo Certo, post-doctoral researcher and Mr Salih Kucuk, PhD Scholar in Mauro lab for their day-to-day guidance, especially in the initial stage, to help me get accustomed to the lab culture and safety regulations whilst teaching me the required lab techniques as well as answering my research questions. I would also like to acknowledge my gratitude towards Dr Jennifer Niven, post-doctoral Researcher in Mauro Lab, for her invaluable contribution to the project, and insightful comments and advice on my lab as well as thesis work.

Special mention goes to Dr Joe Flint for his advice on NanoString nCounter and helping run our samples as well as Dr Christopher Mahony for helping with the analysis of the nCounter data. I would also like to express my deepest appreciation for all staff members at the Institute of Inflammation and Ageing, University of Birmingham, for answering technical queries, providing training and granting access to lab equipment that helped my research massively. Last but not the least, I would like to thank my parents whose constant love, faith and support have been my source of motivation throughout the research course.

TABLE OF CONTENTS

1.0. INTRODUCTION 1

1.1. Obesity: Introduction and Statistics 1

1.2. An overview of the immune system 2

1.3. T-lymphocytes 3

1.3.1. T cell-development and maturation 3
1.3.2. TCR signalling and T cell activation 6
1.3.3. Conventional T-cell subsets 8

1.4. Inflammation 11

1.5. Diet-induced obesity 13

1.6. Obesity and chronic inflammation 14

1.6.1. Obesity-induced metabolic syndrome 14
1.6.2. Adipose tissue dysfunction in obesity 15

1.7. Obese $CD4^+$ T lymphocytes 20

1.8. Fatty acid-driven epigenetic imprinting in obesity 25

1.9. Autophagy, Senescence and Obesity 31

1.9.1. Autophagy in Obesity 31
1.9.2. Senescence in Obesity 38
1.9.3. Crosstalk between Autophagy and Senescence 41

1.10. Isocitrate dehydrogenase (NAD(+)) 3 non-catalytic subunit gamma (IDH3G) 43

1.11. Research hypothesis and aims 45

2.0. MATERIALS AND METHODS 48

2.1. Murine study 48

2.2. In vivo T-cell activation 48

2.3. Tissue preparation and isolation of murine $CD4^+$ T cells 49

2.4. Human $CD4^+$ T cell isolation and culture 50

2.4.1. Isolation of human Peripheral Blood Mononuclear Cells (PBMCs) 50
2.4.2. Human $CD4^+$ T cell isolation, treatment and activation *in vitro* 51
2.4.3. Preparation of adipose-conditioned media (ACM) 52
2.4.4. Preparation of fatty acids 52
2.4.5. Autophagy inhibition 53

2.5. RNA isolation and real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) 53

2.6. NanoString nCounter analysis *57*

2.7. Western blot *57*

2.8. Statistical analysis *60*

3.0. RESULTS **61**

3.1. In vivo *61*

3.1.1. Expression of candidate genes in murine lymph nodes and spleen 61
3.1.2. Expression of candidate proteins in murine lymph nodes and spleen 64
3.1.3. NanoString nCounter analysis of mRNA expression in CD, HFD and HFD-RE mice 68

3.2. In vitro *71*

3.2.1. Co-culturing human $CD4^+$ T cells with adipose-conditioned media 71
3.2.2. Co-culturing human $CD4^+$ T cells with different fatty acids 76

4.0. DISCUSSION **84**

4.1. In vivo and in vitro confirmation of STK26 up regulation *86*

4.1.1. STK26 is induced in HFD mice and does not return to basal levels even after weight loss. 86
4.1.2. Treatment with obese adipose-conditioned media induces the expression of *STK26.* 89
4.1.3. Confirmation of *STK26* gene and protein up-regulation in human $CD4^+$ T cells treated with palmitic acid. 90
4.1.4. Detection of autophagy markers in human $CD4^+$ T cells treated with fatty acids through western blot. 92

4.2. Treatment of human $CD4^+$ T cells with palmitic and stearic acids induces the expression of CDKN1C. *94*

4.3. In vivo and in vitro confirmation of IDH3G expression not changing upon high fat feeding or fatty acid treatment. *94*

4.4. The HFD-RE cluster co-relates with HFD, not CD and differentially expressed genes between HFD/HFD-RE and CD indicate an increased potential for cellular senescence *95*

4.5. Limitations *97*

4.7. Conclusion *100*

BIBLIOGRAPHY **102**

LIST OF FIGURES AND TABLES

Page No

Chapter-1: Introduction

Table 1.1: Different human helper-T cell subsets…….....10-11

Figure 1.1: Differences in immune cell type between lean and obese adipose tissue…….....19

Figure 1.2: Obese $CD4^+$ T cells preferentially differentiate into a pro-inflammatory effector memory-like phenotype…….22

Figure 1.3: *In vivo* mice HFD experiment…….....23

Figure 1.4: Obese $CD4^+$ T cells preferentially differentiate into a pro-inflammatory effector memory phenotype even after weight loss…….24

Figure 1.5: Genome-wide DNA methylation analysis of naïve and memory $CD4^+$ cells isolated from experimental mice in Figure 1.3…….30

Figure 1.6: STK26 is an inducer of autophagy……....37

Figure 1.7: Targeted pyrosequencing analysis to confirm percentage methylation of candidate genes…….45

Chapter-2: Materials and Methods

Table 2.1: Master mix preparation for TaqMan RT-qPCR (2.25 uL cDNA per well) …….54-55

Table 2.2: List of primers (human)…….....55-56

Table 2.3: Master mix preparation for Syber green PCR (1 uL cDNA per well)…….56

Table 2.4: List of primary antibodies…….....59-60

Chapter-3: Results

Figure 3.1: Gene expression changes in mice lymph nodes (bulk) and splenic $CD4^+$ T cells……..…63

Figure 3.2: Protein expression changes in the peripheral lymph node of mice…..66
Figure 3.3: Protein expression changes in the mesenteric lymph node of mice…67
Figure 3.4: Protein expression changes in $CD4^+$ T cells isolated from mice splenocytes……67
Figure 3.5: NanoString nCounter analysis of mRNA expression in CD, HFD and HFD-RE mice……70
Figure 3.6: Expression of candidate genes on co-culturing human $CD4^+$ T cells with adipose-conditioned media……..73
Table 3.1: The BMIs of osteoarthritis patients and corresponding relative changes in gene experiment in the first experiment with adipose tissue-conditioned media…….....74
Table 3.2: The BMIs of osteoarthritis patients and corresponding relative changes in gene expression in the second (repeat) experiment with adipose tissue-conditioned media……75
Figure 3.7: Gene expression analysis after co-culturing human $CD4^+$ T cells with different fatty acids……....78
Figure 3.8: Comparing protein expression levels in human $CD4^+$ T cells treated with different fatty acids…….80
Figure 3.9: Comparing the expression levels of autophagy markers in human $CD4^+$ T cells treated with different fatty acids……..83

LIST OF ABBREVIATIONS

α-KG	α-ketoglutarate
ACM	Adipose-conditioned media
AMPK	Adenosine monophosphate—activated protein kinase
AP1	Activator protein
AT	Adipose tissue
ATM	Ataxia telangiectasia mutated.
Atg	Autophagy-related.
ATMs	Adipose tissue macrophages
BAT	Brown adipose tissue
β-ME	Beta-mercaptoethanol
BMI	Body mass index
BSA	Bovine serum albumin
CD	Chow diet
CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent kinase 4
CDK 6	Cyclin-dependent kinase 6
CDKN1C	Cyclin dependent kinase inhibitor 1C
CDKIs	Cell-cycle dependent kinase inhibitors
CQ	Chloroquine
CRP	C-reactive protein
Ct	Cycle threshold
CTLA-4	Cytotoxic T-lymphocyte associated protein-4

CTLs	Cytotoxic T-lymphocytes
ECM	Extracellular matrix
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ERN	Ethical review committee
FABP5	Fatty acid binding protein 5
FCS	Foetal calf serum
GABARAP	y-aminobutyric acid receptor associated protein
HDL	High-density lipoprotein
HFD	High-fat diet
HFD-RE	High-fat diet-recovery (recovery diet)
HLA-DR	Human leukocyte antigen-DR
HS	High sensitivity
ICAM-1	Intercellular adhesion molecule 1
IDH	Isocitrate dehydrogenase
IDH3G	Isocitrate dehydrogenase (NAD(+)) 3 non-catalytic subunit gamma
IFN-γ	Interferon-gamma
IL1	Interleukin-1
IL2	Interleukin 2
IL6	Interleukin-6
ILC2	Innate lymphoid cell 2
IR	Insulin resistance
ITAM	Immunoreceptor tyrosine-based activation motif
LAG 3	Lymphocyte activation gene 3

LC3	Light chain 3
LDL	Low-density lipoprotein
LFA-1	Lymphocyte function associated antigen 1
lncRNAs	Long non-coding RNA
MAP1	Microtubule-associated protein
mATG8	Mammalian ATG 8
MCP-1	Monocyte chemoattractant protein 1
MLR	Mixed lymphocyte reaction
MUFA	Mono-unsaturated Fatty Acid
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
OA	Oleic acid
OSCC	Oral squamous cell carcinoma
PA	Palmitic acid
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCA	Principal component analysis
PE	Phosphatidylethanolamine
PD-1	Programmed cell death protein 1
pRB	Retinoblastoma protein.
PtdIns(3)P	Phosphatidylinositol 3-phosphate
PIK3C3	Phosphatidylinositol 3-phospate kinase complex
PUFA	Poly-unsaturated fatty acid
RBC	Red blood cell

RRBS	Reduced representation bisulfide sequencing
RT	Room temperature
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SA	Stearic acid
SASP	Senescence-associated secretory phenotype
SAT	Subcutaneous adipose tissue
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
STK26	Serine/threonine kinase 26
TBS	Tris buffered saline
TCA	Tricarboxylic acid cycle
TCM	Central memory T cell
TCR	T cell- receptor
T_{EM}	Effector memory T Cell
T_{EMRA}	Terminally differentiated effector memory T Cell
T_{FH}	Follicular helper T cell
T_{H}	T-helper cell
TLR4	Toll-like receptor 4
TNFα	Tumour necrosis factor α
T_{REG}	Regulatory T Cell
T_{RM}	Tissue-resident memory T cell
UNG	Uracil-N-glycosylase
UT	Untreated
VAT	Visceral adipose tissue

WAT	White adipose tissue
WHO	World health organization

1.0. INTRODUCTION

1.1. Obesity: Introduction and Statistics

Obesity has been a major health burden worldwide - to the extent that it is even used as a measure of morbidity and mortality. It is particularly worse in the modern world because of overconsumption of diets rich in sugars and fats, and poor lifestyle choices like lack of physical exercise, frequent alcohol consumption, smoking and disturbed sleep patterns. The World Health Organization (WHO) defines obesity as abnormal or excessive accumulation of fat that poses a risk to health (1). It is measured in terms of Body Mass Index (BMI), wherein a BMI of over 25 is classed as overweight and that over 30 is considered obese (1). Although it is more prevalent in developing and under-developed countries, the statistics of obese people in western countries have soared over the years; according to recent health estimates, around 25.9% of people aged 16 or above in England are obese and if we also take into consideration overweight adults, the percentage rises to 66.7 (2). Furthermore, around 23.4% of children aged 10-11 and 10.1% in age-group 4-5 are obese (2) and more than half of these obese children go on to become obese in their adulthood (3). Obesity affects people of all age groups, however, those aged 45-74 are more severely affected (2). It is not merely a weight-gain issue as it may sound; obesity increases the likelihood of developing severe health problems like Insulin Resistance (IR) and type 2 diabetes, cardiovascular problems,

cancer, arthritis, acute pancreatitis, and liver and lung complications (4). The NHS spends £6 billion per year on obesity-related illnesses and it is predicted that this expenditure will rise to £9.7 billion annually by the year 2050, which reiterates it is reaching pandemic proportions and calls for some serious interventions (5).

1.2. An overview of the immune system

The immune system in mammals is complex and multi-factorial consisting of various different cell types, proteins and functions that coordinate together as well as with other organ systems in the body to protect against infections and injury and fight intrinsic changes disturbing the body homeostasis like cancer. With rapid advancements in Immunology research, it is well-established that the role of the immune system extends far beyond these canonical functions of self-defence. As well as sensing complex signals from the neuronal system, the immune system can respond to changes in diet and metabolism, regulating physiological processes crucial for maintaining homeostasis like regulation of neuronal function, metabolic state, tissue repair etc (6). The mammalian immune system has two main arms- i) The innate immune system acts as the first line of host defence and mounts a general or broad response to antigens, compromising of physical barriers (e.g., skin, mucous membranes etc.), the complement system and various cell types like phagocytic cells (e.g., neutrophils, monocytes, macrophages), granulocytes (e.g., neutrophils, eosinophils,

basophils, mast cells), innate lymphoid cells (e.g., Natural killer (NK) cells), antigen-presenting dendritic cells, and ii) The adaptive-immune system broadly encompassing B and T lymphocytes, is slower to respond but highly antigen-specific and offers prolonged protection through memory formation (7,8). In the adaptive arm of immunity, there are two main lines of defence: humoral immunity, which is mediated by antibody-producing B lymphocytes and T lymphocyte-mediated cellular immunity. For this thesis, we will focus primarily on T-lymphocytes.

1.3. T-lymphocytes

T-lymphocytes, constituting 51-88% of circulating lymphocytes, are the chief effector cells of the immune system. There are different types of T cells, a characteristic that allows for a diverse set of immune roles including recognising antigens, killing infected cells, producing cytokines, activating other immune cells, and possessing immunogenic memory as well as immune suppressive roles. T-cell dysfunction has been implicated in numerous diseases of the immune system, including auto-immune and allergic disorders, cancer and aging-related disorders (9–12).

1.3.1. T cell-development and maturation

T-lymphocytes originate from hematopoietic stem cells in the bone marrow that differentiate into lymphoid progenitor cells, which in turn migrate to the thymus to develop into fully

functional T cells. The T cell progenitors in the thymus undergo a series of maturation steps, including the expression of the T cell-receptor (TCR) that confers T-cells with the ability to recognise specific antigens, and co-receptors CD2, CD3 and either CD4 or CD8 that promotes differentiation into either T-helper or cytotoxic T-cells. Most progenitor cells that enter the thymus meet an apoptotic death fate and a few go on to develop into mature thymocytes. The TCR is heterogenous and undergoes TCR gene re-arrangement to give rise to either αβ -T cells, comprising 95% of the T-cell population, or the less-studied γδ subset (remaining 5%) which over the years has emerged as a class of unconventional T-lymphocytes with implications in human diseases (13–15). The first step in the maturation process is the expression of CD2, the point at which the progenitor cells have not started re-arranging the TCR genes or expressing the glycoprotein receptor CD4 or CD8 yet and are called double-negative cells (16–18). Some of these cells divide into γδ-T cells and the rest are on the path of becoming mature αβ -T cells (19). These αβ -T committed cells go through four stages of the differentiation process, depending on the expression of CD44, a cell-surface adhesion receptor and CD25, the alpha chain of the IL2 receptor, in the order: CD44+CD25-, CD44+CD25+, CD44-CD25+ and CD44-CD25- (20). It is during this transition that these cells re-arrange their TCR-β chain which

is followed by the expression of CD3 and a pre-TCR α chain (21). Downstream of the pre-TCR α chain development, rapid cell proliferation gives rise to double-positive (expressing both CD4 and CD8) cells and replacement by a fully functional α chain (19). These double positive αβ -T cells then interact with self-antigens on histocompatibility (MHC) class I and class 2 molecules in the thymus cortex and undergo positive or negative selection, which determines their fate (19). Positive selection selects for only those double-positive αβ -T cells that interact with self MHC I or MHC II molecules and further these cells are scrutinized by negative selection- most cells binding 'self' MHC molecules strongly undergo apoptosis and some are selected to be regulatory T cells (T_{reg}), while the remaining exit the thymus as naïve $CD4^+$ or $CD8^+$ T cells (19). Although maturation into $CD4^+$ and $CD8^+$ T cells occurs in the thymus, these cells are still naïve (i.e., they have not encountered antigen yet) and undergo differentiation into various effector and memory T cell subsets in the periphery based on different activation stimuli. The T cell subgroups differ from each other in the pattern of cytokines produced upon stimulation and hence the functions they are associated with.

1.3.2. TCR signalling and T cell activation

T-cell activation is a complex phenomenon supported by the interaction of TCR with antigens and other co-stimulatory molecules, chemokines, integrins and metabolites, promoting a cascade of signalling events that ultimately decide the fate of activated T cells. The differentiation of naïve T cells into effector T cells is signalled by changes in the expression of cell surface markers. Typically, naïve T cells express the long isoform of the receptor-linked protein tyrosine phosphatase CD45- CD45RA and upon activation switch to the low molecular weight CD45RO isoform, although the switch from CD45RO to CD45RA is reversible in some circumstances (22–24). Activated T cells express various other cell-surface receptors including CD25, CD44, CD71 (the transferrin receptor), human leukocyte antigen-DR (HLA-DR) and CD69 (type II C-leptin receptor), and co-stimulatory molecules. (CD26, CD27, CD28, CD30, CD154 or CD40L, and CD134) (25–28). Additionally, the chemokine receptor CXCR3 is highly expressed as a naïve T cell differentiates into its effector counterpart and is important for the trafficking of T cells into inflamed organs (29). Priming of naïve T cells occurs normally in secondary lymphoid organs and requires three explicit signals for activation, proliferation and finally differentiation into specific subsets. The initial activation signal is antigen-specific and requires the engagement of TCR with antigens presented by MHC class I or

II molecules on APCs (30). This triggers a signalling cascade, initiated by the phosphorylation of Immunoreceptor tyrosine-based activation motif (ITAM) on the CD3/TCR complex by Lck kinase, which causes the recruitment and Lck-dependent phosphorylation of Zap70, which in turn phosphorylates downstream adapter proteins, including the key adaptor molecule linker for activation of T cells (LAT) (30). Signal 2 is provided by binding of co-stimulatory receptor CD28 on T-cell to ligands B7.1 (CD80) or B7.2 (CD86) on APCs, that initiates T-cell proliferation (31,32). Additionally, the co-receptor lymphocyte function-associated antigen–1 (LFA-1) plays an important role in the stabilisation of the transient T cell–APC complex, achieved through its binding to the highly expressed cell adhesion molecule intercellular adhesion molecule–1 (ICAM-1) (33,34). Conversely, co-inhibitory receptors including cytotoxic T-lymphocyte associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), or lymphocyte activation gene 3 (LAG-3) act to counteract the activation process, maintaining a steady population of proliferating T cells (35). Finally, the third signalling event is driven by the cytokine milieu, that enhances T-cell clonal expansion and promotes differentiation into specific effector subsets (36–38). In-vitro, T cell activation can be mimicked by using a minimum of two signals- stimulation by anti-CD3 and anti-CD28 antibodies (39).

1.3.3. Conventional T-cell subsets

- **$CD4^+$ T cell** – As previously mentioned, $CD4^+$ T cells get their identity in the thymus, which is facilitated by binding to MHC class II molecules. A large proportion of the naive $CD4^+$ T cells upon activation differentiates into effector T cells which eventually die and only a small percentage persists as long-lived memory cells. The effector $CD4^+$ T cells are also called helper T cells in the sense that they play a 'helping' role in the immune system by activating other immune cells including macrophages, B cells and cytotoxic T cells. Depending on the cytokine profile and regulation at the transcription level, T-helper cells can differentiate into various subsets- Th (T helper)1, Th2, Th9, Th17, Th22, Treg (regulatory T cells), and T_{FH} (follicular helper T cells) (40). Table 1 summarises the different helper-T cell subsets, their differentiation signal, cytokine profiles, transcriptional factors, chemokine receptors and effector functions.
- **$CD8^+$ T cell-** In contrast to $CD4^+$, $CD8^+$ T cells signal by binding to class I MHC molecules. Effector $CD8^+$ T cells are also called cytotoxic T lymphocytes (CTLs) owing to their cytotoxic killing abilities. They are involved in the direct killing of infected and other dysfunctional cells, including tumour cells. CTLs can be characterised into two main subtypes based on their cytokine profiles- i) Tc1 cells that secrete type 1 cytokines like Interferon-

gamma (IFN-γ) , Tumour necrosis factor α (TNFα) and Interleukin 2 (IL2) and ii) Tc2 cells that secrete type 2 cytokines - IL- 4, IL-5 and IL-10 (41,42). These cytokines together with chemokines released by CTLs help in the recruitment of other immune cells like monocytes to the sites of infection. Like $CD4^+$ T cells, $CD8^+$ T cells can also generate a small subset of CD8+ memory T cells, although the process of memory cell formation is different between the two subsets.

- **Memory T cell-** There are two important subclasses of memory T cells for both CD4 and CD8 populations distinguished on the basis of expression of the lymphocyte homing receptor CCR7 and CD62L, a cell adhesion molecule important for lymphocytes trafficking to lymphoid tissues and sites of inflammation: $CD45R0^+CD62^+CCR7^+$ central memory T-cell (T_{CM}) found in lymphoid organs and blood, and $CD45R0^+CD62^-CCR7^-$ effector memory T cell (T_{EM}) that are found in circulation as well as have the ability to traffic to peripheral organs (43). T_{EM} cells can further differentiate into terminally differentiated effector memory cells (T_{EMRA}) (mostly studied in the $CD8^+$ subset) which unlike conventional T_{EM} cells lose CD45RO and switch back to CD45RA (44). Apart from the circulating memory T cells, there are tissue-resident memory T cells (T_{RM}) expressing CD69, a marker of tissue retention which allows for permanent residence in tissues (45).

Table 1.1. Different human helper-T cell subsets

T cell subtype	Phenotype	Differentiation signal	Master transcription factor	Cytokine profile	Chemokine receptors	Effector functions	References
T_{FH}	$CD4^+$ $CXCR5^+$ $CD40L^+$ $ICOS^+$	IL-6, IL-21	BCl6	IL-4, IL-10, IL-17, IL-21, IFN-γ	CXCR5	• Survival and proliferation of germinal centre B cells	(46)
Th1	$CD4^+$ $CXCR3^+$ $CCR5^+$ $IL12R\beta2^+$	IL-12, IFN-γ	T-bet	IFN-γ, IL-2, TNF-α, TNF-β	CXCR3, CCR1, CCR2, CCR5	• Host defense against bacteria and virus •Pathogenesis of chronic inflammatory and autoimmune diseases	(47)
Th2	$CD4^+$ $CCR4^+$ $IL12R\beta2^-$	IL-2, IL-4	GATA-3	IL-4, IL-5, IL-9, IL-17, IL-17E/IL-25	CCR4, CCR3, CCR2, CCR1	• Host defense against last extracellular pathogens • Humoral immunity • Allergic Inflammation	(48)
Th9	$CD4^+$ $CCR3^+$ $CCR6^+$	IL-4, TGF-β	PU.1	IL-9	CCR3, CXCR3, CCR6	• Host defense against parasites • Allergic asthma pathogenesis • Tumoricidal activity	(49)
Th17	$CD4^+$ $CCR6^+$ $CCR4^+$ $NK1.1^+$	IL-2, IL-4	RORC2	IL-17A, IL-17F, IL-21, IL-22, IL-26	CCR4, CCR6	• Host defense against specific fungi and extracellular bacteria • Pathogenesis of inflammatory and autoimmune diseases	(50)
Th22	$CD4^+$ $CCR10^+$ $CCR4^+$	IL-1-β, IL-6, TGF-β	RORγt	IL-10, IL-13, IL-21, IL-22,	CCR4, CCR6, CCR10	• Protection against microbial skin	(51)

	$CCR6^+$			TNF-α		infections • May be implicated in tissue/repair modelling • Pathogenesis of inflammatory and autoimmune diseases	
Treg	$CD4^+$ $CD127^+$ $CD25^+$ $CTLA\text{-}4^+$	IL-2, TGF-β	FOXP3	Galectin-1, IL-10, IL-35, TGF-β1	CCR4, CCR8	• Immune homeostasis and self-tolerance • Protection against autoimmune diseases	(52)

1.4. Inflammation

Inflammation, a mechanism of the innate immune response, is generally understood to be a protective immune response against harmful stimuli like foreign invaders, allergens, damaged cells and injured tissues. Typically, the body sends out immune cells to the injured/affected sites in order to eliminate the cause of injury, get rid of damaged cells and tissues and initiate the process of tissue repair. Although the process of inflammation is integral for maintaining normal tissue homeostasis, prolonged effects of the inflammatory response can be associated with a large number of detrimental outcomes. Based on the duration of the inflammatory response, inflammation can be classified into either acute or chronic. Acute inflammation is an immediate response to infections or tissue injury lasting for some minutes to days, characterised by the release of inflammatory cytokines

allowing the passage of immune cells, mainly neutrophils from blood to sites of inflammation (53,54). The classical symptoms of acute inflammation include redness, swelling, pain and heat (55). However, if acute inflammation is not resolved in days, it switches to chronic inflammation which can last for months or even years. While most of the features of acute inflammation are retained as chronic inflammation takes over, the predominant immune cell type at the site of inflammation is macrophages, not neutrophils, and there is also an increased trafficking of lymphocytes like T-cells, B-cells, plasma cells to sites of injury and the release of inflammatory cytokines, growth factors, enzymes which overall contribute to the progression of tissue damage and secondary repair, but over time can start damaging healthy cells and tissues (56). The most prominent markers in inflammatory diseases are TNFα, Interleukin-1 (IL1), and the chemokine (C-X-C motif) ligand (CXCL) subfamily of cytokines (55,57). Apart from persistent acute inflammation, chronic inflammation can be triggered by other factors such as hypersensitivity to allergens, long-term exposure to irritants like industrial chemicals, auto-immune diseases like rheumatoid arthritis and autoinflammatory diseases like Familial Mediterranean Fever (56). Chronic inflammation has been linked to the pathogenesis of numerous severe health conditions including obesity-associated diabetes, cancers, cardiovascular diseases, respiratory syndromes, chronic kidney diseases, autoimmune and neurodegenerative disorders, which account for more than 50% of deaths worldwide (58).

1.5. Diet-induced obesity

Although there are several risk factors associated with obesity including genetic factors, underlying medical conditions, side effects of certain medications and psychological conditions like stress, it is mainly caused by an overconsumption of diet rich in calories. There has been a long ongoing debate on whether it is the intake of fats or sugars that contributes to higher rates of obesity. Nevertheless, an increasingly large number of studies conducted in humans as well as animals has shown that high-fat diet can easily induce obesity and regulate energy metabolism (59–64). As a result, a high fat-diet model has long been the most popular choice in studying obesity (65). Fatty acids can be classified into either saturated or un-saturated, depending on the presence of single or double bond. The most common dietary saturated fatty acid is palmitic acid (PA) followed by stearic acid (SA). The unsaturated fatty acids can be further divided into mono-unsaturated (MUFA) (e.g., oleic acid [OA]) and poly-unsaturated (PUFA) (e.g., omega-3, omega-6). A high fat diet typically comprises of at least 35% total daily calorie intake from fatty acids. Westernized diets are rich in saturated- and trans-fats (an unsaturated fatty acid found in high levels in processed foods; e.g., elaidic acid) while they have a low content of MUFA and PUFA (66). Essentially, saturated and trans fats have been associated with high levels of low-density lipoprotein (LDL) or bad cholesterol and increased risk of heart diseases, while MUFA and PUFA are considered to be healthier in that they help lower levels of

LDL (67–69). A similar trend can be seen in regard to obesity; there is mounting evidence to support that dietary saturated fatty acids like PA directly trigger obesity-associated inflammatory changes while unsaturated fatty acids mainly omega-3 (e.g., Eicosapentaenoic acid [EPA]) and OA have been shown to have a protective effect in obesity (70–76).

1.6. Obesity and chronic inflammation

1.6.1. Obesity-induced metabolic syndrome

Metabolic syndrome is defined as a group of at least three out of five conditions- increased blood pressure, obesity, elevated serum triglycerides, high blood sugar level, low serum high-density protein (HDL or good cholesterol) and IR, that increases the risk of developing severe health problems including diabetes, coronary heart diseases and stroke (77). Obesity is the most commonly observed risk factor leading to metabolic syndrome. Additionally, fatty acids have been directly linked to the pathogenesis of metabolic syndrome (78–81). The close relationship between immune and inflammatory changes in the body to changes in tissue metabolism has been long recognised and it is indeed a state of chronic low-grade inflammation and altered immune system accompanying obesity that plays a crucial role in the development of metabolic syndrome and contributes to other obesity-associated morbidities (82–84).

Maintenance of metabolic homeostasis requires cross talk between multiple organs, including the brain, liver, muscle and adipose tissue. Over the years, the perspective of adipose tissue has changed from being simply an energy store to a highly specialised endocrine organ and a critical regulator of systemic energy homeostasis (85,86). Dysfunction of the adipose tissue is the key driver of chronic inflammation in obesity and is associated with a shift from an anti-inflammatory to a pro-inflammatory adipose environment (87,88). Long-chain SFAs, elevated in obesity, exert direct effects on adipose tissue inflammation through different pathways (89). One such important mechanism involves the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) through Toll-like receptor 4 (TLR4) signalling, stimulating enhanced production of pro-inflammatory mediators by adipocytes (90,91).

1.6.2. Adipose tissue dysfunction in obesity

There are two main types of adipose tissue (AT)- white adipose tissue (WAT), that stores excess energy as free fatty acids, and brown adipose tissue (BAT), that releases stored energy as heat (92). WAT can be further grouped into- subcutaneous adipose tissue (SAT), found under the skin, and visceral adipose tissue (VAT), lining internal organs (93). Essentially, an excess of macronutrients in an obese environment promotes VAT

enlargement both in number (hypertrophy) and size (hyperplasia), in order to compensate the need for increased lipid storage (94). This is followed by local (VAT) inflammation, which finally progresses to a state of systemic inflammation, spreading to liver, skeletal muscle and other tissues (95). As the adipocyte population expands, they start showing classical signs of inflammation- necrotic cell death and an enhanced secretion of pro-inflammatory cytokines (adipokines) including TNFα, interleukin (IL)-6, monocyte chemoattractant protein-1 (MCP-1) and leptin (96–98). On the other hand, there is lesser production of adiponectin, an anti-inflammatory adipokine normally produced by lean adipocytes to regulate energy homeostasis (99). Hypertrophic adipocytes induce a state of local hypoxia, that accelerates adipose tissue scarring and is considered to be one of the major regulators of adipose tissue dysfunction (100,101). In addition, these dysfunctional adipocytes show increased basal lipolysis, resulting in increased export of free fatty acids into ectopic tissues not meant for lipid storage like liver and muscles, triggering systemic lipolysis, which ultimately culminates in Endoplasmic Reticulum (ER) stress and inflammation (96,102–104). This obesity-induced ER stress further alleviates lipid accumulation, IR, liver and adipose tissue damage, and the vicious loop of obesity and ER stress continues (105,106).

In the lean state, the adipose tissue hosts different immune cell populations required for homeostasis, but with obesity, the adipose tissue switches to a pro-inflammatory immune environment (Figure 1.1). Infiltration of immune-inflammatory cells is one of the primary features of VAT inflammation, mediated by concerted effects of hypoxia, ER stress and the release of cytokines like MCP-1 by adipocytes (107,108). Adipose tissue macrophages (ATMs), comprising up to 40% of all accumulated immune cells in obese adipose tissue, are a key mediator of AT inflammation (109). Normally, macrophages can be polarized into classically activated M1 (pro-inflammatory) and alternatively activated M2 (anti-inflammatory phenotype) phenotypes. While lean AT hosts M2 macrophages with immune suppressive roles that regulate adipose tissue homeostasis, in obesity, there is a switch to a pro-inflammatory phenotype, albeit distinguished from the classical M1 phenotype, referred to as Me, that is, they are activated by metabolic stimuli like free fatty acids, high glucose and high insulin (110,111). These pro-inflammatory macrophages form crown like aggregates around necrotic cells, secrete pro-inflammatory cytokines including TNF-α , IL-6 and IL-1B, and also upregulate genes associated with lipid metabolism (111–114). TNF-α stimulates the production of leptin, which enhances the phagocytic function of obese

macrophages and co-relates with an increase in the circulating levels of the C-reactive protein (CRP), an established marker of systemic inflammation (115,116). Furthermore, ATMs provide the necessary signals required for stimulation of naïve $CD4^+$ T cells into Th1 effector cells (117). Other innate immune cell mediators of AT inflammation include neutrophils, dendritic cells, eosinophils, mast cells, NK cells and innate lymphoid cell 2 (ILC2) (118). In regard to the adaptive immune system, B cell recruitment, although less studied in the context of obesity, occurs shortly after the onset of obesity and precedes the accumulation of other immune cells; they promote the recruitment of neutrophils, monocytes, T cells, and also modulate the pro-inflammatory activation of AT macrophages and T-cells (119,120). T cells are the second largest population of immune cells infiltrating obese AT. There is an increased recruitment of $CD8^+$ T cells in obese AT as they are involved in the polarization of ATMs towards a pro-inflammatory phenotype (121). Furthermore, there is a decline in the numbers of anti-inflammatory Th2 and T_{regs} in obese AT although healthy AT is enriched with them (122). At the same time, there is an increase in the population of pro-inflammatory Th1 cells, that secrete IFN-γ, the main cytokine driving the switch to pro-inflammatory ATMs while also enhancing their antigen presenting abilities (123,124). The role of pro-inflammatory Th17 cells in obesity is

controversial, with some studies suggesting that Th17 and IL17 contribute to chronic inflammation in obesity while others implicating a protective role of Th17 and IL-17 in reducing obesity and insulin resistance (125–128).

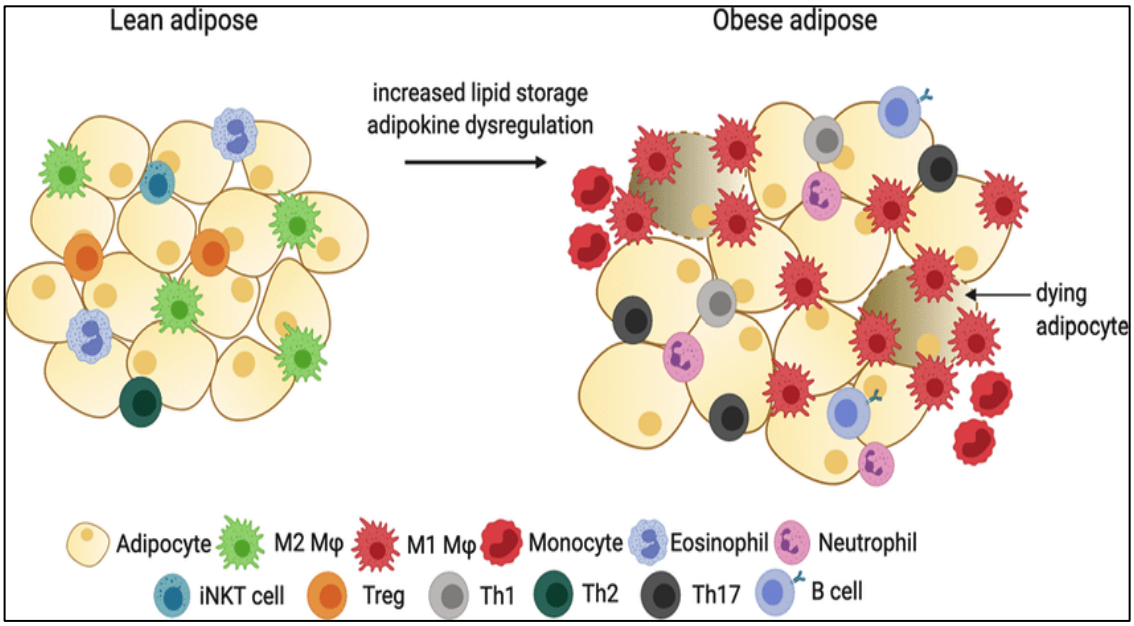


Figure 1.1. Differences in immune cell type between lean and obese adipose tissue

While lean adipose tissue is dominated with anti-inflammatory immune cells like M2 macrophages, Th2 cells, Treg, iNKT cells and Tregs, obese adipose tissue shows increased infiltration of monocytes, pro-inflammatory M1 macrophages, Th1, Th17 cells, B cells and neutrophils.
Figure reprinted from *The complex role of adipokines in obesity, inflammation, and autoimmunity* (P. 3), by Erin B Taylor et al., March 2021, Clinical Sciences, 135(6):731-752, copyright 2021 under the Creative Commons Attribution License 4.0 (CC BY-NC-ND) (110)

1.7. Obese $CD4^+$ T lymphocytes

Although it is well-documented that T-lymphocyte dysfunction is a major source of chronic inflammation in obesity, the mechanisms governing enhanced T lymphocyte activation in VAT and chronic inflammation remain elusive. However, Mauro et al. in 2017 found that immune priming following high-fat diet (HFD) in mice programs a biased differentiation of $CD4^+$ T cells into a pro-inflammatory $CD44^{hi}$- $CCR7^{lo}$-$CD62L^{lo}$-$CXCR3^+$-$LFA1^+$ effector memory-like phenotype, leading to an increased expansion of pro-inflammatory effector $CD4^+$ memory T cells infiltrating adipose tissue and other inflamed sites, with no change in the central memory pool (129) (Figure 1.2). Moreover, palmitic acid was shown to be directly involved in mediating the biased pro-inflammatory phenotype of obese $CD4^+$ T cells, via increased activation of a PI3K p110δ-Akt-dependent pathway. These findings were also confirmed in a human cohort. Following up from this study, the question that was prompted was whether this preferential pro-inflammatory phenotype of obese $CD4^+$ T cells is rescued upon weight loss. To address this, mice were categorised into three different diet groups- HFD and chow diet (CD) as already implemented in the 2017 study but a 14-week diet instead of 8 weeks and an additional group of mice that was put on a recovery diet (CD) following the HFD regimen to facilitate weight loss (figure 1.3). Intriguingly, it was observed that although the recovery (HFD-RE) mice lose weight comparable to the CD group, they still had an expanded pro-inflammatory effector memory $CD4^+$ T cell population

similar to the HFD group, hinting at long-lasting effects of obesity on the phenotype of $CD4^+$ T cells (figure 1.4) (unpublished data).

Importantly, one of the major challenges with maintaining a healthy weight following obesity is that obese patients find it difficult to lose weight especially in the first year and even worse, a majority of obese individuals who have lost weight go on to regain even more weight (130–134). There has been strong evidence linking chronic systemic inflammation and a dysregulated immune system in obesity to weight re-gain, however the underlying mechanisms are not well understood (135–140). Interestingly, $CD4^+$ T cells were identified to play a key role in possessing obesity memory and promoting weight regain in a study by Zou and his colleagues. It was found that depletion of $CD4^+$ T cells prevented the development of obesity memory and furthermore, the transfer of previously obese $CD4^+$ T cells into immunodeficient mice kept on a high fat diet experienced an accelerated weight regain (141). This is in line with the observation that $CD4^+$ T cells are skewed towards a pro-inflammatory phenotype even after weight loss. However, the mechanisms that confer $CD4^+$ T cells with an obesity memory have not been explored.

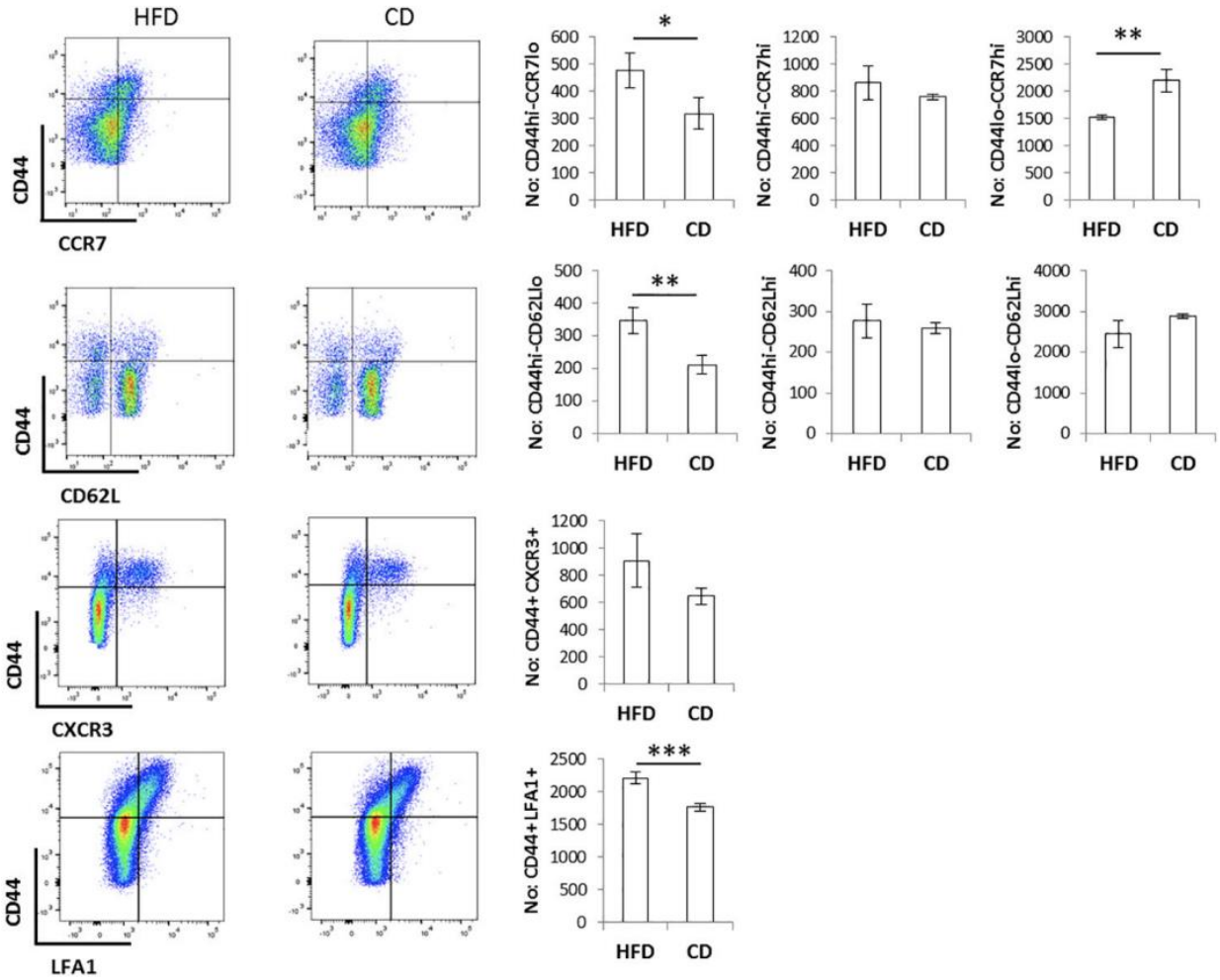


Figure 1.2. Obese CD4⁺ T cells preferentially differentiate into a pro-inflammatory effector memory-like phenotype.

Flow cytometry quantification: Cell surface staining of CCR7, CD62L, CXCR3 and LFA1 in in-vivo-primed CD4⁺ T cells isolated from pooled lymph nodes of the HFD and CD mice (3-6 independent donors) shows that obese CD4⁺ T cells preferentially differentiate into a CD44hi-CCR7lo-CD62Llo-CXCR3+ -LFA1+ effector memory-like phenotype, while there is no change in in the CD44hi-CCR7hiCD62Lhi central memory population (Mauro et al., 2017) (111)

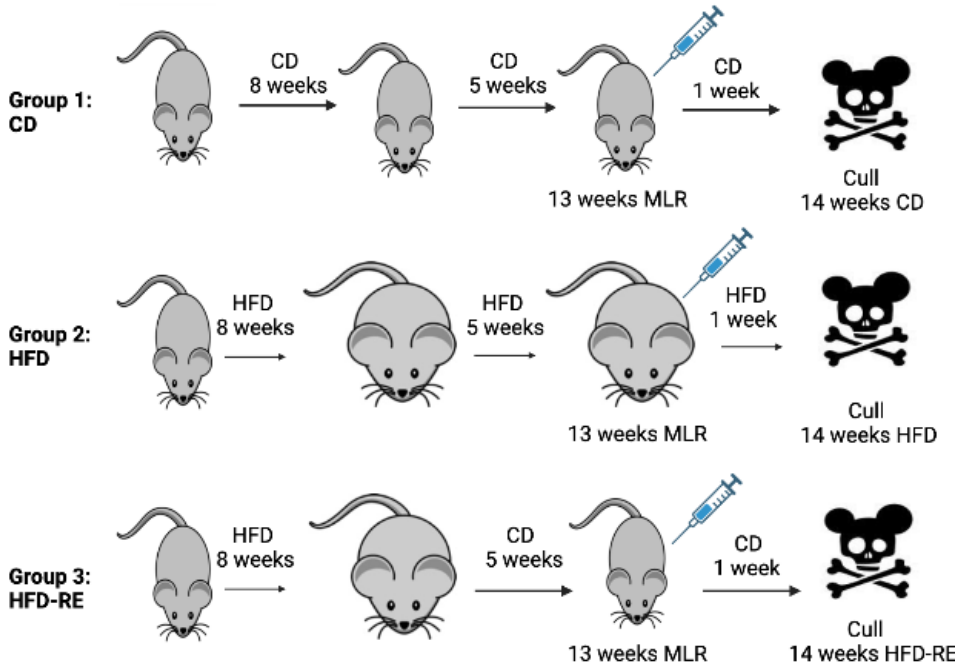


Figure 1.3. *In vivo* mice HFD experiment

Mice were categorised into three different diet groups: CD, HFD and HFD-RE. CD and HFD mice were on 14 weeks of CD and HFD respectively. HFD-RE mice were on HFD for 8 weeks followed by CD for the remaining 6 weeks. On week 13, mice were allo-immunised, via i.p. injection of mixed splenocytes from CBA and Balb/c mouse strains. They were culled at the end of 14 weeks, and spleen, lymph nodes and adipose tissues were collected for analysis.
Abbreviations: MLR, Mixed Lymphocyte Reaction

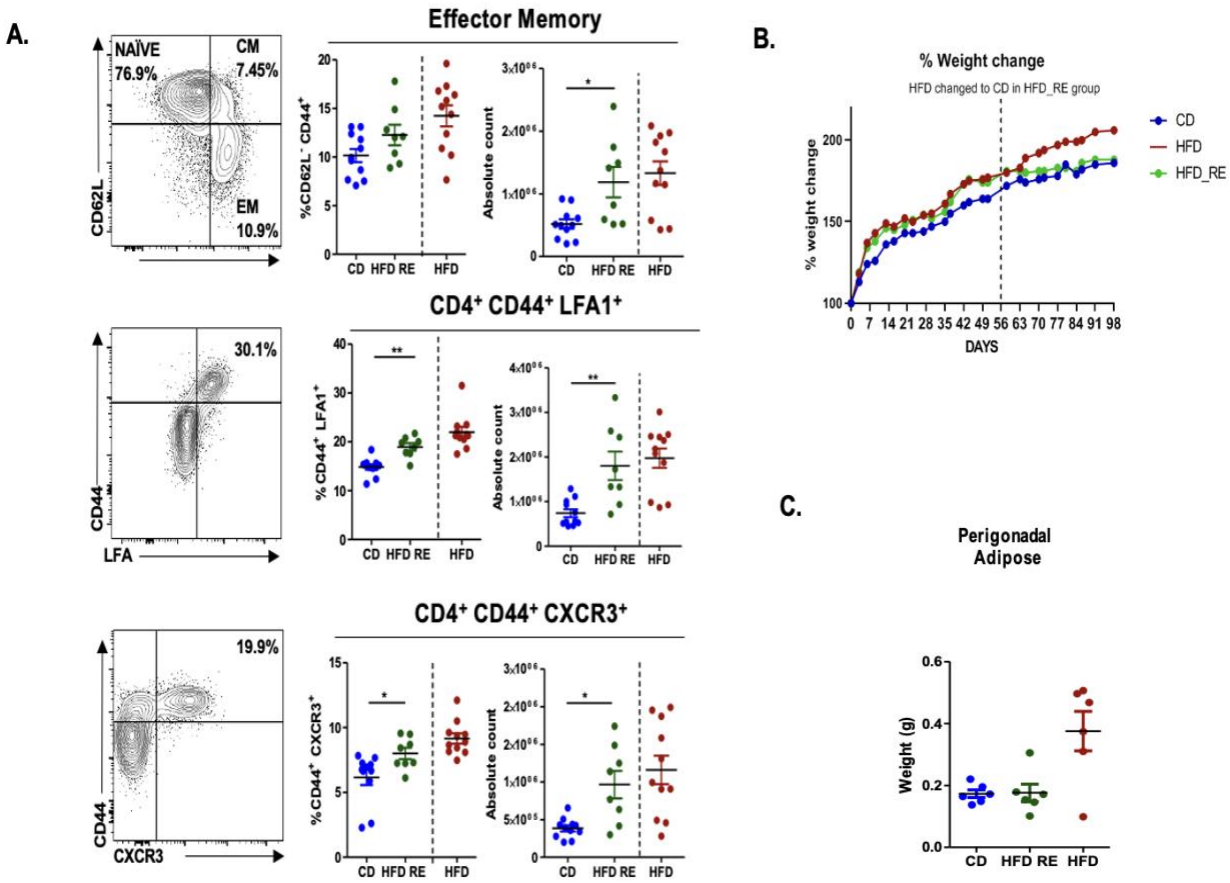


Figure 1.4. Obese CD4⁺ T cells preferentially differentiate into a pro-inflammatory effector memory phenotype even after weight loss.

(A) Flow cytometry quantification- Cell surface staining of CD62L, LFA1 and CXCR3 in in-vivo-primed CD4⁺ T cells isolated from pooled lymph nodes of CD, HFD and HFD-RE mice (6 independent donors), showing both absolute counts (upper) and percentage change (lower). Data reported as a combination of 2 independent experiments mean ± SEM; n=10-11 mice/group; *p<0.05; **p<0.01 (Mann Whitney test). This shows that the pro-inflammatory effector memory CD4⁺ T cell population expanded excessively even when alloimmunisation occurred after weight loss and this is comparable to the phenotype seen in the HFD group. (B) Percentage weight change of mice under different experimental diets. (C) Weight of perigonadal adipose tissue in grams isolated from mice under different experimental diets. (Niven et al., unpublished data)

1.8. Fatty acid-driven epigenetic imprinting in obesity

Epigenetics refers to the study of stable and heritable changes in the genome induced by external factors like environment and lifestyle, that do not involve any alterations in the underlying nucleotide sequence. They are most commonly manifested as changes in gene expression levels, responsible for switching on or off genes and controlling the fates of transcription and translation. Epigenetic changes are important for regulating biological processes crucial for normal development of cells and maintaining the body's homeostasis as well as adaption to changing environmental conditions throughout the lifetime of an organism. However, aberrant epigenetic changes can lead to abnormal activation or silencing of genes and have implications in several inflammatory diseases including cancer, diabetes, asthma, autoimmune diseases and neurodegenerative disorders, making Epigenetics a fast growing field of research in recent years (142–145). The major mechanisms in epigenetic regulation include DNA methylation, histone modifications and RNA based interferences (146–152). DNA methylation, the most extensively studied epigenetic mechanism, encompasses the covalent binding of a methyl group to the C5 position of cytosine residues at CpG sites, associated with essential developmental processes including genomic imprinting, X-chromosome inactivation and transcriptional gene silencing and linked to a number of diseases like cancer, age-associated disorders, metabolic, autoimmune and neurological disorders (153–155). Histone modification,

responsible for regulating the structure and function of chromatin, involves post-translational modification of histone proteins through different processes including phosphorylation, ubiquitination, acetylation and methylation (156,157). Histone marks are closely associated with several inflammatory diseases including type 2 diabetes, atherosclerosis, Alzheimer's diseases, psoriasis, asthma, multiple chronic lung diseases and inflammatory bowel disease (158). Non-coding RNAs, comprising of around 98% of the total transcriptional yield in humans, are categorised into two main types based on size- short chain non-coding RNAs (including siRNAs, miRNAs, and piRNAs) and long non-coding RNA (lncRNAs) (159). Over the years, non-coding regulatory RNAs, especially lncRNAs, have garnered significant attention in terms of epigenetic regulation at the transcriptional and translational levels and their disruption has been implicated in inflammatory, cardiovascular, neurological and non-neoplastic diseases (151,160–162).

Interestingly, dietary fatty acids play a key role in regulating the epigenetic landscape of chronic illnesses including obesity, type 2 diabetes, cardiovascular diseases and cancer (163). In recent years, several studies have demonstrated the roles of dietary fatty acids ranging from MUFA and PUFA to saturated and trans fats in modifying epigenetic processes in diseases. For instance, omega-3 has been shown to mediate hyper-methylation of genes associated with

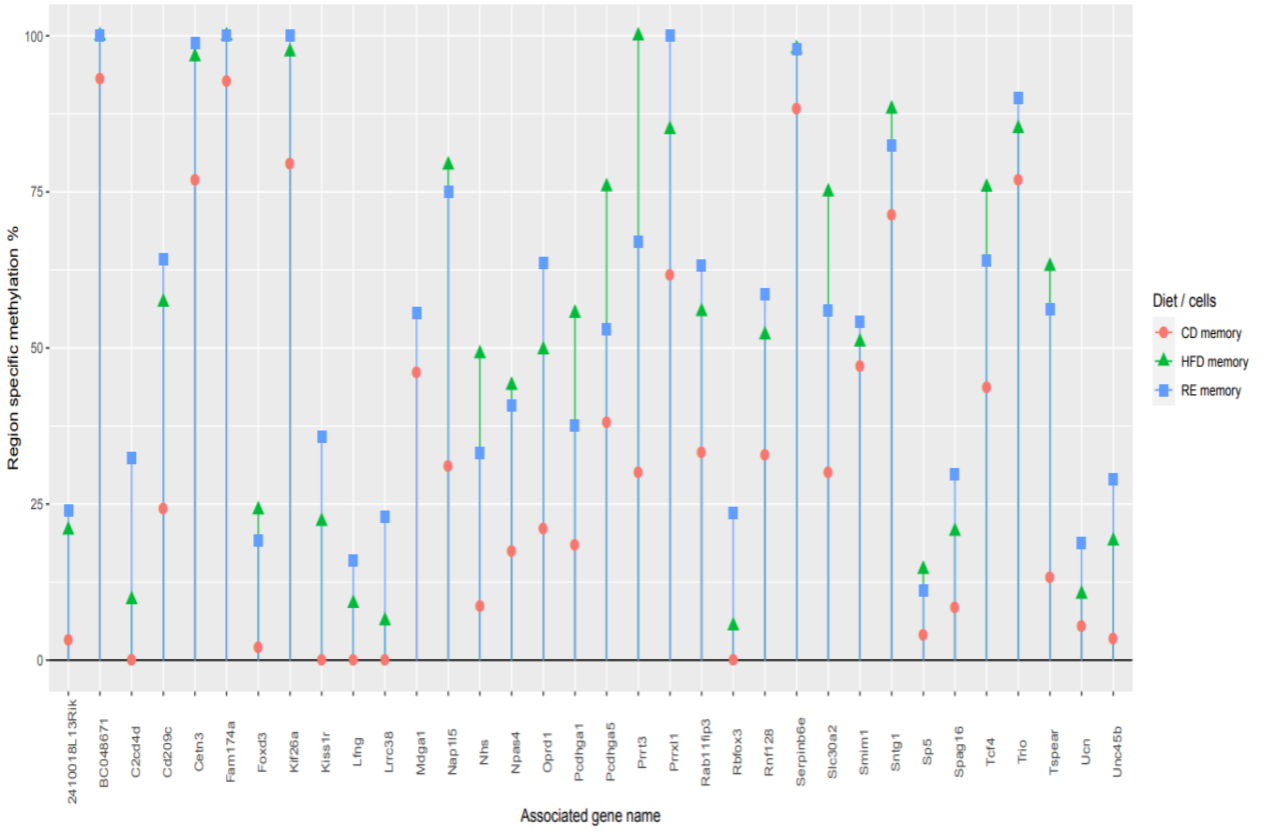
inflammatory and immune responses, lipid metabolism, T2D, and cardiovascular signalling, which in part contributes to the beneficial effects of the fatty acid (164). On the contrary, saturated- and trans-fat-mediated epigenetic changes have detrimental effects in diseases. There have been many studies till date elucidating the contribution of saturated fats, mainly palmitic acid, to epigenetic modifications in the context of obesity, IR, type-2 diabetes and altered lipid metabolism (165–169). Utilising models of IR, it has been shown that circulating palmitate induces a metabolic memory through increased di-methylation of histone H3 at the 36th lysine residue (H3K36me2), associated with transcriptional activation and decreased tri-methylation of histone H3 at the 27th lysine residue (K27me3), linked to the downregulation of nearby genes, on promoter region of FOXO1, increasing its activity and hence favouring IR-induced gluconeogenesis and hyperglycaemia, that persists even after circulating fatty acid levels have been normalised (165). Another study analysing the effect of PA on the genome wide DNA methylation in human pancreatic islets identified altered DNA methylation patterns in 290 genes, of which 73 were linked to BMI (166). PA-mediated epigenetic changes have implications in cancer too; recently, it was shown to induce a pro-metastatic memory in human oral squamous cell carcinoma (OSCC) cells, by increasing the abundance of H3K4me3 (tri-methylation at the 4th lysine residue of H3 that activates transcription) on neural-related genes, which stimulates intra-tumoural Schwann cells and innervation,

eventually leading to long term stimulation of metastasis (170). More recently, it was observed that HFD, stearic acid in particular, triggers persistent epigenetic reprogramming of ATMs in the promoter region of *tnf* gene and at Activator protein (AP1) binding sites, pre-disposing ATMs to neuroinflammation by enriching pathways involved in angiogenesis, cytokine production, and inflammatory response (171). Although there are many studies demonstrating fatty- acid driven epigenetic imprinting in obesity and other diseases, none of them focus on epigenetic reprogramming of the adaptive immune system which plays a key role in mediating obesity-associated chronic low-grade inflammation.

Knowing that the effects of obesity on the immune system, especially $CD4^+$ T lymphocytes are long lasting and $CD4^+$ T cells play an important role in mediating obesity memory and promoting weight regain, the obvious question that was prompted was whether obesity or HFD precisely leads to the epigenetic imprinting of naïve $CD4^+$ T cells making them biased towards differentiation into a pro-inflammatory effector memory like phenotype. Investigation of this was done by our laboratory (Niven et al., unpublished data) in which naïve and memory $CD4^+$ T (CD44 low and high respectively) cells were isolated from the three groups of mice that were on HFD, CD and HFD-RE and genome-wide T-lymphocyte DNA methylation changes were accessed using unbiased high-throughput Reduced Representation Bisulfide Sequencing (RRBS) datasets. Deep bioinformatic analysis of the RRBS

datasets revealed 70 differentially hypo-methylated and 23 hyper-methylated genes in the high-fat diet and recovery groups compared to the chow diet group, underpinning the differential phenotypes of $CD4^+$ T cells in the HFD and RE groups (figure 1.5) (unpublished data).

A.



B.

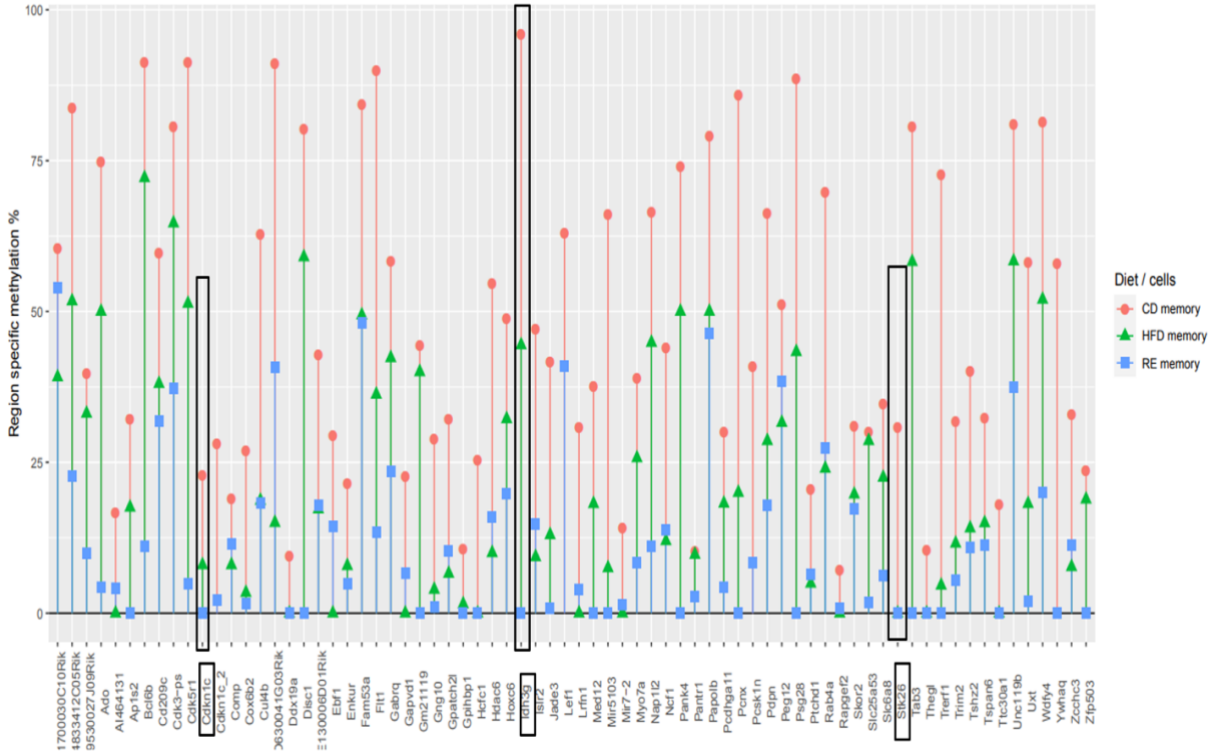


Figure 1.5. Genome-wide DNA methylation analysis of naïve and memory CD4⁺ T cells isolated from experimental mice in Figure 1.3.

(A) Graph showing percentage methylation (represented by the length of each vertical line segment) of differentially 23 hypermethylated genes in the HFD and HFD-RE groups in compared to the CD group. (B) Graph representing percentage methylation of 70 differentially hypo-methylated genes in the HFD and HFD-RE groups related to the CD group. Highlighted in boxes are the candidate genes selected from the DNA methylation screen.

1.9. Autophagy, Senescence and Obesity

1.9.1. Autophagy in Obesity

Autophagy is a highly conserved cellular degradation process in which the body's cells clear out damaged, dysfunctional or unnecessary components through a lysosome-dependent degradation mechanism to ensure homeostasis, differentiation, development and survival (172). There are three major types of autophagy in eukaryotes- macro-autophagy (sequestration of cytosolic components into double membraned autophagosomes that subsequently fuse with lysosomes), micro-autophagy (degradation of cytosolic components by sequestering them directly into the lysosome) and chaperone-mediated autophagy (selective degradation of soluble cytosolic proteins selected and delivered to the lysosome by a chaperone complex), involving different molecular mechanisms but ending in delivery of cargo to the lysosome for degradation and recycling (173–176). Autophagy, first discovered in yeast as a survival mechanism to combat nutrient deprivation, has emerged as a tightly regulated process with critical roles in normal development and maintenance of organism's health, that declines with age and is dysregulated in several diseases including cancer, neurodegenerative disorders and metabolic diseases including obesity, IR, diabetes and atherosclerosis (177–181). Macro-autophagy, the main autophagy pathway, studied extensively in

ageing and human diseases, is mediated by a number of conserved Atg (autophagy-related) proteins and involves five sequential steps: i) initiation, ii) Double-membrane nucleation and formation of pre-autophagosome or phagophore formation, iii) Pre-autophagosome elongation and sequestration of cytoplasmic cargo, iv) Fusion of autophagosome to lysosome and v) Degradation of sequestered cargo in the auto-lysosome (177,182,183). In mammals, nutrient sensors mTOR and AMP-activated kinase (AMPK) are the main upstream regulators of this elaborate pathway that regulate the process through inhibitory phosphorylation of the Unc-51-like kinases ULK1 and ULK2 (mammalian homologue of yeast Atg1) (184). Autophagy initiates with dephosphorylation and activation of the ULK kinases, which in turn phosphorylate Beclin-1 (mammalian homologue of yeast Atg6), part of the Vps34/class III phosphatidylinositol 3-phosphate kinase complex (PIK3C3), generating phosphatidylinositol 3-phosphate (PtdIns(3)P) at early autophagic structures, a key event in autophagosome biogenesis (185). Both ULK and Beclin-1 are recruited to the phagophore where they participate in the activation of downstream autophagy proteins (186,187). Other key families of autophagy proteins include i) the transmembrane protein ATG9 that shuttles to and from the sites of autophagosome formation and its trafficking machinery required for membrane delivery to

the expanding phagophore, and ii) the ubiquitin-like mammalian ATG8 (mATG8) homologues of the microtubule-associated protein (MAP1) light chain 3 (LC3) and γ-aminobutyric acid receptor associated proteins (GABARAP), required for autophagy substrate selection and autophagosome biogenesis and used commonly to monitor autophagy flux (188–191). Membrane formation in autophagy is mediated by two ubiquitin-like conjugate steps. In the first step, Atg7 (a ubiquitin E1-like enzyme), and Atg10 (a ubiquitin E2-like enzyme) conjugate ubiquitin-like protein Atg12 to the lysine residue in Atg5, and the resulting Atg12–Atg5 conjugate forms a complex with Atg16 to form a ubiquitin E3-like enzyme ATG5-12-16L1 complex which is needed for downstream conjugation of LC3/GABARAP (192). Following this, LC3/GABARAP family proteins localize to autophagosomal membranes as pro-LC3/GABARAP where they are activated through C-terminal proteolytic cleavage by the ATG4 family of proteins to become fully functional LC3/GABARAP (also called LC3A/LC3I) (193). LC3-I is conjugated to phosphatidylethanolamine (PE) on the surface of nascent autophagosomes by the action of ATG7, ATG3 (a ubiquitin E2-like enzyme) and the ATG5-12-16L1 complex to form lipidated LC3, also called LC3B/LC3II, which then contribute to the elongation and closure of the autophagosome, and recognition and recruitment of cargo to the autophagosome by interacting

with various autophagy receptors like the ubiquitin-binding protein receptor p62 (also called sequestosome-1) or cargo receptors bound to proteins or organelles (194–196). Finally, the completed autophagosome fuses with a lysosome, mediated by the action of many proteins, including SNARE and UVRAG (197–200). Once the fusion occurs, LC3 on the inner side of the vesicle are degraded along with cargo while that on the outer side are cleaved off (de-lipidation) by Atg4 and recycled (201). This is followed by degradation of the contents of the autolysosome by the action of proteases, lipases, nucleases and glycosidases and subsequent release of the breakdown products into the cytosol by lysosomal permeases (202).

The exact impact of autophagy and its upregulation or inhibition in the context of obesity is controversial and not understood very well. Since starvation stimulates autophagy, it was initially thought that autophagy is suppressed during obesity due to excess nutritional uptake, known to inhibit AMPK and subsequently upregulate mTOR, the autophagy inhibitor (203,204). In line with this notion, several evidences indicate reduced expression of autophagy-related proteins ATG5 and ATG7 and subsequent inhibition in autophagosome biogenesis as well as reduced AMPK signalling resulting in decreased autophagosome formation (205–208). On the contrary, other

studies involving obese mice and human subjects showed increased autophagosome accumulation in adipose and liver tissues, indicating that there is an autophagy induction in response to obesity (209–212). It is likely from these observations that autophagy is triggered as a protective mechanism in response to ER or oxidative stress associated with chronic low-grade inflammation in obesity, to maintain cellular homeostasis (213–215). However, the major challenge in these studies is the assumption that obesity-associated accumulation of autophagosome results from an increase in autophagy flux while the accumulation could simply be due to a defect in degradation stemming from impaired autophagy flux. In most of these studies, an accumulation of autophagy substrates was found, while in reality, functional autophagy should result in decreased accumulation of autophagy substrates. Importantly, autophagy has diverse roles across different organs in the context of obesity- the liver and hypothalamus show reduced autophagic activity in conditions of prolonged obesity while pro-inflammatory cytokines in an obese VAT have been shown to stimulate autophagy in adipocytes (216–218). It needs to be understood that even in conditions of autophagy induction during obesity, prolonged inflammatory stress can eventually impair autophagy, so the relationship between obesity and autophagy is not so straight-forward and there is no definitive finding to

conclude if it is a protective effect or ameliorates ER and inflammatory stress in obesity. Changes in autophagy may also be influenced by various other factors including the model of obesity being studied, duration of obesity, age of patients and the use of *in vivo* or *in vitro* models (219). Moreover, monitoring autophagic flux is complicated and changes in commonly used markers like P62 and LC3 could be misleading (220).

Recently, STK26/MST4 (serine/threonine kinase 26), a member of the mammalian sterile-20 like kinase family, implicated in normal development as well as cancer progression and metastasis, was identified as an inducer of autophagy (221). Amongst the four isoforms of ATG4, ATG4B displays the highest selectivity towards LC3B (222). STK26/MST4 phosphorylates ATG4B at serine residue 383, stimulating ATG4B activity and increasing autophagy flux (figure 1.6) (221). STK26 is interesting in the context of our research because *STK26/MST4* was identified as one of the 70 hypomethylated genes in the DNA methylation screening of obese $CD4^+$ T cells that had a pro-inflammatory phenotype even after normalisation of weight. Targeted pyrosequencing analysis was done to confirm hypomethylation of *stk26* (figure 1.7) (unpublished data). Notably, autophagy plays a critical role in regulating $CD4^+$ T cell development, proliferation, energy metabolism and homeostasis (223,224). Moreover, it plays an integral role in the generation of

memory CD4$^+$ T cells and their survival against the toxic effects of mitochondrial activity and toxic overload, and is associated with the pathogenesis of rheumatoid arthritis (225). Clearly, the relevance of autophagy to memory CD4$^+$ T cell development and survival besides its significance in obesity have been the consensus for choosing *stk26* as one of our candidate genes.

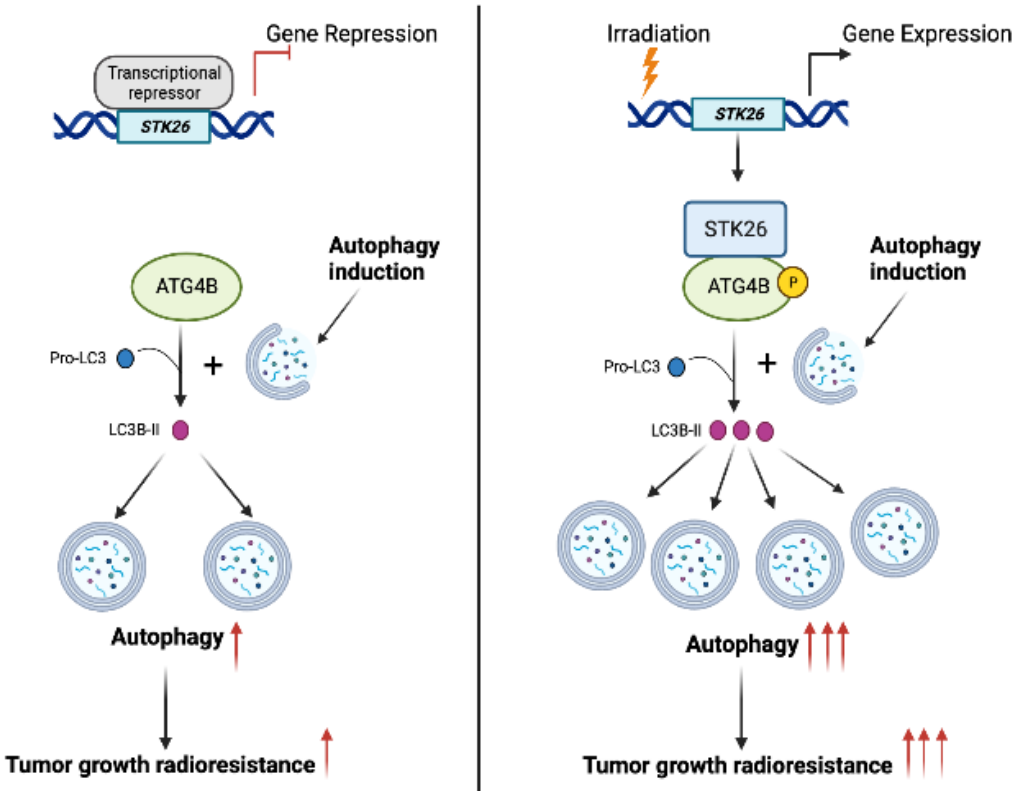


Figure 1.6. STK26 is an inducer of autophagy.

STK26 is an inducer of autophagy that phosphorylates ATG4B, which stimulates ATG4B activity and increases autophagic flux. Low STK26 levels result in reduced autophagic flux through reduced phosphorylation of its substrate ATG4B as a result of which the tumour growth radio-resistance of glioblastoma cells is reduced. inhibition suppresses autophagy and the tumorigenicity of glioblastoma cells. On the other hand, radiation-induced high expression of STK26 promotes ATG4B phosphorylation, increasing the autophagic flux and significantly reducing tumorigenicity of glioblastoma cells.
Figure adapted from *MST4 Phosphorylation of ATG4B Regulates Autophagic Activity, Tumorigenicity, and Radioresistance in Glioblastoma* (Graphical Abstract), by Tianzhi Huang et al., November 2017, Cancer Cell, 32(6): 840–855.e8, Copyright 2017 by the authors under the CC BY-NC-ND license (204).

1.9.2. Senescence in Obesity

Cellular senescence is a process by which cells undergo stable irreversible cell-cycle arrest, accompanied by distinctive chromatin and secretome changes and tumour suppressor activation (226–229). Cells can undergo senescence in response to various developmental signals and external or internal stress factors, including progressive telomere shortening and erosion, irreparable DNA damage, genomic or epigenomic stress, oxidative stress, oncogenic activation, aberrant mitogenic signalling and mitochondrial dysfunction (230–235). Originally discovered as an anti-cancer mechanism, cellular senescence has since been implicated in many physiological processes including tissue homeostasis, tissue remodelling, tissue repair and wound healing (236–238). Regardless of losing their proliferative potential, senescent cells remain metabolically active and secrete diverse growth factors, cytokines, chemokines and extracellular matrix (ECM) components, which comprise the senescence-associated secretory phenotype (SASP) (239). Depending on the biological context, senescent and SASP factors could have either beneficial or detrimental effects. More recently, cellular senescence has emerged as a hallmark of ageing; the altered SASP profiles of senescent cells have been implicated in numerous age-related diseases including osteoarthritis, pulmonary fibrosis, atherosclerosis,

diabetes, Alzheimer's and Parkinson's diseases (240–245). Cellular senescence can be triggered by two main mechanisms both linked to tumour suppressor pathways, the p53/ p21 and $p16^{INK4a}$/pRB pathways, the key difference between the two being the $p16^{INK4a}$/pRB pathway results in an irreversible growth arrest while p53/ p21 might not necessarily induce an irreversible growth arrest (246–248). Both p53 and $p16^{INK4a}$ activate retinoblastoma protein (pRB), which in its active, phosphorylated form prevents progression from the G1 to S phase of the cell-cycle through transcriptional repression of E2F1, a master cell cycle regulator (247). The mechanism of pRB activation is different between the two pathways. p53 activates p21 (CDKN1A), a member of the Cip/Kip family of Cell-cycle dependent kinase inhibitors (CDKIs) (249). Activation of pRB requires inactivation of cyclin-dependent kinase 2 (CDK2), which is facilitated by p21, capable of inactivating all CDKs (250,251). $P16^{INK4a}$, on the other hand, activates pRB by inactivating cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6) (252,253).

Cellular senescence is one of the important mechanisms driving chronic low-grade inflammation in chronic inflammatory diseases (254). The SASP of senescent cells creates a chronic inflammatory milieu, driving both local and systemic inflammation

(255–258). There has been accumulating evidence backing up both mice and human models to suggest that the onset of obesity is linked to an increase in the number of senescent cells in adipose tissue and other organs including liver, pancreatic beta cells and brain (259–262). There is an accumulation of senescent cells in the WAT and an increase in the SASP profile with ageing and more importantly obesity (263,264). Increased ROS production in response to oxidative stress in obesity is one of the key drivers of adipose senescence (265). The use of senolytics to target increasing senescent cells in obesity holds great therapeutic potential to treat obesity-associated metabolic dysfunction through increased adipogenic potential, reduced adipocyte hypertrophy and macrophage infiltration, and enhanced insulin sensitivity (266).

Cyclin dependent kinase inhibitor 1C (CDKN1C/P57 KIP2), a member of the Kip family of CDKIs along with p21 and p27, involved in the regulation of many cellular processes including embryogenesis and tissue differentiation, is a known inducer of cellular senescence that causes cell cycle arrest mostly in the G1 phase (267). It mainly functions by inhibiting CDK2, CDK3 and CDK4, and to a lesser degree CDK1 and CDK6 (268–271). *CDKN1C* plays an important role in the development of brown adipose tissue (271). Essentially, a two-fold increase in the expression of the gene is sufficient to protect against age-related

weight gain, glucose intolerance and insulin sensitivity and furthermore, loss of *cdkn1c* imprinting protects against diet-induced obesity (272). *CDKN1C*, strikingly, was one of the differentially methylated (hypo-methylated) genes identified between the CD and HFD, HFD-RE groups in our DNA methylation screen, and the changes in gene expression were confirmed through pyrosequencing (figure 1.7) (unpublished data).

1.9.3. Crosstalk between Autophagy and Senescence

Autophagy and senescence, although distinct cellular homeostasis processes, are intertwined in many ways. Autophagy has been known to play a protective role in aging, where the decline in autophagy flux promotes senescence and accelerates the aging process (273). Several studies have demonstrated the role of autophagy as an anti-senescence mechanism, showing both the suppression of cellular senescence with an increase in autophagy flux and an induction of cellular senescence with prolonged inhibition of autophagy (274–282). However, research over the years, has shown a rather intricate relationship between the two processes, revealing instances where activation of autophagy is linked with an induction of cellular senescence, suggesting that autophagy plays dual roles in regulating cellular senescence (283–290).The

first causal relationship between autophagy and senescence was established from studies by Liu et al. and Huang et al., which showed that autophagic activity was required for radiation-induced senescence in cancer cells (291,292). Subsequent studies have identified autophagy as a pro-senescence mechanism, particularly in the context of cancer (293–295). Moreover, emerging evidence has shown that an inhibition of autophagy is accompanied by a delay in cellular senescence (296–298). It appears that autophagy facilitates cellular senescence by mediating the synthesis of the SASP (287). Goehe et al. reported that autophagy and senescence occur in parallel and are regulated by overlapping signalling pathways involving the generation of ROS, activation of DNA damage inducer protein kinase ataxia-telangiectasia mutated (ATM), induction of P53 and CDKN1A/P21, and inhibition of Rb (299). Importantly, depending on the biological context, the type of autophagy in question- general or selective autophagy, the timing of action i.e., under normal conditions or in stress and the cell type, autophagy could either trigger or inhibit cellular senescence to regulate cellular homeostasis (300).

It would be interesting to evaluate if a cross talk exists between autophagy-inducer *stk26* and senescence implicated *cdkn1c* found epigenetically altered in our HFD and HFD Re populations, given how closely linked the two processes are.

1.10. Isocitrate dehydrogenase (NAD(+)) 3 non-catalytic subunit gamma (IDH3G)

Isocitrate dehydrogenases (IDH) are essential citric cycle or tricarboxylic acid cycle (TCA) enzymes that catalyse the oxidative decarboxylation of isocitrate to 2-oxoglutarate. These enzymes belong to two important subclasses - one that utilises NAD+ as the electron acceptor and the other utilises NADP(+). IDH3, one of the three isocitrate dehydrogenase enzymes in human, is a heterodimer consisting of two catalytic 37-kDa alpha subunits (IDH3α) encoded by the *IDH3A* gene, one regulatory 39-kDa beta subunit (IDH3β) encoded by the *IDH3B* gene, and one regulatory 39-kDa gamma subunit (IDH3γ), encoded by the *IDH3G* gene (301–303). IDH3, localised in the mitochondria, relies on NAD(+) as its electron acceptor and catalyses the allosterically rate-limiting step in the TCA cycle, involving the irreversible oxidative decarboxylation of isocitrate to yield α-ketoglutarate (α-KG) and CO_2 (301,302,304). IDH3 is subject to allosteric regulation, whereby it is activated by ADP in conditions when cells need energy, and inhibited by ATP and NADH, when energy is no longer required (301).

Being the primary source of cellular energy that generates intermediates for various essential metabolic pathways in the body, it is fathomable how the TCA cycle dysfunction could lead to severe diseases (305). Impairment in the TCA enzymes has been implicated in many diseases including atherosclerosis, various cancers, neurological

and neurometabolic disorders (306–312). Evidence has shown an elevated TCA cycle function in diet-induced hepatic insulin resistance and fatty liver, contributing to the pathogenesis of mitochondrial respiratory dysfunction, hepatic oxidative stress and inflammation (313). Additionally, altered *idh3g* levels have been associated with cardiovascular diseases, Alzheimer's disease, diabetes, HIV-1 infection and cancer (314–317). Moreover, decreased TCA cycle flux, previously associated with type-2 diabetes, has been recently observed in the skeletal muscles of severely obese patients in an attempt to elucidate the mechanism leading to impaired glucose partitioning in obesity (318,319).

IDH3G was one of the 70 hypomethylated genes identified in our DNA methylation screen and after confirmation of gene expression changes by pyrosequencing (figure 1.7), it was chosen as the third candidate gene in our study because of its significance in the TCA cycle.

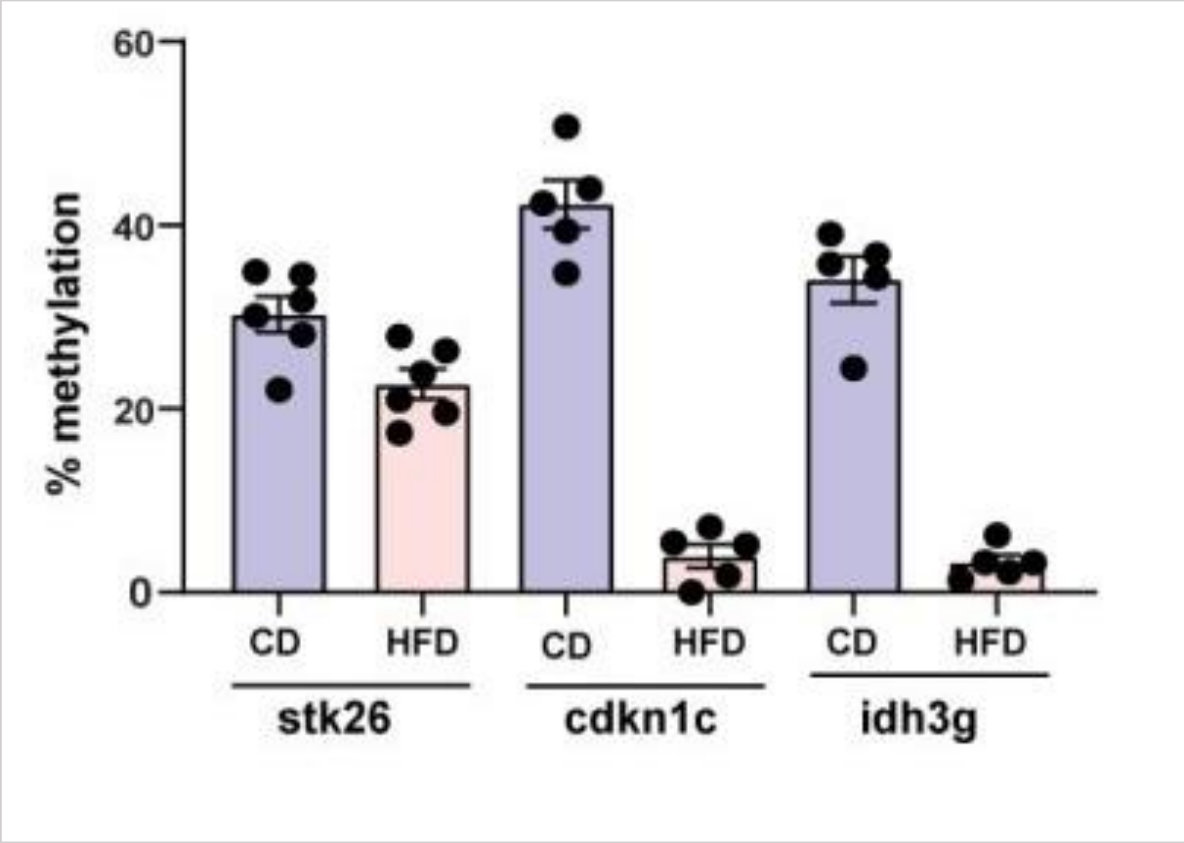


Figure 1.7. Targeted pyrosequencing analysis to confirm percentage methylation of candidate genes

Graph shows the differences in percentage methylation of three candidate genes between CD and HFD experimental mice groups. Three candidate genes-*STK26*, *CDKN1C* and *IDH3G* were chosen from the initial DNA methylation screen and targeted pyrosequencing was done to confirm gene methylation changes. (Mauro et al., unpublished data)

1.11. Research hypothesis and aims

Maintaining a healthy body weight is indispensable for a long and sustainable life. With obesity reaching pandemic proportions, it has become increasingly important to understand how weight gain, weight loss and the immune system are entwined over the course of life. Obesity has long lasting effects on the immune system which are in part responsible for weight re-gain and obesity-associated pathologies even after weight loss. As shown previously by Mauro et al. and our lab's recent pilot data, high-fat diet promotes a biased activation of

naïve $CD4^+$ T cells into a pro-inflammatory effector memory-like phenotype which is not rescued even after normalisation of body weight. Deep bioinformatic analysis of our global DNA methylation dataset generated from naïve and memory $CD4^+$ T cells of mice on chow diet, high-fat diet and recovery diet, has revealed 70 hypomethylated and 23 hypermethylated genes differentially expressed between the high fat diet and recovery groups relative to the chow diet group. Out of the 70 hypomethylated genes identified, we have chosen three candidate genes namely *STK26/MST4*, *CDKN1C/P57KIP2* and *IDH3G* because of their significance in autophagy, senescence and the TCA cycle respectively- processes that are found dysregulated or impaired in obesity. Our hypothesis is that long lasting DNA methylation alterations in these genes may underpin the exacerbated pro-inflammatory like phenotype of obese $CD4^+$ T cells. Confirmation of these potential epigenetic changes through genomic and proteomic analyses utilising both *in vivo* and *in vitro* models and finally understanding how these metabolic processes control the exacerbated response of obese $CD4^+$ T lymphocytes could help support interventions for the return of immune homeostasis upon weight loss. To address this hypothesis, we set the following aims and objectives-

1. *In vivo* studies: a) Confirming candidate gene and protein expression changes in mice fed chow diet, high fat diet or recovery diet through Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis and b) Nano-string

analysis of mRNA isolated from the memory $CD4^+$ T cells of mice spleen to investigate potential inflammatory and immune changes underpinning the obesity-driven dysregulation of $CD4^+$ T lymphocytes.

2. *In vitro* studies: a) Establishing an *in vitro* model of obesity by co-culturing human $CD4^+$ T cells with adipose-conditioned media from different osteoarthritis patients with healthy and obese BMIs followed by TCR stimulation b) Confirming gene expression changes.
3. *In vitro* studies: a) Investigating the direct effects of fatty acids on human $CD4^+$ T lymphocytes by co-culturing them with different fatty acids followed by TCR stimulation and b) Confirming candidate gene and protein expression changes through RT-qPCR and western blot analysis.
4. *In vitro* studies: a) Co-culturing human $CD4^+$ T cells with adipose-conditioned media from different osteoarthritis patients with healthy and obese/overweight BMIs and b) Confirming gene expression changes, to evaluate if there is a similar effect to when $CD4^+$ T cells are treated with fatty acids *in vitro* and activated.

2.0. MATERIALS AND METHODS

2.1. Murine study

All murine experiments were conducted in accordance to the United Kingdom Home Office, Animals (Scientific procedures) Act of 1986; under the project licence PDA36EFFD9. Wild-type (WT) C57BL/6J female mice (5 weeks old, Charles River Laboratories, Bristol, UK) were allocated to three groups of 7 animals each. They were fed either a i) chow diet (CD) (CRM-P, Special Diet Services, LBS Biotech, London, UK), a ii) high-fat diet (HFD) (D12492, Rodent Diet with 60 kcal% Fat, Research Diets, Inc, New Brunswick, USA) for 14 weeks and the third group iii) HFD-RE were fed HFD for 8 weeks followed by CD for the remaining 6 weeks. Mice were immunised in week 13. At the end of 14 weeks, all animals were euthanised via rising concentrations of CO_2 (Schedule 1 procedure), and spleen, mesenteric lymph nodes, peripheral lymph nodes, adipose tissue from lower peritoneum (perigonadal fat) and subcutaneous fat from the animals were harvested.

2.2. *In vivo* T-cell activation

Mice were given allogenic immunisation in order to elicit an immune response and generate memory T cells. All mice except one (non-injected control) per group were intra-peritoneally injected an equal

mixture (1:1 ratio) of splenocytes from WT Balb/c and CBA male mice strains (Charles River Laboratories, Bristol, UK) , in week 13, i.e., one week prior to cull.

2.3. Tissue preparation and isolation of murine $CD4^+$ T cells

Spleen and lymph nodes (peripheral and mesenteric) were collected from all experimental mice. To obtain lymph node and splenocyte single cell suspensions, tissues were smashed in petri-dish with phosphate buffered saline (PBS) containing 2% foetal calf serum (FCS) (Sera Laboratories International, London, UK) and filtered through a 50 µm strainer. Red Blood Cells (RBCs) were removed from splenocytes using the Hybri-Max red blood cell lysis buffer (Sigma-Aldrich, Merck, UK) according to manufacturer's instructions. Splenocytes were then re-filtered through a 50 µm strainer to ensure single cell suspension was achieved. Murine bulk or memory $CD4^+$ T cells were isolated from splenocyte suspension using commercially available isolation kits (EasySep™ Mouse $CD4^+$ T Cell Isolation Kit or EasySep™ Mouse Memory $CD4^+$ T Cell Isolation Kit, Stemcell Technologies, UK), according to manufacturer's instructions. Isolated cells were snap frozen for western blot, while for RNA isolation, the cell pellets were dissolved in 350 µL RLT buffer (QIAGEN, UK) and vortexed before freezing them to -80 °C.

2.4. Human CD4$^+$ T cell isolation and culture

2.4.1. Isolation of human Peripheral Blood Mononuclear Cells (PBMCs)

Human blood cones were obtained from the Birmingham blood donor and transfusion centre under ethical approval from the NHS West Midlands Research Ethics Committee (12/WM/0077) and the University of Birmingham Life and Health Sciences Ethical Review Committee (ERN_10-1246). Human peripheral blood nuclear cells (PBMC) were isolated from blood cones using Ficoll-Paque plus density gradient centrifugation. Firstly, the blood cone was cut into a 50 mL falcon tube and diluted at a 1:1 ratio with PBS. To achieve a Ficoll-paque density gradient, 20 mL of the blood solution was layered carefully on top of 20 mL of Ficoll-Paque™ Plus (GE Healthcare, Buckinghamshire, UK). The gradient was then centrifuged at 400 g for 30 minutes at 18 °C to 20 °C with no brake and an acceleration of 1. Post centrifugation, the cloudy middle band (this is where PBMCs reside) was drawn off and pulled into a separate 50 mL falcon tube. Cells were washed twice with PBS at 400 g for 10 minutes at 18 °C to 20 °C with the acceleration and deceleration rates set at 9. Following two washes, the supernatant was discarded, and cells were re-suspended in PBS, following which they were counted using a haemocytometer. To check for PBMC viability, the trypan blue dye (1:10 dilution) was used during counting that

excludes membrane-intact live cells and makes the membrane-compromised dead cells dark blue. The average PBMC yield ranged from 1 x 10^6 to 3 x 10^6 /mL of blood and no major cell death was observed.

2.4.2. Human $CD4^+$ T cell isolation, treatment and activation *in vitro*

Bulk $CD4^+$ T cells were isolated from human PBMCs using the Stemcell EasySepTM human $CD4^+$T cell isolation Kit (Stemcell Technologies, Cambridge, UK) according to the manufacturer's instructions. Isolated $CD4^+$ T cells were suspended in media consisting of RPMI-1640 (RO883, Sigma Aldrich, UK), 10% FCS, 1% nonessential amino acids, 1% penicillin and streptomycin, 1% L-glutamine and 0.1% beta-mercaptoethanol (β-ME). They were then plated in 24-well cell culture plates (Corning™ 3524, Thermo Fisher Scientific, UK), treated with either adipose-conditioned media or different fatty acids (preparation described below) and cultured at 37 °C, 5% CO_2 for 24 hours. Following treatment, the cells were activated using Dynabeads™ Human T-Activator CD3/CD28 (Gibco™, Thermo Fisher Scientific, UK) and cultured for an additional 48 hours at 37 °C, 5% CO_2 before collecting and snap-freezing them for RNA isolation and western blot.

2.4.3. Preparation of adipose-conditioned media (ACM)

Subcutaneous adipose tissue (SAT) was obtained from osteoarthritis patients undergoing total hip and knee replacement surgery at the Queen Elizabeth Hospital, with their consent. These patients had either a normal BMI or were obese/overweight. To prepare ACM, SAT samples were incubated in myotube differentiation media (HAMS F-10 media supplemented with 6% horse serum) at a ratio of 1 g tissue to 10 mL media at 37 °C, 5% CO_2 for 24 hours. After 24 hours, the ACM was removed, sterile filtered, aliquoted into 5 mL sample containers and stored at -80ºC.

2.4.4. Preparation of fatty acids

For treating $CD4^+$ T cells with PA, SA, OA and EPA in-vitro, sodium palmitate (Sigma-Aldrich, USA), sodium stearate (Sigma-Aldrich, USA), sodium oleate (Cayman Chemical, Michigan, USA) and EPA (Cambridge Bioscience, Cambridge, UK) were prepared as a 10 mM stock solution by dissolving in a 1:4 solution of Bovine Serum Albumin (BSA) and ethanol, and sonicating for 15 minutes at 37 °C. The stock solutions were sterile filtered and stored at -20 °C. Plated $CD4^+$ T cells were treated with 0.5% ethanol (untreated [UT]), 50 µM PA, 50 µM

SA, 50 µM OA or 20 µM EPA in complete RPMI-1640 media for 24 hours at 37 °C, 5% CO_2.

2.4.5. Autophagy inhibition

To inhibit autophagy and monitor it through western blotting, $CD4^+$ T cells, post lipid treatment and TCR activation, were treated with Chloroquine (CQ) (Cell Signalling Technology, USA). The stock concentration of CQ was 50 mM. Cells were dosed with either 50 µM CQ + complete RPMI-1640 media (CQ treated) or 0.05 µL water (since the CQ was dissolved in it) + complete RPMI-1640 media (CQ untreated) and cultured at 37 °C, 5% CO_2 for either 4 hours or 12 hours.

2.5. RNA isolation and real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cells in RLT buffer using the RNeasy mini kit (Qiagen, Manchester, UK) according to manufacturer's instructions. RNA quality and concentration were measured on NanoDrop 2000 (Thermo Scientific™, Thermo Fisher Scientific). Following RNA extraction, 250 or 500 ng of total RNA was transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem™, Thermo Fisher Scientific), according to manufacturer's instructions. cDNA samples were stored at -20 °C.

For mice samples, RT-qPCR was performed in triplicate using TaqMan™ gene expression assays (FAM labelled) (Applied biosystems™ ,Thermo Fisher Scientific) and the TaqMan™ Gene Expression Master Mix (Applied biosystems™ , Thermo Fisher Scientific). The following TaqMan probes were used:

1. Stk26, TaqMan Assay ID: Mm00505199_m1
2. Cdkn1c, TaqMan Assay ID Mm00438170_m1
3. Idh3g, TaqMan Assay ID: Mm00599689_m1
4. Cd36, TaqMan Assay ID: Mm00432403_m1
5. 18s rRNA, TaqMan Assay ID: Hs99999901_s1

The master mix was prepared per 2.25 uL of cDNA (diluted 1 in 5 with nuclease-free water) according to table 2.1. To perform TaqMan qPCR, the CFX384 Touch Real-Time PCR Detection System (Bio-rad, UK) was used, and the reaction conditions were: 50 °C for 2 min (Uracil-N-glycosylase (UNG) activation), 95 °C for 10 min (Taq activation) and then 40 cycles at two steps: 95 °C for 15 sec for denaturation, and 60 °C for 1 min for annealing and extension. Results were analysed using the comparative Ct method in which Ct values of target genes were normalised against the Ct value of the housekeeping gene 18s rRNA (Eukaryotic 18S rRNA Endogenous Control, Applied biosystems™, Thermo Fisher Scientific). Data were expressed as mean ± Standard Error of Mean (SEM) relative to CD.

Table 2.1. Master mix preparation for TaqMan RT-qPCR (2.25 uL cDNA per well)

Reagent	Volume per well (uL)
TaqMan Gene Expression Master Mix (2x)	2.5
TaqMan Gene Expression Assay (20x)	0.25

For human samples, RT-qPCR was performed in triplicate using TB Green Premix Ex Taq II (Tli RNase H Plus) (Takara Bio, California, USA). Primers for the target genes were purchased from Sigma-Aldrich. Primers for the housekeeping gene β-Actin were purchased from Invitrogen. Primers were supplied as 100 uM stocks from which a working concentration of 10 uM was made. The list of primers and their sequences is illustrated in table 2.2. The master mix was prepared per 1 uL of diluted cDNA according to table 2.3. RT-PCR was performed in a CFX384 Touch Real-Time PCR Detection System, and the reaction conditions were initial denaturation phase at 95 °C for 10 minutes, amplification phase- 40 cycles of 95 °C for 10 seconds and 60 °C for 45 seconds, followed by melting curves phase at 95 °C for 10 seconds, 65 °C for 5 seconds and 95 °C for 0 second. Cycle threshold (Ct) values were recorded. Results were analysed using the comparative Ct method in which Ct values of target genes were normalised against the Ct value of the housekeeping gene β-actin.

Table 2.2. List of primers (human)

Gene	Orientation	Sequence (5'-3')
ACTB	Forward	AGTTGCCTTACACCCTTTCTTG
ACTB	Reverse	TCACCTTCACCGTTCCAGTTT
CD36	Forward	CCTCCTTGGCCTGATAGAAA
CD36	Reverse	GTTTGTGCTTGAGCCAGGTT
CDKN1C	Forward	ACCAGAACCGCTGGGATT
CDKN1C	Reverse	CACCGTCTCGCGGTAGAA
IDH3G	Forward	CCCTCAGATCACCTTCGAGA
IDH3G	Reverse	GGCATCACCATGACATCAAA
IL2RA	Forward	ACGGGAAGACAAGGTGGAC
IL2RA	Reverse	TGCCTGAGGCTTCTCTTCA
STK26	Forward	GAAAGTACAGAATGGGGCAGA
STK26	Reverse	CTGATTCCTGCTAGCGTTATTCT

Table 2.3. Master mix preparation for Syber green PCR (1 uL cDNA per well)

Reagent	Volume per well (uL)
TB Green Premix Ex Taq II (2x)	2.5
Forward Primer (10 uM)	0.1
Reverse Primer (10 uM)	0.1
Nuclease-free water	4

2.6. NanoString nCounter analysis

Total RNA was isolated from the splenic memory $CD4^+$ T cells of mice using the RNeasy micro kit (Qiagen, UK) according to manufacturer's instructions. Quality control check of RNA was done by the Genomics faculty at the University of Birmingham using the Qubit RNA High Sensitivity (HS) and TapeStation HS assays. Total RNA (40 ng) from 12 samples (four each from CD, HFD and HFD-RE groups) were run for mRNA-expression analysis on the nCounter-based NanoString instrument (NanoString Technologies, Seattle, USA) using the nCounter® Mouse PanCancer Immune Profiling Panel (770 targets) (NanoString technologies), by the BTA team at the University of Birmingham. After exporting the raw digital count of expression from nSolver v4.0 software, normalisation to 20 housekeeping genes (included in the panel) and downstream analyses were done using R-programming.

2.7. Western blot

Pelleted cells were lysed in RIPA lysis buffer (Sigma Aldrich, UK) containing protease inhibitor (Sigma Aldrich, UK). The samples were incubated on ice for 30 mins and vortexed every 10 minute after which they were centrifuged at 12,700 rpm for 10 minutes and the supernatant was collected. The protein concentration of each sample was determined using the Bradford assay (Bio-rad, Watford, UK). Prior to loading, protein samples were prepared in 4X Laemmli Sample

Buffer (Bio-rad, USA) containing 2-Mercaptoethanol (Sigma Aldrich, UK) and boiled at 95 °C for 10 minutes. Equal amounts of protein were loaded on 10% or 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel (hand-cast gel made using TGX Stain Free FastCast Acrylamide solutions, Bio-rad, UK) depending on the molecular weight of the target proteins. The gel was run initially at 80V and when the proteins entered the resolving gel, the voltage was increased to 120V. Proteins were transferred onto a 0.2 µm nitrocellulose membrane (GE Healthcare Life Science, Germany) using the Trans-blot Turbo Transfer System (Bio-rad, UK). Blots were blocked for one hour at room temperature (RT) with 5% milk (Marvel, UK) in Tris Buffered Saline (TBS) (Fisher bioreagents, Thermo Fisher Scientific, USA) containing 0.1% Tween-20 (Fisher bioreagents, Thermo Fisher Scientific, UK). Blots were incubated overnight or over the weekend at 4 °C with primary antibodies diluted in 5% BSA (Sigma Aldrich)-TBST. Table 2.2 lists all the primary antibodies, companies they were purchased from, host species and dilutions used. Post incubation with primaries, blots were probed with anti-rabbit or anti-mouse IgG HRP-linked secondary antibody (Cell Signalling Technology, Massachusetts, USA) diluted 1:2000 in 5% milk-TBST for one hour at RT. For visualisation, Clarity Western ECL substrate (Biorad, UK) was used: equal amounts of Clarity Western Peroxide Reagent and Clarity Western Luminol/Enhancer Reagent were added. For all the washes, blots were incubated 3X for 5 minutes each at RT with TBST. Band densities were

measured with the ChemiDoc MP imaging system (Bio-Rad, UK) and Image lab software. Protein expression was quantified using using ImageJ software (National Institutes of Health, Maryland, USA) and protein band intensities were normalised against the β-actin loading control.

Table 2.4. List of primary antibodies

Antibody	Code	Company	Host Species	Dilution
MST4	3822	Cell Signalling Technology, USA	Rabbit	1:1000
p57 Kip2	2557	Cell Signalling Technology, USA	Rabbit	1:1000
p57 Kip2	MA5-11309	Invitrogen, Thermo Fisher Scientific, USA	Mouse	1:250
IDH3G polyclonal antibody	PA5-51668	Invitrogen, Thermo Fisher Scientific, USA	Rabbit	1:1000
Anti-p62 lck ligand	610832	BD Transduction Laboratories™, USA	Mouse	1:1000
LC3A/B	4108	Cell Signalling Technology, USA	Rabbit	1:1000

β-Actin	4970	Cell Signalling Technology, USA	Rabbit	1:5000

2.8. Statistical analysis

For the *in vivo* experiments, we have powered our studies using the Experimental Design Tool supported by NC3Rs, to be able to detect a minimum difference of 6% in the measurement of effector memory T lymphocyte expansion upon mouse immunisation between HFD and chow diet. This effect size is of interest since it supports the detrimental links between a dysregulated adaptive immune response and ill health in obesity. We have performed a power calculation ($p<0.05$, power 0.85, two-sided test, mean = 5 and SD = 3) based on data from prior investigations (Mauro et al. Cell Metabolism 2017) to ascertain that 7 mice are needed per group. The human *in vitro* study was a pragmatic/pilot study that we decided to perform with a minimum of four donors (biological replicate). Unless otherwise mentioned, statistical analyses were performed using GraphPad Prism v.9 (GraphPad Software, San Diego, California, USA). Data distribution was checked using Shapiro-wilk test. Data were analysed by Mann-Whitney or one-tailed Student's *t*-test. Details of statistical tests used can be found in the figure legends. Statistical significance was accepted at $P \leq 0.05$.

3.0. RESULTS

3.1. *In vivo*

3.1.1. Expression of candidate genes in murine lymph nodes and spleen

As previously described (section 2.3), whole lymph node cell suspensions were obtained from the peripheral and mesenteric lymph nodes, and bulk $CD4^+$ T cells were isolated from splenocytes. The RRBS dataset was generated using naïve and memory $CD4^+$ T cells isolated from spleen of mice on CD, HFD and HFD-RE and pyrosequencing was done in the lymph nodes , so we wanted to confirm candidate gene expression changes in these tissues. Since hypomethylation is commonly associated with gene up-regulation, we predicted to see higher levels of mRNA transcripts of the genes that were hypomethylated in the HFD and HFD-RE groups compared to the CD group. After extracting total RNA from the lymph node cells and splenic $CD4^+$ T cells , RT-qPCR was performed to quantitate changes in the expression of the three candidate genes- *Stk26*, *Cdkn1c* and *Idh3g* as well as *Cd36*, encoding the fatty acid transporter, between CD, HFD and HFD-RE groups.

In the peripheral lymph node (Figure 3.1A), we observed that the HFD mice had a significantly reduced expression of *Stk26* (P value = 0.0087) as compared to CD mice and while *Stk26* expression in the HFD-RE group was lower than that in the CD group, there was no significant difference between the two. No significant difference was observed in the expression of *Cdkn1c*, *Idh3g* and *Cd36* between the three groups.

In the mesenteric lymph node (Figure 3.1B), there was no significant difference in the expression of *Stk26*, *Cdkn1c* and *Cd36* between the three groups. However, there was a significant reduction in the expression of *Idh3g* in the HFD (P value = 0.0043) and HFD-RE (P value = 0.0152) groups relative to CD.

In spleen (Figure 3.1C), there was no significant difference observed in the expression of any of the genes between the three groups.

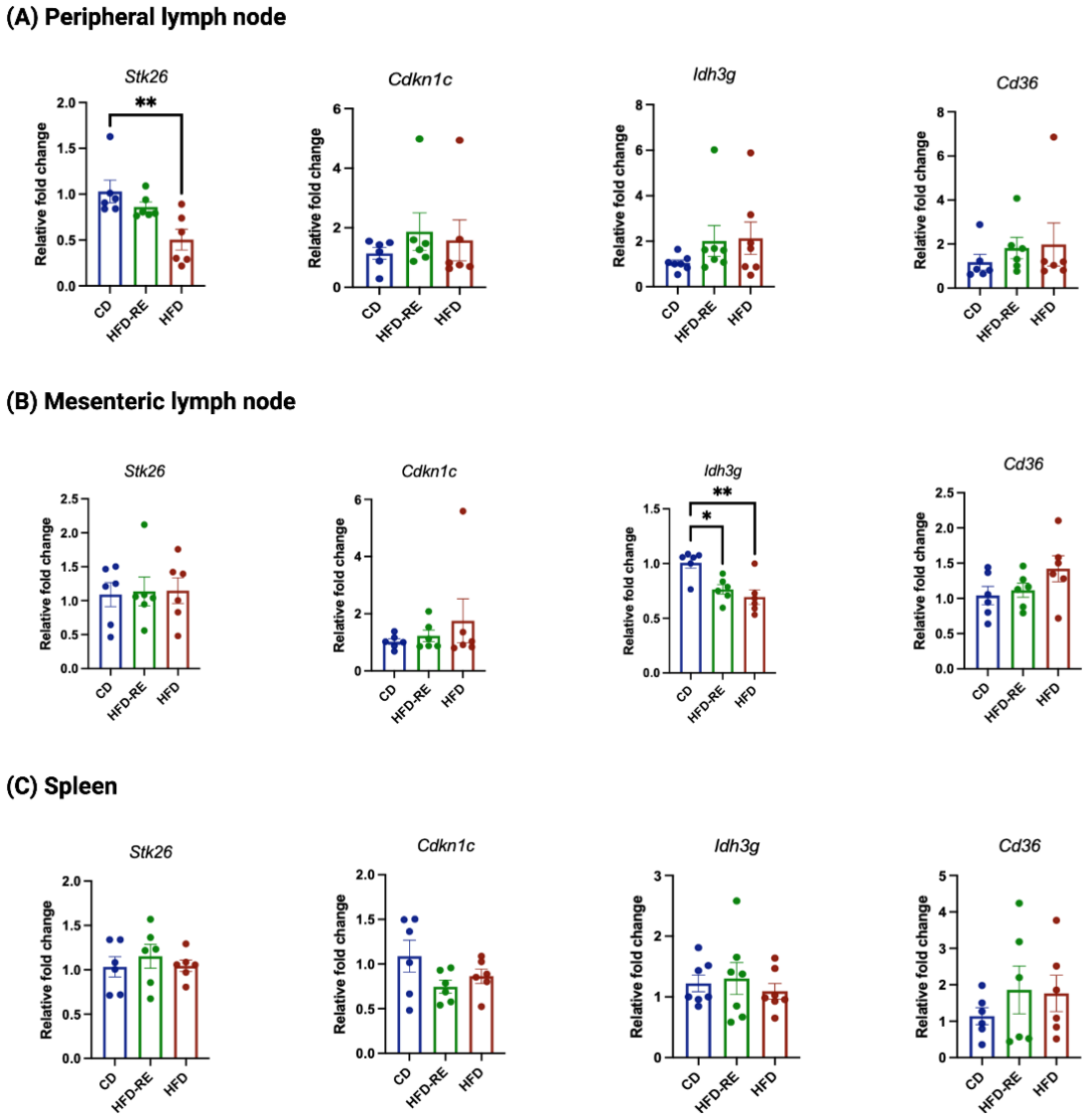


Figure 3.1. Gene expression changes in mice lymph nodes (bulk) and splenic CD4⁺ T cells. Comparison of mRNA expression levels of genes *Stk26*, *Cdkn1c*, *Idh3g* and *Cd36* between mice fed CD, HFD and HFD RE for 14 weeks (n=6 in each group) in the **(A)** peripheral lymph nodes, **(B)** mesenteric lymph nodes, **(C)** splenic CD4⁺ T cells. Total RNA was extracted using the Qiagen RNeasy mini kit and quantified using TaqMan RT-qPCR. Gene expression was normalised against *18s rRNA* and data are expressed as mean ± SEM relative to CD. Differences between groups were analysed by the Mann-Whitney test. Abbreviations: CD, Chow Diet; HFD, High-fat Diet; HFD-RE, High-fat diet-recovery (recovery diet); RNA, Ribonucleic Acid; RT-qPCR, Quantitative Reverse Transcription Polymerase Chain Reaction; mRNA, Messenger RNA; rRNA, Ribosomal RNA; SEM, Standard Error of Mean.

3.1.2. Expression of candidate proteins in murine lymph nodes and spleen

Since differentially expressed mRNA usually positively co-relates with protein expression, we wanted to validate if there is an increase in the expression of proteins encoded by the hypo-methylated candidate genes in the HFD and HFD-RE groups relative to CD (320). After isolating proteins from splenic $CD4^+$ T cells and bulk lymph node cells, western blot was done to compare the expression levels of STK26 and IDH3G between the three diet groups.

Two different gels were used to accommodate all the protein samples from seven mice each in the three diet groups. When band intensities of samples from both the membranes (Figures 3.2A and 3.2B) were analysed together, no significant difference was observed in the expression of either STK26 (Figure 3.2C) OR IDH3G (Figure 3.2D) in the HFD or HFD-RE group in comparison to the CD group. However, on analysing the band intensities of samples from only one membrane (Figure 3.3B alone), mice in the HFD group had a higher expression of STK26 (P value = 0.0140) as compared to the CD group (figure 3.2E). Although, there was no significant difference observed in the expression of STK26 between CD and HFD-RE mice (P value = 0.0980), there is a

trend showing elevated STK26 levels in the HFD-RE mice which is more comparable to the HFD group rather than the CD group.

In the mesenteric lymph node (figure 3.3) and spleen (figure 3.4), no significant difference was observed in the expression of STK26 and IDH3G between CD, HFD and HFD-RE mice.

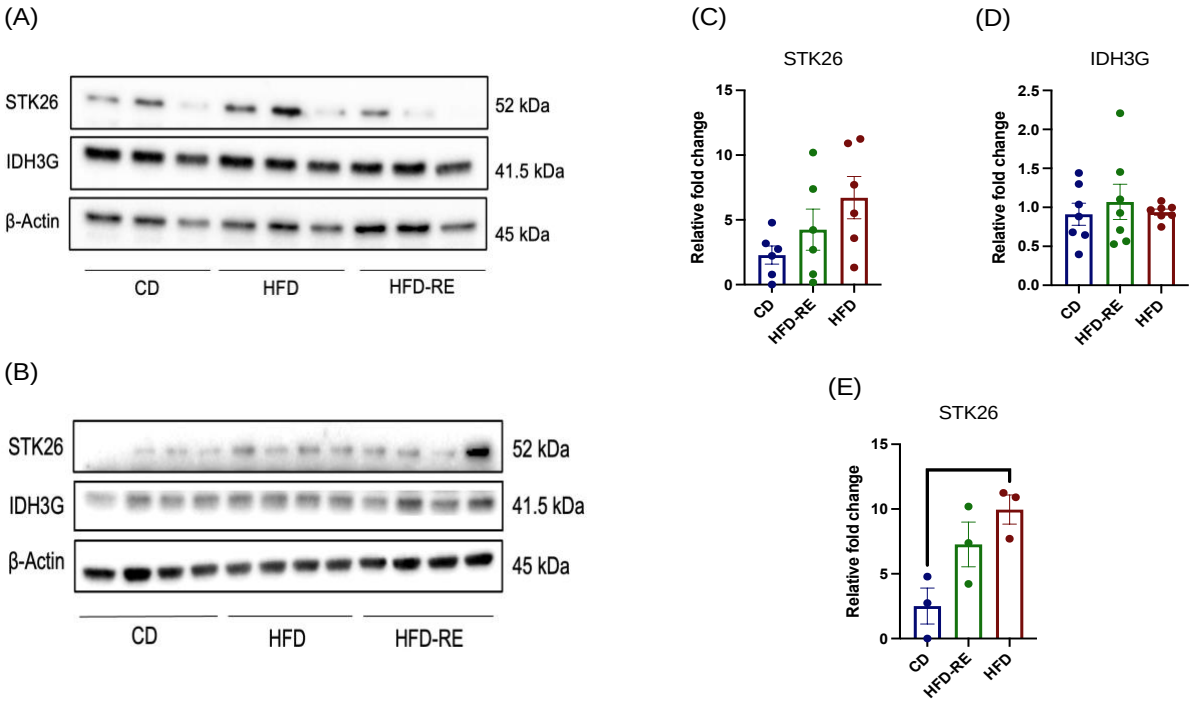


Figure 3.2. Protein expression changes in the peripheral lymph node of mice. (A-B) Representative western blots showing the expression of STK26, IDH3G and β-actin in the peripheral lymph node of mice fed CD, HFD or HFD-RE for 14 weeks (n = 7 in each group). The 4th, 8th, and 12th lane in (B) had proteins from the non-injected control and have been excluded from the densitometry analysis. **(C-D)** Densitometry analysis of STK26 and IDH3G expression from **(A)** and **(B)**. Target protein levels were normalised against the loading control β-actin. Data are expressed as mean ± SEM relative to CD of 6 mice per group. Mann-Whitney test was used to analyse the data **(E)** Densitometry analysis of STK26 expression from **(B)**. Target protein levels were normalised against β-actin. Data are expressed as mean ± SEM relative to CD of 3 mice per group. For statistical analysis, one-tailed Student's T test was used.
Abbreviations: CD, Chow Diet; HFD, High-fat Diet; HFD-RE, High-fat diet- recovery (recovery diet); SEM, Standard Error of Mean.

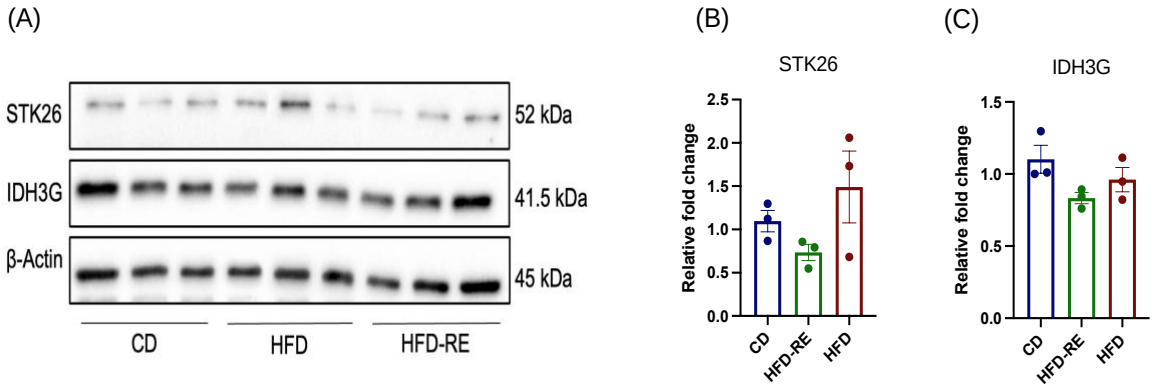


Figure 3.3. Protein expression changes in the mesenteric lymph node of mice. (A) Representative western blot showing the expression of STK26, IDH3G and β-actin in the mesenteric lymph node of mice fed CD, HFD or HFD-RE for 14 weeks (n = 3 in each group). **(B-C)** Densitometry analysis of STK26 and IDH3G expression. Target protein levels were normalised against the loading control β-actin. Data represented as mean ± SEM relative to CD and one-tailed Student's T test was used to analyse differences between groups. Abbreviations: CD, Chow Diet; HFD, High-fat Diet; HFD-RE, High-fat diet-recovery (recovery diet); SEM, Standard Error of Mean.

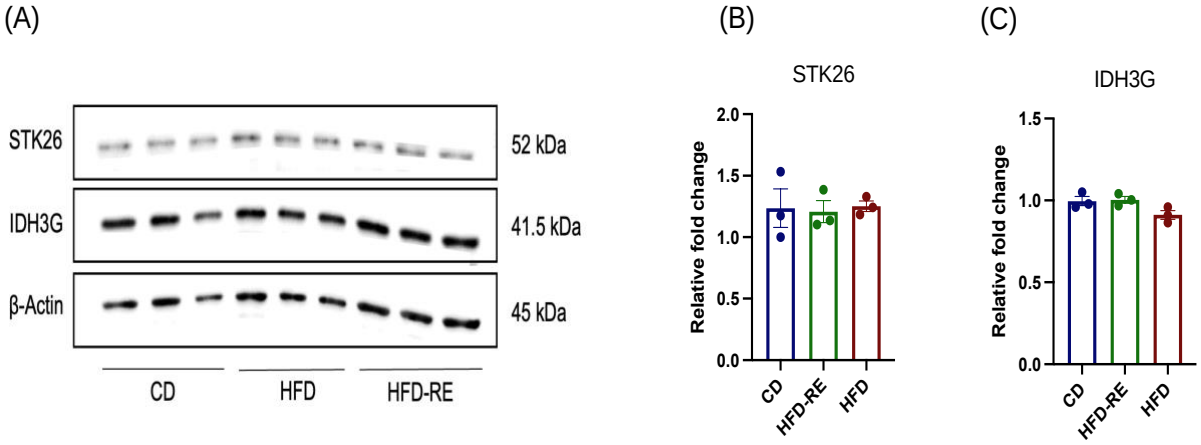


Figure 3.4. Protein expression changes in CD4⁺ T cells isolated from mice splenocytes. (A) Representative western blot showing the expression of STK26, IDH3G and β-actin in bulk CD4⁺ T cells isolated from splenocytes of mice fed CD, HFD or HFD-RE for 14 weeks (n = 3 in each group). **(B-C)** Densitometry analysis of STK26 and IDH3G expression. Target protein levels were normalised against the loading control β-actin. Data represented as mean ± SEM relative to CD and one-tailed Student's T test was used to analyse differences between groups. Abbreviations: CD, Chow Diet; HFD, High-fat Diet; HFD-RE, High-fat diet-recovery (recovery diet); SEM, Standard Error of Mean.

3.1.3. NanoString nCounter analysis of mRNA expression in CD, HFD and HFD-RE mice

The NanoString nCounter analysis was used to compare inflammatory markers in splenic memory $CD4^+$ T cells isolated from CD, HFD and HFD-RE mice.

Figure 3.5A represents the flow cytometry plots showing the percentages of naïve ($CD62L^{high}$ $CD44^{low}$), central memory ($CD62L^{high}$ $CD44^{high}$) and effector memory ($CD62L^{low}$ $CD44^{high}$) populations gated within live $CD4^+$ T cells, pre (left) and post (right) isolation of memory $CD4^+$ T cells from mice spleen. As expected, the percentage of naïve $CD4^+$ T cells dropped from 30 to 2% and most of the cells belong to the effector memory CD4 population (72%), post isolation.

The Principal Component Analysis (PCA) plot (figure 3.5B) shows that the HFD-RE group is clustering with HFD, not CD. Although four mice samples per group were run on the nCounter system, one from each group was an outlier and hence excluded from downstream analyses.

The heat-map (figure 3.5C) showing all normalised gene changes (normalised to 20 housekeeping genes) depicts that

the expression levels of genes in the HFD and HFD-RE groups are similar to each other but different from CD.

Figure 3.5D represents the heat-map of differentially expressed genes that have significantly changed between CD and HFD/HFD-RE groups. There are 13 genes that have significantly different expression between CD and HFD/HFD-RE groups- *Klra4*, *Vegfa*, *Ccl4*, *Kit*, *Ifna8*, *Il18*, *Cfp, Vcam1*, *C1qb*, *C1qa*, *Lrp1*, *Csf1r* and *Cxcr6*.

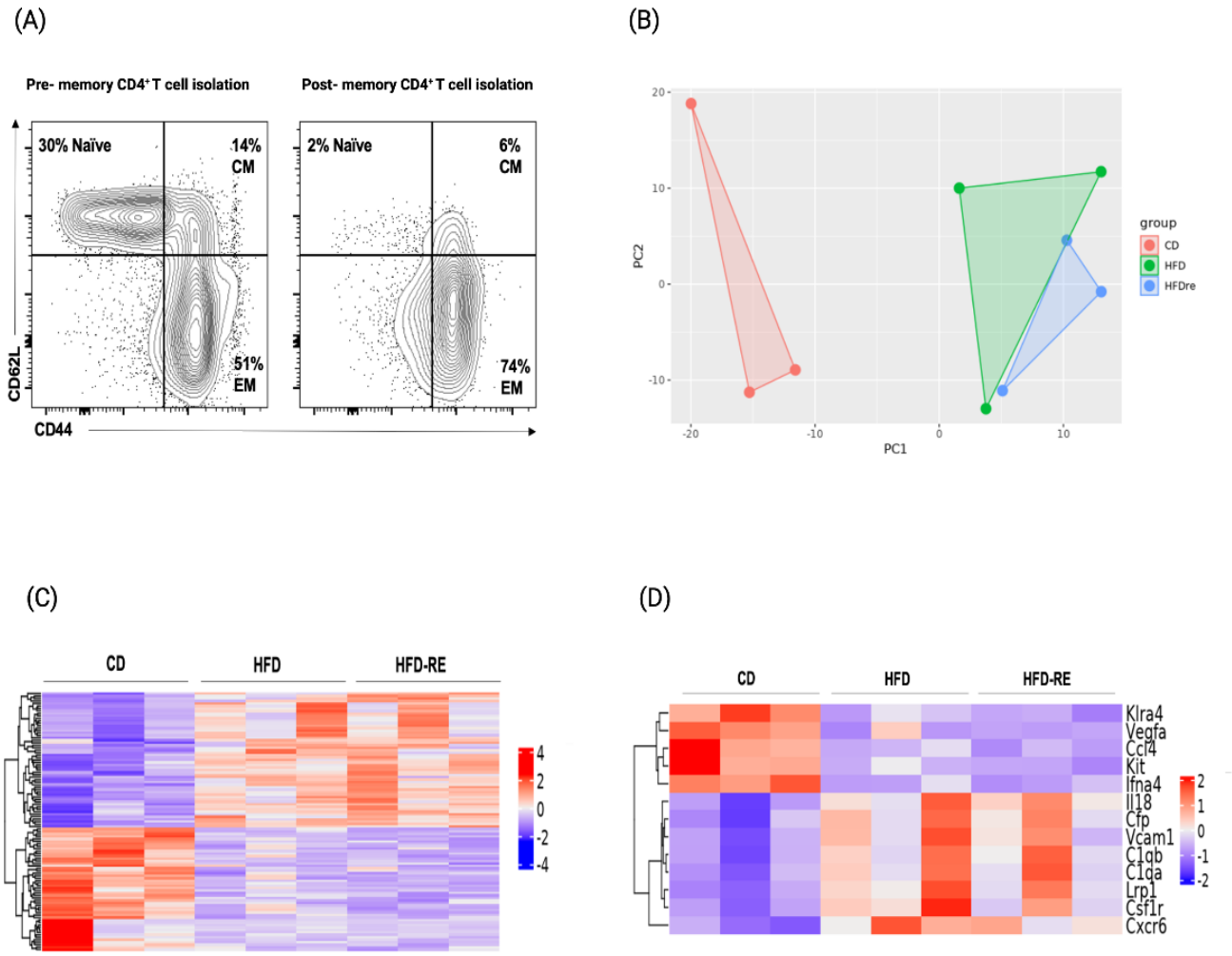


Figure 3.5. NanoString nCounter analysis of mRNA expression in CD, HFD and HFD-RE mice. (A) On the left, representative flow cytometry plot showing the percentages of naïve ($CD62L^{high}$ $CD44^{low}$), CM ($CD62L^{high}$ $CD44^{high}$) and EM ($CD62L^{low}$ $CD44^{high}$) populations gated within live $CD4^+$ T cells, pre- isolation of memory $CD4^+$ T cells from mice spleen. On the right, representative flow cytometry plot showing percentages of naïve, CM and EM cells gated on live $CD4^+$ T cells, post isolation of splenic memory $CD4^+$ T cells.

Post isolation of memory $CD4^+$ T cells, total RNA was extracted using the Qiagen RNeasy micro kit and run on the nCounter system via the PanCancer Immune Profiling Panel. Raw gene counts were exported from the nSolver v4.0 software after QC checks and 'R' was used to normalise gene counts and generate the plots. **(B)** PCA plot showing clustering of samples (n = 9; 3 per group) from CD, HFD and HFD-RE mice. **(C)** Heat-map showing all normalised gene changes (normalised to 20 housekeeping genes) in CD, HFD and HFD-RE mice. **(D)** Heat-map showing genes that are significantly different in expression between CD and HFD/HFD-RE groups. P-values are un-adjusted and have been calculated using Wald test. Abbreviations: CD, Chow Diet; HFD, High-fat Diet; HFD-RE, High-fat diet- recovery (recovery diet); CM, Central Memory; EM, Effector Memory; RNA, Ribonucleic Acid; mRNA, Messenger RNA; QC, Quality Control; PCA, Principal Component Analysis.

3.2. *In vitro*

3.2.1. Co-culturing human $CD4^+$ T cells with adipose-conditioned media

3.2.1.1. Treatment with adipose-conditioned media induces the expression of *STK26.*

When $CD4^+$ T cells were co-cultured with adipose-conditioned media from six osteoarthritis patients, three from healthy weight and three from obese patients and RT-qPCR was done to compare the expression of candidate genes between healthy weight and obese patients, we saw that treatment with culture media from obese patients showed an upward trend in the expression of *STK26*, *CDKN1C* and *IDH3G* relative to patients with healthy weight (figures 3.6A, 3.6B and 3.6C). However, the number of data points (three) per group was not sufficient to achieve statistical significance in this case so the experiment was repeated with six new samples (three from healthy weight and three from obese patients) in addition to the six samples used earlier. The analysis from the repeat experiment revealed that the culture media from tissues from obese patients had a significant increase in the expression of *STK26* (P value = 0.0173) compared to patients with a healthy

weight (figure 3.6D). There was no significant difference observed in the expression of *CDKN1C* and *IDH3G* between obese and healthy weight patients (figures 3.6E and 3.6F). Figure 3.6D represents that the obese patients had a significantly higher BMI (P value = 0.0022) compared to the healthy weight patients).

Table 3.1. shows the BMIs of the osteoarthritis patients and relative fold changes in the expression of the three genes in the first experiment and that of the repeat experiment is demonstrated in table 3.2. There is no direct co-relation observed between the BMIs of patients and the degree of expression of the three candidate genes.

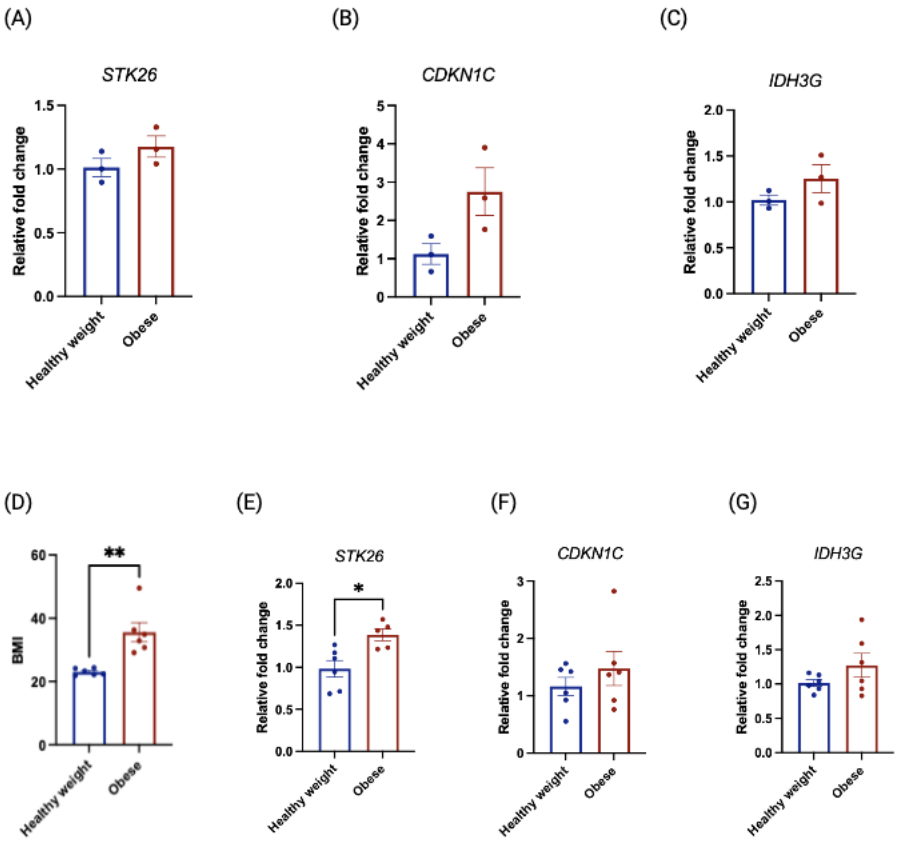


Figure 3.6. Expression of candidate genes on co-culturing human CD4⁺ T cells with adipose-conditioned media. Bulk CD4⁺ T cells isolated from human PBMCs were treated with adipose-conditioned media from osteoarthritis patients either with a healthy weight or obese for 24 hours, followed by CD3/CD28 activation for 48 hours. Total RNA was isolated using the Qiagen RNeasy mini kit and quantified using RT-qPCR. **(A-C)** Comparing mRNA expression levels of candidate genes *STK26*, *CDKN1C* and *IDH3G* between healthy weight and obese patients (n=3 in each group). **(D)** Comparison of BMI between the healthy weight and obese patients. **(E-G)** Comparing mRNA expression levels of candidate genes *STK26*, *CDKN1C* and *IDH3G* between healthy weight and obese patients (n=6 in each group); experimental repeat of **(A-C)** with three additional samples in each group. Gene expression was normalised against β-actin housekeeping gene and data expressed as mean ± SEM relative to healthy weight. Statistical analyses were done using the Mann-Whitney test. Abbreviations: PBMCs, Peripheral Blood Mononuclear Cells; RNA, Ribonucleic Acid; mRNA, Messenger RNA; RT-qPCR, Quantitative Reverse Transcription Polymerase Chain Reaction; BMI, Body Mass Index; SEM, Standard Error of Mean.

Table 3.1. The BMIs of osteoarthritis patients and corresponding relative changes in gene expression in the first experiment with adipose tissue-conditioned media.

Sample type (healthy/obese/ overweight)	BMI	Age	Sex	Relative fold change in *STK26* expression	Relative fold change in *CDKN1C* expression	Relative fold change in *IDH3G* expression
Healthy (1)	22.2	73	Male	1	0.66	0.93
Healthy (2)	21.9	75	Female	0.90	1.11	1.01
Healthy (3)	24.3	84	Male	1.14	1.59	1.12
Obese (1)	49.5	70	Female	1.33	3.90	1.27
Obese (2)	30.7	52	Female	1.16	1.76	0.99
Overweight	29.1	80	Female	1.04	2.59	1.51

Table 3.2. The BMIS of osteoarthritis patients and corresponding relative changes in gene expression in the second (repeat) experiment with adipose tissue-conditioned media.

Sample type (healthy/obese/ overweight)	BMI	Age	Sex	Relative fold change in *STK26* expression	Relative fold change in *CDKN1C* expression	Relative fold change in *IDH3G* expression
Healthy (1)	22.2	73	Male	0.69	1.42	1.13
Healthy (2)	21.9	75	Female	1.11	0.93	0.93
Healthy (3)	24.3	84	Male	1.27	0.56	0.98
Healthy (4)	22.4	53	Female	0.94	1.46	1.16
Healthy (5)	24.1	83	Male	1.17	1.05	1.04
Healthy (6)	23.3	82	Female	0.71	1.56	0.88
Obese (1)	49.5	70	Female	1.57	1.42	1.31
Obese (2)	30.7	52	Female	1.22	0.92	0.93
Obese (3)	34.9	62	Female	1.44	1.57	1.60
Obese (4)	36.3	55	Female	0.98	1.37	1.05
Obese (5)	32.9	59	Female	1.23	2.82	1.94
Overweight	29.1	80	Female	1.48	0.76	0.83

3.2.2. Co-culturing human CD4$^+$ T cells with different fatty acids

3.2.2.1. Treatment with palmitic and stearic acids induce the expression of *STK26* and *CDKN1C* in CD4$^+$ T cells.

RNA was isolated from CD4$^+$ T cells treated with PA, SA, OA, PA + EPA and SA + EPA. Since EPA is known to have anti-inflammatory properties, we thought that its addition could ameliorate the pro-inflammatory effects of PA and SA. RT-qPCR was done to validate change in expression of candidate genes with different fatty acid treatments.

IL2RA, encoding the α-chain of the IL2 receptor, is induced highly upon TCR stimulation (321). Since we had both (+) CD3/CD28 (TCR activated) and (-) CD3/CD28 (non-activated) CD4$^+$ T cells in our experiment, *IL2RA* expression was compared between them to validate that the activation has successfully worked. As expected, activated samples had higher levels of *IL2RA* compared to non-activated samples (figure 3.7A).

Treatment with PA resulted in a significant increase in the expression of *STK26* both in the activated (P value = .0002) and non-activated (P value = .0002) groups (figure 3.7B). Similarly, SA treatment also induced the expression of *STK26* in both activated (P value = 0.002) and non-activated groups (P value = 0.0011) (figure 3.7B). Interestingly, addition of EPA to PA significantly reduced the expression of *STK26* (P value = .0293) in the activated group (figure 3.7B). Adding EPA to SA also significantly reduced the expression of *STK26* in both activated (P value = 0.0080) and non-activated groups (P value = 0.0040) (figure 3.7B).

CDKN1C expression was induced upon palmitate treatment in both the activated (P value = 0.0093) and non-activated groups (P value = 0.0002) (figure 3.7C). In the same vein, SA treatment induced the expression of *CDKN1C* in both activated (P value = 0.0104) and non-activated groups (P value = 0.0207) (figure 3.7C). However, addition of EPA did not ameliorate the effects of PA and SA treatment.

A significant increase in the expression of *IDH3G* was observed on treatment of activated $CD4^+$ T cells with

SA (P value = .0207) and addition of EPA to SA reduced the expression of *IDH3G* significantly (P value = .0293) (figure 3.7D).

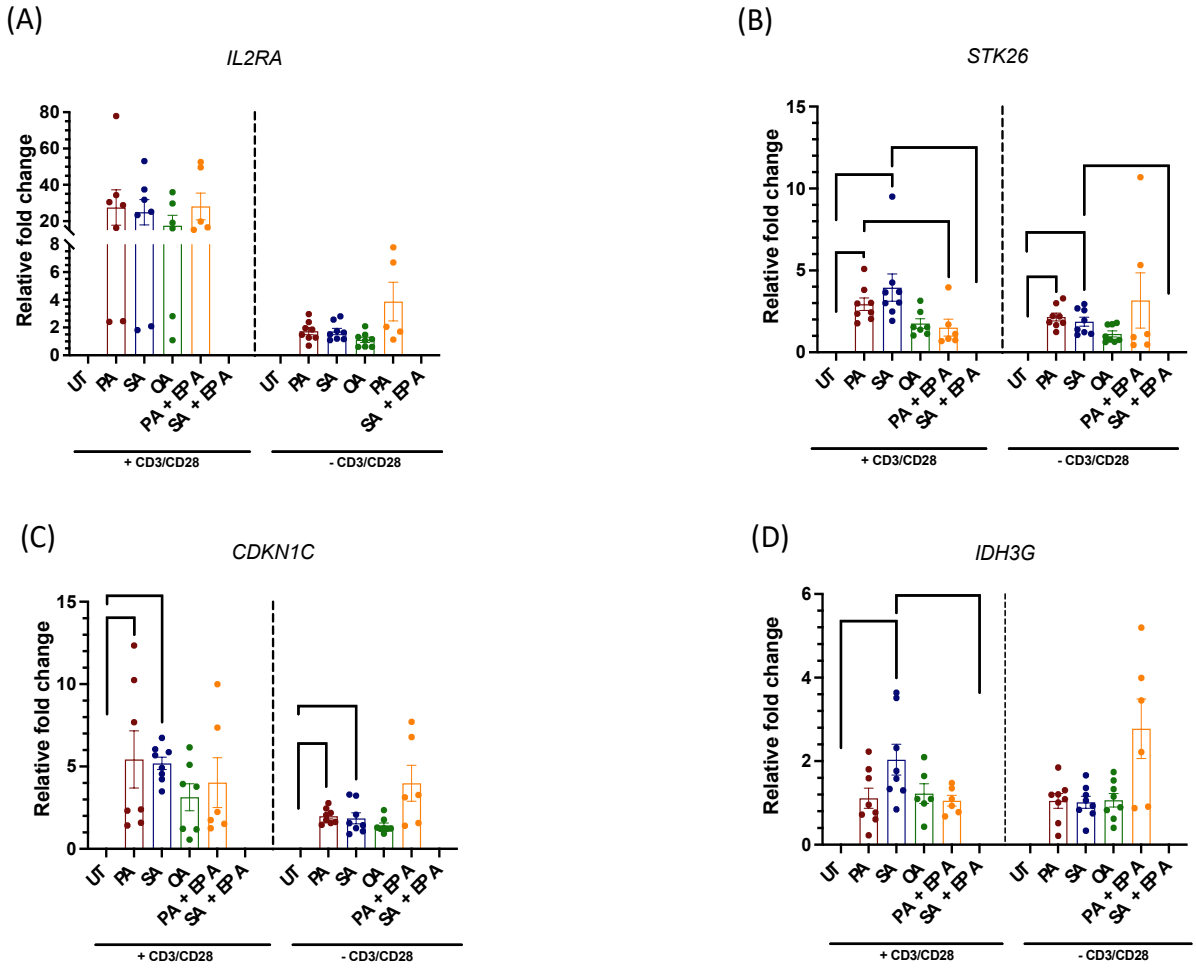


Figure 3.7. Gene expression analysis after co-culturing human CD4⁺ T cells with different fatty acids. Bulk CD4⁺ T cells were isolated from human PBMCs and treated with 0.5% ethanol (UT), 50 µM PA, 50 µM SA, 50 µM OA, 50 µM PA + 20 µM EPA or 50 µM SA + 20 µM EPA for 24 hours and activated with anti-CD3/CD28 beads for 48 hours. Total RNA was isolated using the Qiagen RNeasy mini kit and quantified using RT-qPCR. **(A-D)** Comparing mRNA expression levels of *IL2RA*, *STK26*, *CDKN1C* and *IDH3G* respectively with different fatty acid treatments (n = 8 for UT, PA, SA and OA and n = 6 for PA + EPA and SA + EPA). (+ CD3/CD28) represent the TCR activated samples and (- CD3/CD28) represent the non-activated samples. Gene expression was normalised against β-actin housekeeping gene and data expressed as mean ± SEM relative to UT. Statistical analysis was done using the Mann-Whitney test.
Abbreviations: PBMCs, Peripheral Blood Mononuclear Cells; UT, Untreated; PA, Palmitic Acid; SA, Stearic Acid; OA, Oleic Acid; EPA, Eicosapentaenoic acid; RNA, Ribonucleic Acid; RT-qPCR, Quantitative Reverse Transcription Polymerase Chain Reaction; TCR, T-cell Receptor; SEM, Standard Error of Mean.

3.2.2.2. Palmitate treatment induces the expression of STK26 in TCR-activated $CD4^+$ T cells.

Proteins were isolated from $CD4^+$ T cells treated with different fatty acids and western blotting was done to validate protein expression changes with different fatty acid treatments relative to untreated.

When looking at the western blot images from three out of the four biological replicates (figure 3.8A, 3.8C and 3.8D), we can clearly see that the band intensities of STK26 with palmitate treatment in the activated samples are higher than that in the untreated. Therefore, the analysis for the relative fold change in STK26 was done taking into account these three replicates, excluding the fourth one (figure 3.8B). Consistent with the RT-qPCR data, it was observed that treatment with PA induces the expression of STK26 when $CD4^+$ T cells are activated (P value = 0.0116) but there was no change observed in the non-activated state (figure 3.8E). Although, addition of EPA to PA seems to be reducing the expression level of STK26, no significant difference was observed (figure 3.8E). No other fatty acid treatment had any effect in the expression of STK26 (figure 3.8E).

As opposed to STK26, IDH3G protein levels did not change with any fatty acid treatment (figure 3.8F).

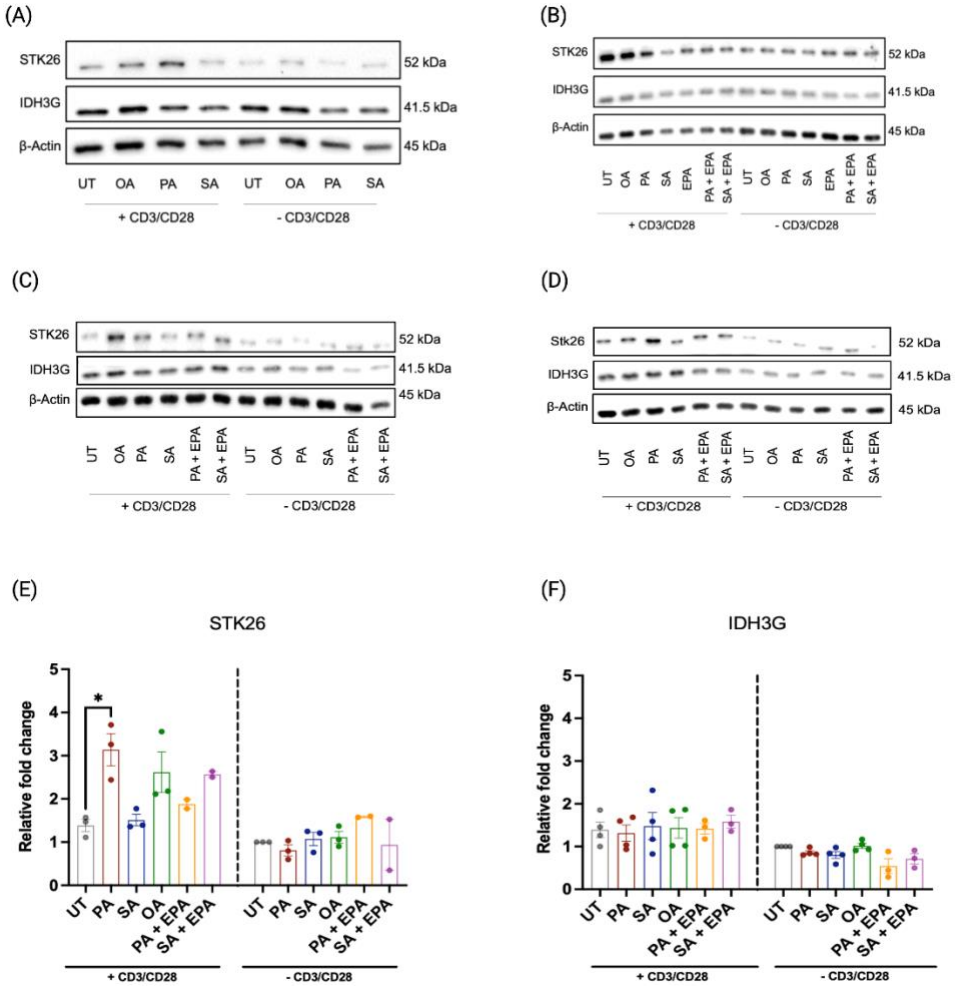


Figure 3.8. Comparing protein expression levels in human CD4⁺ T cells treated with different fatty acids. (A-D) Representative western blots showing the expression of STK26, IDH3G and β-actin in bulk CD4⁺ T cells isolated from human PBMCs and treated with 0.5% ethanol (UT), 50 µM PA, 50 µM SA, 50 µM OA, 50 µM PA + 20 µM EPA or 50 µM SA + 20 µM EPA for 24 hours, followed by CD3/CD28 activation for 48 hours (n = 4). **(E)** Densitometry analysis of STK26 expression from **(A)**, **(C)** and **(D)** (n = 3 for UT, PA, SA and OA, n = 2 for PA + EPA and SA + EPA). Target protein levels were normalised against the loading control β-actin. **(F)** Densitometry analysis of IDH3G expression from **(A)**, **(B), (C)** and **(D)** (n = 4 for UT, PA, SA and OA, n = 3 for PA + EPA and SA + EPA). Target protein bands were normalised against β-actin. Data are represented as mean ± SEM relative to UT and analysed using one-tailed Student's T test.
Abbreviations: PBMCs, Peripheral Blood Mononuclear Cells; UT, Untreated; PA, Palmitic Acid; SA, Stearic Acid; OA, Oleic Acid; EPA, Eicosapentaenoic Acid; SEM, Standard Error of Mean.

3.2.2.3. Analysis of autophagy markers by western blotting

STK26 is an inducer of autophagy, so any changes in its expression level should reflect on the autophagic flux. After confirming the upregulation of the *STK26* gene in the *in vitro* samples treated with palmitate as well as the STK26 protein in the *in vivo* and *in vitro* systems, we wanted to quantify the autophagic flux in human $CD4^+$ T cells treated with fatty acids and activated *in vitro*. LC3BII and P62 are the two most common autophagy markers and to quantitate their expression levels, western blotting was used. To monitor autophagic flux, it is important to use a lysosomal inhibitor which inhibits autophagy by preventing the autophagosome-lysosome fusion, and for this purpose, Chloroquine (CQ) was used. As previously described, human $CD4^+$ T cells, post treatment with different fatty acids and TCR activation, were treated with CQ for 4 or 12 hours. Beside CQ treated samples, we had samples that have not been treated with CQ but water as controls. Tests for statistical significance could not be conducted in this case because n = 1 . However, some trends can be seen which look promising.

The treatment with CQ seems to have saturated the system to an extent that we do not see much variation in the expression of P62 and LC3BII with fatty acid treatment. However, we can see a slightly upward trend in the expression of LC3BII with SA treatment in the 12-hour treatment group (figure 3.9C).

In case of the samples that had not treated with CQ, at 4 hours, PA seems to be reducing the expression of P62 and at 12 hours, P62 expression is lower with both PA and SA treatment, compared to untreated (figure 3.9E), which resonates well with our hypothesis since decreased levels of P62 are observed upon induction of autophagy. Analysis of the LC3BII expression shows a slightly upward trend in the expression of LC3BII at 4 hours with PA treatment and at 12 hours, the induction of LC3BII with both PA and SA treatments (figure 3.9F) looks promising and in line with our hypothesis since an induction of autophagy should result in increased levels of LC3BII.

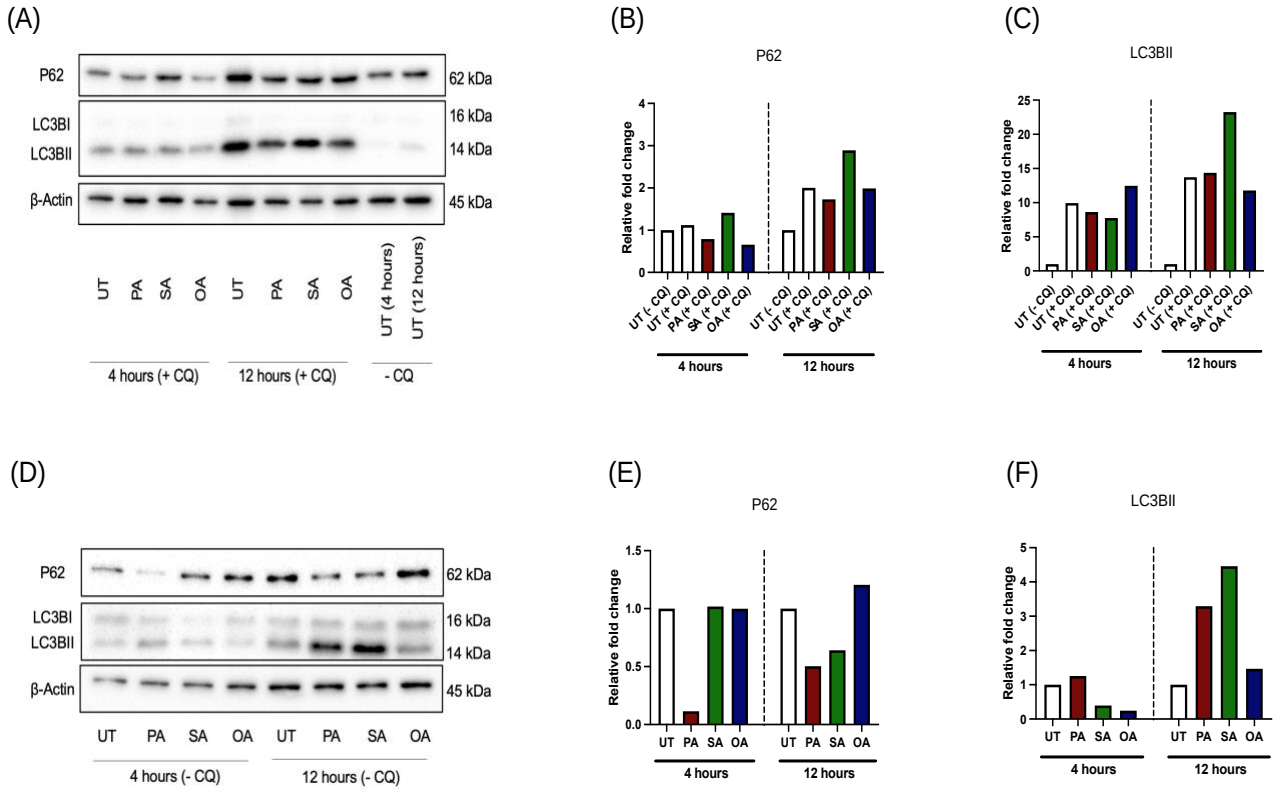


Figure 3.9. Comparing the expression levels of autophagy markers in human CD4⁺ T cells treated with different fatty acids. Bulk CD4⁺ T cells were isolated from human PBMCs and treated with different fatty acids as mentioned earlier. Post treatment and TCR activation, they were treated with CQ for 4 or 12 hours. **(A)** Representative western blot showing P62, LC3BI, LC3BII and β-actin expression in CQ (+ CQ) treated samples (n = 1). Lanes 9 and 10 had proteins from samples that were treated with water instead of CQ (- CQ) for 4 and 12 hours respectively, for normalisation. **(B-C)** Densitometry analysis of P62 and LC3BII expression. Target protein bands were normalised against the loading control β-actin. Data are expressed as mean ± SEM relative to UT (- CQ). **(D)** Representative western blot showing P62, LC3BI, LC3BII and β-actin expression in samples treated with water (- CQ) (n = 1). **(E-F)** Densitometry analysis of P62 and LC3BII expression. Target protein bands were normalised against the loading control β-actin. Data are expressed as mean ± SEM relative to UT. Since n = 1, no statistical analysis was done in this case. Abbreviations: PBMCs, Peripheral Blood Mononuclear Cells; TCR, T-cell Receptor; UT, Untreated; PA, Palmitic Acid; SA, Stearic Acid; OA, Oleic Acid; EPA, Eicosapentaenoic acid; CQ, Chloroquine.

4.0. DISCUSSION

Obesity has become increasingly common in the modern era; the worldwide obesity rate has nearly tripled in the past 40 years (322). The main concerns with a high BMI are the risks for developing severe health problems including diabetes, cardiovascular diseases, cancers, osteoarthritis and several other chronic diseases (323,324). There is a growing need for interventions and policies to mitigate the high rates of obesity worldwide and knowing that the detrimental effects of obesity on the immune system manifests as chronic inflammation-associated disorders, it is imperative to understand the changes that lead to long-lasting immune dysregulation in obesity.

Given the important role of $CD4^+$ T lymphocytes in driving obesity-associated complexities, Mauro et al.'s research pioneered an exacerbated response of $CD4^+$ T cells upon high-fat diet, showing a direct role of the fatty acid Palmitate in driving the effect (129). Following up from the study, a series of pilot experiments done by Mauro's group showed that HFD results in long-lasting DNA methylation alterations in $CD4^+$ T cells, that underpin the exacerbated response of this cell type upon allo-immunisation, which is not rescued even after weight loss. Deep bioinformatics analysis of the DNA methylation dataset suggested the involvement of three key processes- autophagy, senescence, and the TCA cycle, represented by the *MST4*/*STK26*. *CDKN1C* and *IDH3G* genes respectively, in driving the exacerbated pro-inflammatory phenotype of $CD4^+$ T cells. Our study, therefore, aimed at confirming the upregulation of

these genes as well as proteins encoded by them, using both *in vivo* and *in vitro* models of obesity, in an effort to dive deeper into the mechanisms governing the enhanced activation of obese naïve $CD4^+$ T cells into pro-inflammatory effector memory $CD4^+$ T cells.

Obesity in early adulthood is linked to over twice the odds of reduced physical functioning and increased risk of chronic diseases in mid and old life (325,326). Consequently, we investigated the obesity-associated immune dysregulation in young adults- in our study, mice that were 4-week-old (corresponding to a 15-year-old human) were fed CD/HFD for 8 weeks (corresponding to a 23-year-old human), followed by 6 weeks of CD/HFD (corresponding to a 28-year-old human). To stimulate obesity *in vitro* and mimic the mice HFD model, we first treated human $CD4^+$ T cells with adipose-conditioned media or different fatty acids and then activated the TCR response, since mice were on diet first and then given allogenic immunisation. With regard to fatty acid treatment, we used palmitic acid and stearic acid, the most common long-chain saturated fatty acids found in western diet, that have been shown to modulate the epigenetic landscape of chronic illnesses, culminating in detrimental outcomes (163,327). In addition, we incorporated oleic acid and eicosapentaenoic acid into our studies owing to their anti-inflammatory properties (328–331).

Analysis of the gene and/or protein expression levels in *in vivo* and *in vitro* samples confirmed the upregulation of *STK26*/*MST4* and *CDKN1C*, the

autophagy inducing and senescence implicated genes, however, the third candidate TCA cycle gene, *IDH3G*, would be excluded from future studies because no significant changes in its gene or protein expression levels with obesity were observed.

4.1. *In vivo* and *in vitro* confirmation of STK26 up regulation

4.1.1. STK26 is induced in HFD mice and does not return to basal levels even after weight loss.

Analysis of western blot from the peripheral lymph nodes of the first nine mice on CD, HFD and HFD-RE respectively did not reveal any significant difference in the expression of STK26. However, when the blot (figure 3.2B) from the nine other mice was analysed, the HFD mice showed an elevated expression of STK26 compared to the CD group, and although the HFD-RE mice did not have a significantly higher expression of the protein compared to the CD group, there is still a trend suggesting that the protein expression has not gone back to its basal levels even after weight loss. The discrepancies in the results could have arisen from the mice tissues being processed on two separate days. Alternatively, the loading control i.e., β-actin levels in figure 3.2A do not look very consistent between the lanes, which could have led to mis-interpreting the actual target protein levels in samples.

Neither the mesenteric lymph node nor splenic $CD4^+$ T cells showed any significant difference in the expression of STK26 between the groups. The only significant results we have are from the peripheral lymph node, which is in line with the flow cytometry data (pilot experiments) showing an elevated pro-inflammatory effector memory $CD4^+$ T cell population in the HFD and HFD-RE mice relative to CD, that had been generated from $CD4^+$ T cells isolated from pooled peripheral lymph nodes of mice. Additionally, the pyrosequencing analysis to confirm gene hypomethylation had been done using $CD4^+$ T cells from the lymph nodes. However, in this case, we used bulk peripheral lymph node for the analysis, which contain other cell types including B cells and $CD8^+$ T cells in addition to $CD4^+$ T cells, so the effect that we see is cumulative of all such cell types. A better way for validation would be to isolate $CD4^+$ T cells specifically from peripheral lymph node and quantitate protein expression changes in them.

The RT-qPCR analysis of RNA from the peripheral lymph node of mice on CD, HFD and HFD-RE, showed a significant reduction in the expression of *Stk26*/*Mst4* in the HFD and HFD-RE groups, relative to CD. Under normal circumstances, this is what we would expect from mice on HFD, i.e., the autophagy levels to be suppressed since autophagy is induced under

conditions of starvation. But this is against our hypothesis, since hypo-methylation of *Stk26* in the HFD and HFD-RE groups relative to CD, would generally indicate an increased potential for its expression. However, there is an increased expression of the STK26 protein in the HFD group so the lower expression of the *Stk26* gene in the HFD group could stem from a negative feedback loop suppressing the expression of the gene when protein levels are abundant (333–335). Additionally, we did not notice any significant differences in the expression of *Cd36* between the groups in either of the tissues. This is in consonance with the study by Couturier et al., that showed that CD36 is upregulated in adipose tissue lymphocytes and further increased during high fat feeding in mice, but the same had not been observed in blood, lymph node or spleen lymphocytes (336). If not CD36, there must be a different transporter acting on the uptake of fatty acids in these tissues. Ongoing research in the lab is focussed on investigating lipid rafts in our *in vitro* set-up; lipid rafts are highly ordered, dynamic assemblies of lipids and proteins in the plasma membrane harbouring receptor and regulatory molecules to facilitate signal transduction, that have been known to impact epigenetic changes (337,338).

One limitation of this RT-qPCR experiment is the use of 18s rRNA as a housekeeping gene. Although 18s rRNA is a widely

used housekeeping gene, it is not considered ideal in certain circumstances because of the following difficulties associated : i) it has high expression and reduced degradation compared to mRNA transcripts of genes of interest; ii) different rRNA:mRNA ratios between groups and samples (339–342). In our study, 18s rRNA was detected at 5-6 Ct cycles, while the genes of interest *Stk26*, *Cdkn1c*, *Idh3g*, and *Cd36* were detected at Ct cycles ranging from 18-30. Clearly, the expression levels of the housekeeping and target genes were very different which possibly led to inconclusive results in relative gene expression analysis in this case. This could be overcome by using a different housekeeping gene, whose expression level is similar to that of our genes of interest.

4.1.2. Treatment with obese adipose-conditioned media induces the expression of *STK26.*

We observed that treatment of human $CD4^+$ T cells with adipose-conditioned media from obese patients stimulated the expression of *STK26* as compared to that from patients with healthy weight. In addition to validating gene expression levels, it would have been ideal to validate the expression of the STK26 protein through western blot, but due to the limited availability of the adipose-conditioned media, we could stimulate only enough $CD4^+$ T cells for RT-qPCR alone.

Adipose-conditioned media contains various secreted products from adipose tissue, which other than fatty acids, also include exosomes, miRNAs, cytokines, peptide and steroid hormones (343). Fatty acid binding protein 5 (FABP5) is a known regulator of fatty acid uptake and oxidation in T cells (344). In order to validate that it is indeed the fatty acids and not something else in the culture media that stimulate the expression of *STK26*, it could be a good idea to compare the expression of *STK26* upon treatment with culture media from obese patients in the presence and absence of a FABP5 inhibitor.

4.1.3. Confirmation of *STK26* gene and protein up-regulation in human $CD4^+$ T cells treated with palmitic acid.

Treatment of human $CD4^+$ T cells with both palmitic and stearic acids induces the expression of *STK26* in both activated and non-activated conditions, indicating that it is not only palmitic acid, as earlier established in Mauro et al.'s study (129), but stearic acid could also be playing an equally important inflammatory role. Addition of EPA to both palmitic and stearic acids ameliorated the effects of these long-chain saturated fatty acids on the expression of *STK26*, reinforcing EPA's anti-inflammatory effects.

The western blot analyses revealed a significant increase in the expression of STK26 with palmitic acid treatment and in the

activated state alone. Out of the four biological replicates, STK26 upregulation was observed in three but one which is likely because of the variability in blood cones given information on the sex, age, or medical history of donors is not known. Unlike the RT-qPCR data, we did not observe any changes in the expression of STK26 with addition of EPA to Palmitic acid, but this could be because we had only two replicates for PA + EPA treatment but possibly needed more to establish statistical significance. Additionally, stearic acid treatment did not induce the expression of STK26 which could also be accounted to the lesser number of replicates in the protein analysis study v/s mRNA analysis. The discrepancy in the level of mRNA and protein expression, especially the upregulation of *STK26* in non-activated $CD4^+$ T cells treated with palmitic acid and an absence of a similar increase in the expression of the protein with PA treatment in the non-activated state is likely because of post-transcriptional modifications controlling the fate of synthesised mRNA since not all mRNA get translated to proteins or temporal differences in mRNA and protein synthesis in eukaryotes or other factors affecting the synthesis and degradation rates of proteins (345,346).

4.1.4. Detection of autophagy markers in human $CD4^+$ T cells treated with fatty acids through western blot.

Confirming the protein upregulation *in vivo* and both mRNA and protein up-regulation *in vitro* strengthened our hypothesis that STK26-induced autophagy has a role in maintaining the $CD4^+$ T-cell mediated inflammatory conditions in obesity which are not rescued even after weight loss. This led us to quantifying the common autophagy markers LC3II and p62 blot in our *in vitro* set up, viz., $CD4^+$ T cells treated with different fatty acids in the presence or absence of an autophagy inhibitor (Chloroquine), through western blot. So far, we have data from only one replicate and need future experiments to test for statistical significance.

Since the accumulation of LC3II could be indicative of either an autophagy induction or a blockade in the downstream steps, it is important to measure the autophagic flux or LC3II turnover, i.e., the differences in the amounts of LCII in the presence and absence of an autophagy inhibitor (347,348). However, we have not shown LC3II turnover here, because it seems that in our case chloroquine treatment has saturated the system to an extent that we do not see any effect of the fatty acid treatment on the expression of LC3II, so the LC3II turnover in this case is not very different from the expression of LC3II in the presence of

chloroquine. However, in the absence of chloroquine, we see that both palmitic and stearic acids treatment induce the expression of LC3II. Although this is not sufficient to confirm the induction of autophagy, our data also demonstrate that p62 expression is reduced with palmitic and stearic acid treatments and given p62 is known to be degraded by autophagy, it is most likely a scenario of autophagy induction (349). Nevertheless, we would need to repeat the experiment with more biological replicates and also optimise the concentration and duration of chloroquine treatment, so that we could see an upregulation of LC3II with palmitic and stearic acid treatment even in the presence of chloroquine, to confirm that treatment with these fatty acids causes an activation of autophagy. Additionally, detection of ATG4B in these assays would be helpful to validate that the autophagy induction we see is mediated via the action of STK26 on ATG4B. One limitation of this study could arise from the fact that we are not detecting nuclear LC3II, which has gained considerable importance in recent years, for being the major source of membrane conjugated LC3 and having a critical role in starvation-induced autophagy (350).

4.2. Treatment of human CD4$^+$ T cells with palmitic and stearic acids induces the expression of *CDKN1C.*

We observed a significant increase in the expression of *CDKN1C* with palmitic and stearic acid treatment as compared to untreated in both activated and non-activated CD4$^+$ T cells. Unfortunately, we do not have any data on the expression of the protein since the western blot for CDKN1C did not work. We had tried antibodies from two different companies, varying concentrations of primary antibody and lengths of incubation time but did not get any bands. One important thing to infer from high Ct values in the RT-qPCR analyses is that *CDKN1C* is a low-expressed gene so possibly the total protein level (30 µg) in our samples which is normally sufficient to detect most proteins of moderate to high expression was not enough to detect CDKN1C. Consequently, future attempts to detect the protein should involve loading higher amounts of total protein (e.g., 40 µg, 50 µg, 60 µg etc.,) per sample.

4.3. *In vivo* and *in vitro* confirmation of IDH3G expression not changing upon high fat feeding or fatty acid treatment.

Despite *IDH3G* being chosen as a candidate gene following DNA methylation and pyrosequencing analyses, the gene levels were not found to be differentially regulated in the peripheral lymph nodes of HFD and HFD-RE mice or in human CD4$^+$ T cells treated with palmitic acid. Although, we saw a reduced expression of the gene in the

mesenteric lymph nodes of HFD and HFD-RE mice compared to CD, the western blot analyses did not reveal any differences in the protein expression in the lymph nodes or spleen between the three diet groups. Moreover, we did not observe any differences in the expression of IDH3G upon treatment with fatty acids that have an inflammatory role. Taken together, these results indicated that IDH3G was not differentially regulated upon high fat feeding or fatty acid treatment, so we decided to exclude it from future studies.

4.4. The HFD-RE cluster co-relates with HFD, not CD and differentially expressed genes between HFD/HFD-RE and CD indicate an increased potential for cellular senescence.

As seen with the altered pro-inflammatory immune phenotype of $CD4^+$ T cells with HFD that is not reversed even in the HFD-RE group, i.e., after weight loss, we observe a similar trend when analysing around 770 inflammation-associated genes in the three diet groups. PCA plot and heatmap analysis of the nCounter data revealed that the HFD-RE group clusters with the HFD, not CD, and the expression pattern of inflammation-associated genes is similar between HFD and HFD-RE, but different from CD. Clearly, the inflammation associated phenotypes seen with high-fat feeding are not reversed even after weight loss, reflecting the long-lasting effects of obesity on the immune system.

Our dataset point to two key processes-autophagy and senescence altered with obesity that potentially contribute to the pro-inflammatory phenotype of obese $CD4^+$ T cells that persists even after weight loss. Although analysis of the differentially expressed genes between the CD and HFD/HFD-RE groups did not uncover any significance differences in the expression of autophagy-related genes *Atg5*, *Atg7*, *Atg10*, *Atg12* or *Atg16l1*, there is some indication for altered cellular senescence. Amongst the differentially expressed genes between CD and HFD/HFD-RE, *Vegf*, *Cxcr6* and *Il18* have known links to cellular senescence. Importantly, vascular endothelial growth factor (VEGF) has been known to delay the onset of senescence as well as reverse senescence in microvascular endothelial cells and our heat-map shows significantly reduced expression of *Vegf* in the HFD and HFD-RE groups relative to CD (351). CXCR6, found up-regulated in the HFD and HFD-RE groups, mediates the $CD4^+$ T cell-dependent surveillance of senescent hepatocytes (352). There is also an upregulation of the gene encoding Interleukin-18 (IL18) in the HFD/HFD-RE group compared to CD, which in a study by Zhang et al., has been shown to promote fibroblast senescence in pulmonary fibrosis (353). Additionally, genes *C1qa* and *C1qb*, encoding the a and b chains respectively of the complement protein C1q which promotes aging-associated phenotypes, are found upregulated in the HFD/HFD-RE group (354). Taken

together, these gene changes indicate a potential for increased cellular senescence in the HFD and HFD-RE groups compared to CD.

We would be re-analysing the NanoString data, with stricter normalisation parameters, to unravel potential autophagy and senescence-related gene changes, that may have gone un-noticed because of our current strategy of normalisation to all 20 housekeeping genes.

4.5. Limitations

The rationale for using female only mice for the *in vivo* study was that when female mice are caged together, they are less likely to fight compared to male mice. However, the experiment was later repeated with male mice and similar results were observed.

Student's T test, instead of one-way ANOVA, was used to evaluate changes in protein expression in sections 3.2. and 3.8. since all the comparisons were made with respect to one particular group i.e., Chow Diet (in 3.2) or Untreated (in 3.8) and not between all the groups. It is understood that this is one of the limitations of this study since a T-test assumes normal distribution of data which is unlikely to happen with small datasets, therefore, to have a bigger confidence in the results and use a non-parametric test like Mann-Whitney, we would need to repeat the experiment with more mice or donors.

All the fatty acids were used within their physiological range in human serum (the plasma concentrations of palmitic acid range from 300 to

4100 μM, stearic acid from 100 to 1000 μM and oleic acid from 30 to 3200 μM) (332) . The dosage of 50 μM was chosen from literature and although some studies have used higher dosage of fatty acids, mainly palmitic acid, we found that higher dosages were toxic for our $CD4^+$ T cells (170, 171) .

4.6. Future Work

Given the detrimental outcomes obesity is accompanied by, it is important to understand how weight-gain, weight-loss and the immune system are inter-linked over the course of life. Knowing that obesity in childhood increases the likelihood of a person being obese in their adulthood, we need to understand whether childhood obesity has long-term impact on the immune system even when weight loss has been achieved in adulthood or if it is only a matter of time after which the immune system goes back to function normally. We have shown that even after obese mice go on to lose weight, the inflammatory effects of $CD4^+$ T cells persist. The next important question is whether the exacerbated T-cell response returns to homeostasis if a longer time for recovery is allowed. Therefore, the high-fat diet experiment has been repeated, i.e., mice on 8 weeks of HFD were fed CD for 12 weeks instead of 6 weeks to allow a longer time for recovery. The flow cytometry analysis of T lymphocyte responses of mice from this experiment are underway.

Having confirmed the upregulation of STK26 in *in vivo* and *in vitro* models of obesity, the next important step is to confirm our hypothesis and establish whether autophagy induction via STK26 is playing an important role in the maintenance of pro-inflammatory effector memory $CD4^+$ T cell population. To achieve that, as previously mentioned, we need to repeat the western blot for evaluating changes in autophagy markers under different fatty acid treatments, having optimised the concentration and duration of chloroquine treatment. Additionally, the assessment of autophagy would be carried out by using the Luminex Guava® Autophagy LC3 Antibody-based Detection Kit (Flow Cytometry) and confocal microscopy. The impact of senescence would be accessed via markers of T lymphocyte ageing (KLRG1), proliferation (ki67) and IL-2 levels using flow cytometry and ELISA. More importantly, *STK26* knocked-out mice are being bred in our facilities and T-lymphocyte responses will be accessed to evaluate whether homeostatic responses are restored in the absence of STK26. Additionally, senescence markers will be accessed in these knock-out mice to establish the crosstalk between autophagy and senescence that may exist to maintain inflammatory effector memory $CD4^+$ T cell populations in obesity.

Finally, through collaboration with other research labs, we would have access to the adipose tissues of patients with alström syndrome (associated with higher risk of obesity) and osteoarthritis (with healthy or obese BMIs). More importantly, we would have access to adipose

tissues of patients undergoing bariatric surgery and pharmacological treatment (before and after the surgery). We would test the translational impact of our study by accessing markers of autophagy and cellular senescence in these tissues.

4.7. Conclusion

In summary, we have stimulated obesity *in vitro* and mimicked the *in vivo* high-fat diet model by treating human $CD4^+$ T cells with different fatty acids and activating the TCR response *in vitro*. In addition to palmitic acid as already established to be important in the maintenance of pro-inflammatory $CD4^+$ T cell responses, our study has identified stearic acid to be playing a similar pro-inflammatory role. Through the use of both *in vivo* and *in vitro* models, this study has confirmed the upregulation of STK26, the autophagy inducer, in obesity. Additionally, the senescence implicated *CDKN1C* gene upregulation has been confirmed *in vitro* and investigation is underway to confirm protein levels. Taken together, our data suggest that STK26-induced autophagy and CDKN1C-induced senescence may play a role in maintaining $CD4^+$ T cell-mediated inflammatory programs upon weight loss. More causal studies will be required to directly demonstrate these links. Future studies involving *STK26* knockout and investigation of $CD4^+$ T cell phenotype, autophagy and senescence markers in the knockout model would provide a deeper insight into the role of STK26-induced autophagy in altered T-cell response, unravelling mechanistic

links and a crosstalk between autophagy and senescence that may exist to maintain pro-inflammatory $CD4^+$ T cell effector memory population upon weight loss. Finally, the translational impact of this study would be accessed by investigating markers of autophagy and senescence in adipose tissue from cohorts of patients detailed in section 4.5. Once the mechanistic links between autophagy, senescence, and the maintenance of $CD4^+$ T cell inflammatory program upon weight-loss are uncovered, pharmacological targeting of these processes would potentially provide means of restoration of immune homeostasis with a return to healthy weight, eliminating potential immune-related risks of obesity and ensuring long-term health.

BIBLIOGRAPHY

1. WHO. World Health Organization. 2021 [cited 2021 Apr 4]. Obesity. Available from: https://www.who.int/news-room/facts-in-pictures/detail/6-facts-on-obesity

2. Baker C. Obesity statistics [Internet]. House of Commons; 2023 Jan [cited 2023 Mar 23]. Available from: https://commonslibrary.parliament.uk/research-briefings/sn03336/

3. Simmonds M, Llewellyn A, Owen CG, Woolacott N. Predicting adult obesity from childhood obesity: a systematic review and meta-analysis. Obesity Reviews. 2016;17(2):95–107.

4. Pi-Sunyer X. The Medical Risks of Obesity. Postgrad Med. 2009 Nov;121(6):21–33.

5. New obesity treatments and technology to save the NHS billions [Internet]. 2022 [cited 2023 Mar 23]. Available from: https://www.gov.uk/government/news/new-obesity-treatments-and-technology-to-save-the-nhs-billions

6. Rankin LC, Artis D. Beyond Host Defense: Emerging Functions of the Immune System in Regulating Complex Tissue Physiology. Cell. 2018 Apr 19;173(3):554–67.

7. Chaplin DD. Overview of the Immune Response. J Allergy Clin Immunol. 2010 Feb;125(2 Suppl 2):S3-23.

8. Parkin J, Cohen B. An overview of the immune system. The Lancet. 2001 Jun 2;357(9270):1777–89.

9. Zhao TV, Sato Y, Goronzy JJ, Weyand CM. T-Cell Aging-Associated Phenotypes in Autoimmune Disease. Front Aging. 2022 Apr 25;3:867950.

10. Schwartz DM, Burma AM, Kitakule MM, Luo Y, Mehta NN. T Cells in Autoimmunity-Associated Cardiovascular Diseases. Frontiers in Immunology [Internet]. 2020 [cited 2023 Mar 24];11. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2020.588776

11. Gunasinghe SD, Peres NG, Goyette J, Gaus K. Biomechanics of T Cell Dysfunctions in Chronic Diseases. Front Immunol. 2021 Feb 25;12:600829.

12. Xia A, Zhang Y, Xu J, Yin T, Lu XJ. T Cell Dysfunction in Cancer Immunity and Immunotherapy. Front Immunol. 2019 Jul 19;10:1719.

13. Kronenberg M, Siu G, Hood LE, Shastri N. The Molecular Genetics of the T-Cell Antigen Receptor and T-Cell Antigen Recognition. Annual Review of Immunology. 1986;4(1):529–91.

14. Fowlkes BJ, Pardoll DM. Molecular and cellular events of T cell development. Adv Immunol. 1989;44:207–64.

15. Lawand M, Déchanet-Merville J, Dieu-Nosjean MC. Key Features of Gamma-Delta T-Cell Subsets in Human Diseases and Their Immunotherapeutic Implications. Frontiers in Immunology [Internet]. 2017 [cited 2023 Mar 23];8. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2017.00761

16. Duplay P, Lancki D, Allison JP. Distribution and ontogeny of CD2 expression by murine T cells. J Immunol. 1989 May 1;142(9):2998–3005.

17. Owen MJ, Jenkinson EJ, Brown MH, Sewell WA, Krissansen GW, Crumpton MJ, et al. Murine CD2 gene expression during fetal thymus ontogeny. Eur J Immunol. 1988 Jan;18(1):187–9.

18. Law DA, Spruyt LL, Paterson DJ, Williams AF. Subsets of thymopoietic rat thymocytes defined by expression of the CD2 antigen and the MRC OX-22 determinant of the leukocyte-common antigen CD45. Eur J Immunol. 1989 Dec;19(12):2289–95.

19. Robey E, Fowlkes BJ. Selective events in T cell development. Annu Rev Immunol. 1994;12:675–705.

20. Godfrey DI, Kennedy J, Suda T, Zlotnik A. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J Immunol. 1993 May 15;150(10):4244–52.

21. von Boehmer H, Fehling HJ. Structure and Function of the Pre-T Cell Receptor. Annual Review of Immunology. 1997;15(1):433–52.

22. Akbar AN, Terry L, Timms A, Beverley PC, Janossy G. Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. J Immunol. 1988 Apr 1;140(7):2171–8.

23. Beverley PC. Functional analysis of human T cell subsets defined by CD45 isoform expression. Semin Immunol. 1992 Feb;4(1):35–41.

24. Michie CA, McLean A, Alcock C, Beverley PCL. Lifespan of human lymphocyte subsets defined by CD45 isoforms. Nature. 1992 Nov;360(6401):264–5.

25. DeGrendele HC, Kosfiszer M, Estess P, Siegelman MH. CD44 activation and associated primary adhesion is inducible via T cell receptor stimulation. J Immunol. 1997 Sep 15;159(6):2549–53.

26. Graham VA, Marzo AL, Tough DF. A role for CD44 in T cell development and function during direct competition between CD44+ and CD44– cells. European Journal of Immunology. 2007;37(4):925–34.

27. Caruso A, Licenziati S, Corulli M, Canaris A d., De Francesco M a., Fiorentini S, et al. Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. Cytometry. 1997;27(1):71–6.

28. Shipkova M, Wieland E. Surface markers of lymphocyte activation and markers of cell proliferation. Clinica Chimica Acta. 2012 Sep 8;413(17):1338–49.

29. Groom JR, Luster AD. CXCR3 in T cell function. Exp Cell Res. 2011 Mar 10;317(5):620–31.

30. Brownlie RJ, Zamoyska R. T cell receptor signalling networks: branched, diversified and bounded. Nat Rev Immunol. 2013 Apr;13(4):257–69.

31. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. Annu Rev Immunol. 1996;14:233–58.

32. Sharpe AH, Freeman GJ. The B7–CD28 superfamily. Nat Rev Immunol. 2002 Feb;2(2):116–26.

33. Dustin ML, Springer TA. T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature. 1989 Oct 19;341(6243):619–24.

34. Chirathaworn C, Kohlmeier JE, Tibbetts SA, Rumsey LM, Chan MA, Benedict SH. Stimulation through intercellular adhesion molecule-1 provides a second signal for T cell activation. J Immunol. 2002 Jun 1;168(11):5530–7.

35. T Cell Co-inhibitory Receptors-Functions and Signalling Mechanisms [Internet]. [cited 2023 Mar 23]. Available from: https://www.longdom.org/open-access/t-cell-coinhibitory-receptorsfunctions-and-signalling-mechanisms-48434.html

36. Curtsinger JM, Schmidt CS, Mondino A, Lins DC, Kedl RM, Jenkins MK, et al. Inflammatory Cytokines Provide a Third Signal for Activation of Naive CD4+ and CD8+ T Cells1. The Journal of Immunology. 1999 Mar 15;162(6):3256–62.

37. Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. J Immunol. 2005 Apr 15;174(8):4465–9.

38. Curtsinger JM, Mescher MF. Inflammatory Cytokines as a Third Signal for T Cell Activation. Curr Opin Immunol. 2010 Jun;22(3):333–40.

39. Trickett A, Kwan YL. T cell stimulation and expansion using anti-CD3/CD28 beads. Journal of Immunological Methods. 2003 Apr 1;275(1):251–5.

40. Luckheeram RV, Zhou R, Verma AD, Xia B. CD4+T Cells: Differentiation and Functions. Clin Dev Immunol. 2012;2012:925135.

41. Mosmann TR, Li L, Sad S. Functions of CD8 T-cell subsets secreting different cytokine patterns. Seminars in Immunology. 1997 Apr 1;9(2):87–92.

42. Sad S, Marcotte R, Mosmann TR. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. Immunity. 1995 Mar;2(3):271–9.

43. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999 Oct 14;401(6754):708–12.

44. Carrasco J, Godelaine D, Van Pel A, Boon T, van der Bruggen P. CD45RA on human CD8 T cells is sensitive to the time elapsed since the last antigenic stimulation. Blood. 2006 Nov 1;108(9):2897–905.

45. Mueller SN, Mackay LK. Tissue-resident memory T cells: local specialists in immune defence. Nat Rev Immunol. 2016 Feb;16(2):79–89.

46. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity. 2014 Oct 16;41(4):529–42.

47. Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S. Human T helper type 1 dichotomy: origin, phenotype and biological activities. Immunology. 2015 Mar;144(3):343–51.

48. Walker JA, McKenzie ANJ. TH2 cell development and function. Nat Rev Immunol. 2018 Feb;18(2):121–33.

49. Jia L, Wu C. Differentiation, regulation and function of Th9 cells. Adv Exp Med Biol. 2014;841:181–207.

50. McGeachy MJ, Cua DJ. Th17 Cell Differentiation: The Long and Winding Road. Immunity. 2008 Apr 11;28(4):445–53.

51. Jia L, Wu C. The biology and functions of Th22 cells. Adv Exp Med Biol. 2014;841:209–30.

52. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. Annu Rev Immunol. 2012;30:531–64.

53. Hannoodee S, Nasuruddin DN. Acute Inflammatory Response [Internet]. StatPearls [Internet]. StatPearls Publishing; 2022 [cited 2023 Mar 23]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK556083/

54. Ahmed AU. An overview of inflammation: mechanism and consequences. Front Biol. 2011 Aug 1;6(4):274–81.

55. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008 Jul 24;454(7203):428–35.

56. Pahwa R, Goyal A, Jialal I. Chronic Inflammation [Internet]. StatPearls [Internet]. StatPearls Publishing; 2022 [cited 2023 Mar 23]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK493173/

57. Kochumon S, Madhoun AA, Al-Rashed F, Azim R, Al-Ozairi E, Al-Mulla F, et al. Adipose tissue gene expression of CXCL10 and CXCL11 modulates inflammatory markers in obesity: implications for metabolic inflammation and insulin resistance. Ther Adv Endocrinol Metab. 2020;11:2042018820930902.

58. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017 - The Lancet [Internet]. [cited 2023 Mar 23]. Available from: https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(18)32203-7/fulltext

59. Jéquier E. Pathways to obesity. Int J Obes Relat Metab Disord. 2002 Sep;26 Suppl 2:S12-17.

60. Hill JO, Melanson EL, Wyatt HT. Dietary fat intake and regulation of energy balance: implications for obesity. J Nutr. 2000 Feb;130(2S Suppl):284S-288S.

61. Bray GA, Popkin BM. Dietary fat intake does affect obesity! The American Journal of Clinical Nutrition. 1998 Dec 1;68(6):1157–73.

62. Ghibaudi L, Cook J, Farley C, van Heek M, Hwa JJ. Fat intake affects adiposity, comorbidity factors, and energy metabolism of sprague-dawley rats. Obes Res. 2002 Sep;10(9):956–63.

63. Takahashi M, Ikemoto S, Ezaki O. Effect of the fat/carbohydrate ratio in the diet on obesity and oral glucose tolerance in C57BL/6J mice. J Nutr Sci Vitaminol (Tokyo). 1999 Oct;45(5):583–93.

64. Hu S, Wang L, Yang D, Li L, Togo J, Wu Y, et al. Dietary Fat, but Not Protein or Carbohydrate, Regulates Energy Intake and Causes Adiposity in Mice. Cell Metabolism. 2018 Sep 4;28(3):415-431.e4.

65. Hariri N, Thibault L. High-fat diet-induced obesity in animal models. Nutrition Research Reviews. 2010 Dec;23(2):270–99.

66. Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins BA, et al. Origins and evolution of the Western diet: health implications for the 21st century. Am J Clin Nutr. 2005 Feb;81(2):341–54.

67. Nettleton JA, Brouwer IA, Geleijnse JM, Hornstra G. Saturated Fat Consumption and Risk of Coronary Heart Disease and Ischemic Stroke: A Science Update. Ann Nutr Metab. 2017 Apr;70(1):26–33.

68. Giuseppe DD, Wallin A, Bottai M, Askling J, Wolk A. Long-term intake of dietary long-chain n-3 polyunsaturated fatty acids and risk of rheumatoid arthritis: a prospective cohort study of women. Annals of the Rheumatic Diseases. 2014 Nov 1;73(11):1949–53.

69. Willett WC, Sacks F, Trichopoulou A, Drescher G, Ferro-Luzzi A, Helsing E, et al. Mediterranean diet pyramid: a cultural model for healthy eating. Am J Clin Nutr. 1995 Jun;61(6 Suppl):1402S-1406S.

70. Calder PC, Ahluwalia N, Brouns F, Buetler T, Clement K, Cunningham K, et al. Dietary factors and low-grade inflammation in relation to overweight and obesity. Br J Nutr. 2011 Dec;106 Suppl 3:S5-78.

71. Cnop M. Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes. Biochem Soc Trans. 2008 Jun;36(Pt 3):348–52.

72. Korbecki J, Bajdak-Rusinek K. The effect of palmitic acid on inflammatory response in macrophages: an overview of molecular mechanisms. Inflamm Res. 2019;68(11):915–32.

73. Ravaut G, Légiot A, Bergeron KF, Mounier C. Monounsaturated Fatty Acids in Obesity-Related Inflammation. Int J Mol Sci. 2020 Dec 30;22(1):330.

74. Shahidi F, Ambigaipalan P. Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits. Annu Rev Food Sci Technol. 2018 Mar 25;9:345–81.

75. DiNicolantonio JJ, O'Keefe JH. Good Fats versus Bad Fats: A Comparison of Fatty Acids in the Promotion of Insulin Resistance, Inflammation, and Obesity. Mo Med. 2017;114(4):303–7.

76. Tutunchi H, Ostadrahimi A, Saghafi-Asl M. The Effects of Diets Enriched in Monounsaturated Oleic Acid on the Management and Prevention of Obesity: a Systematic Review of Human Intervention Studies. Adv Nutr. 2020 Jul;11(4):864–77.

77. Harmonizing the Metabolic Syndrome | Circulation [Internet]. [cited 2023 Mar 23]. Available from: https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.109.192644

78. Park YW, Zhu S, Palaniappan L, Heshka S, Carnethon MR, Heymsfield SB. The metabolic syndrome: prevalence and associated risk factor findings in the US population from the Third National Health and Nutrition Examination Survey, 1988-1994. Arch Intern Med. 2003 Feb 24;163(4):427–36.

79. Fujita T. Insulin resistance and salt-sensitive hypertension in metabolic syndrome. Nephrol Dial Transplant. 2007 Nov;22(11):3102–7.

80. Roche HM. Fatty acids and the metabolic syndrome. Proc Nutr Soc. 2005 Feb;64(1):23–9.

81. Wakil SJ, Abu-Elheiga LA. Fatty acid metabolism: target for metabolic syndrome. J Lipid Res. 2009 Apr;50 Suppl(Suppl):S138-143.

82. Ellulu MS, Patimah I, Khaza'ai H, Rahmat A, Abed Y. Obesity and inflammation: the linking mechanism and the complications. Arch Med Sci. 2017 Jun;13(4):851–63.

83. Raval FM, Nikolajczyk BS. The Bidirectional Relationship between Metabolism and Immune Responses. Discoveries (Craiova). 1(1):e6.

84. Kominsky DJ, Campbell EL, Colgan SP. Metabolic Shifts in Immunity and Inflammation. J Immunol. 2010 Apr 15;184(8):4062–8.

85. Friedman JM, Leibel RL, Siegel DS, Walsh J, Bahary N. Molecular mapping of the mouse ob mutation. Genomics. 1991 Dec;11(4):1054–62.

86. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994 Dec;372(6505):425–32.

87. Francisco V, Pino J, Gonzalez-Gay MA, Mera A, Lago F, Gómez R, et al. Adipokines and inflammation: is it a question of weight? Br J Pharmacol. 2018 May;175(10):1569–79.

88. Kang YE, Kim JM, Joung KH, Lee JH, You BR, Choi MJ, et al. The Roles of Adipokines, Proinflammatory Cytokines, and Adipose Tissue Macrophages in Obesity-Associated Insulin Resistance in Modest Obesity and Early Metabolic Dysfunction. PLoS One. 2016;11(4):e0154003.

89. Zhou H, Urso C, Jadeja V. Saturated Fatty Acids in Obesity-Associated Inflammation. J Inflamm Res. 2020 Jan 6;13:1–14.

90. Rodriguez-Pacheco F, Gutierrez-Repiso C, Garcia-Serrano S, Alaminos-Castillo MA, Ho-Plagaro A, Valdes S, et al. The pro-/anti-inflammatory effects of different fatty acids on visceral adipocytes are partially mediated by GPR120. Eur J Nutr. 2017 Jun;56(4):1743–52.

91. Ajuwon KM, Spurlock ME. Palmitate activates the NF-kappaB transcription factor and induces IL-6 and TNFalpha expression in 3T3-L1 adipocytes. J Nutr. 2005 Aug;135(8):1841–6.

92. Cinti S. The adipose organ at a glance. Dis Model Mech. 2012 Sep;5(5):588–94.

93. Shen W, Wang Z, Punyanita M, Lei J, Sinav A, Kral JG, et al. Adipose Tissue Quantification by Imaging Methods: A Proposed Classification. Obes Res. 2003 Jan;11(1):5–16.

94. Chait A, den Hartigh LJ. Adipose Tissue Distribution, Inflammation and Its Metabolic Consequences, Including Diabetes and Cardiovascular Disease. Frontiers in Cardiovascular Medicine [Internet]. 2020 [cited 2023 Mar 24];7. Available from: https://www.frontiersin.org/articles/10.3389/fcvm.2020.00022

95. Berg AH, Scherer PE. Adipose Tissue, Inflammation, and Cardiovascular Disease. Circulation Research. 2005 May 13;96(9):939–49.

96. Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW, DeFuria J, Jick Z, et al. Adipocyte death, adipose tissue remodeling, and obesity complications. Diabetes. 2007 Dec;56(12):2910–8.

97. Lafontan M. Fat cells: afferent and efferent messages define new approaches to treat obesity. Annu Rev Pharmacol Toxicol. 2005;45:119–46.

98. Obradovic M, Sudar-Milovanovic E, Soskic S, Essack M, Arya S, Stewart AJ, et al. Leptin and Obesity: Role and Clinical Implication. Frontiers in Endocrinology [Internet]. 2021 [cited 2023 Mar 23];12. Available from: https://www.frontiersin.org/articles/10.3389/fendo.2021.585887

99. Ricci R, Bevilacqua F. The potential role of leptin and adiponectin in obesity: a comparative review. Vet J. 2012 Mar;191(3):292–8.

100. Rasouli N. Adipose tissue hypoxia and insulin resistance. J Investig Med. 2016 Apr;64(4):830–2.

101. Lee YS, Kim JW, Osborne O, Oh DY, Sasik R, Schenk S, et al. Increased adipocyte O2 consumption triggers HIF-1α, causing inflammation and insulin resistance in obesity. Cell. 2014 Jun 5;157(6):1339–52.

102. Wueest S, Rapold RA, Rytka JM, Schoenle EJ, Konrad D. Basal lipolysis, not the degree of insulin resistance, differentiates large from small isolated adipocytes in high-fat fed mice. Diabetologia. 2009 Mar;52(3):541–6.

103. Slawik M, Vidal-Puig AJ. Lipotoxicity, overnutrition and energy metabolism in aging. Ageing Research Reviews. 2006 May 1;5(2):144–64.

104. Rutkowski JM, Stern JH, Scherer PE. The cell biology of fat expansion. J Cell Biol. 2015 Mar 2;208(5):501–12.

105. Özcan U, Yilmaz E, Özcan L, Furuhashi M, Vaillancourt E, Smith RO, et al. Chemical Chaperones Reduce ER Stress and Restore Glucose Homeostasis in a Mouse Model of Type 2 Diabetes. Science. 2006 Aug 25;313(5790):1137–40.

106. Park HW, Park H, Ro SH, Jang I, Semple IA, Kim DN, et al. Hepatoprotective role of Sestrin2 against chronic ER stress. Nat Commun. 2014 Jun 20;5:4233.

107. Vieira-Potter VJ. Inflammation and macrophage modulation in adipose tissues. Cellular Microbiology. 2014;16(10):1484–92.

108. Rull A, Camps J, Alonso-Villaverde C, Joven J. Insulin Resistance, Inflammation, and Obesity: Role of Monocyte Chemoattractant Protein-1 (orCCL2) in the Regulation of Metabolism. Mediators of Inflammation. 2010 Sep 23;2010:e326580.

109. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003 Dec;112(12):1796–808.

110. Kratz M, Coats BR, Hisert KB, Hagman D, Mutskov V, Peris E, et al. Metabolic dysfunction drives a mechanistically distinct pro-inflammatory phenotype in adipose tissue macrophages. Cell Metab. 2014 Oct 7;20(4):614–25.

111. Reilly SM, Saltiel AR. Adapting to obesity with adipose tissue inflammation. Nat Rev Endocrinol. 2017 Nov;13(11):633–43.

112. Lumeng CN, DeYoung SM, Bodzin JL, Saltiel AR. Increased Inflammatory Properties of Adipose Tissue Macrophages Recruited During Diet-Induced Obesity. Diabetes. 2007 Jan 1;56(1):16–23.

113. Han JM, Levings MK. Immune Regulation in Obesity-Associated Adipose Inflammation. The Journal of Immunology. 2013 Jul 15;191(2):527–32.

114. Xu X, Grijalva A, Skowronski A, van Eijk M, Serlie MJ, Ferrante AW. Obesity Activates a Program of Lysosomal-Dependent Lipid Metabolism in Adipose Tissue Macrophages Independently of Classic Activation. Cell Metabolism. 2013 Dec 3;18(6):816–30.

115. Finck BN, Johnson RW. Tumor necrosis factor (TNF)-α induces leptin production through the p55 TNF receptor. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2000 Feb;278(2):R537–43.

116. Hribal ML, Fiorentino TV, Sesti G. Role of C Reactive Protein (CRP) in Leptin Resistance. Curr Pharm Des. 2014 Feb;20(4):609–15.

117. Morris DL, Cho KW, DelProposto JL, Oatmen KE, Geletka LM, Martinez-Santibanez G, et al. Adipose Tissue Macrophages Function As Antigen-Presenting Cells and Regulate Adipose Tissue CD4+ T Cells in Mice. Diabetes. 2013 Aug;62(8):2762–72.

118. Michailidou Z, Gomez-Salazar M, Alexaki VI. Innate Immune Cells in the Adipose Tissue in Health and Metabolic Disease. J Innate Immun. 2021 Apr 13;14(1):4–30.

119. Duffaut C, Galitzky J, Lafontan M, Bouloumié A. Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity. Biochemical and Biophysical Research Communications. 2009 Jul 10;384(4):482–5.

120. Winer DA, Winer S, Shen L, Wadia PP, Yantha J, Paltser G, et al. B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies. Nat Med. 2011 May;17(5):610–7.

121. Rausch ME, Weisberg S, Vardhana P, Tortoriello DV. Obesity in C57BL/6J mice is characterized by adipose tissue hypoxia and cytotoxic T-cell infiltration. Int J Obes (Lond). 2008 Mar;32(3):451–63.

122. Zhou H, Liu F. Regulation, Communication, and Functional Roles of Adipose Tissue-Resident CD4+ T Cells in the Control of Metabolic Homeostasis. Front Immunol. 2018 Aug 30;9:1961.

123. Geng YJ, Hansson GK. Interferon-gamma inhibits scavenger receptor expression and foam cell formation in human monocyte-derived macrophages. J Clin Invest. 1992 Apr;89(4):1322–30.

124. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep. 2014 Mar 3;6:13.

125. Jagannathan-Bogdan M, McDonnell ME, Shin H, Rehman Q, Hasturk H, Apovian CM, et al. Elevated Proinflammatory Cytokine Production by a Skewed T Cell Compartment Requires Monocytes and Promotes Inflammation in Type 2 Diabetes. The Journal of Immunology. 2011 Jan 15;186(2):1162–72.

126. Zeng C, Shi X, Zhang B, Liu H, Zhang L, Ding W, et al. The imbalance of Th17/Th1/Tregs in patients with type 2 diabetes: relationship with metabolic factors and complications. J Mol Med. 2012 Feb 1;90(2):175–86.

127. Zúñiga LA, Shen WJ, Joyce-Shaikh B, Pyatnova EA, Richards AG, Thom C, et al. IL-17 Regulates Adipogenesis, Glucose Homeostasis, and Obesity. The Journal of Immunology. 2010 Dec 1;185(11):6947–59.

128. Hong CP, Park A, Yang BG, Yun CH, Kwak MJ, Lee GW, et al. Gut-Specific Delivery of T-Helper 17 Cells Reduces Obesity and Insulin Resistance in Mice. Gastroenterology. 2017 Jun 1;152(8):1998–2010.

129. Mauro C, Smith J, Cucchi D, Coe D, Fu H, Bonacina F, et al. Obesity-Induced Metabolic Stress Leads to Biased Effector Memory CD4 + T Cell Differentiation via PI3K p110δ-Akt-Mediated Signals. Cell Metabolism. 2017 Mar;25(3):593–609.

130. Kraschnewski J, Boan J, Esposito J, Sherwood N, Lehman E, Kephart D, et al. Long-term weight loss maintenance in the United States. Int J Obes (Lond). 2010 Nov;34(11):1644–54.

131. Svetkey LP, Stevens VJ, Brantley PJ, Appel LJ, Hollis JF, Loria CM, et al. Comparison of strategies for sustaining weight loss: the weight loss maintenance randomized controlled trial. JAMA. 2008 Mar 12;299(10):1139–48.

132. Wadden TA, Neiberg RH, Wing RR, Clark JM, Delahanty LM, Hill JO, et al. Four-year weight losses in the Look AHEAD study: factors associated with long-term success. Obesity (Silver Spring). 2011 Oct;19(10):1987–98.

133. Eight-Year Weight Losses with an Intensive Lifestyle Intervention: The Look AHEAD Study. Obesity (Silver Spring). 2014 Jan;22(1):5–13.

134. Long-term pharmacotherapy in the management of obesity. National Task Force on the Prevention and Treatment of Obesity. JAMA. 1996 Dec 18;276(23):1907–15.

135. Anderson EK, Gutierrez DA, Kennedy A, Hasty AH. Weight Cycling Increases T-Cell Accumulation in Adipose Tissue and Impairs Systemic Glucose Tolerance. Diabetes. 2013 Sep;62(9):3180–8.

136. Kong LC, Wuillemin PH, Bastard JP, Sokolovska N, Gougis S, Fellahi S, et al. Insulin resistance and inflammation predict kinetic body weight changes in response to dietary weight loss and maintenance in overweight and obese subjects by using a Bayesian network approach. Am J Clin Nutr. 2013 Dec;98(6):1385–94.

137. Winer S, Chan Y, Paltser G, Truong D, Tsui H, Bahrami J, et al. Normalization of obesity-associated insulin resistance through immunotherapy. Nat Med. 2009 Aug;15(8):921–9.

138. Schmitz J, Evers N, Awazawa M, Nicholls HT, Brönneke HS, Dietrich A, et al. Obesogenic memory can confer long-term increases in adipose tissue but not liver inflammation and insulin resistance after weight loss. Mol Metab. 2016 Jan 11;5(5):328–39.

139. Kliewer KL, Ke JY, Stout MB, Cole R, Samuel VT, Shulman GI, et al. Short-term food restriction followed by controlled refeeding promotes gorging behavior, enhances fat deposition, and diminishes insulin sensitivity in mice. J Nutr Biochem. 2015 Jul;26(7):721–8.

140. Ambeba EJ, Styn MA, Kuller LH, Brooks MM, Evans RW, Burke LE. Longitudinal Effects of Weight Loss and Regain on Cytokine Concentration in Obese Adults. Metabolism. 2013 Sep;62(9):1218–22.

141. Zou J, Lai B, Zheng M, Chen Q, Jiang S, Song A, et al. CD4+ T cells memorize obesity and promote weight regain. Cell Mol Immunol. 2018 Jun;15(6):630–9.

142. Lund AH, Lohuizen M van. Epigenetics and cancer. Genes Dev. 2004 Jan 10;18(19):2315–35.

143. Keating S, El-Osta A. Epigenetic changes in diabetes. Clinical Genetics. 2013;84(1):1–10.

144. Ho SM. Environmental epigenetics of asthma: An update. Journal of Allergy and Clinical Immunology. 2010 Sep 1;126(3):453–65.

145. Mazzone R, Zwergel C, Artico M, Taurone S, Ralli M, Greco A, et al. The emerging role of epigenetics in human autoimmune disorders. Clinical Epigenetics. 2019 Feb 26;11(1):34.

146. Holliday R, Pugh JE. DNA modification mechanisms and gene activity during development. Science. 1975 Jan 24;187(4173):226–32.

147. Riggs AD. X inactivation, differentiation, and DNA methylation. Cytogenet Cell Genet. 1975;14(1):9–25.

148. Strahl BD, Allis CD. The language of covalent histone modifications. Nature. 2000 Jan 6;403(6765):41–5.

149. Fischle W, Wang Y, Allis CD. Binary switches and modification cassettes in histone biology and beyond. Nature. 2003 Oct 2;425(6957):475–9.

150. Bernstein E, Allis CD. RNA meets chromatin. Genes Dev. 2005 Jul 15;19(14):1635–55.

151. Costa FF. Non-coding RNAs, epigenetics and complexity. Gene. 2008 Feb 29;410(1):9–17.

152. Mattick JS, Amaral PP, Dinger ME, Mercer TR, Mehler MF. RNA regulation of epigenetic processes. BioEssays. 2009;31(1):51–9.

153. Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. Nature. 2007 May;447(7143):425–32.

154. Weber M, Hellmann I, Stadler MB, Ramos L, Pääbo S, Rebhan M, et al. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet. 2007 Apr;39(4):457–66.

155. Jin Z, Liu Y. DNA methylation in human diseases. Genes Dis. 2018 Jan 31;5(1):1–8.

156. Kouzarides T. Chromatin modifications and their function. Cell. 2007 Feb 23;128(4):693–705.

157. Martin C, Zhang Y. The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol. 2005 Nov;6(11):838–49.

158. Lin Y, Qiu T, Wei G, Que Y, Wang W, Kong Y, et al. Role of Histone Post-Translational Modifications in Inflammatory Diseases. Frontiers in Immunology [Internet]. 2022 [cited 2023 Mar 23];13. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2022.852272

159. Zaratiegui M, Irvine DV, Martienssen RA. Noncoding RNAs and Gene Silencing. Cell. 2007 Feb 23;128(4):763–76.

160. Amaral PP, Dinger ME, Mercer TR, Mattick JS. The eukaryotic genome as an RNA machine. Science. 2008 Mar 28;319(5871):1787–9.

161. Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. Nat Rev Genet. 2009 Feb;10(2):94–108.

162. Esteller M. Non-coding RNAs in human disease. Nat Rev Genet. 2011 Dec;12(12):861–74.

163. González-Becerra K, Ramos-Lopez O, Barrón-Cabrera E, Riezu-Boj JI, Milagro FI, Martínez-López E, et al. Fatty acids, epigenetic mechanisms and chronic diseases: a systematic review. Lipids Health Dis. 2019 Oct 15;18:178.

164. Tremblay BL, Guénard F, Rudkowska I, Lemieux S, Couture P, Vohl MC. Epigenetic changes in blood leukocytes following an omega-3 fatty acid supplementation. Clin Epigenetics. 2017 Apr 26;9:43.

165. Kumar S, Pamulapati H, Tikoo K. Fatty acid induced metabolic memory involves alterations in renal histone H3K36me2 and H3K27me3. Mol Cell Endocrinol. 2016 Feb 15;422:233–42.

166. Hall E, Volkov P, Dayeh T, Bacos K, Rönn T, Nitert MD, et al. Effects of palmitate on genome-wide mRNA expression and DNA methylation patterns in human pancreatic islets. BMC Med. 2014 Jun 23;12:103.

167. Ishikawa K, Tsunekawa S, Ikeniwa M, Izumoto T, Iida A, Ogata H, et al. Long-Term Pancreatic Beta Cell Exposure to High Levels of Glucose but Not Palmitate Induces DNA Methylation within the Insulin Gene Promoter and Represses Transcriptional Activity. PLoS One. 2015 Feb 6;10(2):e0115350.

168. Maples JM, Brault JJ, Shewchuk BM, Witczak CA, Zou K, Rowland N, et al. Lipid exposure elicits differential responses in gene expression and DNA methylation in primary human skeletal muscle cells from severely obese women. Physiological Genomics. 2015 May;47(5):139.

169. Wang X, Cao Q, Yu L, Shi H, Xue B, Shi H. Epigenetic regulation of macrophage polarization and inflammation by DNA methylation in obesity. JCI Insight. 1(19):e87748.

170. Pascual G, Domínguez D, Elosúa-Bayes M, Beckedorff F, Laudanna C, Bigas C, et al. Dietary palmitic acid promotes a prometastatic memory via Schwann cells. Nature. 2021 Nov;599(7885):485–90.

171. Hata M, Andriessen EMMA, Hata M, Diaz-Marin R, Fournier F, Crespo-Garcia S, et al. Past history of obesity triggers persistent epigenetic changes in innate immunity and exacerbates neuroinflammation. Science. 2023 Jan 6;379(6627):45–62.

172. Parzych KR, Klionsky DJ. An Overview of Autophagy: Morphology, Mechanism, and Regulation. Antioxid Redox Signal. 2014 Jan 20;20(3):460–73.

173. Parzych KR, Klionsky DJ. An Overview of Autophagy: Morphology, Mechanism, and Regulation. Antioxid Redox Signal. 2014 Jan 20;20(3):460–73.

174. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. Cell Res. 2014 Jan;24(1):24–41.

175. Li W wen, Li J, Bao J ku. Microautophagy: lesser-known self-eating. Cell Mol Life Sci. 2012 Apr;69(7):1125–36.

176. Dice JF. Chaperone-mediated autophagy. Autophagy. 2007;3(4):295–9.

177. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008 Jan 11;132(1):27–42.

178. Huang J, Klionsky DJ. Autophagy and human disease. Cell Cycle. 2007 Aug 1;6(15):1837–49.

179. Dikic I, Elazar Z. Mechanism and medical implications of mammalian autophagy. Nat Rev Mol Cell Biol. 2018 Jun;19(6):349–64.

180. Rocchi A, He C. Emerging roles of autophagy in metabolism and metabolic disorders. Front Biol. 2015 Apr 1;10(2):154–64.

181. Xu J, Kitada M, Ogura Y, Koya D. Relationship Between Autophagy and Metabolic Syndrome Characteristics in the Pathogenesis of Atherosclerosis. Front Cell Dev Biol. 2021 Apr 15;9:641852.

182. Klionsky DJ. The molecular machinery of autophagy: unanswered questions. Journal of Cell Science. 2005 Jan 1;118(1):7–18.

183. Suzuki K, Ohsumi Y. Molecular machinery of autophagosome formation in yeast, Saccharomyces cerevisiae. FEBS Letters. 2007;581(11):2156–61.

184. Chan EY. Regulation and Function of Uncoordinated-51 Like Kinase Proteins. Antioxidants & Redox Signaling. 2012 Sep;17(5):775–85.

185. Russell RC, Tian Y, Yuan H, Park HW, Chang YY, Kim J, et al. ULK1 induces autophagy by phosphorylating Beclin-1 and activating Vps34 lipid kinase. Nat Cell Biol. 2013 Jul;15(7):741–50.

186. Di Bartolomeo S, Corazzari M, Nazio F, Oliverio S, Lisi G, Antonioli M, et al. The dynamic interaction of AMBRA1 with the dynein motor complex regulates mammalian autophagy. J Cell Biol. 2010 Oct 4;191(1):155–68.

187. Hara T, Takamura A, Kishi C, Iemura S ichiro, Natsume T, Guan JL, et al. FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. J Cell Biol. 2008 May 5;181(3):497–510.

188. Orsi A, Razi M, Dooley HC, Robinson D, Weston AE, Collinson LM, et al. Dynamic and transient interactions of Atg9 with autophagosomes, but not membrane integration, are required for autophagy. MBoC. 2012 May 15;23(10):1860–73.

189. Feng Y, Klionsky DJ. Autophagic membrane delivery through ATG9. Cell Res. 2017 Feb;27(2):161–2.

190. Tanida I, Ueno T, Kominami E. LC3 and Autophagy. Methods Mol Biol. 2008;445:77–88.

191. Lee YK, Lee JA. Role of the mammalian ATG8/LC3 family in autophagy: differential and compensatory roles in the spatiotemporal regulation of autophagy. BMB Rep. 2016 Aug 31;49(8):424–30.

192. Nakatogawa H. Two ubiquitin-like conjugation systems that mediate membrane formation during autophagy. Lane JD, editor. Essays in Biochemistry. 2013 Sep 27;55:39–50.

193. Bento CF, Renna M, Ghislat G, Puri C, Ashkenazi A, Vicinanza M, et al. Mammalian Autophagy: How Does It Work? Annual Review of Biochemistry. 2016;85(1):685–713.

194. Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. Journal of Cell Science. 2004 Jun 1;117(13):2805–12.

195. Park S, Choi SG, Yoo SM, Son JH, Jung YK. Choline dehydrogenase interacts with SQSTM1/p62 to recruit LC3 and stimulate mitophagy. Autophagy. 2014 Oct 30;10(11):1906–20.

196. Bento CF, Renna M, Ghislat G, Puri C, Ashkenazi A, Vicinanza M, et al. Mammalian Autophagy: How Does It Work? Annual Review of Biochemistry. 2016;85(1):685–713.

197. Fujita N, Hayashi-Nishino M, Fukumoto H, Omori H, Yamamoto A, Noda T, et al. An Atg4B Mutant Hampers the Lipidation of LC3 Paralogues and Causes Defects in Autophagosome Closure. Mol Biol Cell. 2008 Nov;19(11):4651–9.

198. Fader CM, Sánchez DG, Mestre MB, Colombo MI. TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2009 Dec 1;1793(12):1901–16.

199. Furuta N, Fujita N, Noda T, Yoshimori T, Amano A. Combinational Soluble N-Ethylmaleimide-sensitive Factor Attachment Protein Receptor Proteins VAMP8 and Vti1b Mediate Fusion of Antimicrobial and Canonical Autophagosomes with Lysosomes. Mol Biol Cell. 2010 Mar 15;21(6):1001–10.

200. Kim YM, Jung CH, Seo M, Kim EK, Park JM, Bae SS, et al. mTORC1 phosphorylates UVRAG to negatively regulate autophagosome and endosome maturation. Mol Cell. 2015 Jan 22;57(2):207–18.

201. Satoo K, Noda NN, Kumeta H, Fujioka Y, Mizushima N, Ohsumi Y, et al. The structure of Atg4B–LC3 complex reveals the mechanism of LC3 processing and delipidation during autophagy. EMBO J. 2009 May 6;28(9):1341–50.

202. Rabinowitz JD, White E. Autophagy and Metabolism. Science. 2010 Dec 3;330(6009):1344–8.

203. Dann SG, Selvaraj A, Thomas G. mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. Trends Mol Med. 2007 Jun;13(6):252–9.

204. Galluzzi L, Pietrocola F, Levine B, Kroemer G. Metabolic control of autophagy. Cell. 2014 Dec 4;159(6):1263–76.

205. Yang L, Li P, Fu S, Calay ES, Hotamisligil GS. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. Cell Metab. 2010 Jun 9;11(6):467–78.

206. Wen H, Gris D, Lei Y, Jha S, Zhang L, Huang MTH, et al. Fatty acid-induced NLRP3-PYCARD inflammasome activation interferes with insulin signaling. Nat Immunol. 2011 May;12(5):408–15.

207. Cho CS, Lombard DB, Lee JH. SIRT3 as a regulator of hepatic autophagy. Hepatology. 2017 Sep;66(3):700–2.

208. Dann SG, Selvaraj A, Thomas G. mTOR Complex1–S6K1 signaling: at the crossroads of obesity, diabetes and cancer. Trends in Molecular Medicine. 2007 Jun 1;13(6):252–9.

209. Jansen HJ, van Essen P, Koenen T, Joosten L a. B, Netea MG, Tack CJ, et al. Autophagy activity is up-regulated in adipose tissue of obese individuals and modulates proinflammatory cytokine expression. Endocrinology. 2012 Dec;153(12):5866–74.

210. Kovsan J, Blüher M, Tarnovscki T, Klöting N, Kirshtein B, Madar L, et al. Altered Autophagy in Human Adipose Tissues in Obesity. The Journal of Clinical Endocrinology & Metabolism. 2011 Feb 1;96(2):E268–77.

211. Mei S, Ni HM, Manley S, Bockus A, Kassel KM, Luyendyk JP, et al. Differential roles of unsaturated and saturated fatty acids on autophagy and apoptosis in hepatocytes. J Pharmacol Exp Ther. 2011 Nov;339(2):487–98.

212. Nuñez CE, Rodrigues VS, Gomes FS, Moura RF de, Victorio SC, Bombassaro B, et al. Defective regulation of adipose tissue autophagy in obesity. Int J Obes (Lond). 2013 Nov;37(11):1473–80.

213. Ogata M, Hino S ichiro, Saito A, Morikawa K, Kondo S, Kanemoto S, et al. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol Cell Biol. 2006 Dec;26(24):9220–31.

214. Filomeni G, De Zio D, Cecconi F. Oxidative stress and autophagy: the clash between damage and metabolic needs. Cell Death Differ. 2015 Mar;22(3):377–88.

215. Qian M, Fang X, Wang X. Autophagy and inflammation. Clin Transl Med. 2017 Dec;6(1):24.

216. Liu HY, Han J, Cao SY, Hong T, Zhuo D, Shi J, et al. Hepatic autophagy is suppressed in the presence of insulin resistance and hyperinsulinemia: inhibition of FoxO1-dependent expression of key autophagy genes by insulin. J Biol Chem. 2009 Nov 6;284(45):31484–92.

217. Ignacio-Souza LM, Bombassaro B, Pascoal LB, Portovedo MA, Razolli DS, Coope A, et al. Defective regulation of the ubiquitin/proteasome system in the hypothalamus of obese male mice. Endocrinology. 2014 Aug;155(8):2831–44.

218. Ju L, Han J, Zhang X, Deng Y, Yan H, Wang C, et al. Obesity-associated inflammation triggers an autophagy–lysosomal response in adipocytes and causes degradation of perilipin 1. Cell Death Dis. 2019 Feb 11;10(2):1–16.

219. Zhang Y, Sowers JR, Ren J. Targeting autophagy in obesity: from pathophysiology to management. Nat Rev Endocrinol. 2018 Jun;14(6):356–76.

220. Yoshii SR, Mizushima N. Monitoring and Measuring Autophagy. Int J Mol Sci. 2017 Aug 28;18(9):1865.

221. Huang T, Kim CK, Alvarez AA, Pangeni RP, Wan X, Song X, et al. MST4 Phosphorylation of ATG4B Regulates Autophagic Activity, Tumorigenicity, and Radioresistance in Glioblastoma. Cancer Cell. 2017 Dec 11;32(6):840-855.e8.

222. Li M, Hou Y, Wang J, Chen X, Shao ZM, Yin XM. Kinetics Comparisons of Mammalian Atg4 Homologues Indicate Selective Preferences toward Diverse Atg8 Substrates. J Biol Chem. 2011 Mar 4;286(9):7327–38.

223. Pua HH, Dzhagalov I, Chuck M, Mizushima N, He YW. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. J Exp Med. 2007 Jan 22;204(1):25–31.

224. Hubbard VM, Valdor R, Patel B, Singh R, Cuervo AM, Macian F. Macroautophagy regulates energy metabolism during effector T cell activation. J Immunol. 2010 Dec 15;185(12):7349–57.

225. Murera D, Arbogast F, Arnold J, Bouis D, Muller S, Gros F. CD4 T cell autophagy is integral to memory maintenance. Sci Rep. 2018 Apr 13;8(1):5951.

226. Campisi J. Aging, Cellular Senescence, and Cancer. Annu Rev Physiol. 2013;75:685–705.

227. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. Genes Dev. 2010 Nov 15;24(22):2463–79.

228. Adams PD. Healing and hurting: molecular mechanisms, functions, and pathologies of cellular senescence. Mol Cell. 2009 Oct 9;36(1):2–14.

229. Tchkonia T, Zhu Y, van Deursen J, Campisi J, Kirkland JL. Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. J Clin Invest. 2013 Mar 1;123(3):966–72.

230. Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, et al. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature. 2006 Nov 30;444(7119):638–42.

231. Ewald JA, Desotelle JA, Wilding G, Jarrard DF. Therapy-induced senescence in cancer. J Natl Cancer Inst. 2010 Oct 20;102(20):1536–46.

232. Petrova NV, Velichko AK, Razin SV, Kantidze OL. Small molecule compounds that induce cellular senescence. Aging Cell. 2016 Dec;15(6):999–1017.

233. Herranz N, Gil J. Mechanisms and functions of cellular senescence. J Clin Invest. 2018 Apr 2;128(4):1238–46.

234. Fedarko NS. The biology of aging and frailty. Clin Geriatr Med. 2011 Feb;27(1):27–37.

235. Campisi J, Robert L. Cell senescence: role in aging and age-related diseases. Interdiscip Top Gerontol. 2014;39:45–61.

236. Yun MH, Davaapil H, Brockes JP. Recurrent turnover of senescent cells during regeneration of a complex structure. Elife. 2015 May 5;4:e05505.

237. Ramakrishna G, Anwar T, Angara RK, Chatterjee N, Kiran S, Singh S. Role of cellular senescence in hepatic wound healing and carcinogenesis. Eur J Cell Biol. 2012 Oct;91(10):739–47.

238. Demaria M, Ohtani N, Youssef SA, Rodier F, Toussaint W, Mitchell JR, et al. An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. Dev Cell. 2014 Dec 22;31(6):722–33.

239. Coppé JP, Desprez PY, Krtolica A, Campisi J. The Senescence-Associated Secretory Phenotype: The Dark Side of Tumor Suppression. Annual review of pathology. 2010;5:99.

240. Farr JN, Xu M, Weivoda MM, Monroe DG, Fraser DG, Onken JL, et al. Targeting cellular senescence prevents age-related bone loss in mice. Nat Med. 2017 Sep;23(9):1072–9.

241. Schafer MJ, White TA, Iijima K, Haak AJ, Ligresti G, Atkinson EJ, et al. Cellular senescence mediates fibrotic pulmonary disease. Nat Commun. 2017 Feb 23;8:14532.

242. Roos CM, Zhang B, Palmer AK, Ogrodnik MB, Pirtskhalava T, Thalji NM, et al. Chronic senolytic treatment alleviates established vasomotor dysfunction in aged or atherosclerotic mice. Aging Cell. 2016 Oct;15(5):973–7.

243. Narasimhan A, Flores RR, Robbins PD, Niedernhofer LJ. Role of Cellular Senescence in Type II Diabetes. Endocrinology. 2021 Oct 1;162(10):bqab136.

244. Musi N, Valentine JM, Sickora KR, Baeuerle E, Thompson CS, Shen Q, et al. Tau protein aggregation is associated with cellular senescence in the brain. Aging Cell. 2018 Dec;17(6):e12840.

245. Martínez-Cué C, Rueda N. Cellular Senescence in Neurodegenerative Diseases. Frontiers in Cellular Neuroscience [Internet]. 2020 [cited 2023 Mar 23];14. Available from: https://www.frontiersin.org/articles/10.3389/fncel.2020.00016

246. Lowe SW, Cepero E, Evan G. Intrinsic tumour suppression. Nature. 2004 Nov;432(7015):307–15.

247. Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence. The International Journal of Biochemistry & Cell Biology. 2005 May 1;37(5):961–76.

248. Beauséjour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P, et al. Reversal of human cellular senescence: roles of the p53 and p16 pathways. EMBO J. 2003 Aug 15;22(16):4212–22.

249. d'Adda di Fagagna F. Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer. 2008 Jul;8(7):512–22.

250. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell. 1993 Nov 19;75(4):805–16.

251. d'Adda di Fagagna F. Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer. 2008 Jul;8(7):512–22.

252. Wiedemeyer WR. Resistance Mechanisms to Cyclin-Dependent Kinase Inhibitors. In: Yarden Y, Elkabets M, editors. Resistance to Anti-Cancer Therapeutics Targeting Receptor Tyrosine Kinases and Downstream Pathways [Internet]. Cham: Springer International Publishing; 2018 [cited 2023 Mar 23]. p. 181–210. (Resistance to Targeted Anti-Cancer Therapeutics). Available from: https://doi.org/10.1007/978-3-319-67932-7_8

253. Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence. The International Journal of Biochemistry & Cell Biology. 2005 May 1;37(5):961–76.

254. Freund A, Orjalo AV, Desprez PY, Campisi J. Inflammatory Networks during Cellular Senescence: Causes and Consequences. Trends Mol Med. 2010 May;16(5):238–46.

255. Ortiz-Montero P, Londoño-Vallejo A, Vernot JP. Senescence-associated IL-6 and IL-8 cytokines induce a self- and cross-reinforced senescence/inflammatory milieu strengthening tumorigenic

capabilities in the MCF-7 breast cancer cell line. Cell Communication and Signaling. 2017 May 4;15(1):17.

256. Vernot JP. Senescence-Associated Pro-inflammatory Cytokines and Tumor Cell Plasticity. Frontiers in Molecular Biosciences [Internet]. 2020 [cited 2023 Mar 23];7. Available from: https://www.frontiersin.org/articles/10.3389/fmolb.2020.00063

257. Davalos AR, Coppe JP, Campisi J, Desprez PY. Senescent cells as a source of inflammatory factors for tumor progression. Cancer Metastasis Rev. 2010 Jun 1;29(2):273–83.

258. Sun Y, Wang X, Liu T, Zhu X, Pan X. The multifaceted role of the SASP in atherosclerosis: from mechanisms to therapeutic opportunities. Cell & Bioscience. 2022 May 31;12(1):74.

259. Chaib S, Tchkonia T, Kirkland JL. Obesity, Senescence, and Senolytics. Handb Exp Pharmacol. 2022;274:165–80.

260. Palmer AK, Jensen MD, Tchkonia T, Kirkland JL. Chapter 11 - Senescence in obesity: causes and consequences. In: Serrano M, Muñoz-Espín D, editors. Cellular Senescence in Disease [Internet]. Academic Press; 2022 [cited 2023 Mar 23]. p. 289–308. Available from: https://www.sciencedirect.com/science/article/pii/B9780128225141000092

261. Smith U, Li Q, Rydén M, Spalding KL. Cellular senescence and its role in white adipose tissue. Int J Obes. 2021 May;45(5):934–43.

262. Conley SM, Hickson LJ, Kellogg TA, McKenzie T, Heimbach JK, Taner T, et al. Human Obesity Induces Dysfunction and Early Senescence in Adipose Tissue-Derived Mesenchymal Stromal/Stem Cells. Frontiers in Cell and Developmental Biology [Internet]. 2020 [cited 2023 Mar 23];8. Available from: https://www.frontiersin.org/articles/10.3389/fcell.2020.00197

263. Tchkonia T, Morbeck DE, Von Zglinicki T, Van Deursen J, Lustgarten J, Scrable H, et al. Fat tissue, aging, and cellular senescence. Aging Cell. 2010 Oct;9(5):667–84.

264. Liu Z, Jin L, Yang JK, Wang B, Wu KKL, Hallenborg P, et al. The Dysfunctional MDM2-p53 Axis in Adipocytes Contributes to Aging-Related Metabolic Complications by Induction of Lipodystrophy. Diabetes. 2018 Nov;67(11):2397–409.

265. Minamino T, Orimo M, Shimizu I, Kunieda T, Yokoyama M, Ito T, et al. A crucial role for adipose tissue p53 in the regulation of insulin resistance. Nat Med. 2009 Sep;15(9):1082–7.

266. Palmer AK, Xu M, Zhu Y, Pirtskhalava T, Weivoda MM, Hachfeld CM, et al. Targeting senescent cells alleviates obesity-induced metabolic dysfunction. Aging Cell. 2019 Jun;18(3):e12950.

267. Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. [Internet]. [cited 2023 Mar 23]. Available from: https://genesdev.cshlp.org/content/9/6/639.short

268. Lee MH, Reynisdóttir I, Massagué J. Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev. 1995 Mar 15;9(6):639–49.

269. Matsuoka S, Edwards MC, Bai C, Parker S, Zhang P, Baldini A, et al. p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. Genes Dev. 1995 Mar 15;9(6):650–62.

270. Reynaud EG, Guillier M, Leibovitch MP, Leibovitch SA. Dimerization of the amino terminal domain of p57Kip2 inhibits cyclin D1-Cdk4 kinase activity. Oncogene. 2000 Feb;19(9):1147–52.

271. Van De Pette M, Tunster SJ, McNamara GI, Shelkovnikova T, Millership S, Benson L, et al. Cdkn1c Boosts the Development of Brown Adipose Tissue in a Murine Model of Silver Russell Syndrome. PLoS Genet. 2016 Mar 10;12(3):e1005916.

272. Van de Pette M, Tunster SJ, John RM. Loss of Imprinting of Cdkn1c Protects against Age and Diet-Induced Obesity. Int J Mol Sci. 2018 Sep 12;19(9):2734.

273. Barbosa MC, Grosso RA, Fader CM. Hallmarks of Aging: An Autophagic Perspective. Frontiers in Endocrinology [Internet]. 2019 [cited 2023 Mar 23];9. Available from: https://www.frontiersin.org/articles/10.3389/fendo.2018.00790

274. Kang HT, Lee KB, Kim SY, Choi HR, Park SC. Autophagy Impairment Induces Premature Senescence in Primary Human Fibroblasts. PLoS One. 2011 Aug 8;6(8):e23367.

275. García-Prat L, Martínez-Vicente M, Perdiguero E, Ortet L, Rodríguez-Ubreva J, Rebollo E, et al. Autophagy maintains stemness by preventing senescence. Nature. 2016 Jan 7;529(7584):37–42.

276. Wen X, Klionsky DJ. Autophagy is a key factor in maintaining the regenerative capacity of muscle stem cells by promoting quiescence and preventing senescence. Autophagy. 2016 Apr 6;12(4):617–8.

277. Han X, Tai H, Wang X, Wang Z, Zhou J, Wei X, et al. AMPK activation protects cells from oxidative stress-induced senescence via autophagic flux restoration and intracellular NAD + elevation. Aging Cell. 2016 Jun;15(3):416–27.

278. Tai H, Wang Z, Gong H, Han X, Zhou J, Wang X, et al. Autophagy impairment with lysosomal and mitochondrial dysfunction is an important characteristic of oxidative stress-induced senescence. Autophagy. 2016 Oct 28;13(1):99–113.

279. Dalle Pezze P, Nelson G, Otten EG, Korolchuk VI, Kirkwood TBL, von Zglinicki T, et al. Dynamic Modelling of Pathways to Cellular Senescence Reveals Strategies for Targeted Interventions. PLoS Comput Biol. 2014 Aug 28;10(8):e1003728.

280. Nopparat C, Sinjanakhom P, Govitrapong P. Melatonin reverses H2 O2 -induced senescence in SH-SY5Y cells by enhancing autophagy via sirtuin 1 deacetylation of the RelA/p65 subunit of NF-κB. J Pineal Res. 2017 Aug;63(1).

281. Goto N, Makino S, Fujiwara K. Neuropathogenicity of mutant strains of mouse hepatitis virus, 1A and 2C, from DBT cells persistently infected with JHM strain. Adv Exp Med Biol. 1987;218:439–40.

282. Kang HT, Park JT, Choi K, Kim Y, Choi HJC, Jung CW, et al. Chemical screening identifies ATM as a target for alleviating senescence. Nat Chem Biol. 2017 Jun;13(6):616–23.

283. Levine B, Kroemer G. Autophagy in the Pathogenesis of Disease. Cell. 2008 Jan 11;132(1):27–42.

284. Rubinsztein DC, Mariño G, Kroemer G. Autophagy and aging. Cell. 2011 Sep 2;146(5):682–95.

285. Young ARJ, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JFJ, et al. Autophagy mediates the mitotic senescence transition. Genes Dev. 2009 Apr 1;23(7):798–803.

286. Gewirtz DA. Autophagy and senescence. Autophagy. 2013 May 1;9(5):808–12.

287. Narita M, Young ARJ, Arakawa S, Samarajiwa SA, Nakashima T, Yoshida S, et al. Spatial Coupling of mTOR and Autophagy Augments Secretory Phenotypes. Science. 2011 May 20;332(6032):966–70.

288. Cayo A, Segovia R, Venturini W, Moore-Carrasco R, Valenzuela C, Brown N. mTOR Activity and Autophagy in Senescent Cells, a Complex Partnership. International Journal of Molecular Sciences. 2021 Jan;22(15):8149.

289. Capparelli C, Chiavarina B, Whitaker-Menezes D, Pestell TG, Pestell RG, Hulit J, et al. CDK inhibitors (p16/p19/p21) induce senescence and autophagy in cancer-associated fibroblasts, "fueling" tumor growth via paracrine interactions, without an increase in neo-angiogenesis. Cell Cycle. 2012 Oct 1;11(19):3599–610.

290. Nam HY, Han MW, Chang HW, Kim SY, Kim SW. Prolonged autophagy by MTOR inhibitor leads radioresistant cancer cells into senescence. Autophagy. 2013 Oct;9(10):1631–2.

291. Liu H, He Z, Simon HU. Autophagy suppresses melanoma tumorigenesis by inducing senescence. Autophagy. 2014 Feb;10(2):372–3.

292. Huang YH, Yang PM, Chuah QY, Lee YJ, Hsieh YF, Peng CW, et al. Autophagy promotes radiation-induced senescence but inhibits bystander effects in human breast cancer cells. Autophagy. 2014 Jul 1;10(7):1212–28.

293. Horikawa I, Fujita K, Jenkins LMM, Hiyoshi Y, Mondal AM, Vojtesek B, et al. Autophagic degradation of the inhibitory p53 isoform Δ133p53α as a regulatory mechanism for p53-mediated senescence. Nat Commun. 2014 Aug 21;5:4706.

294. Dou Z, Xu C, Donahue G, Shimi T, Pan JA, Zhu J, et al. Autophagy mediates degradation of nuclear lamina. Nature. 2015 Nov 5;527(7576):105–9.

295. Dou Z, Ivanov A, Adams PD, Berger SL. Mammalian autophagy degrades nuclear constituents in response to tumorigenic stress. Autophagy. 2015 Dec 10;12(8):1416–7.

296. Mosieniak G, Adamowicz M, Alster O, Jaskowiak H, Szczepankiewicz AA, Wilczynski GM, et al. Curcumin induces permanent growth arrest of human colon cancer cells: link between senescence and autophagy. Mech Ageing Dev. 2012 Jun;133(6):444–55.

297. S P, J C, A P, N M, J C, D P, et al. Lipid mediators of autophagy in stress-induced premature senescence of endothelial cells. American journal of physiology Heart and circulatory physiology [Internet]. 2008 Mar [cited 2023 Mar 23];294(3). Available from: https://pubmed.ncbi.nlm.nih.gov/18203850/

298. Singh K, Matsuyama S, Drazba JA, Almasan A. Autophagy-dependent senescence in response to DNA damage and chronic apoptotic stress. Autophagy. 2012 Feb;8(2):236–51.

299. Goehe RW, Di X, Sharma K, Bristol ML, Henderson SC, Valerie K, et al. The Autophagy-Senescence Connection in Chemotherapy: Must Tumor Cells (Self) Eat Before They Sleep? J Pharmacol Exp Ther. 2012 Dec;343(3):763–78.

300. Kwon Y, Kim JW, Jeoung JA, Kim MS, Kang C. Autophagy Is Pro-Senescence When Seen in Close-Up, but Anti-Senescence in Long-Shot. Molecules and Cells. 2017 Sep 9;40(9):607.

301. Soundar S, O'Hagan M, Fomulu KS, Colman RF. Identification of Mn2+-binding Aspartates from α, β, and γ Subunits of Human NAD-dependent Isocitrate Dehydrogenase *. Journal of Biological Chemistry. 2006 Jul 28;281(30):21073–81.

302. Zeng L, Morinibu A, Kobayashi M, Zhu Y, Wang X, Goto Y, et al. Aberrant IDH3α expression promotes malignant tumor growth by inducing HIF-1-mediated metabolic reprogramming and angiogenesis. Oncogene. 2015 Sep;34(36):4758–66.

303. National Library of Medicine [Internet]. 2023 [cited 2023 Mar 23]. IDH3G isocitrate dehydrogenase (NAD(+)) 3 non-catalytic subunit gamma [Homo sapiens (human)] - Gene - NCBI. Available from: https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=ShowDetailView&TermToSearch=3421

304. Soundar S, Park JH, Huh TL, Colman RF. Evaluation by Mutagenesis of the Importance of 3 Arginines in α, β, and γ Subunits of Human NAD-dependent Isocitrate Dehydrogenase *. Journal of Biological Chemistry. 2003 Dec 26;278(52):52146–53.

305. Kang W, Suzuki M, Saito T, Miyado K. Emerging Role of TCA Cycle-Related Enzymes in Human Diseases. Int J Mol Sci. 2021 Dec 2;22(23):13057.

306. Baardman J, Verberk SGS, Velden S van der, Gijbels MJJ, Roomen CPPA van, Sluimer JC, et al. Macrophage ATP citrate lyase deficiency stabilizes atherosclerotic plaques. Nature Communications [Internet]. 2020 [cited 2023 Mar 23];11. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7722882/

307. Raimundo N, Baysal BE, Shadel GS. Revisiting the TCA cycle: signaling to tumor formation. Trends Mol Med. 2011 Nov;17(11):641–9.

308. Gibson GE, Park LCH, Sheu KFR, Blass JP, Calingasan NY. The α-ketoglutarate dehydrogenase complex in neurodegeneration. Neurochemistry International. 2000 Feb 1;36(2):97–112.

309. Hassinen IE. Signaling and Regulation Through the NAD+ and NADP+ Networks. Antioxid Redox Signal. 2019 Feb 20;30(6):857–74.

310. Lenzen KP, Heils A, Lorenz S, Hempelmann A, Sander T. Association analysis of malic enzyme 2 gene polymorphisms with idiopathic generalized epilepsy. Epilepsia. 2005 Oct;46(10):1637–41.

311. Kremer DM, Nelson BS, Lin L, Yarosz EL, Halbrook CJ, Kerk SA, et al. GOT1 inhibition promotes pancreatic cancer cell death by ferroptosis. Nat Commun. 2021 Aug 11;12:4860.

312. Broeks MH, Shamseldin HE, Alhashem A, Hashem M, Abdulwahab F, Alshedi T, et al. MDH1 deficiency is a metabolic disorder of the malate-aspartate shuttle associated with early onset severe encephalopathy. Hum Genet. 2019 Dec;138(11–12):1247–57.

313. Elevated TCA cycle function in the pathology of diet-induced hepatic insulin resistance and fatty liver - PubMed [Internet]. [cited 2023 Mar 23]. Available from: https://pubmed.ncbi.nlm.nih.gov/22493093/

314. Myers SA, Nield A, Chew GS, Myers MA. The Zinc Transporter, Slc39a7 (Zip7) Is Implicated in Glycaemic Control in Skeletal Muscle Cells. PLoS One. 2013 Nov 12;8(11):e79316.

315. Landi A, Vermeire J, Iannucci V, Vanderstraeten H, Naessens E, Bentahir M, et al. Genome-wide shRNA screening identifies host factors involved in early endocytic events for HIV-1-induced CD4 down-regulation. Retrovirology. 2014 Dec 13;11:118.

316. Liu T, Wang Y, Zhao M, Jiang J, Li T, Zhang M. Differential expression of aerobic oxidative metabolism-related proteins in diabetic urinary exosomes. Front Endocrinol (Lausanne). 2022 Sep 14;13:992827.

317. Jiang J, Huang D, Jiang Y, Hou J, Tian M, Li J, et al. Lactate Modulates Cellular Metabolism Through Histone Lactylation-Mediated Gene Expression in Non-Small Cell Lung Cancer. Front Oncol. 2021 Jun 2;11:647559.

318. Gaster M. Reduced TCA flux in diabetic myotubes: A governing influence on the diabetic phenotype? Biochemical and Biophysical Research Communications. 2009 Oct 2;387(4):651–5.

319. Zou K, Hinkley JM, Park S, Zheng D, Jones TE, Pories WJ, et al. Altered tricarboxylic acid cycle flux in primary myotubes from severely obese humans. Int J Obes. 2019 Apr;43(4):895–905.

320. Koussounadis A, Langdon SP, Um IH, Harrison DJ, Smith VA. Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system. Sci Rep. 2015 Jun 8;5(1):10775.

321. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995 Aug 1;155(3):1151–64.

322. Obesity and overweight [Internet]. [cited 2023 Jul 16]. Available from: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight

323. Dai H, Alsalhe TA, Chalghaf N, Riccò M, Bragazzi NL, Wu J. The global burden of disease attributable to high body mass index in 195 countries and territories, 1990–2017: An analysis of the Global Burden of Disease Study. PLoS Med. 2020 Jul 28;17(7):e1003198.

324. Larsson SC, Burgess S. Causal role of high body mass index in multiple chronic diseases: a systematic review and meta-analysis of Mendelian randomization studies. BMC Med. 2021 Dec 15;19(1):320.

325. Norris T, Blodgett JM, Rogers NT, Hamer M, Pinto Pereira SM. Obesity in early adulthood and physical functioning in mid-life: Investigating the mediating role of c-reactive protein. Brain Behav Immun. 2022 May;102:325–32.

326. de Mutsert R, Sun Q, Willett WC, Hu FB, van Dam RM. Overweight in Early Adulthood, Adult Weight Change, and Risk of Type 2 Diabetes, Cardiovascular Diseases, and Certain Cancers in Men: a Cohort Study. Am J Epidemiol. 2014 Jun 1;179(11):1353–65.

327. Ervin RB, Wright JD, Wang CY, Kennedy-Stephenson J. Dietary intake of fats and fatty acids for the United States population: 1999-2000. Adv Data. 2004 Nov 8;(348):1–6.

328. Carrillo C, Cavia M del M, Alonso-Torre S. Role of oleic acid in immune system; mechanism of action; a review. Nutr Hosp. 2012;27(4):978–90.

329. Oliveira V, Marinho R, Vitorino D, Santos GA, Moraes JC, Dragano N, et al. Diets Containing α-Linolenic (ω3) or Oleic (ω9) Fatty Acids Rescues Obese Mice From Insulin Resistance. Endocrinology. 2015 Nov;156(11):4033–46.

330. Calder PC. Omega-3 Fatty Acids and Inflammatory Processes. Nutrients. 2010 Mar 18;2(3):355–74.

331. Crupi R, Cuzzocrea S. Role of EPA in Inflammation: Mechanisms, Effects, and Clinical Relevance. Biomolecules. 2022 Feb 1;12(2):242.

332. Abdelmagid SA, Clarke SE, Nielsen DE, Badawi A, El-Sohemy A, Mutch DM, et al. Comprehensive Profiling of Plasma Fatty Acid Concentrations in Young Healthy Canadian Adults. PLOS ONE. 2015 Feb 12;10(2):e0116195.

333. Alon U. Network motifs: theory and experimental approaches. Nat Rev Genet. 2007 Jun;8(6):450–61.

334. Savageau MA. Comparison of classical and autogenous systems of regulation in inducible operons. Nature. 1974 Dec 13;252(5484):546–9.

335. Becskei A, Serrano L. Engineering stability in gene networks by autoregulation. Nature. 2000 Jun;405(6786):590–3.

336. Couturier J, Nuotio-Antar AM, Agarwal N, Wilkerson GK, Saha P, Kulkarni V, et al. Lymphocytes upregulate CD36 in adipose tissue and liver. Adipocyte. 2019 Apr 30;8(1):154–63.

337. Simons K, Toomre D. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol. 2000 Oct;1(1):31–9.

338. Patra SK, Bettuzzi S. Epigenetic DNA-methylation regulation of genes coding for lipid raft-associated components: A role for raft proteins in cell transformation and cancer progression (Review). Oncology Reports. 2007 Jun 1;17(6):1279–90.

339. Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun. 2004 Jan 23;313(4):856–62.

340. Brunner AM, Yakovlev IA, Strauss SH. Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol. 2004 Aug 18;4:14.

341. Bustin SA, Benes V, Nolan T, Pfaffl MW. Quantitative real-time RT-PCR--a perspective. J Mol Endocrinol. 2005 Jun;34(3):597–601.

342. Paolacci AR, Tanzarella OA, Porceddu E, Ciaffi M. Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. BMC Mol Biol. 2009 Feb 20;10:11.

343. Fasshauer M, Blüher M. Adipokines in health and disease. Trends Pharmacol Sci. 2015 Jul;36(7):461–70.

344. Jin R, Hao J, Yu J, Wang P, Sauter ER, Li B. Role of FABP5 in T Cell Lipid Metabolism and Function in the Tumor Microenvironment. Cancers. 2023 Jan;15(3):657.

345. de Sousa Abreu R, Penalva LO, Marcotte EM, Vogel C. Global signatures of protein and mRNA expression levels. Mol Biosyst. 2009 Dec;5(12):1512–26.

346. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat Rev Genet. 2012 Apr;13(4):227–32.

347. Levine B, Kroemer G. Autophagy in the Pathogenesis of Disease. Cell. 2008 Jan 11;132(1):27–42.

348. Wong E, Cuervo AM. Autophagy gone awry in neurodegenerative diseases. Nat Neurosci. 2010 Jul;13(7):805–11.

349. Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Øvervatn A, et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol. 2005 Nov 21;171(4):603–14.

350. Huang R, Liu W. Identifying an essential role of nuclear LC3 for autophagy. Autophagy. 2015 May 6;11(5):852–3.

351. Watanabe Y, Lee SW, Detmar M, Ajioka I, Dvorak HF. Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) delays and induces escape from senescence in human dermal microvascular endothelial cells. Oncogene. 1997 May;14(17):2025–32.

352. Mossanen JC, Kohlhepp M, Wehr A, Krenkel O, Liepelt A, Roeth AA, et al. CXCR6 Inhibits Hepatocarcinogenesis by Promoting Natural Killer T- and CD4+ T-Cell-Dependent Control of Senescence. Gastroenterology. 2019 May;156(6):1877-1889.e4.

353. Zhang LM, Zhang J, Zhang Y, Fei C, Wang L, Yi ZW, et al. Interleukin-18 promotes fibroblast senescence in pulmonary fibrosis through down-regulating Klotho expression. Biomedicine & Pharmacotherapy. 2019 May 1;113:108756.

354. Naito AT, Sumida T, Nomura S, Liu ML, Higo T, Nakagawa A, et al. Complement C1q Activates Canonical Wnt Signaling and Promotes Aging-Related Phenotypes. Cell. 2012 Aug 3;150(3):659–60.