

The role of dietary fat in anti-tumour immunity



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Declaration

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Hannah Prendeville B.Sc.

For my Mam and Dad

Summary

Obesity is a risk factor for cancer development, which is due in part to impaired immunosurveillance. Obesity disrupts immunometabolic pathways in NK and CD8 T cells, resulting in metabolic paralysis, reduced IFN γ production, and killing capacity. To improve NK and CD8 T cell functional responses in obesity, there is a need to understand the mechanisms by which obesity interferes with systemic and cellular immunometabolism.

Immune effector functions are regulated by cellular metabolic pathways which are influenced by nutrients and metabolites in the cell's microenvironments. Nutrient availability is dictated by diet, and thus, diet composition shapes the immune response by regulating cellular metabolism. Therefore, we hypothesised that the source of dietary fat (animal vs. plant) would differentially affect anti-tumour NK and CD8 T cell responses through the impact of these diets on systemic nutrient availability. To explore this, we used mouse models of B16 melanoma and diet induced obesity (DIO) where mice were fed high-fat diets (HFDs) from animal and plant sources which included lard, butter (animal), palm oil, peanut oil, and coconut oil (plant). Indeed, tumour growth dynamics were dependent on the source of dietary fat. We found that animal derived HFDs increased the growth of B16 tumours, whereas plant HFDs did not. Furthermore, IFN γ production by tumoral NK and CD8 T cells was reduced when mice were fed animal but not plant derived HFDs. To further explore the mechanism(s), we focused on butter and palm oil HFDs which resulted in the most striking differences in tumour growth and infiltration of NK and CD8 T cells.

HFDs disrupt the plasma acylcarnitine profile, resulting in increased abundance of long chain acylcarnitines (LCACs), due to mitochondrial metabolic overload and incomplete fatty acid oxidation (FAO). Therefore, we performed metabolomics analysis of the blood plasma to understand how these HFDs affect systemic nutrient and metabolite availability. While both diets altered the plasma metabolome, the LCAC octadecanoyl-carnitine (C18) was only increased when mice were fed lard or butter, not palm oil. Indeed, we found that a HFD from butter disrupts the carnitine shuttle in metabolic organs which controls the oxidation of long chain fatty acids (LCFAs). C18 is derived from stearic acid (SA) which is enriched in lard and butter diets, and thus C18 accumulation in the plasma indicates incomplete oxidation of SA. Furthermore, when mice were fed a standard fat diet (SFD) following a lard HFD to induce weight loss (WL), the plasma metabolome was re-established, and peripheral NK and CD8 T cell

responses were restored. These data further demonstrate that dietary composition dictates nutrient and metabolite availability in the plasma, which likely affects NK and CD8 T cell effector functions.

LCACs promote inflammation in type 2 diabetes (T2D) by increasing IL-17 production from Th17 cells, but to date, the effects of different acylcarnitines on anti-tumour NK and CD8 T cell functions have not been investigated. In this study, we show that acylcarnitines decreased IFN γ production by CD8 T cells in a chain length dependent manner, with C18 inducing the most significant defect. Furthermore, C18 causes mitochondrial depolarisation and metabolic paralysis in CD8 T cells. In contrast, NK cell functional and metabolic responses were not affected by any of the acylcarnitines tested. B16 and MC38 tumour microenvironments (TMEs) are rich in acylcarnitines, which may further increase when mice are fed HFDs from animal sources. This work reveals that LCACs impair CD8 T cell immunosurveillance and anti-tumour immunity and may explain why animal HFDs accelerate tumour growth.

The Lynch lab previously demonstrated that lipids interfere with mTORC1 signalling in NK cells, resulting in metabolic paralysis and reduced IFN γ production. In this project, we revealed that NK cells accumulate lipids only when mice are fed animal HFDs. Additionally, NK cells from mice fed butter had decreased rates of glycolysis and oxidative phosphorylation (oxphos) and increased levels of cellular reactive oxygen species (ROS), possibly due to lipotoxicity. On the other hand, NK cells from mice fed palm oil activate antioxidant defence pathways involving Nrf1 and Nnt, which protect against excess lipids and decrease ROS levels. The intricacies of this mechanism will require further investigation, but it is likely that these protective pathways are induced by the specific lipid cocktail of a palm oil HFD. Furthermore, complete oxidation of dietary lipids from a palm oil diet ensures that LCACs do not accumulate when mice are fed palm oil, and CD8 T cells are thus functionally and metabolically intact. Indeed, work is now emerging that tumours favour specific lipid cocktails to support their growth and metastasis. In this project, we have built on this concept and also show that anti-tumour immune cells are also influenced by lipid compositions of the diet and how these dietary lipids are metabolised systemically.

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Publications

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

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UCP1 governs liver extracellular succinate and inflammatory pathogenesis.

Mills EL, Harmon C, Jedrychowski MP, Xiao H, Garrity R, Tran NV, Bradshaw GA, Fu A, Szpyt J, Reddy A, **Prendeville H**, Danial NN, Gygi SP, Lynch L, Chouchani ET. *Nature Metabolism*, 2021.

Cysteine 253 of UCP1 regulates energy expenditure and sex-dependent adipose tissue inflammation.

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

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Abstract

Obesity increases the risk of many cancers and impairs immunosurveillance. However, little is known about whether the type of dietary fat affects tumour growth and anti-tumour immune responses. Here, we show that a high-fat diet (HFD) from animal sources (lard or butter), but not plant sources (palm, coconut, peanut oil) accelerates tumour growth, despite similar levels of obesity and adipose distribution. Animal-derived HFDs impaired NK and CD8 T cell metabolism by two distinct cell specific mechanisms, both leading to mitochondrial dysfunction and loss of IFN γ production. The animal derived butter versus plant derived palm oil yielded the most different results and were used to investigate the mechanism in depth. Butter fed mice had significantly accelerated tumour growth while palm fed mice were completely protected from obesity-induced tumour progression despite equal obesity. Using *in vivo* whole body metabolic analysis and multiomic profiling, we found that HFDs caused a systemic metabolic switch to β -oxidation and impaired glucose handling regardless of fat source. However, at the metabolome level, critical differences were revealed which impacted tumour growth and immunity. Animal fat, but not plant fat, caused accumulation of intracellular lipids in NK cells, increased reactive oxygen species, metabolic paralysis, and impaired IFN γ production. However, in plant derived HFDs, NK cells activated antioxidant pathways involving Nrf1 and Nnt, protecting from lipotoxicity and retaining cytotoxic function despite obesity. Furthermore, animal derived HFDs result in incomplete fatty acid oxidation in key metabolic organs, causing systemic accumulation of circulating long chain acylcarnitines, particularly octadecanoyl-carnitine which completely impaired CD8 T cell metabolism and function. Weight loss restored the plasma metabolome and peripheral NK and CD8 T cell functional responses; however, weight loss did not protect against tumour growth, and tumoral NK and CD8 T cells were still defective. These data reveal that excess dietary fat from animal sources is more harmful to anti-tumour immunity and these functional defects persist after weight loss. This may inform the nutritional regime for patients alongside traditional cancer therapies, particularly immunotherapy.

Abbreviations

2-DG	2-deoxy-D-glucose
25/27-HC	25/27-hydroxycholesterol
β-me	2-mercaptoethanol
ACC	Acetyl-CoA carboxylase
ACADVL	Very long chain acyl-CoA dehydrogenase
ACAT	Acyl cholesterol acyltransferase
ACLY	ATP citrate lyase
ADCC	Antibody-dependent cellular cytotoxicity
Anti-A	Antimycin-A
APC	Antigen presenting cell
AS	ATP synthase
AT	Adipose tissue
ATP	Adenosine triphosphate
APS	Ammonium persulfate
BCAA	Branched chain amino acid
BFA	Brefeldin A
BSA	Bovine serum albumin
C8	Octanoyl-carnitine
C14	Myristoyl-carnitine
C16	Palmitoyl-carnitine
C18	Octadecanoyl-carnitine
CACT	Carnitine acylcarnitine translocase
CAR	Chimeric antigen receptor
cCD8 T cell	Circulating CD8 T cell
CLP	Common lymphoid progenitor
CMS	Citrate malate shuttle
cNK cell	Circulating NK cell
CPT1a	Carnitine palmitoyltransferase 1A
cRPMI	Complete RPMI
C _t	Cycle threshold
CTL	Cytotoxic lymphocyte
CTV	Cell trace violet

CVD	Cardiovascular Disease
DHA	Docosahexaenoic acid
DIO	Diet induced obesity
DLCBL	Diffuse large B cell lymphoma
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
DCFDA	2',7'-dichlorofluorescein diacetate
DCF	2',7'-dichlorofluorescein
DC	Dendritic cell
DGAT1	Diglyceride acyltransferase 1
DISC	Death-inducing signalling complex
ECAR	Extracellular acidification rate
ETC	Electron transport chain
FABP	Fatty acid binding protein
FACS	Fatty acyl-CoA synthetase
FACS	Fluorescent-activated cell sorting
FADH ₂	Flavin adenine dinucleotide
FAME	Fatty acid methyl ester
FAO	Fatty acid oxidation
FASL	Fas ligand
FASN	Fatty acid synthase
FCS	Foetal calf serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine
FA	Fatty acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Glut1/3	Glucose transporter 1/3
GPX4	Glutathione peroxidase 4
GTT	Glucose tolerance test
GzB	Granzyme B
H ₂ DCFDA	2',7'-dichlorodihydrofluorescein
HBSS	Hanks balanced salt solution
Hex2	Hexokinase 2
HFD	High-fat diet

HIF-1 α	Hypoxia inducible factor 1 α
HPRA	The health products regulatory authority
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunotyrosine-based inhibitory motifs
ITSM	Immunoreceptor tyrosine-based switch motifs
KD	Ketogenic diet
KIR	Killer immunoglobulin-like receptor
LA	Linoleic acid
LAMP-1/2	Lysosomal-associated membrane protein
LCFA	Long chain fatty acid
LC/MS	Liquid chromatography / mass spectrometry
LD	Lipid droplet
LDHA	Lactate dehydrogenase A
LN	Lymph node
LTG	LipidTOX Green
MA	Myristic acid
mAB	Monoclonal antibody
MACs	Magnetic activated cell separation
MCT	Monocarboxylate transporter
MHC	Major histocompatibility complex
MICA/B	MHC class 1 polypeptide-related sequence A/B
MIF	Macrophage inhibitory factor
MRR	Maximum respiratory rate
MTG	Mitotracker Green
MTOC	Microtubule organising centre
mTORC1	Mammalian target of rapamycin complex 1
MUFA	Monounsaturated fatty acid
NADH	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NCR	Natural cytotoxicity receptor
NEFA	Non-esterified fatty acid
NK cell	Natural Killer cell
NR	Nicotinamide riboside
NSCLC	Non-small-cell lung cancer

OA	Oleic acid
OAA	Oxaloacetate
OCR	Oxygen consumption rate
Oligo	Oligomycin
OxLDL	Oxidised low density lipoprotein
Oxphos	Oxidative phosphorylation
PA	Palmitic acid
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PDH	Pyruvate dehydrogenase
PKD-1	Phosphoinositide-dependent kinase 1
PEP	Phosphoenolpyruvate
PGC-1 α	Peroxisome proliferation receptor- γ co-activator- α
PHD	Prolyl hydroxylase
PI(3,4,5)P ₃	Phosphatidylinositol 3,4,5-triphosphate
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome proliferation receptor
PPP	Pentose phosphate pathway
P/S	Penicillin-streptomycin
PUFA	Polyunsaturated fatty acids
RER	Respiratory exchange ratio
ROS	Reactive oxygen species
Rot	Rotenone
RPMI-1640	Roswell Park memorial institute-1640 medium
RT	Room temperature
S6	Ribosomal protein S6
S6K	P70-S6 kinase
SA	Stearic acid
SCD	Stearoyl-CoA desaturase
sCD8 T cell	Splenic CD8 T cell
SDS	Sodium dodecyl sulphate
SFA	Saturated fatty acid

SFD	Standard fat diet
Slc7a5	Solute carrier family 7 membrane 5
S/MCFA	Short/medium chain fatty acid
sNK	Splenic NK cell
SREBP	Sterol regulatory element-binding protein
T2D	Type 2 diabetes
TAG	Triacylglycerol
TBST	Tris-buffered saline (TBS) and tween 20
TCA cycle	Tricarboxylic acid cycle
TCR	T cell receptor
TEMED	Tetramethylethylenediamine
TIF	Tumour interstitial fluid
TIL	Tumour infiltrating lymphocyte
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TME	Tumour microenvironment
TMRM	Tetramethylrhodamine, methyl ester, perchlorate
WT	Wild-type
γc	Common gamma chain

Chapter 1. Introduction

1.1 The anti-tumour immune response

In the 1900s, Paul Ehrlich first predicted that cells of the immune system could recognise and protect against spontaneous tumour growth¹. Burnet and Thomas revisited this concept of immunosurveillance in the 1950s, a time when immunologists had a greater understanding and appreciation for tumour biology, transplant immunology, and immune tolerance. They hypothesised that immune cells could recognise specific neo-antigens and eliminate immunogenic tumours, similar to homograft rejection^{2,3}. The cancer immunosurveillance hypothesis was accepted in the 1960s with the discovery of mouse tumour-derived antigens⁴ and identification of T cells, which were branded as the effector cells mediating cancer immunosurveillance⁵. The goal of cancer immunosurveillance is to detect and kill transformed cells and the primary immune populations that carry out this role are cytotoxic natural killer (NK) and CD8 T cells⁶.

1.1.1 Natural Killer (NK) cells

NK cells are granular lymphocytes that represent between 5-20% of circulating lymphocytes in humans⁷ and 2-5% of splenocytes of laboratory mice⁸. They are commonly characterised by the expression of CD56 in humans and NKp46 and NK1.1 in mice as well as the absence of CD3⁷. NK cells arise from the common lymphoid progenitor (CLP) derived from haematopoietic stem cells in the bone marrow (BM), where they predominantly develop. IL-15 is essential for NK cell development as NK cell numbers are reduced in mice lacking IL-15 or the IL-15R α -chain^{9,10}. IL-15 signalling also controls NK cell homeostasis and survival in the periphery, where it is thought to prevent apoptosis¹¹. Mature NK cells circulate in the blood and reside in various organs such as the uterus and liver where they have tissue-specific roles such as supporting immune tolerance and ensuring tissue homeostasis^{12,13}. Mature NK cells express a myriad of activating and inhibitory receptors and are poised for activation¹⁴ against virally infected and tumour cells.

NK cell activation

Unlike their B or T cell counterparts, NK cells do not express specific antigen receptors. Rather, NK cells express an array of germline encoded activating and inhibitory

receptors to detect malignant or virally infected cells. NK cell activation is determined by the combination of signals from both these receptor repertoires, which govern whether the NK cell kills the target cell. NK cell cytotoxicity is inhibited by the absence of activating signals and/or engagement of inhibitory receptors, which ensures that NK cells are tolerant to healthy host cells (Figure 1.1).

Activating receptors

NK cells are equipped with a host of activating receptors to detect inducible ligands that are upregulated on the surface of stressed cells such as virally infected and tumour cells. The natural cytotoxicity receptors (NCRs) including NKp30 (NCR3) and the prototypical activating receptor NKG2D (encoded by *Klrk1*), bind B7-H6 and MHC class 1 polypeptide-related sequence A/B (MICA/B) in humans¹⁵ and H60 in mice¹⁶. These ligands are expressed by tumour cells early in tumorigenesis and are not expressed by healthy cells in steady-state conditions, ensuring that NK cells do not compromise the integrity of normal cells. NKG2D is crucial for NK cell immunosurveillance as NKG2D-deficient mice cannot control the growth of prostate adenocarcinomas and E μ -Myc-induced lymphomas¹⁷. Activating receptors contain short intracellular domains with no signalling properties. NCRs associate with immunoreceptor tyrosine-based activation motifs (ITAMs) which recruit the tyrosine kinases Syk and ZAP70, while NKG2D associates with DAP10/12 adaptor proteins and recruits PI3K¹⁸. These kinases propagate strong activating signals to induce cytokine secretion and NK cell degranulation. In addition, engagement of activating receptors initiates calcium signalling which promotes cytoskeletal rearrangement and localisation of the microtubule organising centre (MTOC) at the NK cell-target cell interface. This ensures that lytic granules are positioned and released appropriately into the immunological synapse, resulting in specific killing of target cells^{19,20} (Figure. 1.1).

Inhibitory receptors

NK cell activation is tightly regulated by inhibitory receptors which tolerise NK cells towards healthy host cells and prevent damage (Figure 1.1). Inhibitory receptors recognise and bind MHC class 1 molecules, constitutively expressed by healthy cells. To enable self vs non-self discrimination, NK cells are “educated” in the BM by engaging inhibitory receptors with MHC class 1, generating functional, mature NK cells that are informed of the host MHC repertoire. Killer-cell immunoglobulin-like receptors (KIRs) are expressed by human NK cells and recognise HLA-C expression, while CD94-NKG2A

heterodimer is expressed by both human and murine NK cells and recognise non-classical HLA-E molecules. Inhibitory receptors also enable “missing self-recognition” of malignant cells that commonly downregulate MHC class 1 during their transformation as an immune escape mechanism from cytotoxic lymphocytes (CTLs). Reduced ligation of inhibitory receptors in the presence of activating signals triggers NK cell activation to promote tumour cell killing²¹. Engagement of inhibitory receptors activates cytosolic immunotyrosine-based inhibitory motifs (ITIMs), resulting in dephosphorylation of downstream signalling molecules to suppress NK cell activation²². ITIM signalling also decreases calcium influx and destabilises the formation of the immunological synapse between NK and target cells²³. Importantly, ligation of receptors does not result in a bipolar state of NK cell activation or inhibition. Rather, NK cells exist along a spectrum of activation and inhibitory states that depend on expression levels of activating vs inhibitory receptors. In short, if the target cell expresses sufficient inhibitory ligands which surpass ligation of activation receptors, NK cell cytotoxicity will not be triggered.

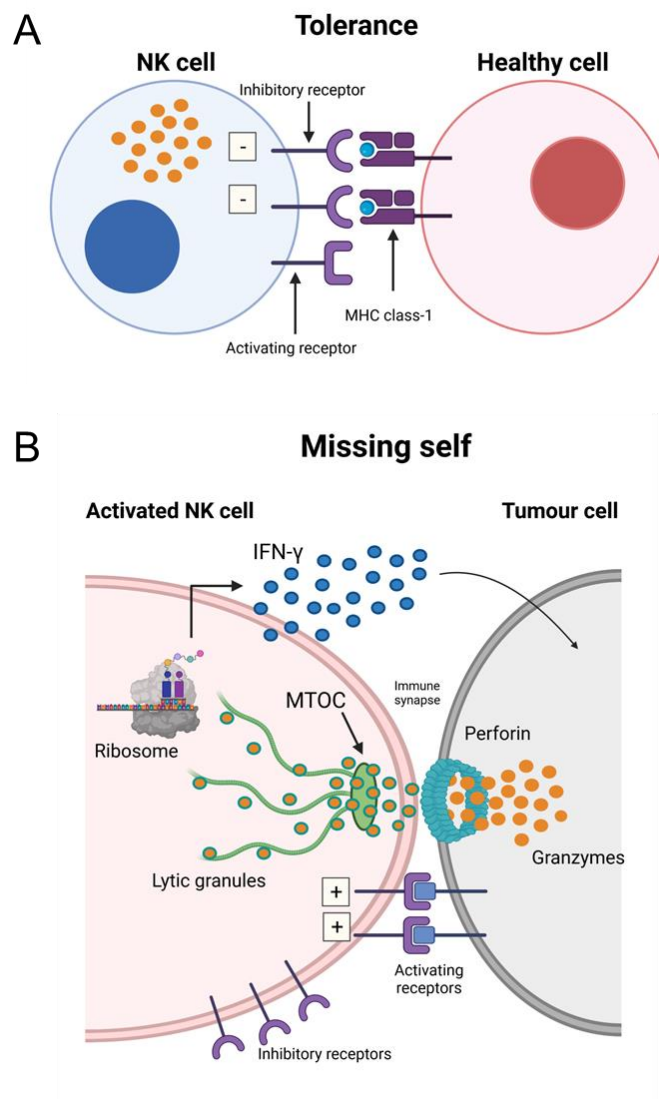


Figure 1.1 Activating and inhibitory receptors coordinate NK cell tolerance or activation

NK cells express activating and inhibitory receptors that tolerise NK cells towards healthy host cells but induce highly specific killing of virally infected or malignant cells. Inhibitory receptors recognise and bind MHC class 1 molecules expressed on the surface of healthy host cells, ensuring that NK cell cytotoxicity is not triggered (A). On the other hand, activating receptors recognise and bind stress inducible ligands that are upregulated on the surface of malignant or virally infected cells. This induces pro-inflammatory cytokine secretion and polarisation of the microtubule organising centre (MTOC) and lytic granules towards the NK cell membrane. Perforins and granzymes are released into the immune synapse which induce target cell death. Malignant cells often downregulate the expression of MHC class 1 molecules. Decreased ligation of inhibitory receptors also initiates lytic granule release to induce specific target cell death (B).

Activation of NK cells by cytokines

The magnitude of the NK cell response is heavily influenced by cytokines in the environment which act in isolation and synergistically²⁴. IL-15 controls NK cell priming to ensure complete effector capacity. IL-15 is cross-presented to NK cells by monocytes or dendritic cells (DCs) and initiates the transcription of cytotoxic perforins and granzymes for rapid secretion upon activation²⁵. Alongside IL-12, and IL-18, IL-15 also controls the transcription of IFN γ mRNA in NK cells, which can be rapidly translated upon activation. IL-15 interacts with a receptor complex containing the common gamma chain (γ_c), a component of the IL-2R. However, IL-2 is dispensable for NK cell homeostasis, possibly due to the lack of IL-2R α at steady-state. IL-12 induces the expression of IL-2R α , enabling NK cells to respond to low doses of IL-2, which is important for IFN γ production, but not NK cell cytotoxicity²⁴. Newly primed CD4 T cells secrete IL-2, which synchronises NK cell responses with adaptive T cells. Given these responses, IL-2, IL-12, and IL-15 are commonly used *in vitro* to induce NK cell proliferation and production of effector molecules including IFN γ and granzyme B.

NK cell effector functions

NK cells possess both cytotoxicity and cytokine producing effector functions, which enables highly specific killing of transformed and virally infected cells. NK cells are primed with IL-15 which “pre-arms” cells with perforin and granzymes, and IFN γ mRNA, allowing for an effective cytotoxic response within minutes of activation²⁵. Ligation of activating receptors triggers the rapid release of preformed lytic granules into the immune synapse. However, additional cytokine signals are required to induce protein translation for optimal IFN γ production²⁶.

NK cell cytotoxicity

To exert their cytotoxicity, NK cells must establish direct contact with their target cell and form an immunological synapse, into which the components of cytolytic granules are released. NK cell killing occurs in a controlled, stepwise fashion and results in highly specific killing of target cells, without affecting surrounding by-stander cells. Work from Jordan Orange’s lab identified the key steps involved in NK cell killing. Following docking of the NK cell to the target and formation of the immunological synapse, the NK cell actin cytoskeleton is rearranged. This allows the MTOC and secretory lysosomes to polarise towards the lytic synapse. Cytolytic granules fuse with the NK cell membrane and the

granule contents are released into the synapse, a process known as degranulation²⁷. During this process, lysosomal-associated membrane protein-1/2 (LAMP-1/2 a.k.a. CD107a/b) transiently appears on the NK cell surface, and are commonly used to measure NK cell cytotoxicity²⁸. Fusion of lytic granules with the NK cell membrane increases presynaptic membrane packing which creates local lipid shields to protect the NK cell from self-destruction following degranulation²⁹. The main components of NK cell cytolytic granules are perforins and granzymes which act in synergy to kill target cells. Perforin is a pore-forming protein that polymerises at the target cell membrane, forming a transmembrane channel. Originally, it was thought that perforin functions to enable granzyme entry into the target cell to enhance granzyme-mediated cell death. However, granzyme uptake into the target cell has been shown to efficiently occur by receptor-mediated endocytosis³⁰. Nevertheless, perforin is critical for NK cell cytotoxicity, as NK cells from perforin-deficient mice are defective at killing targets *in vitro*³¹. Evidence suggests that perforin induces the free efflux of ions and polypeptides from target cells, which disrupts osmotic balance and activates pro-apoptotic pathways³². Granzymes are serine proteases that initiate caspase-dependent and independent cell death pathways by cleaving proteins after aspartate residues³³. To protect the NK cell from death by its own cargo, cytolytic granules are maintained at a low pH (pH 5). Perforins are active at pH 7 and thus only activated upon release into the lytic synapse³⁴.

NK cell cytokine secretion

Activated NK cells also produce pro-inflammatory cytokines such as IFN γ which mediates tumour killing by directly activating pro-apoptotic pathways³⁵. Ligation of NKG2D or IL-12 signalling induces IFN γ production by NK cells. NK cell derived cytokines also regulate the development of an effective adaptive immune response. In response to various chemokines, NK cells are recruited to lymph nodes where they are proximal to the site of T cell priming. IFN γ and TNF released by NK cells can influence DC maturation and augment their antigen presenting functions and IL-12 production³⁶. This contributes to the polarisation and proliferation of Th1 CD4 T cells, which are crucial for an effective anti-tumour response. NK cell cytokine production thus links the innate and adaptive arms of the immune response and informs an effective anti-tumour strategy.

The Lynch lab has shown that lipids and HFDs inhibit NK cell effector functions *in vivo*, resulting in accelerated growth of B16 and MC38 tumours³⁷ (described in detail in Section 1.3.1). Work from this thesis shows that circulating NK cell functions are restored when mice lose weight and additionally, NK cell function and metabolism are not affected

when mice are fed a HFD from plant derived fats. These data suggest that NK cells are sensitive to dietary manipulation and that acquisition of effector functions are determined by the nutrients and metabolites in NK cell microenvironments (Section 1.2.6).

NK cell immunotherapeutic strategies

NK cells are an attractive target for cancer immunotherapies as they are rapidly activated and not restricted by antigen specificity. NK cells are currently being harnessed in the clinic to treat many cancers. For example, Fc receptors on the surface of NK cells recognise and engage monoclonal antibodies (mAbs) that bind the surface of cancer cells, activating antibody-dependent cellular cytotoxicity (ADCC) to induce target cell death. Currently, mAbs have been approved for the treatment of leukaemias and solid tumours. Examples include Trastuzumab which targets Her2, is approved for the treatment of Her2⁺ breast cancer and Her2⁺ metastatic gastric adenocarcinoma, and Cetuximab which targets EGFR, is approved for the treatment of metastatic colorectal cancers³⁸.

NK cell adoptive transfer is a promising cellular therapy that replenishes and improves NK cell functions in cancer patients. To ensure optimal cell numbers, NK cells are expanded *in vitro* with activating cytokines such IL-2 and IL-15 prior to transfer, which also acts to prime NK cells for robust activation *in vivo*³⁹. Adoptive transfer of autologous NK cells (derived from the patient) showed very little anti-tumour effects, possibly due to inhibitory receptors that matched self MHC class 1 molecules on tumour cells. Allogenic NK cells (derived from a third party or NK cell line) do not express HLA-specific inhibitory receptors, and have proven effective in the treatment of haematological malignancies without causing graft-versus-host disease⁴⁰. Indeed, adoptive transfer of allogenic NK cells, in combination with chemotherapy, has been shown to cause complete remission in some patients with poor prognosis acute myeloid leukaemia⁴¹. Lately, focus has been on the genetic manipulation of allogenic NK cells to express chimeric antigen receptors (CARs) which bind certain proteins on cancer cells. Currently, 17 CAR-NK cell trials are being conducted worldwide, which includes glioblastoma-specific CAR-NK-92 cells for the treatment of glioblastoma⁴².

1.1.2 CD8 T cells

CD8 T cells represent a large portion of the adaptive immune system, constituting ~30% of peripheral blood mononuclear cells (PBMCs) in humans. CD8 T cells are characterised by the cell surface expression of CD3, CD8, and an $\alpha\beta$ T cell receptor (TCR). Through their TCR, CD8 T cells recognise specific antigens presented by MHC class 1 molecules to initiate a robust effector response involving cytokine production and direct killing of targets. CD8 T cells arise from the CLP derived from haematopoietic stem cells in the BM. To become committed T cell precursors, lymphoid progenitors migrate through the bloodstream to the thymus, where they can give rise to either $\alpha\beta$ or $\gamma\delta$ TCR-expressing mature T cells following positive and negative selection. Mature CD8 T cells circulate in the blood and lymphatics where they survey MHC class 1 molecules for their TCR-specific antigens⁴³. CD8 T cells are cytotoxic T cells, capable of killing malignant or virally infected cells.

CD8 T cell activation

CD8 T cell activation is dependent on several simultaneous interactions between the T cell and antigen presenting cells (APC) (signal 1 and 2), as well as the surrounding cytokine milieu (signal 3) and these three signals are required for full T cell activation (Figure 1.2). Through their TCR, CD8 T cells recognise specific peptide fragments presented by MHC class 1 molecules on the APC surface (signal 1). CD8 co-receptors stabilise this interaction by binding MHC class 1, activating the tyrosine kinase Lck⁴⁴ which propagates downstream activation pathways. CD28 on the CD8 T cell surface binds to CD80 and CD86 proteins on the APC which provides co-stimulation and licenses the T cell to respond to antigen (signal 2). Without CD28-CD80/CD86 interactions, the T cell is unable to mount a proper immune response and becomes anergic. Inflammatory cytokines such as IL-12 enhance T cell survival, promote clonal expansion, and induce the expression of effector molecules such as IFN γ , granzymes, and perforins (signal 3). Naïve T cells require these three signals to avoid tolerance and promote their clonal expansion, survival, and production of effector molecules following antigen recognition (Figure 1.2). Following activation, effector CD8 T cells can be identified by the expression of cell surface markers CD25, CD69, and KLRG1.

IL-2 in CD8 T cell activation

IL-2 is a member of the $\gamma\epsilon$ receptor family of cytokines and plays an important role in the proliferation and differentiation of cytotoxic CD8 T cells, at the expense of memory cell

formation⁴⁵. IL-2 is produced by CD8 T cells following activation through the TCR and is essential for the survival of surrounding effector CD8 T cells through the induction of anti-apoptotic genes *bcl-2* and *bcl-xl*⁴⁶. Once targets cells are eliminated, reduced TCR signalling decreases the levels of IL-2, resulting in CD8 T cell apoptosis and resolution of an effective inflammatory response. In addition, IL-2 directs the transcriptional program of effector CD8 T cells and controls the expression of key cytolytic effector and adhesion molecules including IFN γ , perforins, granzymes, CD62L and CCR7^{45,47}. IL-2 signalling also sustains nutrient transport and energy production to support the biosynthetic demands of activated cytotoxic lymphocytes⁴⁸ (discussed in section 1.2.7). In the context of CD8 T cells, IL-2 signalling leads to the accumulation of phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P₃) which activates a series of serine/threonine protein kinases including Akt. In response to IL-2, Akt signalling controls the expression of IFN γ , perforin, and key cytokine receptors, establishing a transcriptional profile typical of activated CD8 T cells⁴⁷. Akt also controls CD8 T cell trafficking in response to IL-2 by downregulating the expression of CCR7 and CD62L adhesion molecules. CCR7 directs T cell migration into lymphoid organs and CD62L binds GlyCAM-1 on high endothelial venules, enabling T cells to adhere to lymph nodes (LNs). IL-2 signalling therefore decreases T cell homing to lymphoid tissues upon activation, promotes CD8 T cell survival, and drives the expression of key cytolytic effector molecules⁴⁷.

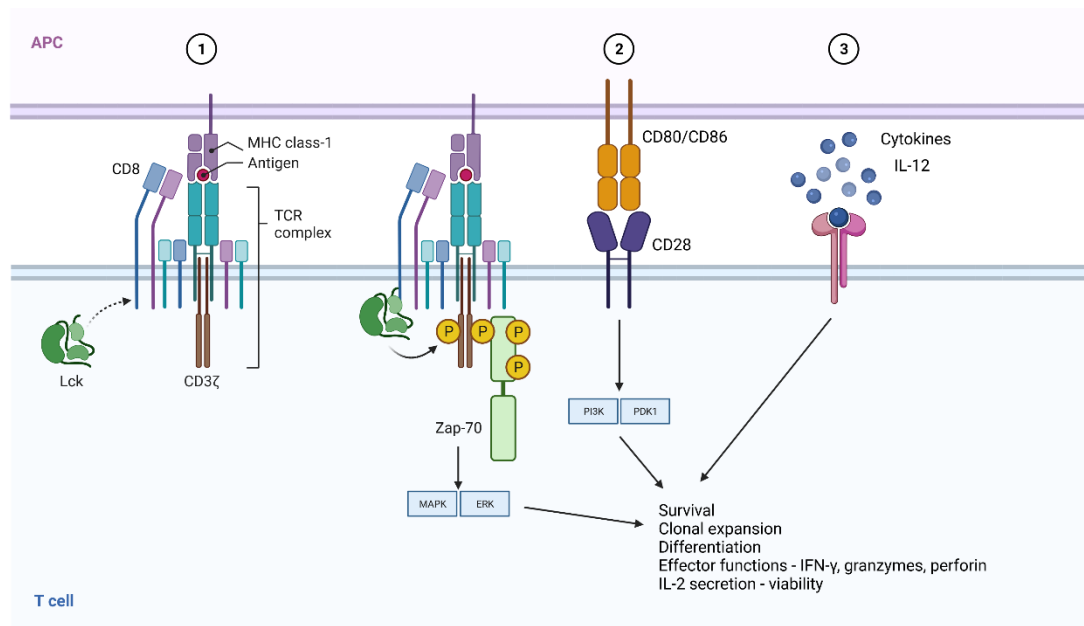


Figure 1.2 T cell receptor (TCR) signalling

Mature CD8 T cells are activated by several interactions between the T cell and antigen presenting cell (APC). Specific peptide fragments are loaded onto an MHC class 1 molecule on APCs and recognised by the T cell receptor (TCR) on CD8 T cells (Signal 1). This interaction is stabilised by CD8 which activates the tyrosine kinase Lck. Lck phosphorylates and activates the intracellular immunoreceptor tyrosine-based activation motifs (ITAM) of CD3 ζ which recruits ZAP70 leading to the activation of downstream pathways. Simultaneously, CD28 on the T cell surface interacts with CD80 and CD86 on the APC cell surface which provides co-stimulation and prevents T cell anergy (Signal 2). In addition, cytokine signalling enhances CD8 T cell survival, promotes clonal expansion and differentiation, and induces the expression of effector molecules (signal 3). In particular, IL-2 signalling activates Akt signalling in CD8 T cells that promotes trafficking from secondary lymphoid organs and the transcription of effector molecules.

CD8 T cell anti-tumour effector functions

Like NK cells, CD8 T cells exert their cytotoxicity against malignant and virally infected cells through inflammatory cytokine production and direct killing of target cells. CD8 T cells are primed in the LNs where they recognise tumour-specific neo-antigens and self-antigens. In response to CXCL9 and CXCL10 produced by tumoral dendritic cells, effector T cells invade the primary tumour bed and rapidly produce IFN γ and TNF. IFN γ induces the expression of TNF receptors on target cells which mediate target cell death upon TNF ligation⁴⁹. IFN γ also coordinates an effective adaptive immune response by increasing the expression of MHC class 1 on tumour cells, making them better targets and also enhancing the anti-tumour abilities of other surrounding cells⁵⁰. Upon activation, CD8 T cells increase the expression of Fas ligand (FasL) which cross-links with the cell surface death receptor Fas expressed on target cells. Fas-FasL initiates the formation of an intracellular death-inducing signalling complex (DISC), inducing caspase-dependent apoptosis of target cells⁵¹. Like NK cells, CD8 T cells can also directly kill tumour cells through the release of cytotoxic perforins and granzymes. This mechanism requires the formation of a tumour-immune synapse into which granzymes, and perforins are released, ensuring highly specific death of tumour cells. Effector CD8 T cells can form numerous immunological synapses at once to allow for the simultaneous killing of multiple cells. These distinct contacts are independent events, occur at different time points, and in different areas of the cell, ensuring the most efficient killing of targets⁵².

CD8 T cell exhaustion and dysfunction in cancer

In 1968, it was first suggested by Erik Hellstrom that tumour specific CD8 T cells become dysfunctional over the course of tumorigenesis (The Hellstrom paradox)⁵³. Indeed, it is now accepted that persistent exposure to neoantigens and inflammatory stimuli can induce a dysfunctional state resulting in T cell exhaustion. Although T cell exhaustion occurs to limit damage caused by the immune response, exhausted CD8 T cells function at a suboptimal level and fail to limit tumour progression. T cell exhaustion is characterised by a progressive loss of proliferative capacity and effector functions, altered cellular metabolism, increased expression of multiple inhibitory checkpoint receptors (e.g. PD-1) and exhaustion-associated transcription factors (such as TOX)⁵⁴. T cell exhaustion is a major barrier in the anti-tumour immune response. Cancer immunotherapies such as immune checkpoint blockade using anti-PD-1 antibodies re-invigorate adaptive immune responses and have revolutionised the treatment of certain cancers such as melanoma and non-small cell lung cancer^{55,56}. Indeed, James Allison and Tasuku Honjo received the Nobel Prize in Physiology or Medicine in 2018 “for their discovery of cancer therapy by inhibition of negative immune regulation”. Understanding the molecular mechanisms that promote exhaustion may help identify strategies to restore self-renewal and maintain effector functions in exhausted T cells.

Inhibitory receptors are negative regulators of adaptive immunity and are essential to maintain self-tolerance and prevent autoimmunity. Although effector CD8 T cells transiently express inhibitory receptors following activation, it is the sustained, high expression of inhibitory receptors, such as PD-1 that is a hallmark of exhausted T cells⁵⁷. Engagement of PD-1 initiates a signalling cascade involving intracellular ITIM and immunoreceptor tyrosine-based switch motifs (ITSM) which recruit and activate phosphatases. This attenuates phosphorylation-dependent signalling pathways initiated by TCR and co-stimulatory signalling which suppresses CD8 T cell activation, proliferation, cytotoxicity, metabolic reprogramming, and cytokine production^{58,59}. The severity of cellular dysfunction is controlled by the co-expression patterns of multiple inhibitory receptors, which include CTLA-4, TIM-3, LAG-3 and 2B4⁶⁰, commonly upregulated on exhausted tumoral CD8 T cells.

Activated CD8 T cells require high levels of exogenous glucose to support cell proliferation and the transcription of effector molecules^{61,62} (described in section 1.2.7). Cancer cells outcompete CD8 T cells for glucose, which results in CD8 T cell metabolic and functional exhaustion. Decreased glycolysis limits phosphoenolpyruvate (PEP) which is necessary to sustain NFAT signalling and tumoricidal activities of CD8 T cells,

by blocking Ca^{2+} reuptake by SERCA⁶³. Reduced glucose uptake also impedes mTORC1 activation and IFN γ production by CD8 T cells which contributes to accelerated tumour growth *in vivo*⁶⁴. During times of nutrient deprivation, cells often increase mitochondrial biogenesis to support the metabolic demands of proliferation and effector cytokine production. However, reduced mitochondrial fitness and mitochondrial depolarisation are also features of exhausted intra-tumoral CD8 T cells⁶⁵. Indeed, chronic antigen stimulation impairs mitochondrial ATP production in tumoral-CD8 T cells, which promotes terminal differentiation and limits T cell self-renewal. This metabolic defect is associated with expression of PD-1, the exhaustion-associated transcription factor TOX, and reduced production of TNF and IFN γ ⁶⁶. To preserve energy homeostasis and ATP production, limited access to glucose may force a metabolic switch towards fatty acid (FA) uptake and metabolism in CD8 T cells. However, this metabolic adaptation is also linked to CD8 T cell dysfunction across many human cancer types and mouse models (described in section 1.4.3). In this thesis, we demonstrate that tumoral CD8 T cells are impaired when mice are fed HFDs from animal but not plant sources, suggesting that CD8 T cells are sensitive to dietary manipulation and that nutrients and metabolites in the cell microenvironments control CD8 T cell effector functions.

CD8 T cell immunotherapeutic strategies

Immunotherapy is clinically validated for the treatment of many cancers. Immune checkpoint blockade aims to harness anti-tumour CD8 T cells, either by stimulating effector functions or opposing inhibitory mechanisms induced by the tumour microenvironment (TME). Cytotoxic CD8 T cells are the most prominent and powerful effector cell type, and their degree of activation determines the success of these therapies.

Tumour cells express ligands that can bind inhibitory receptors on tumour specific CD8 T cells to induce immune tolerance and dampen effector functions in the TME⁶⁷. Immune checkpoint therapy refers to the use of mABs that target specific inhibitory receptors on the CD8 T cell surface e.g., PD-1. Indeed, targeting the PD-1 – PD-L1 inhibitory axis greatly improves clinical outcomes in several cancers by enhancing CD8 T cell function and revitalising exhausted T cells. In particular, the treatment of metastatic melanoma has been revolutionised by anti-PD-1 therapies which result in long-lasting responses in over 50% of patients. Increased PD-L1 expression and the presence of tumour-

infiltrating CD8 T cells is positively associated with good clinical outcomes and therapeutic efficacy of PD-1 blockade. The combination of checkpoint blockade and traditional cancer therapies such as chemotherapy have been shown to improve response rates for many cancers. Currently, the anti-PD-1 agent pembrolizumab alongside cytotoxic chemotherapies is approved for first-line treatment of metastatic non-small-cell lung cancer (NSCLC)⁶⁸.

1.2 Immunometabolism

Immunometabolism describes the interactions between metabolism and the immune system. At the cellular level, immune effector functions are regulated by specific metabolic configurations that adapt to nutrients and metabolites in the cell's microenvironments. Additionally, tissue resident immune populations can influence whole-body metabolism by regulating tissue-specific functions. For example, IL-17 producing $\gamma\delta$ T cells in adipose tissues regulate body temperature and promote thermogenesis in response to cold⁶⁹. Our lab is particularly interested in the interdependence of cellular and systemic immunometabolism and how systemic metabolic perturbations such as obesity can affect immune cell metabolism and the effectiveness of the anti-tumour immune response.

Basic metabolic configurations

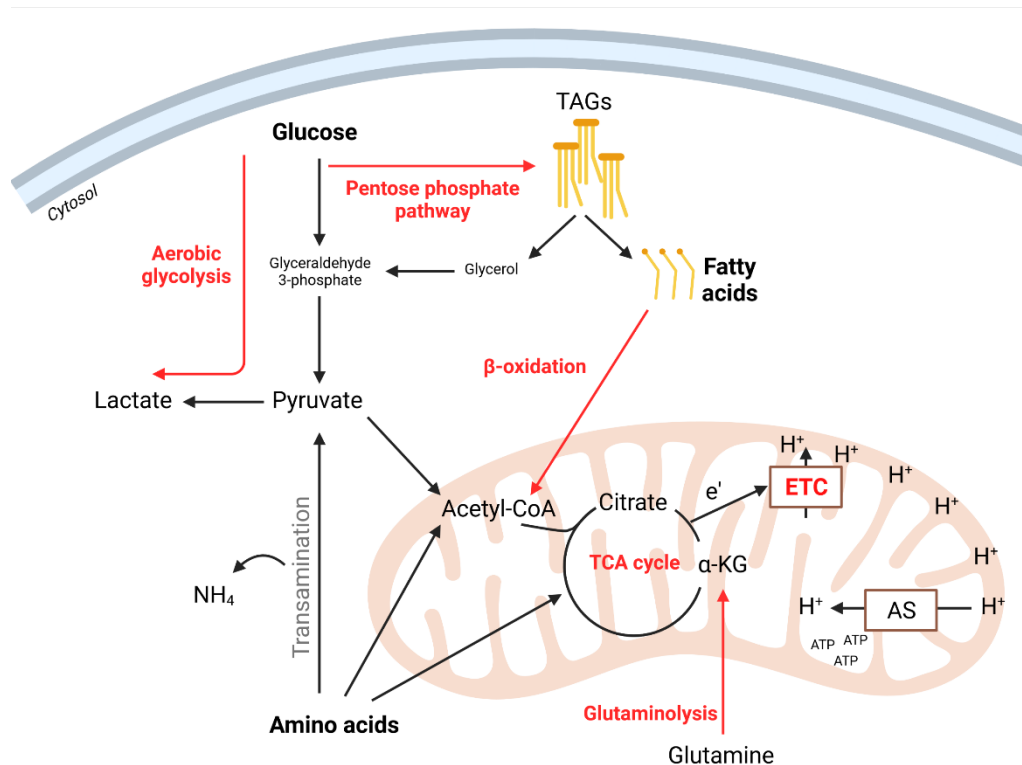
In response to infection, inflammation, or other insults, quiescent immune cells become activated and initiate a developmental program characterised by blastogenesis, proliferation, migration, and synthesis of effector molecules. To support these energy demanding processes, activated immune cells change from catabolic to anabolic metabolism whereby nutrients are incorporated into biomass to support macromolecular synthesis⁷⁰. Different immune populations rely on distinct metabolic configurations to regulate specific effector functions including cytokine production and proliferation⁷¹. Additionally, some metabolites have specific immunomodulatory properties and can regulate the activity of transcription factors and the production of effector molecules⁷². Activity between different metabolic pathways is heavily influenced by nutrient and O₂ availability⁷⁰. Metabolic flexibility dictates the success of an immune response in the context of the TME for example, where nutrients and O₂ are limiting (Figure 1.3).

1.2.1 The glycolytic metabolic pathway (Glycolysis)

Glycolysis is a cytosolic catabolic pathway that enzymatically metabolises glucose into pyruvate. In the presence of O_2 , pyruvate dehydrogenase converts pyruvate into acetyl-CoA which is further metabolised in the tricarboxylic acid cycle (TCA cycle)⁷³. When O_2 is limiting/not present, pyruvate is converted into lactate by lactate dehydrogenase and exported. Even when O_2 is present, some cells such as activated immune cells (CD8 T cells⁷⁴, NK cells⁸) and cancer cells⁷⁵ preferentially use glycolysis – a process known as aerobic glycolysis or Warburg metabolism. In this context, heightened rates of glycolysis are maintained by reducing glucose-derived pyruvate into lactate which replenishes NAD^+ levels - a crucial co-factor for the upstream glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Although glycolysis is energetically inefficient, there are many benefits to glycolysis other than energy generation. Glycolytic intermediates can be used as substrates for the synthesis of lipids, nucleotides, and amino acids to support anabolic growth. For example, glucose-6-phosphate can be siphoned into the pentose phosphate pathway (PPP) where its metabolism generates ribose 5-phosphate, a precursor for nucleotide synthesis, and NADPH, a reducing agent required for lipid synthesis. Given these pro-anabolic features of glycolysis, it is unsurprising that many pro-growth signalling pathways such as PI3K and mitogen-activated protein kinase (MAPK) pathways activate glycolytic metabolism in cells. Therefore, glycolysis is essential to support the rapid proliferation of activated immune cells and cancer cells⁷⁶.

1.2.2 The tricarboxylic acid cycle (TCA cycle)

The TCA cycle is a network of enzymes in the mitochondrial matrix that generates NADH and $FADH_2$ from multiple nutrient inputs. Acetyl-CoA, primarily from glucose-derived pyruvate and FAs, enters the TCA cycle and condenses with oxaloacetate (OAA) to form citrate. Glutamine is an anaplerotic substrate that is directly converted into the TCA cycle intermediate α -ketoglutarate by glutamate dehydrogenase. As these intermediates are further metabolised, high energy reducing equivalents, NADH and $FADH_2$ are generated, which donate electrons to the electron transport chain (ETC) to fuel oxphos and generate ATP. The TCA cycle, coupled with oxphos, is a highly efficient method for ATP generation and is the major metabolic pathway used by quiescent and non-proliferating cells such as memory T cells, whose chief needs are to support longevity and survival. TCA cycle intermediates can also be diverted into biosynthetic reactions



for biomass production (cataplerosis)⁷⁶. For example, mitochondrial citrate can be exported into the cytosol where it is cleaved by ATP citrate lyase (ACLY) yielding OAA and acetyl-CoA. Cytosolic acetyl-CoA is the major substrate for FA synthase (FASN) for the synthesis of FAs.

Figure 1.3 Basic metabolic configurations for energy homeostasis and biosynthesis

Cells can configure their metabolic pathways to generate energy in the form of ATP from a variety of nutrient inputs including glucose, fatty acids, and amino acids. Glucose can be metabolised to pyruvate through glycolysis. Glycolytic intermediates are siphoned into biosynthetic pathways such as the pentose phosphate pathway to generate biomass in the form of lipids. When O_2 is limiting, or in activated immune cells/cancer, pyruvate is converted into lactate which is exported as metabolic waste. Alternatively, when O_2 is available, pyruvate is converted into acetyl-CoA which enters the tricarboxylic acid (TCA) cycle, generating NADH and $FADH_2$ reducing equivalents. NADH and $FADH_2$ donate electrons to the electron transport chain (ETC), which are transferred through ETC protein complexes. Protons are pumped from the matrix into the mitochondrial intermembrane space generating an electrochemical gradient. The flow of protons down this concentration gradient into the matrix through ATP synthase (AS) is coupled to the synthesis of ATP. Cytosolic triacylglycerols (TAG) can be hydrolysed by lipases to yield glycerol and free fatty acids. Glycerol can feed into glycolysis and fatty acids can be oxidised to generate

acetyl-CoA which enters the TCA cycle. Additionally, amino acids can be transaminated to generate pyruvate, acetyl-CoA, or different TCA cycle intermediates which are further metabolised in the mitochondria.

1.2.3 The citrate malate shuttle (CMS)

Glucose-derived carbons can also enter the CMS, another important cycle for generating NADH. Mitochondrial citrate can be transported into the cytosol through Slc25a1, an obligate citrate/malate transporter. Cytosolic citrate is converted into OAA and acetyl-CoA by ACLY. OAA can be converted to malate, a reaction that also generates NAD⁺. Malate can enter the mitochondria through Slc25a1 where it is re-converted into OAA, yielding an NADH molecule. OAA can react with another glucose-derived acetyl-CoA to form citrate which continues the cycle. Alongside NADH which fuels oxphos, NAD⁺ generated through the CMS supports high glycolytic flux through GAPDH. Indeed, activated NK cells use this metabolic configuration to support both glycolysis and oxphos⁷⁷.

1.2.4 Fatty acid oxidation (FAO, β -oxidation)

In the 1950s, Eugene Kennedy and Albert Lehninger demonstrated that FAs are oxidised in the mitochondria⁷⁸. However, it is now understood that peroxisomes initiate the metabolism of very long chain FAs (≥ 22 carbons) which are then transported to the mitochondria as short (≤ 6 carbons), medium (6-12 carbons), or long chain (14-20 carbons) FAs to complete their oxidation⁷⁹. In the cytosol, long chain fatty acids (LCFAs) are first activated by acyl-CoA synthases, yielding a fatty acyl-CoA molecule. To enter the mitochondrial matrix where FAO occurs, long chain fatty acyl-CoAs require the carnitine shuttle, consisting of the enzymes carnitine palmitoyltransferase (CPT) 1 and 2 and the transporter carnitine acyltransferase (CACT). CPT1 conjugates the fatty acyl to carnitine, forming acylcarnitine which is transported into the mitochondrial matrix through CACT. Here, acylcarnitine is converted back to fatty acyl-CoA by CPT2 and β -oxidation of the fatty acyl chain can commence. In contrast to their LCFA counterparts, short and medium chain FAs (S/MCFAs) diffuse across the mitochondrial membranes in their non-esterified forms and are activated by acyl-CoA synthases in the mitochondrial matrix⁸⁰.

β -oxidation is a recurring sequence of oxidation, hydration, oxidation, and thiolysis reactions that generates a FA chain shortened by two carbons, acetyl-CoA, FADH_2 , and NADH with each round (Figure 1.4). Acetyl-CoA is further metabolised in the TCA cycle and FADH_2 and NADH donate electrons to the ETC for ATP production⁸¹. Overall, FAO is extremely energy efficient, with one 16 carbon fatty acid (palmitic acid) yielding over 100 molecules of ATP⁷⁶. Consequently, energy demanding tissues such as the heart and skeletal muscle primarily rely on β -oxidation.

In obesity, mitochondrial lipid overload can result in the development of skeletal muscle insulin resistance, due to excessive FAO. Flux through the TCA cycle is essential to maintain optimal rates of FAO. However, depletion of several TCA cycle intermediates and high rates of FA catabolism in obesity results in incomplete β -oxidation where FAs entering the mitochondria are only partially degraded. This leads to the accumulation of incompletely oxidised acylcarnitines which interfere with insulin signalling and promote inflammation associated with T2D^{82,83}.

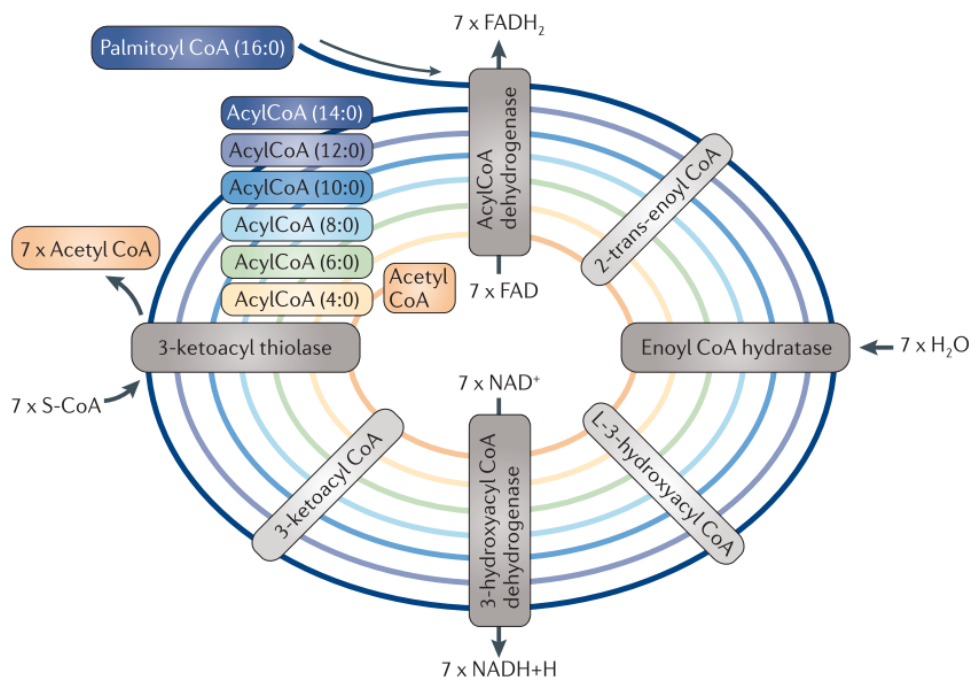


Figure 1.4 β -oxidation of palmitic acid in the mitochondria

Mitochondrial fatty acyl-CoAs undergo a cyclic series of dehydration, hydration, and thiolysis reactions that results in the progressive shortening of the FA chain. Each round of β -oxidation generates NADH and FADH_2 reducing equivalents, acetyl-CoA, and a fatty acyl-CoA shortened by two carbons.

1.2.4.1 Acylcarnitines

Acylcarnitines are intermediate lipid metabolites comprising of a fatty acyl chain esterified to a carnitine molecule. Most acylcarnitines arise from the action of CPT1 to enable the transport of LCFAs into the mitochondria for oxidation (Figure 1.5). However, acylcarnitines can also be formed from the degradation of amino acids including lysine, leucine, valine, isoleucine, and tryptophan. Two transporters have been implicated in the transport of acylcarnitines across cellular membranes – OCTN2, and the monocarboxylate transporter (MCT) Slc16a9. Efflux of acylcarnitines seems largely dependent on their intracellular concentration, however the exact nature of this transport is unknown. Plasma acylcarnitines are widely used as clinical biomarkers to diagnose in-born errors of FAO that affect the metabolism of LCFAs⁸⁴. In addition, acylcarnitines are implicated in obesity associated insulin resistance by disrupting components of the insulin signalling pathways and increasing inflammation^{82,83}. It was recently reported that murine tumour interstitial fluids (TIFs) are rich in a diverse array of acylcarnitines⁸⁵. In this thesis, we tested the hypothesis that acylcarnitines alter NK and CD8 T cell functions, which may contribute to increased tumour growth in obesity.

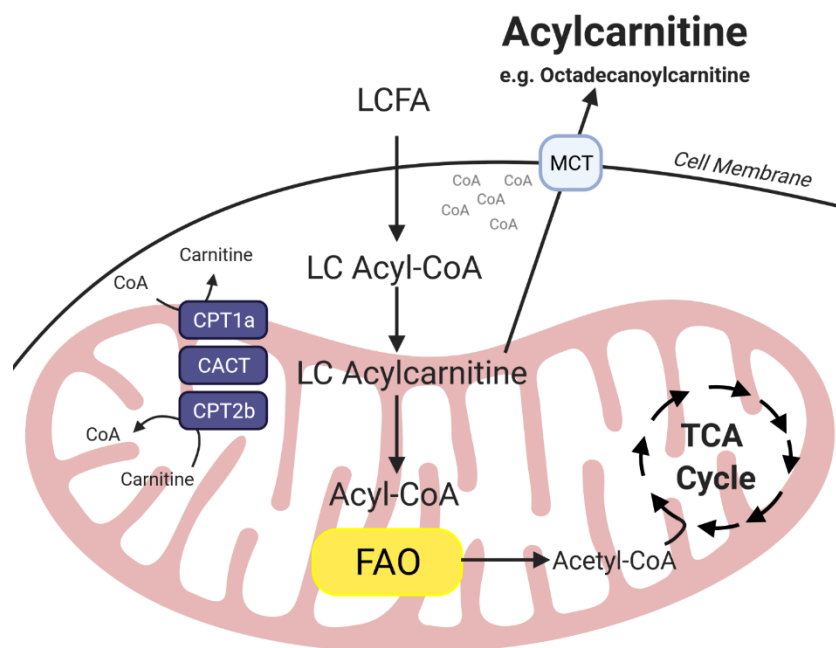


Figure 1.5 Long chain fatty acids require the carnitine shuttle to enter the mitochondria

Long chain fatty acids (LCFAs) react with free CoA in the cytosol, yielding fatty acyl-CoA. Carnitine palmitoyl-transferase 1 (Cpt1), located on the outer mitochondrial membrane, exchanges the CoA adduct for a carnitine molecule, forming acylcarnitine. Acylcarnitines are transported into the mitochondrial matrix through the carnitine acyl-carnitine translocase (CACT), where they are re-converted back to fatty acyl-CoA by the action of Cpt2. Once inside the matrix, β -oxidation of the fatty acyl-CoA is initiated, which generates molecules of acetyl-CoA which enter the tricarboxylic acid (TCA) cycle. Alternatively, in times of obesity when mitochondrial lipids are increased relative to flux through the TCA cycle, or when the Cpt1a:CACT ratio is increased, acylcarnitines can be exported from the cell through monocarboxylate transporters (MCT) where they are detected in the plasma.

1.2.5 Oxidative phosphorylation

Oxphos is a process in which electrons are passed through a sophisticated series of mitochondrial enzymes and coenzyme complexes that work in tandem to synthesise ATP. Electrons are transferred from NADH and FADH₂ through ETC complexes until O₂, the terminal electron acceptor, is reduced to H₂O. Two specialised electron carriers, CoQ (ubiquinone) and cytochrome c, are involved in ferrying electrons through the complexes of the ETC. The transfer of electrons leads to the pumping of protons (H⁺) from the mitochondrial matrix into the intermembrane space through three electron-driven proton pumps – complex 1, 3, and 4. This generates an electrochemical gradient across the inner mitochondrial membrane which creates a proton-motive force. Protons flow down the potential energy gradient, into the mitochondrial matrix through AS, a process called chemiosmosis. Proton flow forces the rotation of AS which synthesises ATP from ADP and Pi⁸⁶. Oxphos is a highly efficient method for ATP generation. Each molecule of glucose metabolised through the TCA cycle and oxphos generates a net of 34 ATP, compared to 2 ATP generated through glycolysis.

Immune cell metabolism

Immune cells rewire their metabolism when transitioning between quiescent and activated states. Appropriate activation requires sufficient uptake and precise distribution of nutrients into specific metabolic pathways, which directly impact immune effector functions. NK and CD8 T cells in particular rely on glucose and amino acids to control the production of inflammatory cytokines and cytolytic effector molecules.

1.2.6 NK cell metabolism

Resting NK cells maintain low levels of glycolysis and oxphos which is sufficient to support acute NK cell responses⁸⁷. However, overnight stimulation of NK cells with IL-2 and IL-12 induces robust metabolic changes characterised by heightened rates of glycolysis and oxphos, which are supported by increased expression of glucose transporters and glycolytic enzymes and increased mitochondrial mass^{8,77}. Limiting the rates of glycolysis or oxphos with the metabolic inhibitors 2DG or oligomycin reduces the production of IFN γ and GzB⁸, and impairs the ability of NK cells to kill target cells *in vitro*⁸⁸. Glucose-derived pyruvate is primarily exported as lactate; however, some pyruvate enters the mitochondria where it is converted into citrate. NK cells export citrate into the cytosol through Slc25a1, where it enters the CMS, an alternative cycle which generates NADH to fuel oxphos, and NAD⁺ to facilitate heightened glycolytic flux⁷⁷. Unlike the TCA cycle, the CMS is exclusively fuelled by glucose. Indeed, NK cells do not use anaplerotic substrates like glutamine to fuel oxphos, and inhibition of glutaminase, the first enzyme in glutaminolysis, does not affect the rates of NK cell oxphos or effector functions⁸⁹.

NK cell metabolic reprogramming is controlled by a number of key metabolic regulators and transcription factors (Figure 1.6). mTORC1 is a serine/threonine protein kinase that is robustly activated in cytokine-stimulated NK cells and controls the expression of nutrient transporters, glycolytic enzymes, and effector molecules. Inhibiting mTORC1 activation with rapamycin decreases the rates of NK cell glycolysis and limits the production of IFN γ and GzB⁸. mTORC1 induces the expression of SREBP and c-Myc transcription factors which control NK cell metabolic machinery. Conventionally, SREBPs are master regulators of *de novo* lipid synthesis⁹⁰. However, SREBP maintains glucose metabolism in NK cells by controlling the transcription of Slc25a1 and ACLY – two key factors required for CMS activity. Inhibiting SREBP reduces the rates of NK cell glycolysis and oxphos and impairs IFN γ and GzB production⁷⁷. Additionally, c-Myc controls the expression of glucose transporters and glycolytic machinery and is required for mitogenesis to ensure optimal mitochondrial mass to support oxphos. c-Myc is rapidly induced in NK cells following cytokine stimulation. Initially, c-Myc expression is dependent on mTORC1 activity, however, sustained expression of c-Myc relies on the uptake of amino acids through the amino acid transporter Slc7a5 to cancel the continuous degradation of c-Myc protein. Although glutamine is not an important fuel for NK cells, glutamine regulates amino acid uptake through Slc7a5 and thus is essential to maintain optimal levels of c-Myc. Indeed, glutamine withdrawal results in rapid loss of c-

Myc protein, reduces rates of NK cell glycolysis, oxphos, and production of effector molecules⁸⁹.

Although NK cell function is dependent on glucose metabolism, metabolic flexibility of NK cells determines their anti-tumour responses in the TME. Oxidative stress causes NK cell metabolic impairment in human ovarian cancer, which results in loss of function. However, NK cells can be rescued by the anti-oxidant Nrf2 which induces metabolic flexibility in these nutrient-deprived conditions⁹¹. Therefore, targeting antioxidant pathways to infer metabolic flexibility may be used to harness NK cell responses in times of metabolic jeopardy.

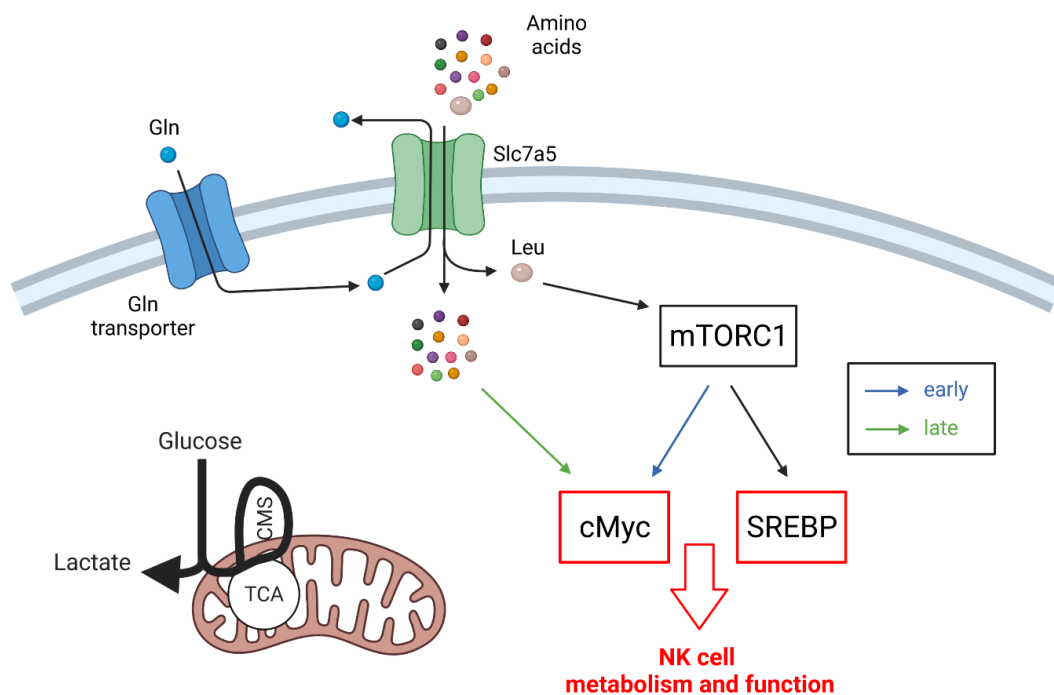


Figure 1.6 Amino acid regulation of NK cell function and metabolism

cMyc and SREBP transcription factors regulate NK cell metabolism and functions. SREBP activation requires mTORC1 signalling whereas cMyc is controlled by mTORC1 and amino acid uptake through Slc7a5. Leucine (Leu) uptake through Slc7a5 activates mTORC1 signalling which promotes the synthesis of cMyc protein minutes after NK cell activation. However, at later time points, amino acid uptake through Slc7a5 is essential to sustain cMyc protein expression and mTORC1 activity is not required. Amino acid uptake is regulated by glutamine export through Slc7a5; therefore, NK cells increase the expression of the glutamine transporter (Gln transporter) to maintain an intracellular pool of glutamine. SREBP controls the expression of Slc25a1 and ATP citrate lyase which are crucial components of the citrate malate shuttle (CMS). cMyc

increases the expression of glucose transporters and glycolytic enzymes that support high flux through glycolysis. SREBP and cMyc also control the expression of proinflammatory cytokines and NK cell cytotoxic machinery.

1.2.7 CD8 T cell metabolism

Activated CD8 T cells significantly increase glucose uptake and rewire their metabolism toward aerobic glycolysis, which provides energy for cell proliferation and synthesis of effector molecules⁹². This initial glycolytic switch is controlled by the transcription factor c-Myc in response to antigen receptor stimulation⁹³. High levels of extracellular glucose and IL-2 signalling sustain this glycolytic phenotype by activating phosphoinositide-dependent kinase 1 (PDK1), mTORC1 and HIF transcription factors that control the expression of glucose transporters and glycolytic enzymes in antigen activated CD8 T cells⁹⁴. Many non-metabolic “moonlighting” functions of glycolytic enzymes have been described, which may further explain why activated CD8 T cells heavily rely on glycolysis. For example, GAPDH controls the translation of IFN γ mRNA by binding to 3' UTR regions, resulting in IFN γ mRNA degradation. Upon activation, GAPDH engages in glycolysis, enabling unopposed IFN γ translation and effector cytokine production⁹⁵.

Although activated CD8 T cells primarily metabolise glucose through glycolysis, mitochondrial mass increases upon TCR stimulation and correlates with anti-tumour activity. Interestingly, it was recently demonstrated by the Griffiths lab that mitochondrial translation controls the de novo synthesis of cytosolic cytotoxic proteins including granzyme B, perforin, and IFN γ which are essential for CD8 T cell cytotoxicity. Indeed, loss of the mitochondrial protein Usp30 or culturing CD8 T cells with doxycycline to selectively inhibit mitochondrial translation attenuated the translation of cytolytic proteins and impaired target cell killing. It's believed that Usp30 controls the expression of metabolic enzymes with moonlighting properties which fine-tune the translation of cytosolic effector proteins, allowing for an effective anti-tumour response⁹⁶.

Alongside heightened glucose metabolism, activated CD8 T cells increase the expression of system L amino acid and glutamine transporters⁹⁷ to support rapid proliferation and production of effector molecules by providing amino acids for protein synthesis. Amino acid transport is dependent on sustained exposure to antigen and IL-2 signalling and is essential to maintain expression levels of the metabolic regulator c-Myc. Indeed, T cells from Slc7a5 knock out mice cannot upregulate c-Myc-controlled glycolytic machinery that is required for CD8 T cell differentiation⁹⁸. In addition, an

optimal supply of amino acids is essential to maintain mTORC1 activity. Leucine and glutamine activate RagA GTPases at the lysosome which recruit mTORC1 where it interacts with Rheb to stimulate its kinase activity⁹⁹. This leads to downstream activation of HIF and transcription of glucose transporters and glycolytic enzymes⁹⁴. Therefore, CD8 T cells must increase the uptake and metabolism of glucose and amino acids to support their proliferation and acquisition of effector functions.

1.3 Obesity

Obesity is defined by the World Health Organisation (WHO) as a body mass index (BMI) ≥ 30 and affects ~13% of the adult population worldwide. BMI is a simple classification used for both sexes and all adult ages and is calculated by a person's weight in kg divided by the square of their height in m (kg/m^2). BMI does not account for fat vs lean mass and therefore is sometimes not useful for predicting disease risk. Increased intake of foods that are high in fats and sugars, coupled with physical inactivity due to the sedentary nature of many forms of work and modes of transport contribute to the increased rates of obesity¹⁰⁰. However, current approaches targeting lifestyle and behaviour to reduce calorie intake and increase energy expenditure have shown limited effectiveness in inducing and/or maintaining weight loss, possibly due to persistent hormonal and metabolic abnormalities associated with obesity as well as difficulties in maintaining lifestyle changes long term¹⁰¹.

Obesity increases the risk of many metabolic diseases such as T2D, fatty liver, and cardiovascular diseases (CVDs)¹⁰¹. Excessive accumulation of fat in adipose tissues, liver, and particularly in and around the abdomen, leads to changes in systemic metabolism that accompany obesity, including hypertension, impaired glucose tolerance, and dyslipidaemia¹⁰². These metabolic abnormalities contribute to the development of the metabolic syndrome, which significantly increases the risk of developing T2D and CVDs. It was recently reported that an individual's plasma metabolome is a better indicator of metabolic health than adiposity and can predict metabolic disease risk. Indeed, metabolically healthy individuals with obesity have a reduced risk of cardiac events compared to lean individuals with "unhealthy" plasma metabolomes¹⁰³. The phenomenon of metabolically healthy obesity is relatively new and not well understood and may be influenced by many factors, including diet, physical activity, and/or genetics.

Obesity is a risk factor for at least 14 types of cancers including endometrial, colorectal, liver, and breast cancers^{104,105}. The cause for increased risk of cancer in obesity is multifactorial and includes the overproduction of growth hormones and adipokines which can directly promote tumour cell proliferation^{106,107}. Indeed, women with obesity have an increased risk of developing postmenopausal breast cancer, due in part to increased bioavailability of oestradiol, testosterone¹⁰⁸, and leptin, which activate pro-proliferative PI3K and MAPK signalling pathways to enhance tumour growth and survival¹⁰⁹. Furthermore, obesity is associated with increased plasma non-esterified fatty acids (NEFAs), which strongly predict cancer mortality¹¹⁰. NEFAs can freely diffuse into the liver where they directly contribute to the pathogenesis of non-alcoholic fatty liver disease (NAFLD)¹¹¹, a common co-morbidity of obesity that is strongly associated with the development of hepatocellular carcinoma (HCC)¹¹². Moreover, work from the Lynch lab and others has demonstrated that obesity impairs immunosurveillance which contributes to accelerated tumour growth^{37,113,114}.

1.3.1 Obesity impairs anti-tumour immunity

1.3.1.1 NK cells are defective in obesity

NK cells play a crucial role in protective immunity against cancer. However in obese mice and humans, the number and function of NK cells are reduced, which likely contributes to an increased risk of cancer in obesity^{115,37}. In response to lipid rich environments NK cells accumulate lipids and activate peroxisome proliferator-activated receptor (PPAR) dependent lipid metabolism pathways. Upregulation of PPAR pathways inhibits mTORC1 activation in NK cells which is crucial for metabolic reprogramming and acquisition of effector functions⁸. As a result, NK cells from individuals with obesity fail to increase the rates of glycolysis and oxphos, produce less IFN γ and GzB, and kill fewer target cells compared to NK cells from lean controls. Interestingly, obesity does not impair NK cell recognition of or docking to tumour cells, but rather interferes with lytic granule polarisation and release into the synapse, which is essential for efficient killing of targets. Culturing NK cells with palmitic acid (PA) *in vitro* or activating PPARs directly recapitulates these metabolic and functional defects, demonstrating that FAs and lipid metabolism pathways likely promote NK cell dysfunction in obesity³⁷. In addition, the Lynch lab demonstrated that obesity interferes with NK cell infiltration and IFN γ production in B16 tumours *in vivo* which leads to accelerated tumour growth in mouse models³⁷ (Figure 1.7).

In contrast, NK cells isolated from children with obesity are highly activated and metabolically stressed. Basally, NK cells from children with obesity have elevated levels of glycolysis and mitochondrial ROS but have reduced capacity to increase their glycolytic rates following cytokine stimulation, and thus produce less GzB and kill less targets compared to lean controls. Together, these data demonstrate that the negative effects of obesity on anti-tumour immune responses are present early in life, before the development of malignancies¹¹⁶.

NK cells possess memory-like features and display enhanced IFN γ production upon restimulation after initial cytokine training¹¹⁷. NK cell metabolic training is dependent on increased flux through glycolysis and oxphos during the initial activation period. It was recently reported that NK cells from individuals with obesity are not capable of cytokine-induced training *in vitro* and do not display features of memory formation compared to NK cells from lean controls¹¹⁸. NK cell immunotherapies rely on the *ex vivo* activation of allogenic NK cells with cytokines to induce robust effector responses *in vivo*. However, these data suggest that autologous NK cell directed immunotherapies may be less efficient for individuals with obesity. In addition, it is currently unknown if WL can reverse the metabolic vulnerabilities of NK cells, which may allow for NK cell memory formation.

1.3.1.2 CD8 T cells are defective in obesity

CD8 T cells are a prominent and powerful anti-tumour immune population that exerts their cytotoxicity against malignant cells through direct target cell killing and the production of inflammatory cytokines. However, constant exposure to neoantigens and reduced availability of nutrients in the TME can cause CD8 T cell exhaustion, resulting in impaired cytotoxicity and failure to limit tumour progression. Obesity increases PD-1 expression on CD8 T cells which accelerates immune dysfunction systemically and in multiple tumour models. HFD increased the numbers of PD-1⁺ CD8 T cells in the blood, liver, and spleen which proliferated less and produced less IFN γ and TNF. CD8 T cells in B16 and 4T1 tumours also displayed features of exhaustion when mice were fed a HFD, characterised by increased expression of the immune checkpoints PD-1, TIM-3, and LAG-3, increased EOMES and reduced T-bet expression¹¹⁹. Importantly, exhausted CD8 T cells were reinvigorated by anti-PD-1 therapy, which augmented the production of IFN γ and TNF, leading to reduced B16 tumour burden and enhanced survival of mice fed a HFD¹²⁰. In addition, anti-PD-1 therapy improved the progression free and overall

survival of obese patients with colorectal cancer, illustrating the benefits of immune checkpoint blockade in patients with obesity¹²⁰.

In contrast to this report where obesity increased PD-1 expression on CD8 T cells and accelerated systemic immune dysfunction, we and others have recently reported that obesity does not cause global CD8 T cell dysfunction in humans or mice. Rather, the effects of obesity on CD8 T cells are mainly centred on the TME. Mice fed a HFD had reduced numbers of tumoral CD8 T cells in B16 and MC38 tumours, likely due to decreased expression of CXCR3 and CD49d, which control CD8 T cell extravasation and homing to the tumour bed^{121,122}. Residual CD8 T cells produced less IFN γ , TNF, and GzB and proliferated less when mice were fed a HFD. However, PD-1 expression was lower on tumoral CD8 T cells from mice fed a HFD, indicating that obesity interferes with CD8 T cell activation¹¹³. Indeed, we and others have demonstrated that obesity targets two distinct metabolic pathways in tumoral CD8 T cells, resulting in impaired activation and reduced effector cytokine production. CD98 expression and amino acid uptake through Slc7a5 is impaired in tumoral CD8 T cells from obese mice, resulting in reduced mTORC1 activation and IFN γ production¹¹³. Additionally, obesity induces a metabolic tug of war between tumour cells and CD8 T cells for lipids. HFD reprograms tumour cells to accumulate lipids and activate lipid metabolism and oxphos pathways rather than glycolysis, which enhances their proliferation and survival. FA uptake by tumours deprives CD8 T cells of FAs, resulting in impaired activation and cytotoxicity which contributes to tumour growth¹¹⁴. Importantly, both these studies show that tumoral CD8 T cell metabolism and function can be restored by supplying glutamine or targeting FA metabolism in tumour cells, which increases lipid availability for CD8 T cells^{113,114}. Targeting immunometabolic pathways may therefore improve CD8 T cell function in combination with traditional cancer therapies in obesity (Figure 1.7).

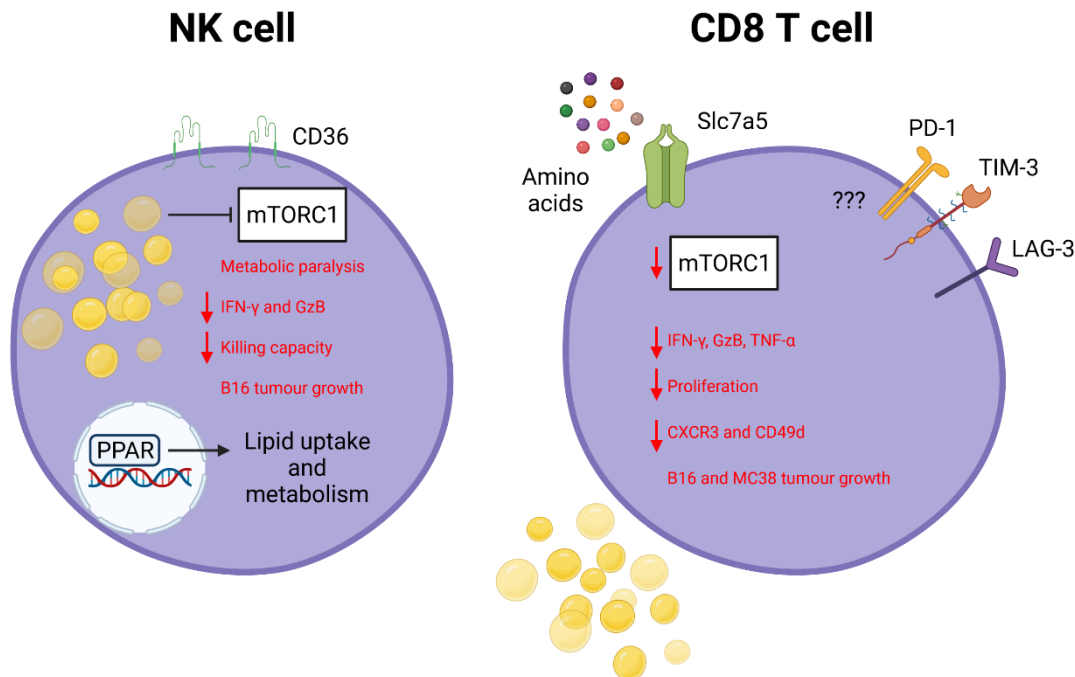


Figure 1.7 NK and CD8 T cells are impaired in obesity

Obesity alters immunometabolic pathways in NK and CD8 T cells which impairs their immunosurveillance. NK cells from obese mice increase the surface expression of the lipid transporter CD36, leading to intracellular lipid accumulation and activation of peroxisome proliferator-activated receptor (PPAR) dependent lipid metabolism pathways which inhibit mTORC1. As a result, NK cells fail to increase the rates of glycolysis and oxphos resulting in metabolic paralysis, reduced production of IFN γ and granzyme B (GzB) and fail to control tumour growth in vivo. CD8 T cell infiltration into the tumours of mice fed a high-fat diet (HFD) is impaired, likely due to decreased expression of CXCR3 and CD49d which regulate T cell trafficking and homing. CD8 T cells in tumours of mice fed a HFD do not accumulate lipids, due to metabolic competition for lipids between tumour cells. Rather, HFD impairs amino acid uptake through Slc7a5 in CD8 T cells resulting in decreased mTORC1 activation and reduced production of IFN γ and GzB. Some studies report that HFD increases the expression of checkpoint markers PD-1, TIM-3, and LAG-3 on CD8 T cells, however this may depend on the length of HFD feeding.

1.3.2 Weight loss restores anti-tumour functions in obesity

Bariatric surgery is commonly used to induce WL in individuals with obesity¹²³ and has beneficial effects on the anti-tumour functions of NK and CD8 T cells. Roux-en-Y gastric

bypass surgery enhances NK cell cytotoxicity against tumour cells 6 months after surgery. Additionally, bariatric surgery increases the production of IL-12, IL-18, and IFN γ from whole PBMCs, which may contribute to enhanced NK cell cytotoxicity¹²⁴. Interestingly, we recently reported that vertical sleeve gastrectomy can lead to tumour regression in obese patients with endometrial cancer. WL following surgery resulted in CD8 T cell infiltration and tumour regression in 6 out of 9 patients who received surgical intervention. These results confirm that WL following bariatric surgery has beneficial effects on anti-tumour NK and CD8 T cell responses which may reduce the incidence or risk of cancer in individuals with obesity. However, less is known about the effects of WL through dietary intervention on NK and CD8 T cell metabolism and functional responses, and thus was an aim for this thesis.

1.4 Lipids – an enemy of an anti-tumour environment

The rate of cancer growth and effectiveness of anti-tumour immune responses are determined by nutrient availability in the TME. There is vast literature on the metabolic environment of tumours, on one hand describing tumours as metabolically sparse with low levels of glucose and glutamine¹²⁵, while other studies describe high glucose levels in the tumour¹²⁶. Recently however, several studies have begun to shed light onto the lipid composition of TIFs. Lipid abundance is now recognised as a feature of many tumours including melanoma¹²⁷, pancreatic cancer¹²⁸, and others, and lipids have emerged as a potential enemy of an anti-tumour environment.

1.4.1 Introduction to lipid biology

Lipids are a heterogeneous pool of water insoluble metabolites that mainly comprise cholesterol and FAs. Cholesterol is a cyclic sterol that can be obtained through diet or synthesised via the mevalonate pathway. Endogenous cholesterol production primarily occurs in the liver and intestine, although all mammalian cells are capable of cholesterol synthesis. FAs are derived from the diet or synthesised *de novo* in the liver in response to insulin signalling after feeding¹²⁹. FAs represent a major source of energy for cells but are also key components of complex structural lipids including phospholipids (PLs) and sphingolipids, which together with cholesterol comprise the major components of cell membranes¹³⁰. In times of low energy demand, adipose tissues store excess FAs in the

form of TAGs, which, along with cholesterol esters, are the main components of intracellular lipid droplets (LDs)¹³⁰. LDs are dynamic organelles that promote lipid homeostasis by sequestering excess lipids to prevent lipotoxicity. TAGs in LDs can be mobilised through the action of lipases, releasing FAs which can be used to construct PLs for membrane formation or as substrates for β -oxidation¹³¹. An optimal supply of FAs is therefore essential for highly proliferative cells, such as cancer cells, to provide energy and building blocks to support their rapid growth. Figure 1.8 depicts an overview of lipid metabolism.

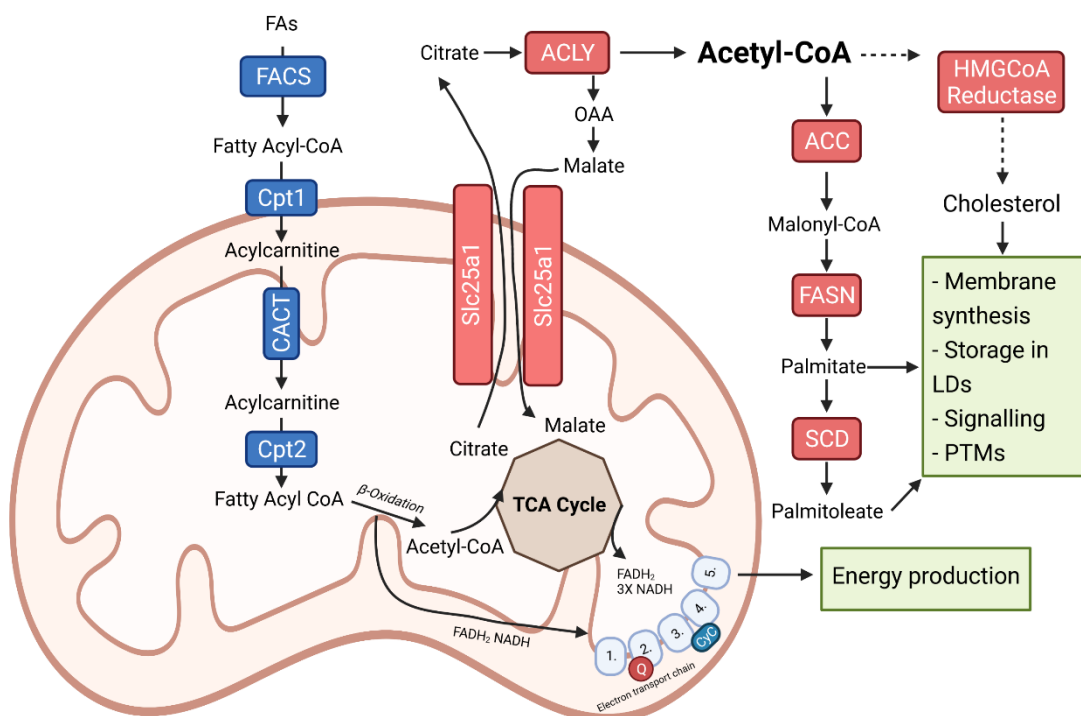


Figure 1.8 Overview of lipid metabolism

Lipid metabolism consists of β -oxidation (blue) and lipid synthesis (red) which are controlled by PPARs and SREBPs, respectively. In the cytosol, long chain fatty acids (LCFAs) are activated by fatty acyl-CoA synthetase (FACS), yielding fatty acyl-CoA. At the mitochondria, carnitine palmitoyl-transferase 1 (Cpt1), exchanges the CoA adduct for carnitine, forming acylcarnitine, which is transported into the matrix through carnitine acyl-carnitine translocase (CACT). Cpt2 re-converts acylcarnitine into fatty acyl-CoA and β -oxidation begins. Mitochondrial citrate is exported to the cytosol through Slc25a1. ATP citrate lyase (ACLY) converts cytosolic citrate into acetyl-CoA, which is converted into malonyl-CoA by acetyl-CoA carboxylase (ACC), together forming the substrates for fatty acid synthase (FASN). FASN catalyses the synthesis of palmitic acid which may be converted to palmitoleate by stearoyl-CoA desaturase (SCD). Cytosolic acetyl-CoA

can also enter the mevalonate pathway, producing cholesterol and other steroids. Fatty acids and cholesterol play an important role in membrane synthesis and signalling and can be stored in lipid droplets to be used as a fuel source.

1.4.2 Lipids and cancer

Given the dynamic roles of lipids in membrane production and energy generation, it is not surprising that lipid metabolism plays a significant role in cancer. To date, most evidence suggests that lipids promote the survival and spread of cancer¹³⁰. In murine models, the TIF of B16 melanoma tumours contains many FA species, which increase in abundance with tumour progression¹²⁷. This lipid phenotype is common across other models of melanoma including patient-derived melanoma xenografts and human melanoma metastases¹²⁷. Similarly in mouse models of pancreatic cancer, LDs accumulate LCFAs which promote a pro-tumorigenic environment¹²⁸. The TIF of B16 and MC38 tumours also contains many acylcarnitines, ceramides, phospholipids and esterified cholesterol (Figure 1.9), highlighting the diverse nature of lipids in the TME⁸⁵. Consequently, many cancers rewire their metabolism towards lipogenesis and β -oxidation to support key oncogenic functions. Understanding how lipids accumulate in tumours may provide therapeutic opportunities to inhibit lipid uptake or metabolism.

1.4.2.1 Exogenous lipid uptake

Cancer cells express a myriad of receptors and binding proteins to facilitate lipid uptake and traffic, including CD36 and fatty acid binding proteins (FABPs).

CD36 is a scavenger receptor involved into the recognition and delivery of a host of lipids to cells, including LCFAs and oxidised low-density lipoprotein (OxLDL)¹³². Lipid uptake via CD36 and β -oxidation boosts the metastatic potential of human oral carcinomas which is inhibited by CD36 antibody therapy in orthotopic mouse models of human oral cancer. CD36 expression correlates with poor disease-free survival in patients with squamous cell lung carcinoma, bladder, and breast cancers possibly because the predisposition to accumulate and metabolise lipids initiates metastasis¹³³.

FABPs are intracellular lipid chaperone proteins that coordinate lipid trafficking and lipolysis¹³⁴. Human ovarian cancer cells metastasise and home to the omentum where they express high levels of FABP4 to assist FA transfer from local adipocytes, providing a proliferative advantage¹³⁵. Inhibition of FABP4 prevented adipocyte-mediated invasion

and lipid accumulation in human ovarian cancer cells *in vitro*, while FABP4 deficiency resulted in fewer metastatic nodules in the ovarian bursa in a mouse model of ovarian cancer¹³⁵. These studies establish a tumour-initiating and metastasis-promoting role for lipid uptake.

1.4.2.2 *De novo* fatty acid synthesis

In addition to the uptake of exogenous lipids, many cancer cells are equipped to synthesise lipids endogenously (Figure 1.9). Cytosolic acetyl-CoA is the major substrate for the synthesis of FAs, primarily from glucose-derived carbons¹³⁶. Mitochondrial citrate is shuttled into the cytosol via Slc25a1¹³⁷, where it is cleaved by ACLY yielding OAA and acetyl-CoA¹³⁸. Human breast carcinoma tissue and multiple mouse and human breast cancer cell lines have high expression of Slc25a1¹³⁹ which likely favours a shift towards FA synthesis when extracellular glucose is limiting¹⁴⁰. It is also possible that in times of glucose abundance, glucose is directed towards pyruvate and citrate synthesis to make acetyl-CoA via Slc25a1, and thus, lipid metabolism could be favoured in the tumour regardless of glucose levels.

Carboxylation of cytosolic acetyl-CoA to malonyl-CoA by ACC is the rate-limiting step in FA synthesis. ACC expression is high in both advanced human breast carcinomas and pre-neoplastic lesions, highlighting the importance of FA synthesis in multiple stages of cancer development^{141,142}. The multi-enzyme FASN catalyses the condensation of acetyl-CoA and malonyl-CoA to PA. High expression of FASN is a feature of many cancers including colon¹⁴³, prostate¹⁴⁴, and breast¹⁴⁵ and is associated with poor prognosis and disease-free survival.

Similar to the endogenous synthesis of FAs in the liver, neoplastic lipogenesis is controlled by the SREBP transcription factors. SREBPs are master regulators of lipogenesis and control the transcription of FASN in response to multiple environmental signals including growth factor receptor activation of PI3K and MAPK pathways – pathways that are commonly hyper-activated in many cancers including breast, endometrium and ovarian¹⁴⁶. Together, these metabolic characteristics favour lipid synthesis for membrane formation to support aberrant proliferation, as well as energy production by β -oxidation in many cancer cells.

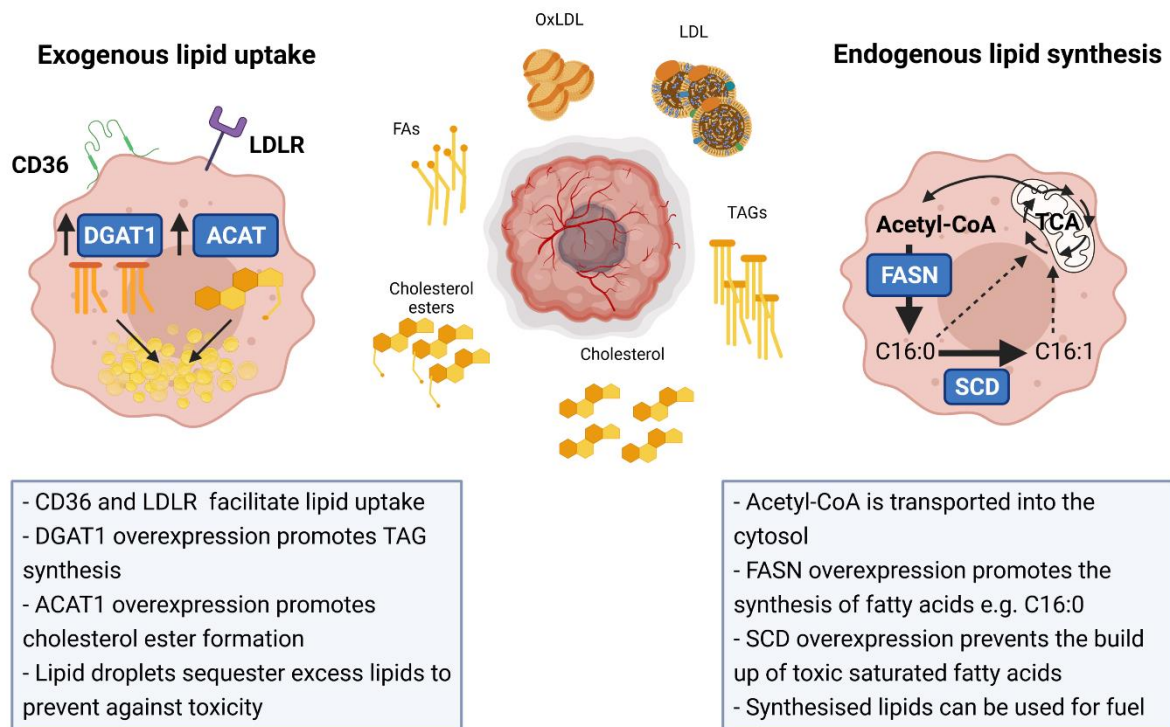


Figure 1.9. Tumour interstitial fluids (TIFs) contain a diverse array of lipids which promote tumour growth and metastasis

The tumour interstitial fluid is rich in a diverse array of lipids, including free fatty acids (FAs), low density lipoproteins (LDL), oxidised LDL (OxLDL), cholesterol, cholesterol esters and triacylglycerols (TAGs). Tumour cells express a range of cell surface receptors to facilitate the uptake of lipids, including the scavenger receptor CD36 and the LDL receptor. Overexpression of Diglyceride acetyltransferase 1 (DGAT1) is a common feature of many tumours. DGAT1 is responsible for the synthesis of TAGs from FAs, which can then be stored in lipid droplets (LDs). Acyl cholesterol acyltransferase (ACAT) is also overexpressed in many tumours. ACAT plays a key role in cholesterol homeostasis by catalysing the formation of cholesterol esters from free cholesterol and FAs. Together, TAGs and cholesterol esters form the major components of LDs. LDs are a feature of many tumour cells and promote lipid homeostasis by sequestering excess intracellular lipids, to prevent against lipotoxicity, peroxidation, and ferroptosis. Fatty acids may also be synthesised endogenously from cytosolic acetyl-CoA. Fatty acid synthase (FASN) expression is a feature of many cancers and catalyses the production of palmitic acid. Palmitic acid can be converted to the monounsaturated fatty acid (MUFA) palmitoleic, to protect against the toxic effects of saturated fatty acids (SFAs).

1.4.2.3 β -oxidation in tumour initiation, maintenance, and dissemination

Tumour cells heavily rely on aerobic glycolysis (i.e. the Warburg effect) to facilitate biomass production and support their rapid proliferation¹⁴⁷. However, the role of lipids and β -oxidation has recently gained traction in cancer biology. As cells proliferate and tumour volume expands, the metabolic landscape changes, resulting in vast areas devoid of nutrients, particularly glucose and glutamine¹⁴⁸, while maintaining heightened levels of lipids¹²⁷. To meet high bioenergetic demands, maintain ATP levels, and importantly proliferate, many tumour cells increase the expression of transcription factors and enzymes involved in β -oxidation. β -oxidation is transcriptionally regulated by PPAR transcription factors (Figure 1.8) that sense FA signals derived from dietary lipids and FA metabolites¹⁴⁹.

In mice, Lgr5⁺ intestinal stem cells proliferate and remodel the intestine in response to cues from the diet. Beyaz *et al.* found that in response to a HFD Lgr5⁺ cells activate PPAR δ signalling pathways, leading to enhanced proliferation and stemness, resulting in the spontaneous formation of adenomas¹⁵⁰. Loss of PPAR δ or blocking β -oxidation through inhibition of Cpt1a¹⁵¹, blocks the proliferative effects of dietary lipids on intestinal stem cells *in vivo*. These results highlight the role of lipid metabolism in initiating colon cancer in these models. As previously described, lipid uptake through CD36 (a PPAR target gene) and induction of β -oxidation is also critical for the survival and spread of squamous cell carcinomas¹³³. Targeting PPARs could therefore impair both cancer initiation and spread by limiting the ability to acquire and metabolise lipids, inducing a metabolically vulnerable state.

Many enzymes involved in β -oxidation are also dysregulated in cancer and contribute to high rates of proliferation, survival, and metastatic spread. Cpt1 overexpression is a feature of many cancers including human lung cancer and offers a survival advantage by supporting ATP production through β -oxidation and resistance to glucose deprivation¹⁵². β -oxidation is an aerobic process, thus tumour cells relying on lipid metabolism require a vascular network to support this process. β -oxidation supports *de novo* nucleotide synthesis for DNA replication and proliferation of human endothelial cells, and blocking Cpt1 depletes nucleotide precursors in endothelial cells, inhibiting angiogenesis¹⁵³. Therefore, other cells in the TME harness lipids from the TIF to fuel tumour promoting processes.

1.4.2.4 Cancer cells escape lipotoxicity

Excess FAs and heightened β -oxidation can generate ROS which can trigger lipid peroxidation – the oxidative degradation of lipids by free radicals¹⁵⁴. Polyunsaturated fatty acids (PUFAs) are prime targets for lipid peroxidation reactions due to the high proportion of carbon-carbon double bonds that house reactive hydrogen atoms¹⁵⁵. Accumulation of lipid-based ROS triggers ferroptosis, a form of programmed cell death. Anti-oxidant molecules and pathways such as glutathione peroxidase 4 (GPX4) can suppress ferroptosis by reducing lipid hydroperoxides in biological membranes¹⁵⁴. Overexpression of GPX4 is a feature of many cancers, offering protection against oxidative stress-induced cell death¹⁵⁶. Inhibition of GPX4 promotes ferroptosis of triple negative breast cancer cells *in vitro* and increases sensitivity to EGFR inhibitors *in vivo* in xenograft tumours, suggesting that dual therapy with GPX4 inhibitors may enhance anti-cancer effects of the treatment of lipid rich tumours¹⁵⁷.

To prevent against lipotoxicity, cells can sequester FAs in LDs in the form of TAGs¹³¹. Indeed, LDs are a feature of many cancers including glioblastoma¹⁵⁸, prostate¹⁵⁹, colon¹⁶⁰, and renal¹⁶¹ cancers. Diglyceride acyltransferase (DGAT1) is responsible for the synthesis of TAGs and is highly expressed in glioblastoma patient tissues, correlating with poor patient survival¹⁵⁸. Inhibiting DGAT1 in patient derived glioblastoma cells *in vitro* triggered the accumulation of acylcarnitines and production of ROS resulting in apoptosis. Indeed, genetic inhibition of DGAT1 *in vivo* suppresses tumour growth and prolongs the survival of glioblastoma-bearing mice¹⁵⁸. LDs preferentially store n-3 and n-6 PUFAs in cancer cells, possibly due to their capacity to undergo peroxidation. Excess PUFAs that surpass LD storage exert cytotoxic effects in cancer cells by inducing ferroptosis *in vitro*¹⁶². Interestingly, a fish oil-based diet, high in the PUFA docosahexaenoic acid (DHA) induced ferroptosis and delayed tumour growth *in vivo* in a mouse model of colorectal cancer. Inhibiting DGAT further increased lipid peroxidation in mice fed a DHA-rich diet¹⁶², highlighting a role for specific dietary regimes when used in combination with traditional cancer therapies.

1.4.3 Lipids and immunity

The metabolic challenges imposed by the TME mediates immune cell dysfunction and exhaustion which drives tumour progression. The current dogma is that tumour cells comfortably outcompete immune cells for glucose⁶⁴ and glutamine¹⁴⁸, resulting in vast areas devoid of these nutrients⁶³, while lipids are readily available for use^{127,85}. A lipotoxic

TME is emerging as a pro-tumour environment, by promoting tumour proliferation while also impairing cytotoxicity and promoting immunosuppression.

Lipids suppress anti-tumour immunity

In established tumours often reported to have low levels of glucose, NK and CD8 T cells commonly rewire their metabolism towards FA uptake and oxidation to preserve energy homeostasis and ATP production. This metabolic adaptation is consequently linked to cellular dysfunction across many human cancer types and mouse models (Figure 1.10).

NK cells

NK cells produce IFN γ and rapidly kill tumour cells¹⁶³. NK cell proliferation and effector functions are controlled by SREBP which is inhibited by cholesterol and oxidised forms of cholesterol – oxysterols including 25-hydroxycholesterol (25-HC) and 27-HC¹⁶⁴. Oxysterols are a common signature of TMEs and can be secreted by cancer cells themselves^{165,166}. 25-HC prevents NK cell metabolic reprogramming which impairs their ability to produce IFN γ and kill tumour cells *in vitro*⁷⁷. 27-HC is elevated in ER⁺ breast cancer tissue which promotes tumour cell proliferation and facilitates breast cancer metastasis^{167,168}. Peripheral NK cells from patients with metastatic breast cancer are functionally defective and metabolically impaired resulting in reduced killing capacity¹⁶⁹, possibly due to 27-HC.

The anabolic protein complex mTORC1 regulates NK cell metabolic reprogramming and is essential for the production of IFN γ and Granzyme B⁸. LCFAs decrease mTORC1 activity in NK cells and activate PPAR-dependent lipid metabolism. This transcriptional switch induces a state of metabolic paralysis, impairs IFN γ production, and reduces NK cell anti-tumour activity *in vivo* in a B16 model of melanoma³⁷. Additionally, NK cells derived from lymphoma-bearing mice and patients with diffuse large B cell lymphomas (DLBCL) display increased lipid metabolism, impaired mTORC1 activation, and produce less IFN γ . The lymphoma environment is rich in lipids which disrupt an effective anti-tumour response¹⁷⁰. These results further demonstrate how a metabolic adaptation to a lipid-rich environment is consequently linked to NK cell dysfunction. Metabolically rewiring NK cells away from β -oxidation may improve cancer outcomes, in the context of a lipotoxic environment.

CD8 T cells

Pancreatic ductal adenocarcinoma (PDAC) is an example of a lipid-rich tumour with a poor overall survival rate¹⁷¹. In mouse models of PDAC, CD8 T cells increase the expression of CD36, accumulate LCFAs, and acquire features of functional exhaustion, characterised by the upregulation of PD-1 and TIM-3 and reduced production of IFN γ and granzyme B. Despite heightened lipid uptake, intra-tumoral CD8 T cells fail to engage oxphos and downregulate very long chain acyl-CoA dehydrogenase (ACADVL), which catalyses the first step of β -oxidation, leading to metabolic exhaustion. Increasing ACADVL expression improved the survival and metabolic fitness of CD8 T cells in this metabolically hostile TME *in vivo*, highlighting the possible importance of coupling lipid uptake with β -oxidation to promote CD8 T cell survival in the context of lipid-rich tumours¹²⁸.

Similarly, CD8 T cells from B16 tumours progressively increase the expression of CD36 which is associated with increased PD-1 and TIM-3 expression, and decreased production of IFN γ and TNF^{85,172}. CD36 mediates the uptake of oxidised lipids including OxLDL which enhance lipid peroxidation, resulting in ferroptosis. Overexpression of GPX4 to prevent lipid peroxidation or direct inhibition of ferroptosis enhanced IFN γ and TNF production by CD8 T cells *in vivo*⁸⁵. While tumour cells have developed mechanisms to overcome lipotoxicity and peroxidation¹⁵⁶, these studies reveal that intra-tumoral CD8 T cells are sensitive to ferroptosis and GPX4 maybe a novel target for CD8 T cell-based therapies.

In contrast to these reports demonstrating the deleterious effects of lipids on CD8 T cell function and metabolism, FAs have also been implicated in supporting CD8 T cell function within a metabolically challenging TME. In B16 tumours, where FAs are abundant, promoting β -oxidation in CD8 T cells improves T cell polyfunctionality and delays tumour growth¹²⁷. Different metabolic TMEs and tumour types may explain these opposing findings on the role of β -oxidation in promoting or suppressing CD8 T cell anti-tumour responses. The TIF of B16 tumours contains a diverse array of lipids, including FAs, acylcarnitines, cholesterol and triacylglycerols⁸⁵ and it is thus possible that these contrasting reports are due to different cocktails of lipids in the TME. Distinct FA species differentially affect tumorigenesis and may also differentially regulate anti-tumour immunity. Finding the “sweet spot” of lipids in the TME to harness an effective anti-tumour response would prove beneficial for many cancers in which lipids are central to tumour and T cell metabolism.

Lipids support immuno-suppression

Recent evidence suggests that excess lipids in the TME support suppressive or regulatory immune cells which further promote a pro-tumour environment (Figure 1.10).

Regulatory T cells (Tregs)

Tregs are a major immunosuppressive population and high infiltration of Tregs is associated with poor clinical outcome in many mouse and human cancers including colorectal and ovarian cancers^{173,174,175}. Like other cells in the TME, Tregs adjust their metabolic configuration to support their survival and function in a hostile environment. Intra-tumoral Tregs from patients with melanoma, breast, and non-small-cell lung carcinoma express high amounts of the FA scavenger receptor CD36. CD36 provides lipids to induce PPAR-dependent lipid metabolism which promotes Treg viability and suppressive functions. Indeed, Treg-specific genetic ablation of CD36 attenuated tumour growth in a mouse model of melanoma, promoted Treg apoptosis while reinforcing CD8 T cell infiltration and effector cytokine production. Importantly, CD36 KO did not promote peripheral autoimmunity demonstrating that this metabolic configuration involving lipid uptake and metabolism is unique to intra-tumoral Tregs¹⁷⁶.

It was recently reported that intra-tumoral Tregs also upregulate SREBPs and synthesise FAs *de novo*. SREBPs are essential to maintain Treg viability and immunosuppression in the TME as inhibiting the expression of SREBP target genes increased the frequency of IFN γ ⁺ Tregs and number of polyfunctional CD8 T cells in the tumour. Together, these effects significantly reduced MC38 and B16 tumour growth, highlighting the importance of lipid synthesis for Treg immunosuppression in multiple cancer models¹⁷⁷. Importantly, lipid metabolism is dispensable for Treg mediated immune homeostasis in the periphery and these studies identified a context-specific lipid signature for Tregs in the TME. Targeting lipid metabolism in Tregs would therefore unleash an effective anti-tumour immune response without affecting peripheral Tregs.

$\gamma\delta$ T cells

$\gamma\delta$ T cells are a small population of poised innate lymphocytes capable of producing the key effector cytokines – IFN γ or IL-17. In mice, $\gamma\delta^{\text{IFN}\gamma}$ cells mediate an effective anti-tumour response through the production of IFN γ and direct tumour cell killing via perforin and granzymes. In contrast, many studies have described a pro-tumour role for $\gamma\delta^{17}$ cells in cancer, which recruit other immunosuppressive immune cells including neutrophils to enhance tumour growth¹⁷⁸ and metastasis¹⁷⁹. Like other effector cells, $\gamma\delta^{\text{IFN}\gamma}$ cells almost

exclusively use glycolysis. However, $\gamma\delta^{17}$ cells engage in both glycolysis and oxidative metabolism which is supported by a higher mitochondrial mass and potential¹⁸⁰. $\gamma\delta^{17}$, but not $\gamma\delta^{\text{IFN}\gamma}$ cells are increased when mice are fed high fat (HF)¹⁸⁰ and ketogenic diets (KD)¹⁸¹, suggesting that a lipid-rich environment supports $\gamma\delta^{17}$ expansion. Indeed, immunosuppressive $\gamma\delta^{17}$ cells are enriched in B16 tumours which harbour a diverse array of lipids^{127,85}. HFDs further increased $\gamma\delta^{17}$ tumour infiltration, correlating with enhanced tumour growth. Together, these data highlight that lipid-rich environments support the accumulation of immunosuppressive $\gamma\delta^{17}$ cells which promote tumour growth.

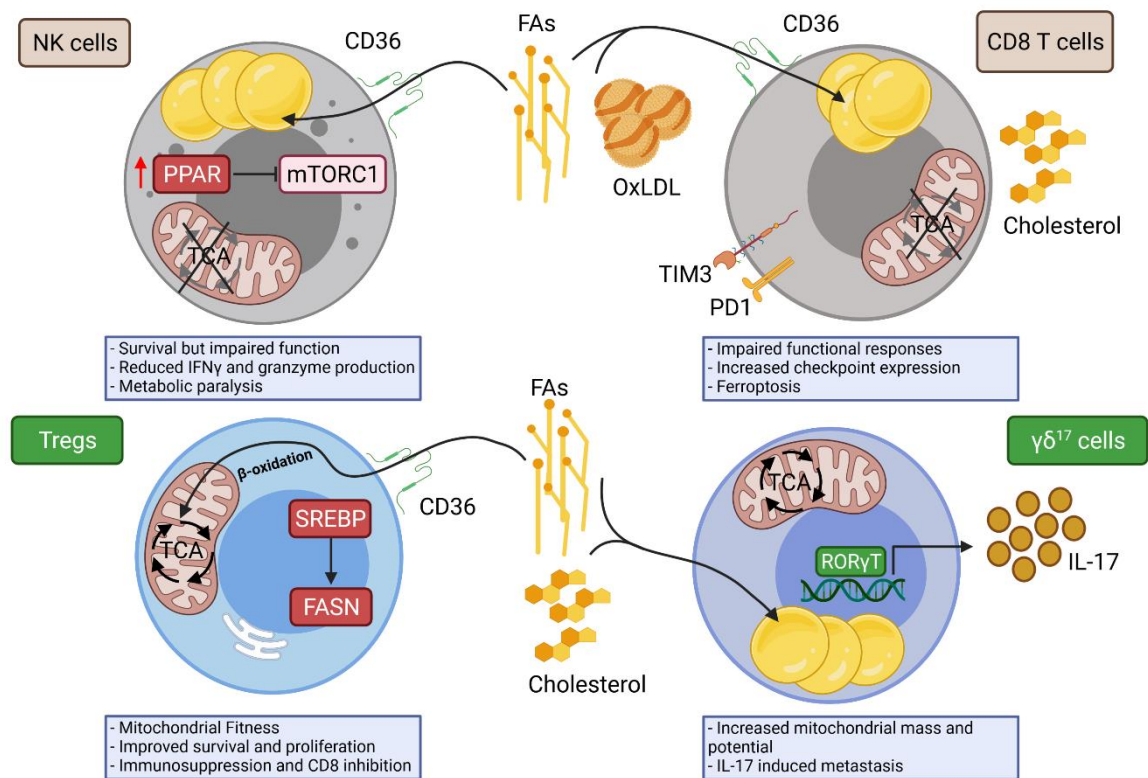


Figure 1.10. Lipids differentially affect immune populations while overall promoting an immunosuppressive phenotype

Lipids suppress cytotoxic anti-tumour NK and CD8 T cells and promote the survival and pro-tumorigenic roles of immunosuppressive Tregs and $\gamma\delta^{17}$ cells. In the presence of fatty acids (FAs), NK cells increase the expression of CD36 and accumulate lipids which activates a PPAR-dependent lipid metabolism program and inhibits mTORC1. Loss of mTORC1 results in metabolic paralysis, reduced cytotoxicity and impaired IFN γ and granzyme B production. In lipid rich

tumours, CD8 T cells increase the expression of CD36 which transports FAs and oxidised low density lipoprotein (OxLDL). OxLDL enhances lipid peroxidation, resulting in ferroptosis and impaired functional responses. On the other hand, lipid uptake and synthesis promote Treg immunosuppressive functions in many tumour models. Tumoral Tregs express CD36 and activate a PPAR-dependent lipid metabolism program which promotes their survival, proliferation, and pro-tumor functions. Intra-tumoral Tregs also increase the expression of SREBPs, leading to *de novo* synthesis of FAs and cholesterol. Similarly, $\gamma\delta^{17}$ cells display increased mitochondrial mass and potential to support heightened rates of lipid metabolism which is essential for IL-17 production to promote neutrophil-mediated cancer metastasis.

1.4.4 Dietary lipids and cancer

It is becoming increasingly evident that the nutritional state of a patient may be important in their response to cancer therapy. Preclinical evidence suggests that dietary restriction of particular nutrients including glucose, fructose, methionine and serine may enhance cancer therapy¹⁸². Given that lipids are emerging as key nutrients in fuelling cancer proliferation, it is not surprising that dietary intake of fat is also gaining much interest in cancer studies. The link between dietary fat and cancer incidence was first described in the 1930s by demonstrating that a butter diet accelerated subcutaneous tumour growth in mice¹⁸³. Indeed, we and others have shown that HFD-induced obesity accelerates the growth of B16 melanoma and MC38 adenocarcinoma tumours^{37,114}. However, little is known about the role of individual dietary lipid components or how the source of dietary fat regulates tumour growth and antitumour immunity. As distinct FAs differentially affect signalling pathways involved in cell proliferation¹⁸⁴, it is likely that the FA composition of dietary fats may play a role in tumour growth and progression. Indeed, it is now emerging that individual FAs distinctly affect tumour growth *in vitro* and *in vivo*.

PA is the most abundant saturated FA (SFA) in the human body and is enriched in many common fat sources including palm oil, butter, and lard¹⁸⁵. Many studies have demonstrated that the pro-tumorigenic effects of a HFD are due to increased abundance of PA. PA and HFD activate a PPAR-dependent lipid metabolism program in Lrg5⁺ intestinal stem cells which increases their stemness and proliferation, resulting in spontaneous adenoma formation *in vivo*¹⁵⁰. Similarly, a recent paper demonstrated that a palm oil HFD, rich in PA, epigenetically reprograms oral carcinoma cells to adopt a pro-regenerative state which increases metastasis through the secretion of a specialised extracellular matrix. In contrast, tumours from mice fed a HFD from olive oil, rich in the monounsaturated FA (MUFA) oleic acid (OA), were less metastatic, illustrating the

differential effects of these FAs on tumour growth *in vivo*¹⁸⁶. Indeed, olive oil is a main component of the Mediterranean diet¹⁸⁷, which is renowned for its health benefits and is inversely associated with cancer mortality from liver, breast and colorectal cancers¹⁸⁸. However, these differences between PA and OA are likely not due to saturation profile alone as evidence suggests a protective role for SA, a longer SFA, against some cancers. Dietary intake of SA is inversely associated with the risk of breast cancer development in post-menopausal women¹⁸⁹. Indeed, SA inhibits the proliferation of human breast cancer cells¹⁹⁰ and dietary SA reduces tumour burden in mouse models of breast cancer by inhibiting key cell cycle checkpoints and increasing the expression of the cell cycle inhibitors p21 and p27¹⁹¹.

Work from the Lynch lab and others has shown that dietary FAs, particularly PA can inhibit anti-tumour NK and CD8 T cell functions. PA may therefore function as a double-edged sword by promoting tumorigenesis and dampening an anti-tumour immune response *in vivo*. PA interferes with mTORC1 activation in NK cells, resulting in metabolic paralysis and reduced tumour cell killing *in vitro*, and treating NK cells with PA impairs their ability to control tumour growth *in vivo*³⁷. Interestingly, OA could not rescue NK cells from the detrimental effects of PA and a combination of OA and PA further impaired NK cell metabolism and function. However, little is known about the effects of SA on the immune system. Given that SA is another long chain SFA, it is possible that SA may also impair anti-tumour immunity however this hypothesis has not yet been examined in detail.

The TME is rich in a diverse array of FAs, acylcarnitines, cholesterol esters and structural lipids, which increase in abundance with tumour progression. It would be interesting to investigate how specific lipid cocktails regulate NK and CD8 T cells and whether this corresponds to functional responses and metabolic configurations as the tumour progresses. In addition to FAs and cholesterol, acylcarnitines have emerged as potential regulators of metabolism in obesity. Incomplete β -oxidation of FAs is a feature of HFD-induced obesity that leads to the accumulation of medium- and long-chain acylcarnitines in the plasma of mice⁸². Acylcarnitines activate pro-inflammatory signalling pathways in macrophages in an acyl-chain length and concentration-dependent manner, resulting in increased production of TNF and IL-1 β ¹⁹². Whether NK or CD8 T cell responses are affected by acylcarnitines is unknown and thus was a main aim for this thesis.

1.5 Thesis Aims

It is now established that obesity negatively affects NK and CD8 T cell anti-tumour functions which contributes to increased cancer risk. Work from the Lynch lab and others has shown that the dietary lipid PA negatively affects NK and CD8 T cell immunometabolic responses in obesity^{37,113,128}. At the same time, PA boosts cancer initiation and metastasis in different cancer models^{133,150}, further highlighting the negative effects of dietary lipids. However, little is known about the effects of specific dietary components of common HFDs on tumour growth and anti-tumour immunity. In this project, we tested the **hypothesis that the source of dietary fat would differentially affect tumour growth and anti-tumour immunity *in vivo***. In addition, we postulated that WL through dietary intervention could reverse some metabolic defects that are associated with obesity, and thus restore or improve anti-tumour immune functions.

1.5.1 Specific Objectives:

1. To investigate if WL can improve NK and CD8 T cell anti-tumour immune functions, through restoring systemic and subsequently, cellular metabolism
2. To investigate the differential effects of dietary lipids from animal vs plant sources on anti-tumour immune responses *in vivo*
3. To identify potential immunomodulatory metabolite(s) that are increased in obesity and examine their effects on NK and CD8 T cell responses *in vitro*

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

1,4-Dithiothreitol, 2-Deoxy-D-Glucose (2DG), 2-Mercaptoethanol (β -Me), ammonium chloride (NH_4Cl), ammonium persulfate (APS), antimycin-A, bovine serum albumin (BSA), fatty acid free BSA, brefeldin A (BFA), bromophenol blue, DNase 1, dimethyl sulfoxide (DMSO), Dulbecco's phosphate buffered saline (PBS), fluoro-carbonyl cyanide phenylhydrazide (FCCP), glucose, glycerol, glycine, sulfuric acid (H_2SO_4), hanks buffered saline solution (HBSS), hydrochloric acid (HCl), igepal NP-40, immobilon western chemiluminescent horse radish peroxidase (HRP) substrate, ionomycin, isopropanol, L-glutamine solution, myristoyl-carnitine, octadecanoyl-carnitine, octanoyl-carnitine, oligomycin, palmitic acid, palmitoyl-carnitine, penicillin-streptomycin (P/S), phorbol 12-myristate 13-acetate (PMA), ponceau S, polyvinylidene difluoride (PVDF) membrane, rotenone, Roswell Park memorial institute (RPMI-140), skim milk powder, sodium bicarbonate (NaHCO_3), sodium chloride (NaCl), sodium deoxycholate, sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH), N,N,N',N' -Tetramethylethylenediamine (TEMED), tris, trypan blue, trypsin-EDTA and tween® 20 were purchased from **Merck**. Ammonium chloride potassium (ACK) lysis buffer, pierce bicinchoninic acid assay (BCA) protein assay kit, cell-tak™ cell and tissue adhesive, eBioscience™ intracellular (IC) fixation buffer, eBioscience™ IC fixation & permeabilization buffer set, eBioscience™ permeabilization buffer (10X), OneComp eBeads™ compensation beads, protease inhibitor cocktail (100X), RNase OUT recombinant ribonuclease inhibitor, PureLink Mini RNA kit, spectra multi-colour broad range protein ladder, ultra-pure water nuclease free and TRIzol reagent were purchased from **Fisher Scientific**. Collagenase 2 and fetal calf serum (FCS) were purchased from Gibco. Collagenase D was purchased from **Roche**. EasySep™ mouse CD8a positive selection kit, EasySep™ mouse NK cell isolation kit and lymphoprep™ were purchased from **Stem Cell**. Ethylenediaminetetraacetic acid (EDTA) and virkon powder were purchased from **VWR International**. 2-propanol and ethanol were purchased from **school stores**. Glycocholate-d4, Inosine- $^{15}\text{N}_4$ and Thymine-d4 were purchased from **Cambridge Isotope Laboratories**. High-capacity cDNA reverse transcription kit and nuclease-free water were purchased from **Biosciences Ltd**. Oleic acid and stearic acid were purchased from **Cayman Chemical Ltd**. PBS tablets were purchased from **Medical Supplies Company**. Phosphatase inhibitor cocktail 2 was purchased from **Med**

Chem Express. Precision count beads were purchased from **Biolegend**. Protogel (30%) was purchased from **National Diagnostics**. Rodent high-fat diets (HFDs) were purchased from **Research Diets**. Seahorse XF calibrant solution and seahorse XF RPMI media (without phenol red) were purchased from **Agilent Technologies**. SYBR™ Select Master Mix was purchased from **eBioscience**.

2.1.2 Equipment

Falcon tubes (15 ml, 50 ml), sterile tissue culture plates (6, 12, 24, 48, 96), sterile tissue culture flasks (T25, T75, T175), pipette tips (p10, p200, p1000), petri dishes (15 mm, 60 mm), syringes (2ml, 5 ml, 20 ml), Eppendorf tubes (0.5 ml, 1.5 ml), 1.5 ml microtubes, serological pipettes (5 ml, 10 ml, 25 ml) and 2 ml cryovials were all purchased from **Cruinn Diagnostics Ltd**. Pipette sets (p2, p10, p20, p200, p100), aerosol barrier pipette tips (10µl, 20µl, 200µl, 1000µl), transfer pipettes, pipette boy, cell strainers (70 µm, 100 µm), FACs tubes (with and without lids), PCR strip tubes, 0.2 µm, 70µm and 100µm filters, needles (18 g, 23 g, 27 g), Mr. Frosty, scalpels, dissection tools (scissors, tweezers), steel razor blades, heparin/EDTA micro tubes, HiPPR Detergent Removal Column kit, MicroAmp Optical 96-well and 384-well reaction plates, QuantStudio™ 5 real-time PCR system, StepOnePlus™ real time PCR system, Ultimate 3000 RSLCnano system, Easy-Spray PepMap RSLC C18 column and Q-Exactive Plus Mass Spectrometer were all purchased from **Fisher Scientific**. Seahorse Analyzer (XFp, XFe96), Seahorse cell culture plates, Seahorse cartridges were all purchased from **Agilent Technologies**. Centrifuges (5424 R, 5425 R, 5804 R) were purchased from Eppendorf. Water bath, vortex, mini-fuge and multi-channel pipettes were purchased from **Medical Supplies Company**. Cell culture hood, steri-cycle CO₂ incubator, nanodrop 2000 UV spectrophotometer, ultrasonic cleaner, Q-Exactive™ HF-X mass spectrometer, and TraceFinder software version 4.1 were purchased from **Thermo Fisher Scientific**. Haemocytometer was purchased from **Hausser Scientific**. Glass scintillation vials were purchased from **Kimble**. LSR Fortessa, FACs CANTO, MiniAmp Plus Thermal Cycler and MiniAmp optical adhesive film were purchased from **BD Biosciences**. FlowJo v10 software was purchased from **Treestar INC**. Gel Documentation System and Image lab analysis software were purchased from **Bio-Rad**. TissueLyser 2 was purchased from Qiagen. Luna-amino column was purchased from **Phenomenex**. MARS 6 Express 50 positron microwave reaction system was purchased from **CEM corporation**. Blood glucose monitor and strips were purchased from the **Lombard Pharmacy**. Promethion metabolic cages were purchased from **Stable**

Systems International. UltiMate 3000 HPLC system was purchased from **Waters**. Olympus BX51 upright microscope was purchased from **Olympus**.

2.1.3 Mice

2.1.3.1 Ethics and animal license

All mice used for this project were C57BL/6 mice between 8 and 20 weeks of age. Mice were bred in-house and maintained under specific pathogen-free conditions in accordance with Irish and European Union regulations. Experiments were carried out in compliance with the Lynch lab project license, with ethical approval from the Trinity College Dublin university ethics committee and the Animal Research Ethics Committee from the Health Products Regulatory Authority (HPRA). Animals were culled by CO₂ inhalation or cervical dislocation. Organs were harvested in the comparative medicine unit (CMU) and carcasses were disposed of in accordance with the guidelines described by the TCD CMU.

2.1.4 Cell lines

B16-F10, murine melanoma cell line was purchased from The American Type Culture Collection (ATCC). MC38, murine colon adenocarcinoma cell line was kindly gifted by Arlene Sharpe, Harvard University.

2.1.5 Cell culture medium

2.1.5.1 Natural killer (NK) cell and T cell culture medium

Complete RPMI (cRPMI) cell culture medium was prepared by supplementing RPMI-1640 with 20μM L-glutamine, 10% FCS, 1% P/S and 55 μM β-Me.

2.1.5.2 B16/MC38 tumor cell culture medium

Complete DMEM (cDMEM) cell culture medium was prepared by supplementing DMEM with 10% FCS and 1% P/S.

2.1.6 Cell culture stimulants / inhibitors

Reagent	Vehicle	Stock concentration	Desired concentration
2DG	Seahorse Media or H ₂ O	1M	30mM
Anti-CD28	PBS	1mg/ml	3µg/ml
Anti-CD3	PBS	1mg/ml	1µg/ml
Antimycin A	Ethanol	5mM	4µM
BFA	DMSO	1mg/ml	5µg/ml
FCCP	Ethanol	10.1µM	1µM
Ionomycin	DMSO	1mg/ml	1µg/ml
Myristoyl-carnitine (C14)	Methanol	50mM	25-60µM
Octadecanoyl-carnitine (C18)	Methanol	50mM	25-60µM
Octanoyl-carnitine (C8)	Methanol	50mM	25-60µM
Oleic Acid (OA/ C18:1)	Ethanol	23mg/ml	50-200µM
Oligomycin	Ethanol	2mM	2µM
Palmitoyl-carnitine (C16)	Methanol	50mM	25-60µM
Palmitic Acid (PA/ C16:0)	Ethanol	23mg/ml	50-200µM
PMA	DMSO	1mg/ml	10ng/ml
Rapamycin	Ethanol	2mM	20nM
Recombinant mouse IL-2	dH ₂ O + 0.1% BSA	100µg/ml	20ng/ml
Recombinant mouse IL-12	dH ₂ O + 0.1% BSA	20µg/ml	10ng/ml
Recombinant mouse IL-15	dH ₂ O + 0.1% BSA	20µg/ml	10ng/ml
Rotenone	DMSO	1mM	0.1µM
Stearic Acid (SA/ C18:0)	Ethanol	20mg/ml	50-200µM

Table 2.1 List and concentrations of stimulants and inhibitors used in cell culture

2.1.7 Buffers

Buffer	Composition
Freezing medium	20% DMSO in cRPMI (2.1.6.1.)
Thawing medium	cRPMI without β -me + 50 μ g/ml DNase 1
MACs cell separation buffer	PBS + 2% FCS + 1mM EDTA
3% BSA medium	50ml: 1.5g BSA in cRPMI
3% FACS buffer	50ml: 1.5ml FCS + 48.5ml PBS
1X Perm Butter	50ml: 5ml 10X perm buffer + 45ml dH ₂ O
Cell-Tak (3.5μg/ml final concentration)	2986.1 μ l Sodium bicarbonate (0.1M pH 8), 9.29 μ L cell tak (1.85mg/ml), 4.56 μ l 1M NaOH
Seahorse Medium	RPMI medium without phenol red + 10mM glucose + 2mM glutamine, warmed to 37°C, pH 7.4.
5M NaCl	100ml: 29.2g NaCl in dH ₂ O
10% sodium deoxycholate	100ml: 10g sodium deoxycholate in dH ₂ O
10% SDS	100ml: 10g SDS in dH ₂ O
RPIA Buffer	25mM Tris (pH 7.4), 1.5Mm NaCl, 1% Igepal (NP-40), 0.5% sodium deoxycholate, 10% SDS
5X Sample loading buffer	5ml Glycerol, 10ml 10% SDS, 10mg Bromophenol Blue, 6.25ml 1M Tris-HCl (pH6.8), 28.75ml H ₂ O, 250ml β -mercaptoethanol per ml
10% APS	1ml: 100mg in dH ₂ O
1.5M Tris pH 8.8	For 1L: 181.71g in dH ₂ O
1M Tris pH 6.8	For 1L: 121.14g in dH ₂ O
1M Tris-Cl pH 6.8	For 1L: 121.14g Tris base, 700ml dH ₂ O, adjust to pH 7.0 with concentrated HCl. Bring final volume to 1L with dH ₂ O
SDS-PAGE 10X Running Buffer	For 1L: 30.3g Tris, 144g Glycine, 10g SDS in dH ₂ O

10X Transfer Buffer	For 1L: 30.28g Tris, 150.14g Glycine in dH ₂ O
10X Tris Buffered Saline – Tween (0.05%) (TBST)	For 1L: 12.11g Tris, 87.6g NaCl, in dH ₂ O. 5ml Tween-20, pH 8-8.5 before adding tween-20
Blocking Buffer (5% milk)	50ml= 2.5g skim milk powder in 1X TBST
Primary and secondary antibody dilution buffer (5% BSA)	50ml= 2.5g BSA in 1X TBST
Tumour digestion buffer	RPMI + 20U/ml DNase 1 + 1mg/ml collagenase D
Metabolomics extraction buffer	80% methanol containing inosine- ¹⁵ N ₄ , thymine-d ₄ and glycocholate-d ₄
Proteomics lysis buffer	4% SDS, 50mM triethylammonium bicarbonate (TEAB) pH 8.5, 10mM tris 2-carboxyethyl phosphine (TCEP)
Proteomics digest buffer	0.1% SDS, 50mM TEAB pH 8.5, 1mM CaCl ₂
Proteomics elution buffer A	10mM ammonium formate/2% acetonitrile, pH 9.0
Proteomics elution buffer B	10mM ammonium formate/80% acetonitrile, pH 9.0
Proteomics separation buffer A	0.1% formic acid
Proteomics separation buffer B	0.1% formic acid/80% acetonitrile

Table 2.2 List and composition of commonly used buffers

2.1.8 Flow cytometry antibodies / stains

2.1.8.1 Mouse antibodies

Target	Color	Clone	Supplier
NK1.1	BV421	PK136	Biolegend
	PerCpCy5.5	PK136	Biolegend

CD3	FITC	17A2	Biolegend
	BV650	17A2	Biolegend
	APC	17A2	Biolegend
	BUV395	17A2	Biolegend
IFNγ	BV785	XMG1.2	Biolegend
	PE-CF594	XMG1.2	BD Biosciences
	PerCpCy5.5	XMG1.2	eBioscience
GzB	PeCy7	NGZB	eBioscience
	AF700	QA16A02	Biolegend
CD71	PE	R17217	Biolegend
	BV510	C2	BD Biosciences
CD69	APC-Cy7	H1.2F3	Biolegend
pS6	PE	D57.2.2E	CST
CD98	PE	RL388	Biolegend
CD4	AF700	RM4-5	Biolegend
	APCeF780	RM4-5	eBioscience
CD8	BV650	53-6.7	Biolegend
	PeCy7	53-6.7	Biolegend
	APC-Cy7	53-6.7	Biolegend
	APCeF780	53-6.7	eBioscience
CD19	PeCy7	6D5	Biolegend
PD-1	BV711	29F.1A12	Biolegend
TIM-3	BV421	B8.2C12	Biolegend
TNF-α	BV785	MP6-XT22	Biolegend
CD45	BV605	30-F11	Biolegend

Table 2.3 List of mouse flow cytometry antibodies

2.1.8.2 Flow cytometry stains

Stain	Stock concentration	Desired concentration	Supplier
CellTrace™ Violet (CTV)	5mM	1μM	Invitrogen
Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM)	100μM	100nM	Invitrogen
MitoTracker™ Green FM (MTG)	1mM	1μM	Invitrogen
2',7'-dichlorodihydrofluorescein (H ₂ DCFDA)	5mM	2μM	Invitrogen
MitoSOX™ Red	5mM	2.5μM	Invitrogen
Zombie Aqua Fixable Viability Dye	1000X solution in DMSO	Dilute 1:1000	Biolegend
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain	1000X solution in DMSO	Dilute 1:1000	Invitrogen
HCS LipidTOX™ Green Neutral Lipid Stain	1000X solution in DMSO	Dilute 1:1000	Invitrogen

Table 2.5 List of flow cytometry stains

2.1.9 Western Blot Antibodies

Primary Antibody	Dilution	Secondary Antibody	Supplier
β-Actin	1:10,000	Mouse	Cell Signaling Technologies
c-Myc	1:1000	Rabbit	Cell Signaling Technologies
pS6	1:1000	Rabbit	Cell Signaling Technologies
Total S6	1:1000	Rabbit	Cell Signaling Technologies

Table 2.6 List of western blot primary antibodies

2.1.10 Resolving and stacking gels for SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Component	Volume (ml)
H ₂ O	5.9
30% acrylamide mix	5
1.5M Tris (pH 8.8)	3.8
10% SDS	0.15
10% Ammonium persulfate	0.15
TEMED	0.006

Table 2.7 List of components to prepare 15ml 10% resolving gel for SDS-PAGE

Component	Volume (ml)
H ₂ O	3.4
30% acrylamide mix	0.83
1M Tris (pH 6.8)	0.63
10% SDS	0.05
10% Ammonium persulfate	0.05
TEMED	0.005

Table 2.8 List of components to prepare 5ml 5% stacking gel for SDS-PAGE

2.1.11 PCR primers

Primer sequences for quantitative real time PCR (qRT-PCR) were obtained from the Harvard PrimerBank available at <https://pga.mgh.harvard.edu/primerbank/>. NCBI gene IDs were obtained from the NCBI website located at: <https://www.ncbi.nlm.nih.gov/>. Gene IDs were loaded into the PrimerBank and a list of validated forward and reverse primers were obtained. Sequences were verified using BLASTn algorithm available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch.

Gene	Gene ID	Forward Sequence	Reverse Sequence
<i>Cpt1a</i>	12894	CTCCGCCTGAGCCATGAAG	CACCAGTGATGATGCCATTCT
<i>Cact</i>	57279	GACGAGCCGAAACCCATCAG	AGTCGGACCTTGACCGTGT
<i>PPARα</i>	19013	ACCACTACGGAGTTCACGCATG	GAATCTTGACGCTCCGATCACAC
<i>PPARγ</i>	19016	GTACTGTCGGTTTCAGAAGTGCC	ATCTCCGCCAACAGCTTCTCCT
<i>Slc2a1</i>	20525	GGAATCGTCGTTGGCATCCT	CGAAGCTTCTTCAGCACACTC
<i>Slc2a3</i>	20527	ATGGGGACAACGAAGGTGAC	GTCTCAGGTGCATTGATGACTC
<i>Hex2</i>	15277	TCGCCTGCTTATTCACGGAG	CCATCCGGAGTTGACCTCAC
<i>Gapdh</i>	14433	CATGGCCTTCCGTGTTCTTA	CCTGCTTCACCACCTTCTTGAT
<i>Pkm1</i>	18746	AAACAGCCAAGGGGGACTAC	TTATAAGAGGCCTCCACGCTG
<i>Ldha</i>	16828	CTGGGAGAACATGGCGACTC	ATGGCCCAGGATGTGTAACC
<i>Slc16a1</i>	20501	TGTTAGTCGGAGCCTTCATTC	CACTGGTCGTTGCACTGAATA
<i>Actb</i>	11461	GCTCTGGCTCCTAGCACCAT	GCCACCGATCCACACCGCGT
<i>Ppib</i>	19035	GGAGATGGCACAGGAGGAA	GCCCGTAGTGCTTCAGCTT

Table 2.9 List of mouse primer sequences for qPCR

2.2 Methods

2.2.1 Mouse model of diet induced obesity (DIO)

A mouse model of DIO was used in this study to investigate the effects of obesity on tumour growth and cytotoxic lymphocyte function and metabolism. Mice were fed a HFD where 45% / 60% of calories were from fat. A SFD where 12% of calories were from fat (soybean oil) was used as a control diet. The duration of the HFD regimen is indicated in each figure legend. The source of fat used included lard, butter, palm oil, coconut oil, or peanut oil and is indicated in each figure legend. Mice were weighed before starting a HFD regimen and then once every 4 weeks. To induce WL following HFD feeding, mice were fed a SFD for 2 weeks.

2.2.2 Tumour Models

2.2.2.1 Preparing cells

B16.F10 or MC38 cells were cultured in a T125 flask in cDMEM (2.1.5.2) for ~4 days. Cells were harvested during the log growth phase when they reached 70% confluency. Medium was discarded, and cells were washed with sterile PBS. Cells were incubated with trypsin-EDTA for 15min at 37°C. Harvested cells were washed three times with sterile PBS, counted, and the cell concentration adjusted to 2×10^6 cells per ml in PBS. The flanks of mice were shaved and 100µl of the tumour cell suspension was injected subcutaneously into the left flank of female mice. Tumour growth was monitored, and tumour size was measured every 2-3 days using calipers, measuring the diameters (d) of the tumour. Tumour size was calculated using the following formula – $(d1)^2 \times (D2/2)$. D1 being the smaller value. Mice were sacrificed when the tumour size exceeded 15mm or tumours became ulcerous.

2.2.2.2 Isolating tumour cells and infiltrating leukocytes

Tumours were removed from tumour-bearing mice. Tumours were chopped into small pieces and digested with tumour digestion buffer containing DNase 1 and collagenase D in RPMI medium (Table 2.2) for 1 hr. Subsequently, tumour cells were filtered through 100µm nylon mesh with cRPMI (2.1.5.1). Single cell suspensions were stimulated with PMA, ionomycin, and BFA (Table 2.1) for 3 hr and subsequently stained for intracellular cytokines (2.2.1).

2.2.3 Fasting blood glucose and glucose tolerance test (GTT)

Mice were fasted overnight. The tails were cut, and fasting blood glucose levels were measured using a blood glucose monitor. 100µl of glucose in PBS was injected intra-peritoneally at a concentration of 2g / kg body weight. Blood glucose levels were measured 10, 20, 45, 60, 90 and 120 minutes after injection.

2.2.4 Analysis of animal behavior and systemic metabolism

The Promethion metabolic cage system was used to assess the systemic metabolism and behavior of mice. Briefly, mice were singly housed for 48 hr and O₂ consumption, CO₂ emission, body weight, food and water intake, and locomotor activity were

monitored throughout. Data was analyzed using the CalR version 1.3 online software available at: <https://calrapp.org/>.

2.2.5 Metabolomics analysis

2.2.5.1 Sample preparation for metabolite profiling

Mice were culled immediately by cervical dislocation. Blood was taken via cardiac puncture, collected into a microtainer tube lined with lithium heparin and centrifuged for 10 min, max speed, at 4°C. Blood plasma was subsequently snap frozen. Liver and calf muscle tissues were isolated and snap frozen. Metabolomics analysis was carried out by Dr. Evanna Mills at Dana Farber Cancer Institute (DFCI), Harvard Medical School, as previously described¹⁹³. Metabolites were extracted from plasma by adding cold extraction buffer (Table 2.2) in a 1:4 ratio plasma to extraction buffer. Samples were then centrifuged twice for 5 min, max speed, at 4°C and supernatants collected. Snap frozen tissues were quickly weighed and homogenized in cold extraction buffer (Table 2.2) at a 4:1 volume to wet ratio. Samples were then centrifuged twice for 5 min, max speed, at 4°C and supernatants collected.

2.2.5.2 Metabolite analyses by mass spectrometry

Metabolite extracts (10 µl) were loaded onto a Luna-amino column using an UltiMate-3000 TPLRS LC as previously described¹⁹³. Subsequent analysis was carried out using a Q-Exactive™ HF-X mass spectrometer as previously described¹⁹³. Targeted processing of a subset of known metabolites was conducted using TraceFinder software version 4.1. Compound identities were confirmed using reference standards. In all case metabolite abundance was normalized using internal standards and relative changes were assessed by comparison with metabolites extracted from the same sample type (i.e., plasma). Metabolomics data was analysed and PCA plots and heatmaps were generated using MetaboAnalyst 5.0 software.

2.2.6 Murine cell culture

2.2.6.1 Splenocyte isolation

Murine spleens were removed and splenocytes were isolated by gentle mashing through 70µm filters into cRPMI (2.1.5.1). Erythrocytes were lysed by resuspending the cells in

ACK lysing buffer for 2 min. Lymphocytes were re-suspended in 10 ml cRPMI and cells were counted using a haemocytometer.

2.2.6.2 NK cell culture

2.2.6.2.1 NK cell purification

NK cells were purified using the EasySep™ murine NK cell isolation kit. This protocol is based on immunomagnetic negative selection to yield highly purified murine NK cells from splenocytes. Splenocytes were washed in PBS and re-suspended in MACs cell separation buffer (Table 2.2) at a concentration of 10×10^8 cells per ml in a 5ml polystyrene round-bottomed tube. Cells were incubated with streptavidin-labelled antibodies for 10 min RT, which bind all cells excluding NK cells. Biotin-labelled magnetic beads were then added to the cell suspension for 5 min. MACs buffer was added to the tube to reach a final volume of 2.5ml and the tube was placed into a magnet for 5 min. Cells labelled with magnetic beads were attracted to the walls of the tube, while the pure NK cells remained in suspension. After 5 min, the magnet and tube were inverted in one motion, yielding a pure population of NK cells. NK cell purity was verified by flow cytometry.

2.2.6.2.2 NK cell cytokine stimulation

NK cells were plated in cRPMI (2.1.5.1) at a concentration of 2×10^6 cells per ml. NK cells were activated with IL-2 (10ng/ml), IL-12 (20ng/ml), and IL-15 (5ng/ml) for 18-20 hr in 37°C, 21% O₂ and 5% CO₂. For the final four hours of the experiment, BFA (5µg/ml) was added to cells to prevent cytokine exocytosis for analysis by flow cytometry.

2.2.6.3 Murine T cell culture

2.2.6.3.1 CD8 T cell purification

CD8 T cells were purified using the EasySep™ mouse CD8a Positive Selection kit 2. This protocol is based on immunomagnetic positive selection to yield highly purified murine CD8 T cells from splenocytes. In brief, splenocytes were washed in PBS and re-suspended in MACs cell separation buffer (Table 2.2) at a concentration of 10×10^8 cells per ml in a 5ml polystyrene round-bottomed tube. Cells were incubated with an FcR blocker and a selection cocktail for 5 min RT, which bind CD8a⁺ T cells. Magnetic RapidSpheres were added to the cell suspension for 3 min RT. MACs buffer was added

to the tube to reach a final volume of 2.5ml and the tube was placed into a magnet for 5 min. Labelled CD8 T cells were attracted to the walls of the tube, and other cell populations remained in suspension. After 5 min, the magnet and tube were inverted in one motion, to discard CD8⁺ cells. CD8 T cell purity was verified by flow cytometry.

2.2.6.3.2 T cell stimulation

T cells were plated in cRPMI (2.1.5.1) at a concentration of 1×10^6 cells per ml and cultured with plate-bound anti-CD3 (1 μ g/ml), soluble anti-CD28 (3 μ g/ml), and 5ng/ml IL-2 for 1-3 days in 37°C, 21% O₂ and 5% CO₂. For the final four hours of the experiment, BFA (5 μ g/ml) was added to prevent cytokine exocytosis for analysis by flow cytometry.

2.2.7 Lipid preparation

Unconjugated OA, PA, and SA FAs were re-suspended in ethanol to a concentration of 100mM and stored at -20°C. Lipids were heated to 37°C in a water bath before use. 400 μ M working stocks were prepared by pipetting 8 μ l of lipid onto the edge of a glass vial. The ethanol evaporated under the culture hood leaving the lipid attached to the edge of the glass. Lipids were dissolved in 8ml cRPMI (2.1.5.1) + 0.5mM L-Carnitine + 3% FA-free BSA. Lipids were heated and sonicated for 2 min using a water bath sonicator, to break up the lipid aggregates and prevent the formation of lipid micelles. Lipids were diluted in cRPMI and pipetted directly onto cells to yield final desired concentrations of 50 μ M, 100 μ M, and 200 μ M.

2.2.8 Proteomic analysis

For proteomic analysis, NK cells were purified *ex vivo* from mouse spleens by immunomagnetic cell selection (2.2.6.2.1). NK cells were stimulated with IL-2 (10ng/ml) and IL-12 (20ng/ml) in cRPMI for 18 hr. Live NK cells were FACs sorted into FACs buffer (Table 2.2). Cells were washed in HBSS X2, and cell pellets were snap frozen. Cell pellets were stored at -80°C until further preparation. Proteomics analysis was carried out by Dr. Linda Sinclair at the University of Dundee.

2.2.8.1 Proteomics sample prep

Sample processing was done using the SP3 method¹⁹⁴. Cell pellets were lysed in 400µl SDS lysis buffer (Table 2.2), with samples processed as previously described¹⁹⁵. Samples were digested overnight at 37°C with LysC in a 1:100 ratio LysC to protein, then digestion was repeated with trypsin in a 1:100 ratio trypsin to protein. Peptide clean-up was performed as previously described¹⁹⁵.

2.2.8.2 Fractionation

For fractionation, samples were adjusted to 5% formic acid and separated into 16 fractions using a high pH reverse phase method on an UltiMate 3000 HPLC system on a 2.1 mm x 150 mm XBridge Peptide BEH C18 column with 3.5 µm particles (Waters) column. Samples were separated over a 25 min elution gradient between buffer A (Table 2.2) and buffer B (Table 2.2) with a flow rate of 0.3 mL/min as follows: 0 min, 10% B; 11 min, 50% B; 12-17 min, 100% B; 18-25 min, 10% B. Fractions were combined to form 8 fractions and dried down in a GeneVac EZ-2 evaporator.

2.2.8.3 Mass spec analysis

Mass spectrometry analysis was performed by the Fingerprints Proteomics Facility, University of Dundee. Samples were resuspended in 5% formic acid. Every 8th sample was processed with the HiPPR Detergent Removal Column kit (Thermo Scientific) before analysis. For analysis, 15µl of each sample was injected onto an Ultimate 3000 RSLCnano system (Thermo Scientific) HPLC. Peptides were trapped on an Acclaim PepMap 100 column (C18, 100 µm x 2cm) with 0.1% TFA loading buffer and separated on an Easy-Spray PepMap RSLC C18 column (75 µm x 50 cm, Thermo Scientific). The following buffers were used during separation: buffer A (Table 2.2) and buffer B (Table 2.2), with a flow rate of 0.3 µl/min. The gradient was as follows: 0-5 min, 2% B; 5-130 min, 5-35% B; 130-132 min, 35-98% B; 132-152 min, 98% B; 153-170 min, 2% B. Following separation, peptides were electrosprayed by an Easy-Spray source set at 50°C and source voltage 2.0 kV to a Q-Exactive Plus Mass Spectrometer (Thermo Scientific), which was operated in positive ion mode. Data was acquired in a DDA (data dependent acquisition) mode using a top15 method with a 150 min runtime. Mass scan range was set to 350-1600 m/z and resolution to 70,000.

2.2.9.4 Data analysis

Raw files were searched using MaxQuant version 1.6.2.6. Files were searched against the reviewed mouse proteome database (downloaded August 2017) and the MaxQuant contaminant file. A reverse library was used to identify false matches and the peptide and protein FDR was set to 1%. Minimum peptide length was set to 6. Cysteine carbamidomethylation was included as a fixed modification and methionine oxidation, protein N-terminal acetylation, deamination of asparagine and glutamine and N-terminal conversion of glutamine to pyro-glutamic acid were included as variable modifications. Match between runs was deselected. The proteingroups.txt output file was loaded into the Perseus program, and reverse hits, potential contaminants and proteins identified by site were removed before the calculation of estimated protein copy numbers and cell weights using the proteomic ruler function¹⁹⁶. Proteomics data was analysed using MetaboAnalyst 5.0 software and KEGG pathway analysis was performed using DAVID.

2.2.10 Flow cytometry analysis

Cells were harvested into labelled FACs tubes. 1ml PBS was added to each tube and cells were washed by centrifugation for 5 min. The supernatants were removed, and cell pellets were disrupted (hereafter implied when cells are washed) and stained for analysis using the following protocol. The following control cell samples were prepared: Unstained (for each treatment), single-stained unstimulated CTV, live/dead, TMRM, Mitotracker Green (MTG), Lipidtox Green (LTG), MitoSox and DCF. Single-stained bead controls were prepared for each antibody used.

2.2.10.1 Cell Trace Violet (CTV) Staining

To track the proliferation of T cells *in vitro*, a CTV assay was performed. Before the cells were plated and stimulated, cells were re-suspended in 10 ml PBS and transferred to a clean, dry 50 ml falcon tube. CTV dye was pipetted onto the wall of the falcon tube (Table 2.5). Cells were vortexed and stained for 15 min at 37°C. Some cells were not stained with CTV for other single cell controls. Cells were plated and stimulated for 72 hr as described in 2.2.6.3.2. Analysis of the sequential loss of the CTV dye indicates the round of proliferation.

2.2.10.2 TMRM and Mitotracker Green (MTG) co-staining

For quantification of mitochondrial mass and membrane potential, cells were co-stained with MTG and TMRM dyes. An MTG / TMRM master mix was prepared in warm RPMI medium (Table 2.5). Cells were transferred to labelled FACs tubes, washed with 1ml PBS, and re-suspended in 200µl of the MTG/TMRM cocktail. Cells were stained for 30 min at 37°C. Cells were then washed X1 with PBS for further staining. Cells stained with TMRM and MTG were not fixed and acquired live on the LSR Fortessa cytometer.

2.2.10.3 MitoSox staining

To quantify the production of mitochondrial ROS, cells were stained with MitoSox dye. MitoSox was diluted in warm RPMI medium (Table 2.5). Cells were transferred to labelled FACs tubes, washed with 1ml PBS, and re-suspended in 200µl of the MitoSox master mix. Cells were stained for 15 min at 37°C. Cells were then washed X1 with PBS for further staining. Cells stained with MitoSox were not fixed and acquired live on the FACs Canto cytometer.

2.2.10.4 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) staining

To quantify the production of cellular ROS, cells were stained with H₂DCFDA dye. H₂DCFDA was diluted in warm RPMI. Cells were transferred to labelled FACs tubes, washed with 1ml PBS, and re-suspended in 200µl of the H₂DCFDA master mix. Cells were stained for 15 min at 37°C. H₂DCFDA is deacetylated by cellular esterases to form 2', 7'-dichlorodihydrofluorescein (H₂DCF). In the presence of ROS, H₂DCF is rapidly oxidized to 2', 7'-dichlorofluorescein (DCF) which is highly fluorescent. Cells were then washed X1 with PBS for further staining. Cells stained with H₂DCFDA were not fixed and acquired live on the FACs Canto or LSR Fortessa cytometers.

2.2.10.5 Live cell staining

To ensure gating on live cells only, a live/dead stain was prepared in PBS (Table 2.5). Cells were re-suspended in 50µl of the live/dead master mix and incubated in the dark at room temperature (RT) for 20 min. For single-stain and unstained controls, 50µl of PBS was added. Cells were washed with 1ml PBS for further staining.

2.2.10.6 Extracellular staining and cell fixing

An extracellular fluorochrome-labelled antibody cocktail with Fc block was prepared in FACs buffer (Table 2.2). Cells were re-suspended in 50µl of the master mix and

incubated in the dark at RT for 30 min. For unstained controls, 50µl of FACs buffer was added. Cells were washed with 1ml FACs buffer for further staining or re-suspended in 150µl of FACs buffer prior to acquisition on a FACs Canto or FACs Fortessa. For experiments involving intracellular staining, 100µl of IC fixation buffer was added to each tube and cells were fixed for 30 min at 4°C.

2.2.10.7 Intracellular staining

After cellular fixation, cells were washed X2 with 1ml 1X perm buffer (Table 2.2). An intracellular fluorochrome-labelled antibody cocktail was prepared in 1X perm buffer. Cells were incubated with this master mix at a final volume of 50µl per tube for 25 min. For unstained controls, 50µl of 1X perm buffer was added. Cells were washed with 1X FACs buffer for further staining or re-suspended in 150µl of FACs buffer prior to acquisition on a FACs Canto or LSR Fortessa cytometers.

2.2.10.8 Lipidtox green (LTG) staining

LTG neutral lipid stain was used to determine total cellular lipid levels (intracellular lipids and membrane lipids). A LTG master mix was prepared in PBS (Table 2.5). Cells were re-suspended in 100µl of LTG master mix and incubated at 4°C until acquired on a FACs Canto or LSR Fortessa cytometers. For single-stain and unstained controls, 100µl of PBS was added. LTG stain was not washed off the cells prior to acquisition.

2.2.11 Metabolic Analysis by Seahorse XF analyser. Metabolic phenotyping

A Seahorse XFe-96 Analyzer was used to determine the metabolic phenotype of pure NK or CD8 T cells. Analysis of the extracellular acidification rate (ECAR) of the media is used as a surrogate for glycolytic flux. Cellular oxygen consumption rate (OCR) was measured in real time to indicate oxphos.

2.2.11.1 Cartridge hydration

One day prior to the analysis, the Seahorse XFe-96 sensor cartridge was hydrated overnight with Agilent XF calibrant buffer in a non-CO₂ incubator at 37°C.

2.2.11.2 Cell preparation

Cells were harvested, counted, and washed X2 with seahorse medium (Table 2.2) to remove any pH buffers in the medium the cells were previously cultured in. The seahorse cell culture plate was lined with 200µl of cell-tak (Table 2.2) and incubated for 30 min RT, before being washed X2 with filter-sterilised dH₂O. Cells were re-suspended in seahorse medium at a concentration of 200,000 cells per 180µl. 200,000 cells were added to the wells of the cell-tak coated 96-well XF96 cell culture microplate. 180µl of Seahorse medium was pipetted into remaining wells. To ensure an even monolayer of cells, the cell culture plate was centrifuged for 2 min, 200g, break 0 and acceleration 0. The cell culture plate was placed into a non-CO₂ incubator at 37°C for 20 min before the beginning of the Seahorse run. Meanwhile, working stocks of the metabolic inhibitors – oligomycin, fluoro-carbonyl cyanide phenylhydrazine (FCCP), rotenone, antimycin-A and 2DG – were prepared in Seahorse medium, as per table 2.10. 20µl or 22µl of inhibitor was added to the designated ports. The loaded Seahorse cartridge and utility plate were placed in the Seahorse XFe-96 analyzer and calibrated for 20 min. When the calibration was complete, the utility plate was swapped for the cell culture plate for analysis of cellular metabolism in real time.

Compound	Stock conc	Final conc	Vehicle (Seahorse media) (µl)	Inhibitor (µl)
Oligomycin	2mM	2µM	2970	30
Fccp	1mM	1µM	2980	30
Rot/Anti-A	1mM / 5mM	0.1µM / 4µM	2967	3.6 / 29
2DG	1M	30mM	1819	1181

Table 2.10 List and concentration of inhibitors used in Seahorse analysis

2.2.12 Protein Analysis by Western blotting

Western blotting was performed to determine protein expression in purified NK and CD8 T cells.

2.2.12.1 Protein extraction from purified NK cells or CD8 T cells

Cells were harvested, washed in PBS, and counted. Cells were re-suspended and lysed in 1X sample loading buffer (Table 2.2) containing 20mM DTT, phosphatase and protease inhibitors, at a concentration of 10×10^6 cells per ml. Cell lysates were sonicated for 30 seconds to shear the DNA and subsequently boiled at 99°C for 5 min to denature the proteins. Cell lysates were stored at – 80°C until the day of the experiment and boiled once more before use.

2.2.12.2 SDS-PAGE

Resolving and stacking polyacrylamide gels were prepared as per the recipe (Table 2.7 and 2.8). The proteins in the cell lysates were resolved, based on their molecular weight, through the gel in 1X running buffer (Table 2.2) at 80 V.

2.2.12.3 Protein transfer

A wet transfer was used to electrotransfer the resolved proteins onto a PVDF membrane, activated in methanol. A gel-membrane sandwich was assembled, with each component soaked in 1X transfer buffer (Table 2.2) + 10% methanol and placed into the transfer cassette. The cassettes were loaded into the transfer apparatus and submerged in 1X transfer buffer +10% methanol. An ice block was positioned in the unit to prevent overheating. Proteins were transferred onto the membrane over 2 hr at 45 V. Membranes were incubated with Ponceau S dye for 30 seconds to clarify the quality of the transfer, which was subsequently washed with dH₂O.

2.2.12.4 Membrane blocking, antibody incubation and imaging

To prevent non-specific antibody binding, membranes were incubated with blocking buffer (Table 2.2) for 1 hr at RT with moderate agitation. Membranes were washed X3 with 1X TBST (hereafter implied) for 5 min at RT. Membranes were incubated with primary antibodies diluted in primary antibody dilution buffer (Table 2.2) over night at 4°C with gentle agitation. Membranes were washed and incubated with HRP-conjugated secondary antibodies diluted in secondary antibody dilution buffer (Table 2.2) for 1hr at RT. Membranes were washed and incubated with ECL substrate. Images were developed on a Biorad Gel Doc imaging system, based on the luminescence produced by HRP-ECL chemical reaction.

2.2.13 RNA analysis

To minimize RNA degradation, all steps were performed on ice unless otherwise stated, the centrifuge was cooled to 4°C, all tubes used were RNase / DNase free, all pipette tips were sterile and filtered, equipment and tools were sprayed with ethanol and 1% SDS before working with RNA and a lab coat was always worn.

2.2.13.1 RNA isolation from cells

For gene expression analysis by qPCR, NK or CD8 T cells were first purified by magnetic separation and stimulated as described (2.2.6.2 and 2.2.6.3). Following stimulation, cells were harvested, supernatants discarded, and cells were washed X2 in ice cold PBS. Cells were lysed in 300µl RNA lysis buffer and RNA lysates were stored at -80°C until purification. The PureLink™ RNA Mini Kit was used to isolate RNA according to the manufacturer's instructions. Isolated RNA samples were stored at -80°C.

2.2.13.2 RNA isolation from liver and adipose tissues

Tissues were snap frozen in liquid nitrogen and stored at -80°C. Tissues were defrosted at RT on a shaker and transferred to a 2ml tube containing a 5mm stainless steel bead. 1ml trizol reagent was added to each tube. Tissues were homogenized in a tissue lyser for 2 min, 25 pulses / sec. Homogenized tissues were transferred to a clean Eppendorf tube. 400µl chloroform was added to each tube without re-suspending. Tubes were inverted once and left at RT for 2-3 min. Tubes were centrifuged at 12,000×g for 15 min. The aqueous phase containing RNA was transferred into a new Eppendorf tube. 500µl isopropanol was added to each tube to precipitate the RNA. Tubes were inverted X10 and left at RT for 10 min. Tubes were centrifuged at 12,000×g for 10 min. Supernatants were discarded and 1ml 75% ethanol, diluted in RNase free dH₂O, was added to each tube. Tubes were centrifuged at 12,000×g for 5 min. Supernatants were removed by pipetting. The RNA pellet was left to dry at RT. The pellets were re-suspended in 50µl RNase free water. RNA was left on ice for 30 min and samples were stored at -80°C.

2.2.13.3 Quantification of RNA and quality assessment

RNA quality and concentration was determined using a Nanodrop 2000 UV spectrophotometer. The nanodrop was cleaned with lint-free tissue and blanked with RNase free water. 1µl of RNA sample was ran and the concentration (ng/µl), and the absorbance (nm) ratios at 260 / 280 and 260 / 230 were recorded. A 260 / 280 and 260

/ 230 ratio of ~ 2 indicates a pure RNA yield. Lower ratios may indicate DNA or phenol contamination from the extraction protocol.

2.2.13.4 cDNA synthesis

20µl of cDNA was synthesized from 1µg of isolated RNA using the high-capacity cDNA reverse transcription kit. RNA was normalized and diluted to a final volume of 13.2µl in RNase free water and added to 6.8µl of reverse transcription mix (Table 2.11). Samples were centrifuged at 500×g for 20 sec and placed into a MiniAmp Plus thermal cycler and ran according to the program in Table 2.12. cDNA was diluted 1:10 with RNase free water. Samples were stored at -20°C until qPCR was performed.

Component	Volume per reaction (µl)
10X reverse transcription buffer	2
25X 100mM dNTP	0.8
10X random primers	2
RNase inhibitor	1
Reverse transcriptase	1

Table 2.11. List of components in reverse transcription mix for cDNA synthesis from mRNA

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

Table 2.12. Thermal cycler program for reverse transcription of mRNA

2.2.13.5 Quantitative real time PCR (qPCR)

To quantify the relative mRNA expression of genes of interest, qPCR was performed in a 96 or 384 well plate format using SYBR Green based detection. 2µl of cDNA was pipetted into a MicroAmp optical reaction plate in triplicate. 8µl of qPCR master mix

(Table 2.13) was added to each well. β -actin, PPIB or RpLp0 were used as housekeeping genes for mRNA normalization. The plate was sealed with MicroAmp optical adhesive film and centrifuged at 300×g for 30 sec. The plates were run on a StepOnePlus™ or QuantStudio™ 5 real-time PCR system (Table 2.14). Relative mRNA levels were calculated using the $\Delta\Delta$ cycle threshold ($\Delta\Delta C_t$) method. Threshold cycle values (C_t) of target genes were normalized to corresponding endogenous controls (ΔC_t). The ΔC_t value of control samples were subtracted from ΔC_t values of experimental samples ($\Delta\Delta C_t$). A fold change in expression of the target gene relative to the control sample was calculate by $2^{(-\Delta\Delta C_t)}$.

Reagent	Volume (μl)
Forward Primer	0.4
Reverse Primer	0.4
SYBR Green FastMix	5
RNAse free H ₂ O	2.2
cDNA	2

Table 2.13. SYBR Green qPCR master mix

Stage	Temperature	Time	Cycles
1	95°C	1 min	1
2	95°C	1 sec	35
2	57°C	20 sec	35
3	95°C	15 sec	1
3	55°C	30 sec	1
3	95°C	15 sec	1

Table 2.14. qPCR run set up

2.2.14 Fatty acid analysis

Fatty acid methyl esters (FAMES) were prepared using the MARS 6 express 40 position microwave reaction system and quantified by gas chromatography (GC) as previously

described¹⁹⁷. Briefly, diet pellets were minced and 0.5g of sample was added to an Xpress vessel tube containing a 10 mm stir bar. 10ml 2.5% potassium hydroxide (KOH) in methanol and 100µl internal standard (ISTD) (10mg/ml C23:0-Methyl Ester in heptane) was added to each tube. Samples were loaded into the microwave reaction system for saponification. The reaction vessels were heated to 130°C over 4 min and held at 130°C for a further 4 min. Samples were removed and cooled on ice for 5 min. 15ml 5% acetyl chloride in methanol was added to each tube. Samples were loaded into the microwave reaction system for esterification. The reaction vessels were heated to 120°C over 4 min and held at 120°C for a further 2 min. Samples were removed and cooled on ice. To extract FAMES, 10 ml of pentane was added to each reaction vessel and gently mixed. 20ml of saturated NaCl solution in dH₂O was added to each tube and gently mixed, allowing the aqueous (bottom) and organic (top, pentane) layers to separate. 1ml of the top, pentane layer was transferred into a GC vial containing 0.2g anhydrous Na₂SO₄.

Quantification of FAMES was performed using an Agilent 7890B gas chromatograph fitted with a flame ionisation detector (FID). Analytes were separated using a CP-Sil 88 capillary with a 100 m × 0.25 mm internal diameter × 0.2µm film thickness column. The oven was heated to 220°C from 80°C at 6.2°C/min, held for 3.2min, heated to 240°C at 6.3°C/min, where it was held for 6.5 min. The injector temperature was 270°C and 0.5µl of sample was injected with the split set to 10:1. The carrier gas used was hydrogen with a constant flow rate of 1.25 ml/min. The temperature of the FID was maintained at 270°C. Compounds were identified by comparing their retention times with FAME Supelco standards. Peak area analysis was conducted using Agilent OpenLAB CDS 2.1 Workstation. The content of each fatty acid was calculated according to the following equation:

$$(\text{Peak Area (FAME)} / \text{peak area (ISTD)}) \times (\text{Weight ISTD} / \text{weight sample}) \times (\text{ISTD purity}) \times 10 = \text{content (mg/g of diet pellet)}.$$

2.2.15 Histological analysis of liver sections

Oil red o staining of liver sections was performed to analyse lipid and triglyceride levels. Oil red o stock solution was prepared in isopropanol (300mg/100ml) one day prior to experimentation. Livers were washed in PBS and frozen in OCT cryomolds. Frozen tissues were sectioned to 5µm thickness using a cryostat and air dried on a microscope slide for 30 min. Sections were fixed in 10% formalin for 10 min, washed in 60%

isopropanol in dH₂O and stained with oil red o solution (30ml of oil red o stock solution in 20ml dH₂O) for 15 min. Slides were washed with 60% isopropanol and dH₂O before staining nuclei with haematoxylin for 3 min. Slides were dipped in dH₂O X10 and a coverslip was mounted with aqueous mounting gel. Tissue images were developed using an Olympus BX51 upright microscope. Images were analysed using ImageJ software to quantify the % area of oil red o-stained lipids.

2.2.16 Statistical Analysis

GraphPad Prism 9.3.0 was used for statistical analysis. For all experiments, a 95% confidence interval was used and a p-value ≤ 0.05 was considered statistically significant. A D'Agostino-Pearson omnibus normality test was first performed to test if the data was normally distributed (Gaussian distribution). If the n number was too small to perform a D'Agostino-Pearson omnibus normality test, normal distribution was assumed. If data was normally distributed, parametric testing was performed. If data was not normally distributed, non-parametric testing was performed. When comparing 2 groups, an unpaired two-tailed student's t-test was used. When comparing more than 2 groups, an ordinary one-way ANOVA with Dunnett test was used. When comparing data with two variables, a two-way ANOVA with Bonferroni test was used. For correlation analysis, simple linear regression was used. In all figures, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

2.2.17 Gating strategies

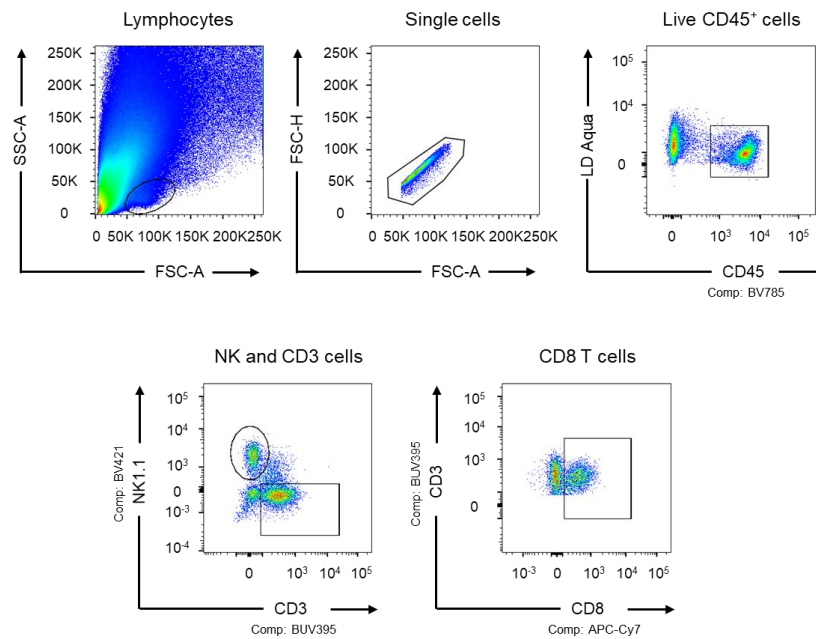


Figure 2.1 Gating strategy to identify tumoral NK and CD8 T cells

Tumours were isolated and cells were stained for flow cytometry as described in 2.2.2.2 and 2.2.10. Single lymphocytes were selected based on forward and side scatter. Dead cells and CD45⁻ non-immune cells were removed. NK cells were selected as NK1.1⁺CD3⁻ and CD8 T cells were selected as CD3⁺CD8⁺ cells.

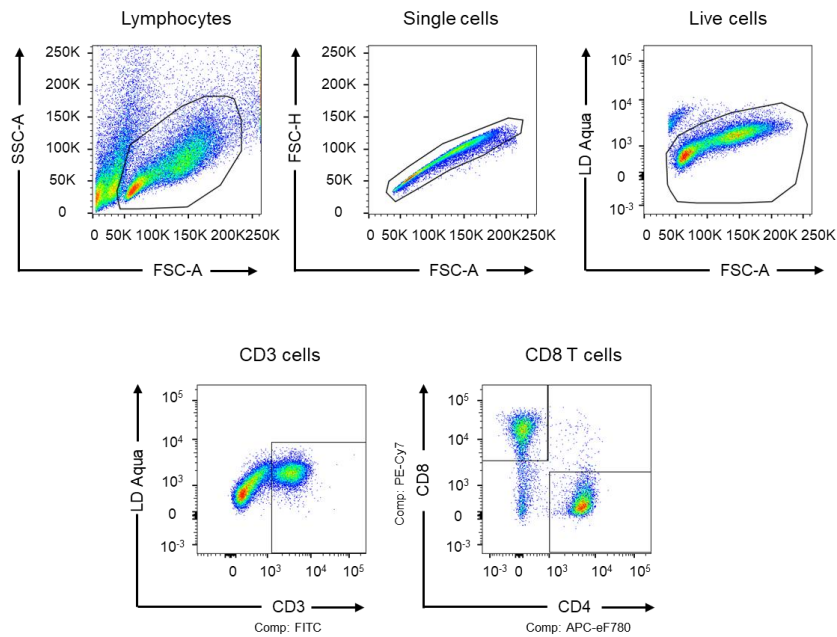


Figure 2.2 Gating strategy to identify CD8 T cells following *in vitro* culture

Single lymphocytes were selected based on forward and side scatter. Dead cells were removed, and CD8 T cells were selected as CD3⁺CD8⁺CD4⁻ cells.

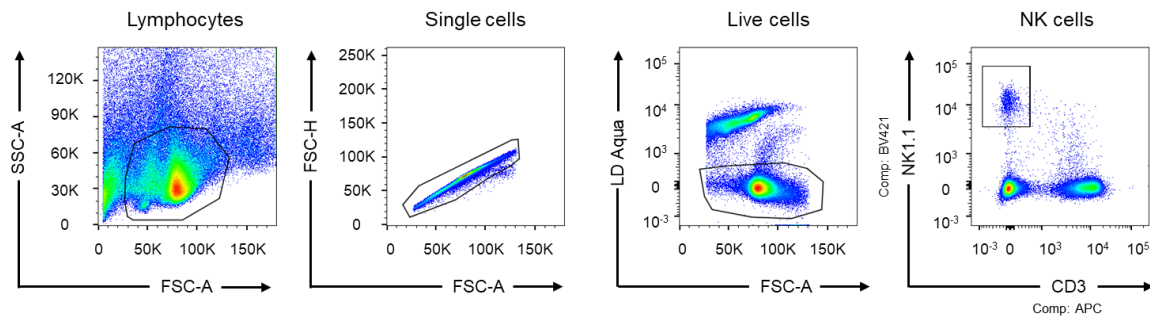


Figure 2.3 Gating strategy to identify NK cells following *in vitro* culture

Single lymphocytes were selected based on forward and side scatter. Dead cells were removed, and NK cells were selected as CD3⁻NK1.1⁺ cells.

Chapter 3. High-fat diet alters the plasma
metabolome of mice, which is restored by weight
loss

3.1 Introduction

Obesity is an established risk factor for the development of at least 14 types of cancers including endometrial, oesophageal, and liver cancers^{104,105}. Whole body metabolic changes including increased production of adipokines, impaired glucose handling, and fatty liver all favour tumour growth and metastasis in obesity¹⁹⁸. In addition, we and others found that obesity inhibits NK and CD8 T cell function leading to impaired immunosurveillance^{37,113}. NK cells from obese mice and humans accumulate lipids resulting in metabolic paralysis and reduced capacity to produce IFN γ and kill tumour cells³⁷. Similarly, CD8 T cells in the obese tumour are functionally and metabolically impaired and fail to control tumour growth^{114,113}. Lipids are also abundant in many tumours⁸⁵ and can fuel tumour cells to enhance their proliferation and metastasis while also fostering an pro-tumour immune environment¹⁹⁹. NK and CD8 T cells adapt to these lipid rich environments by activating lipid metabolism transcriptional programs and this is consequently linked to their dysfunction^{37,85}.

Bariatric surgery is commonly used to induce WL in individuals with obesity¹²³ and has beneficial effects on the anti-tumour functions of NK and CD8 T cells by enhancing their proliferation, cytotoxicity and IFN γ production¹²⁴. However, less is known about the effects of WL through dietary intervention on NK and CD8 T cell function and metabolism. Immune cells rewire their cellular metabolism in response to nutrients in their microenvironment and this is shaped by the diet¹⁸². Here we hypothesised that a HFD would alter systemic metabolism, and this may impair anti-tumour NK and CD8 T cell responses. In addition, we tested the hypothesis that WL can remodel the systemic metabolome and therefore may restore some anti-tumour functions of NK and CD8 T cells.

3.2 Weight loss following high fat diet restores systemic metabolic flexibility in mice

To study the effects of HFD and WL on systemic metabolism and anti-tumour immune responses, we used a mouse model of DIO and WL. Wild-type mice were fed a 60% HFD from animal lard or a SFD, as per Fig 3.1. 8 week HFD feeding caused significant weight gain and significantly increased the weight of the visceral, subcutaneous, and brown adipose depots. To induce WL, mice received a SFD after 6 weeks of HFD feeding which resulted in a similar body weight to mice fed SFD at the endpoint. Mice fed a HFD for 1-week gained weight but did not become obese (Fig. 3.2 A-E).

We next investigated the effects of HFD and WL on food consumption, activity, and systemic metabolism using Promethion metabolic cages. Mice fed a HFD consumed less food than mice fed a SFD, whereas mice fed a HFD for 1 week consumed significantly more food than mice fed a SFD. Mice that lost weight consumed a similar amount of food to mice fed a SFD (Fig. 3.3). Mice fed a HFD were less active than mice fed a SFD and mice that lost weight, which had a similar level of activity (Fig. 3.4). These data confirm that WL is not due to changes in food consumption or activity, but rather changes in diet. Despite reduced activity, mice fed a HFD expended more energy than mice fed a SFD or mice that lost weight, likely due to heightened lipid metabolism (Fig. 3.5). Typically, mice fed a SFD alternate between carbohydrate and lipid metabolism during the dark-light cycle, which can be measured by the RER¹⁸⁰. Indeed, HFD disrupts the metabolic circadian rhythm of mice, resulting in reduced RER, indicating a switch to lipid metabolism over the full 24hrs. WL reversed this metabolic defect and restored metabolic flexibility, allowing for oxidation of both lipids and carbohydrates (Fig. 3.6).

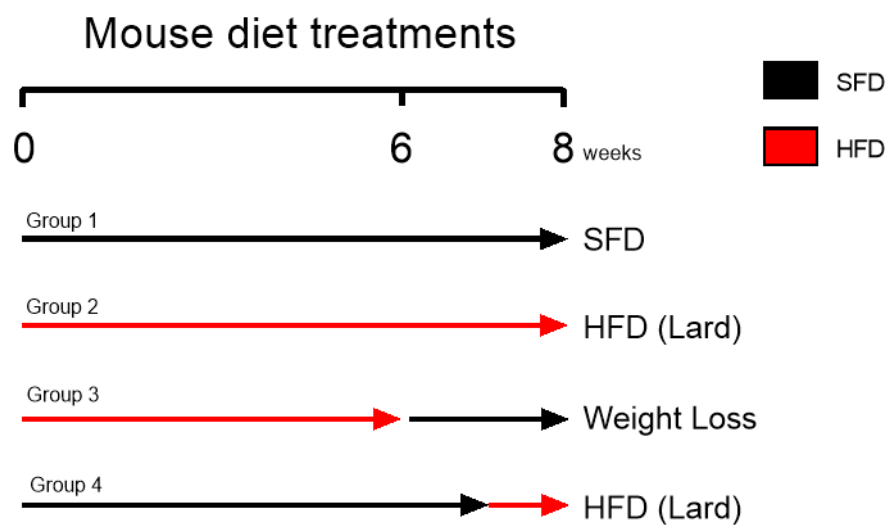


Figure 3.1 Murine weight loss experimental design

Mice were fed a high-fat diet (HFD, 60% fat) from lard for 8 weeks. After 6 weeks, one group of mice were fed a standard fat diet (SFD, 12% fat) in place of a HFD to induce weight loss. Another group of mice were fed a HFD for 1 week.

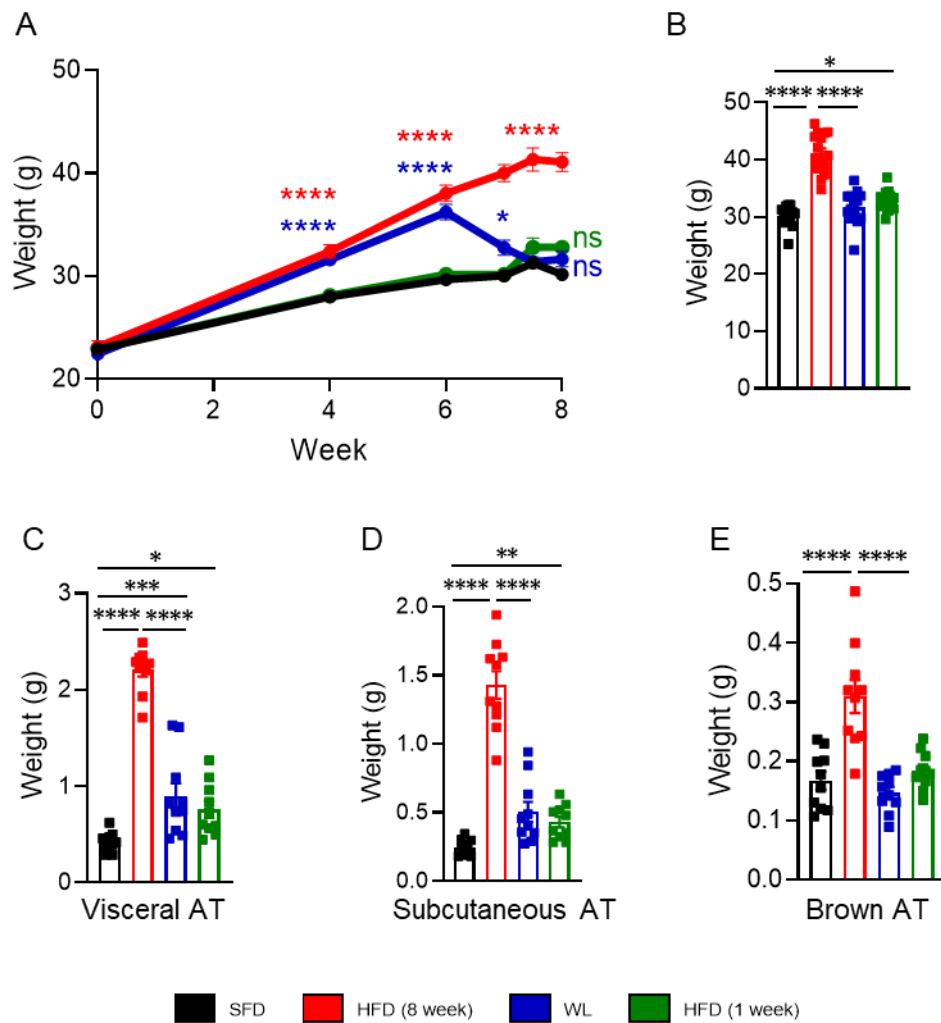


Figure 3.2 HFD-induced weight gain is rapidly reversed when mice are fed a SFD for 2 weeks

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Graphs show weight gain over 8 weeks **(A)**, final body weight **(B)**, and weight of visceral, subcutaneous, and brown adipose tissues (AT) **(C-E)**. Data are mean $\pm$ S.E.M, pooled data from N=3/2 independent experiments, n=10/15 mice per group. Squares represent individual mice. Significance was calculated using two-way ANOVA with Bonferroni test **(A)**, an ordinary one-way ANOVA with Dunnett test **(B-E)**. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

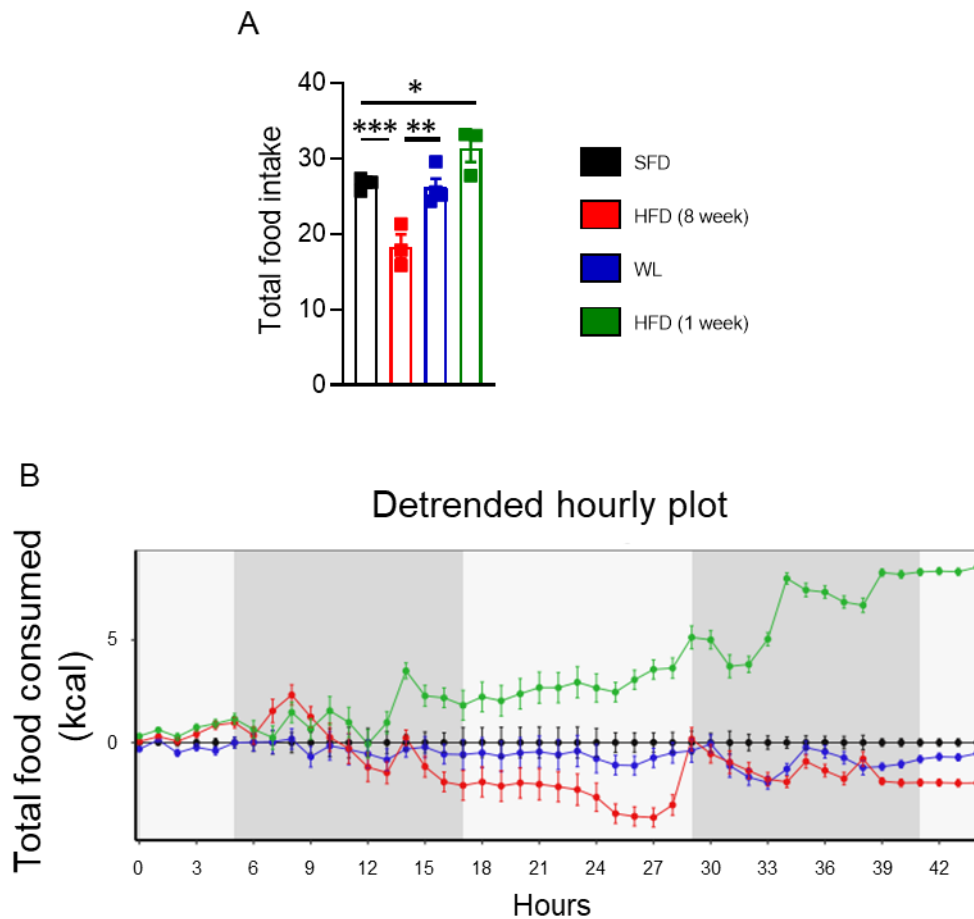


Figure 3.3 HFD feeding disrupts the food consumption of mice, which is restored when mice lose weight

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Mice were singly housed in Promethion metabolic cages for 2 days. Graphs show total food consumed (kcal) in one day (**A**), or over 45 hr (**B**). Data are mean $\pm$ S.E.M from 1 experiment, $n=4$ mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

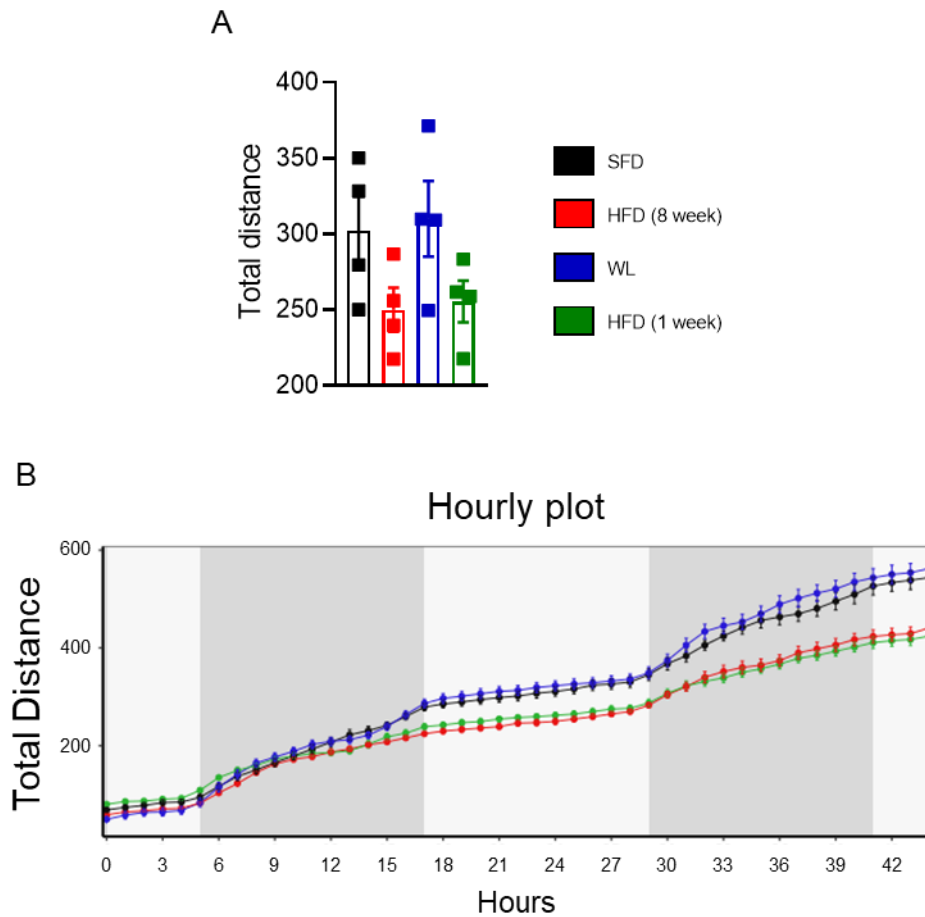


Figure 3. 4 Mice fed a HFD are less active than mice fed a SFD and mice that lost weight

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Mice were singly housed in Promethion metabolic cages for 2 days. Graphs show total distance covered in one day (**A**), or over 45 hr (**B**). Data are mean $\pm$ S.E.M from 1 experiment, $n=4$ mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test.

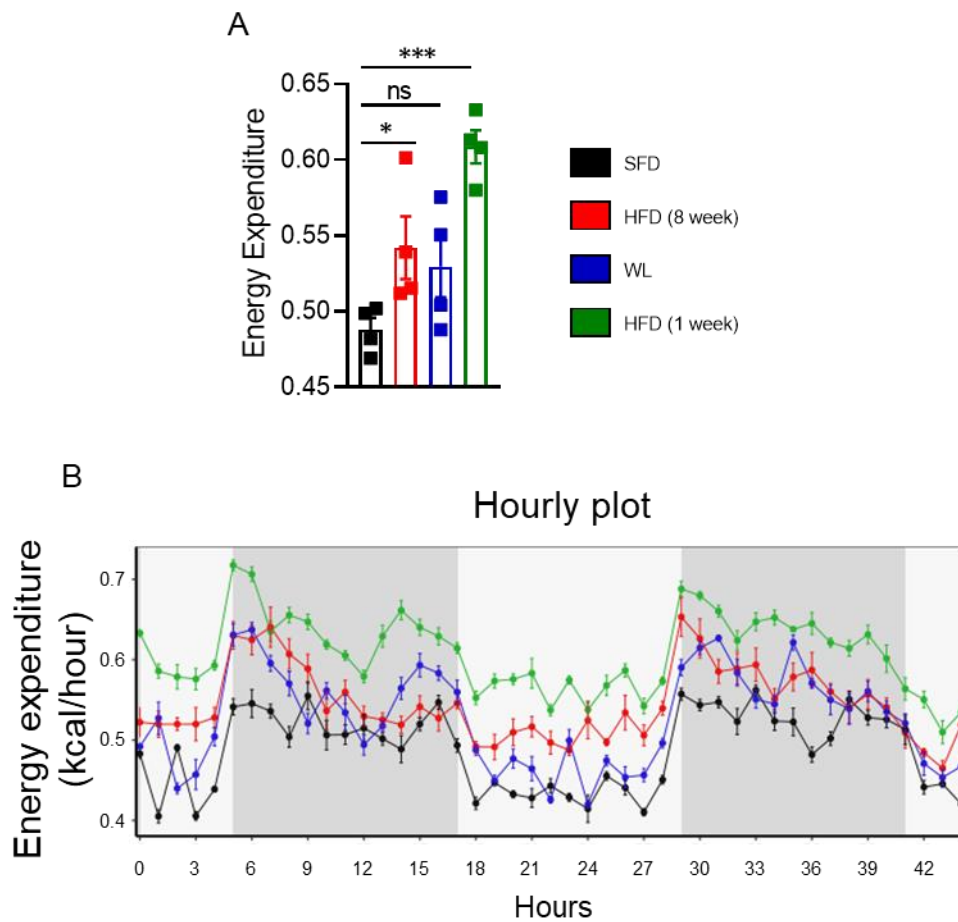


Figure 3. 5 Mice fed a HFD expend more energy than mice fed a SFD

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Mice were singly housed in Promethion metabolic cages for 2 days. Graphs show energy expenditure (kcal/hour) in one day (**A**), or over 45 hr (**B**). Data are mean $\pm$ S.E.M from 1 experiment, $n=4$ mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. ns= not significant, * $p \leq 0.05$, *** $p \leq 0.001$.

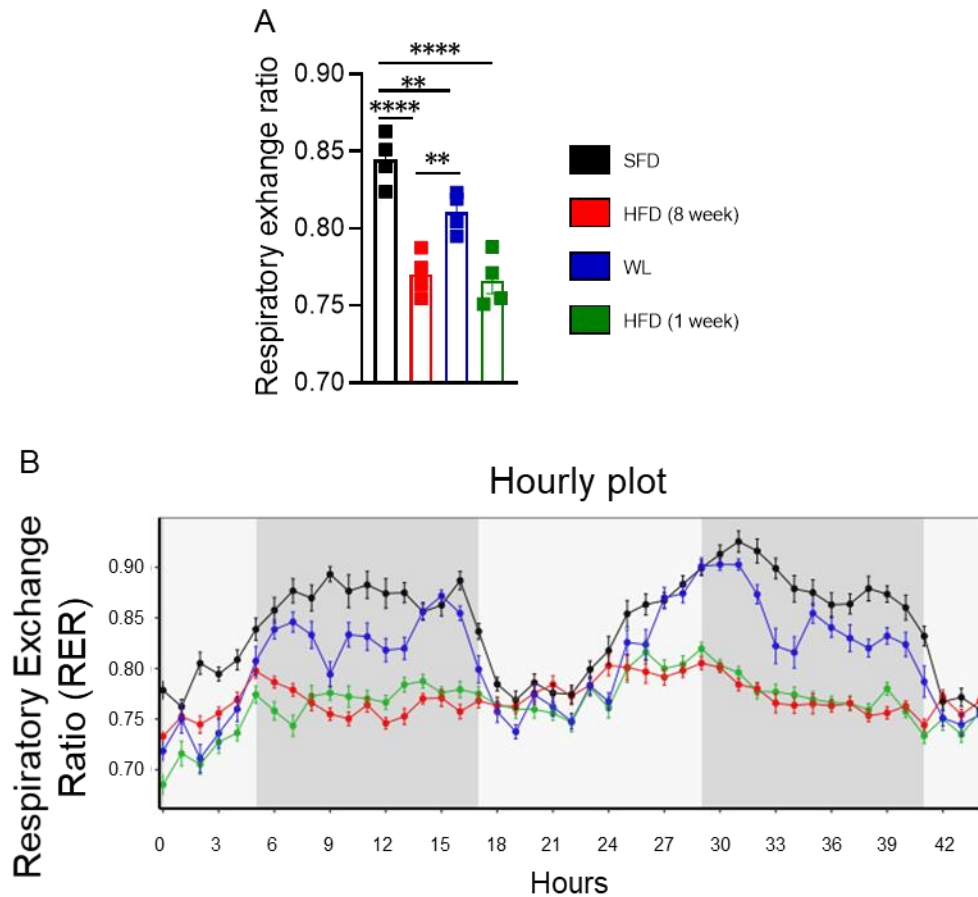


Figure 3. 6 HFD feeding reduces the respiratory exchange ratio of mice, which is partially restored when mice lose weight

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Mice were singly housed in Promethion metabolic cages for 2 days. Graphs show respiratory exchange ratio (RER) in one day **(A)**, or over 45 hr **(B)**. Data are mean $\pm$ S.E.M from 1 experiment, $n=4$ mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. ns= not significant, ** $p \leq 0.01$, **** $p \leq 0.0001$.

3.3 Splenic NK cells from mice fed a high fat diet do not accumulate lipids and are functionally and metabolically intact

The Lynch lab has shown that NK cells from obese mice and humans accumulate lipids which interferes with mTORC1 activation, leading to impaired IFN γ and Granzyme B (GzB) production³⁷. Therefore, we next investigated if WL could restore NK cell immunometabolic responses. Surprisingly, HFD, or WL did not affect the expression of the activation marker CD69, IFN γ , or GzB by splenic NK cells (sNK) (Fig. 3.7).

To support the production of effector molecules, NK cells increase the rates of glycolysis and oxphos^{8,77}. However, NK cell metabolism is blunted when cells are cultured with lipids or isolated from individuals with obesity³⁷. HFD or WL did not affect the rates of sNK cell glycolysis (Fig. 3. 8) or oxphos (Fig. 3. 9). In contrast, 1 week HFD feeding increased the rates of sNK cell glycolysis and oxphos (Fig. 3.8 and 3.9).

NK cell metabolic reprogramming is controlled by the transcription factor c-Myc, which is regulated by mTORC1 and amino acid transport through Slc7a5⁸⁹. Activation of PPAR-dependent lipid metabolism pathways inhibits mTORC1 and cMyc in NK cells from obese mice and humans³⁷. However, HFD did not affect the levels of c-Myc and pS6, a readout of mTORC1 activation, in sNK cells (Fig. 3.10). cMyc and S6 phosphorylation were higher in sNK cells from mice fed a HFD for 1 week (3.10), aligning with increased rates of glycolysis and oxphos. Furthermore, HFD or WL did not affect the expression of CD71, a known c-Myc target gene, or CD98, a component of system L-amino acid transporters, on sNK cells (Fig. 3.11), together demonstrating that HFD does not affect the function or metabolism of sNK cells.

As we did not observe any functional or metabolic defects in sNK cells, we hypothesised that sNK cells do not accumulate lipids when mice are fed a HFD. Indeed, HFD, or WL did not alter the lipid levels of sNK cells (Fig. 3.12). Given that NK cells isolated from the blood of patients with obesity stain strongly for lipids, we next examined the lipid levels in circulating NK cells (cNK) vs sNK cells from mice fed a HFD. Again, sNK cells did not accumulate lipids when mice were fed a HFD (Fig. 3.13 A, B). In contrast, HFD feeding significantly increased the lipid content of cNK cells by 2-fold compared to cNK cells from mice fed a SFD (Fig. 3.13 C, D).

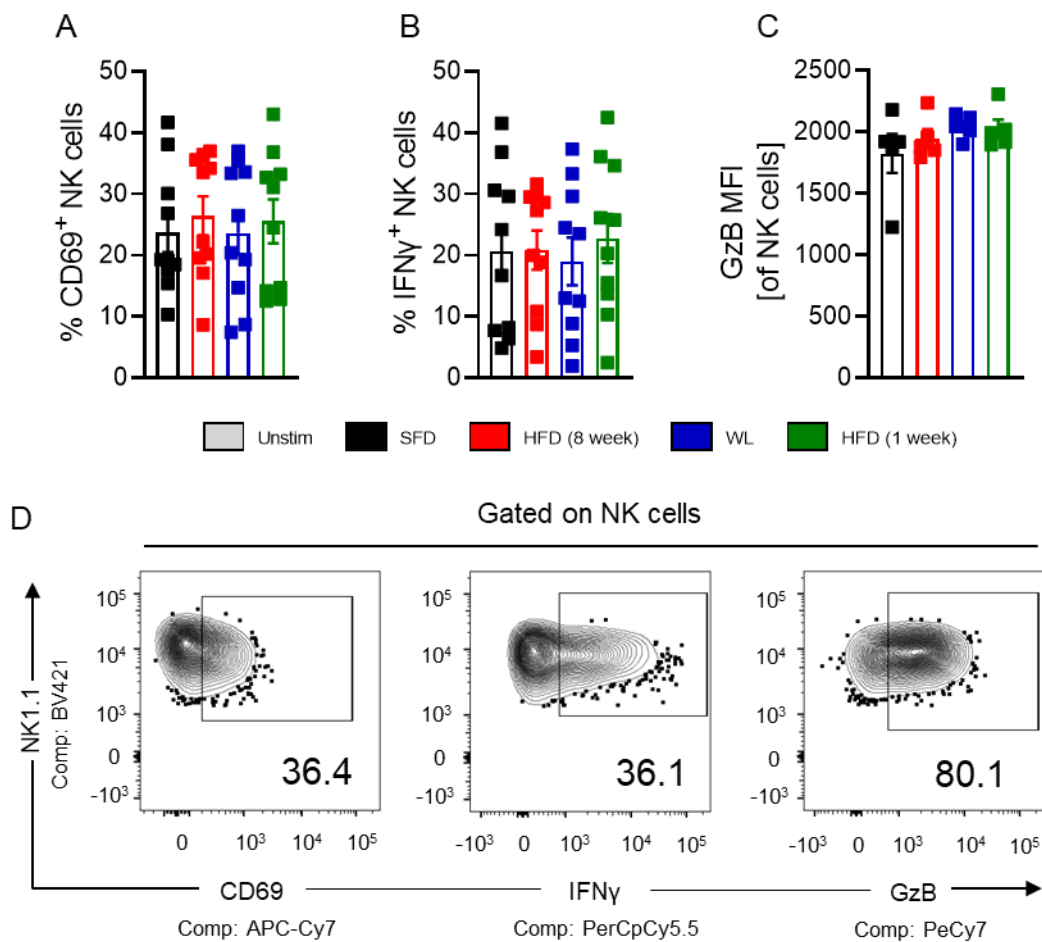


Figure 3. 7 HFD feeding does not affect splenic NK cell activation or expression of IFN γ and granzyme B

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Splenocytes were stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr and NK cells were analysed by flow cytometry. Graphs show percentage of CD69⁺, IFN γ ⁺ NK cells and GzB mean fluorescence intensity (MFI) (**A-C**), and representative flow plots (**D**). Data are mean $\pm$ S.E.M from N=1/2 independent experiments, n=5/10 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

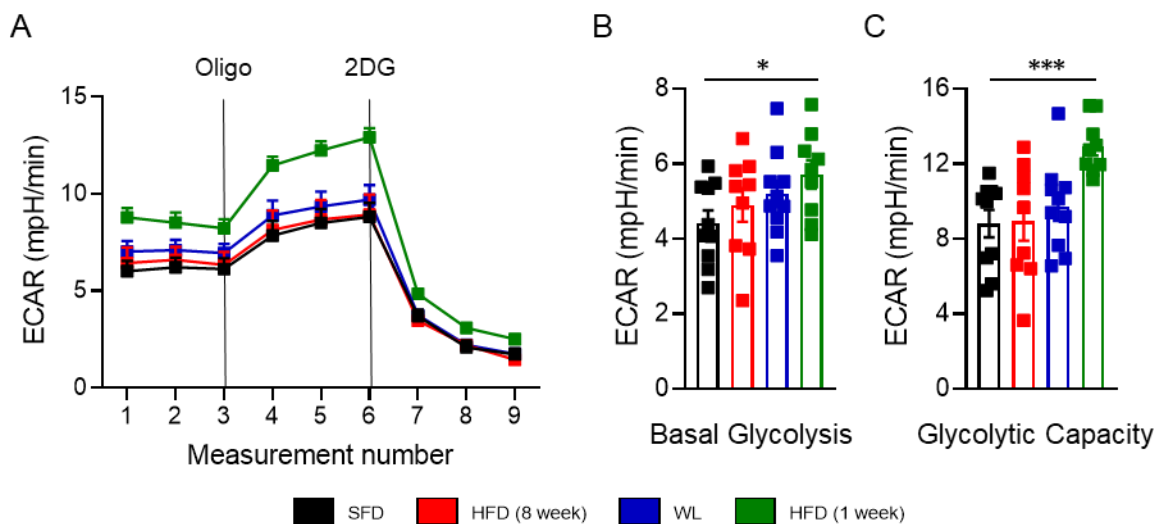


Figure 3.8 Short-term HFD feeding increases the rates of glycolysis in splenic NK cells

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 13% fat) for 8 weeks as per experimental design (Fig. 3.1). Splenic NK cells were purified and stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr. Extracellular acidification rate (ECAR) was measured by Seahorse analysis following injection of the metabolic inhibitors oligomycin (oligo) and 2-deoxyglucose (2DG). Graphs show representative trace **(A)** and pooled measurements for basal glycolysis **(B)** and glycolytic capacity **(C)**. Data are representative **(A)** or mean $\pm$ S.E.M **(B, C)** of N=2 independent experiments. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, *** $p \leq 0.001$.

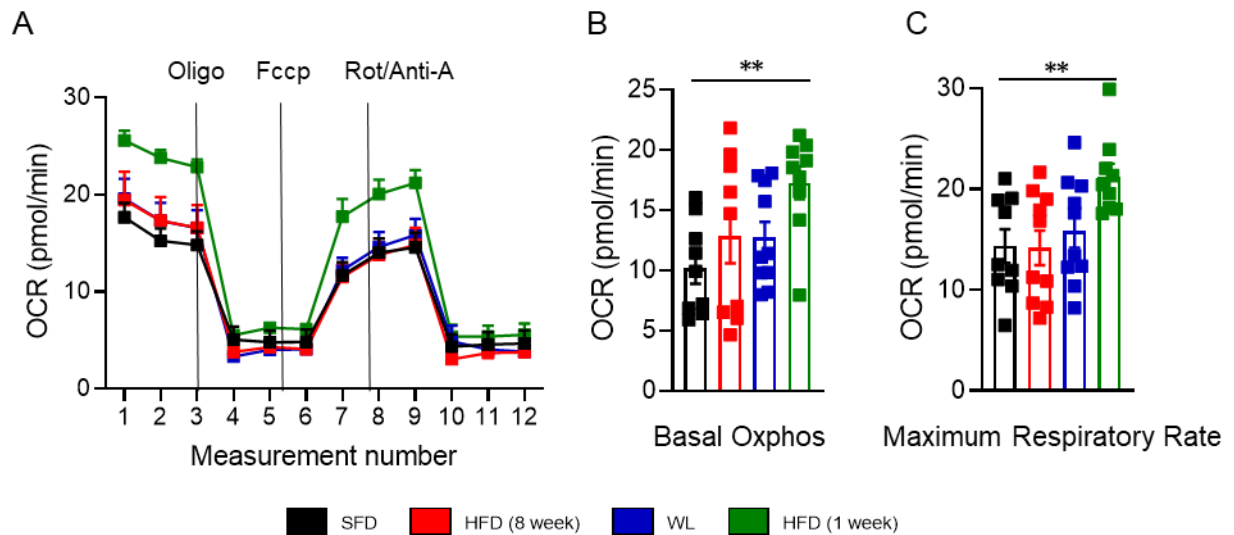


Figure 3.9 Short-term HFD feeding increases the rates of oxphos in splenic NK cells

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Splenic NK cells were purified and stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr. Oxygen consumption rate (OCR) was measured by Seahorse analysis following injection of the metabolic inhibitors oligomycin (oligo), FCCP, rotenone and antimycin A (Rot/Anti-A). Graphs show representative trace **(A)** and pooled measurements for basal oxphos **(B)** and maximum respiratory rate **(C)**. Data are representative **(A)** or mean $\pm$ S.E.M **(B, C)** of N=2 independent experiments. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. ** $p \leq 0.01$.

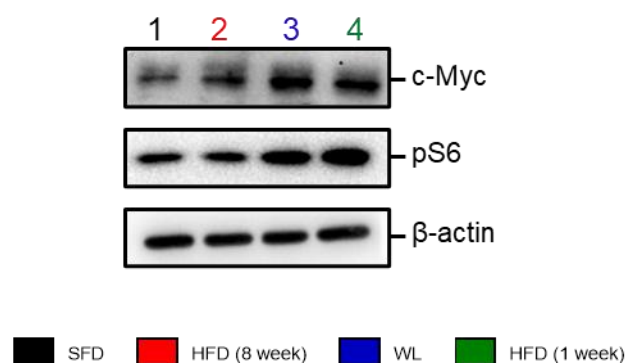


Figure 3.10 HFD does not affect the protein expression of c-Myc or pS6 in splenic NK cells

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Figure 3.1). Splenic NK cells were isolated and stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr. Expression of c-Myc (57-70 kDa) and pS6 (32 kDa) were analysed by western blotting. β-actin (42kDa) was used as a loading control. Graphs show representative blot for N=2 independent experiments, n=10 mice per group.

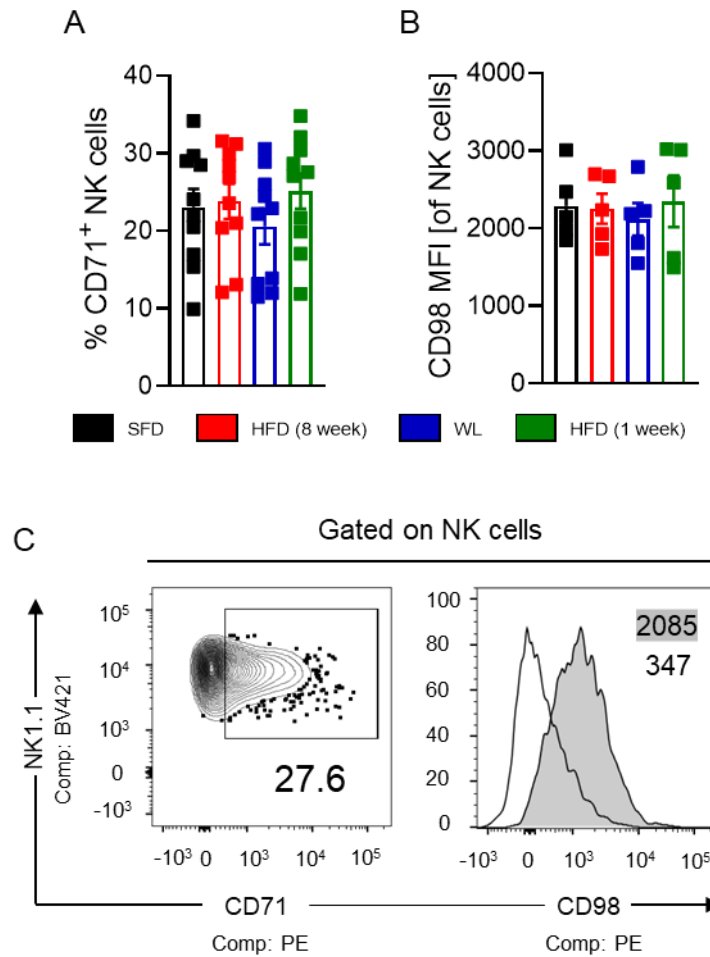


Figure 3.11 HFD does not affect the expression of CD71 or CD98 in splenic NK cells

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Figure 3.3). Splenocytes were stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr. NK cells were analysed by flow cytometry. Graphs show percentage of CD71⁺ NK cells (**A**), CD98 mean fluorescence intensity (MFI) of NK cells (**B**), and representative flow plot and histogram (**C**). Data are mean $\pm$ S.E.M from N=1/2 independent experiments, n=5/10 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

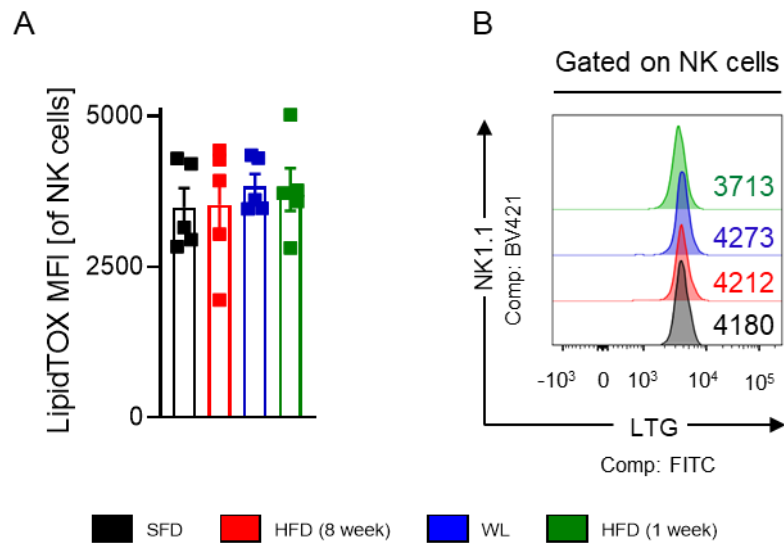


Figure 3.12 HFD-feeding does not alter the lipid content of splenic NK cells

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Splenocytes were stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr. LipidTOX Green (LTG) staining was used to analyse total lipid levels of NK cells by flow cytometry. Graphs show LTG mean fluorescence intensity (MFI) of NK cells **(A)**, and representative histogram **(B)**. Data are mean $\pm$ S.E.M from N=1 experiment, n=5 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

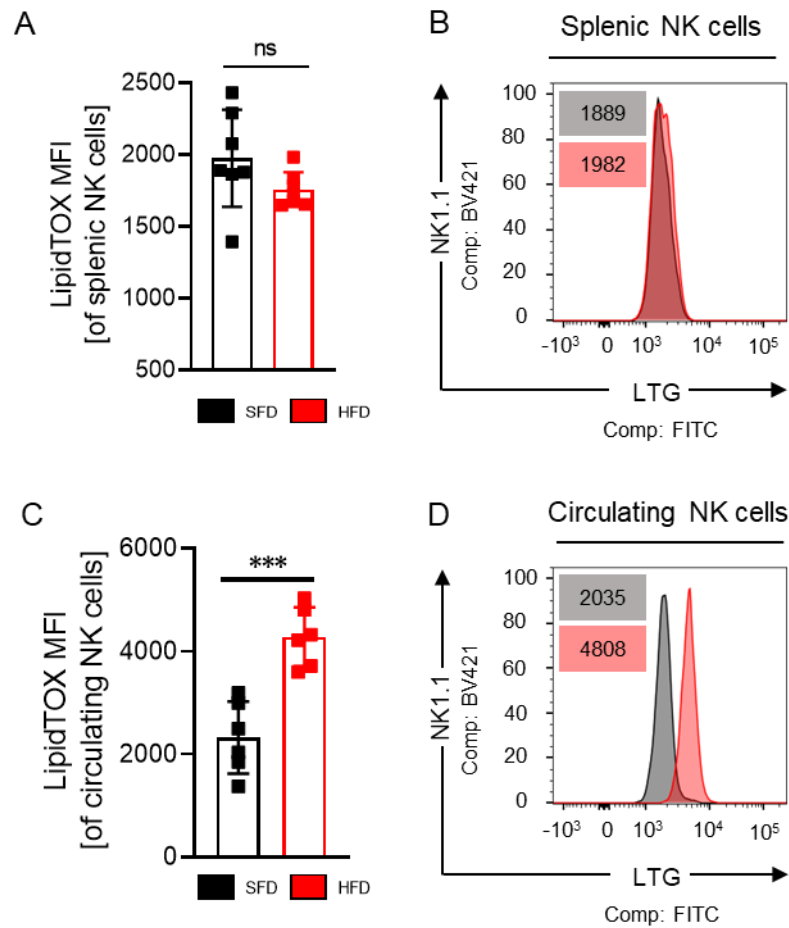


Figure 3.13 Circulating, but not splenic NK cells stain strongly for lipids when mice are fed a HFD

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 13% fat) for 8 weeks as per experimental design (Fig. 3.1). Splenocytes were stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr. Circulating cells were stimulated with PMA and ionomycin for 4hr. LipidTOX Green (LTG) was used to analyse total lipid levels by flow cytometry. Graphs show LTG mean fluorescence intensity (MFI) of matched splenic and circulating NK cells (**A**, **C**), and representative histograms (**B**, **D**). Data are mean $\pm$ S.E.M from N=1 experiment, n=6 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ns= not significant, *** $p \leq 0.001$. *Experiment performed with Dr Lydia Dyck.*

3.4 Circulating NK cells have reduced phosphorylation of S6 when mice are fed a HFD, which is restored by weight loss

The total lipid levels of cNK cells from mice that lost weight was similar to cNK cells from mice fed a SFD and significantly decreased compared to cNK cells from mice fed a HFD. cNK cells from mice fed a HFD for 1 week did not accumulate lipids, indicating that lipid uptake by cNK cells only occurs with chronic HFD feeding (Fig. 3.14).

Given that lipid accumulation impairs NK cell functional responses³⁷, we hypothesised that the functional capacity of cNK cells would be reduced by HFD and restored with WL. There was a slight but non-significant reduction in the number of IFN γ producing cNK cells when mice were fed a HFD, but not when mice lost weight (Fig. 3.15). Furthermore, cNK cells had reduced phosphorylation of S6 when mice were fed a HFD, and this was restored when mice lost weight (Fig. 3.16 A, B). Despite no increase in body weight or cellular lipids, IFN γ expression was slightly decreased and phosphorylation of S6 was reduced in cNK cells when mice were fed a HFD for 1 week (Fig. 3.15, 3.16, 3.2, 3.14). Together, these data indicate that HFD, rather than body weight or cellular lipid levels interferes with IFN γ expression and particularly the phosphorylation of S6 in cNK cells.

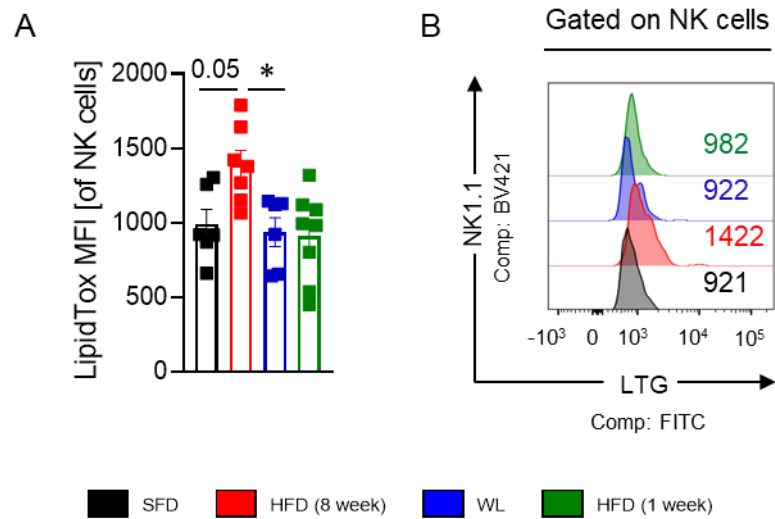


Figure 3.14 Weight loss following HFD feeding decreases the intracellular lipid content of circulating NK cells

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Circulating cells were stimulated with PMA and ionomycin for 4hr. LipidTOX Green (LTG) was used to analyse total lipid levels by flow cytometry. Graphs show LTG mean fluorescence intensity (MFI) of NK cells **(A)**, and representative histogram **(B)**. Data are mean $\pm$ S.E.M from N=1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$.

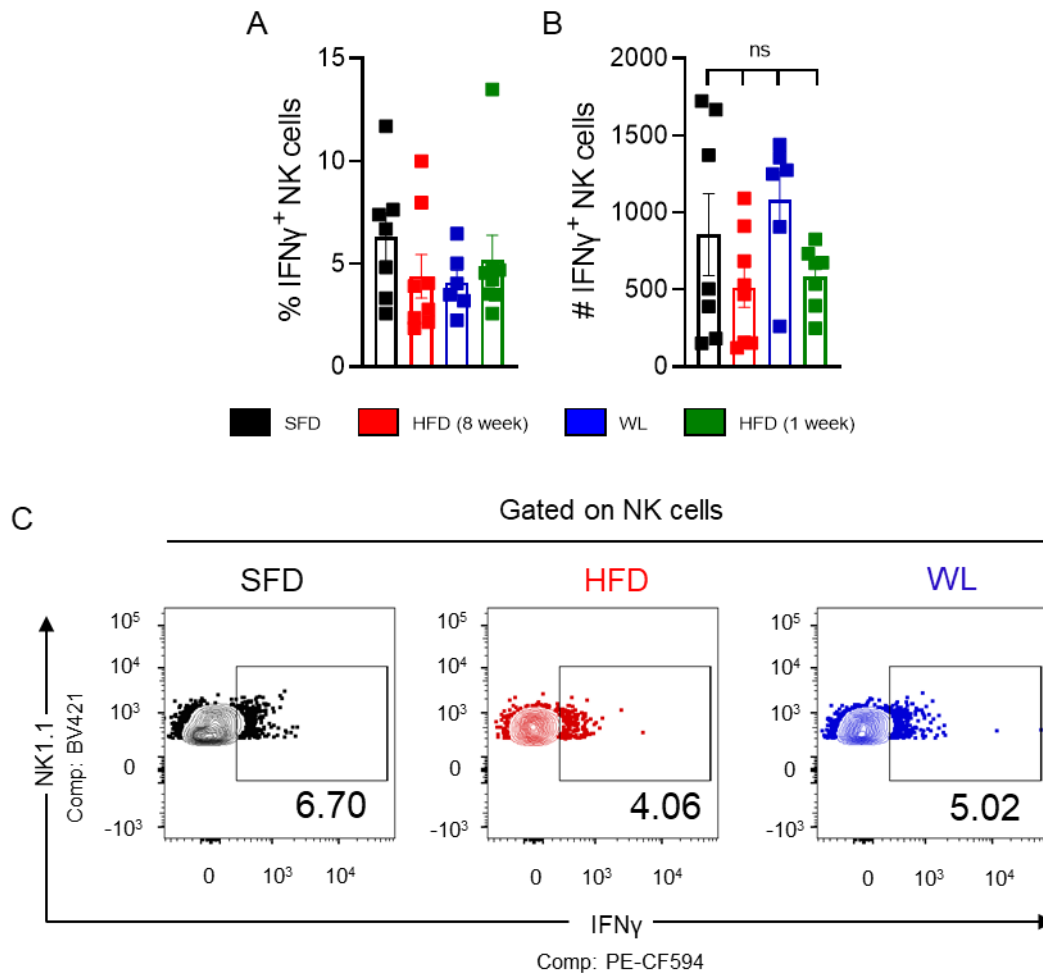


Figure 3.15 Mice that lost weight have a higher number, but not proportion, of circulating IFN γ ⁺ NK cells than mice fed a HFD

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Circulating cells were stimulated with PMA, ionomycin and brefeldin A for 4hr. IFN γ production was analysed by flow cytometry. Graphs show percentage of IFN γ ⁺ NK cells (**A**), number of IFN γ ⁺ NK cells (**B**) and representative flow plots (**C**). Data are mean $\pm$ S.E.M from N=1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ns = not significant.

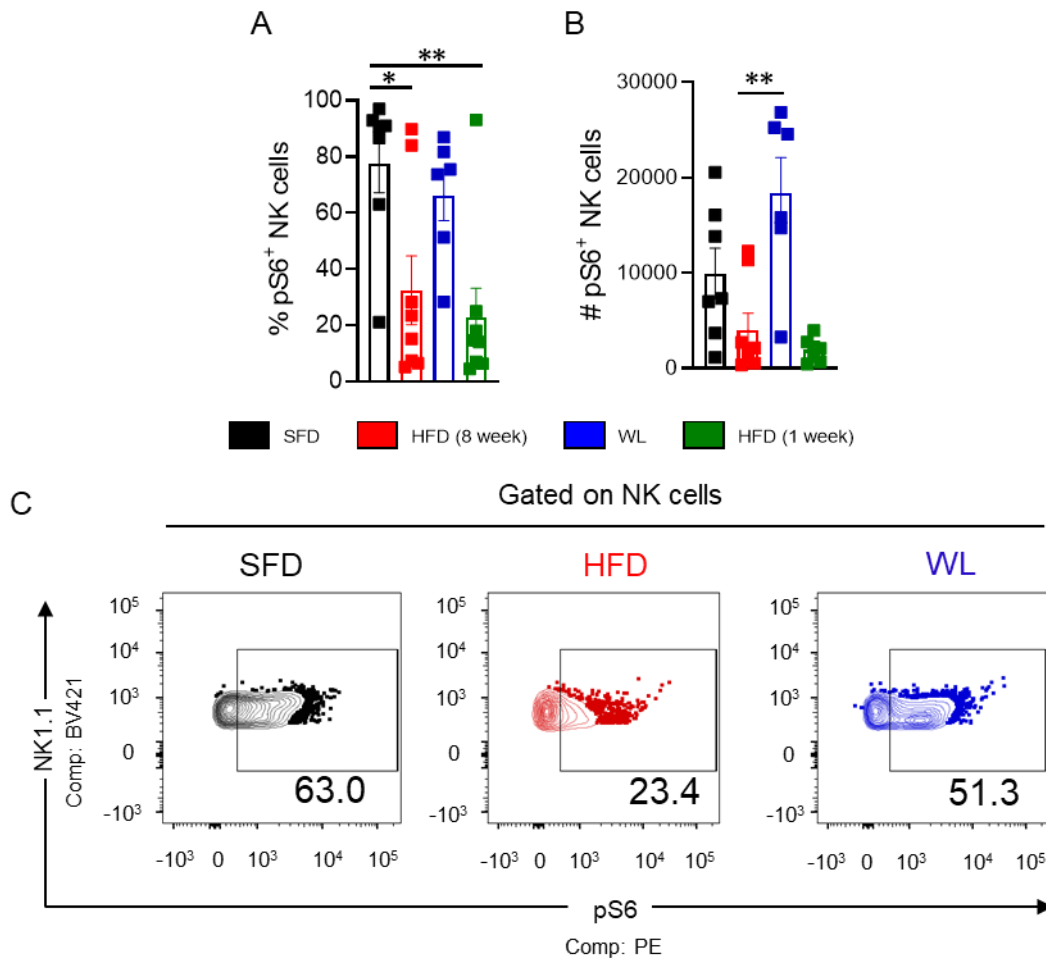


Figure 3.16 Circulating NK cells have reduced phosphorylation of S6 when mice are fed a HFD, and this is restored by weight loss

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Circulating cells were stimulated with PMA, ionomycin and brefeldin A for 4hr. Phosphorylation of S6 was analysed by flow cytometry. Graphs show percentage of pS6⁺ NK cells (**A**), number of pS6⁺ NK cells (**B**) and representative flow plots (**C**). Data are mean $\pm$ S.E.M from N=1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$.

3.5 Circulating CD8 T cells accumulate lipids when mice are fed a high fat diet, but are functionally intact

We and others have shown that obesity impairs CD8 T cells by multiple mechanisms^{120,114,113}. For example, CD8 T cells from obese mice and humans are functionally exhausted, characterised by increased expression of PD-1, reduced production of IFN γ and TNF, and loss of proliferative capacity¹²⁰. Additionally, we recently reported that obesity disrupts amino acid uptake and downstream mTORC1 signalling in CD8 T cells, resulting in impaired activation, IFN γ production, and accelerated tumour growth¹¹³. Therefore, we next investigated if WL could restore CD8 T cell immunometabolic responses. While HFD did not significantly affect IFN γ expression, or phosphorylation of S6 in splenic CD8 T cells (sCD8), IFN γ expression and phosphorylation of S6 were significantly increased when mice lost weight, compared to sCD8 T cells from mice fed a HFD (Fig. 3.17 A, B and Fig. 3.18 A, B). 1 week HFD feeding increased the expression of IFN γ and phosphorylation of S6 in sCD8 T cells (Fig. 3.17 A, B and Fig. 3.18 A, B).

Having shown that cNK but not sNK cells stain strongly for lipids when mice are fed a HFD, we next examined the total lipid content of sCD8 T cells vs circulating CD8 (cCD8) T cells from mice fed a SFD or HFD. cCD8 T cells, but not sCD8 T cells stain strongly for lipids when mice are fed a HFD (Fig. 3.19 A, B). Furthermore, WL significantly reduced the total lipid levels of cCD8 T cells and were similar to cCD8 T cells from mice fed a SFD (Fig. 3.20 A, B). cCD8 T cells from mice fed a HFD for 1 week did not accumulate lipids, again indicating that lipid uptake by circulating immune cells only occurs with chronic HFD feeding (Fig. 3.20 A, B).

We next examined the functional capacity of cCD8 T cells. Despite increased lipid levels, HFD did not affect IFN γ expression by cCD8 T cells (Fig. 3.21 A). There was a slight but non-significant reduction in phosphorylation of S6 in cCD8 T cells from mice fed a HFD and mice that lost weight (Fig. 3.21 B). Together, these data indicate that lipid accumulation and HFD feeding does not affect the functional responses of cCD8 T cells, unlike cNK cells which are sensitive to HFD feeding and lipid levels.

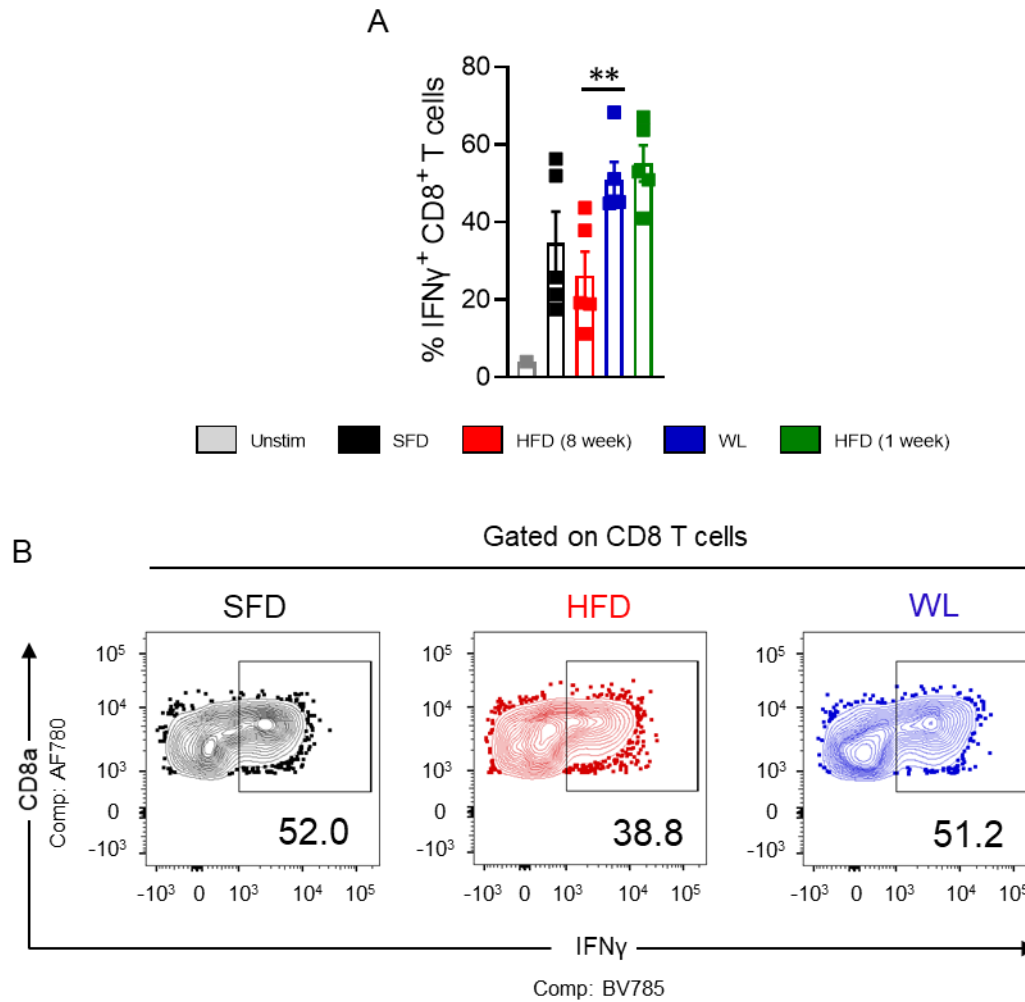


Figure 3.17 IFN γ expression by splenic CD8 T cells is augmented when mice lose weight compared to mice fed a HFD for 8 weeks

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Splenocytes were stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5ng/ml) for 72hr. IFN γ expression by CD8 T cells was analysed by flow cytometry. Graphs show percentage of IFN γ ⁺ CD8 T cells (**A**), and representative flow plots (**B**). Data are mean $\pm$ S.E.M from N=1 experiment, n=5 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ** $p \leq 0.01$.

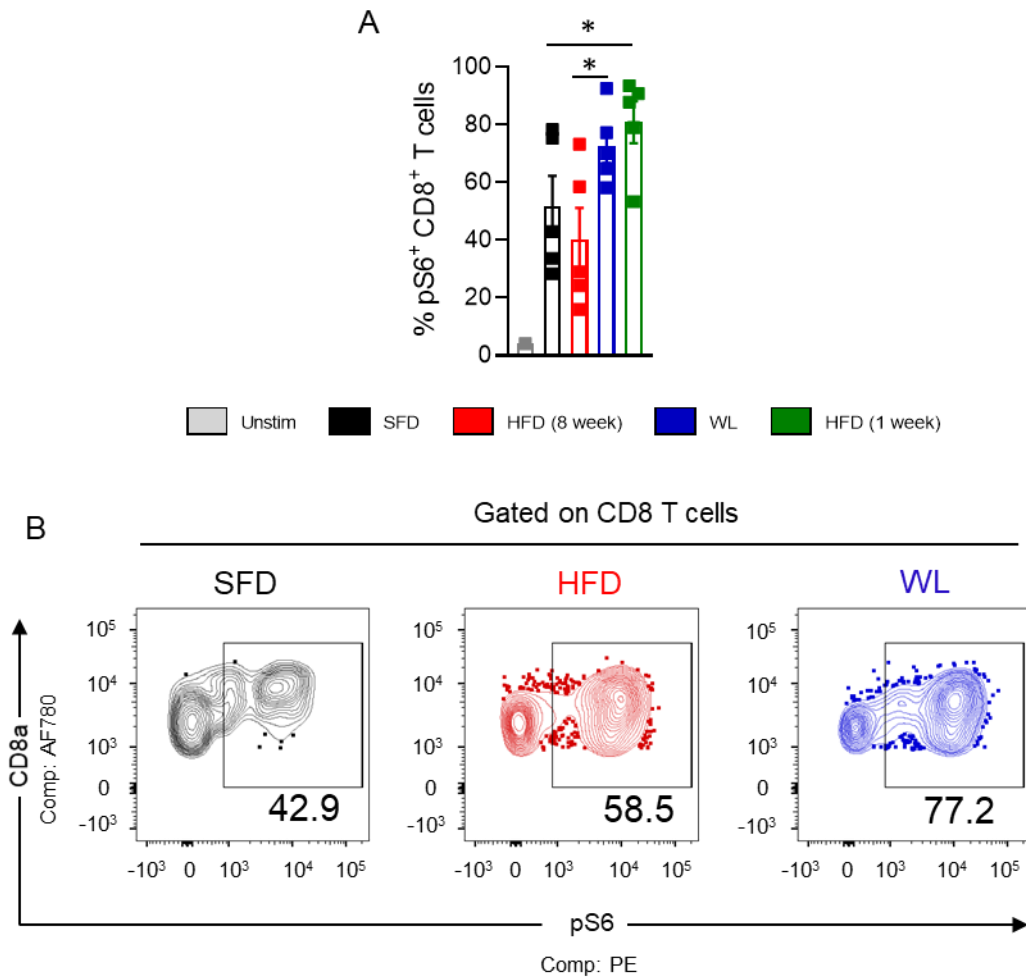


Figure 3.18 Splenic CD8 T cells have increased phosphorylation of S6 when mice lose weight compared to mice fed a HFD for 8 weeks

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Splenocytes were stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5ng/ml) for 72hr. Phosphorylation of S6 by CD8 T cells was analysed by flow cytometry. Graphs show percentage of pS6⁺ CD8 T cells (**A**), and representative flow plots (**B**). Data are mean $\pm$ S.E.M from N=1 experiment, n=5 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$.

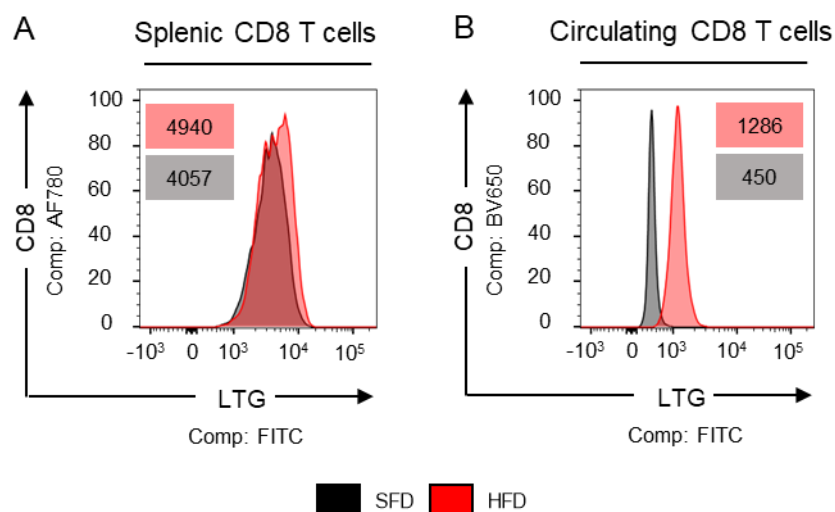


Figure 3.19 Circulating, but not splenic CD8 T cells stain more strongly for lipids when mice are fed a HFD, compared to a SFD

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Splenocytes were stimulated with anti-CD3 (1µg/ml), CD28 (3µg/ml), and IL-2 (5ng/ml) for 48hr. Circulating cells were stimulated with PMA and ionomycin for 4hr. LipidTOX Green (LTG) was used to analyse the total lipid levels of CD8 T cells by flow cytometry. Graphs are representative histograms showing LTG mean fluorescence intensity (MFI) of splenic CD8 cells **(A)** and matched circulating CD8 T cells **(B)**, from n=3 mice, 1 experiment. *Experiment performed with Dr Lydia Dyck.*

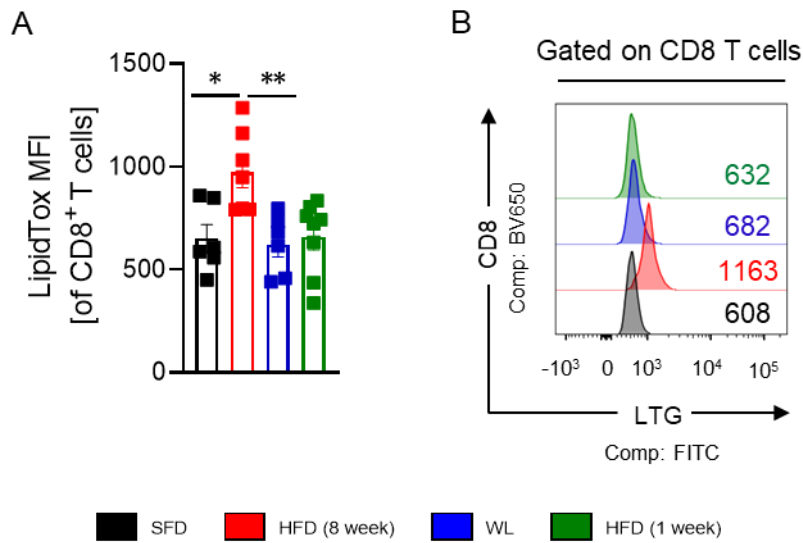


Figure 3.20 Circulating CD8 T cells stain strongly for lipids when mice are fed a HFD for 8 weeks, which is decreased by weight loss

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Circulating cells were stimulated with PMA and ionomycin for 4hr. LipidTOX Green (LTG) was used to analyse total lipid levels by flow cytometry. Graphs show LTG mean fluorescence intensity (MFI) of NK cells (**A**), and representative histogram (**B**). Data are mean $\pm$ S.E.M from N=1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$.

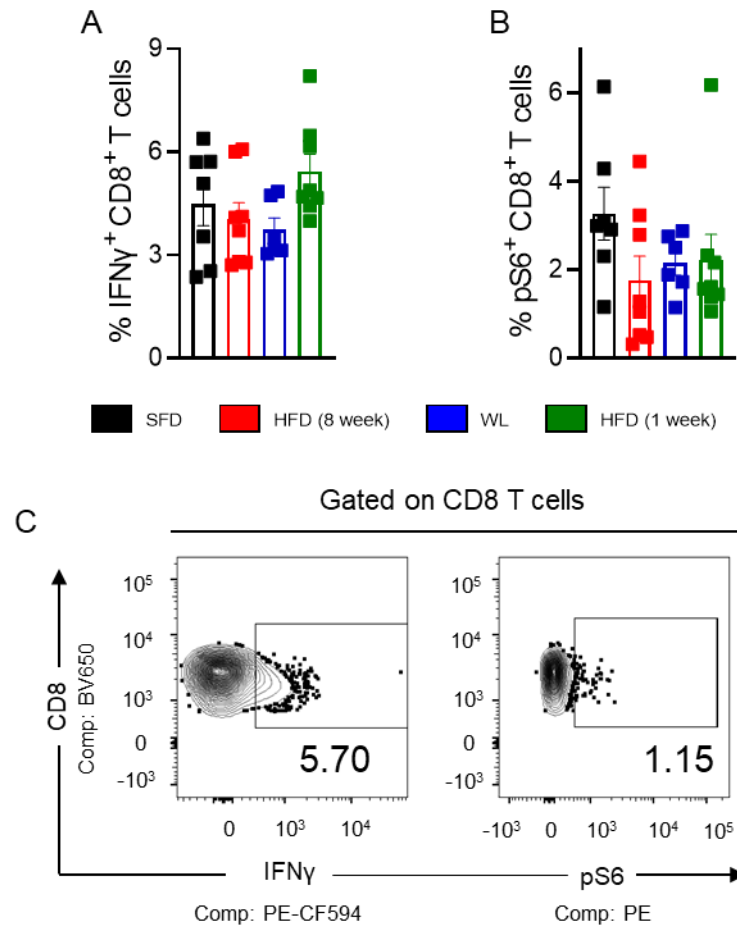


Figure 3.21 HFD does not alter IFN γ expression or phosphorylation of S6 in circulating CD8 T cells

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Circulating cells were stimulated with PMA and ionomycin for 4hr. IFN γ expression and phosphorylation of S6 was analysed by flow cytometry. Graphs show percentage of IFN γ ⁺ CD8 T cells (**A**), percentage of pS6⁺ CD8 T cells (**B**), and representative flow plots (**C**). Data are mean $\pm$ S.E.M from N=1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

3.6 High fat diet alters the plasma abundance of acylcarnitines, which are restored when mice lose weight

Diet composition controls the abundance of nutrients and metabolites in the blood¹⁸². We next hypothesised that HFD feeding alters the systemic metabolome of mice, and this may be restored by WL. Moreover, we speculated that certain metabolites produced in response to HFDs may interfere with immunometabolic pathways in NK and CD8 T cells, contributing to the obesity-induced dysfunction on immunosurveillance. To explore the effects of HFD on the systemic metabolome we performed targeted metabolomics analysis of the blood plasma of mice. HFD markedly remodels the plasma metabolome (Fig. 3.22 A) and 34 metabolites are altered in the plasma when mice are fed a HFD for 8 weeks, compared to mice fed a SFD (Fig. 3.22 B, Table 3.1). WL re-establishes the metabolome signature of mice (Fig. 3.23 A) and restores the plasma abundance of many metabolites that were up/downregulated by HFD (Fig. 3.23 B).

Among the top downregulated metabolites in the plasma of mice fed a HFD vs SFD were aminobenzoic acid and hippurate (Fig. 3.24 A). Aminobenzoic acid, part of the vitamin B₉ folate complex, is synthesised by commensal bacteria²⁰⁰ and hippurate is associated with the microbial degradation of dietary components²⁰¹. These data likely reflect microbiome dysbiosis, that is typical of HFD feeding and obesity. WL restores the plasma abundance of aminobenzoic acid and hippurate suggesting that a diverse microbiome can be re-established (Fig. 3.24).

We recently reported that obesity interferes with amino acid uptake and downstream immunometabolic signalling in CD8 T cells¹¹³. Therefore, we next examined the plasma abundance of amino acids. HFD increased the levels of the branched chain amino acids (BCAAs) valine and leucine (Fig. 3.25 A, B), which are strongly associated with insulin resistance²⁰². In contrast, ornithine, arginine, and glutamine which are vital for T cell survival and metabolic reprogramming^{203,204}, were decreased in the plasma of mice fed HFD (Fig. 3.25 C-E). These data suggest that a perturbed systemic metabolome in obesity potentially contributes to CD8 T cell functional defects, illustrating the interdependence of systemic and cellular immunometabolism. WL restored the abundance of amino acids in the plasma (Fig. 3.25 A-E), which may explain increased sCD8 T cell functional responses when mice lose weight.

Given that mice fed a HFD had reduced RER rhythm (Fig. 3.6), we suspected that glycolytic and TCA cycle metabolites would be altered. Indeed, pyruvate, lactate, and succinate were significantly reduced in the plasma of mice fed a HFD but restored when

mice lost weight (Fig. 3.26 A, B, C). These data reflect a defect in systemic carbohydrate metabolism caused by HFD feeding, which is reversed when mice lose weight.

Given that mice constantly burn lipids when fed a HFD, we next examined the plasma acylcarnitine levels which indicate flux through β -oxidation⁸⁴. Consistent with previous reports⁸², HFD alters the plasma acylcarnitine profile, leading to decreased abundance of short and medium chain acylcarnitines (S/MCACs) (Fig. 3.27 A) and increased long chain acylcarnitines (LCACs), particularly octadecanoyl-carnitine (C18) (Fig. 3.27 B). Of note, octadecanoyl-carnitine was the top upregulated metabolite in the plasma of mice fed a HFD for 8 weeks compared to mice fed a SFD (Fig. 3.22 B). Increased LCACs in the plasma indicate incomplete FAO due to mitochondrial metabolic overload⁸². WL restored the plasma abundance of S/MCACs and LCACs, suggesting that the systemic metabolic defect in lipid metabolism caused by HFD feeding can be reversed.

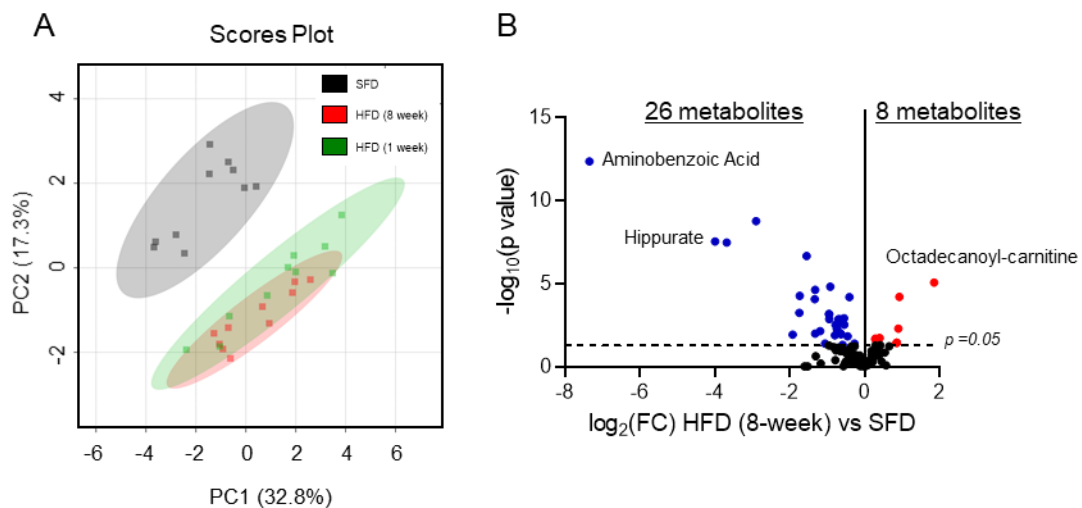


Figure 3.22 HFD feeding alters the plasma metabolome of mice

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Metabolomic analysis of the plasma metabolome was performed by LC/MS. Graphs show PCA analysis of the plasma metabolome **(A)**, and volcano plot showing metabolite differences between mice fed HFD (8 weeks) vs SFD **(B)**. Data is presented as volcano plots where the horizontal line represents a p value ≤ 0.05 . 2 independent experiments, $n=10$ mice per group. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

Metabolite Name	pValue	Fold change
Aminobenzoic acid	4.29E-13	0.006111
Acetyl-carnitine	1.70E-09	0.13371
Propionyl-carnitine	2.86E-08	0.062586
Hippurate	3.34E-08	0.077587
Gluconic acid	2.15E-07	0.34149
Octadecanoyl-carnitine	8.57E-06	3.6127
Allantoin	1.52E-05	0.52969
N-formyl-L-methionine	2.31E-05	0.39845
Kynurenic acid	5.32E-05	0.3006
Proline	6.21E-05	1.8998
Creatinine	6.36E-05	0.75224
4-pyridoxate	8.63E-05	0.39636
Carnitine	0.00068214	0.51702
L-carnitine	0.00068214	0.51702
GABA	0.0011968	0.68109
Pyruvate	0.0013446	0.61561
Cystine	0.0013462	0.51938
Malonate	0.0014236	0.63894
Cis-4-hydroxy-D-proline	0.0023399	0.61189
4-hydroxy-L-proline	0.0023775	0.61204
Ornithine	0.0029743	0.68504
Acetyl-tyrosine	0.005077	1.8617
Butyryl-carnitine	0.0072326	0.43715
Arginine	0.010178	0.64052
Adenine	0.011765	0.26296
3-hydroxybutyric acid	0.014856	0.72815
Lysine	0.01842	1.3167
Betaine	0.020108	1.2202
Valine	0.022307	1.2147
Lauryl-L-carnitine	0.035739	1.8094
Dodecanoyl-carnitine	0.035771	1.8093
Butyric acid	0.039885	0.82669
Hexanoyl-L-carnitine	0.039954	0.47989

Uracil	0.047659	0.66442
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Table 3.1 List of the significantly altered metabolites in the plasma of mice fed a HFD for 8 weeks compared to a SFD.

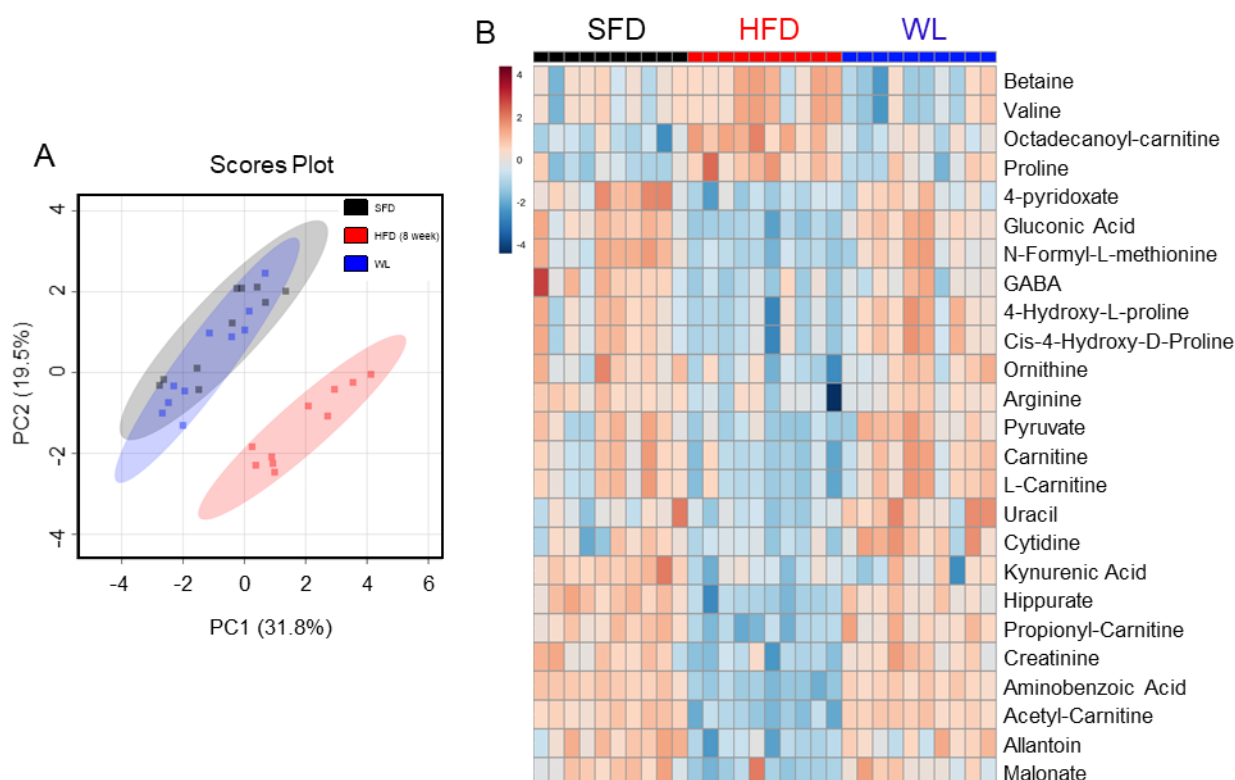


Figure 3.23 WL restores the plasma metabolome of mice

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Metabolomic analysis of the plasma metabolome was performed by LC/MS. Graphs show PCA analysis of the plasma metabolome **(A)**, and heatmap of the top 25 significantly different metabolites between mice fed a HFD (8 week) vs SFD vs mice that lost weight **(B)**. Red and blue indicate metabolites that are increased or decreased, respectively. 2 independent experiments, n=10 mice per group. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

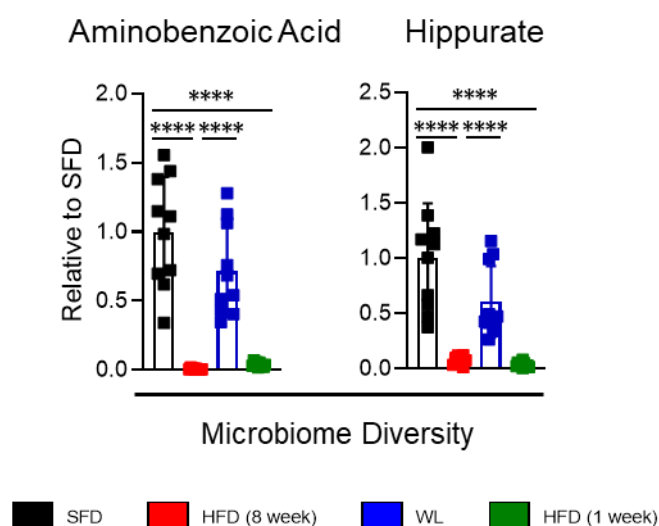


Figure 3.24 HFD decreases the plasma abundance of aminobenzoic acid and hippurate which are restored when mice lose weight

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Metabolomic analysis of the plasma metabolome was performed by LC/MS. Graphs show relative abundance of aminobenzoic acid and hippurate metabolites. Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 2 experiments, $n=10$ mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. **** $p \leq 0.0001$. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

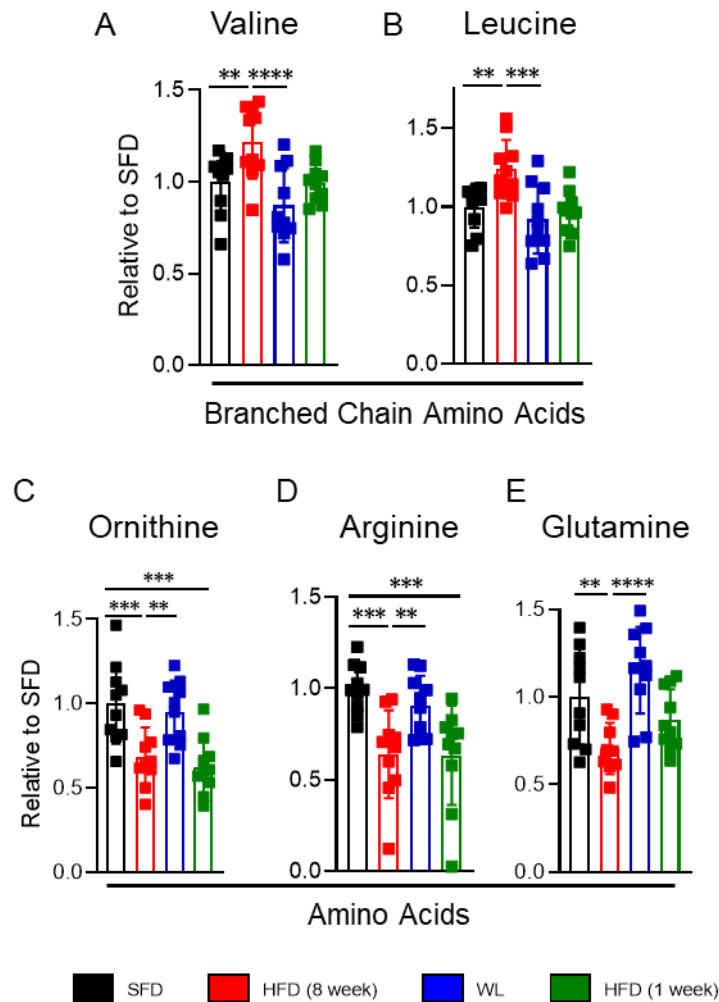


Figure 3.25 HFD alters the plasma abundance of amino acids, which are restored when mice lose weight

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Metabolomic analysis of the plasma metabolome was performed by LC/MS. Graphs show relative abundance of plasma amino acids. Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 2 experiments, $n=10$ mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

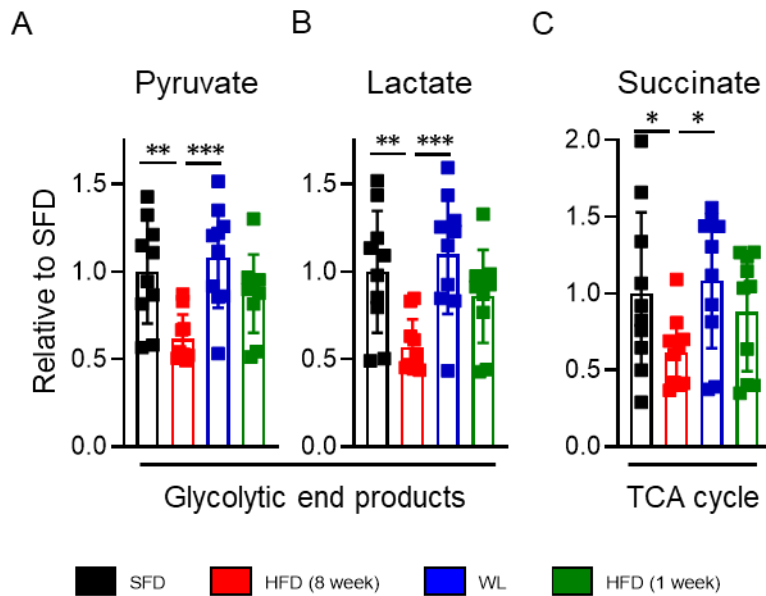


Figure 3.26 HFD decreases the plasma abundance of glycolytic and TCA cycle intermediates which are restored when mice lose weight

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Metabolomic analysis of the plasma metabolome was performed by LC/MS. Graphs show relative abundance of pyruvate, lactate, and succinate metabolites. Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 2 experiments, $n=10$ mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. *Metabolomics experiments were performed by Dr Evanna Mills, DFCL, and analysed by Hannah Prendeville.*

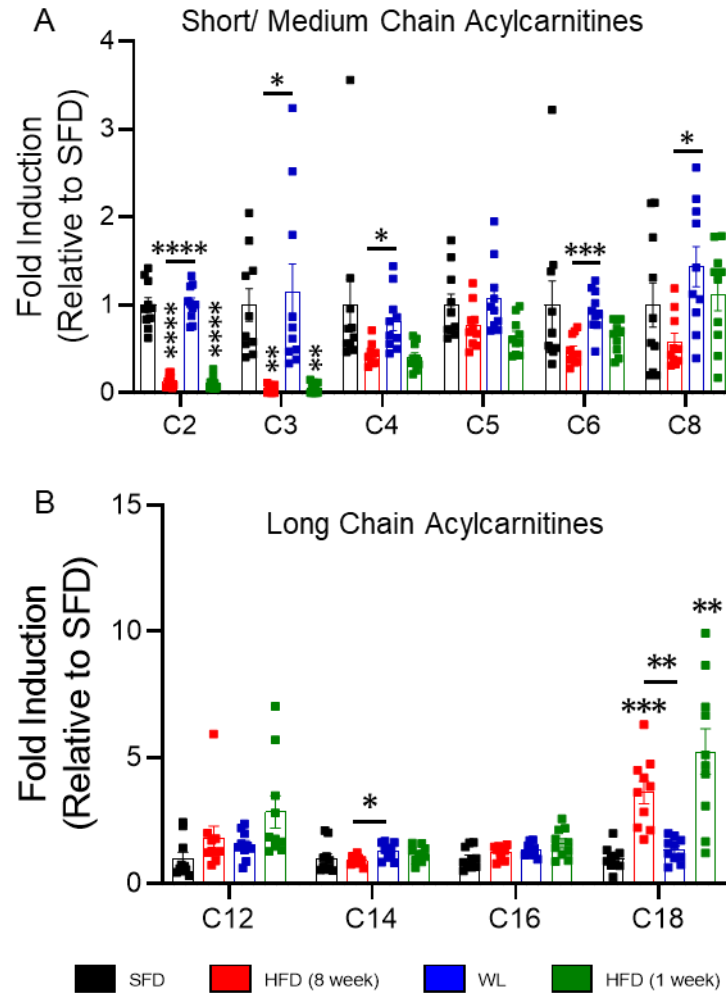


Figure 3.27 HFD alters the plasma acylcarnitine profile, which is restored when mice lose weight

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Metabolomic analysis of the plasma metabolome was performed by LC/MS. Graphs show relative abundance of short/medium chain **(A)** and long chain **(B)** acylcarnitines. Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 2 experiments, $n=10$ mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

3.7 High fat diet feeding disrupts the acylcarnitine profile of the liver and muscle, which are restored when mice lose weight

Our data suggests that HFD feeding may cause incomplete oxidation of fatty acids and this can be reversed by WL. The rate of β -oxidation is determined by the availability of L-carnitine, which is synthesised in the liver and sequestered by the muscle⁸². HFD decreased the abundance of L-carnitine in the skeletal muscle, but not livers of mice, and this is restored when mice lose weight (Fig. 3.28). We questioned if HFD distinctly affects the rates of lipid metabolism in the liver and skeletal muscle. To further elucidate this and examine the effects of WL on key metabolic organs, we next analysed the acylcarnitine profile of the skeletal muscle and livers of mice.

HFD markedly remodels the liver and muscle metabolomes (Fig. 3.29 A and Fig. 3.30 A) which are restored by WL (Fig. 3.29 B and Fig. 3.30 B). The liver metabolome is more distinctly altered than the muscle metabolome after 1-week HFD feeding (Fig. 3.29 A and Fig. 3.30 A), likely suggesting that the liver is more sensitive to HFD than muscle. Indeed, HFD feeding decreased the abundance of S/MCAs C4, C6, and C8 in the liver whereas only C3 was slightly decreased in the muscle (Fig. 3.29 C and Fig. 3.30 C). HFD increased the abundance of the LCAs palmitoyl-carnitine (C16) and octadecanoyl-carnitine (C18) in the liver, and dodecanoyl-carnitine (C12), myristoyl-carnitine (C14), C16 and C18 in the muscle (Fig. 3.29 D and Fig. 3.30 D). WL restores the abundance of S/M/LCAs in the liver and muscle demonstrating that systemic metabolic alterations can be reversed. Together, these results demonstrate that HFD feeding causes a global defect in lipid metabolism resulting in LCA accumulation in metabolic organs which may contribute to increased LCAs in the plasma.

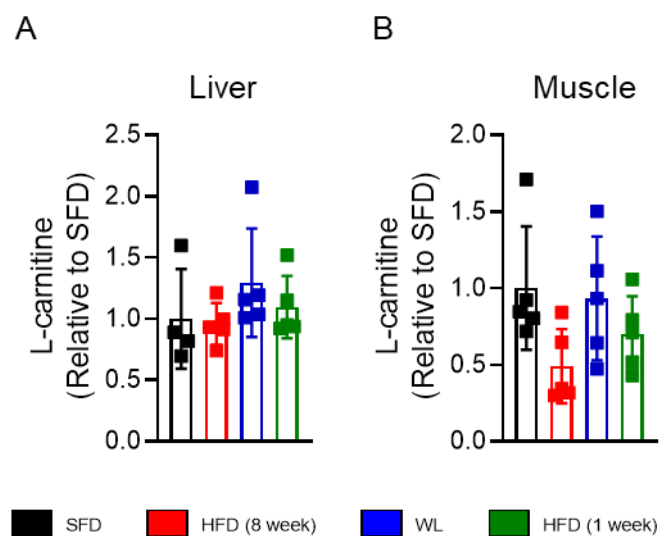


Figure 3.28 L-carnitine levels are decreased in the muscle, but not livers of mice fed a HFD and restored by WL

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Liver and muscle tissues were snap frozen and metabolomics analysis was performed by LC/MS. Graphs show relative abundance of L-carnitine. Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 1 experiment, $n=5$ mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

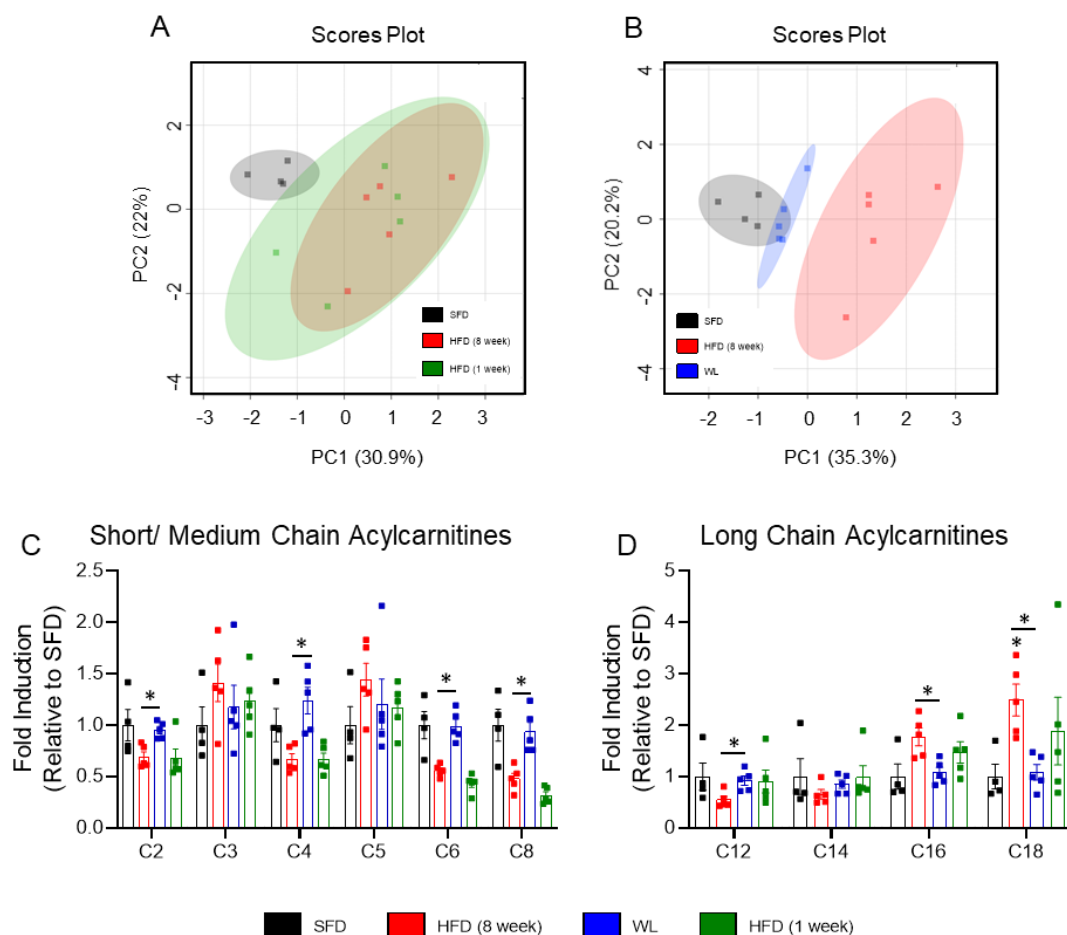


Figure 3.29 HFD alters the liver acylcarnitine profile, which is restored when mice lose weight

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Livers were snap frozen and metabolomics analysis was performed by LC/MS. Graphs show PCA analysis (**A, B**) and relative abundance of acylcarnitines (**C, D**). Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 1 experiment, n=5 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

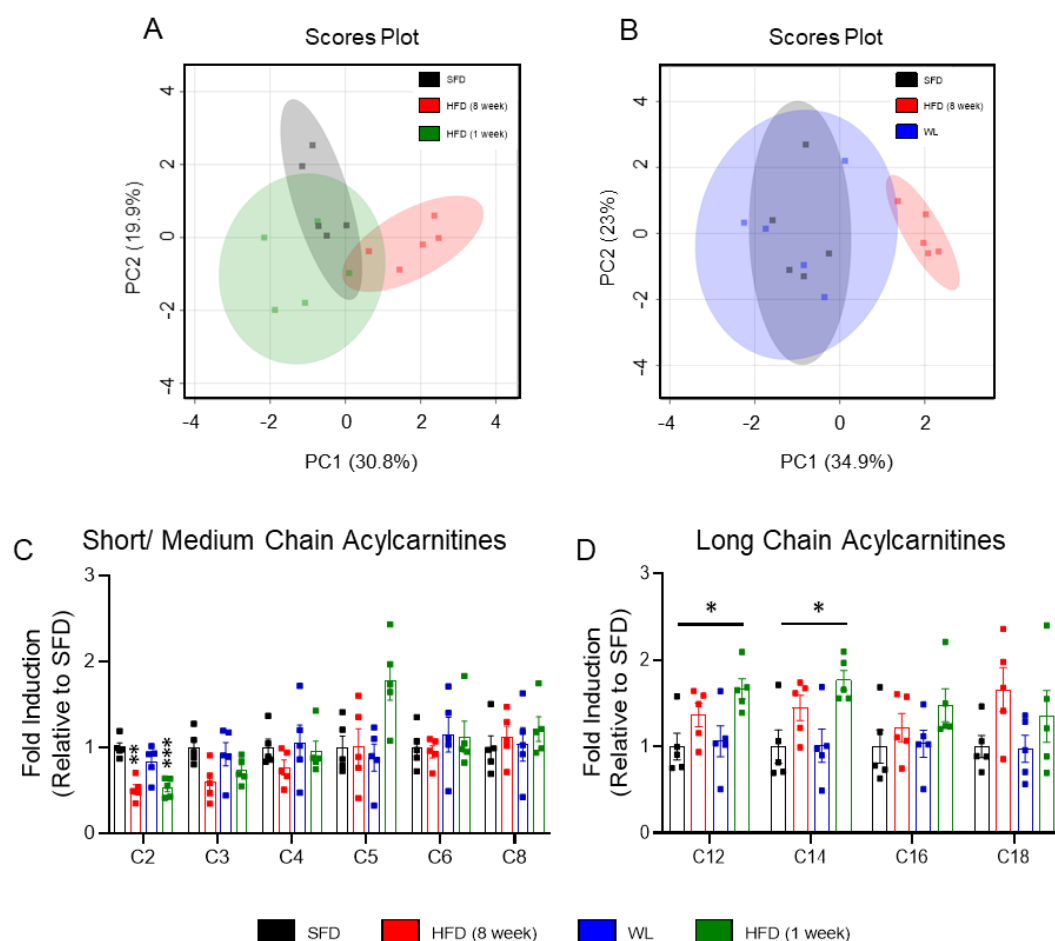


Figure 3.30 HFD alters the muscle acylcarnitine profile, which is restored when mice lose weight

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 8 weeks as per experimental design (Fig. 3.1). Muscles were snap frozen and metabolomics analysis was performed by LC/MS. Graphs show PCA analysis (**A, B**) and relative abundance of acylcarnitines (**C, D**). Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 1 experiment, $n=5$ mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

3.8 High fat diet results in rapid tumour growth and reduced immune infiltration, which is not restored by weight loss

We and others have demonstrated that anti-tumour NK and CD8 T cells are functionally and metabolically impaired in obesity and this accelerates tumour growth^{37,113,114}. In this project, we have built on this concept and shown that immunometabolic defects caused by HFD can be reversed by WL, possibly by restoring systemic metabolism. We next examined the effects of HFD feeding and WL on tumour growth *in vivo*. 4 days after inducing WL, mice were injected subcutaneously with MC38 colorectal adenocarcinoma cells. We chose this time point as mice were actively losing weight when tumours were transplanted (Fig. 3.31 A). Mice fed a SFD or HFD did not lose weight after the tumours were injected, indicating that WL was not caused by tumour transplantation, but changes in the diet (Fig. 3.31 A). At the end of the experiment, mice fed a HFD for 8 weeks were significantly heavier than mice fed a SFD and mice that lost weight. Mice fed a HFD for 1 week did not gain a significant amount of weight (Fig. 3.31 B).

HFD resulted in more rapid tumour development than mice fed a SFD (Fig. 3.32 A-D). However, WL did not protect against the enhanced tumour growth that is associated with DIO and weight gain in mice (Fig. 3.33).

We next examined the tumour immune infiltrates. We recently reported that the number of total CD45⁺ immune cells, NK, CD4 and CD8 T cells is decreased in B16 and MC38 tumours of mice fed a HFD¹¹³. While we did not observe any differences in the proportion or number of CD45⁺ lymphocytes or NK cells (Fig. 3.34 A, B), the proportion and number of intra-tumoral CD8 T cells was significantly decreased in the tumours of mice fed a HFD and mice that lost weight (Fig. 3.34 C). Mice fed a HFD for 1 week had a similar reduction in intra-tumoral CD8 T cells, however this was not significant (Fig. 3.34 C). These data indicate that WL does not protect against defects in tumour immune infiltration that are caused by HFD and obesity.

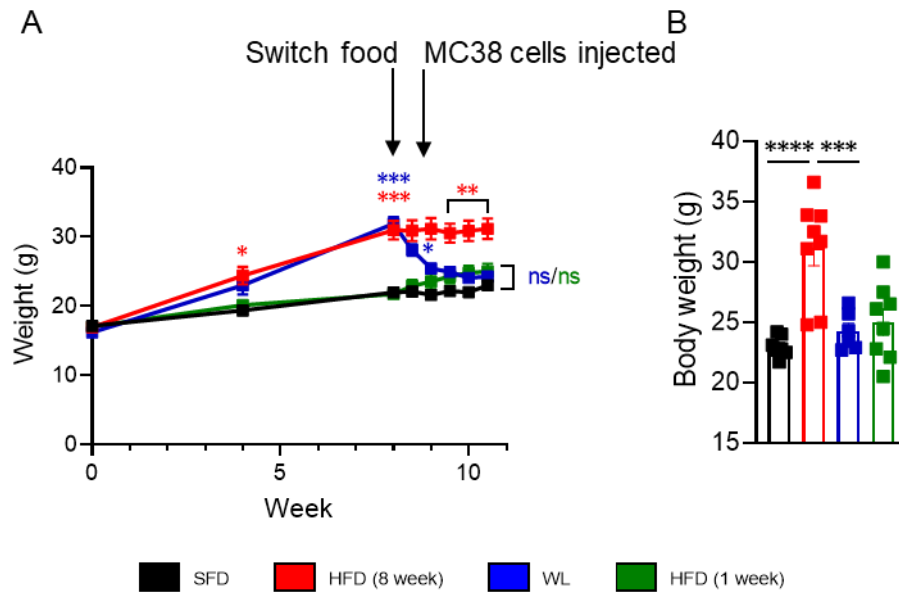


Figure 3.31 HFD-induced weight gain is rapidly reversed when mice are fed a SFD

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. Mice were injected subcutaneously with MC38 cells after 8.5 weeks of feeding. Graphs show weight gain over 11 weeks (**A**), final body weight (**B**). Squares represent individual mice. Significance was calculated using two-way ANOVA with Bonferroni test (**A**), ordinary one-way ANOVA with Dunnett test (**B**). N=1 experiment, n=6/8 mice per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

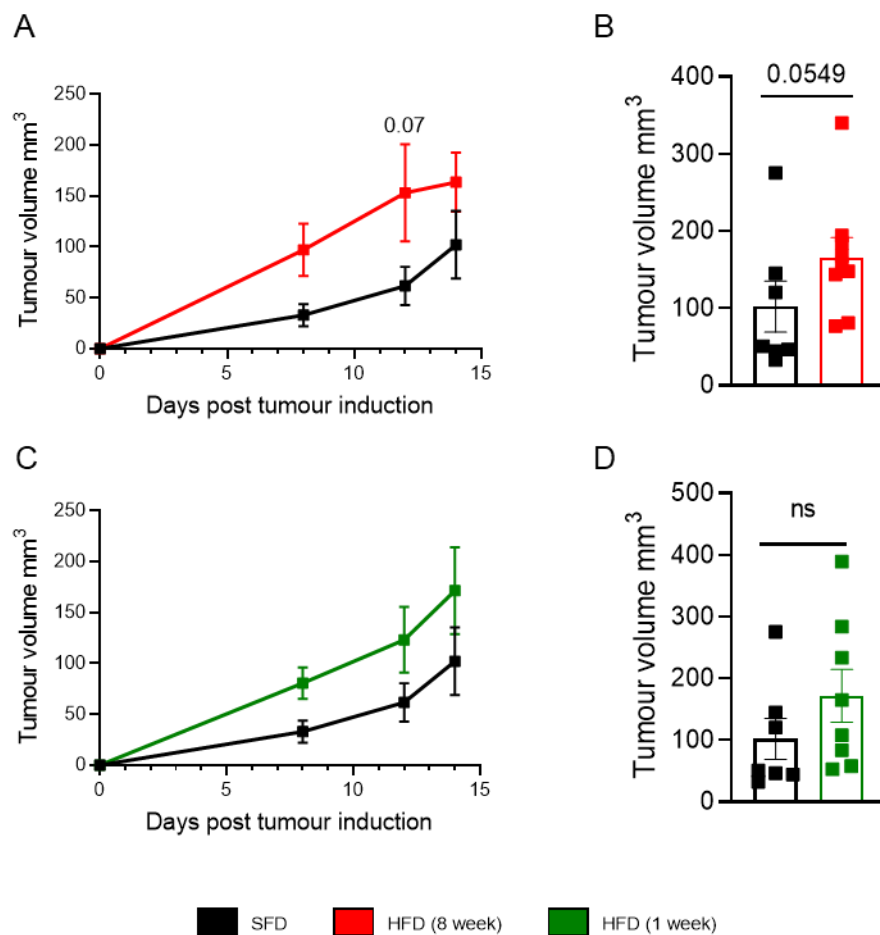


Figure 3.32 HFD accelerates MC38 tumour growth compared to SFD

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8.5 weeks of feeding, mice were injected subcutaneously with MC38 cells and tumour growth was monitored. Graphs show tumour volume on days 8, 12 and 14 (**A, C**) and final tumour volume (**B, D**). Squares represent individual mice. Significance was calculated using two-way ANOVA with Bonferroni test (**A, C**), ordinary one-way ANOVA with Dunnett test (**B, D**). N=1 experiment, n=6/8 mice per group. ns= not significant. *Injections performed by Dr Lydia Dyck. Measurements performed by Hannah Prendeville*

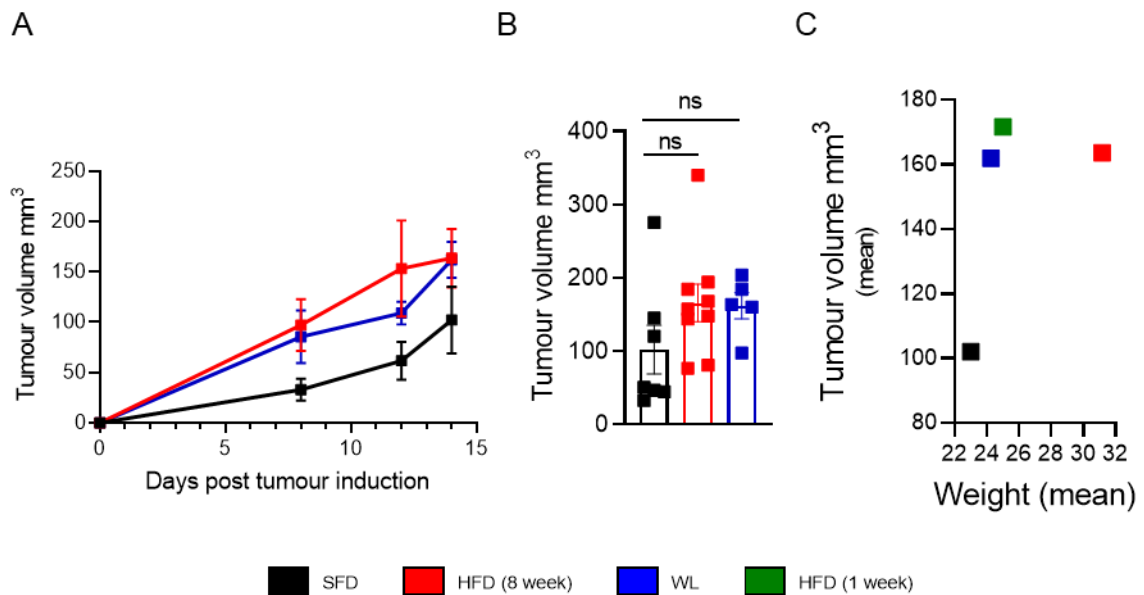


Figure 3.33 Weight loss does not protect against the pro-tumorigenic effects of a HFD

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8.5 weeks of feeding, mice were injected subcutaneously with MC38 cells and tumour growth was monitored. Graphs show tumour volume on days 8, 12 and 14 **(A)**, final tumour volume **(B)**, and correlation between mean tumour volume (mm³) and mean body weight (g) **(C)**. Squares represent individual mice **(B)** or group averages **(C)**. Significance was calculated using two-way ANOVA with Bonferroni test **(A)**, an ordinary one-way ANOVA with Dunnett test **(B)**. N=1 experiment, n=6/8 mice per group. ns= not significant. *Injections performed by Dr Lydia Dyck. Measurements performed by Hannah Prendeville.*

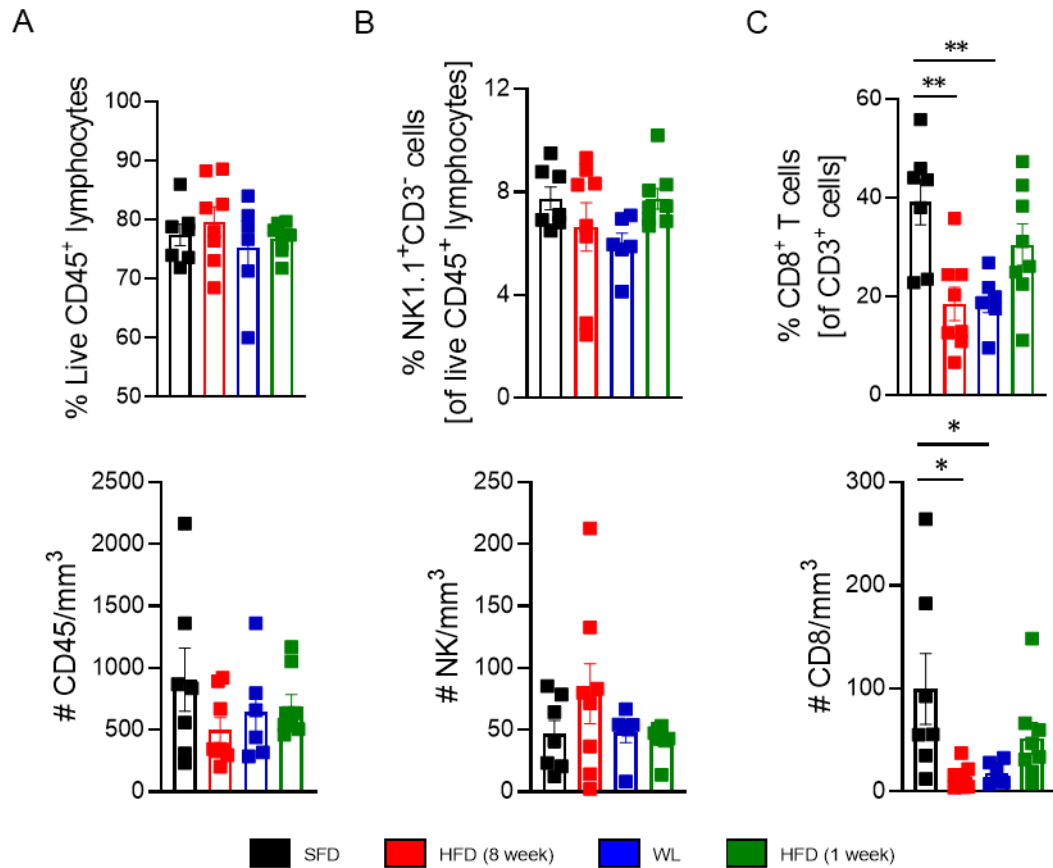


Figure 3.34 HFD reduces the proportion and number of intra-tumoral CD8 T cells which is not restored by weight loss

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8.5 weeks of feeding, mice were injected subcutaneously with MC38 cells. Tumours were isolated and intra-tumoral immune cells were analysed by flow cytometry. Graphs show frequency and density of tumoral CD45⁺ lymphocytes **(A)**, NK cells **(B)**, and CD8 T cells **(C)**. Data are mean $\pm$ S.E.M for 1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$.

3.9 HFD impairs tumoral CD8 T cell effector functions, which are not restored by Weight loss

CD8 T cells control tumour growth by secreting pro-inflammatory effector cytokines such as IFN γ and TNF. HFD led to a reduction in IFN γ , pS6, and TNF expression in CD8 T cells and this was not restored by WL (Fig. 3.35, Fig. 3.36, and Fig. 3.37).

HFD reprograms tumour cells to accumulate lipids and activate lipid metabolism pathways, which enhances their proliferation and survival. Lipid uptake by tumours deprives CD8 T cells of FAs, resulting in impaired activation and cytotoxicity¹¹⁴. In our model, lipid levels of tumoral CD8 T cells were similar between mice fed SFD, HFD, or mice lost weight (Fig. 3.38 A, B), suggesting that differences in cellular lipid content do not explain the functional defects in CD8 T cells observed.

Loss of effector function, particularly IFN γ production, is commonly associated with immune exhaustion and high expression of the immune checkpoint PD-1 on CD8 T cells in the tumour⁵⁷. As described¹¹³, PD-1 expression was higher on tumoral CD8 T cells compared to the draining LNs (Fig. 3.39 A). Consistent with previous reports from our lab¹¹³, tumoral CD8 T cells from mice fed a HFD had reduced levels of PD-1, and this was not restored by WL (Fig. 3.39 B). PD-1 is also a marker of CD8 T cell activation and CD8 T cells that express IFN γ and pS6 are primarily PD-1^{hi} (Fig. 3.40 B and Fig. 3.41 B). PD-1⁺ CD8 T cells express significantly less IFN γ and pS6 when mice are fed a HFD or lose weight (Fig. 3.40 and Fig. 3.41). Together, these data are consistent with reduced CD8 T cell activation in the tumours of mice fed a HFD and mice that lost weight.

Before infiltrating the tumour bed, CD8 T cells are activated in the dLNs. We questioned whether HFD interfered with CD8 T cell priming in the dLN which may explain functional defects in the tumour. CD8 T cells are highly active and express high levels of IFN γ in the dLN when mice are fed a HFD, and this was restored by WL (Fig. 3.42 A). These data demonstrate that WL can reverse some immune defects caused by HFD in the periphery, but not in the tumour.

We next examined the effector functions of tumoral NK cells, which are impaired by HFD³⁷. NK cells from mice fed a HFD and mice that lost weight produced less IFN γ , however this was not significant (Fig. 3.43 A). Phosphorylation of S6 (a readout of mTORC1 activity) was significantly decreased in NK cells from mice fed a HFD and mice that lost weight (Fig. 3.43 B). Together, these data demonstrate that HFD impairs the

functional and metabolic responses of tumoral NK and CD8 T cells, which cannot be restored by WL.

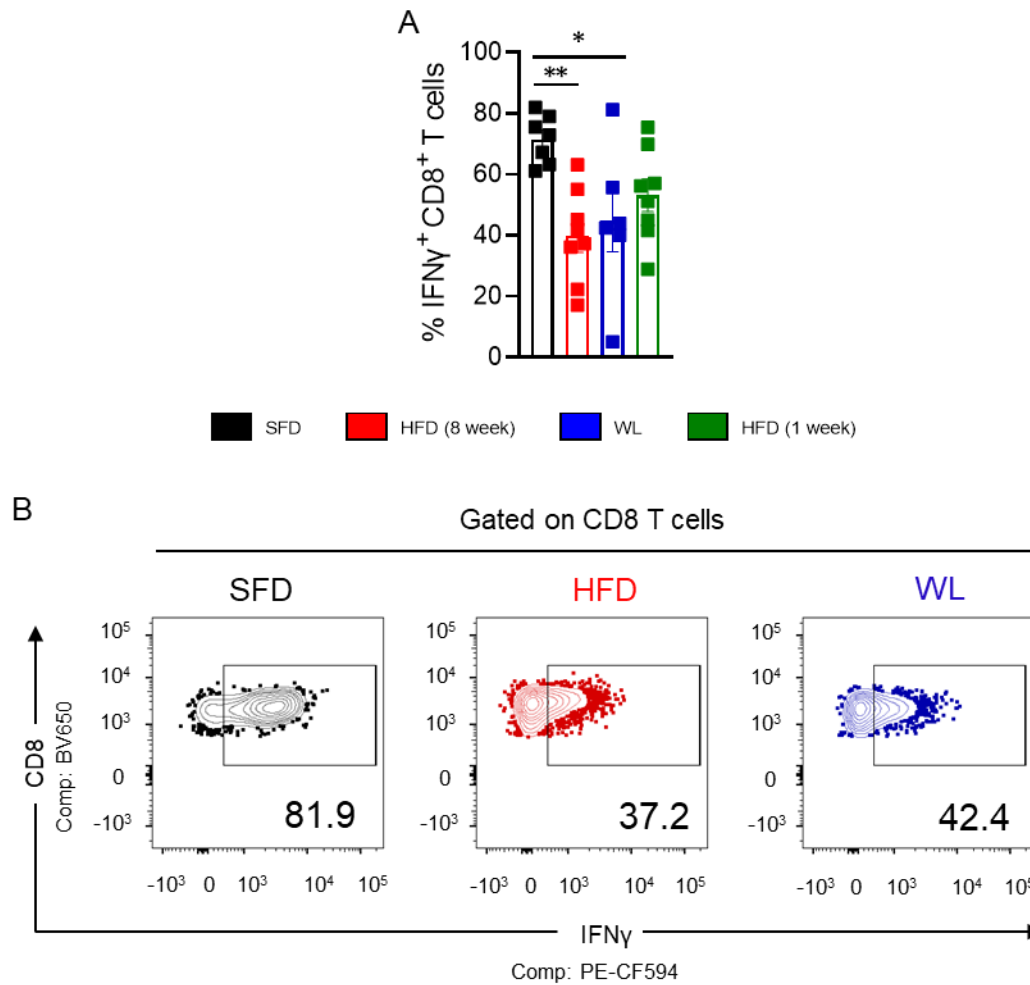


Figure 3.35 HFD decreases IFN γ expression in tumoral CD8 T cells which is not restored by weight loss

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8.5 weeks of feeding, mice were injected subcutaneously with MC38 cells. Tumours were isolated, stimulated with PMA, ionomycin, and brefeldin-A for 4 hr and intra-tumoral immune cells were analysed by flow cytometry. Graphs show frequency of IFN γ ⁺ (**A**), and representative flow plots (**B**). Data are mean $\pm$ S.E.M for 1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$.

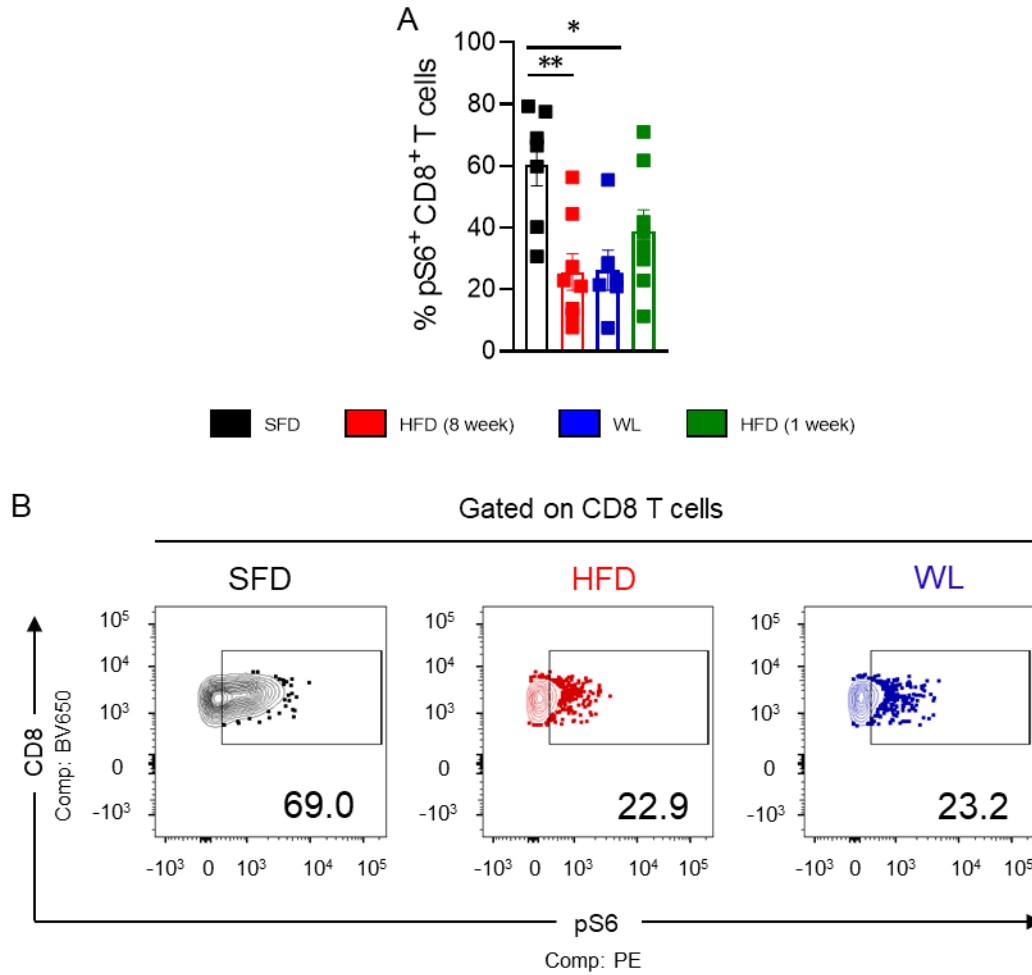


Figure 3.36 HFD decreases the phosphorylation of S6 in tumoral CD8 T cells which is not restored by weight loss

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8.5 weeks of feeding, mice were injected subcutaneously with MC38 cells. Tumours were isolated, stimulated with PMA, ionomycin, and brefeldin-A for 4 hr and intra-tumoral immune cells were analysed by flow cytometry. Graphs show frequency of pS6⁺ CD8 T cells **(A)**, and representative flow plots **(B)**. Data are mean $\pm$ S.E.M for 1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$.

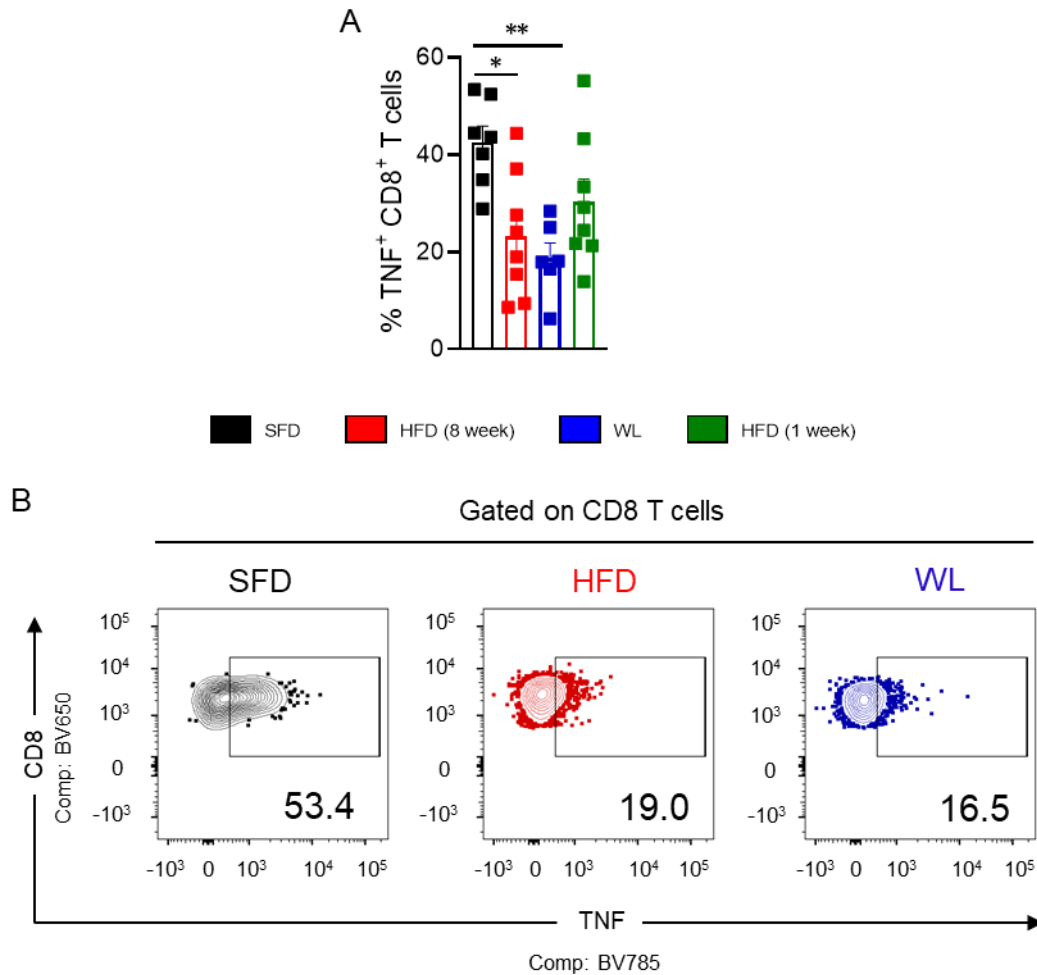


Figure 3.37 HFD decreases TNF expression in tumoral CD8 T cells which is not restored by weight loss

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8.5 weeks of feeding, mice were injected subcutaneously with MC38 cells. Tumours were isolated, stimulated with PMA, ionomycin, and brefeldin-A for 4 hr and intra-tumoral immune cells were analysed by flow cytometry. Graphs show frequency of TNF⁺ (**A**), and representative flow plots (**B**). Data are mean $\pm$ S.E.M for 1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$.

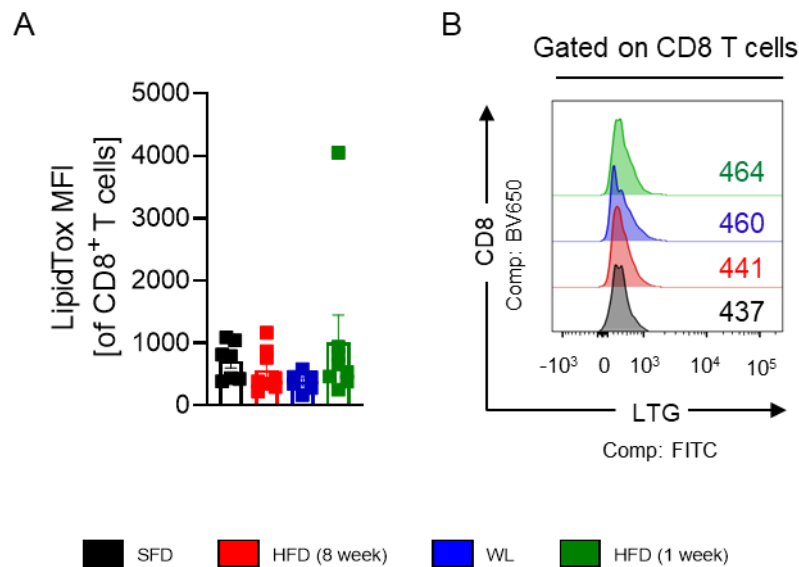


Figure 3.38 HFD does not alter total lipid levels of tumoral CD8 T cells

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8.5 weeks of feeding, mice were injected subcutaneously with MC38 cells. Tumours were isolated, stimulated with PMA, ionomycin, and brefeldin-A for 4 hr and LipidTOX Green (LTG) was used to analyse total lipid levels by flow cytometry. Graphs show LTG mean fluorescence intensity (MFI) of tumoral CD8 T cells **(A)**, and representative histograms **(B)**. Data are mean $\pm$ S.E.M from N=1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

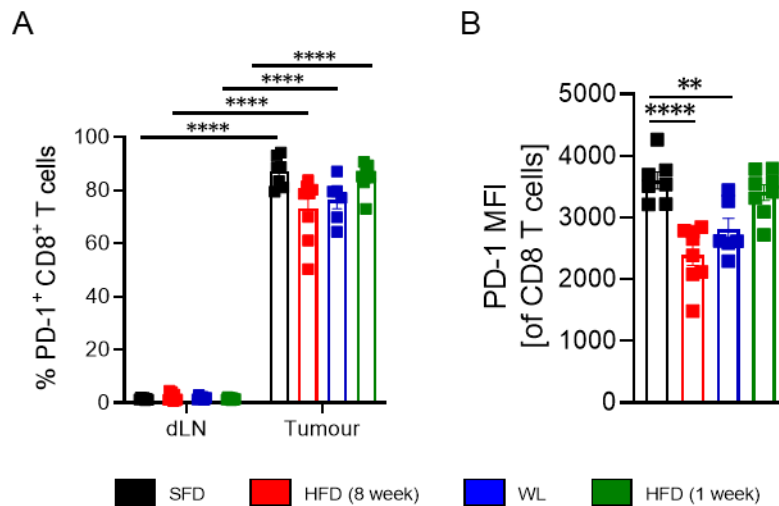


Figure 3.39 HFD decreases the expression of PD-1 on tumoral CD8 T cells, which is not restored by weight loss

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8.5 weeks of feeding, mice were injected subcutaneously with MC38 cells. Draining lymph nodes (dLNs) and tumours were isolated and immune cells were analysed by flow cytometry. Graphs show percentage of PD-1⁺ CD8 T cells in dLNs and tumours **(A)** and PD-1 mean fluorescence intensity (MFI) of tumoral CD8 T cells **(B)**. Data are mean $\pm$ S.E.M from N=1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ** $p \leq 0.01$, **** $p \leq 0.0001$.

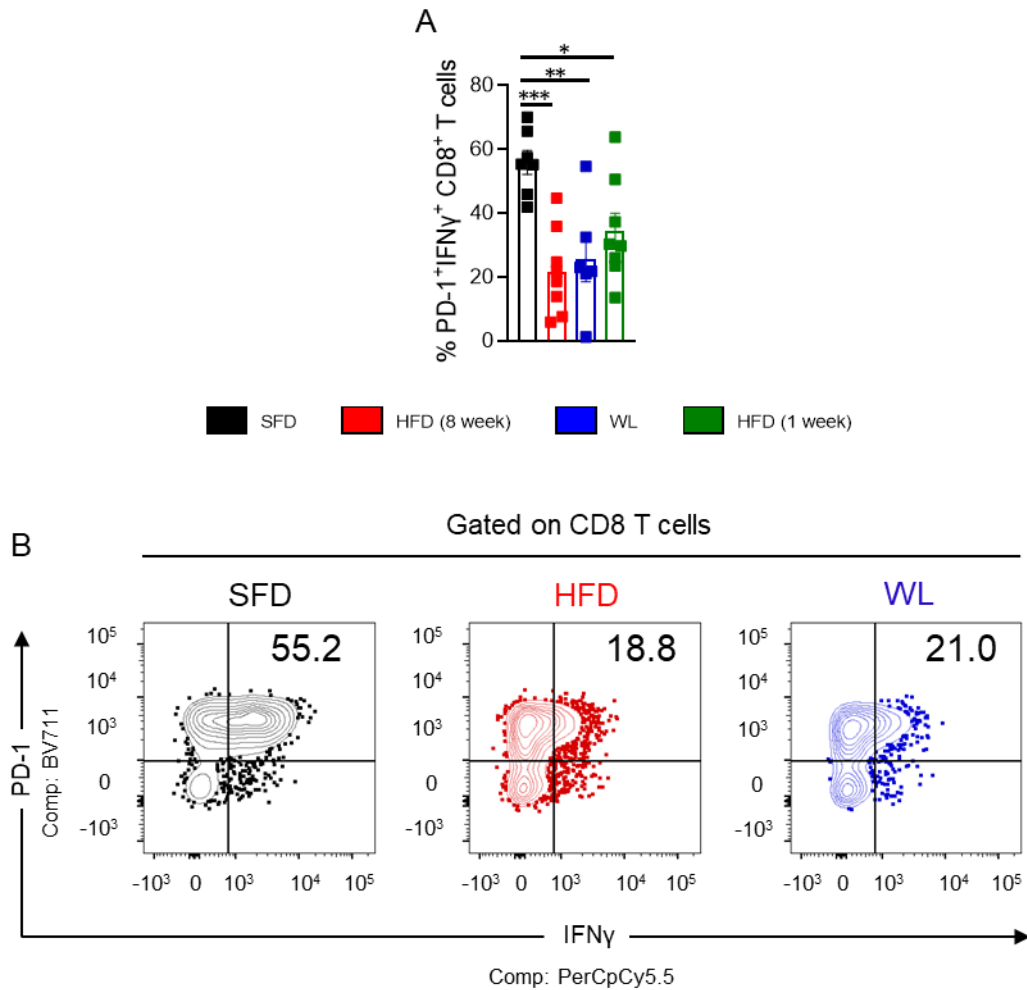


Figure 3.40 HFD decreases the frequency of PD-1⁺ IFNγ⁺ CD8 T cells in the tumour, which are not restored by weight loss

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8.5 weeks of feeding, mice were injected subcutaneously with MC38 cells. Tumours were isolated, stimulated with PMA, ionomycin, and brefeldin-A for 4 hr and immune cells were analysed by flow cytometry. Graphs show percentage of PD-1⁺IFNγ⁺ CD8 T cells (**A**) and representative flow plots (**B**). Data are mean $\pm$ S.E.M from N=1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

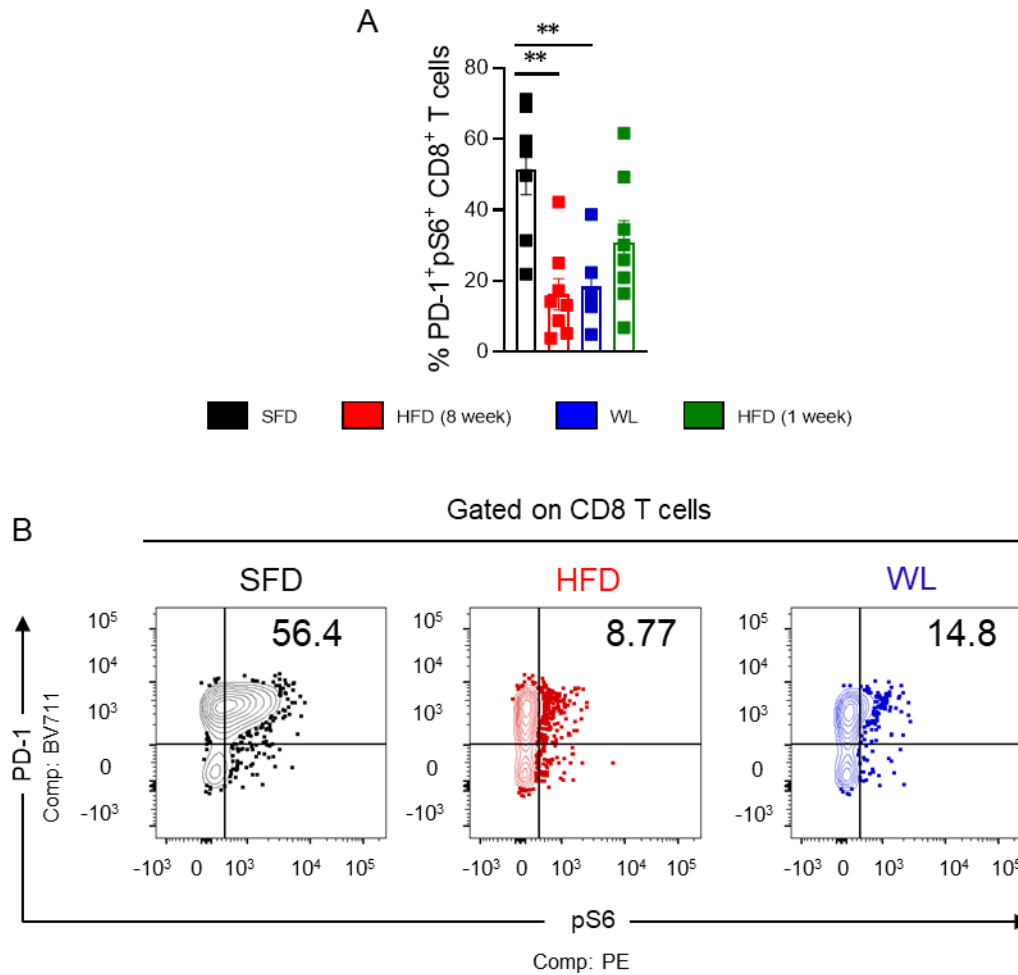


Figure 3.41 HFD decreases the frequency of PD-1⁺ pS6⁺ CD8 T cells in the tumour, which are not restored by weight loss

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8.5 weeks of feeding, mice were injected subcutaneously with MC38 cells. Tumours were isolated stimulated with PMA, ionomycin, and brefeldin-A for 4 hr and immune cells were analysed by flow cytometry. Graphs show percentage of PD-1⁺-pS6⁺ CD8 T cells (**A**), and representative flow plots (**B**). Data are mean $\pm$ S.E.M from N=1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ** $p \leq 0.01$.

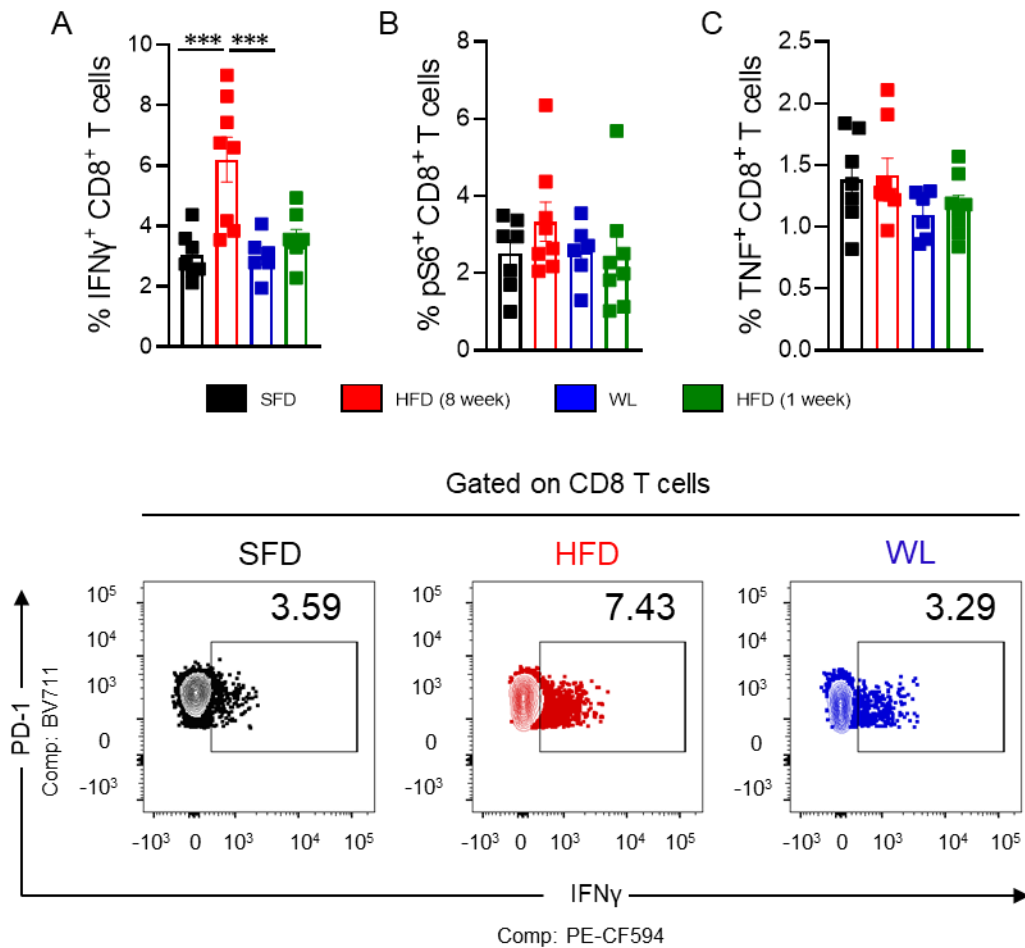


Figure 3.42 CD8 T cells in the draining lymph node produce more IFN γ when mice are fed a HFD, compared to a SFD and mice that lost weight

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8.5 weeks of feeding, mice were injected subcutaneously with MC38 cells. Draining lymph nodes were isolated, stimulated with PMA, ionomycin, and brefeldin-A for 4 hr and immune cells were analysed by flow cytometry. Graphs show frequency of IFN γ ⁺ (**A**), pS6⁺ (**B**) and TNF⁺ (**C**) CD8 T cells, and representative flow plots (**D**). Data are mean $\pm$ S.E.M for 1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. *** $p \leq 0.001$.

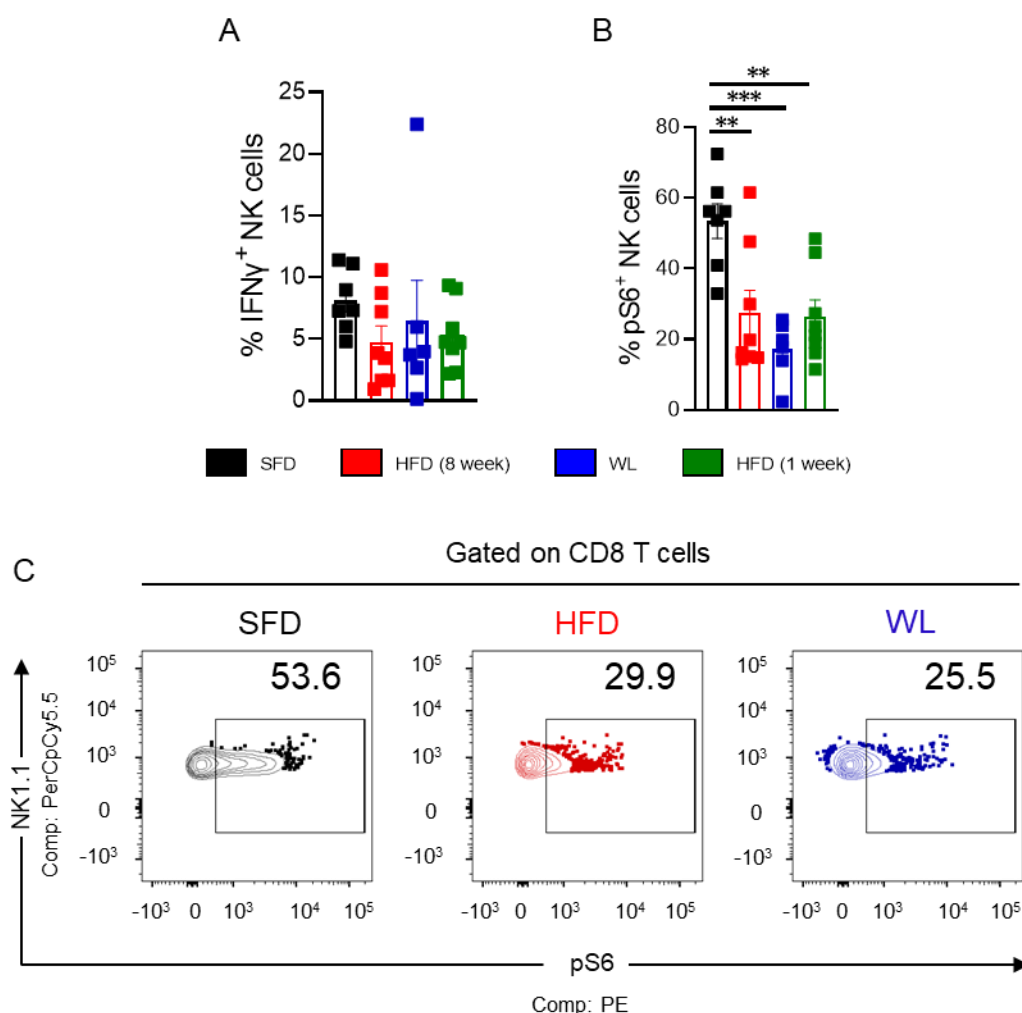


Figure 3.43 HFD reduces the phosphorylation of S6 in tumoral NK cells, and this is not restored by weight loss

Mice were fed a high-fat diet (HFD, 60% fat) or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8.5 weeks of feeding, mice were injected subcutaneously with MC38 cells. Tumours were isolated, stimulated with PMA, ionomycin, and brefeldin-A for 4 hr and intra-tumoral immune cells were analysed by flow cytometry. Graphs show frequency of IFN γ ⁺ (**A**), pS6⁺ (**B**) NK cells, and representative flow plots (**C**). Data are mean $\pm$ S.E.M for 1 experiment, n=6-8 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. ** $p \leq 0.01$, *** $p \leq 0.001$.

3.10 Discussion

Work from this chapter shows that WL can reverse the systemic metabolic defects induced by HFD feeding, by restoring systemic metabolic flexibility and remodelling the plasma metabolome in mice. In addition, we found that the functional responses of peripheral NK and CD8 T cells are augmented when mice lose weight. However, WL does not protect against tumour growth, and tumoral NK and CD8 T cells are defective when mice are fed a HFD or lose weight.

Immune cells adapt to the metabolic environment they are exposed to, which is shaped by the diet¹⁸². Indeed, work from this project shows that HFDs alter the plasma metabolome of mice, and this is restored by WL through dietary intervention. In particular, we show that HFDs alter the plasma acylcarnitine profile, causing decreased abundance of S/MCACs and increased LCACs, which is consistent with previous reports⁸². Acylcarnitines are intermediates of lipid metabolism and serve as a measure of flux through β -oxidation. Although mice fed a HFD had reduced RER, indicative of heightened β -oxidation, increased abundance of LCACs in the plasma reflect incomplete FAO due to mitochondrial metabolic overload of lipids⁸². In particular, mice fed a HFD from lard fat had increased abundance of the LCAC C18 which is derived from the LCFA SA, enriched in lard fat¹⁸⁵. Decreased consumption of dietary lipids in mice that lost weight likely re-establishes the complete oxidation of lipids and therefore decreases the plasma abundance of LCACs such as C18. In addition, acylcarnitines also indicate amino acid flux. Data from this project shows that C3 and C5 acylcarnitines, direct products of BCAA metabolism²⁰², are reduced in the plasma of mice fed a HFD. This is consistent with increased plasma abundance of the BCAAs valine and leucine, suggesting that HFD impairs BCAA metabolism. Indeed, expression of the branched-chain α -keto acid dehydrogenase (BCKDH) complex, which contributes to the circulating BCAA pool by regulating BCAA metabolism, is decreased in the adipose tissue of humans with obesity and mice fed a HFD²⁰⁵. BCAAs are commonly increased in the plasma of obese mice and humans and contribute to insulin resistance by activating mTORC1 and inducing insulin receptor substrate 1 (IRS1)_{ser307} phosphorylation, which interferes with downstream insulin signalling pathways²⁰². HFD feeding therefore results in systemic metabolic defects associated with a perturbed metabolome, and this can be reversed by WL.

In contrast to BCAAs, we show that HFD decreases the plasma abundance of ornithine, arginine, and glutamine. Arginine and glutamine are vital for T cell survival and metabolic reprogramming^{203,204} and we previously reported that obesity interferes with amino acid

uptake and downstream immunometabolic signalling in CD8 T cells, resulting in reduced IFN γ production¹¹³. These data suggest that a perturbed systemic metabolome in obesity potentially contributes to CD8 T cell functional defects, illustrating the interdependence of systemic and cellular immunometabolism.

Work from the Lynch lab has shown that NK cells are impaired in mice and humans with obesity. Lipid accumulation interferes with cellular bioenergetics leading to impaired IFN γ production and killing capacity³⁷. Importantly, this study has revealed that lipid uptake and NK cell dysfunction in obesity is site specific and does not occur globally. Splenic NK cells do not accumulate lipids when mice are fed a HFD, and are functionally and metabolically intact. Although, NK cells from mice fed a HFD for 1 week have increased rates of glycolysis and oxidative phosphorylation. NK cells from children with obesity have elevated levels of glycolysis basally, thus short term HFD feeding may resemble childhood obesity at the cellular level. In contrast to splenic NK cells, circulating NK cells stain strongly for lipids when mice are fed a HFD. Obesity is associated with increased levels of NEFAs and cholesterol in the circulation¹¹⁰. It is possible that splenic NK cells are not in contact with circulating lipids and are thus protected from NEFAs, lipid uptake, and cellular dysfunction. WL following bariatric surgery decreases circulating cholesterol²⁰⁶ and NEFAs²⁰⁷, while also reducing cancer risk²⁰⁸. Indeed, here we show that circulating NK cells do not accumulate lipids when mice lose weight. Additionally, the number of IFN γ ⁺ NK cells and phosphorylation of S6 is increased in NK cells from mice that lost weight, compared to mice fed a HFD, possibly due to decreases in cellular lipids. Interestingly, circulating NK cells from mice fed a HFD for 1 week do not accumulate lipids but express less IFN γ and have reduced phosphorylation of S6 than mice fed a SFD. Therefore, HFD may regulate NK cell function and metabolism by means other than intracellular lipid accumulation. HFD may interfere with the uptake and/or metabolism of other nutrients such as glucose which is an important fuel for NK cells^{8,77}. The Randle cycle describes how lipid derived acetyl-CoA impairs glucose metabolism by inhibiting pyruvate dehydrogenase (PDH) which is critical for the further metabolism of glucose-derived pyruvate in the TCA cycle. There is no evidence to suggest that NK cells can metabolise lipids²⁰⁹, however, HFD increases the expression of CPT and other PPAR dependent lipid metabolism genes in NK cells³⁷ and this metabolic adaptation is consequently linked to their dysfunction. It would be interesting to investigate if increased lipid metabolism in the context of HFD feeding can inhibit PDH in NK cells, resulting in altered glucose metabolism and associated functional defects. In contrast to NK cells, circulating CD8 T cells accumulate lipids when mice are fed a HFD, but retain the ability to express IFN γ and phosphorylate S6. CD8 T cells,

particularly memory cells, are metabolically equipped to oxidise lipids²¹⁰, which may explain why these cells are not affected by lipid uptake in the circulation.

Despite a restored plasma metabolome and improved immune cell functions in the periphery, WL did not protect against accelerated tumour growth. In addition, tumoral NK and CD8 T cells are dysfunctional in mice that lost weight, similar to mice fed a HFD. Consistent with previous reports from the Lynch lab¹¹³, we show that CD8 T cells are less activated in the tumours of mice fed a HFD and mice that lost weight, owing to reduced PD-1 expression. Tumoral immune cells are influenced by nutrients and metabolites in the TIFs which are rich in a diverse array of lipids⁸⁵ and likely increase with HFD feeding. This study did not investigate nutrients or metabolites in the TIF, which may indicate why WL does not restore anti-tumour immune functions. In this study, tumours were transplanted when mice were actively losing weight. Despite reduced dietary intake of fat, lipids that are released from adipose stores during WL may be sequestered by the tumour to promote tumour development and metastasis and impair anti-tumour immune responses¹⁹⁹. It would be interesting to investigate if tumoral immune functions are restored if tumours are transplanted after WL. A western diet has been shown to trigger innate immune memory through long lasting metabolic and epigenetic cellular reprogramming in myeloid cells and myeloid precursors²¹¹. Whether HFD epigenetically reprograms cytotoxic NK and CD8 T cells is an ongoing project in the Lynch lab and will reveal if anti-tumour immune dysfunction can be reversed after WL.

It is well reported that WL following gastric bypass improves the systemic metabolic health of people with obesity and has beneficial effects on the anti-tumour functions of NK and CD8 T cells. In this project we investigated the effects of WL through dietary intervention on systemic and cellular immunometabolism and found that WL can restore the plasma metabolome and improve NK and CD8 T cell functional responses in the periphery. These results suggest that WL may reduce the risk of metabolic diseases such as T2D and fatty liver disease which predispose to cancer formation and trigger tumour growth. While WL did not protect against aberrant tumour growth or restore the functional responses of tumoral NK and CD8 T cells, it is possible that anti-tumor immune functions may be restored if tumours are transplanted post WL, but more work needs to be done to elucidate this.

Chapter 4. The source of dietary fat, rather than obesity, influences anti-tumour immunity

4.1 Introduction

Lipids are abundant in many TMEs^{127,85} and can fuel tumour cells, enhancing their proliferation and metastasis, while fostering a pro-tumour immune environment¹⁹⁹. In pancreatic cancer models, LDs form as the tumour grows, which impairs tumour infiltrating CD8 T cells¹²⁸. Similarly, feeding mice a HFD boosts tumour lipid uptake and metabolism in multiple cancer models, which promotes survival and metastasis, leading to increased tumorigenesis^{150,133}. Alongside this, we and others have reported that HFD impairs the anti-tumour immune functions of NK and CD8 T cells, which contributes to increased tumour growth in obesity^{37,113,114}. Thus, obesity and HFD feeding has a dual effect on cancer, promoting tumour cell metabolism and proliferation, while impairing the anti-tumour immune response. The link between dietary fat and cancer incidence was first described in the 1930s by demonstrating that a butter diet accelerated subcutaneous tumour growth in mice¹⁸³. However, not all fats are the same, and identifying the critical dietary factors linking obesity and cancer would potentially allow us to eliminate or reduce the link between cancer and obesity safely, to promote immunity for patients with obesity and/or cancer.

Studies examining the effects of individual dietary FAs on cancer growth have mainly studied *in vitro* models of cancer cell proliferation and viability. For example, the SFA PA which is enriched in many sources of dietary fat including lard, butter, and palm oil¹⁸⁵, promotes the proliferation of colorectal cancer cells *in vitro*²¹². In contrast, OA, the most abundant MUFA in the human diet¹⁸⁵ has anti-tumour effects, impairing the viability and migration of liver cancer cells *in vitro*²¹³. However, diets are never composed of one type of FA and less is known about the impact of different compositions of dietary fat on tumour growth and tumour immune responses. Furthermore, little is known about the differential effects of dietary lipids on cytotoxic immune cells *in vitro* or *in vivo*. Given the pro-tumour impact of obesity, but the diverging effects of different FAs on cancer growth *in vitro*, we sought to identify particular components of a HFD that promoted tumour growth and impaired immunity *in vivo*. Every immune cell process is supported by intracellular metabolism, and immune cells adapt to the metabolic environment they are exposed to. Thus, diet shapes systemic metabolism which in turn regulates cellular metabolism and shapes the immune response. In this chapter, we tested the hypothesis that the source of dietary fat would differentially affect tumour growth *in vivo*, through the impact of different diets on whole body metabolism which regulates cellular metabolism and function.

Experiments in chapter 4 were carried out with Dr Roisin Loftus and Dr Lydia Dyck, as stated in relevant figure legends.

4.2 Tumour growth is dependent on the source of dietary fat, rather than adiposity

To study the effects of dietary lipids on anti-tumour immune responses, we used a mouse model of DIO where mice were fed a HFD from animal and plant sources. Wild-type mice were fed a 45% HFD derived from lard or butter (animal), or peanut oil, coconut oil, or palm oil (plant) for 13 weeks. All diets were isocaloric and contained the same type and amount of carbohydrates, protein, vitamins, and minerals. The lipid composition of each diet can be found in table 4.1. A SFD where 12% of calories were derived from soybean oil was used as a control diet. Regardless of the fat source, mice fed a HFD gain significant weight (Fig. 4.1 A, B). Moreover, the visceral and subcutaneous fat pad weights were comparable between HFDs and significantly heavier than mice fed a SFD (Fig. 4.2 A, B). After 10 weeks of feeding, mice were injected subcutaneously with B16 melanoma cells. Surprisingly, tumour growth dynamics differed significantly with the source of dietary fat, despite similar levels of obesity. Consistent with previous reports from our lab and others, the commonly used lard HFD significantly accelerated tumour growth compared to mice fed SFD^{37,114} (Fig. 4.3 A). Mice fed another animal derived HFD, butter, also had accelerated tumour growth, even more so than lard (Fig. 4.3 B). There was a slight but non-significant increase in tumour growth from coconut and peanut oil HFDs compared to SFD (Fig. 4.4 A, B), whereas a palm oil HFD did not enhance tumour growth (Fig. 4.4 C). Taken together, these results confirm that the source of dietary fat rather than weight gain and adiposity had a greater influence on tumour burden.

<u>Diet Code</u>	D1245 1	D0512230 1	D1601070 5	D1002040 4	D0708150 1
<u>Ingredient</u>					
Lard	177.5				
Coconut Oil, 101		177.5			
Peanut Oil			177.5		
Butter, Anhydrous				177.5	
Palm Oil, RBD					177.5
Soybean Oil	25	25	25	25	25
<u>Total</u>	202.5	202.5	202.5	202.5	202.5
C2, Acetic	0	0	0	0	0
C4, Butyric	0	0	0	5.7	0
C6, Caproic	0	1.1	0	3.4	0
C8, Caprylic	0	13.7	0	2	0.3
C10, Capric	0.1	10.5	0	4.4	0.3
C12, Lauric	0.2	84.5	0	5	3.2
C14, Myristic	2.1	32	0.1	17.8	2.9
C14:1, Myristoleic, n-9	0	0	0	2.7	0
C15	0.1	0	0	0	0.1
C16, Palmitic	36.88	18	17.2	49.1	77.8
C16:1, Palmitoleic, n-9	2.5	0	0.2	4.1	0.3
C16:2, n-4	0	0	0	0	0
C16:3, n-9	0	0	0	0	0
C16:4, n-4	0	0	0	0	0
C17	0.7	0	0	0	0.2
C17:1	0	0	0	0	0
C18, Stearic	19.8	19.8	5.2	22.4	8.7
C18:1, Oleic, n-9	64.1	7.2	112.5	50.3	68.6
C18:1, Elaidic, Trans	0	0	0	0	0
C18:2, Linoleic	56.2	13	45.5	17	29
C18:2, Trans	0	0	0	0	0
C18:3, Linolenic	4.2	1.9	2.2	4.3	4.7
C18:3, n-6	0	0	0	0	0
C18:3, Trans	0	0	0	0	0
C18:4, Stearidonic	0	0	0	0	0

C20, Arachidic	0.4	0.1	2.2	1.8	0.7
C20:1	1.2	0.1	3.3	0.1	0.3
C20:2	1.4	0	0	0	0
C20:3, n-6	0.2	0	0	0	0
C20:3, n-3	0	0	0	0	0
C20:4, Arachidonic, n-6	0.5	0	0	0	0
C20:4, n-3	0	0	0	0	0
C20:5, Eicosapentaenoic, n-3	0	0	0	0	0
C21:5, n-3	0	0	0	0	0
C22, Behenic	0.1	0.1	5.3	0.1	0.1
C22:1, Erucic	0	0	0.4	0	0
C22:4, Clupanodonic, n-6	0	0	0	0	0
C22:5, Docosapentaenoic, n-3	0.2	0	0	0	0
C22:6, Docosahexaenoic, n-3	0	0	0	0	0
C24, Lignoceric	0	0	3.8	0	0
C24:1	0	0	0	0	0
Total	190.7	201.8	198.0	190.1	197.1
Saturated (g)	62.2	179.7	33.8	111.6	94.3
Monounsaturated (g)	67.7	7.3	116.4	57.1	69.2
Polyunsaturated (g)	62.8	14.8	47.7	21.4	33.6
Saturated (%)	31.6	89	17.1	58.7	47.8
Monounsaturated (%)	35.5	3.6	58.8	30.1	35.1
Polyunsaturated (%)	32.9	7.4	24.1	11.2	17.1
n6	57	13	45.5	17	29
n3	4.4	1.9	2.2	4.3	4.7
n6:n3 ratio	13.1	7	20.6	3.9	6.2

Table 4.1. Fatty acid composition (g) of rodent high fat diets (45%) from lard, coconut oil, peanut oil, butter, or palm oil.

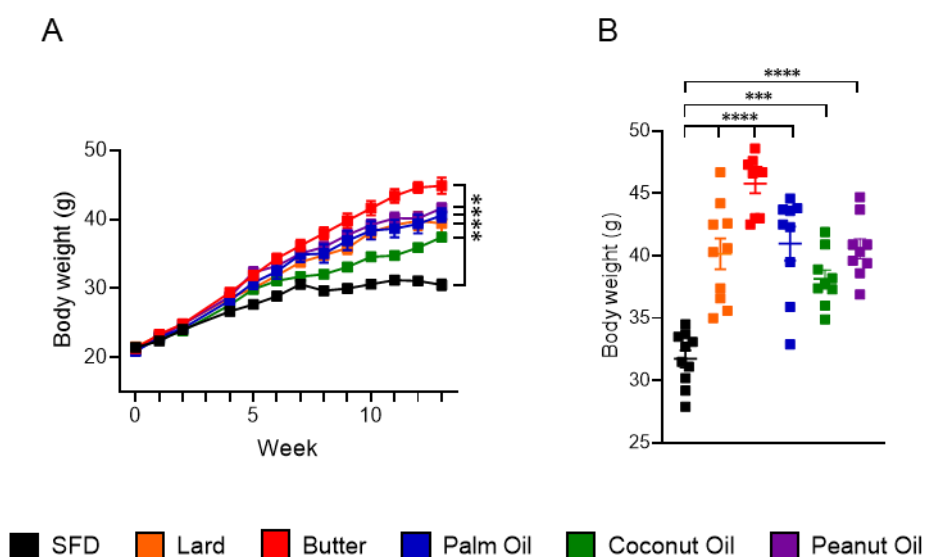


Figure 4.1 HFD feeding causes significant weight gain, regardless of the source of dietary fat

Mice were fed a high-fat diet (HFD, 45% fat) from animal or plant sources or a standard-fat diet (SFD, 12% fat) for 13 weeks. Graphs show weight gain over 13 weeks **(A)** and final body weight **(B)**. Data are representative growth curve **(A)** or mean $\pm$ S.E.M, pooled data **(B)** from N=2 independent experiments, n=10 mice per group. Squares represent individual mice. Significance was calculated using two-way ANOVA with Bonferroni test **(A)**, ordinary one-way ANOVA with Dunnett test **(B)**. **** $p \leq 0.0001$. Experiments performed with Dr Roisin Loftus and Dr Lydia Dyck.

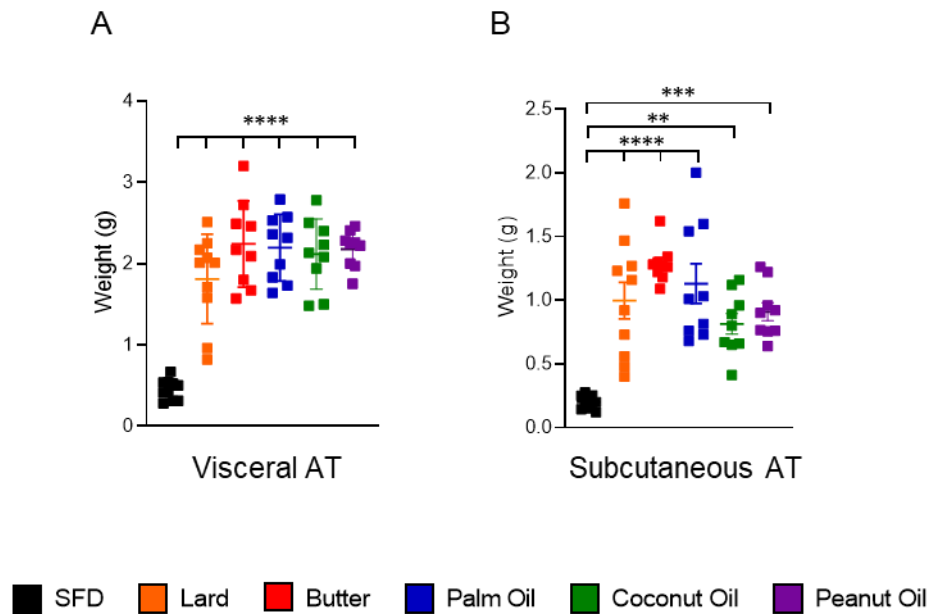


Figure 4.2 HFD feeding increases the weight of the visceral and subcutaneous adipose depots, regardless of the source of dietary fat

Mice were fed a high-fat diet (HFD, 45% fat) from animal or plant sources or a standard-fat diet (SFD, 12% fat) for 13 weeks. Graphs show weight of visceral **(A)** and subcutaneous **(B)** adipose tissues (AT). Data are mean $\pm$ S.E.M, pooled data from N=2 independent experiments, n=10 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test **(B)**.** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. *Experiments performed with Dr Roisin Loftus and Dr Lydia Dyck.*

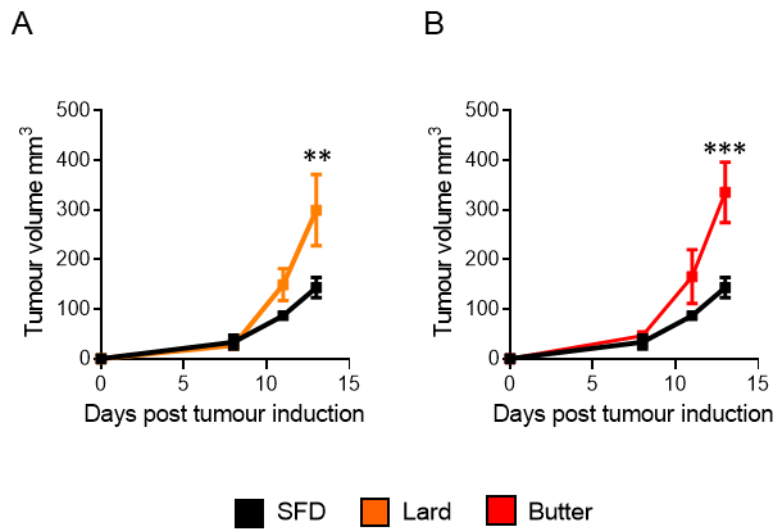


Figure 4.3 Animal HFDs (lard and butter) accelerate B16 tumour growth

Mice were fed a high-fat diet (HFD, 45% fat) from lard or butter or a standard-fat diet (SFD, 12% fat) for 13 weeks. After 10 weeks of feeding, B16-F10 cells were injected subcutaneously into the flanks and tumour growth was monitored over 2 weeks. Graphs show tumour volume on days 8, 11 and 13 (**A, B**). Data are mean $\pm$ S.E.M of 1 experiment, $n=5$ mice per group. Significance was calculated using a two-way ANOVA with Bonferroni test. ** $p \leq 0.01$, *** $p \leq 0.001$. *Injections and measurements performed by Dr Lydia Dyck.*

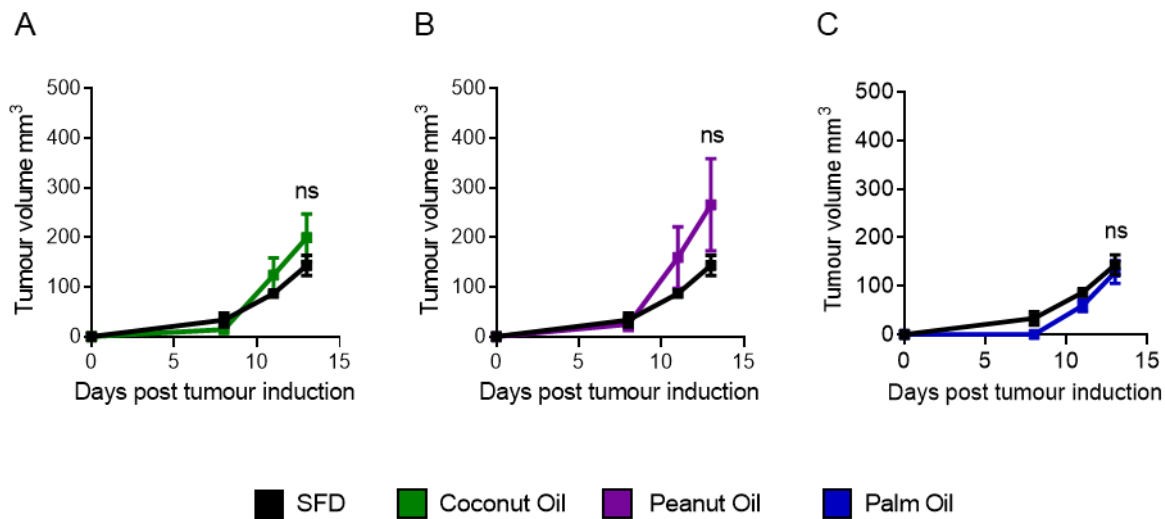


Figure 4.4 Mice fed a HFD from palm oil are completely protected against enhanced tumour growth that is associated with obesity

Mice were fed a high-fat diet (HFD, 45% fat) from coconut oil, peanut oil or palm oil or a standard-fat diet (SFD, 12% fat) for 13 weeks. After 10 weeks of feeding, B16-F10 cells were injected subcutaneously into the flanks and tumour growth was monitored over 2 weeks. Graphs show tumour volume on days 8, 11 and 13 (**A, B**). Data are mean $\pm$ S.E.M of 1 experiment, $n=5$ mice per group. Significance was calculated using a two-way ANOVA with Bonferroni test. ns = not significant. *Injections and measurements performed by Dr Lydia Dyck.*

4.3 Tumour-infiltrating NK and CD8 T cells from mice fed butter, but not palm oil, are functionally impaired

To better understand how some obesogenic diets increase tumour growth while others do not, we focused on the two diets that gave the most different results; animal butter HFD which resulted in the most significant increase in tumour growth, and palm oil HFD which caused no obesity-associated acceleration of tumour growth (Fig. 4.3 B and Fig. 4.4 C). We repeated the experiment with butter and palm oil HFDs twice more and found that butter consistently accelerated tumour growth, while palm oil had no effect, despite equal weight gain (Fig. 4.5 A, B). We and others have shown that obesity impairs tumour infiltration of NK and CD8 T cells which contributes to accelerated tumour growth in mice^{37,114}. Therefore, we next examined the immune infiltrates of B16 tumours from mice fed butter or palm HFDs. The frequency of NK and CD8 T cells was significantly reduced in B16 tumours from mice fed butter, but not when mice were fed palm oil (Fig. 4.6 and Fig. 4.7). IFN γ plays an important anti-tumour role²¹⁴, therefore we next investigated the effects of butter and palm oil on IFN γ production by NK and CD8 T cells. Although not significant, there was a trend towards reduced IFN γ production by NK cells when mice were fed butter, but not palm oil. Tumoral CD8 T cells produced significantly less IFN γ when mice were fed butter, but not palm oil (Fig. 4.8 and Fig. 4.9). Together, these data demonstrate that distinct dietary fat compositions differentially affect NK and CD8 T cell functions in B16 tumours, which may contribute to differences in tumour growth.

These results prompted us to look at the immune infiltrates of all the animal and plant derived diets tested. There were significantly less NK cells and reduced number of CD8 T cells in B16 tumours from mice fed lard (Fig. 4.10 and 4.11), which is consistent with previous reports from our lab and others^{37,113,114}. Similarly, the number of NK cells in B16 tumours from mice fed butter, coconut oil, and peanut oil were significantly reduced compared to mice fed SFD (Fig. 4.10). There was a similar trend of a reduced number of CD8 T cells in tumours from mice fed butter, coconut oil, and peanut oil compared to SFD however this was not significant (Fig. 4.11). In contrast to the other HFDs, the number of tumoral NK or CD8 T cells were not reduced in palm fed mice (Fig. 4.10 and Fig. 4.11). IFN γ production by NK and CD8 T cells was reduced only when mice were fed HFDs from animal sources, though this was not significant (Fig. 4.12). IFN γ production by NK and CD8 T cells was comparable, or in some cases slightly higher, in mice fed plant-derived HFDs compared to mice fed SFD, however this did not reach significance (Fig. 4.12). Thus, animal derived HFDs seem to have a worse impact on

cytotoxic lymphocytes than plant derived fats, with butter and palm oil having the most diverging effects.

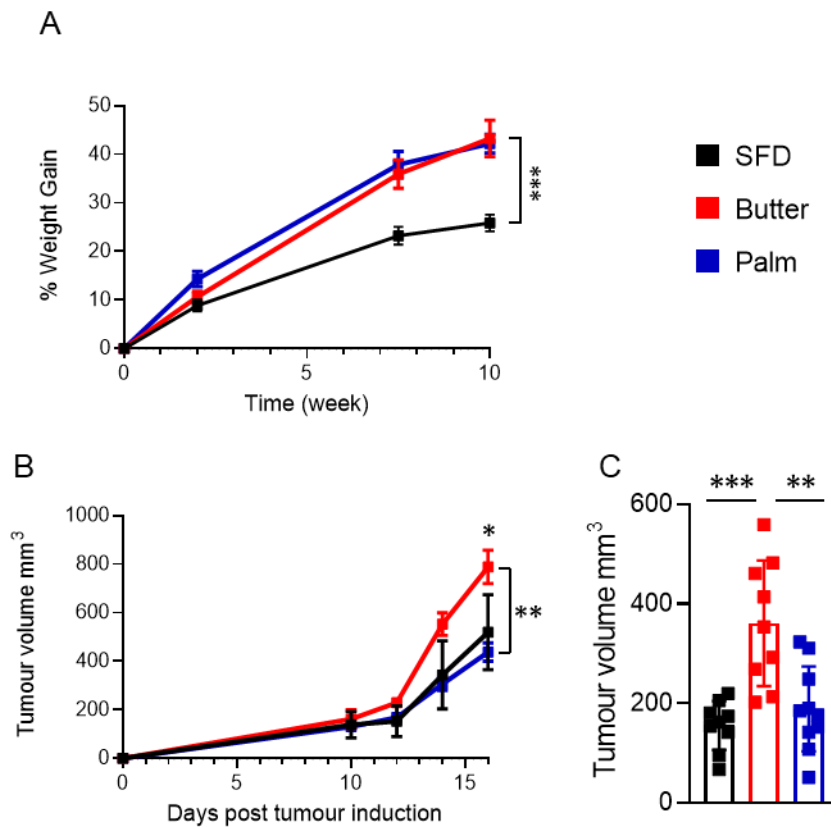


Figure 4.5 Butter HFD, but not palm oil, accelerates B16 tumour growth, despite equal weight gain

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8 weeks of feeding, B16-F10 cells were injected subcutaneously into the flanks and tumour growth was monitored over 2 weeks. Graphs show percentage weight gain over 10 weeks (**A**), tumour volume on days 10, 12, 14 and 16 (**B**), and final tumour volume post tumour induction (**C**). Data are representative growth curve (**A, B**) or mean $\pm$ S.E.M pooled data (**C**) from N=2 independent experiments, n=10 mice per group. Squares represent individual mice. Significance was calculated using a two-way ANOVA with Bonferroni test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. *Injections and measurements performed by Dr Lydia Dyck.*

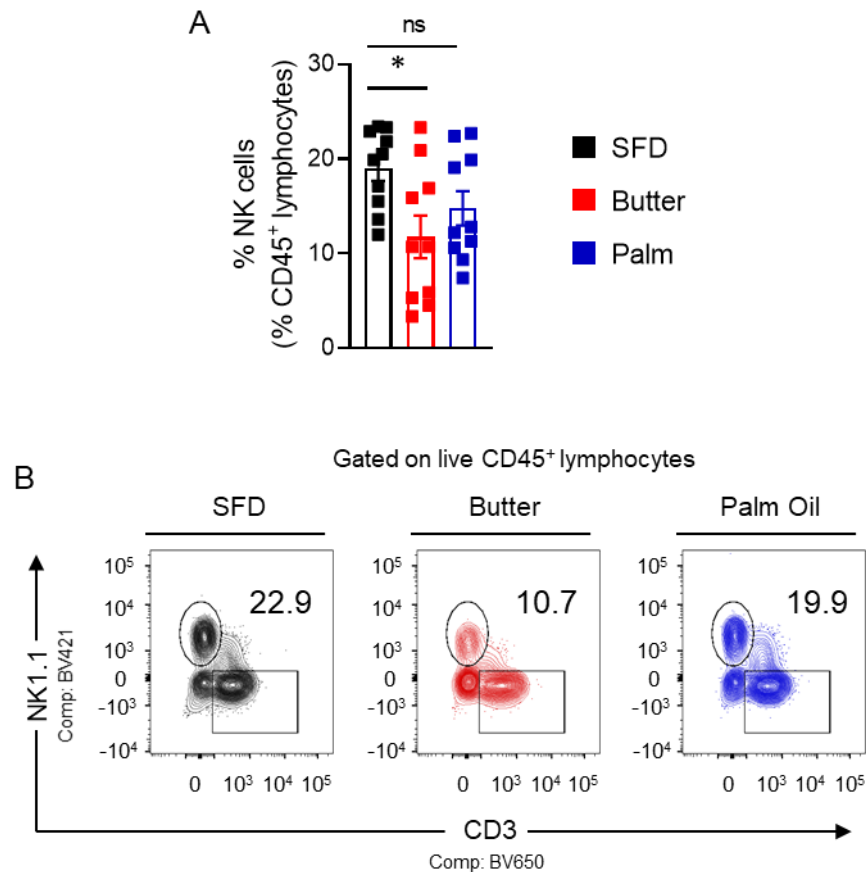


Figure 4.6 Butter HFD, but not palm oil, significantly decreased the frequency of tumoral NK cells in B16 tumours

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8 weeks of feeding, B16-F10 cells were injected subcutaneously into the flanks. Tumours were isolated and intra-tumoral immune cells were analysed by flow cytometry. Graphs show frequency of tumoral NK cells **(A)** and representative flow plot **(B)**. Data mean $\pm$ S.E.M, pooled data from N=2 independent experiments, n=10 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. ns = not significant, * $p \leq 0.05$. *Experiments performed with Dr Roisin Loftus, Dr Lydia Dyck, and Dr Britta Kunkemoeller.*

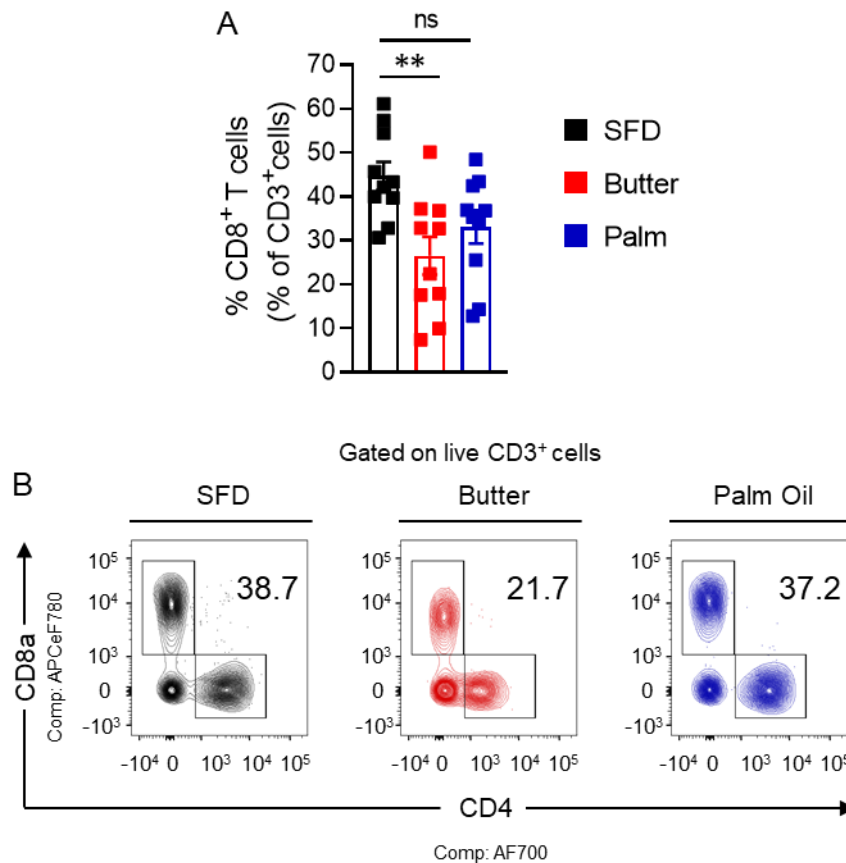


Figure 4.7 Butter HFD, but not palm oil, significantly decreased the frequency of tumoral CD8 T cells in B16 tumours

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8 weeks of feeding, B16-F10 cells were injected subcutaneously into the flanks. Tumours were isolated and intra-tumoral immune cells were analysed by flow cytometry. Graphs show frequency of tumoral CD8 T cells **(A)** and representative flow plot **(B)**. Data are mean $\pm$ S.E.M, pooled data from N=2 independent experiments, n=10 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. ns = not significant, ** $p \leq 0.01$. *Experiments performed with Dr Roisin Loftus, Dr Lydia Dyck, and Dr Britta Kunkemoeller.*

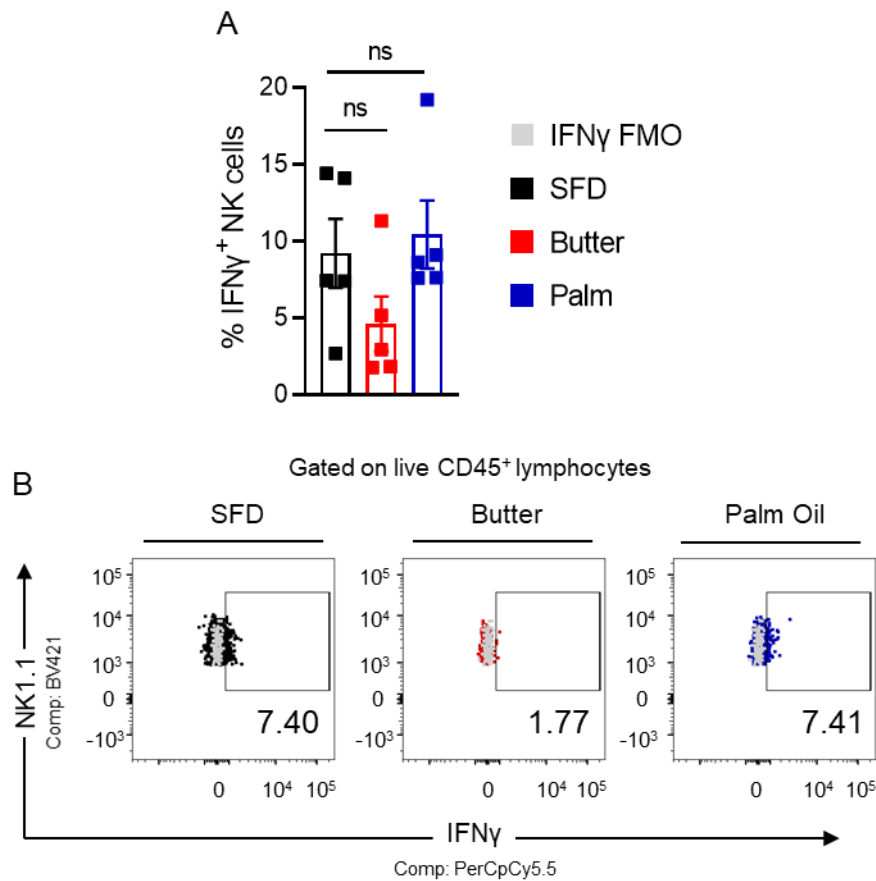


Figure 4.8 Tumoral NK cells produce less IFN γ when mice are fed butter, but not palm oil

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8 weeks of feeding, B16-F10 cells were injected subcutaneously into the flanks. Tumours were isolated, stimulated with PMA, ionomycin and brefeldin A for 4 hr and NK cells were analysed by flow cytometry. Graphs show frequency of IFN γ ⁺ NK cells **(A)** and representative flow plot **(B)**. Data are mean $\pm$ S.E.M for 1 experiment. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. ns = not significant. *Experiments performed with Dr Roisin Loftus and Dr Lydia Dyck.*

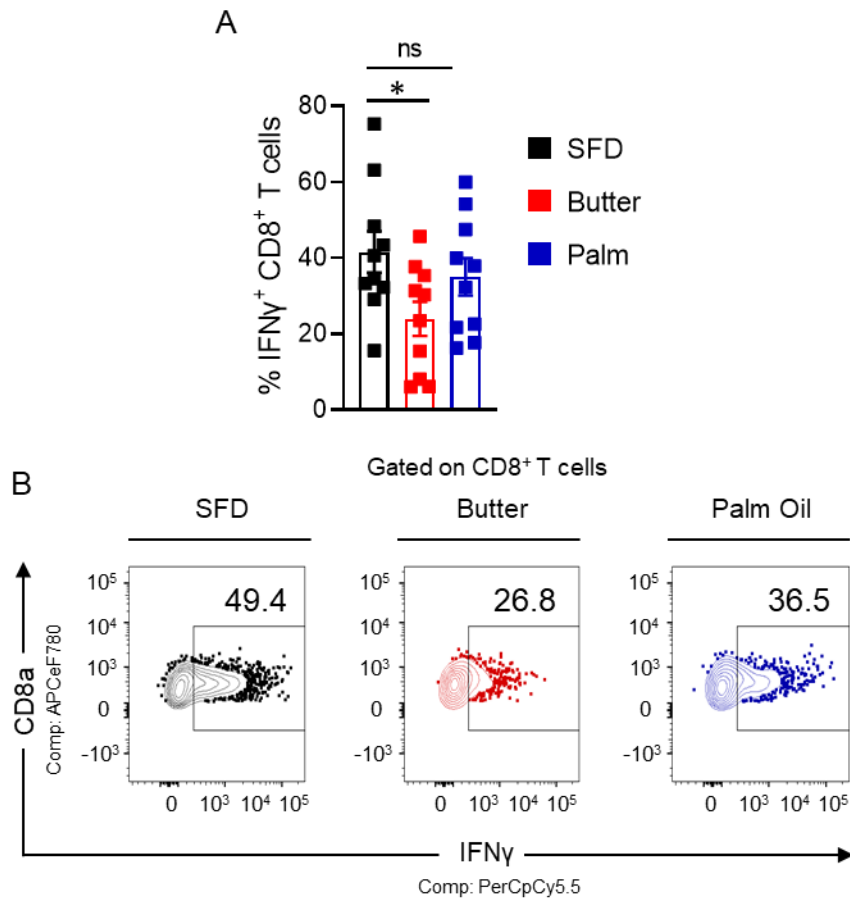


Figure 4.9 Tumoral CD8 T cells produce less IFN γ when mice are fed butter, not palm oil

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 10 weeks. After 8 weeks of feeding, B16-F10 cells were injected subcutaneously into the flanks. Tumours were isolated, stimulated with PMA, ionomycin, and brefeldin A for 4 hr and CD8 T cells were analysed by flow cytometry. Graphs show frequency of IFN γ ⁺ CD8 T cells (**A**) and representative flow plot (**B**). Data are mean $\pm$ S.E.M, pooled data from N=2 independent experiments, n=10 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. ns = not significant, * $p \leq 0.05$. *Experiments performed with Dr Roisin Loftus, Dr Lydia Dyck, and Dr Britta Kunkemoeller.*

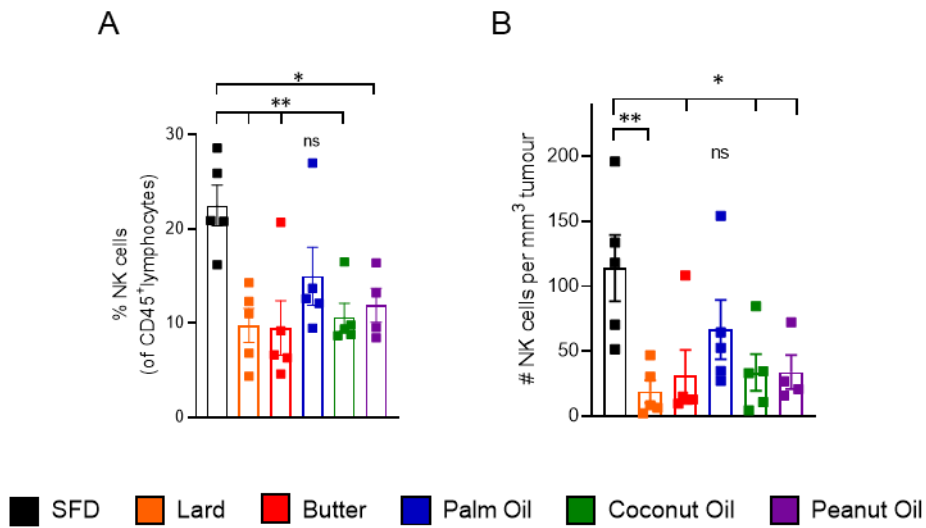


Figure 4.10 All HFDs, except palm oil, significantly decrease the frequency of NK cells and number of NK cells/mm³ in B16 tumours

Mice were fed a high-fat diet (HFD, 45% fat) from animal or plant sources or a standard-fat diet (SFD, 12% fat) for 13 weeks. After 10 weeks of feeding, B16-F10 cells were injected subcutaneously into the flanks. Tumours were isolated and intra-tumoral immune cells were analysed by flow cytometry. Graphs show frequency **(A)** and density **(B)** of tumoral NK cells. Data are mean $\pm$ S.E.M for 1 experiment, n=5 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. ns = not significant, * $p \leq 0.05$, ** $p \leq 0.01$. *Experiments performed with Dr Roisin Loftus and Dr Lydia Dyck.*

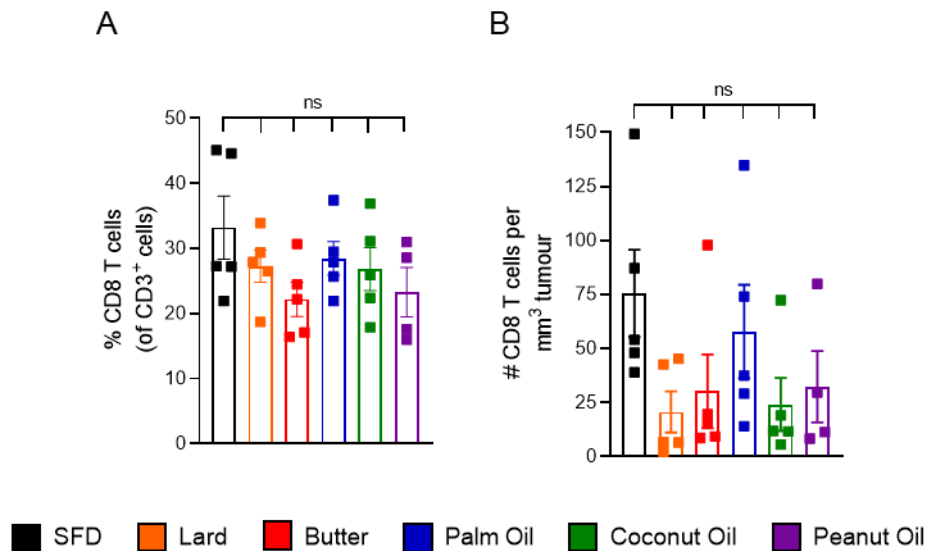


Figure 4.11 HFD does not affect the frequency of tumoral CD8 T cells, but all HFDs, except palm oil, decrease the number of CD8 T cells/mm³ in B16 tumours

Mice were fed a high-fat diet (HFD, 45% fat) from animal or plant sources or a standard-fat diet (SFD, 12% fat) for 13 weeks. After 10 weeks of feeding, B16-F10 cells were injected subcutaneously into the flanks. Tumours were isolated and intra-tumoral immune cells were analysed by flow cytometry. Graphs show frequency **(A)** and density **(B)** of tumoral CD8 T cells. Data are mean $\pm$ S.E.M for 1 experiment, n=5 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. ns = not significant. *Experiments performed with Dr Roisin Loftus and Dr Lydia Dyck.*

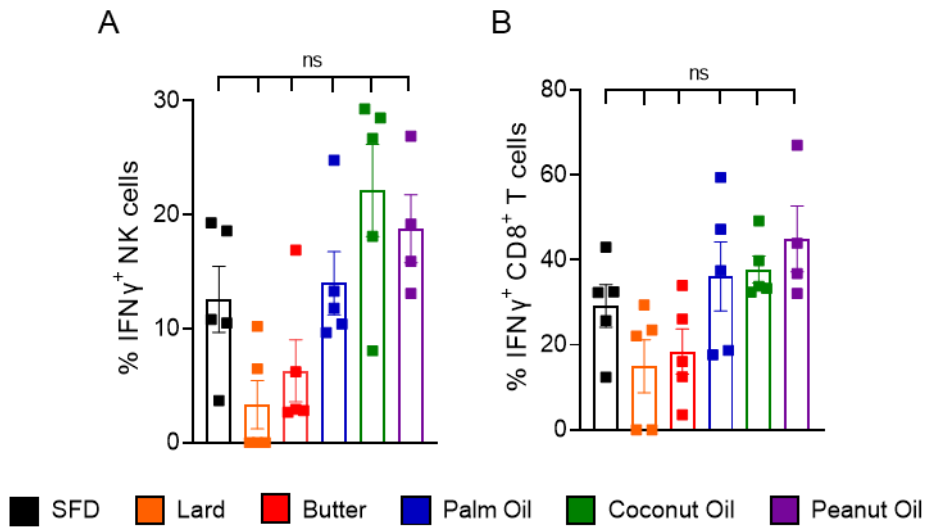


Figure 4.12 IFN γ expression is slightly reduced in tumoral NK and CD8 T cells when mice are fed animal, but not plant HFDs

Mice were fed a high-fat diet (HFD, 45% fat) from animal or plant sources or a standard-fat diet (SFD, 12% fat) for 13 weeks. After 10 weeks of feeding, B16-F10 cells were injected subcutaneously into the flanks. Tumours were isolated and intra-tumoral immune cells were analysed by flow cytometry. Graphs show percentage of IFN γ ⁺ NK cells (**A**) and CD8 T cells (**B**). Data mean $\pm$ S.E.M for 1 experiment, n=5 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. *Experiments performed with Dr Roisin Loftus and Dr Lydia Dyck.*

4.4 Obesity alters systemic metabolism regardless of the source of fat

The striking differences in tumour growth and immune function between butter or palm fed mice led us to question if this was due to differential effects on systemic metabolism caused by the different diets. Obesity is associated with whole body metabolic changes including a switch from carbohydrates to lipids as the main fuel source, impaired glucose handling, and fatty liver, which favour tumour growth¹⁹⁸. To address this, we first examined whole-body substrate utilisation using indirect calorimetry. Typically, mice fed regular chow (SFD) alternate between carbohydrate and lipid metabolism during the dark-light cycle, which can be measured by the RER²¹⁵ (Fig. 4.13). HFD feeding is known to disrupt the metabolic circadian rhythm of mice, resulting in reduced RER, indicating a switch to lipid metabolism over the full 24hrs⁸². Regardless of fat source, both HFDs caused a significant reduction in RER and loss of RER circadian rhythm (Fig. 4.13). Moreover, both HFDs caused increased fasting blood glucose levels and impaired glucose handling compared to SFD (Fig. 4.14).

The liver plays a major role in systemic lipid metabolism and coordinates lipid oxidation under fasting conditions with lipid synthesis from carbohydrate and protein during the fed state¹²⁹. Hepatocyte lipid accumulation is a common feature of obesity and leads to the development of fatty liver disease. Histological analysis of liver sections revealed that LDs accumulate in the livers of mice fed both butter and palm oil HFDs (Fig. 4.15 A). We also found that both HFDs caused an upregulation of PPAR- γ and PPAR- α transcription factors that regulate lipid metabolism in liver tissues (Fig. 4.15 B, C), similar to results from multiple published genetic and diet models of obesity²¹⁶. Together, these data demonstrate that the gross metabolic hallmarks of obesity were not significantly different between mice fed butter or palm oil so were unlikely the cause of the differential tumour growth and impaired immunity between both diets.

It is emerging that diet composition plays a significant role in cancer development, as well as a patient's response to certain cancer therapies¹⁸². Differences in FA saturation contribute to PDAC tumour growth *in vivo*²¹⁷ and FAs differentially affect tumour cell proliferation *in vitro*¹⁹⁹. To investigate the main differences in the FA composition of each diet, we performed FAME analysis of the diet pellets. We first analysed the total levels of SFAs, MUFAs, and PUFAs in each diet. Traditionally, animal-based fats are rich in SFAs, whereas unsaturated fats come largely from plant sources²¹⁸. Both butter and palm HFDs contain a large proportion of SFAs (Fig. 4.16 A, B), and therefore it is unlikely that degree of saturation alone explains the differential tumour growth and immune response. However, the ratio of SFA/MUFA is 2 times higher in butter than SFD and

palm oil (Fig. 4.16 C). We next investigated if there were differences in specific FA components that might affect immunity. The FA profile of butter and palm oil was similar, and both diets were mainly comprised of PA (C16:0), OA (C18:1), and linoleic acid (C18:2) (LA) (Fig. 4.17 A). Differences included significantly more myristic acid (C14:0) (MA) and SA (C18:0) in the butter diet (Fig. 4.17 A), as well as more short and medium chain FAs compared to SFD and palm oil (Fig. 4.17 B). We previously reported that PA impairs metabolic pathways in NK cells *in vitro*, resulting in loss of function and impaired cytotoxicity³⁷. To examine if the degree of FA saturation affects immune responses, we cultured CD8 T cells from spleens with two SFAs – PA and SA, or the MUFA OA. PA, SA, or OA did not affect the viability of CD8 T cells *in vitro* (data not shown), however, PA significantly reduced IFN γ expression in CD8 T cells and SA caused a trend towards reduced IFN γ expression in CD8 T cells, though this was not significant. Phosphorylation of S6 was slightly reduced when CD8 T cells were cultured with high concentrations of PA or SA, but this was not significant. Meanwhile, OA did not affect the levels of pS6 or IFN γ expression in CD8 T cells (Fig. 4.18, 4.19, 4.20). As SA negatively affects CD8 T cells *in vitro*, increased levels in butter could contribute to increased CD8 T cell dysfunction. However, overall, the saturated fat content and FA composition were quite similar, and so it may not be one FA component of the diet alone that is responsible for the differential tumour growth and immunity, but a combination of the ratio of total SFA to MUFA and longer chain SFAs. It was recently reported that a HFD with a high C18:0/C16:0 (SA/PA) ratio induces worse metabolic parameters than an isocaloric HFD with low C18:0/C16:0²¹⁹. The C18:0/C16:0 ratio is significantly higher in butter compared to SFD and palm oil and even lower in the palm oil diet compared to SFD (Fig. 4.21). Importantly however, when mice are fed different diets, nutrients are first encountered in the intestine, and intestinal epithelial cells and the microbiota play a key role in handling and transforming dietary lipids which are then released into the circulation²²⁰. Therefore, to identify more physiological lipid components that the immune system may encounter after HFD feeding, we performed metabolomics analysis of the blood plasma after butter or palm oil HFD feeding.

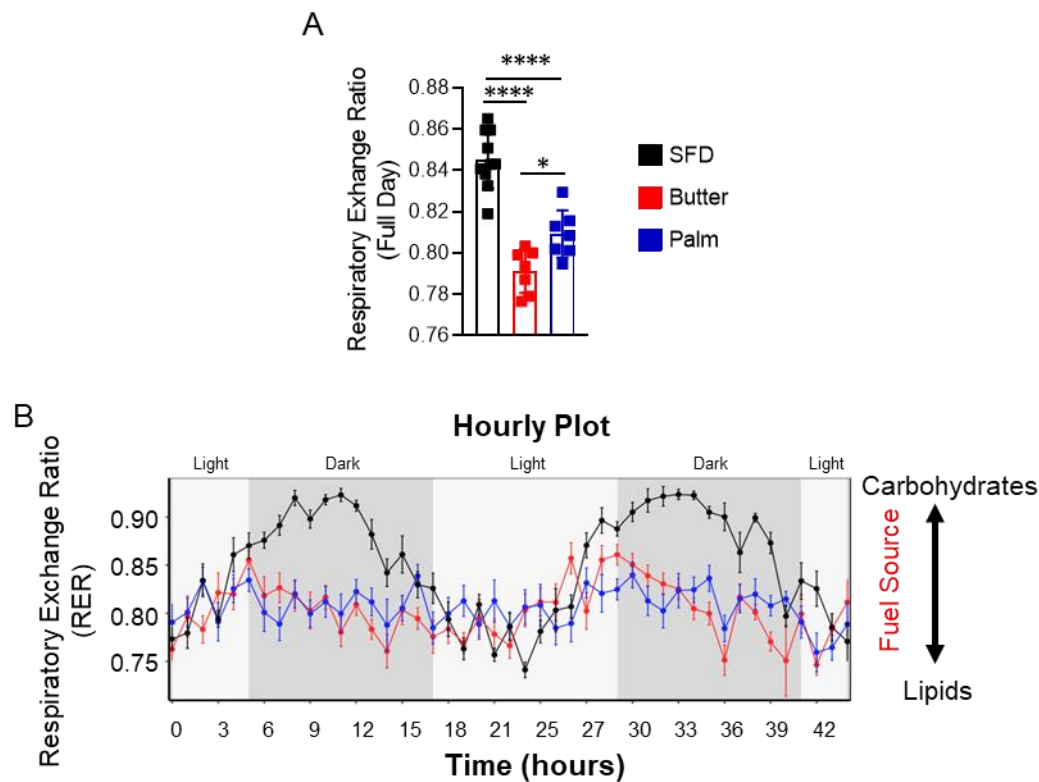


Figure 4.13 Butter and palm oil HFDs reduce the Respiratory Exchange Ratio (RER) of mice

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 1 week. Mice were singly housed in Promethion metabolic cages for 2 days. Graphs show the RER of mice over a full day (**A**) or over 45 hr (**B**). Data are mean $\pm$ S.E.M, pooled data (**A**) or representative plot (**B**) from N=2 independent experiments, n=10 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, **** $p \leq 0.0001$.

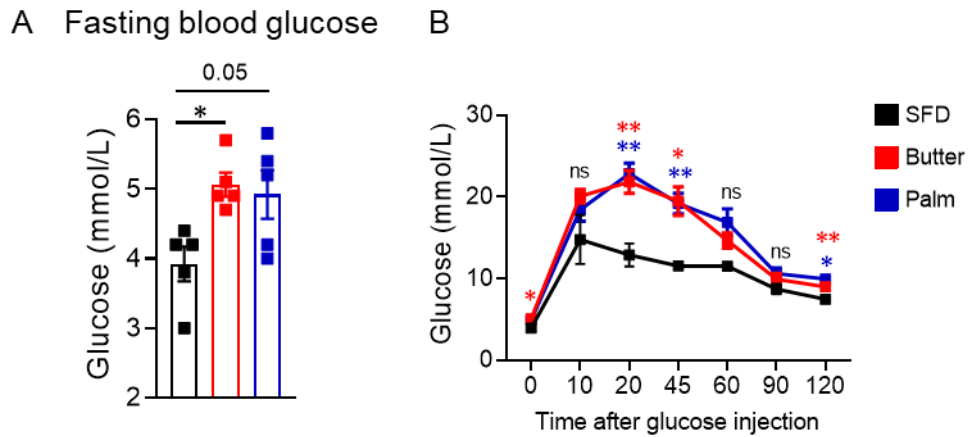


Figure 4.14 Butter and palm oil HFDs increase fasting blood glucose levels and impair glucose tolerance

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 12 weeks. Mice were fasted overnight. Graph **(A)** shows fasting blood glucose levels (mmol/L). Mice were injected intraperitoneally with glucose (2g/kg body weight) and a glucose tolerance test was performed. Graph **(B)** shows blood glucose measurements at 0, 10, 20, 45, 60, 90 and 120 min. Data are mean $\pm$ S.E.M from 1 independent experiment, $n=5$ mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test **(A)** or two-way ANOVA with Bonferroni test **(B)**. * $p \leq 0.05$, ** $p \leq 0.01$. *Experiment performed with Aaron Douglas.*

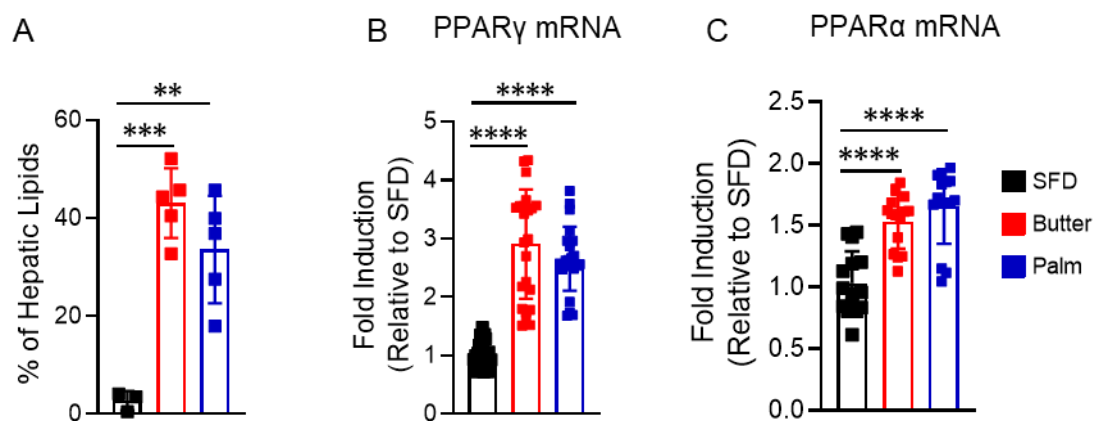


Figure 4.15 Butter and palm oil HFDs cause hepatocyte lipid accumulation and induce PPAR transcription factors in livers

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 12 weeks. Livers were frozen in OCT cryomolds, sectioned to 5 μ m thickness, and stained with oil red o solution to analyse hepatic lipids **(A)**. Livers were snap frozen in liquid nitrogen and qPCR was performed **(B, C)**. Graphs show the % of hepatic lipids **(A)** and the relative expression of PPAR γ and PPAR α transcription factors **(B, C)**. Data are mean $\pm$ S.E.M from 1 independent experiment, n=5 mice per group **(A)** or pooled data from N=2 independent experiments **(B)**. Squares represent individual mice **(A)** or technical replicates **(B, C)**. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. *Oil Red O staining was performed by Dr Cathal Harmon.*

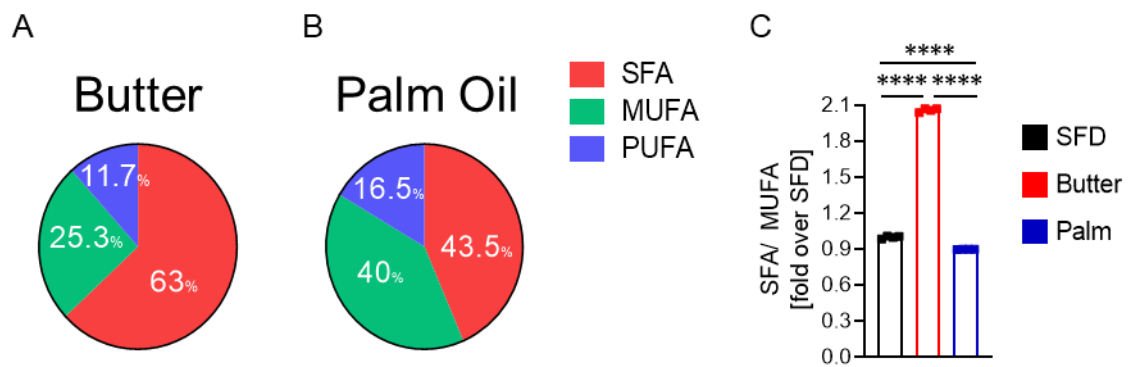


Figure 4.16 Butter diet, but not palm oil diet, has a high saturated / monounsaturated fatty acid ratio

Diet pellets were minced, and fatty acid methyl ester (FAME) analysis was performed. Graphs show frequency pie charts of the levels of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids. % of SFA, MUFA and PUFAs are indicated in white (**A, B**). Ratio of SFA / MUFA relative to SFD (**C**). Data are average (**A, B**) or mean $\pm$ S.E.M for 4 individual diet pellets (**C**). Significance was calculated using ordinary one-way ANOVA with Dunnett test. **** $p < 0.0001$. FAME analysis was performed by Kathleen Mitchelson, UCD, and analysed by Hannah Prendeville.

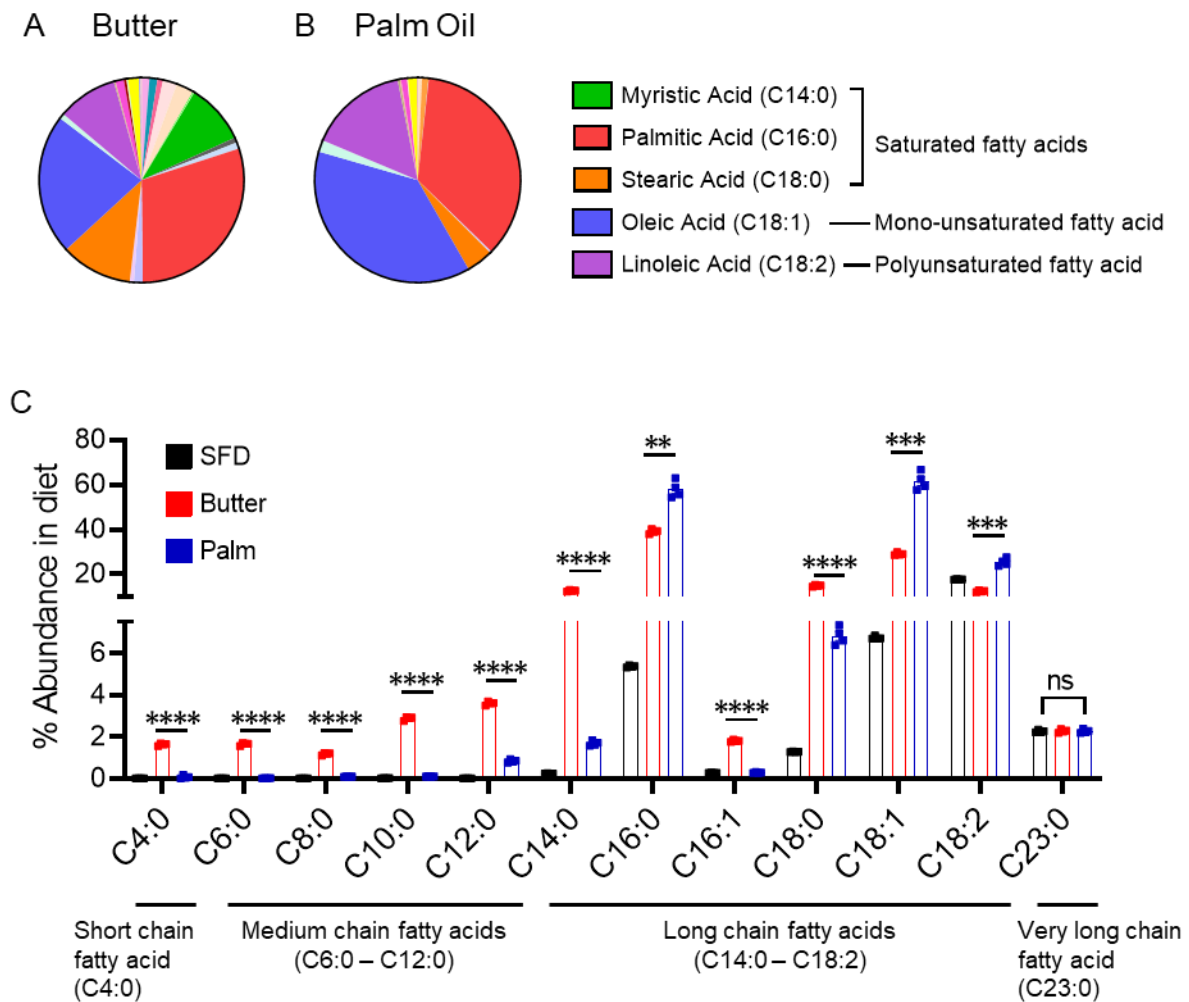


Figure 4.17 Butter diet is rich in short /medium chain saturated fatty acids

Diet pellets were minced, and fatty acid methyl ester (FAME) analysis was performed. Graphs show frequency pie charts of the levels of fatty acids **(A)** or the percentage abundance of short, medium, long, and very-long chain fatty acids **(B)**. Data are average **(A, B)** or mean $\pm$ S.E.M for 4 individual diet pellets **(C)**. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$. FAME analysis was performed by Kathleen Mitchelson, UCD, and analysed by Hannah Prendeville.

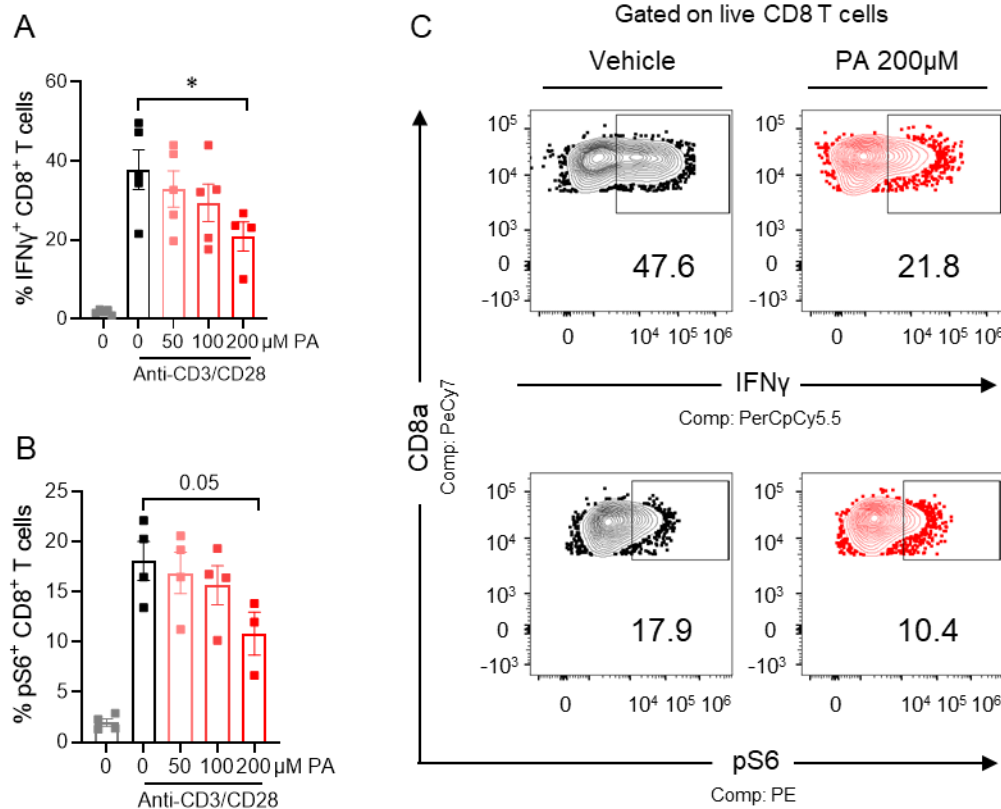


Figure 4.18 IFN γ expression in CD8 T cells is blunted by palmitic acid *in vitro*

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of palmitic acid (PA) (50-200 μ M) and stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5ng/ml) for 48hr. Experiments were performed in technical triplicates which were averaged for each experiment (individual squares). Graphs show percentage of IFN γ ⁺ CD8 T cells **(A)**, pS6⁺ CD8 T cells **(B)** and representative flow plots **(C)**. Data are mean $\pm$ S.E.M from N=3-5 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$

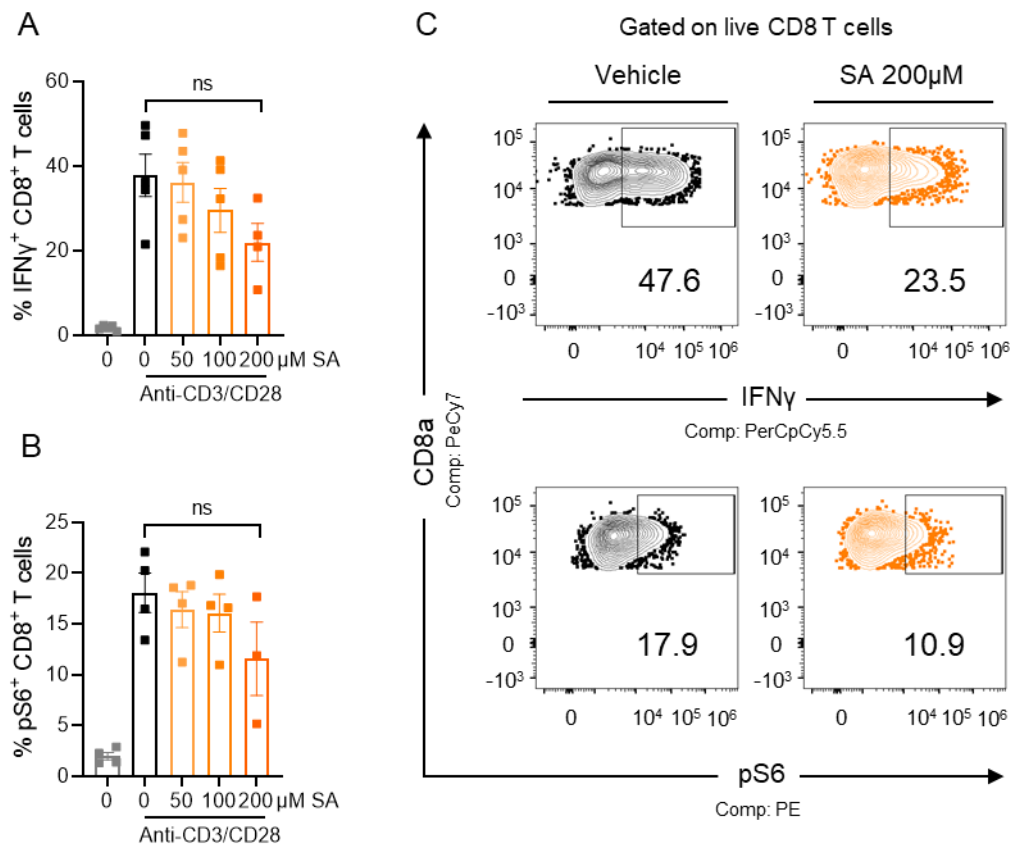


Figure 4.19 Stearic acid slightly reduces IFN γ expression in CD8 T cells *in vitro*

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of stearic acid (SA) (50-200 μ M) and stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5ng/ml) for 48hr. Experiments were performed in technical triplicates which were averaged for each experiment (individual squares). Graphs show percentage of IFN γ ⁺ CD8 T cells (**A**), pS6⁺ CD8 T cells (**B**) and representative flow plots (**C**). Data are mean $\pm$ S.E.M from N=3-5 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

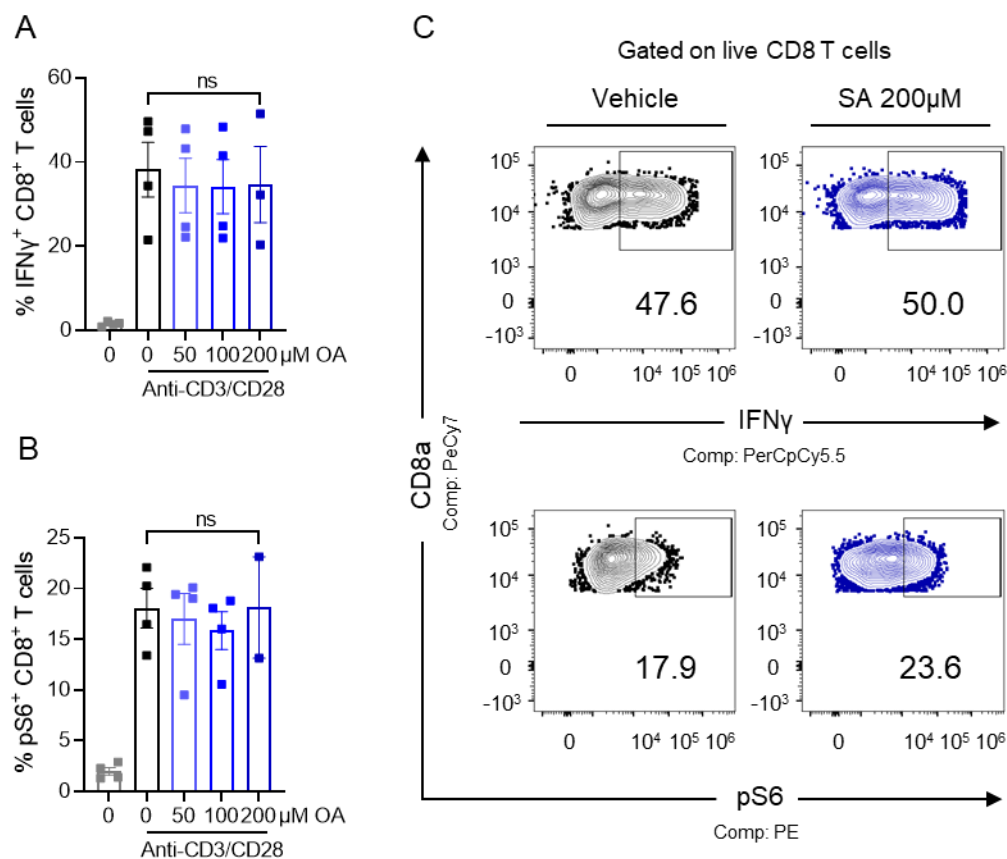


Figure 4.20 Oleic acid does not affect IFN γ expression or phosphorylation of S6 in CD8 T cells *in vitro*

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of oleic acid (OA) (50-200 μM) and stimulated with anti-CD3 (1 μg/ml), CD28 (3 μg/ml), and IL-2 (5 ng/ml) for 48 hr. Experiments were performed in technical triplicates which were averaged for each experiment (individual squares). Graphs show percentage of IFN γ ⁺ CD8 T cells **(A)**, pS6⁺ CD8 T cells **(B)** and representative flow plots **(C)**. Data are mean $\pm$ S.E.M from N=2-4 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ns = not significant.

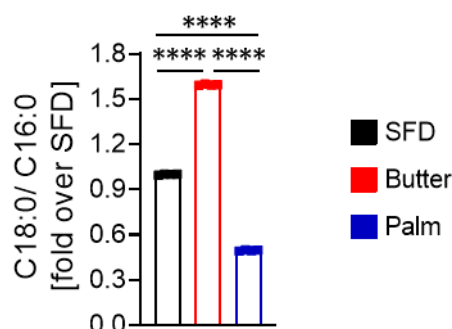


Figure 4.21 Butter diet, but not palm oil diet, has a high stearic acid (C18:0) / palmitic acid (C16:0) ratio

Diet pellets were minced, and fatty acid methyl ester (FAME) analysis was performed. Graph shows the ratio of C18:0 / C16:0 in diet pellets. Data are mean $\pm$ S.E.M relative to SFD, for 4 individual diet pellets. Significance was calculated using ordinary one-way ANOVA with Dunnett test. **** $p \leq 0.0001$. *FAME analysis was performed by Kathleen Mitchelson, UCD, and analysed by Hannah Prendeville.*

4.5 Butter HFD increases the plasma abundance of long-chain acylcarnitines compared to a palm oil HFD

While there were no gross differences in indirect calorimetry or systemic glucose analysis, we found significant differences in the metabolome. Metabolomic analysis of the plasma was performed after mice were fed butter or palm oil HFDs in a non-tumour setting. PCA analysis revealed that the metabolome of mice fed a HFD is distinct from SFD-fed mice, regardless of the source of fat (Fig. 4.22 A). Indeed, many metabolites were reduced in the plasma of mice fed butter or palm oil compared to mice fed SFD (Fig. 4.22 B, C, Table 4.2, Table 4.3). These included aminobenzoic acid, part of the vitamin B9 folate complex, which is synthesised by commensal microbiota²⁰⁰, and hippurate which is associated with microbial degradation of dietary components²⁰¹ (Fig.

4.23 A, B). These data likely reflect microbiome dysbiosis, typical of obesity, which occurs irrespective of the source of dietary fat.

Mice fed butter had reduced abundance of the TCA cycle metabolites fumarate and malate and the glycolytic metabolites 2-phosphoglycerate and 3-phosphoglycerate (Fig. 4.24 A, B). In contrast, there were no differences in TCA or glycolytic metabolites in mice fed palm HFD compared to SFD (Fig. 4.24 A, B). Thus, while we did not observe gross differences in systemic metabolism between mice fed butter and palm oil, carbohydrate metabolism is defective in mice fed butter, but not palm oil.

When comparing the metabolome of mice fed butter vs. palm oil, we identified only 6 differentially expressed metabolites (Fig. 4.25 A, B). These include myristoyl-carnitine (C14) and octadecanoyl-carnitine (C18) which are two LCACs derived from the oxidation of MA (C14:0) and SA (C18:0) FAs (Fig. 4.26 A, B). While PA is abundant in both butter and palm HFDs, MA and SA FAs are selectively enriched in butter (Fig. 4. 17), which correlates with higher levels of the corresponding acylcarnitines C14 and C18 in the plasma of mice fed butter compared to palm oil. These data further confirm that diet composition dictates metabolite abundance in the plasma. Interestingly, C18 is also increased in the plasma of mice fed a lard HFD compared to SFD (Fig. 3.27 B). Analysis of the complete acylcarnitine profile between butter and palm fed mice found that both HFDs slightly reduced the abundance of S/MCACs (C2, C6, C8), while the LCAC C18 is significantly increased in the plasma of mice fed butter, not palm oil. The LCAC C12 is slightly increased in the plasma of mice fed butter, however this is not significant (Fig. 4.27). These data suggests that the metabolism of LCFAs is disrupted when mice are fed animal fats like butter (and lard (Fig. 3.27)), but not palm oil. Plasma acylcarnitines are important indicators of flux through lipid metabolism and are used as clinical biomarkers to diagnose inborn errors of FA metabolism, which leads to the incomplete oxidation of FAs and export of cytosolic acylcarnitines. Thus, accumulation of LCACs indicate incomplete FAO²²¹. Unlike S/MCFAs which passively diffuse into the mitochondria, LCFAs require the carnitine shuttle, consisting of CPT1/2 and CACT⁸⁰ (Fig. 4. 28). LCFAs are first activated by FACS in the cytosol, generating a fatty acyl-CoA molecule. CPT1, located on the outer mitochondrial membrane exchanges the CoA adduct for carnitine, converting the fatty acyl-CoA to acylcarnitine. The CACT transporter facilitates the entry of acylcarnitine into the mitochondrial matrix, where it is enzymatically converted back to fatty acyl-CoA by CPT2. Once inside the mitochondrial matrix, β -oxidation of the fatty acyl-CoA begins²²². Patients with obesity²²³ or T2D⁸³ often have increased expression of CPT1a but not CACT, and thus express a higher CPT1A: CACT protein ratio. When CPT1A: CACT is high, there is disrupted lipid flux and

accumulation of LCACs which are then shuttled out of the cells where they promote inflammation and can be detected systemically⁸³. Therefore, we next analysed the expression ratio of *Cpt1a* to *Cact* mRNA in key metabolic organs that contribute to whole body FAO – the liver and visceral adipose tissue. Despite increased *Cpt1a* in the liver and particularly in the adipose tissue from mice fed butter, there was no increase in *Cact* (Fig. 4.29 A, B, D, E). In contrast, mice fed palm HFD had a striking and significant increase in *Cact* expression in the liver and adipose compared to both butter HFD and SFD, indicating increased lipid flux through the inner mitochondrial membrane and increased lipid oxidation (Fig. 4.29 B, E). Thus, the *Cpt1a:Cact* ratio was significantly increased in the liver and adipose tissue of mice fed butter compared to SFD or palm oil (Fig. 4.29 C, F). This data indicates inefficient transport and incomplete oxidation of LCFAs resulting in the accumulation of LCACs, such as C18, which are then exported through MCTs and detected in the plasma⁸⁴. *Cpt1a* and *Cact* expression is controlled by PPAR transcription factors which are activated and regulated by PPAR γ co-activator- α (PGC-1 α). During times of high lipid influx, effective and efficient oxidation of FAs in the mitochondria depends on the expression levels of PGC1 α which favours a shift from incomplete to complete FAO²²⁴. PPAR α and γ expression was increased in the livers of mice fed butter and palm oil (Fig. 4.15 C, D), however PGC-1 α expression was higher in the livers of mice fed palm oil compared to butter or SFD (Fig. 4.30). The mRNA expression of PPARs or PGC-1 α was not changed in the adipose of mice fed any HFD (data not shown). In summary, systemic lipid metabolism is altered when mice are fed butter (and lard), but not palm oil, which leads to the accumulation of LCACs through incomplete FAO.

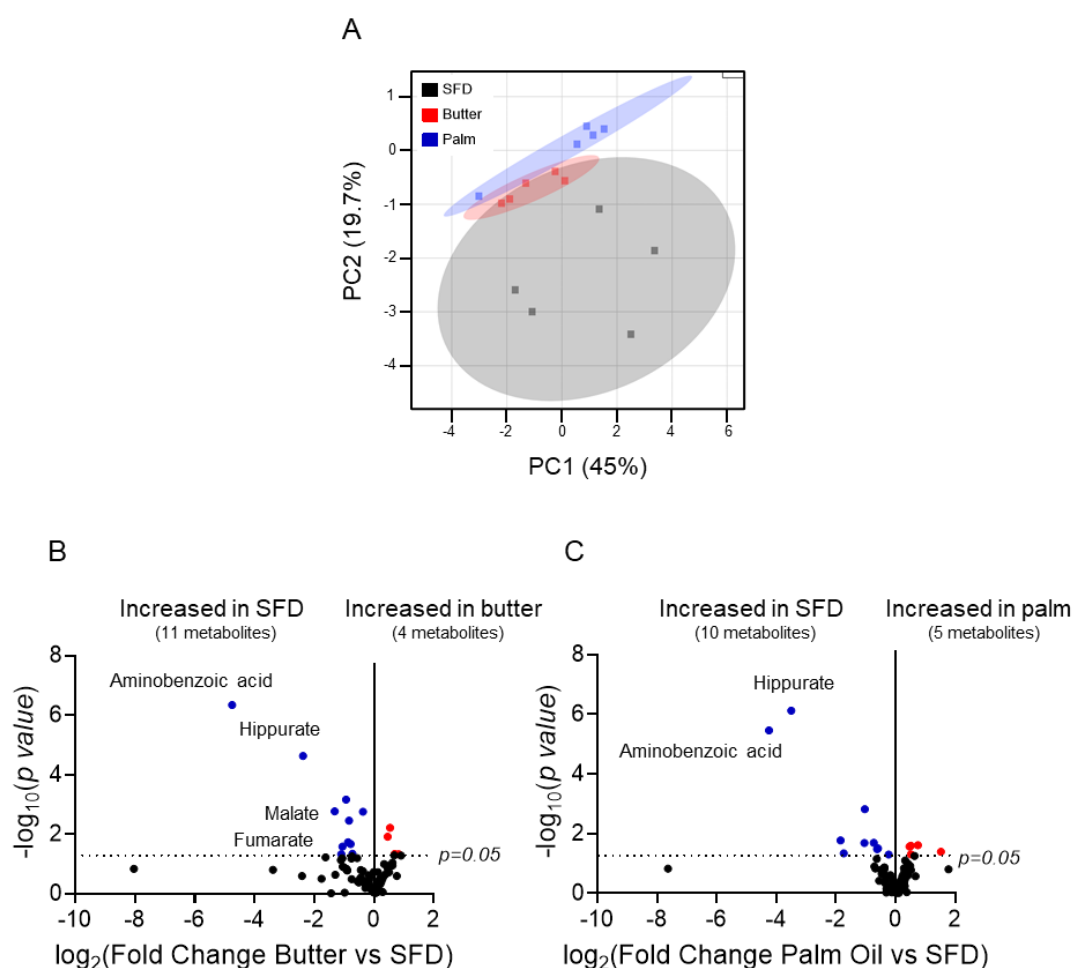


Figure 4.22 HFD feeding perturbs the plasma metabolome regardless of the source of fat

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 1 week. Metabolomic analysis of the plasma metabolome was performed by LC/MS. Graph **(A)** shows PCA analysis of the plasma metabolome. Graphs **(B and C)** show metabolite differences between mice fed butter **(B)** or palm oil **(C)** vs SFD. Metabolites that are significantly increased / decreased in the plasma of mice fed butter or palm oil are highlighted in red or blue, respectively. Data is presented as volcano plots where the horizontal line represents a p value ≤ 0.05 , for 1 experiment, $n=5$ mice per group. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

Metabolite Name	pValue	Fold change
Aminobenzoic Acid	4.55E-07	0.037185
Hippurate	2.33E-05	0.19275
Gluconic Acid	0.000686	0.52297
Linoleic Acid	0.001707	0.40101
Malonate	0.001754	0.7733
Allantoin	0.003502	0.56078
2-Methylglutaric Acid	0.006039	1.4512
Octadecanoyl-carnitine	0.012155	1.3708
Malate	0.018575	0.54738
Acetyl-carnitine	0.021328	0.58122
Pantothenate	0.026105	0.48181
Maleate	0.045447	0.60572
3-Hydroxybutyric Acid	0.046038	1.6103
Alanine	0.046948	0.46673
Asparagine	0.047099	1.7726

Table 4.2 List of significantly altered metabolites in the plasma of mice fed butter relative to SFD.

Metabolite Name	pValue	Fold change
Hippurate	7.54E-07	0.088638
Aminobenzoic Acid	3.44E-06	0.05271
Allantoin	0.001509	0.49042
MDA	0.016726	0.27903
Gluconic Acid	0.020229	0.60241
Linoleic Acid	0.02039	0.48559
Asparagine	0.024357	1.6852
Proline	0.02524	1.4301
2-deoxyguanosine	0.026734	1.382
4-pyridoxate	0.030671	0.66587
Acetyl-carnitine	0.032755	0.65265
Hydroxy palmitoyl-carnitine	0.04019	2.8742
Riboflavin	0.045114	0.30016
Serine	0.048711	1.428
Malonate	0.049447	0.85243

Table 4.3 List of significantly altered metabolites in the plasma of mice fed palm oil relative to SFD.

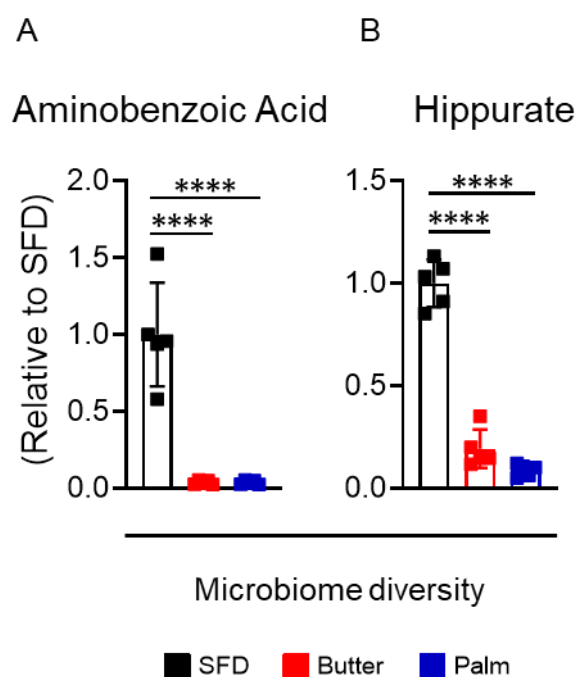


Figure 4.23 Butter and palm oil decrease the plasma abundance of aminobenzoic acid and hippurate

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 1 week. Metabolomic analysis of the plasma metabolome was performed by LC/MS. Graphs show relative abundance of aminobenzoic acid and hippurate metabolites. Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 1 experiment, $n=5$ mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. **** $p \leq 0.0001$. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

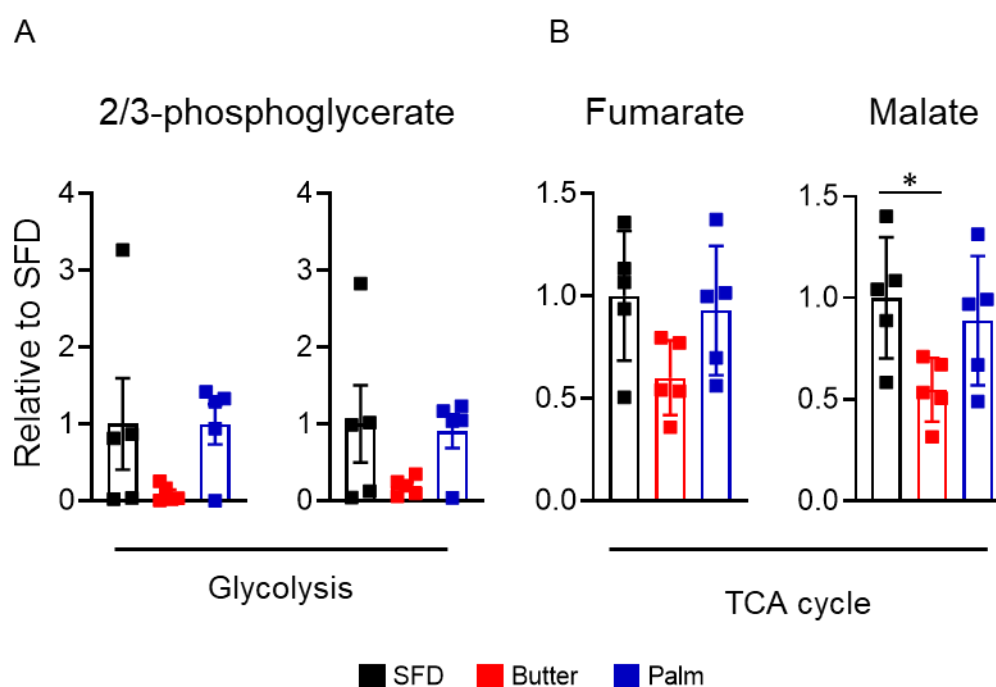


Figure 4.24 Butter, but not palm oil, decreases the plasma abundance of glycolytic and TCA cycle intermediates

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 1 week. Metabolomic analysis of the plasma metabolome was performed by LC/MS. Graphs show relative abundance of 2/3-phosphoglycerate, fumarate, and malate metabolites. Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 1 experiment, $n=5$ mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

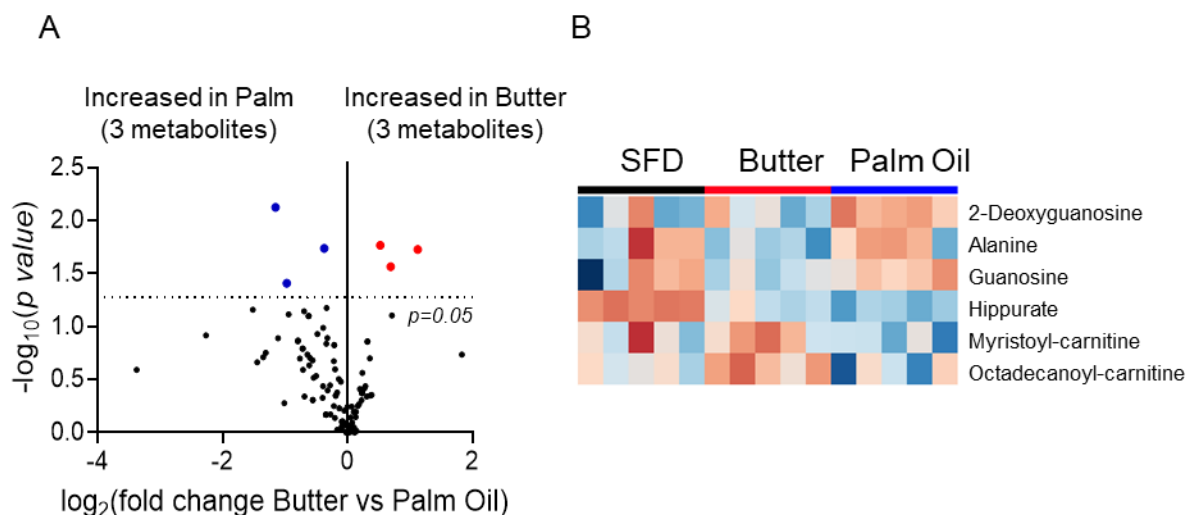


Figure 4.25 6 metabolites are differentially expressed in the plasma of mice fed butter vs palm oil

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 1 week. Metabolomic analysis of the plasma metabolome was performed. Graphs show volcano plot **(A)** or heatmap **(B)** of significantly different metabolites between mice fed butter vs palm oil. Metabolites that are significantly increased / decreased in the plasma of mice fed butter or palm oil are highlighted in red or blue, respectively. Data is presented as volcano plots where the horizontal line represents a $p \text{ value} \leq 0.05$, for 1 experiment, $n=5$ mice per group. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

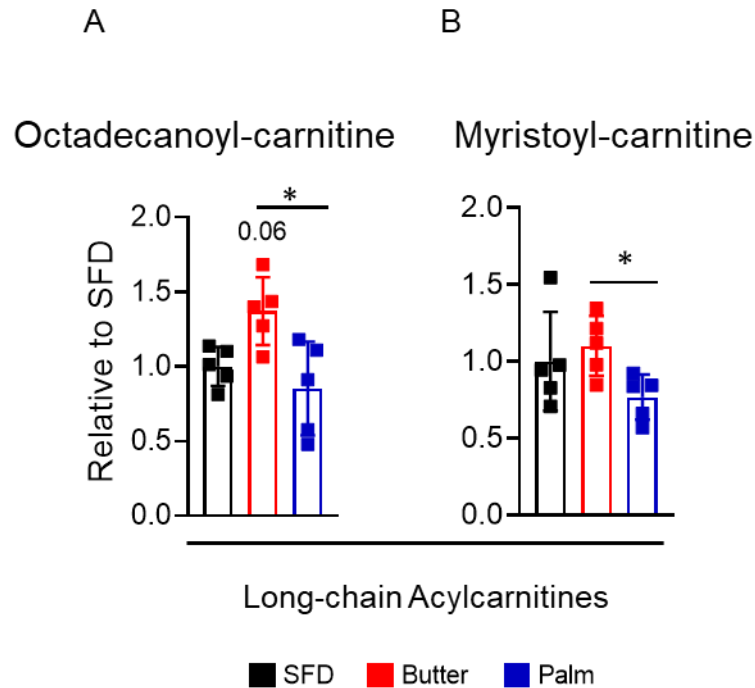


Figure 4.26 Octadecanoyl-carnitine and myristoyl-carnitine are increased in the plasma of mice fed butter compared to mice fed palm oil

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 1 week. Metabolomic analysis of the plasma metabolome was performed. Graphs show relative abundance of octadecanoyl-carnitine and myristoyl-carnitine metabolites. Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 1 experiment, $n=5$ mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

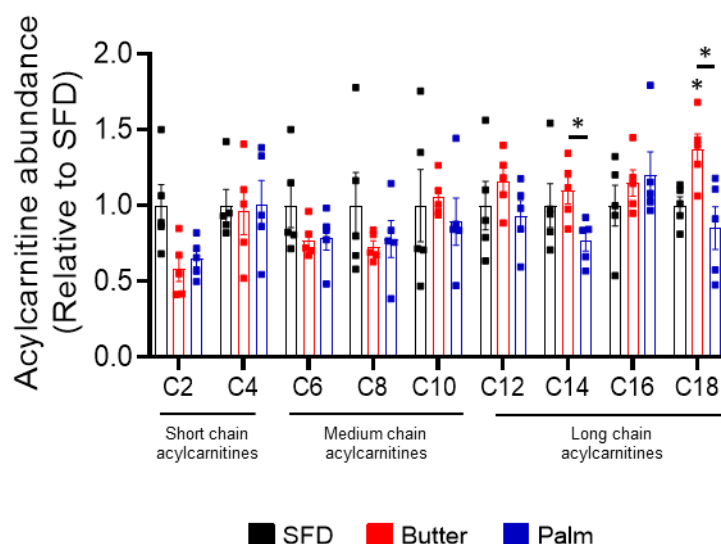


Figure 4.27 Acylcarnitine C18 is increased in the plasma of mice fed butter, not palm oil

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 1 week. Metabolomic analysis of the plasma metabolome was performed. Graphs show relative abundance of short, medium, and long-chain acylcarnitine metabolites. Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 1 experiment, $n=5$ mice per group. Squares represent individual mice. Significance was calculated using ordinary two-way ANOVA with Tukey test. * $p \leq 0.05$. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

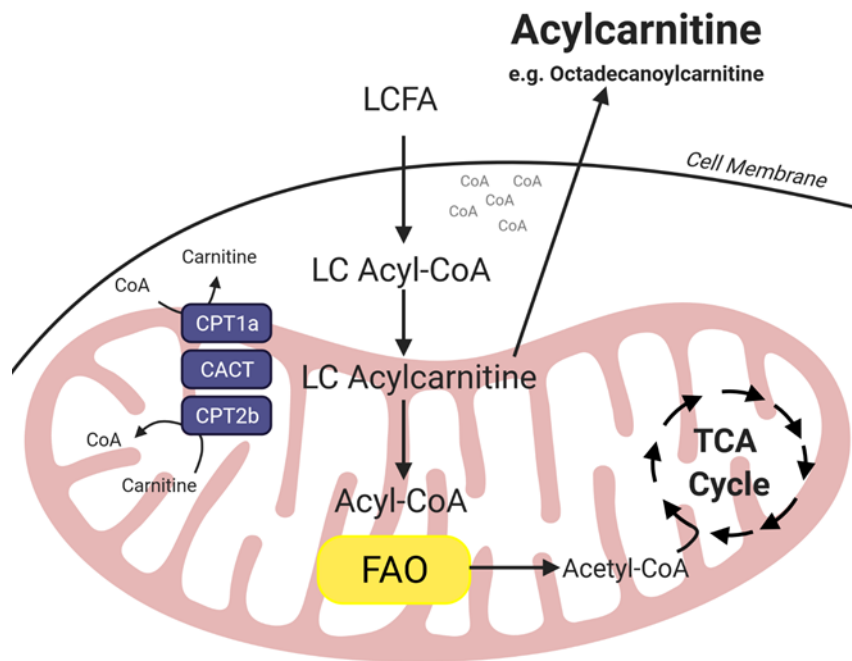


Figure 4.28 Schematic of the carnitine shuttle system and activation of LCFA oxidation

Schematic depicting the carnitine shuttle system used to transport long chain fatty acids into the mitochondria for oxidation. Long chain fatty acid; LCFA, long chain acyl-CoA; LC Acyl-CoA, long chain acylcarnitine; LC Acylcarnitine, carnitine palmitoyl-carnitine 1; Cpt1a, carnitine acylcarnitine translocase; CACT, carnitine palmitoyl-carnitine 2; Cpt2, fatty acid oxidation; FAO, tricarboxylic acid cycle; TCA cycle.

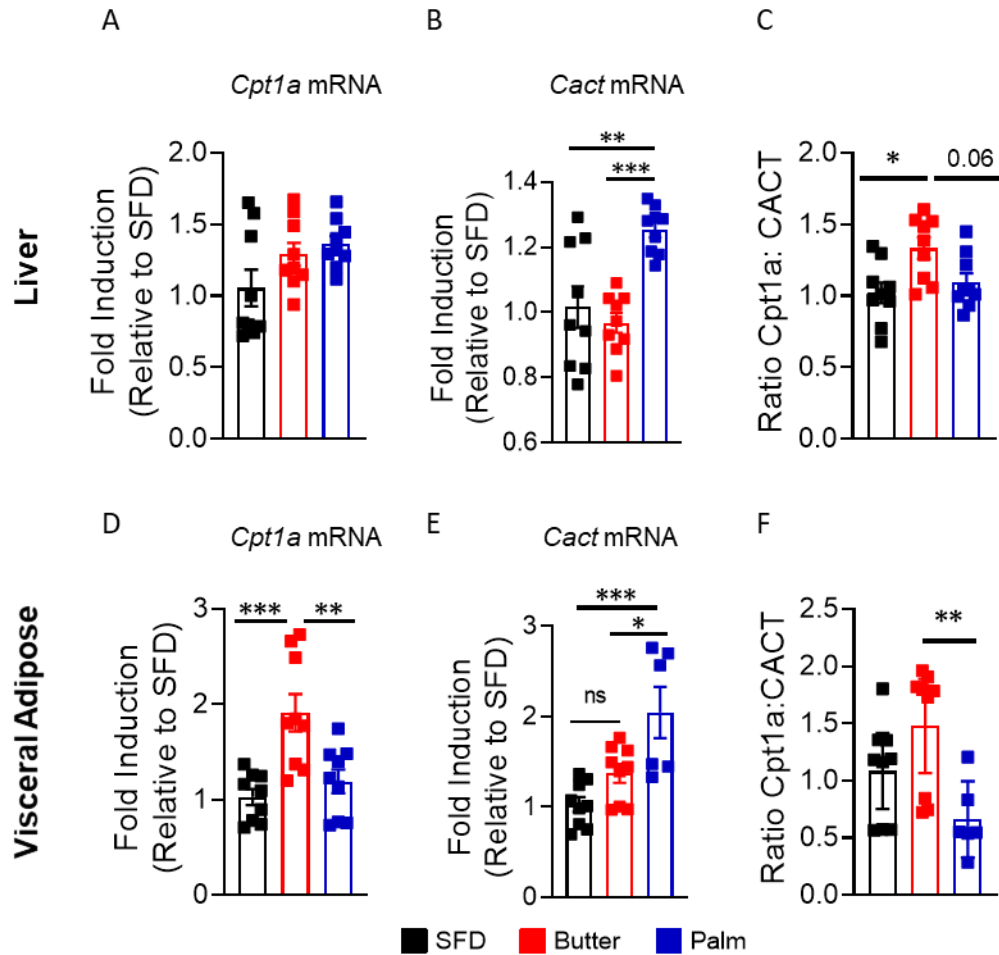


Figure 4.29 Butter, but not palm oil, increases the *Cpt1a*:*Cact* ratio in the liver and visceral adipose tissues

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 12 weeks. Livers were snap frozen in liquid nitrogen and qPCR was performed. Graphs show the relative expression of *Cpt1a* and *Cact* (**A, B, D, E**) or the ratio of *Cpt1a*:*Cact* (**C, F**). Data are mean $\pm$ S.E.M from 1 independent experiment, n=3 mice per group. Squares represent technical replicates. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

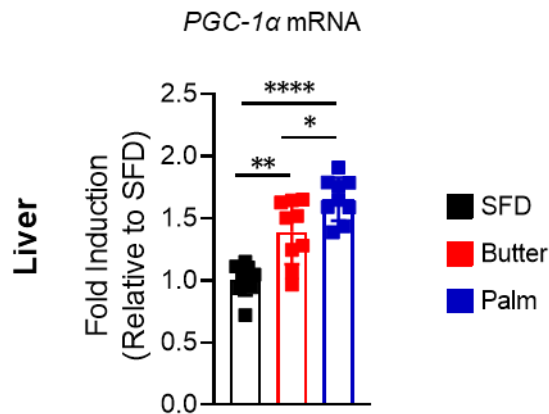


Figure 4.30 PGC-1 α expression is higher in the livers of mice fed palm oil compared to butter or SFD

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 12 weeks. Livers were snap frozen in liquid nitrogen and qPCR was performed. Graph shows the relative expression of PGC-1 α . Data are mean $\pm$ S.E.M from 1 independent experiment, $n=3$ mice per group. Squares represent technical replicates. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.8 NK cells are metabolically paralysed when mice are fed butter, but not palm oil

We previously reported that NK cells from humans with obesity accumulate lipids which interferes with cellular bioenergetics leading to impaired functional responses³⁷, and other groups have subsequently reported similar findings in dysfunctional NK cells that accumulate in human lymphoma¹⁷⁰. Therefore, we next investigated how these different HFDs affect cellular immunometabolic pathways. NK cells from mice fed animal butter and lard HFDs stained strongly for LipidTOX which marks neutral lipids, but not from mice fed palm HFD (Fig. 4.31 A, B, Fig. 3.13 B). Similar findings were seen for CD8 T cells (Fig. 4.31 C, D). Given that lipid accumulation induces NK cell metabolic paralysis³⁷, we hypothesised that NK cells from mice fed butter, but not palm oil would have a blunted metabolic response. Indeed, NK cells from mice fed butter had significantly reduced rates of glycolysis and oxphos, indicating metabolic paralysis. In contrast, NK cell

metabolism is intact when mice are fed palm oil, despite equal obesity in both diets (Fig. 4.32, Fig. 4.33).

To understand how NK cells from mice fed palm oil were protected against the immunometabolic dysfunction associated with obesity, we performed proteomic analysis of NK cells isolated from mice fed butter or palm oil. Regardless of the source of fat, obesity significantly altered the NK cell proteome (Fig. 4.34 A, B). However, 46 proteins were differentially expressed between NK cells from mice fed butter or palm oil (Fig. 4.35 A, B). KEGG pathway analysis of proteomic data found oxphos, TCA cycle, and metabolic pathways were increased in NK cells from mice fed palm oil compared to butter, consistent with our Seahorse data (Fig. 4.36 A). c-Myc is critical for NK cell metabolic reprogramming in response to cytokine stimulation⁸⁹. c-Myc protein expression was reduced in NK cells from mice fed butter but NK cells from mice fed palm oil had optimal levels of c-Myc protein after activation (Fig. 4.36 B). Furthermore, NK cell cytotoxicity pathway was higher in NK cells from palm-fed compared to butter-fed mice (Fig. 4.36 A). Indeed, the activating receptor KLRK1 (NKG2D), which promotes the production of cytolytic molecules and inflammatory cytokines²²⁵, is reduced in NK cells from mice fed butter, not palm oil (Fig. 4.37 A). Macrophage migration inhibitory factor (MIF) downregulates the transcription of KLRK1 leading to impaired NK cell cytotoxicity²²⁶. MIF expression is increased in NK cells from mice fed butter compared to mice fed SFD or palm HFD, which may explain reduced KLRK1 expression (Fig. 4.37 B). In addition, IFN γ protein expression is significantly decreased in NK cells from mice fed butter, but not palm oil (Fig. 3.37 C). Together, these data demonstrate that NK cell cytotoxicity is impaired when mice are fed butter, but not palm oil, which further explains the differences in tumour growth observed *in vivo*.

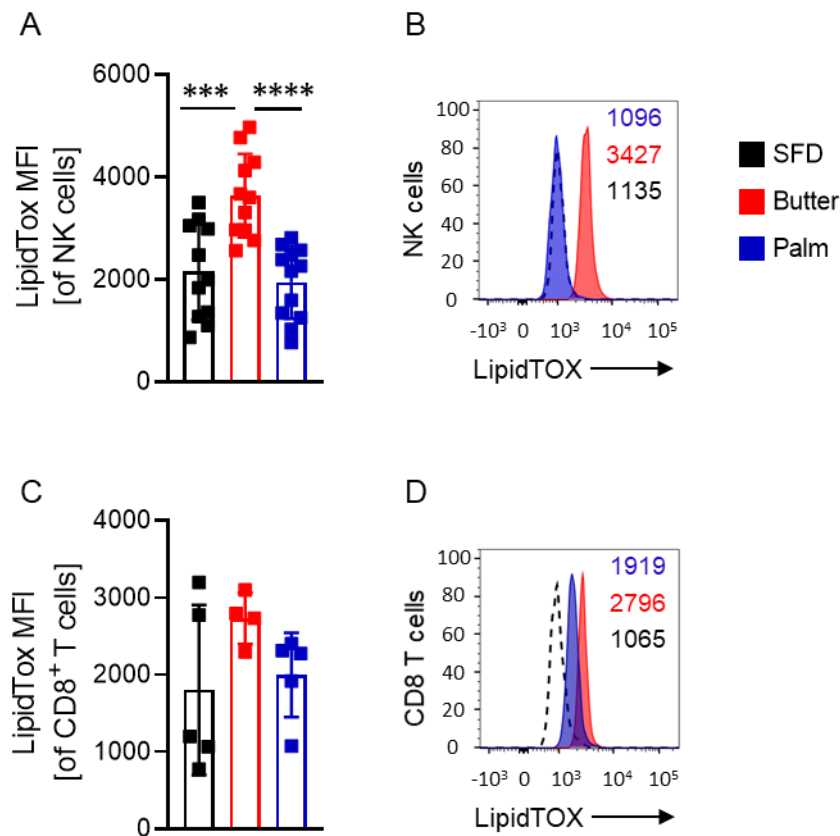


Figure 4.31 Circulating NK and CD8 T cells accumulate lipids when mice are fed butter, not palm oil

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 12 weeks. Graphs show lipid levels in circulating NK and CD8 T cells as assessed by LipidTOX staining. Data are mean $\pm$ S.E.M of LipidTOX mean fluorescence intensity (MFI), pooled data **(A)** or representative histogram **(B)** from 2/1 independent experiments, n=12/5 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. *** p $\leq$ 0.001, **** p $\leq$ 0.0001. *Experiments were performed with Dr Roisin Loftus.*

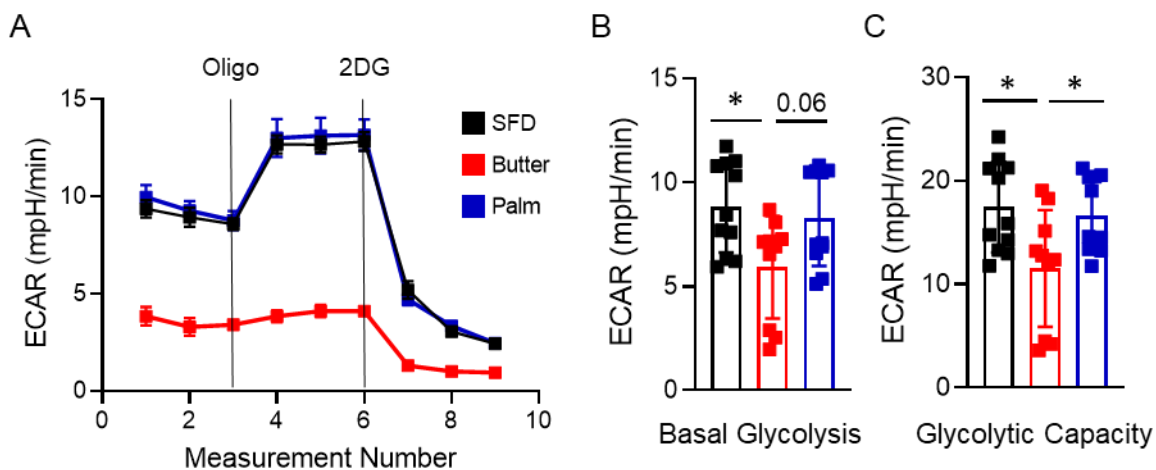


Figure 4.32 The rates of NK cell glycolysis are reduced when mice are fed butter, but not palm oil

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 12 weeks. Splenic NK cells were purified and stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr. Extracellular acidification rate (ECAR) was measured by Seahorse analysis following injection of oligomycin (oligo) and 2-deoxyglucose (2DG). Graphs show representative trace (**A**) and pooled measurements for basal glycolysis (**B**) and glycolytic capacity (**C**). Data are representative (**A**) or mean $\pm$ S.E.M (**B, C**) of N=3 independent experiments. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$. *Experiments were performed with Dr Roisin Loftus.*

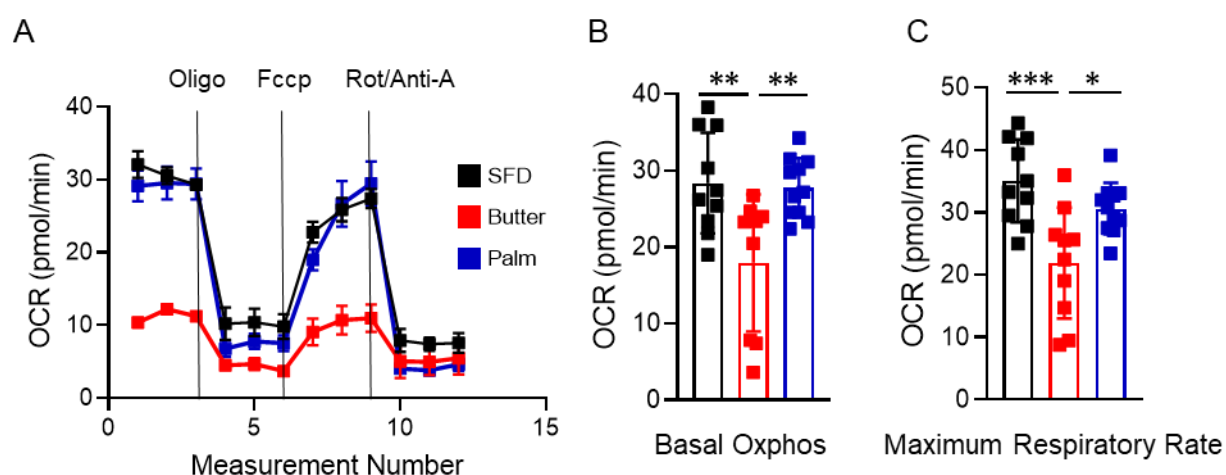


Figure 4.33 The rates of NK cell oxphos are reduced when mice are fed butter, but not palm oil

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 13% fat) for 12 weeks. Splenic NK cells were purified and stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr. Oxygen consumption rate (OCR) was measured by Seahorse analysis following injection of oligomycin (oligo), Fccp, rotenone and anti-mycin A (Rot/Anti-A). Graphs show representative trace (**A**) and pooled measurements for basal oxphos (**B**) and maximum respiratory rate (**C**). Data are representative (**A**) or mean $\pm$ S.E.M (**B, C**) of N=3 independent experiments. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. *Experiments were performed with Dr Roisin Loftus.*

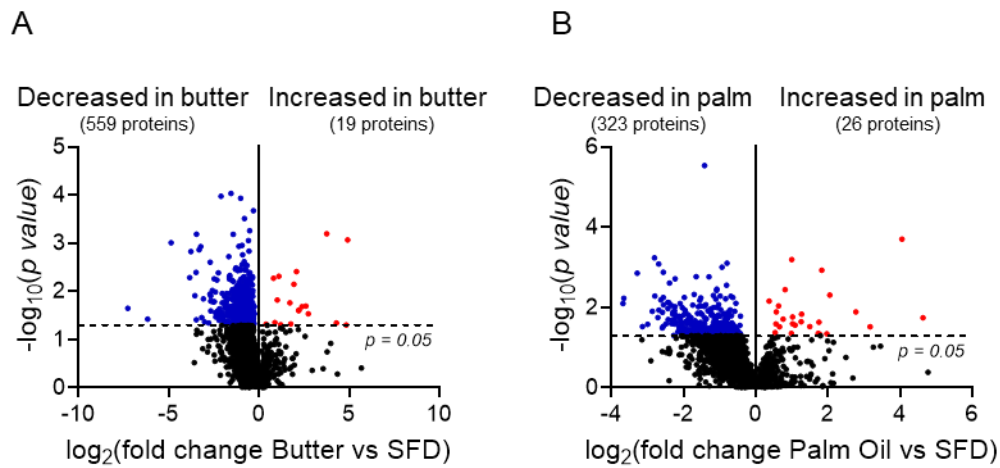


Figure 4.34 HFD feeding alters the NK cell proteome regardless of the source of dietary fat

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 12 weeks. Splenic NK cells were purified and stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr, and proteomic analysis was performed. Graphs show protein abundance differences between NK cells from mice fed a HFD from butter **(A)** or palm oil **(B)** compared to NK cells from mice fed a SFD. Proteins (dots) that are significantly increased/decreased are highlighted in red or blue, respectively. Data is presented as volcano plots where the horizontal line represents a $p \text{ value} \leq 0.05$, for 1 experiment, $n=5$ mice per group. *NK cell purification was performed with Dr Roisin Loftus. Proteomics analysis performed by Dr Linda Sinclair, University of Dundee. Analysis by Hannah Prendeville.*

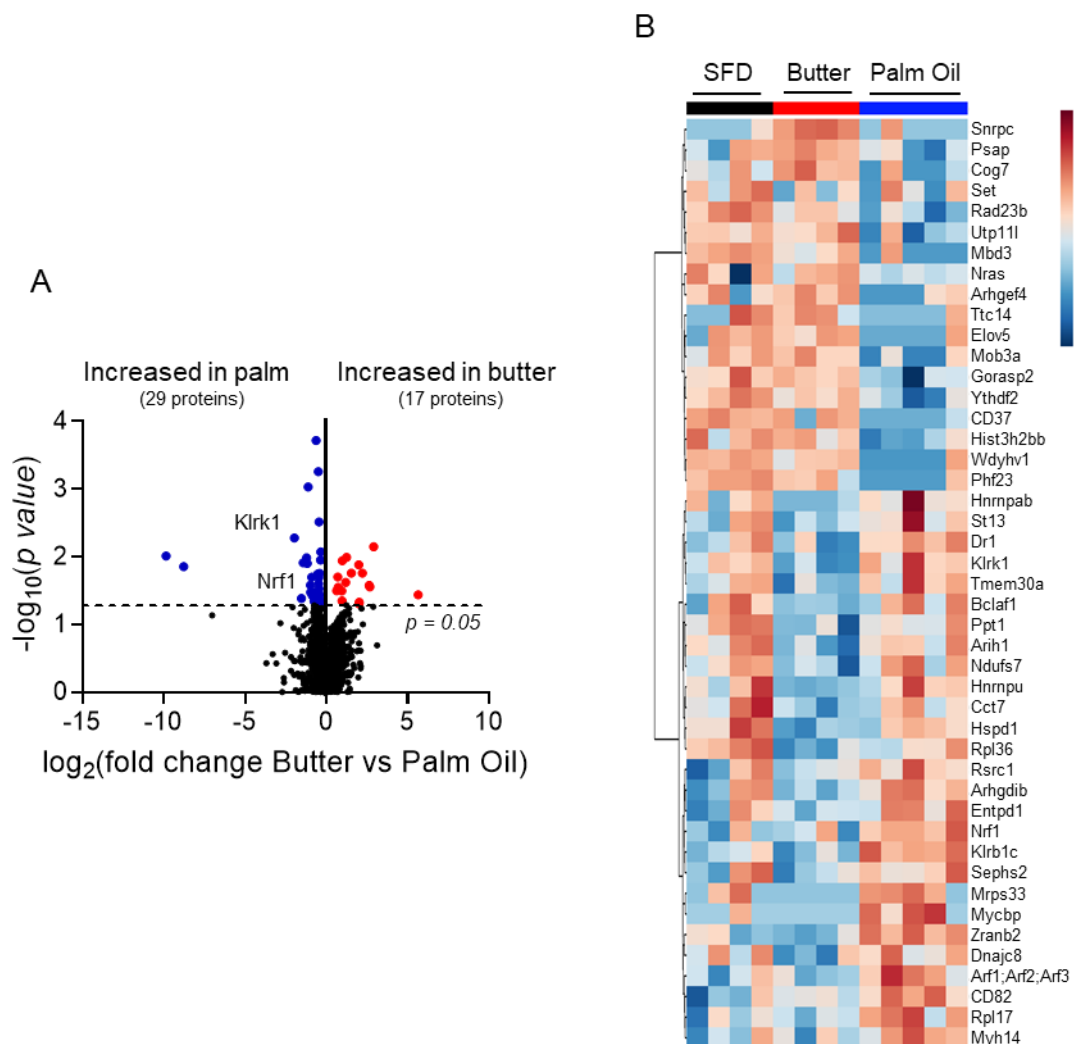


Figure 4.35 Proteins altered in NK cells from mice fed butter vs palm oil

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 12 weeks. Splenic NK cells were purified and stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr, and proteomic analysis was performed. Graphs show protein abundance differences between NK cells from mice fed a HFD from butter vs palm oil (**A**) or a heatmap of differentially expressed proteins between mice fed butter vs palm oil (**B**). Proteins (dots) that are significantly increased/decreased are highlighted in red or blue, respectively. Data is presented as volcano plots where the horizontal line represents a $p \text{ value} \leq 0.05$, for 1 experiment, $n=5$ mice per group. *NK cell purification was performed with Dr Roisin Loftus. Proteomics analysis performed by Dr Linda Sinclair, University of Dundee. Analysis by Hannah Prendeville.*

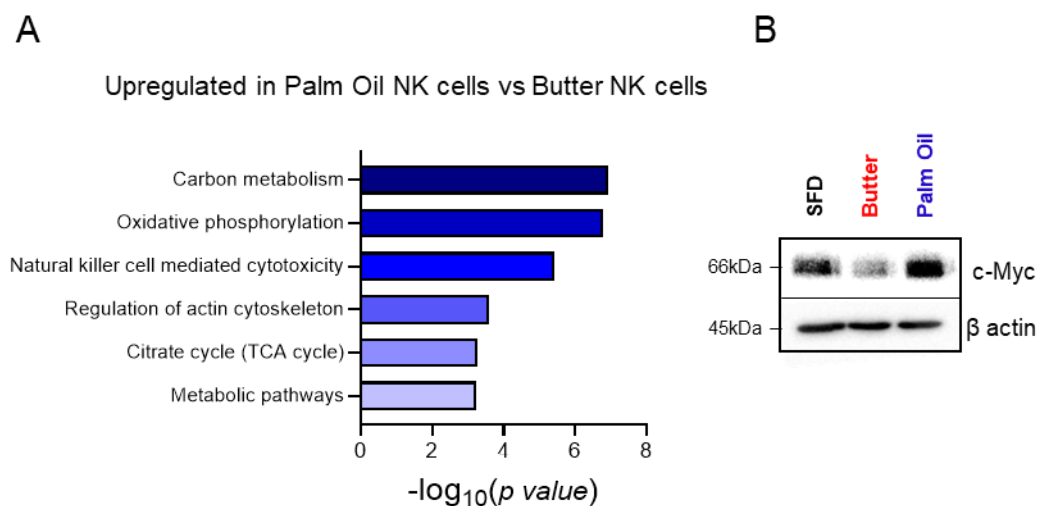


Figure 4.36 Metabolic pathways are activated in NK cells from mice fed palm oil relative to NK cells from mice fed butter

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 12 weeks. Splenic NK cells were purified and stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr. Proteomic analysis **(A)**, or western blot analysis **(B)** was performed. Graph **(A)** shows metabolic pathways that are among the top 30 KEGG pathways identified in NK cells from mice fed palm oil vs butter. Data are presented as $-\log_{10}(p \text{ value})$ for each pathway, for 1 experiment, n=5 mice per group. Graph **(B)** shows c-Myc expression, representative blot from N=3 independent experiments, n=5 mice pooled per group. NK cell was purification performed with Dr Roisin Loftus. Proteomics analysis performed by Dr Linda Sinclair, University of Dundee. Analysis by Hannah Prendeville. Western blot performed by Dr Roisin Loftus.

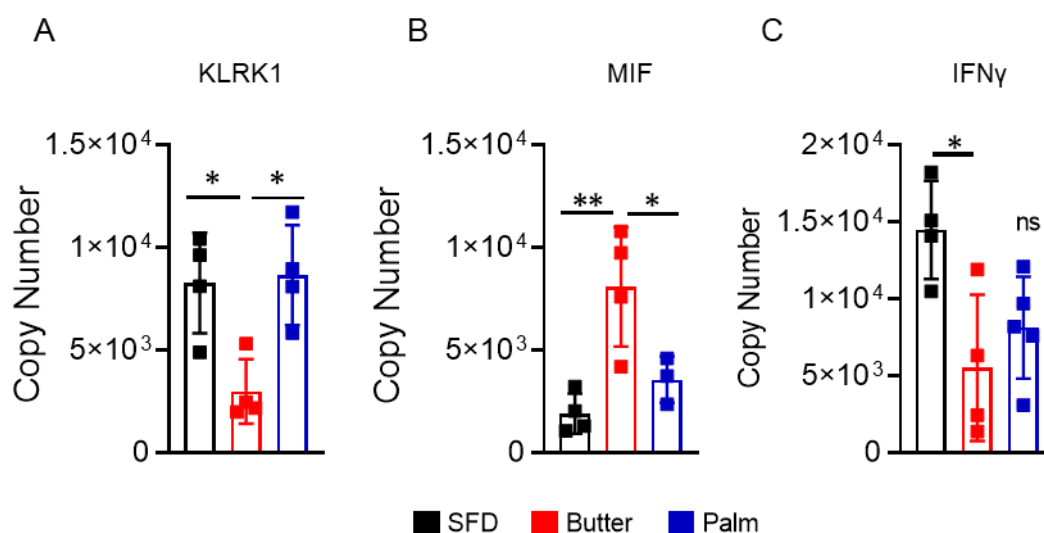


Figure 4.37 Klrk1 expression is reduced in NK cells from mice fed butter, not palm oil

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 13% fat) for 12 weeks. Splenic NK cells were purified and stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr. Proteomic analysis was performed. Graphs show KLRK1 (A), MIF (B), and IFN γ (C) protein copy numbers in NK cells. Data are mean $\pm$ S.E.M for 1 independent experiment, n=5 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. ns = not significant, * $p \leq 0.05$, ** $p \leq 0.01$. NK cell purification performed with Dr Roisin Loftus. Proteomics analysis performed by Dr Linda Sinclair, University of Dundee. Analysis by Hannah Prendeville.

4.9 NK cells from mice fed palm oil activate antioxidant defence pathways to protect against lipid-induced ROS production

Despite being in a high fat environment, NK cells from mice fed palm oil do not lose their effector function or metabolic capacity. We wondered if NK cells activate protective pathways in palm fed mice compared to butter fed mice. Of the differentially expressed proteins, Nrf1 was increased in NK cells from palm-fed mice, compared to both SFD and butter-fed mice (Fig. 4.38 A). Nrf1 is an antioxidant defence protein²²⁷. Furthermore, nicotinamide nucleotide transhydrogenase (Nnt) protein is also increased in NK cells from mice fed palm oil compared to both SFD and butter HFD (Fig. 4.38 B). Nnt regulates mitochondrial redox balance and protects against mitochondrial dysfunction and excessive production of ROS²²⁸. Given these data, we hypothesised that NK cells from mice fed plant based HFDs activate antioxidant defence strategies to maintain cellular homeostasis and protect against lipid-induced ROS production. Therefore, we next examined the levels of ROS in NK cells and found that NK cells from mice fed butter have more cellular ROS compared to mice fed palm oil (Fig. 4.39 A). Interestingly, NK cells from palm oil fed mice have even less cellular ROS compared to mice fed SFD, possibly due to activation of antioxidant pathways (Fig. 4.38 A). CD8 T cells from mice fed butter also have increased cellular ROS compared to mice fed palm oil (Fig. 4.39 B), indicating that palm oil may activate a protective antioxidant defence system in multiple cell types to protect against excess lipids from a HFD.

In conclusion, HFD from animal derived lard and butter impairs cytotoxic lymphocyte function and metabolism. Lipids accumulate within NK cells from mice fed animal fat which is associated with impaired IFN γ and normal cellular metabolic pathways, and also causes an accumulation of cellular ROS. However, in mice fed palm oil, activation of antioxidant pathways protects NK cells from excess lipids and lipotoxicity, despite equal obesity and similarly impaired whole body glucose handling between palm oil and butter-fed mice. Thus, the source of fat matters to immune function and animal fat diets are more harmful to anti-tumour immunity and promote tumour growth.

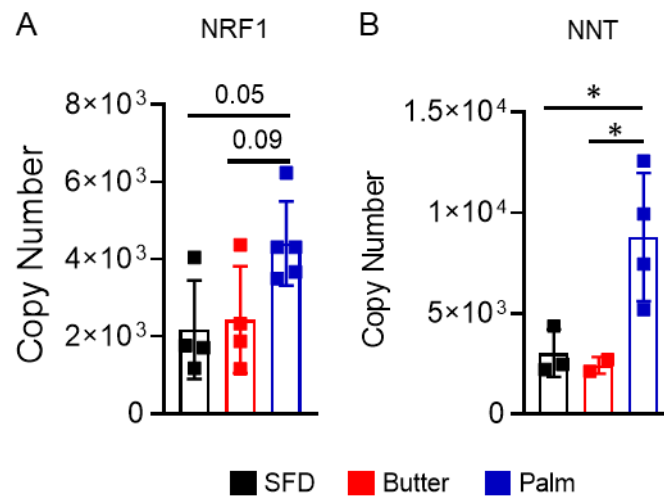


Figure 4.38 Nrf1 and Nnt expression is increased in NK cells from mice fed palm oil, compared to SFD and butter

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 12 weeks. Splenic NK cells were purified and stimulated with IL-2 (20ng/ml) and IL-12 (10ng/ml) for 20 hr. Proteomic analysis was performed. Graphs show NRF1 and NNT protein copy numbers in NK cells. Data are mean $\pm$ S.E.M for 1 independent experiment, n=5 mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$. *NK cell purification performed with Dr Roisin Loftus. Proteomics analysis performed by Dr Linda Sinclair, University of Dundee. Analysis by Hannah Prendeville.*

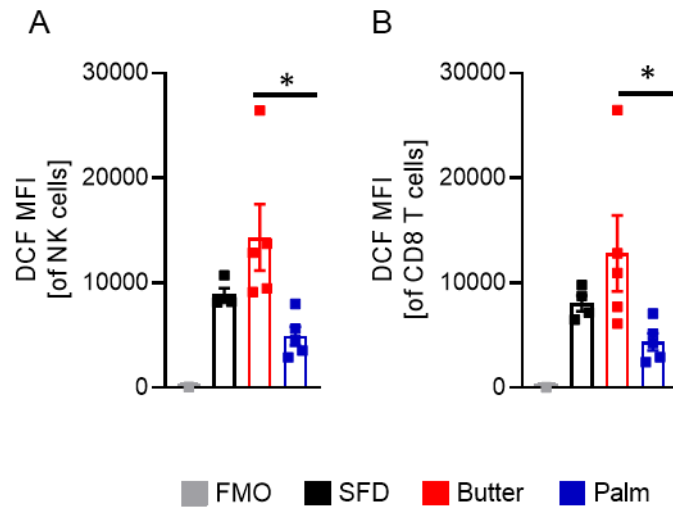


Figure 4.39 Circulating NK and CD8 T cells have more cellular ROS levels when mice are fed butter compared to palm oil

Mice were fed a high-fat diet (HFD, 45% fat) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 6 weeks. Graphs show Dichlorodihydrofluorescein (DCF) MFI, indicating cellular reactive oxygen species (ROS) levels in circulating NK and CD8 T cells. Data are mean $\pm$ S.E.M for 1 independent experiment, $n=5$ mice per group. Squares represent individual mice. Significance was calculated using an ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$.

4.10 Discussion

Work from this chapter demonstrates that tumour growth and cytotoxic immune cell functions are largely dependent on the source of dietary fat, rather than weight gain and adiposity. In particular, HFDs from animal sources accelerate tumour growth and impair intra-tumoral NK and CD8 T cell functional responses. Despite equal obesity, plant derived HFDs, particularly palm oil, do not enhance tumour growth and immune dysfunction.

Diet composition controls the endogenous nutrient and metabolite profiles, which in turn indicate disease risk in lean and obese individuals^{182,103}. Indeed, a recent study found that the plasma metabolome is a better indicator of metabolic health than BMI and can accurately predict an individual's risk of cardiac events or developing insulin resistance¹⁰³. Additionally, evidence is now emerging that metabolic markers and plasma metabolome signatures may indicate those at a higher risk of developing certain cancers including prostate and breast cancers^{229,230}. Consistent with previous reports⁸², we show that HFD feeding increased the abundance of the LCAC C18 in the plasma of mice, owing to incomplete FAO. However, this is not due to obesity itself but is dependent on the source of dietary fat. LCACs including C18 are increased in the plasma of mice fed animal derived HFDs lard and butter, but not palm oil. C18 is derived from SA, which is enriched in butter and lard, not palm oil, confirming that diet composition dictates certain metabolite abundance in the plasma. The rate of FAO is determined by the FA chain length and the carnitine shuttle system. Entry of LCFAs into the mitochondrial matrix is regulated by the carnitine shuttle, however S/MCFAs freely diffuse through the mitochondrial membranes and thus are more easily oxidised⁸⁰. Given that S/MCFAs are enriched in a butter HFD, but not palm oil, it is likely that increased diffusion, and oxidation of S/MCFAs may cause mitochondrial metabolic overload in mice fed butter, leading to disruption of the carnitine shuttle and inefficient oxidation of LCFAs such as SA, which results in LCAC accumulation. LCACs are then exported and accumulate in the plasma, which indicates disrupted lipid flux and decreased LCFA metabolism. Indeed, plasma acylcarnitines are used as clinical biomarkers to diagnose inborn errors of FA metabolism, which commonly involve disruptions to the carnitine shuttle⁸⁴.

The Lynch lab reported that lipid accumulation in NK cells from obese mice and humans results in metabolic paralysis and reduced capacity to produce IFN γ and kill tumour cells³⁷. However, work from this project demonstrates that NK cells accumulate lipids when mice are fed animal HFDs from butter and lard but not HFDs from plant fats such

as palm oil. Lipid uptake and metabolism is controlled by the PPAR family of transcription factors and we previously demonstrated that NK cells from mice fed a HFD from animal lard activate a PPAR transcriptional program, resulting in increased expression of the lipid transporter CD36 and Cpt1a³⁷. PPARs sense FAs and lipid metabolites derived from the diet. The SFAs MA and SA bind PPAR α ¹⁴⁹, one of the PPAR isoforms expressed by NK cells, alongside PPAR δ . Given that a butter HFD is rich in MA and SA, it is possible that PPAR α transcription factors are active in NK cells from mice fed butter, resulting in CD36 expression and associated lipid uptake. Indeed, activation of PPAR α/δ recapitulates the effects of lipids and HFDs in NK cells, resulting in metabolic paralysis and reduced c-Myc expression. Similarly, NK cells from mice fed butter but not palm oil had reduced rates of glycolysis and oxphos and decreased expression of c-Myc. How these diets distinctly affect PPAR lipid metabolism pathways in NK cells was not examined in this project and may reveal why NK cells accumulate lipids when mice are fed butter or lard, but not palm oil. Efficient oxidation of FAs in the mitochondria depends on the expression levels of PGC1 α which regulates PPAR transcription factors and controls the transcription of mitochondrial genes. It was recently reported that PGC-1 α is required for optimal NK cell effector functions and loss of PGC-1 α reduces NK cell metabolism and IFN γ production, resulting in increased metastasis of B16 melanoma cells to the lung²³¹. Given that PGC-1 α is increased in the livers of mice fed palm oil compared to butter, PGC-1 α may also be increased in NK cells, which retains metabolic flexibility and functional responses in a high fat environment. LipidTOX is a neutral lipid stain and cannot differentiate between different lipid species. NK cells may accumulate acylcarnitines that are increased in the plasma when mice are fed butter or lard, not palm oil. It is also arguable that heightened flux and complete oxidation of lipids in mice fed palm oil, due to increased levels of Cact, may result in fewer circulating lipids for NK cells to accumulate. However, more work is needed to confirm why NK cells accumulate lipids when mice are fed animal fats, but not plant fats.

Excess FAs and heightened β -oxidation can generate ROS which can trigger lipid peroxidation, cellular damage, ER stress and apoptosis¹⁵⁴. Cancer cells have adopted many strategies to prevent against lipotoxicity-induced cellular damage, particularly in lipid rich tumours. For example, overexpression of GPX4 is a feature of many cancers, which suppresses ferroptosis, a form of programmed cell death induced by lipid-based ROS¹⁵⁶. However, immune cells are sensitive to ferroptosis in lipid rich tumours, likely due to increased production of ROS, and inhibition of ferroptosis enhanced IFN γ and TNF production by CD8 T cells *in vivo*⁸⁵. Work from this chapter shows that NK cells from mice fed butter have more cellular ROS compared to mice fed palm oil. FAs can

also be oxidised in the peroxisomes which results in high levels of ROS production due to the absence of an ETC²³². It was recently revealed that oxidation of PA in liver peroxisomes is associated with increased production of ROS²³³. Given that butter induces mitochondrial overload and reduced lipid oxidation at the systemic level, excess FAs may be oxidised in peroxisomes in NK cells which is consequently linked to increased production of ROS and metabolic dysfunction. In contrast, NK cells from palm oil fed mice do not accumulate ROS and have even less cellular ROS compared to mice fed SFD. We found that NK cells from mice fed palm oil were protected against lipotoxicity and ROS-induced metabolic dysfunction by activating antioxidant pathways involving Nrf1 and Nnt which regulate mitochondrial redox balance and protect against mitochondrial dysfunction and excessive ROS production²²⁸. PGC-1 α directly interacts with Nrf1 protein and co-activates its transcriptional activity²³⁴. Therefore, PGC-1 α may protect NK cells from mice fed palm oil by promoting antioxidant pathways involving Nrf1, thus regulating energetic and functional responses. It was recently reported that activation of the anti-oxidant protein Nrf2 rescues NK cell metabolism and anti-tumour activity in human ovarian cancer by inducing metabolic flexibility⁹¹. Activating Nrf1 with small molecule stimulators in mice fed butter may therefore reverse the metabolic defects in NK cells, resulting in increased cytokine production and cytotoxicity *in vivo*. These results suggest that NK cells are sensitive to oxidative stress induced by lipids or the TME and may be revigorated by activating antioxidant pathways. Diets rich in plant-based fats may confer metabolic flexibility and efficient cytotoxicity in NK cells by activating antioxidant pathways to allow for optimal anti-tumour responses.

It is becoming clear that the source of dietary fat plays an important role in many aspects of human health and disease²³⁵. Our results show that animal fat diets are more harmful to anti-tumour immunity and promote tumour growth. Interestingly, a recent paper examining cancer incidence in the animal kingdom demonstrated that cancer mortality is associated with diet. Although significantly more work is needed to justify this idea, it was found that carnivorous animals had the highest cancer-related mortality²³⁶, which supports our work. Furthermore, recent reports demonstrate that plant-fat based diets prolong the survival of patients with pancreatic cancer, more-so than diets rich in animal fats²¹⁷. Modifying the source of dietary fat may be an easy and affordable way to harness an effective anti-tumour immune response, potentially without the requirement for WL. Understanding dietary substitutions that could be useful for patients with cancer, alongside traditional cancer therapies could prove beneficial for multi-factorial effects on the tumour and immune system.

Chapter 5. Octadecanoyl-carnitine is an immunomodulatory metabolite that limits anti-tumour immune responses in obesity

5.1 Introduction

Acylcarnitines are intermediate lipid metabolites that indicate flux through lipid metabolism. LCACs accumulate in the plasma of humans with defects in FA metabolism²²² and in mouse models of DIO due to mitochondrial lipid overload resulting in incomplete FAO⁸². However, in this project, we show that plasma LCACs only accumulate when mice are fed HFDs from animal sources (lard and butter), but not palm oil. In addition, B16 and MC38 TIFs are rich in a diverse array of acylcarnitines⁸⁵, which likely increase when mice are fed animal fats. In this chapter, we tested the hypothesis that acylcarnitines, specifically C18, interferes with NK and CD8 T cell anti-tumour responses which may explain the divergent effects of animal and plant derived HFDs on tumour growth and anti-tumour immune responses *in vivo*.

5.2 Acylcarnitines do not affect NK cell functional or metabolic responses *in vitro*

Previous work from our lab showed that FAs can interfere with normal immunometabolic pathways in NK cells, leading to metabolic paralysis and impaired functional responses³⁷. In addition, we also show here that the SFAs PA and SA, but not the MUFA OA impairs CD8 T cell function *in vitro* (Fig. 4.20 – Fig. 4.22). Given that C18 is increased in plasma of mice fed animal fat (lard or butter), but not when mice are fed palm oil or have lost weight (Fig. 5.1 A, B), we next investigated if C18 had immunomodulatory properties, which may further explain the immune dysfunction observed in mice fed butter or lard *in vivo*. We tested a panel of short, medium, and long chain acylcarnitines on NK and CD8 T cell functions *in vitro*. These included octanoyl-carnitine (C8) derived from octanoic acid, myristoyl-carnitine (C14) derived from MA, palmitoyl-carnitine (C16) derived from PA, and C18 derived from SA. C8, C14, and C16 were not significantly changed in the plasma of mice fed a HFD from lard (Fig. 5.2 A-C).

Lipids can be toxic to certain cells²³⁷, but at the concentrations used (25-60µM), acylcarnitines did not affect lymphocyte viability (Figure 5.3 A-D) or NK or CD8 T cell size (Fig. 5.4 A, B) *in vitro*. Splenic NK cells from WT mice fed a SFD were stimulated overnight in the presence of acylcarnitines to assess NK cell function. There was no effect of C18 or other acylcarnitines on IFNγ (Fig. 5.5 A, B) or granzyme B (Fig. 5.6 A, B) expression in NK cells. C18 also had no effect on the rates of NK cell glycolysis or oxphos (Fig. 5.7 and Fig. 5.8). NK cell metabolic reprogramming is controlled by the

transcription factor c-Myc which is regulated by amino acid transport through the system L-amino acid transporter Slc7A5⁷⁷. Acylcarnitines did not affect CD71 expression, a known cMyc target gene (Fig. 5.9), or CD98 expression, a component of heterodimeric system L-amino acid transporters (Fig. 5.10), in NK cells. Together, these data demonstrate that C18 or other acylcarnitines do not affect NK cell functional or metabolic responses *in vitro*.

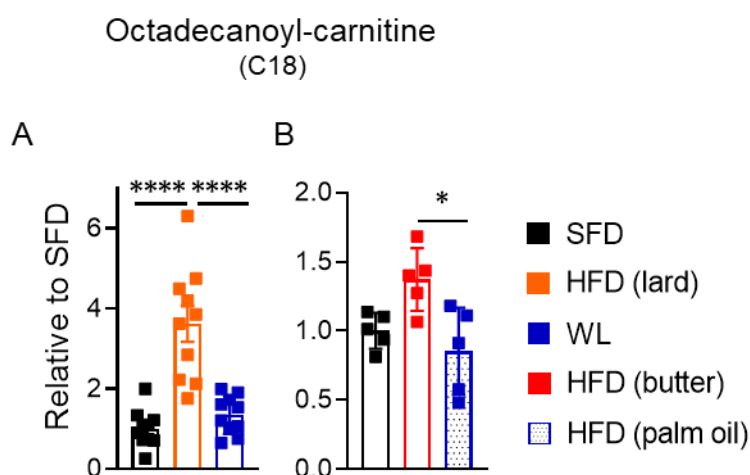


Figure 5.1 Octadecanoyl-carnitine is increased in the plasma of mice fed a HFD from animal sources (lard and butter)

Mice were fed a high-fat diet (HFD, 60%) from lard or a standard-fat diet (SFD, 12% fat) for 8 weeks. After 6 weeks, one group of mice received a SFD in place of a HFD to induce WL (**A**). Naïve C57BL/6 mice were fed a high-fat diet (HFD, 45%) from butter or palm oil or a standard-fat diet (SFD, 12% fat) for 1 week (**B**). Metabolomic analysis of the plasma metabolome was performed by LC/MS. Graphs show relative abundance of octadecanoyl-carnitine (C18) in mice fed lard, butter, palm oil or mice that lost weight. Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 1/2 experiments, n=5/10 mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, **** $p \leq 0.0001$. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

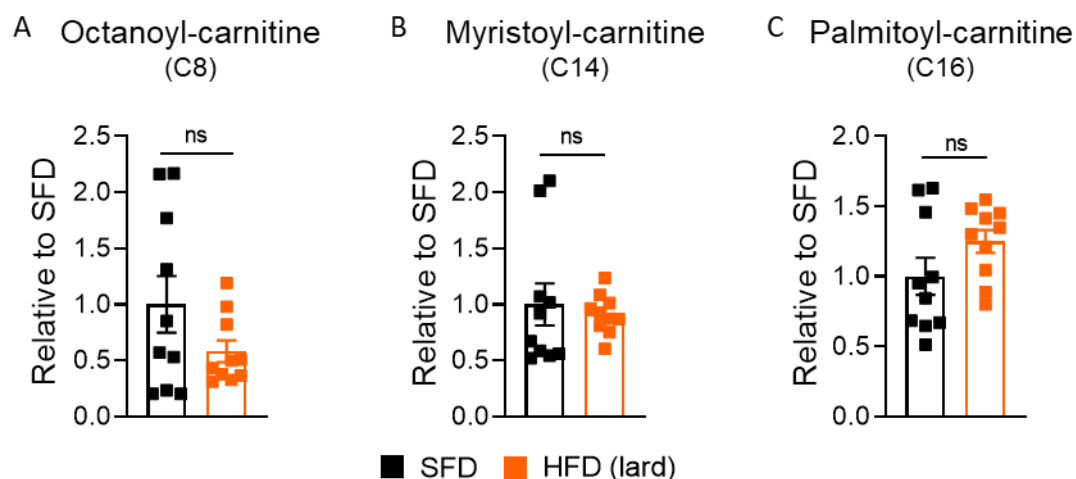


Figure 5.2 Plasma levels of octanoyl-carnitine, myristoyl-carnitine, or palmitoyl-carnitine are not significantly altered by HFD (lard)

Mice were fed a high-fat diet (HFD, 60%) from lard or a standard-fat diet (SFD, 12% fat) for 8 weeks. Metabolomic analysis of the plasma metabolome was performed by LC/MS. Graphs show relative abundance of octanoyl-carnitine (C8), myristoyl-carnitine (C14), and palmitoyl-carnitine (C16) in mice fed lard. Data are mean $\pm$ S.E.M relative to mice fed a SFD, for 2 experiments, $n=10$ mice per group. Squares represent individual mice. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ns = not significant. *Metabolomics experiments were performed by Dr Evanna Mills, DFCI, and analysed by Hannah Prendeville.*

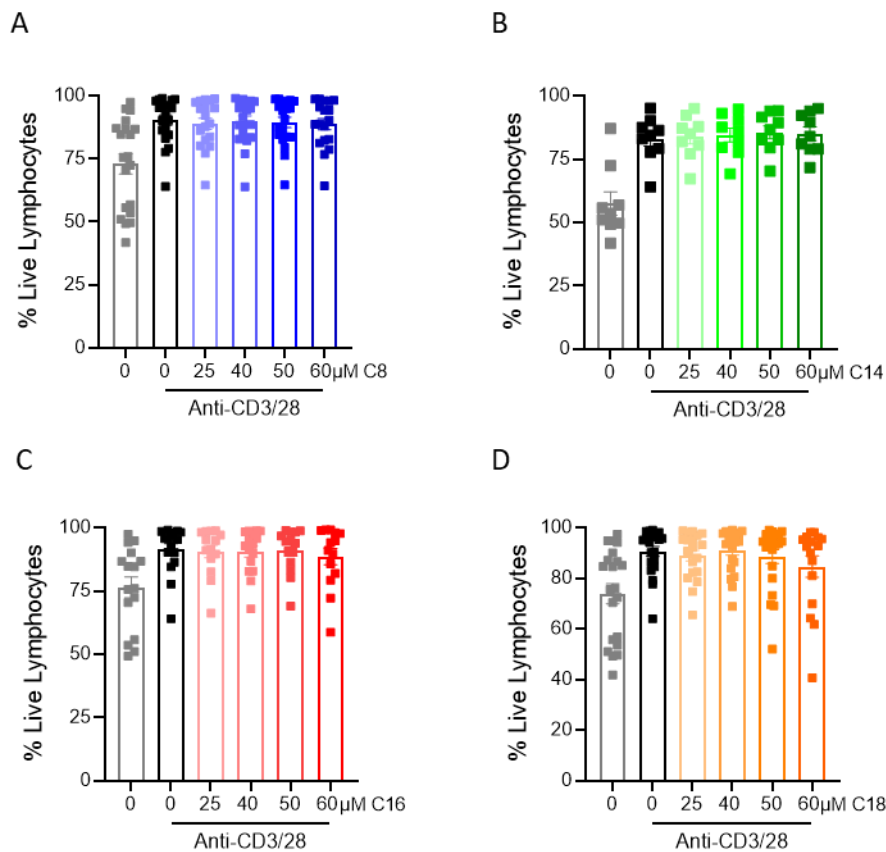


Figure 5.3 Acylcarnitines do not affect lymphocyte viability

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of octanoyl-carnitine (C8), myristoyl-carnitine (C14), palmitoyl-carnitine (C16) or octadecanoyl-carnitine (C18) (hereafter referred to as acylcarnitines) (25-60 μ M) and stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5 ng/ml) for 48 hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual squares). Graphs show percentage of live (zombie aqua^{neg}) lymphocytes. Data are mean $\pm$ S.E.M from N $\sim$ 20 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

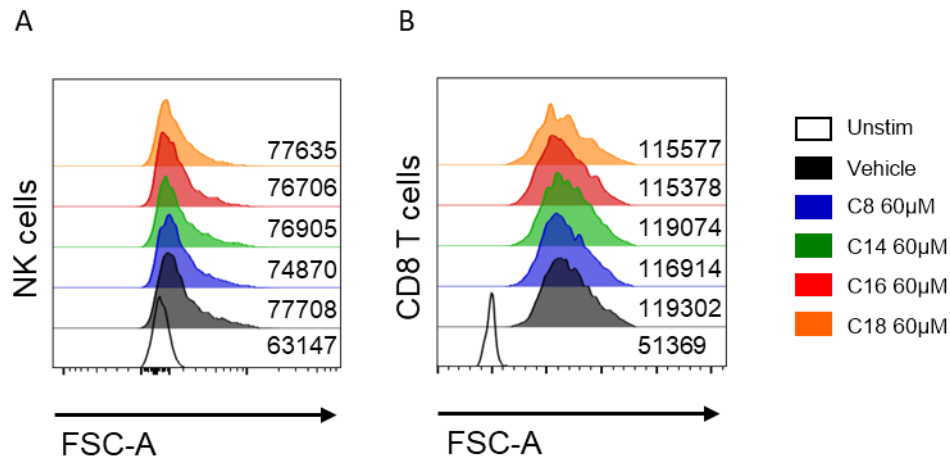


Figure 5.4 Acylcarnitines do not affect NK or CD8 T cell size

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60µM) and stimulated with IL-2 (20ng/ml), IL-12 (10ng/ml) and IL-15 (5ng/ml) for 20 hr **(A)** or anti-CD3 (1µg/ml), CD28 (3µg/ml), and IL-2 (5ng/ml) for 48hr **(B)**. Graphs show representative plot for NK cell size **(A)** and CD8 T cell size **(B)** as depicted by FSC-A, where numbers indicate mean fluorescence intensity. N=~20 independent experiments.

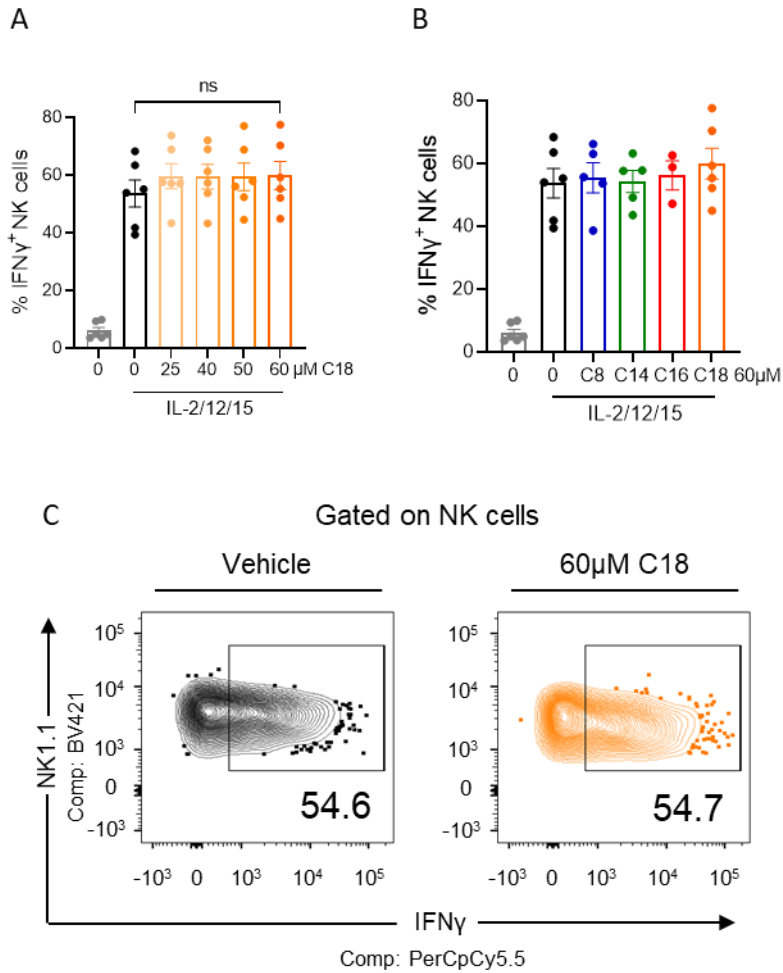


Figure 5.5 Acylcarnitines do not affect IFN γ expression in NK cells

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60 μ M). NK cells were stimulated with IL-2 (20ng/ml), IL-12 (10ng/ml), and IL-15 (5ng/ml) for 20 hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual dots). Graphs show percentage of IFN γ ⁺ NK cells (**A**, **B**) and representative flow plot (**C**). Data are mean $\pm$ S.E.M from N=6 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ns = not significant.

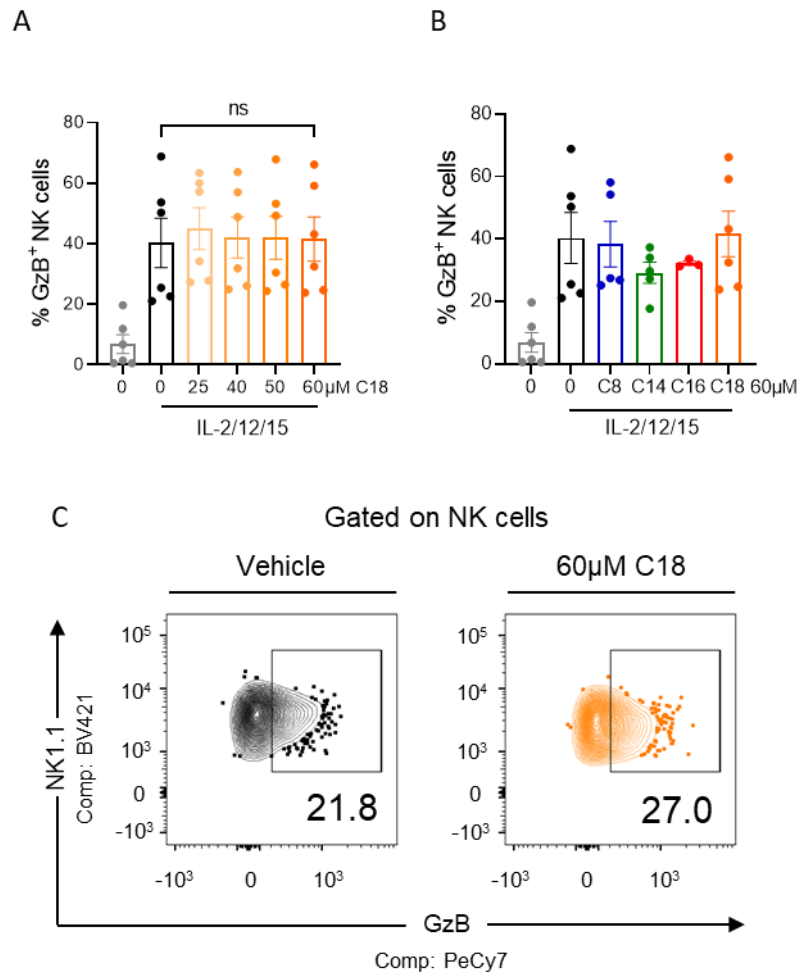


Figure 5.6 Acylcarnitines do not affect granzyme B expression in NK cells

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60 µM). NK cells were stimulated with IL-2 (20 ng/ml), IL-12 (10 ng/ml), and IL-15 (5 ng/ml) for 20 hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual dots). Graphs show percentage of GzB⁺ NK cells (**A**, **B**) and representative flow plot (**C**). Data are mean ± S.E.M from N=6 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ns = not significant.

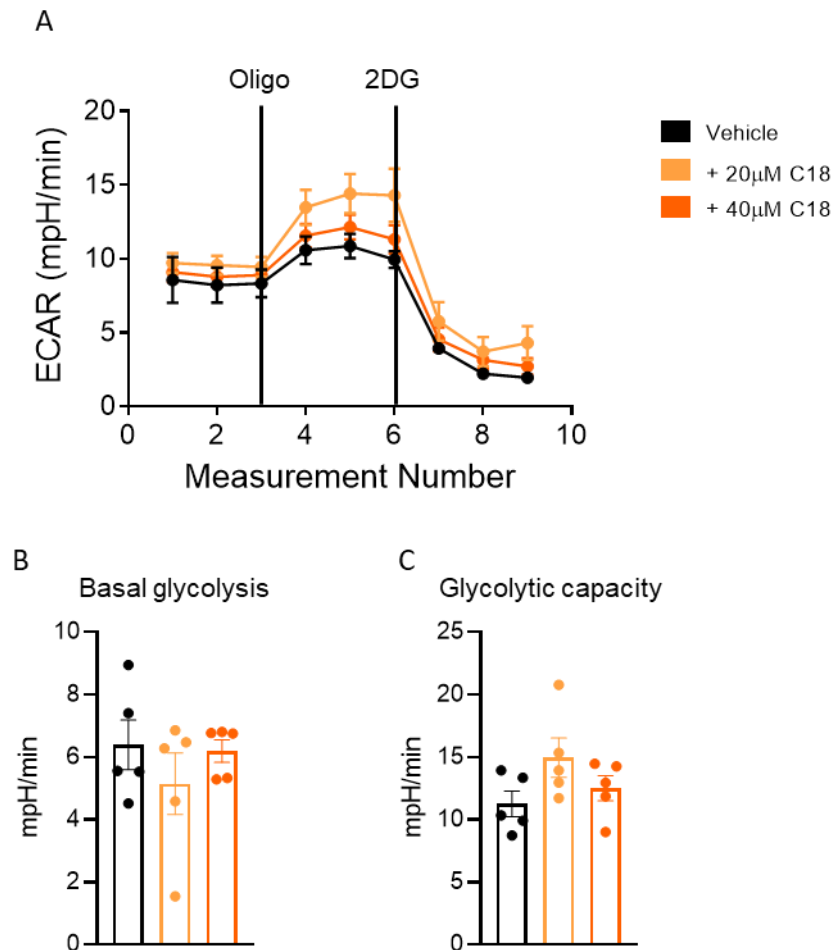


Figure 5.7 Octadecanoyl-carnitine does not affect the rates of NK cell glycolysis

NK cells were isolated from the spleens of naïve mice fed a standard fat diet. Pure NK cells were cultured with increasing concentrations of acylcarnitines (25-60μM) and stimulated with IL-2 (20ng/ml), IL-12 (10ng/ml), and IL-15 (5ng/ml) for 20 hr. Extracellular acidification rate (ECAR) was measured by Seahorse analysis following injection of oligomycin (oligo) and 2-deoxyglucose (2DG). Graphs show representative trace (**A**), basal glycolysis (**B**), and glycolytic capacity (**C**). Data are mean $\pm$ S.E.M from one experiment. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

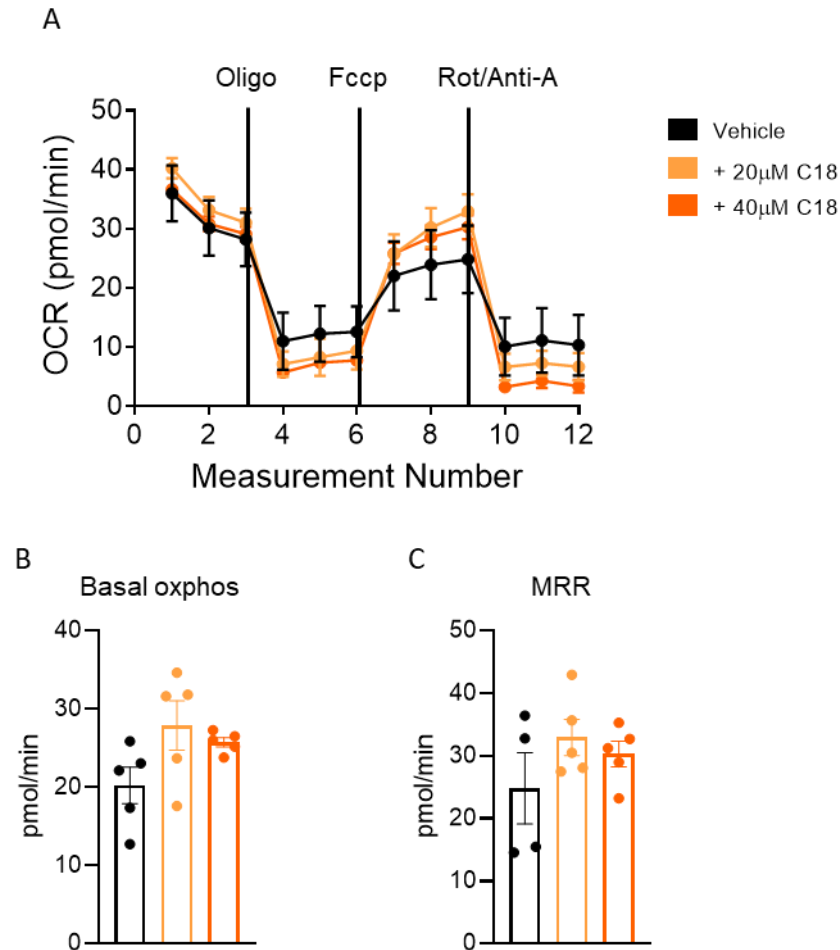


Figure 5.8 Octadecanoyl-carnitine does not affect the rates of NK cell oxphos

NK cells were isolated from the spleens of naïve mice fed a standard fat diet. Pure NK cells were cultured with increasing concentrations of acylcarnitines (25-60μM) and stimulated with IL-2 (20ng/ml), IL-12 (10ng/ml), and IL-15 (5ng/ml) for 20 hr. Oxygen consumption rate (OCR) was measured by Seahorse analysis following injection of oligomycin (oligo), Fccp, rotenone and antimycin-A (Rot/Anti-A). Graphs show representative trace (**A**), basal oxphos (**B**), and maximum respiratory rate (**C**). Data are mean $\pm$ S.E.M from one experiment. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

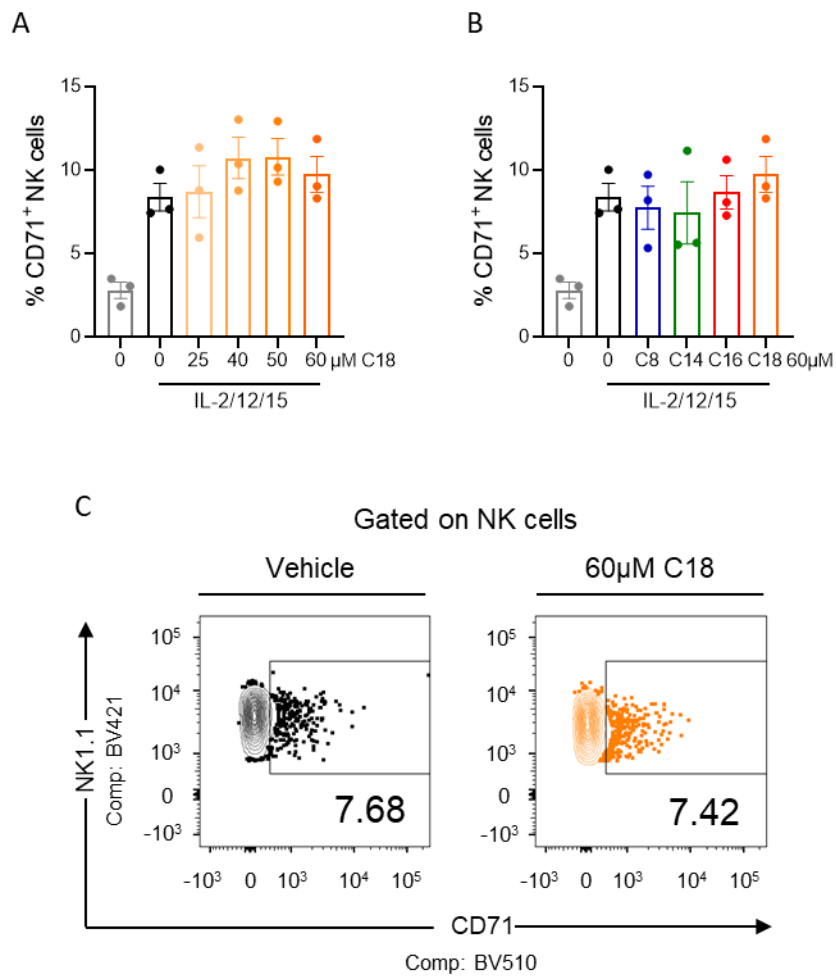


Figure 5.9 Acylcarnitines do not alter CD71 expression in NK cells

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60µM). NK cells were stimulated with IL-2 (20ng/ml), IL-12 (10ng/ml), and IL-15 (5ng/ml) for 20 hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual squares). Graphs show percentage of CD71⁺ NK cells (**A**, **B**) and representative flow plot (**C**). Data are mean $\pm$ S.E.M from N=3 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ns = not significant.

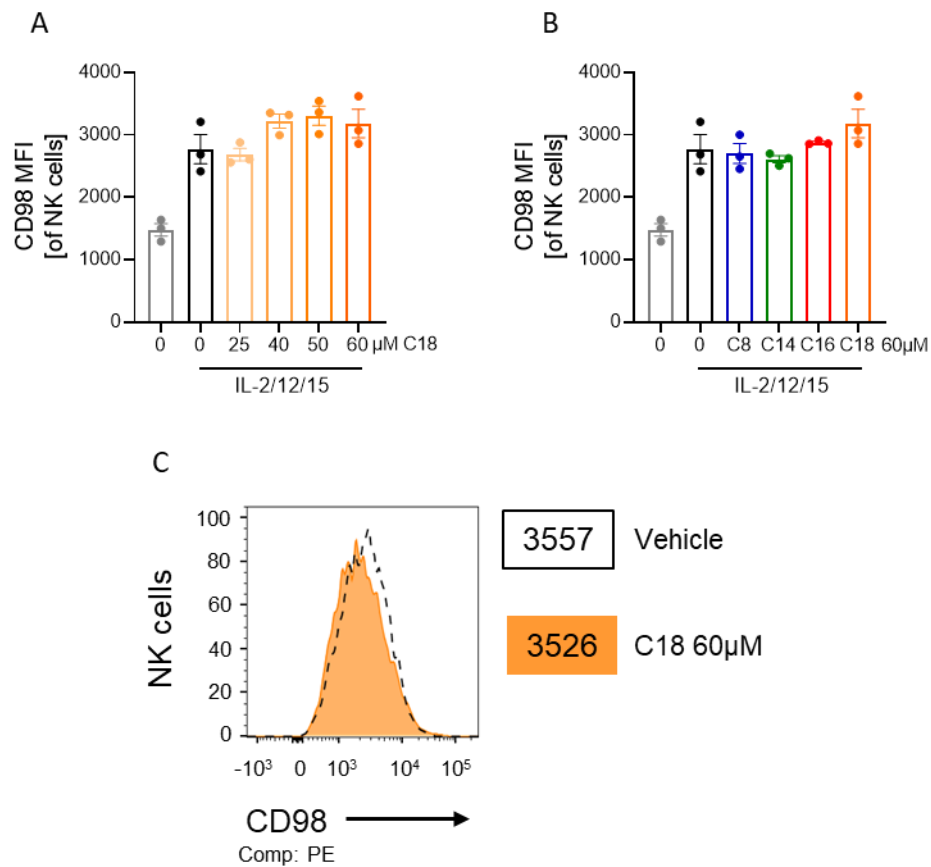


Figure 5.10 Acylcarnitines do not alter CD98 expression in NK cells

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60 μM). NK cells were stimulated with IL-2 (20 ng/ml), IL-12 (10 ng/ml), and IL-15 (5 ng/ml) for 20 hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual squares). Graphs show CD98 mean fluorescence intensity (MFI) of NK cells (**A**, **B**) and representative histogram (**C**). Data are mean $\pm$ S.E.M from N=3 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ns = not significant.

5.2 Octadecanoyl-carnitine impairs IFN γ production by CD8 T cells *in vitro*

Next, we investigated the effects of acylcarnitines on CD8 T cell function and metabolism. Splenic CD8 T cells from WT mice fed a SFD were stimulated for 24, 48, or 72 hr in the presence of acylcarnitines. In contrast to NK cells, CD8 T cell functional responses were greatly impaired by LCACs *in vitro*. C18 decreased the expression of IFN γ by CD8 T cells in a concentration dependent manner (Fig. 5.11 A). Interestingly, acylcarnitines decreased IFN γ expression in CD8 T cells in a chain length dependent manner, with C18 inducing the most significant decrease in IFN γ , followed by C16 and then C14 (Fig. 5.11 B). C18 and C16 also impaired IFN γ expression in CD4 T cells, which was concentration dependent (Fig. 5.12 A, B). In line with these findings, C18 decreased PD-1 and TIM-3 expression on CD8 T cells, indicating reduced activation (Fig. 5.13 and Fig. 5.14). However, acylcarnitines did not affect the expression of CD69 on CD8 T cells (Fig. 5.15 A, B), expression of which indicates that a T cell has responded to antigen²³⁸. CD8 T cell proliferation was not affected by acylcarnitines and slightly decreased by higher concentrations of C18, however this was not significant (Fig. 5.16 A, B). Together, these data suggest that acylcarnitines do not interfere with the ability of CD8 T cells to respond to antigen stimulation, proliferate or blast *in vitro* but rather decrease the strength of activation, leading to impaired IFN γ expression.

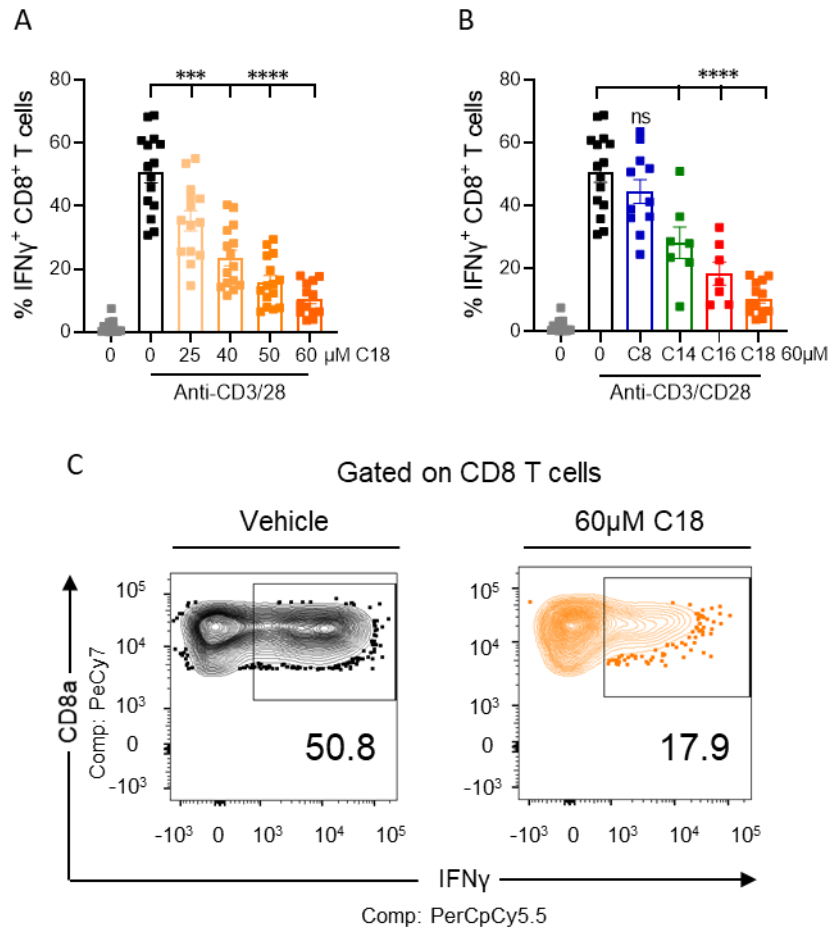


Figure 5.11 Acylcarnitines reduce IFN γ expression in CD8 T cells in a concentration dependent and chain-length dependent manner

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60 μ M). T cells were stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5ng/ml) for 48hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual squares). Graphs show percentage of IFN γ ⁺ CD8 T cells (**A**, **B**) and representative flow plot (**C**). Data are mean $\pm$ S.E.M from N $\sim$ 15 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. *** $p \leq 0.001$, **** $p \leq 0.0001$.

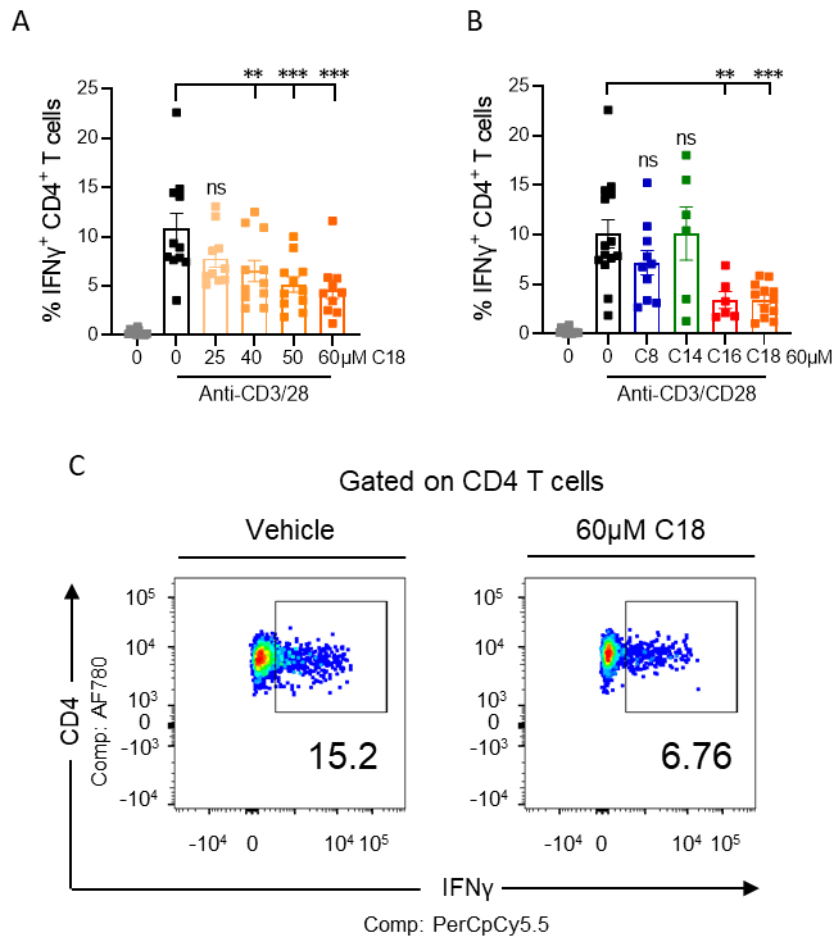


Figure 5.12 Long chain acylcarnitines decrease IFN γ expression in CD4 T cells in a concentration dependent manner

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60 μ M). T cells were stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5 ng/ml) for 48 hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual squares). Graphs show percentage of IFN γ ⁺ CD4 T cells (**A**, **B**) and representative flow plot (**C**). Data are mean $\pm$ S.E.M from N $\sim$ 11 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ** $p \leq 0.01$, *** $p \leq 0.001$.

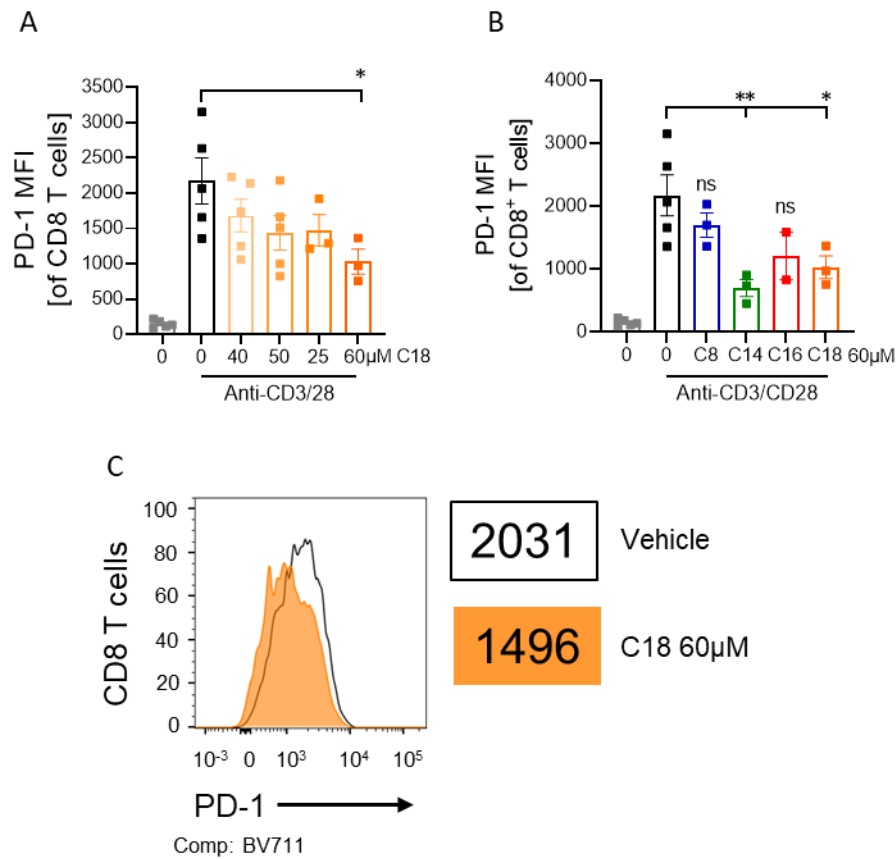


Figure 5.13 Octadecanoyl-carnitine reduces PD-1 expression on CD8 T cells

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60 μM). T cells were stimulated with anti-CD3 (1 μg/ml), CD28 (3 μg/ml), and IL-2 (5 ng/ml) for 48 hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual squares). Graphs show PD-1 mean fluorescence intensity (MFI) of CD8 T cells (**A**, **B**) and representative histogram (**C**). Data are mean $\pm$ S.E.M from N=5 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. *** $p \leq 0.001$, **** $p \leq 0.0001$.

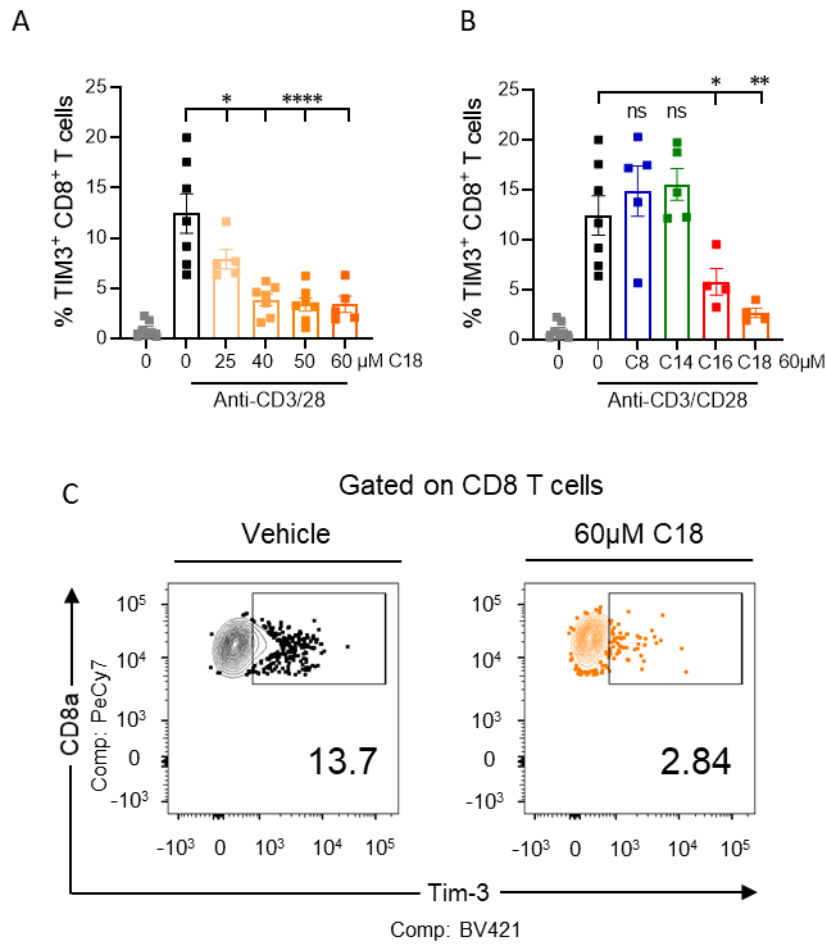


Figure 5.14 Octadecanoyl-carnitine reduces TIM-3 expression on CD8 T cells

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60µM). T cells were stimulated with anti-CD3 (1µg/ml), CD28 (3µg/ml), and IL-2 (5ng/ml) for 48hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual squares). Graphs show percentage of TIM-3⁺ CD8 T cells **(A, B)** and representative flow plot **(C)**. Data are mean +/- S.E.M from N=5 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. *** $p \leq 0.001$, **** $p \leq 0.0001$.

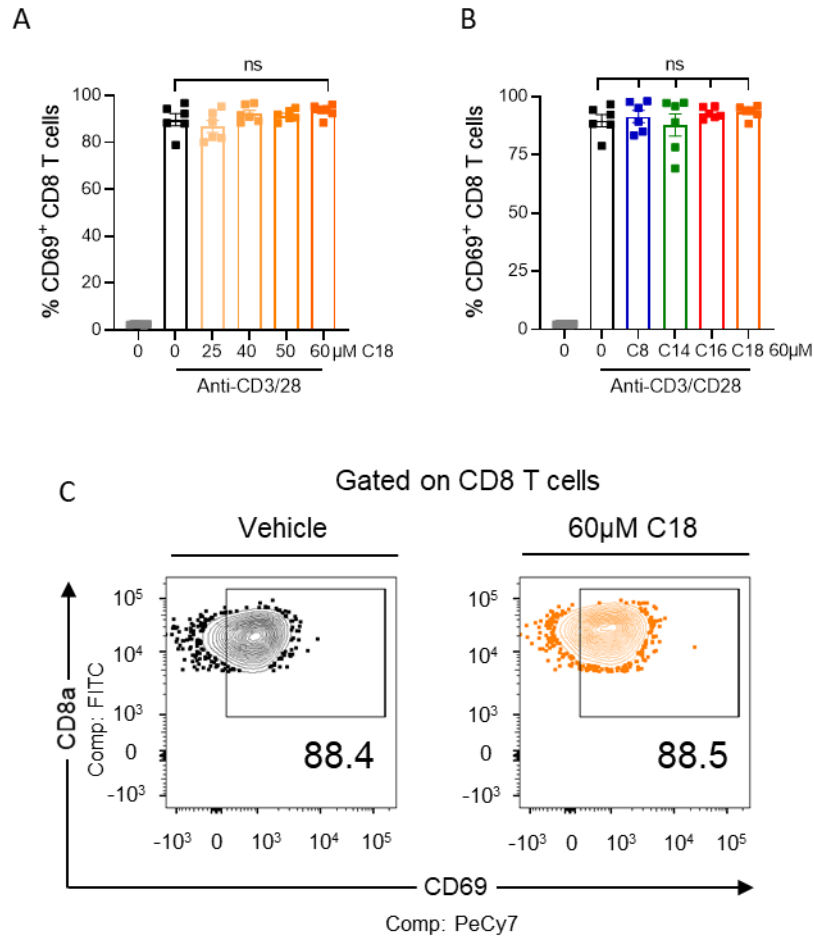


Figure 5.15 Acylcarnitines do not affect CD69 expression on CD8 T cells

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60 μ M). T cells were stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5 ng/ml) for 48 hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual squares). Graphs show percentage of CD69⁺ CD8 T cells (**A**, **B**) and representative flow plot (**C**). Data are mean $\pm$ S.E.M from N=5 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ns= not significant.

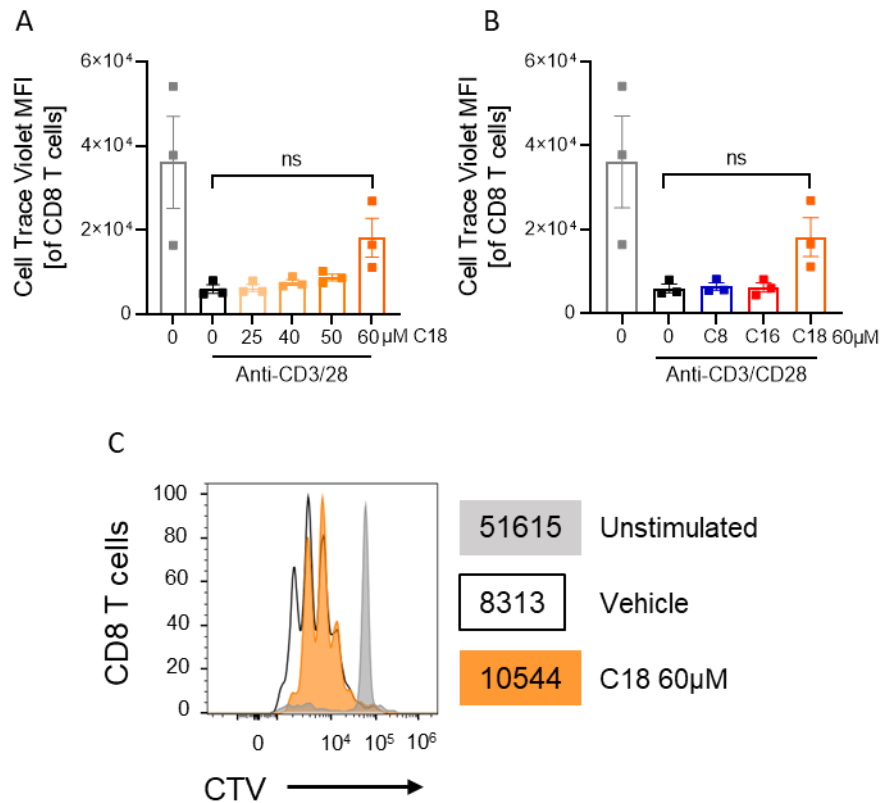


Figure 5.16 Acylcarnitines do not significantly affect CD8 T cell proliferation

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60 µM). T cells were stimulated with anti-CD3 (1 µg/ml), CD28 (3 µg/ml), and IL-2 (5 ng/ml) for 48 hr. Prior to stimulation, splenocytes were stained with 1 µM cell trace violet (CTV). Experiments were performed in technical triplicate which were averaged for each experiment (individual squares). Graphs show CTV mean fluorescence intensity (MFI) of CD8 T cells (**A**, **B**) and representative histogram (**C**). Numbers indicate CTV MFI. Data are mean $\pm$ S.E.M from N=3 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ns= not significant.

5.3 Octadecanoyl-carnitine reduces the rates of CD8 T cell glycolysis *in vitro*

In response to antigen, CD8 T cells increase glucose uptake and the rates of glycolysis. This metabolic phenotype supports T cell blastogenesis and production of effector cytokines such as IFN γ and cytolytic molecules⁹². Therefore, we next examined the effects of acylcarnitines on CD8 T cell glycolytic metabolism by Seahorse analysis. In line with the functional readouts, C16 and C18, but not C8, significantly reduced the rates of glycolysis in CD8 T cells. Again, this was in a chain length dependent manner with C18 inducing the most significant decrease in basal glycolysis and glycolytic capacity (Fig. 5.17 A-C). C18 decreased the mRNA expression of the glucose transporters Slc2a1 (Glut 1) and Slc2a3 (Glut3) in CD8 T cells in a dose dependent manner (Fig. 5.18 A, B) and the expression of the glycolytic enzymes Hex2, Gapdh, and Pkm1 (Fig. 5.19 A-C). To maintain efficient rates of aerobic glycolysis, cells must effectively produce and export the glycolytic product lactate. Consistent with reduced rates of glycolysis, C18 decreased the mRNA expression of lactate dehydrogenase (Idha) and Slc16a1 (Fig. 5.19 D-E).

Glucose uptake, expression of glycolytic enzymes and glycolysis is regulated by the protein kinase complex mTORC1 in CD8 T cells⁹⁴. Given that FAs inhibit mTORC1 activation in mouse and human NK cells, we reasoned that acylcarnitines, especially C18 may tamper with mTORC1 signalling. However, acylcarnitines did not affect mTORC1 activation in CD8 T cells, as assessed by phosphorylation of S6 by flow cytometry and western blot (Fig. 5.20 A-C). Importantly, rapamycin inhibited the phosphorylation of S6 in vehicle and acylcarnitine treated CD8 T cells, confirming that S6 phosphorylation is mTORC1 dependent. Together, these results suggest that C18 reduces the rates of CD8 T cell glycolysis and the expression of glucose transporters and glycolytic enzymes without affecting mTORC1 activation.

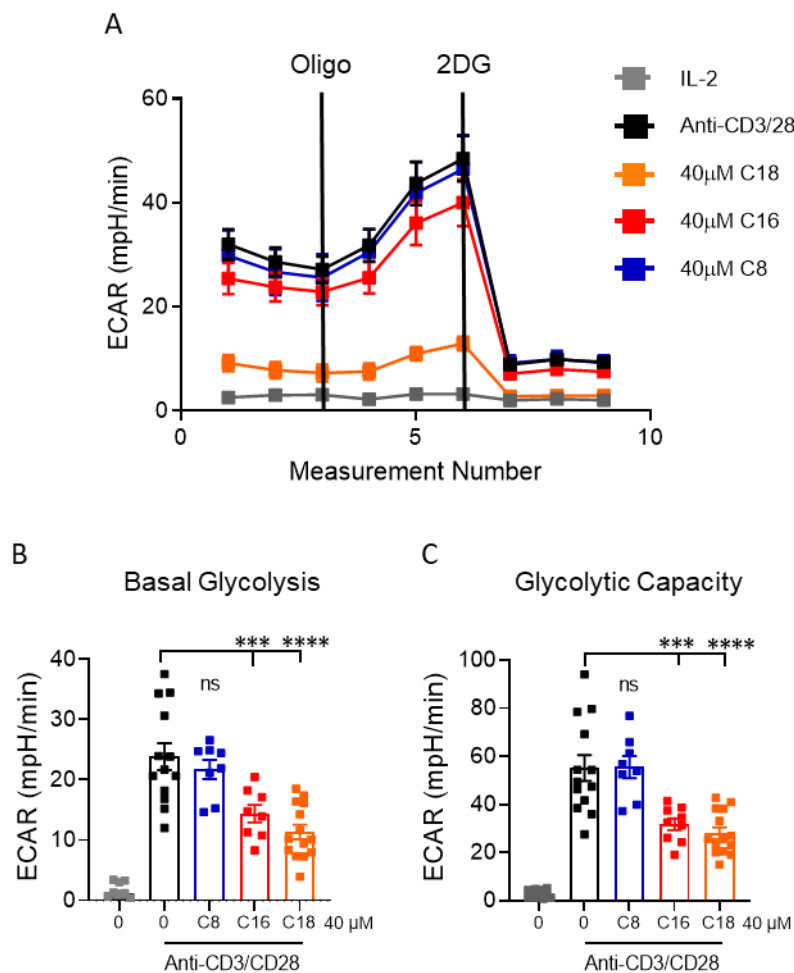


Figure 5.17 Acylcarnitines reduce the rates of CD8 T cell glycolysis in a chain-length dependent manner

CD8 T cells were isolated from the spleens of naïve mice fed a standard fat diet. Pure CD8 T cells were cultured with increasing concentrations of acylcarnitines (25-60 μ M) and stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5ng/ml) for 48hr. Extracellular acidification rate (ECAR) was measured by Seahorse analysis following injection of oligomycin (oligo) and 2-deoxyglucose (2DG). Graphs show representative trace **(A)**, basal glycolysis **(B)**, and glycolytic capacity **(C)**. Data are mean $\pm$ S.E.M from N=3 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ns= not significant, *** $p \leq 0.001$, **** $p \leq 0.0001$.

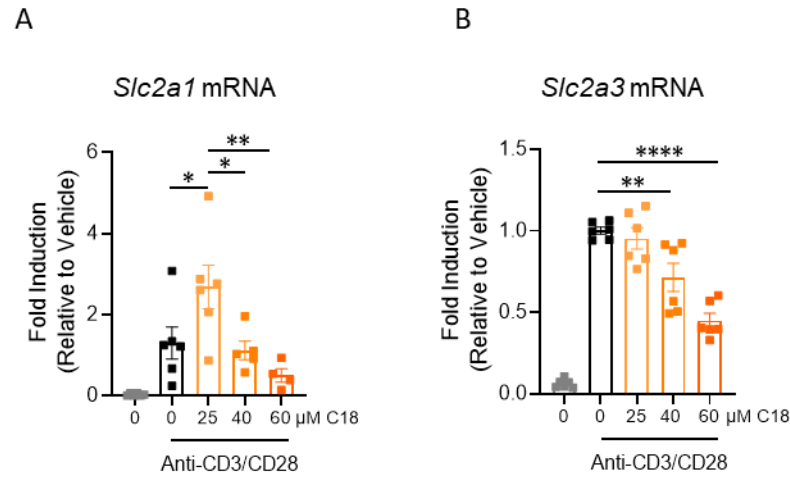


Figure 5.18 Octadecanoyl-carnitine decreases *Slc2a1* and *Slc2a3* glucose transporter expression in CD8 T cells

CD8 T cells were isolated from the spleens of naïve mice fed a standard fat diet. Pure CD8 T cells were cultured with increasing concentrations of acylcarnitines (25-60 μ M) and stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5ng/ml) for 48hr. mRNA was isolated and gene expression of *Slc2a1* and *Slc2a3* relative to vehicle was analysed by qPCR. Data are mean $\pm$ S.E.M from N=2 independent experiments, technical triplicates. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

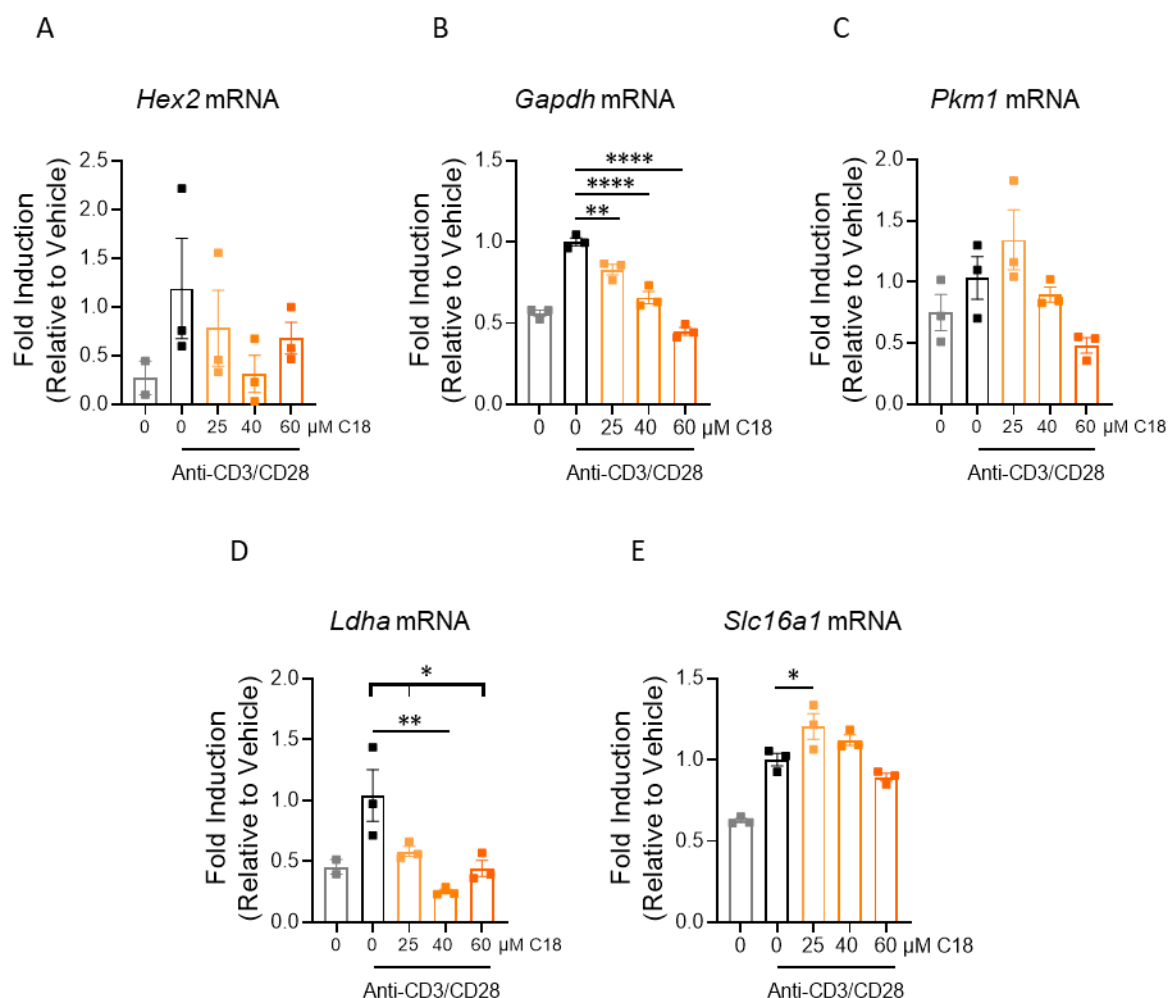


Figure 5.19 Octadecanoyl-carnitine decreases the expression of glycolytic enzymes in CD8 T cells

CD8 T cells were isolated from the spleens of naïve mice fed a standard fat diet. Pure CD8 T cells were cultured with increasing concentrations of acylcarnitines (25-60μM) and stimulated with anti-CD3 (1μg/ml), CD28 (3μg/ml) and IL-2 (5ng/ml) for 48hr. mRNA was isolated and gene expression of *Hex2*, *Gapdh*, *Pkm1*, *Ldha*, and *Slc16a1* relative to vehicle was analysed by qPCR. Data are mean +/- S.E.M from N=1 independent experiment, technical triplicates. Significance was calculated using ordinary one-way ANOVA with Dunnett test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.0001$.

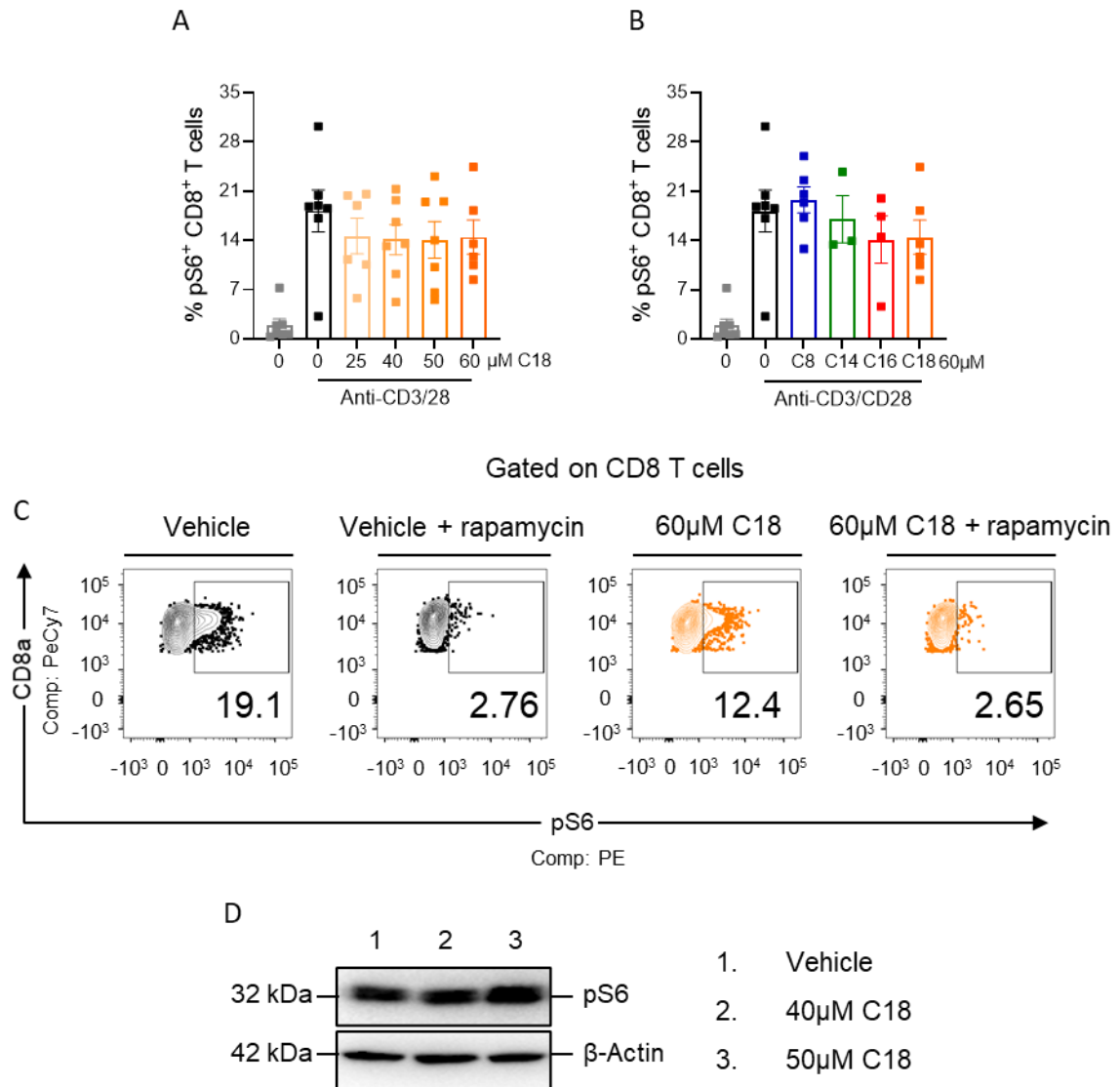


Figure 5.20 Acylcarnitines do not affect mTORC1 activation in CD8 T cells

Splenocytes **(A-B)** or pure CD8 T cells **(D)** from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60 μM). T cells were stimulated with anti-CD3 (1 μg/ml), CD28 (3 μg/ml), and IL-2 (5 ng/ml) for 48 h. Phosphorylation of S6 was analysed by flow cytometry **(A-C)** or western blotting **(D)**. Graphs show percentage of pS6⁺ CD8 T cells **(A, B)**, representative flow plot **(C)**, and representative pS6 blot **(D)**. Data are mean ± S.E.M from N=6 independent experiments **(A-C)** or representative of N=4 independent experiments **(D)**. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

5.4 Octadecanoyl-carnitine causes CD8 T cell mitochondrial depolarisation *in vitro*

Although activated CD8 T cells primarily rely on glycolysis, CTLs maintain abundant amounts of protein machinery for oxphos²³⁹. Therefore, we next investigated the effects of acylcarnitines on CD8 T cell oxphos by Seahorse analysis. C16 and C18 reduced the rates of oxphos, basal oxphos, and the maximum respiratory rate of CD8 T cells. However, C8 did not affect the rates of oxphos in CD8 T cells (Fig. 5.21 A-C). Together, these data indicate that LCACs, in particular C16 and C18 result in CD8 T cell metabolic paralysis which leads to impaired production of effector cytokines such as IFN γ .

We next examined CD8 T cell mitochondrial dynamics following stimulation in the presence of acylcarnitines. While there were no differences in mitochondrial mass (Fig. 5.22 A, B), only C18 caused mitochondrial depolarisation, which was in a concentration dependent manner (Fig. 5.23 A, B). C18 had no effect on the level of cellular ROS as measured by DCF staining and caused a modest but non-significant increase in mitochondrial ROS as measured by MitoSOX (Fig. 5.24 A, B). Together our data demonstrates that LCACs, in particular C18 induce metabolic paralysis in CD8 T cells, characterised by mitochondrial depolarisation and loss of oxidative and glycolytic metabolism, coupled with impaired IFN γ production.

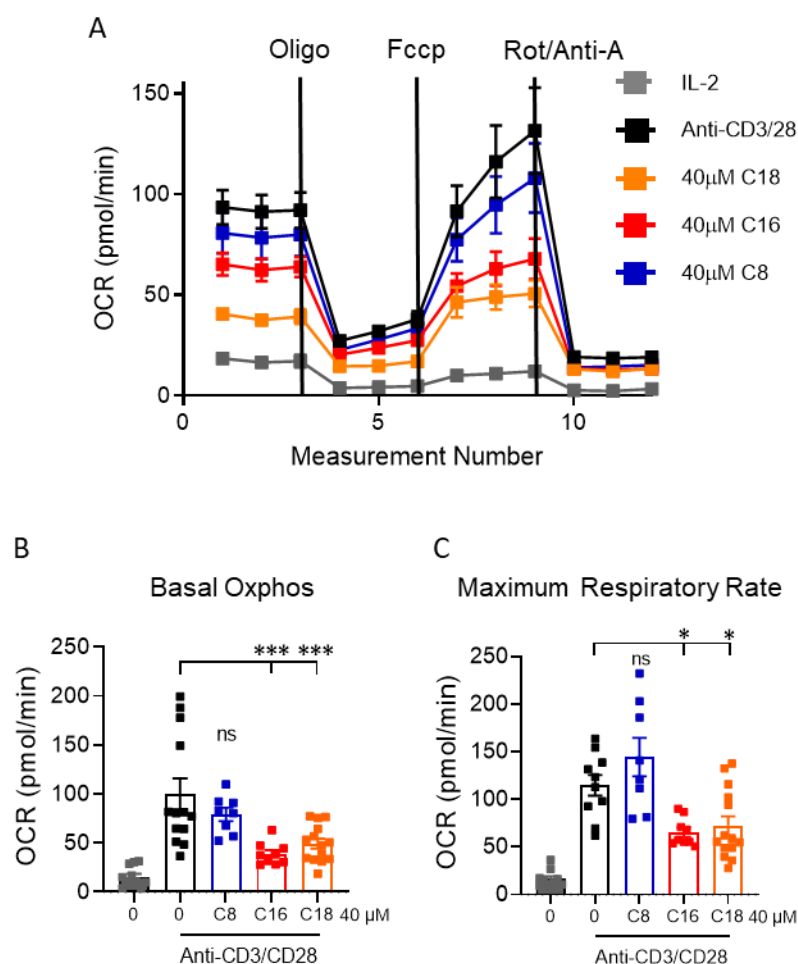


Figure 5.21 Acylcarnitines reduce the rates of CD8 T cell oxphos in a chain-length dependent manner

CD8 T cells were isolated from the spleens of naïve mice fed a standard fat diet. Pure CD8 T cells were cultured with increasing concentrations of acylcarnitines (25-60 μ M) and stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5ng/ml) for 48hr. Oxygen consumption rate (OCR) was measured by Seahorse analysis following injection of oligomycin (oligo), Fccp, rotenone and antimycin-A (Rot/Anti-A). Graphs show representative trace (**A**), basal oxphos (**B**), and maximum respiratory rate (**C**). Data are mean $\pm$ S.E.M from N=3 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test. ns= not significant, * $p \leq 0.05$, *** $p \leq 0.001$.

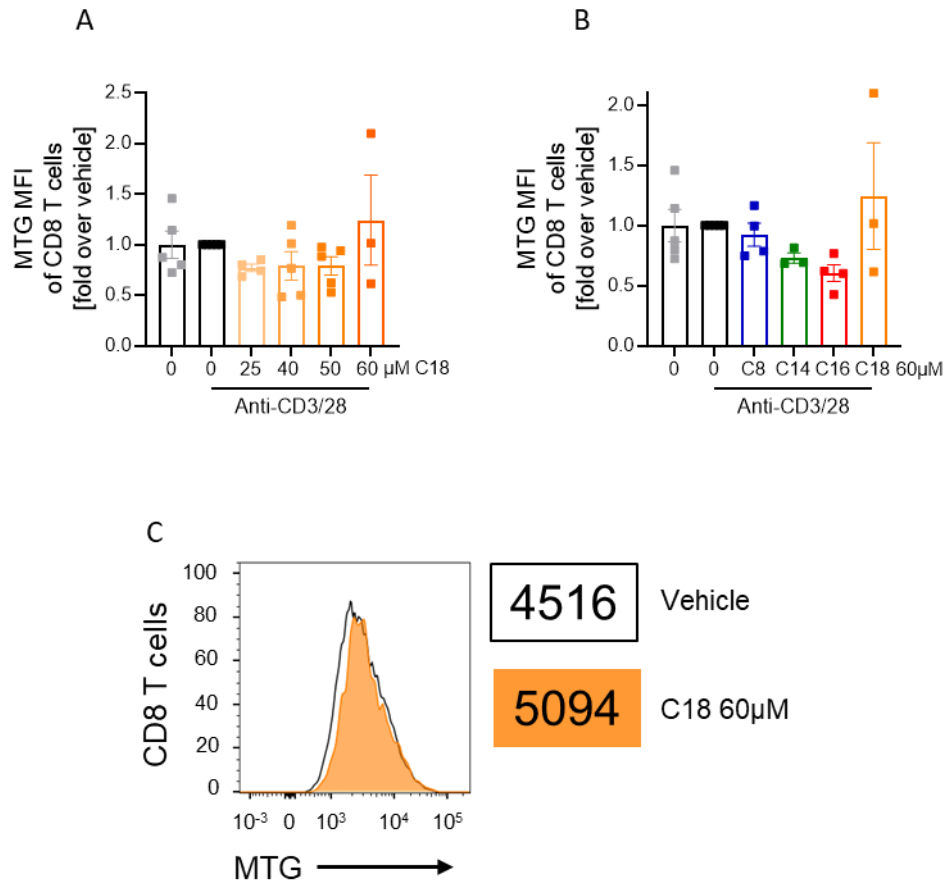


Figure 5.22 Acylcarnitines do not alter CD8 T cell mitochondrial mass

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60 μ M). T cells were stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5ng/ml) for 48hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual squares). Graphs show MitoTracker Green (MTG) mean fluorescence intensity (MFI) of CD8 T cells, relative to vehicle (**A**, **B**) and representative histogram (**C**). Data are mean $\pm$ S.E.M from N=5 independent experiments. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

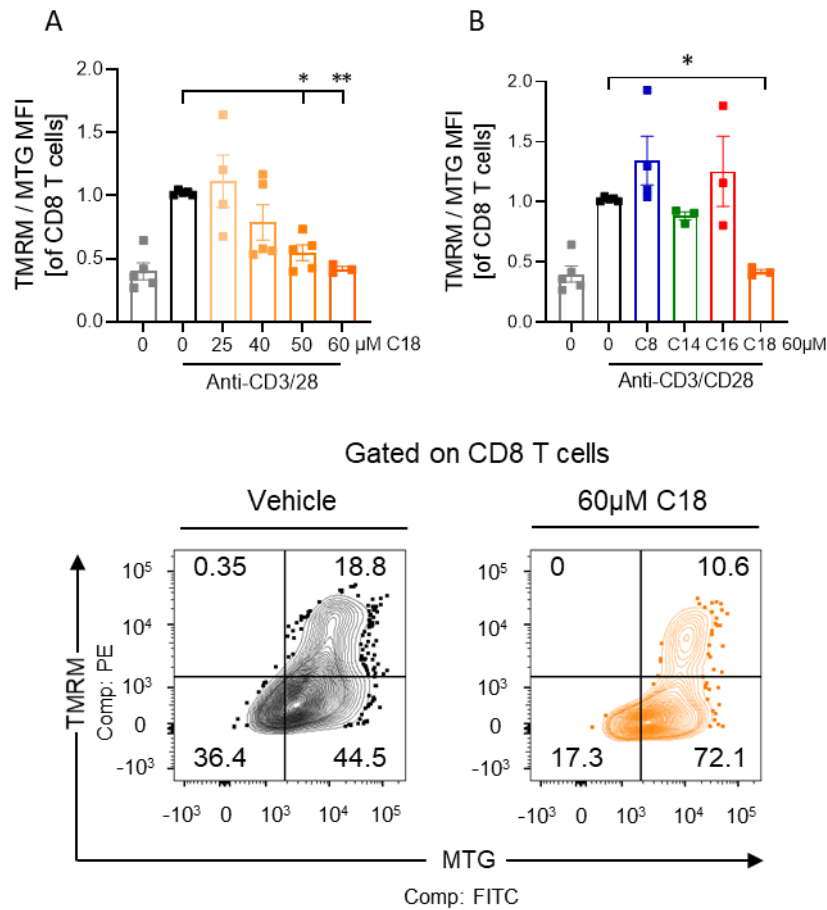


Figure 5.23 Octadecanoyl-carnitine reduces CD8 T cell mitochondrial membrane potential in a concentration dependent manner

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60 μ M). T cells were stimulated with anti-CD3 (1 μ g/ml), CD28 (3 μ g/ml), and IL-2 (5ng/ml) for 48hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual squares). Graphs show the mean fluorescence intensity (MFI) of TMRM / MTG of CD8 T cells, relative to vehicle (**A, B**) and representative flow plot (**C**). Data are mean $\pm$ S.E.M from N=5 independent experiments. Numbers represent percentage of cells. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

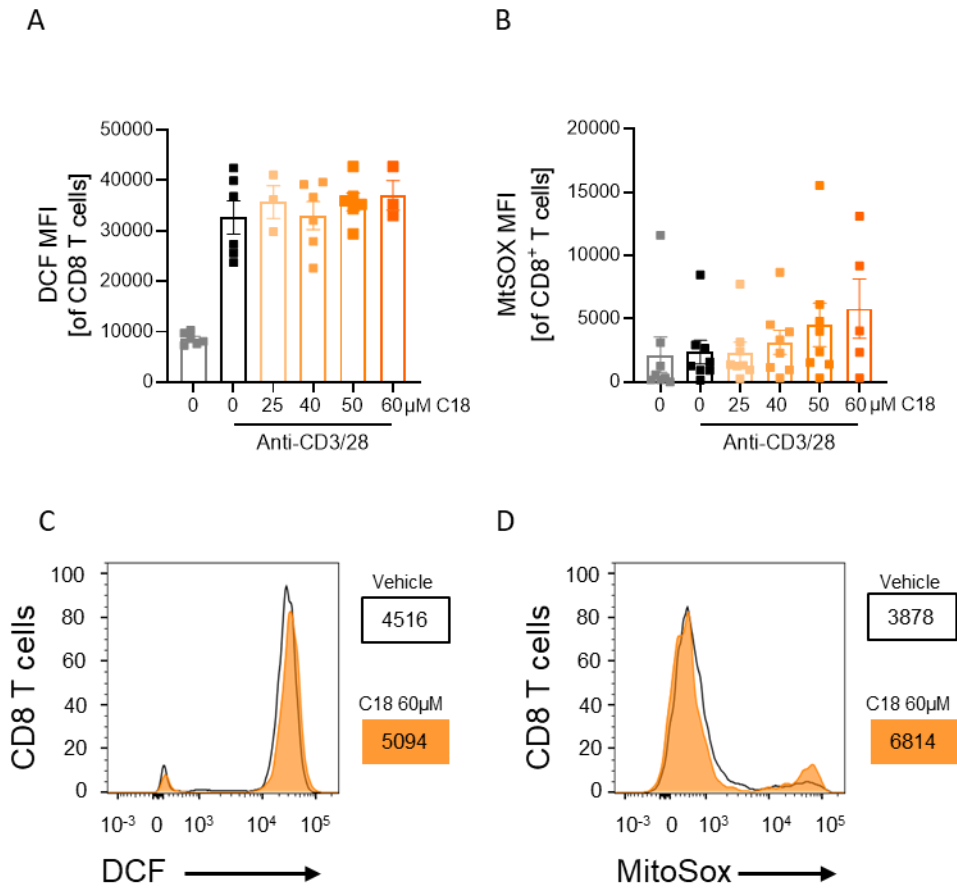


Figure 5.24 Octadecanoyl-carnitine increases mitochondrial reactive oxygen species production by CD8 T cells

Splenocytes from naïve mice fed a standard fat diet were cultured with increasing concentrations of acylcarnitines (25-60µM). T cells were stimulated with anti-CD3 (1µg/ml), CD28 (3µg/ml), and IL-2 (5ng/ml) for 48hr. Experiments were performed in technical triplicate which were averaged for each experiment (individual squares). Graphs show the mean fluorescence intensity (MFI) of DCF and MitoSOX of CD8 T cells (**A, B**) and representative histograms (**C, D**). Data are mean +/- S.E.M from N=6/8 independent experiments. Numbers represent MFI. Significance was calculated using ordinary one-way ANOVA with Dunnett test.

5.5 Discussion

Work from chapter 3 and 4 demonstrated that LCACs, particularly C18, accumulate in the plasma when mice are fed HFDs from animal lard and butter. In contrast, LCACs did not accumulate when mice were fed palm oil or when mice lost weight. Here in chapter 5, we show that LCACs, specifically C18, greatly impair CD8 T cell functional and metabolic responses *in vitro*. Interestingly, NK cell functions or metabolism were not affected, indicating that acylcarnitines directly target and interfere with anti-tumour CD8 T cells. Systemic accumulation of LCACs in mice fed lard and butter likely contributes to increased tumour growth *in vivo*.

Plasma acylcarnitine levels fluctuate in response to diet and increase under fasting conditions⁸². In humans, acylcarnitine efflux is well characterised in patients with a defect in lipid metabolism and FAO²²¹, however the exact nature of this transport is unknown and is thought to be largely dependent on the cellular concentration of acylcarnitines. Two putative transporters – OCTN2 and MCT9 (Slc16a9) are assumed to play a role, but other MCTs may be involved⁸⁴. Interestingly, the protein expression of MCTs (Slc16a1/3/10) is much higher on cytotoxic CD8 T cells and CD8 T cells following TCR stimulation, compared to NK cells (ImmPRes.co.uk). Given these differences, it is possible that CD8 T cells have a greater propensity to transport acylcarnitines intracellularly than NK cells, making NK cells more resistant to their immunomodulatory properties.

Cellular efflux of lactate is controlled by MCTs, in particular Slc16a1, and this process is critical to maintain high flux through glycolysis in activated CD8 T cells. Given that acylcarnitines are transported through MCTs, it is possible that acylcarnitines impede lactate transport, resulting in decreased levels of glycolysis. Indeed, the extracellular acidification rate is a measure of lactate export, which is reduced by C16 and C18 LCACs. Whether this is due to decreased flux through glycolysis or reduced efflux of lactate was not examined. In addition, C18 also reduces mitochondrial metabolism and causes mitochondrial depolarisation in CD8 T cells. It was recently reported that tumoral CD8 T cells with depolarised mitochondria are terminally exhausted and produce less IFN γ and TNF⁶⁵. Supplementing CD8 T cells with nicotinamide riboside (NR) *in vitro* and *in vivo* increased mitochondrial polarisation and boosted the production of IFN γ ⁶⁵. We questioned if acylcarnitines were also causing CD8 T cell terminal exhaustion and speculated that NR may restore IFN γ production by increasing mitochondrial activity. NR also replenishes the levels of NAD⁺ which is essential for GAPDH enzymatic activity to maintain heightened levels of glycolysis. However, in the presence of NR, IFN γ

expression or mitochondrial polarisation were not restored when CD8 T cells were cultured with LCACs (data not shown in thesis). Indeed, it is unlikely that LCACs cause CD8 T cell exhaustion as cells were only stimulated for 24-72hr. Recent reports demonstrate that CD8 T cell self-renewal and effector functions are reduced when cells are chronically stimulated with anti-CD3 for 8 days *in vitro*⁶⁶. In addition, LCACs decreased the expression of PD-1 and TIM-3 on CD8 T cells, indicating reduced activation and response to stimulation, rather than exhaustion. Given that CD69 expression does not change, LCACs do not interfere with the CD8 T cell's ability to respond to antigenic stimulation but may interfere with the magnitude of activation or the ability of the cell to sustain activation signals. In line with these findings, LCACs do not interfere with c-Myc expression, which initiates CD8 T cell metabolic reprogramming in response to initial antigen stimulation⁹³ (data not shown in thesis). Glycolytic metabolism is sustained in cytotoxic lymphocytes by the transcription factor HIF-1 α which is controlled by mTORC1 activation and PDK1⁹⁴. While mTORC1 activity was not affected by LCACs, C18 decreased the expression of the glycolytic enzymes *Hex2*, *Gapdh*, *Pkm1*, and *Ldha* and the glucose transporters *Slc2a1* and *Slc2a3*, which are HIF-1 α target genes. Therefore, it is possible that LCACs, especially C18, interferes with HIF-1 α , downstream of mTORC1 in CD8 T cells, preventing sustained levels of glycolysis, resulting in reduced expression of IFN γ following activation. Indeed, NK cell metabolism is controlled by cMyc, and not HIF-1 α ⁸⁹ which may further explain why acylcarnitines do not interfere with NK cell function or metabolic reprogramming.

Obesity is an immune paradox of both increased inflammation and immunosuppression. Heightened inflammation interferes with insulin signalling, contributing to the development of metabolic disorders such as T2D. Acylcarnitines promote sterile inflammation in obesity by enhancing IL-17F production by Th17 cells, driving T2D and associated metabolic pathologies⁸³. At the same time, immune suppression and impaired immunosurveillance increases the risk of cancer and infection in obesity. Here we show that LCACs, particularly C18 suppress CD8 T cell anti-tumour responses, resulting in metabolic paralysis and reduced IFN γ production *in vitro*. These diverging immunomodulatory properties of acylcarnitines may explain the paradox of increased inflammation and impaired anti-tumour activities in obesity. TMEs are rich in a diverse array of lipids including FAs and acylcarnitines^{127,85} which we show may also contribute to CD8 T cell immunosuppression in this context. Given that nutrients and metabolites in TIF are controlled by diet and the plasma metabolome²⁴⁰, it is likely that the source of dietary fat also influences the profile of acylcarnitines and lipids in the TIF which may explain the divergent effects of animal and plant derived HFDs on tumour growth *in vivo*.

Chapter 6. Overall Discussion

Obesity is a major risk factor for at least 14 types of cancers^{104,105}. It is now well established that obesity increases the risk of cancer by inhibiting NK and CD8 T cell functions, leading to impaired immunosurveillance^{113,114}. Immune effector functions are shaped by intracellular metabolic pathways which are in turn influenced by nutrients and metabolites in the cell's microenvironments⁷⁰. Nutrient availability is dictated by diet and therefore diet composition shapes the immune response by regulating cellular metabolism. For this thesis, we hypothesised that HFDs and obesity alter the plasma metabolome and that certain metabolites increased in obesity may interfere with immunometabolic pathways in NK and CD8 T cells. We further speculated that the source of dietary fat (animal vs plant) would differentially affect tumour growth *in vivo*, through the impact of different HFDs on whole body metabolism which regulates the metabolism and functions of anti-tumour NK and CD8 T cells.

Work from this thesis shows that the plasma metabolome is altered when mice are fed HFDs from both animal and plant sources. These changes occurred rapidly after one week of HFD feeding and were reversed by WL through dietary intervention. Alongside this, WL improved NK and CD8 T cell functional responses in the periphery, possibly due to changes in circulating metabolites and lipid levels. However, WL did not protect against enhanced growth of MC38 tumours and intra-tumoral CD8 T cells were functionally defective in mice that were losing weight. Bariatric surgery is associated with metabolic alterations soon after surgery such as decreased levels of insulin²⁰⁷. However, serum NEFAs only decrease 6 months post-surgery, and some FAs including SA are increased in the serum following surgery²⁰⁷. It is possible that the bolus of lipids released from adipose tissue stores during WL are sequestered by tumours to support their growth. It would be interesting to investigate if the lipid profile of adipose tissues correlates with lipids in the TIF as mice lose weight given that tumour cells can harness lipids from the circulation to support their growth¹⁹⁹.

Although both animal and plant HFDs disrupted the plasma metabolome, only HFDs from animal lard and butter accelerated the growth of B16 tumours and impaired intra-tumoral NK and CD8 T cell functions. Mice fed plant fats, particularly from palm oil, were protected against enhanced tumour growth and immune dysfunction that is commonly associated with HFDs and obesity (Fig. 6.1). It was previously reported that the plasma acylcarnitine signature is altered in mouse models of DIO. LCACs accumulate in the plasma of mice fed a HFD due to mitochondrial lipid overload, resulting in incomplete FAO⁸². However, we show that only animal fat HFDs from butter and lard increased the plasma abundance of the LCAC octadecanoyl-carnitine (C18) which was not altered in the plasma of mice fed palm oil. LCACs contribute inflammation in T2D by enhancing IL-

IL-17 production by Th17 cells⁸³ and it was recently reported that the TIFs of B16 and MC38 tumours are rich in a diverse array of acylcarnitines⁸⁵. Here we report an additional immunomodulatory role for LCACs, especially C18, which causes metabolic paralysis and mitochondrial depolarisation in CD8 T cells, resulting in reduced IFN γ expression *in vitro*. C18 may therefore accelerate tumour growth *in vivo* by inhibiting anti-tumour functions of CD8 T cells when mice are fed a HFD from butter or lard. IL-17 also contributes to tumour initiation, growth, and metastasis by recruiting immunosuppressive neutrophils¹⁷⁹ and remodelling the stromal architecture of the TME to promote tumour dissemination²⁴¹. The Lynch lab recently reported that IL-17 producing $\gamma\delta$ T cells are enriched in LNs and B16 tumours of mice fed a HFD¹⁸⁰. Given that LCACs increase IL-17 production by Th17 cells in diabetes⁸³, it is possible that acylcarnitines promote IL-17 expression and expansion of $\gamma\delta$ T cells when mice are fed animal HFDs, further contributing to tumour growth *in vivo*. Tumours are supplied with nutrients through the vasculature and dietary changes are reflected in nutrient levels in the TIF. Indeed, there is a strong correlation between TIF to plasma ratio of metabolite concentrations between mice on different diets²⁴⁰. Therefore, it is possible that LCACs such as C18 are further increased in the TIFs of mice fed animal, but not plant HFDs, and these may contribute to impaired CD8 T cell functions in this context.

Cancer immunotherapy re-invigorates adaptive immune responses and has revolutionised the treatment of certain cancers. In particular, immune checkpoint blockade using anti-PD-1 antibodies significantly prolongs the survival of patients with melanoma and non-small cell lung cancer^{55,56}. Recently, the Lynch lab reported that anti-PD-1 therapy reduces MC38 and B16 tumour growth in both lean and obese mice. This is associated with increased infiltration of CD8 T cells and partial restoration of CD8 T cell functions, which include increased proliferation and amino acid uptake. However, anti-PD-1 therapy only increased the expression of IFN γ and granzyme B in CD8 T cells in MC38 tumours of lean mice¹¹³. MC38 TIF contains high amounts of lipids, as well as acylcarnitines compared to the plasma of mice⁸⁵, and this likely increases when mice are fed a HFD. The high levels of acylcarnitines in the circulation of mice fed a HFD from animal lard or butter possibly perfuse the tumour to limit CD8 T cell functions and response to therapy. In this project, we demonstrated that NK cell metabolism and effector functions are not affected by acylcarnitines. Therefore, NK cells may be a more favourable immunotherapeutic target for the treatment of cancers in obesity where acylcarnitines are abundant.

The proliferation rate and metabolic requirements of cancer cells is also dictated by diet which controls nutrient sensing pathways associated with oncogenic signalling¹⁸².

Although it was not examined in this project, it is likely that animal and plant HFDs differentially affect the proliferation of tumour cells, which may further explain the divergent effects of these diets on tumour growth *in vivo*. Lipids are abundant in many mouse and human tumours^{127,85}, and fuel cancer growth by inducing proliferation and metastasis¹⁹⁹. Consumption of excess dietary fat is a risk factor for cancer progression¹⁸³, and evidence is now emerging that specific types and groups of FAs promote proliferation and metastasis of cancer cells both *in vitro* and *in vivo*. PA, a SFA enriched in many common fat sources, recapitulates the effects of HFD feeding *in vitro*, by boosting the proliferation of colorectal cancer cell lines²¹². Similarly, PA promotes the stemness of Lgr5⁺ intestinal stem cells, which increases the spontaneous formation of colorectal adenomas *in vivo*¹⁵⁰. In contrast, the MUFA OA reduces the viability, migration, and invasion of Hep3b and Huh7.5 liver cancer cells *in vitro*²¹³ and OA reduced the growth of CAL27 squamous cell carcinoma xenograft tumours *in vivo*²⁴². Interestingly, a recent paper from the Benitah lab demonstrated that a palm oil HFD, rich in PA, epigenetically reprograms oral carcinoma cells to adopt a pro-regenerative state which increases metastasis through the secretion of a specialised extracellular matrix. In contrast, tumours from mice fed a HFD from olive oil, rich in OA, were less metastatic, illustrating the differential effects of these FAs on tumour growth in mouse models of oral carcinoma¹⁸⁶. PA is enriched in both palm oil and butter HFDs and thus unlikely explains the divergent effects of butter and palm oil HFDs on B16 melanoma tumour growth in this project. Different tumour types may display specific FA preferences, which is likely influenced by their anatomical location. Indeed, high levels of OA in lymph nodes induce metastatic competency to melanoma cells that have invaded the lymphatic system²⁴³, contrary to the protective effects of OA in oral carcinoma metastasis¹⁸⁶. However, diets are not composed of one type of FA. It is likely a combined effect of the cocktail of lipids in a butter HFD favours tumour growth over one FA species alone. In addition to the LCFAs MA, PA, and SA, butter is also rich in short and medium chain fatty acids (S/MCFAs). Interestingly, a coconut oil HFD also contains S/MCFAs, even more so than butter, but less PA and SA (Table 4.1). Tumour cells may therefore favour a combination of S/M/ and LCFAs to fuel energy production and promote their proliferation. A recent report in *Nature* revealed that tumours adapt to imbalances in the serum lipid content produced in response to low glycaemic diets. Caloric restriction leads to a decreased ratio of MUFAs : SFAs in the TIF of PDAC tumours, which slows tumour growth. On the other hand, a ketogenic diet increases the availability of both MUFAs and SFAs, which enhances tumour progression²¹⁷. Therefore, these data also suggest that specific lipid species also play a role in tumour growth and thus a combination of all FAs and the

overall saturation profile may be responsible for the differential effects of butter and palm oil HFDs on both tumour growth and the anti-tumour immune response in this project.

Given that acylcarnitines are increased in the TIF of B16 and MC38 tumours of mice fed a SFD⁸⁵, it is possible that high levels of lipids in TIFs cause mitochondrial metabolic overload and incomplete FAO in tumour cells, resulting in acylcarnitine accumulation. It would be interesting to investigate the Cpt1:Cact ratio in different tumours and correlate this with acylcarnitines in the TIF, to see if diet affects the metabolism of lipids in tumours. Tumour cells may harness acylcarnitines in the TIF to support their rapid proliferation or to be used for energy production. Similar to their effects on CD8 anti-tumour functions, it is possible that acylcarnitines may differentially affect tumour growth and proliferation in a chain length manner. Thus, acylcarnitine accumulation in TIFs and from animal HFDs may have a dual effect on cancer, by promoting tumour cell metabolism and proliferation, while impairing infiltrating cytotoxic immune cells.

The World Cancer Research Fund / American Institute for Cancer Research (WCRF/AICR) has issued a set of lifestyle guidelines which aim to help people reduce their risk of cancer. One such recommendation is to follow a healthy dietary pattern, rich in wholegrains, vegetables, fruits, and beans, while limiting the consumption of red and processed meats²⁴⁴. Adhering to these recommendations is associated with a decreased risk of cancer, particularly breast and colorectal cancers, as well as reduced cancer mortality²⁴⁵. In addition, nutritional interventions are now emerging as tools to treat certain cancers alongside traditional cancer therapies. Pre-clinical evidence suggests that dietary restriction of particular nutrients including glucose, fructose, methionine, and serine may enhance cancer therapy¹⁸². While PA has emerged as a FA that promotes tumour initiation, growth, and metastasis^{150,186}, it is unknown whether restriction of PA or another type or group of FAs can reduce tumour growth. Understanding specific metabolic pathways of tumour cells and restricting the dietary intake of certain nutrients can target the metabolic vulnerabilities of tumours by modifying the supply of nutrients that are essential for tumour growth. As diets are rich in a diverse array of lipids that are abundant in many common sources of fat¹⁸⁵, dietary restriction of pro-tumorigenic FAs is a challenge. Therefore, identifying the molecular targets and signalling pathways that are regulated by FAs or combinations of FAs in animal vs plant HFDs may help design effective targeted therapies.

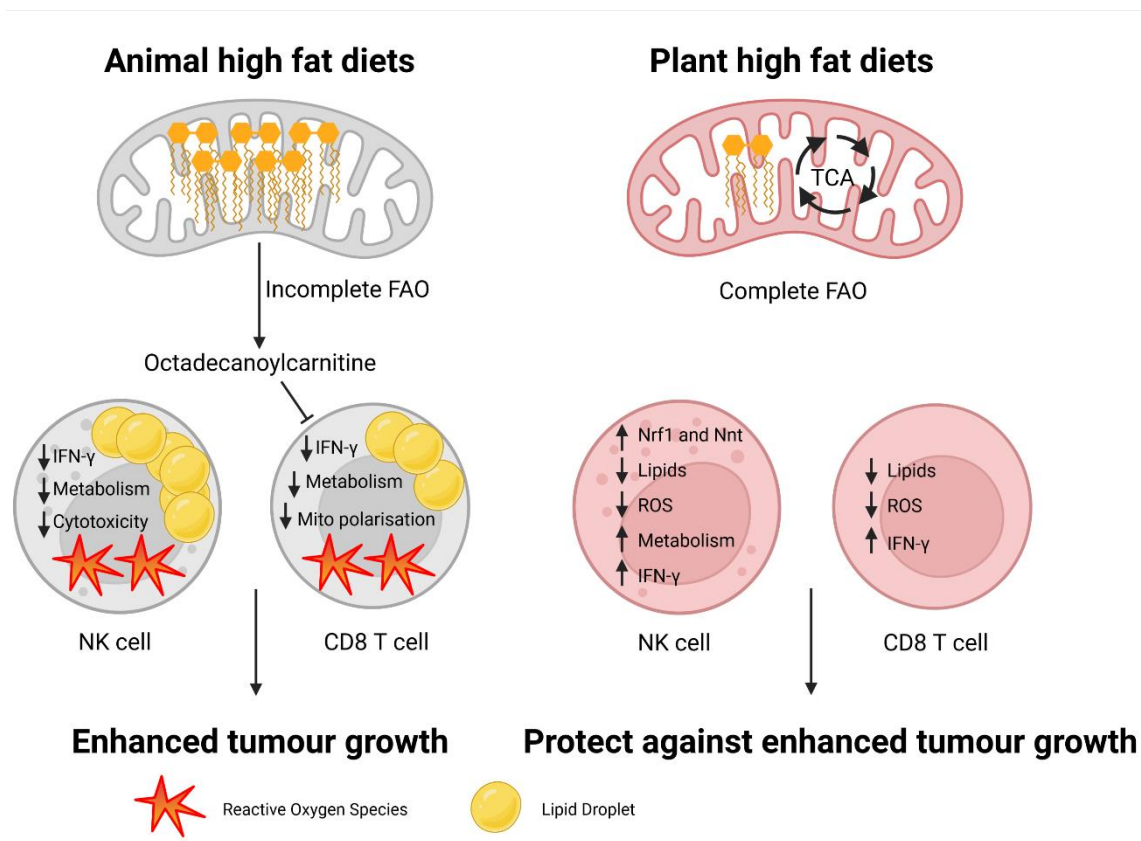


Figure 6.1 Octadecanoyl-carnitine limits CD8 T cell functions when mice are fed a high fat diet from animal sources

High-fat diets (HFD) from animal sources, but not plant sources accelerates tumour growth despite similar levels of obesity and adipose distribution. Long chain acylcarnitines (LCACs), particularly octadecanoyl-carnitine (C18) accumulate in the plasma of mice fed animal, but not plant HFDs due to mitochondrial metabolic overload and incomplete fatty acid oxidation (FAO). C18 does not affect NK cell functions or metabolism but results in CD8 T cell mitochondrial depolarisation and metabolic paralysis, resulting in reduced IFN-γ production. Additionally, NK and CD8 T cells from mice fed animal, but not plant HFDs accumulate lipids intracellularly, resulting in increased reactive oxygen species (ROS), metabolic paralysis, and impaired IFN-γ production. NK cells activate antioxidant pathways involving Nrf1 and Nnt when mice are fed plant HFDs, protecting from lipotoxicity and retaining cytotoxicity.

Chapter 7: Bibliography

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